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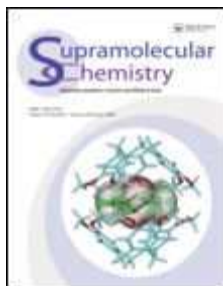
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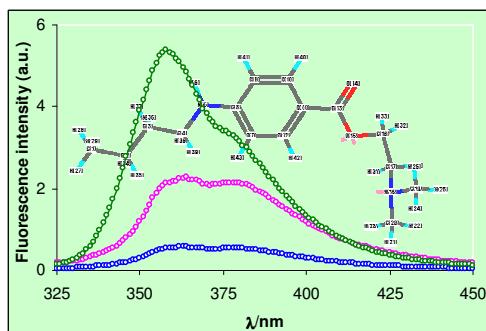


Fluorescence study of tetracaine-cyclodextrin inclusion complexes

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Graphical Abstract



Tetracaine shows dual fluorescence in water-solvent mixtures and in aqueous cyclodextrin solutions; the formation of 1:1 inclusion complexes has been studied in α -, β -, and γ -CD.

Fluorescence study of tetracaine-cyclodextrin inclusion complexes

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The steady state fluorescence emission from of the local anesthetic tetracaine (TCA) in water-solvent mixtures and in the presence of α -, β -, and γ -cyclodextrin (CD) was investigated at various pH values. Emission was observed from the locally (LE) and the intramolecular charge transfer (ICT) excited states. The TCA:CD system found to be characterized by 1:1 associate in every case. The association constants of each complex were determined.

Keywords : Cyclodextrins, tetracaine, fluorescence, stability constants

Introduction

It is generally believed that interaction of the anesthetic molecules with membrane lipids or membrane proteins leads to the inactivation of neuronal ion channel activity.¹ Tetracaine [TCA, 2-dimethylaminoethyl-4(N-butylamine) benzoate] is one of the most widely studied tertiary amine local anesthetic,^{2,3} The absorption and the ability of this drug to cross biological membranes are pH-dependent, since TCA can exist in neutral, singly or doubly positively charged form. The use of natural cyclodextrins as drug carriers provides the solution to many difficulties in drug formulation that includes poor bioavailability, limited shelf life, or restricted utility when the drug is irritating to skin, membranes, tissues, and so on.

Natural cyclodextrins (CDs) are water soluble cyclic-oligosaccharides that behave in aqueous solutions as pre-organized hosts that form hydrophobic cavities with hydrophilic external walls.^{4,5} The formation of inclusion complex means that compatibility is reached between the host and the guest in terms of the polarity and stereochemistry; in this process, the guest molecule experiences a non-polar environment and possesses a decreased freedom for bulk and intramolecular rotations, in the rigid non-polar CD cavity. This ability of CDs to accommodate guest molecules of the appropriate size in their cavities has been utilized to control photophysical and photochemical properties^{6,7} or chemical reactivity^{8,9} of molecules. The restricted shape and size and the hydrophobic nature of the cavities of CDs offer the opportunity to carry out selective phototransformations and to investigate specific aspects of photochemical mechanisms. In addition, in chromophoric species that can be entirely enclosed into de CD cavity, direct protection from quencher can take place as long as the probe remains inside the cavity. The analysis of the changes in physical and chemical properties of the complexed guest provides information about the structure of the complex, the stoichiometry, and stability.

The intramolecular charge transfer (ICT) phenomenon, firstly introduced by Grabowski and coworkers¹⁰ to explain the dual fluorescence emission observed by Lippert *et al.*¹¹ in 4-(dimethyl)amino benzonitrile, DMABN), is strongly dependent on the medium polarity and viscosity and has been the

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3 subject of several studies from the early 1970s.¹²⁻²⁴ On the basis of the fluorescence features, such as
4 maxima of emission bands, fluorescence lifetimes and relative intensities of the short-wavelength (SW)
5 due to locally excited state (LE) and long-wavelength (LW) due to the ICT emissions of DMABN
6 complexes, different geometries were proposed for the inclusion in the α - or β -CD cavities.
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10 Recently, the study of fluorescence emission and of chemical reactivity of novocaine (2-
11 diethylamino-ethyl,4-aminobenzoate) has been carried out in aqueous β -CD solutions.²⁵ In the present
12 study we report on the influence of complexation with α -, β -, and γ -CD on the TCA excited state
13 properties; the obtained results provide information on the effect of environment on LE and ICT
14 emissions and on the stability and geometry of the inclusion complexes.
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18 19 20 **Experimental**

21 High purity tetracaine-HCl and cyclodextrins were purchased from Sigma-Aldrich. Dioxane, DMSO,
22 MeOH, and acetonitrile (uvasol grade) were from Merck. Twice distilled water obtained under
23 potassium permanganate solution, were used for preparing solutions and buffers.
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25 Concentrated stock solution of the hydrochloric anesthetic salt was prepared in water and
26 frequently renewed. The concentration of TCA was determined by weighing the appropriate quantity of
27 salt and the consequent dilution of the stock solution.
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30 Potentiometric measurements were carried out in a jacket cell that was kept at constant
31 temperature (25 °C) by circulating water from a thermostat. Purified nitrogen was bubbled through the
32 solutions to ensure thorough homogenization and CO₂ removal. A Crison microBu 2030 automatic
33 burette furnished with a 2.5 mL syringe for dispensing the titrant was used. The burette was controlled
34 via a computer that was used to read the emf values from a Crison micropH 2000 pHmeter, connected
35 to two electrodes: a glass electrode Radiometer pHG211 and a reference electrode Radiometer REF201.
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40 Absorption spectra were recorded on a Uvikon-Kontron 942 double beam spectrophotometer
41 fitted with thermostated cell holders at 25 °C. The concentration of tetracaine was about 6.5×10^{-5} M.
42 Data acquisition and analysis of UV-vis spectra were performed with software supported by the
43 manufacturer and converted to ASCII format for their analysis with common packet programs.
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47 Steady-state fluorescence spectra were recorded on an SLM Aminco-Bowman series 2
48 spectrofluorimeter fitted with a 150W Xenon lamp at 25 °C. Data acquisition and analysis of
49 fluorescence spectra were performed with the Fluorescence Data Manager Software supported by
50 Aminco. Excitation and emission slits were fixed at 4 and 2 nm, respectively, the excitation wavelength
51 was set at 300 nm, and the emission intensity of fluorescence was recorded at 346 nm. In all
52 measurements the emission spectra, collected from 315 to 400 nm, was obtained from solutions of
53 constant tetracaine concentration of ~ 9 μ M, choose in the way that the absorbance of the sample at 300
54 nm (the excitation wavelength) was lower than 0.2 absorbance units, and variable CD concentration.
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Results

Tetracaine is a tertiary amine that also contains a secondary amine group bonded to the phenyl ring (Scheme 1).

Scheme 1

The absorption spectrum of TCA (62 μM) in water at 25 $^{\circ}\text{C}$ is characterized by two structureless bands with $\lambda_{\text{max}}=226$ nm and $\lambda_{\text{max}}=309$ nm. Both the longer (LW, assigned to the $S_0 \rightarrow S_1$ transition) and the shorter (SW, assigned to the $S_0 \rightarrow S_2$ transition) wavelength bands exhibit a slight blue shift in low dielectric media, Table 1. In strong aqueous acid media ($\text{pH} < 2$) the LW absorption markedly decreases, whereas, in mild acid (or neutral) aqueous media, the maximum was observed at 310 nm, but shifts to 306 nm in alkaline medium. The pK_a values of tertiary and secondary amine groups of TCA are largely different. The corresponding values have been determined by potentiometry at 0.10 M ionic strength NaCl. The results are reported in Table 2 along with the values determined from the spectroscopic titration method²⁶ and from literature data.²⁷ Therefore, three different absorbing species of tetracaine can be considered on varying the pH of the medium, which correspond to TcH_2^{+2} , TcH^+ and Tc (Scheme 1). At very low pH, the strongest absorption was observed at 228 nm (SW, due to $\pi \rightarrow \pi^*$ transition) whereas, either mild acid or alkaline medium favour the low energetic $n \rightarrow \pi^*$ transition (the auxochrome secondary amine group is deprotonated) and a strong absorption at 310/306 nm (LW) was respectively observed for the acid and alkaline medium. A progressively blue shift of LW and SW bands on increasing pH was observed.

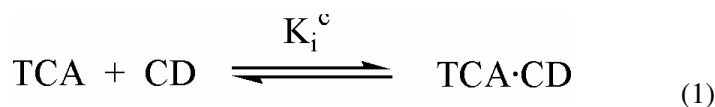
The fluorescence spectrum of tetracaine in water is strongly affected by the medium acidity showing dual fluorescence emission and the intensity is strongly enhanced with the pH of the medium. Figure 1 shows the corresponding spectra recorded in strong acid ($\text{pH} < 1.5$), mild acid ($\text{pH} \sim 5$), basic ($\text{pH} \sim 10$), and in alkaline medium. The different investigated pH regions reflect the appearance of also three different fluorescent species of TCA showing distinct fluorescence quantum yield. As the pH regions in fluorescence are similar to those observed in the ground state, it suggest no significant changes on the pK_a value of TCA in the excited state in accordance with the Förster approximation.^{26,28} In addition, at $\text{pH} > 4$ (existence of TcH^+ or Tc) dual fluorescence is observed. The emitting chromophore in tetracaine is the benzene ring, and emission occurs from both the lowest (π , π^*) singlet state (LE) and the intramolecular charge transfer (ICT) state, which is originated from the N-lone pair of the secondary amine group (acting as the electron donor group) to π -orbitals of the benzene ring or to the carbonyl group that is characterized by a high electron accepting character. An alternative view could attribute the dual emission to a $\pi \rightarrow \pi^*$ transition involving a considerable charge transfer character. The scan [2] in Fig. 1 corresponds to the emission of TcH^+ species, that is, when the N-atom of the secondary amine group is deprotonated. Scans [3] and [4] were obtained when the tertiary amine group is also deprotonated.

The emission through ICT state decreases faster than the LE emission as the non-polar solvent concentration increases. Plotting the fluorescence emission intensity, I_F , for both maximum emission bands, as a function of the solvent concentration, result the Figs. 2a and 2b, respectively to the emission at 357 (LE) and 377 nm (ICT). Two different behaviours are clearly observed; for both dioxane and DMSO solvents, high non-linear fluorescence emission was observed; whereas, in the presence of acetonitrile good straight lines were drawn; methanol can be seen as an intermediate situation. In S_1 state, the polarity of the secondary amine group is further increased due to the increased charge transfer interactions from this group to the carbonyl moiety, and the nitrogen-lone pair of the secondary amine group acts as a better proton donor. Table 1 reports the fluorescence intensity ratio of LE to ICT states measured for the same solvent concentration, along with solvent properties²⁹ and characteristics of the absorption spectra. It can be noted the decisive role of H-bonding with the solvent –the case of water and MeOH– to stabilized the optimal configuration required to observe emission from the ICT state (low ratio of $I_F(\text{LE})/I_F(\text{ICT})$ determined at 10 M solvent concentration).

Another parameter that conditions the ICT state emission is the restricted motion imposed to the fluorophore. The nature of the CD microvessel constraints nuclear motions and may stabilize conformations which are less favoured in free solution. On the other hand, the hydrophobic nature of the cavity can affect photoprocesses which are sensitive to solvent polarity or dielectric properties. For that, we investigated the effect of rigid hosts such as cyclodextrins (CDs) in the emission of fluorescence of tetracaine. As mentioned elsewhere, CDs are very soluble in water but their cavity is hydrophobic. Therefore, hydrophobic species can be “extracted” from water and enclosed in the CDs, with a consequent change in the luminescence properties.

Figure 3a shows the steady-state fluorescence spectra of tetracaine as a function of the acidity of the medium at constant $[\beta\text{-CD}]$; whereas, Fig. 3b shows the effect of $\beta\text{-CD}$ concentration on the fluorescence emission intensity recorded in aqueous solutions of 0.067 M of sodium acetate.

Under all experimental conditions, the addition of $\beta\text{-CD}$ at increasing concentrations (up to 9 mM) induces normal spectral variations characterized by a progressive blue-shift of the maximum to *ca* 357 nm and large enhancement of emission intensity with respect to water (compare Fig. 1 and 3a). In addition, the emission through the LE state is favoured over that of ICT state -the band at 357 nm increases with both the $\beta\text{-CD}$ concentration (Fig. 3b) and with the stability of the complex -. The former statement is, in fact, the consequence of the latter, since as more CD is present in the medium, the quantity of the TCA forming complexes increases in accordance with eq. (1) for the case of 1:1 stoichiometry of the inclusion complexes.

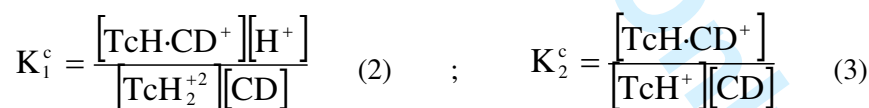


In this sense, the fluorescence emission intensities, read under different experimental conditions and plotted in Fig. 4, increase with $\beta\text{-CD}$ concentration and level off at high cyclodextrin concentration.

This behaviour is indicative of inclusion complexes formation of 1:1 stoichiometry. It can also be seen, the strong effect of pH on the stability of the complex: the highest increase in I_F provoked by the lowest cyclodextrin concentration was observed in aqueous carbonate/bicarbonate buffer.

To understand the concept of stability of the inclusion complexes, one need to know which tetracaine species is included into the CD-cavity. Then, by considering the acid- base equilibrium of tetracaine stated in Scheme 1, and that of cyclodextrin, which includes the ionization of a secondary HO-group in alkaline medium (the pK_a is 12.2)³⁰, four possible different complexes of 1:1 stoichiometry can be postulated that, *a priori*, could be $TcH_2^{+2} \cdot CD$, $TcH^+ \cdot CD$, $Tc \cdot CD$, or $Tc \cdot CD^-$, i.e. neutral CD complexed with dication-, monocation, and neutral forms of tetracaine and ionized CD complexed with neutral tetracaine. The presence of two acid-base equilibrium in TCA molecule, along with the own acid-base equilibrium in cyclodextrin, and four possible complexation equilibrium processes, makes the CD-TCA system a very complex one. Nevertheless, the system results notably simplified when the experiments are performed under determined working conditions; in addition, the main driving forces for complex formation is the hydrophobic effect that considers the exclusion of the hydrophobic guest from the bulk water solvent when intermolecular interactions are not favourable, accompanied by the release of high energy water molecules from the CD cavity. This fact explains why the double protonated TCA molecule (TcH_2^{+2}) does not form inclusion complexes; nevertheless, even in strong acid medium one observes spectral variations over the whole CD concentration range. The Scheme 2 accounts for the experimental facts, in which the TcH^+ species undergoes complexation with CD by the equilibrium process of constant K_1^c or K_2^c expressed by eqs. (2) and (3), respectively. The “extraction” of this species from water and enclosed into de CD cavity, shifts the acid-base equilibrium, K_1^H , towards the basic form of TCA, i.e., TCA is a weaker base in the presence of CD.

Scheme 2



Under the experimental conditions of the work, i.e., $[TCA]_t \ll [CD]$, we must consider firstly, that, in strong acid, HCl, the stoichiometric tetracaine concentration is given by: $[TCA]_0 = [TcH_2^{+2}] + [TcH^+] + [TcH \cdot CD^+]$. Secondly, taking into account that the quantum emission yield of TcH_2^{+2} species (tetracaine dication) is negligible, then the measured fluorescence intensity is due to both the protonated tetracaine in the bulk water phase (TcH^+) and included into the CD-cavity ($TcH \cdot CD^+$). Taking into account the different quantum yield (Φ) for free and included species, the total fluorescence emission is given by $I_F = \varepsilon_w \ell I_0 \Phi_w [TcH^+]_w + \varepsilon_c \ell I_0 \Phi_c [TcH \cdot CD^+]$, where ε is the molar absorption coefficient, $\ell = 1$ cm the path light, and I_0 is the absorbed intensity. This equation can be

converted into eq. (4) by considering together eqs. (2) and (3). In this equation, I_F^w and I_F^c represent the fluorescence intensities corresponding to the TCA free and complexed with CD, respectively, and $K_1^c = K_2^c K_1^H$.

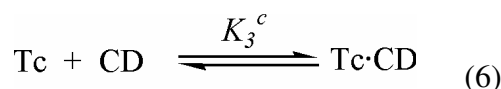
$$I_F = \frac{I_F^w f(H^+) + I_F^c K_2^c f(H^+) [CD]}{1 + K_2^c f(H^+) [CD]} \quad \text{with } f(H^+) = \frac{K_1^H}{K_1^H + [H^+]} \quad (4)$$

Solid curves corresponding to the down two set points displayed in Fig. 4a were drawn in the fit of eq. (4) to the experimental data read at both 357 (filled symbols) and 377 (open symbols). The non linear regression analysis of this equation to the experimental data yield the values for the optimized parameters, I_F^c and K_2^c , listed in Table 3, first arrow.

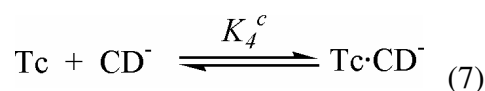
In mild acid medium, *i.e.*, in aqueous buffered solutions of acetic acid-acetate of $\text{pH} > 4$, as the $\text{p}K_1^H$ has been measured equal to 2.27, then the $[H^+] \ll K_1^H$ and eq. (4) simplifies to eq. (5). Solid curves for the upper two set-points in Fig. 4a correspond to the fit of eq. (5) to the experimental data read also at both 357 (filled) and 377 (open) symbols, when the optimized parameters take the values reported in Table 3.

$$I_F = \frac{I_F^w + I_F^c K_2^c [CD]}{1 + K_2^c [CD]} \quad (5)$$

In mild basic medium, *i. e.*, in aqueous buffered solutions of carbonate-bicarbonate buffer of $\text{pH} \sim 10$, the tertiary amine group of tetracaine is deprotonated ($\text{p}K_2^H = 8.39$). As tetracaine is now a neutral molecule, the hydrophobic effect is increased, since the strong ion-dipole interactions are now not possible, and one may expect a higher value for the association constant of TCA with CD-cavity. The equilibrium reaction of eq. (6), lead us to an equation of type (5) where K_2^c must be replaced by K_3^c that corresponds to the equilibrium constant of the inclusion complexes formed between both neutral forms of TCA and CD. The experimental data are displayed in Fig. 4b and the fit to the corresponding eq. (of type 5) results in the values of the optimized parameters listed in Table 3. As expected, the value of K_3^c is nearly 3-fold that of K_2^c ; the difference measures the relative importance of the hydrophobic effect against the ion-dipole interactions (the energy of water release from the cavity is in both cases the same).



Finally, in alkaline medium, tetracaine is a neutral molecule but the host, cyclodextrin, is an anion; that is, a secondary $-\text{OH}$ group is ionized ($\text{p}K_a \sim 12.2$). This group is located at the main entrance of CD cavity, therefore, when it is ionized, the hydration level is more important and difficult the inclusion process of a hydrophobic guest. Thus, the equilibrium reaction of eq. (7) can be proposed.



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Taking into account that the tetracaine concentration is much lower than that of CD and proceeding as in the previous cases, we can easily arrive to an equation of type (5) to relate the changes in fluorescence emission intensity with the cyclodextrin concentration in alkaline medium, where K_2^c must be replaced by K_4^c that corresponds to the stability constant of the inclusion complexes formed between the neutral form of TCA and the ionized cyclodextrin, CD^- . The experimental data are displayed also in Fig. 4b and the fit to the corresponding eq. (of type 5) results in the values of the optimized parameters listed in Table 3. These data indicate that $K_3^c > K_2^c \sim K_4^c$; the K_1^c values measured in the presence of sodium acetate, is, in fact, a combined result of both K_2^c and K_3^c , because under these conditions of $pH \sim pK_2^H$, TCA exists as both TcH^+ and Tc . In the same sense, at $[OH^-]=0.048$ M where the percentage of ionized β -CD is not higher than 80%, the value of $K_1^c=1010$ M^{-1} must be a mixed value of true K_3^c and K_4^c , *i.e.* the true K_4^c should be lower.

As a summary, the preceding mechanistic treatment implies that the variation of I_F as a function of $[CD]$ should follow the eq. (4), or (5) depending on the experimental conditions of the experiments, where K_2^c , K_3^c and K_4^c are the stability equilibrium constants of the inclusion complexes formed between protonated tetracaine and CD ($TcH \cdot CD^+$), neutral tetracaine and CD ($Tc \cdot CD$), and neutral tetracaine and ionized CD ($Tc \cdot CD^-$), respectively, and I_F^w and I_F^c refer to the fluorescence intensity readings obtained, under each experimental conditions, in water and at a CD concentration corresponding to the complete guest inclusion, respectively.

In order to study the effect of the size of the cyclodextrin cavity, we analyzed the effect of both α - and γ -cyclodextrin. Figure 5 shows the variation of the fluorescence emission intensity as a function of the cyclodextrin concentration. In every case, the plot of I_F against $[CD]$ describes saturation curves, due to the inclusion complex formation of 1:1 stoichiometry. The non-linear regression analysis of the experimental data to eq. (5) –valid for experiments performed in either mild acid or mild basic medium– afford the values for the optimized parameters listed in Table 4, along with the results obtained in the presence of β -CD for the sake of comparison. It can be concluded that β -CD cavity combines optimal geometry and polarity conditions to complex TCA molecules, whereas γ -CD yields the less stable complex. It is worthy to mention the restricted motion imposed by the narrow α -CD cavity, in which case the I_F^c due to LE emission is more than 3-fold the value measured in γ -CD cavity, where both the polarity (dielectric constant equal to 70 against 47 for α -CD)³⁰ and the rotation freedom are high.

The experimental data were also fitted to the linearized form of eqs. (4) or (5), that is, the Benesi-Hildebrand relation used for these types of complexation processes and expressed in terms of fluorescence intensity with the assumption that $[CD] \gg [TCA \cdot CD]$, *i.e.*, the reciprocal plot of $(I_F - I_F^w)$ against $1/[CD]$. In every case, perfect straight lines were obtained (plots not shown) and rendering similar values of K_1^c and I_F^c to those obtained from the non linear regression analysis.

Discussion

The UV-vis spectra of TCA indicate no significant changes in the pK_a of the ground state with respect to that of the excited state. Excitation at the lowest energy absorption band ($\lambda_{max} \sim 300$ nm) yields dual fluorescence, which emission intensity increases drastically with both the pH and the apolar character of the medium, represented here by aqueous-solvent mixtures or aqueous cyclodextrins solutions. Tetracaine forms 1:1 inclusion complexes with α -, β -, or γ -cyclodextrin; only the protonated (at the tertiary amine group) or neutral TCA molecules are able to be included into the CD-cavity; however, the highest complex stability was obtained in conditions of neutral host and guest, i. e. at pH ~ 10 in bicarbonate-carbonate buffered solutions. The size of the CD cavity affects both the complex stability and dual fluorescence emission.

In aqueous solvent mixtures up to 80% v/v solvent/water, TCA shows dual non-linear fluorescence, except for the case of acetonitrile, in which medium nearly straight lines of I_F vs [solvent] was obtained. By contrast, in aqueous-cyclodextrin solutions, the I_F vs [CD] increase at low CD-concentrations and levels off at high concentration of the host, when almost all TCA molecules are forming complexes.

In aqueous buffered solutions of acetic acid-acetate, or even in aqueous sodium acetate solutions, the quotient between the fluorescence intensities of LE (~ 357 nm) and of ICT (~ 377 nm) is nearly 1, scan 2 in Fig.1; that is, the ICT emission state is as important as the LE emission. This fact is contrary to the behaviour observed in those fluorophores bearing a dialkylamine group bonded in the *p*-position of the phenyl ring to the acceptor group like, for instance, methyl *p*-dimethylamine benzoate, *p*-(N,N-dimethyl-amine) benzoic acid,²² which in pure water emit only LE fluorescence at short wavelength. We could attribute this difference to the ability of TCA to form H-bonding with water through the secondary amine group, a fact that would enhance the stability of ICT. In this sense, the ratio of I_{LE}/I_{ICT} increases proportional to the solvent concentration in the aqueous mixtures, showing the greatest effect in aqueous dioxane mixtures (Fig. 6a), i.e. in the less polar and aprotic solvent (see Table 2 for solvent properties), in where, the ICT state is not stabilized and TCA emits nearly LE fluorescence.

In aqueous CD-solutions, the plot of I_{LE}/I_{ICT} against [CD] increases also with [CD], but drawing saturation curves that level off at high CD; in addition, the I_{LE}/I_{ICT} vs. [CD] profiles show similar shape for α -, β -, or γ -cyclodextrin; Fig. 6b shows representative data for the case of β -CD. Again, this behaviour is contrary to that observed with e.g. DEABA in which case, firstly, the ICT emission intensity is higher than the LE emission (i.e. I_{ICT}/I_{LE} increases with [β -CD]), and secondly, the observed behaviour in β -CD is opposite to that seen with α -CD.^{22, 23}

Our results indicate that, the CD-cavity provides a less polar and aprotic environment that favours LE emission over the ICT state, because of the destabilization of the latter due to the absence of H-bonding interactions and the constrained microenvironment that difficult the necessary rotation to

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3 achieve the optimal orthogonal position between donor and acceptor groups. The saturation value
4 obtained in I_{LE}/I_{ICT} vs. [CD] plots depends on the nature of TCA molecule forming complexes, *i.e.*
5 singly positively charged on the tertiary amine N-atom or. Geometrical optimization of TCA structure
6 with AM1 method³¹ yields the form and dimensions of TCA displayed in Scheme 3 and compared to
7 that of cyclodextrin.
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11 12 13 Scheme 3

14 15 Conclusions

16 Tetracaine shows dual fluorescence in either water solvent mixtures or aqueous cyclodextrin
17 solutions. The ratio of normal emission intensity to the ICT emission increases with CD concentration
18 and level off at high concentrations, whereas in water–solvent mixtures straight lines were observed.
19 The behaviour is different to the most studied fluorophores of type dimethylaminebenzotrile. On the
20 other hand, the stability of inclusion complex formed between tetracaine and cyclodextrins depends on
21 the pH of the medium and on the cyclodextrin nature; the most stable complex was observed with β -CD
22 in mild basic medium of pH~10. While, with α - and β -CD the results suggest the existence of two
23 different inclusion complexes, with γ -CD one conformation for the inclusion complex suffices to
24 explain the experimental observations.
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CAPTIONS of SCHEMES and FIGURES

Scheme 1. Acid-base ionization equilibrium of tetracaine in water

Scheme 2. Postulated equilibrium steps in TCA/CD system in strong acid medium

Scheme 3. Sizes of minimized energy structure of TCA compare to CDs

Figure 1. Emission spectra of TCA 10.4 μM in aqueous solutions of [1] hydrochloric acid 0.033 M; [2]acetic acid-acetate buffer 0.066 M pH 4.78; [3]carbonate-bicarbonate buffer 0.033 M pH 10.2, and $[\text{OH}^-]=0.048$ M; $\lambda_{\text{ex}}=315$ nm.

Figure 2. Plot of fluorescence intensities read at (a) 357 nm and at (b)377 nm as a function of the solvent concentration in the water-solvent mixtures; symbols correspond to (●)dioxane, (▲)dimethylsulphoxide, DMSO; (▼)acetonitrile, AN, and (◆)methanol, MeOH.

Figure 3. Emission spectra of TCA 10.4 μM in (a)aqueous- β -cyclodextrin (1.29 mM) solutions of [1] hydrochloric acid 0.033 M; [2]acetic acid-acetate buffer 0.066 M pH 4.78; [3]alkaline medium of $[\text{OH}^-]=0.048$ M, and [4]carbonate-bicarbonate buffer 0.033 M pH 10.2, and (b)in aqueous sodium acetate 0.067M as a function of β -cyclodextrin concentration equal to [1] 0; [2]0.026, [3]0.052; [4]0.10, [5]0.16 mM. $\lambda_{\text{ex}}=315$ nm.

Figure 4. Variation of the fluorescence emission intensities of 10.4 μM tetracaine dissolved in (a) (○,●)aqueous HCl 0.033M, and in (▼,▽)aqueous 0.067 M acetic acid-acetate (1:1) and (b) (▲,△)aqueous 0.048 M of NaOH, and in (○,●)aqueous 0.033 M carbonate-bicarbonate (1:1) buffer, as a function of β -cyclodextrin concentration: (open) symbols correspond to 377 nm or ICT emission state, (solid) symbols correspond to 357 nm or LE emission state.

Figure 5. Variation of the fluorescence emission intensities of 10.4 μM TCA dissolved in water (a) (○,●)at 0.033 M carbonate-bicarbonate (1:1) buffer and (▼,▽)at 0.067 M acetic acid-acetate (1:1) as a function of α -CD concentration; the inset shows the indicated expanded plot, and (b) (○,●)at 0.033 M carbonate-bicarbonate (1:1) buffer, as a function of γ -CD concentration; (open) symbols correspond to 377 nm or ICT emission state, (solid) symbols correspond to 357 nm or LE emission state.

Figure 6. Plot of the ratio $I_{\text{LE}}/I_{\text{ICT}}$ as a function of (a)solvent concentration in the aqueous mixture (●)dioxane, (▼)acetonitrile, (▲)methanol; (b) β -CD concentration in (▲)strong acid, HCl; (○)mild acid, acetic acid-acetate; (▽)mild base, carbonate-bicarbonate, and (◆)alkaline medium.

TABLES

Table 1. Solvent properties, values of the ratio of fluorescence emission intensities of LE to ICT states, and UV-vis absorption data.

solvent	ϵ_r ^(a)	μ/D ^(b)	I_{LE} / I_{ICT} ^(c)	λ_{max} (log ϵ) ^(d)
Dioxane	2.2	0.45	2.28	224(4.0); 310(4.5)
DMSO	46.45	4.05	1.77	Cut off; 313(4.5)
Acetonitrile	35.94	3.54	1.60	225(3.9); 308(4.4)
MeOH	32.66	1.71	1.20	223(4.0); 309(4.45)
H ₂ O	78.50	1.78	0.92	226(3.9); 309(4.3)

^(a) Relative solvent dielectric constant; ^(b) dipole moment in Debyes (1D= 3.34×10⁻³⁰ C·m); ^(c) ratio of fluorescence intensities read at 357 (LE) and 377 nm (ICT) in 80% v/v solvent-water solutions; ^(d) maximum wavelength absorption (log extinction coefficient in M⁻¹cm⁻¹).

Table 2. Concentration and mixed dissociation constants of tetracaine determined by potentiometry and spectrophotometric titrations at 25 °C

Method	Potentiometry	Spectrophotometry
pK ₁ ^H	2.241±0.005 ^(a)	2.27±0.02 ^{(b) ref. 26}
pK ₂ ^H	8.390±0.001 ^(a)	8.24±0.28 ^{ref. 27}

^(a) This work, I=0.1 M, NaCl; ^(b) I=0.12 M, buffers, ref. 26

Table 3.-Experimental conditions and optimized parameters obtained in the fitting process of eqs. (5), or (6) to the experimental data, which result in the study of the influence of β -cyclodextrin concentration on the steady-state fluorescence emission of tetracaine.

Medium	[TCA]/ μ M	$\lambda = 357$ nm, LE emission state			$\lambda = 377$ nm, ICT emission state		
		I_F^w	I_F^c	K_i^c/M^{-1}	I_F^w	I_F^c	K_i^c/M^{-1}
HCl 0.035 M	10.4	0.706 ^(a)	4.08 \pm 0.06	1174 \pm 16 ^(a)	0.708 ^(a)	2.80 \pm 0.05	1178 \pm 50 ^(a)
HAc/Ac ⁻ (1:1) 0.048 M ^(b)	3.6	0.258	2.09 \pm 0.02	922 \pm 45 ^(c)	0.212	1.37 \pm 0.01	924 \pm 47 ^(c)
HAc/Ac ⁻ (1:1) 0.167 M ^(b)	3.6	0.255	2.04 \pm 0.02	991 \pm 50 ^(c)	0.225	1.34 \pm 0.02	989 \pm 50 ^(c)
HAc/Ac ⁻ (1:1) 0.067 M ^(b)	10.4	0.451	4.50 \pm 0.02	1090 \pm 20 ^(c)	0.490	3.03 \pm 0.01	1113 \pm 18 ^(c)
NaAc 0.067 M ^(b)	10.4	0.563	5.26 \pm 0.04	1412 \pm 40 ^(c)	0.535	3.40 \pm 0.03	1380 \pm 43 ^(c)
HCO ₃ ⁻ /CO ₃ ²⁻ (1:1) 0.025 M ^(d)	3.6	0.359	3.86 \pm 0.03	3344 \pm 120 ^(e)	0.283	2.10 \pm 0.02	3418 \pm 160 ^(e)
HCO ₃ ⁻ /CO ₃ ²⁻ _(d) (1:1) 0.040 M	3.6	0.322	3.71 \pm 0.04	3354 \pm 200 ^(e)	0.249	2.12 \pm 0.02	3395 \pm 216 ^(e)
HCO ₃ ⁻ /CO ₃ ²⁻ _(d) (1:1) 0.033 M	10.4	0.656	9.02 \pm 0.07	3464 \pm 145 ^(e)	0.63	4.96 \pm 0.03	3530 \pm 130 ^(e)
NaOH 0.048 M	10.4	0.728	9.5 \pm 0.2	940 \pm 60 ^(f)	0.613	5.05 \pm 0.09	1010 \pm 70 ^(f)

^(a)eq. 5 value of $f(H^+)=0.17$; ^(b)buffer solutions of acetic acid (HAc) acetate (Ac⁻); ^(c)eq. 6, K_2^c ; ^(d)buffer solutions of carbonate-bicarbonate, pH 10.2; ^(e)eq. 6, value of K_3^c ; ^(f)eq. 6, value of K_4^c

Table 4.- Experimental conditions and parameters obtained in the study of the influence of cyclodextrins on the fluorescence emission of 10.4 μ M tetracaine aqueous solutions.

Medium	cyclodextrin	$\lambda = 357$ nm, LE emission state			$\lambda = 377$ nm, ICT emission state		
		I_F^w	I_F^c	K_3^c/M^{-1}	I_F^w	I_F^c	K_3^c/M^{-1}
HAc/Ac ⁻ (1:1) 0.067 M ^(a)	α -CD	0.451	4.80 \pm 0.20	46 \pm 4 ^(b)	0.495	2.7 \pm 0.1	49 \pm 6 ^(b)
HCO ₃ ⁻ /CO ₃ ²⁻ buffer (1:1) 0.033 M	α -CD	0.65	15.35 \pm 0.22	125 \pm 4	0.61	6.5 \pm 0.1	141.5 \pm 5.0
HCO ₃ ⁻ /CO ₃ ²⁻ buffer (1:1) 0.033 M	β -CD	0.656	9.02 \pm 0.07	3464 \pm 145	0.63	4.96 \pm 0.03	3530 \pm 130
HCO ₃ ⁻ /CO ₃ ²⁻ buffer (1:1) 0.033 M	γ -CD	0.65	4.7 \pm 0.1	65 \pm 4	0.62	2.60 \pm 0.04	76 \pm 5

^(a)buffer solutions of acetic acid-acetate; ^(b) eq. 6, value of K_2^c

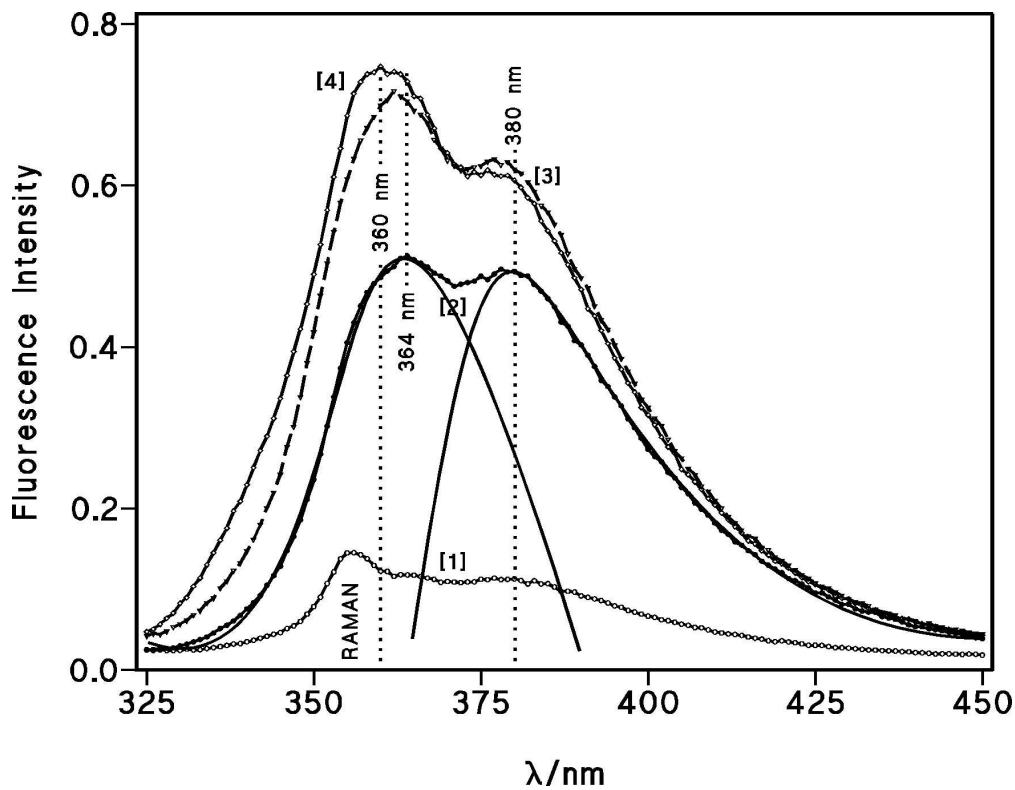


Figure 1. Emission spectra of TCA 10.4 μM in aqueous solutions of [1] hydrochloric acid 0.033 M; [2] acetic acid-acetate buffer 0.066 M pH 4.78; [3] carbonate-bicarbonate buffer 0.033 M pH 10.2, and [4] $[\text{OH}^-] = 0.048 \text{ M}$; $\lambda_{\text{ex}} = 315 \text{ nm}$.
148x114mm (300 x 300 DPI)

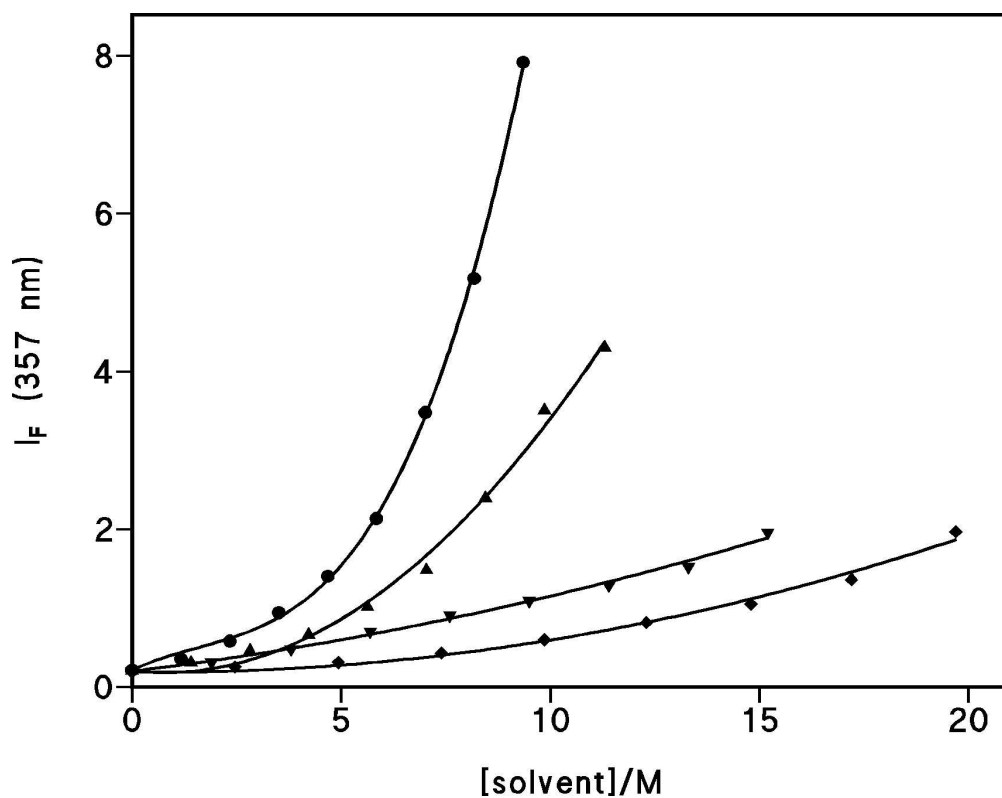


Figure 2. Plot of fluorescence intensities read at (a) 357 nm as a function of the solvent concentration in the water-solvent mixtures; symbols correspond to (●)dioxane, (△)dimethylsulphoxide, DMSO; (▽)acetonitrile, AN, and (◇)methanol, MeOH.
144x113mm (300 x 300 DPI)

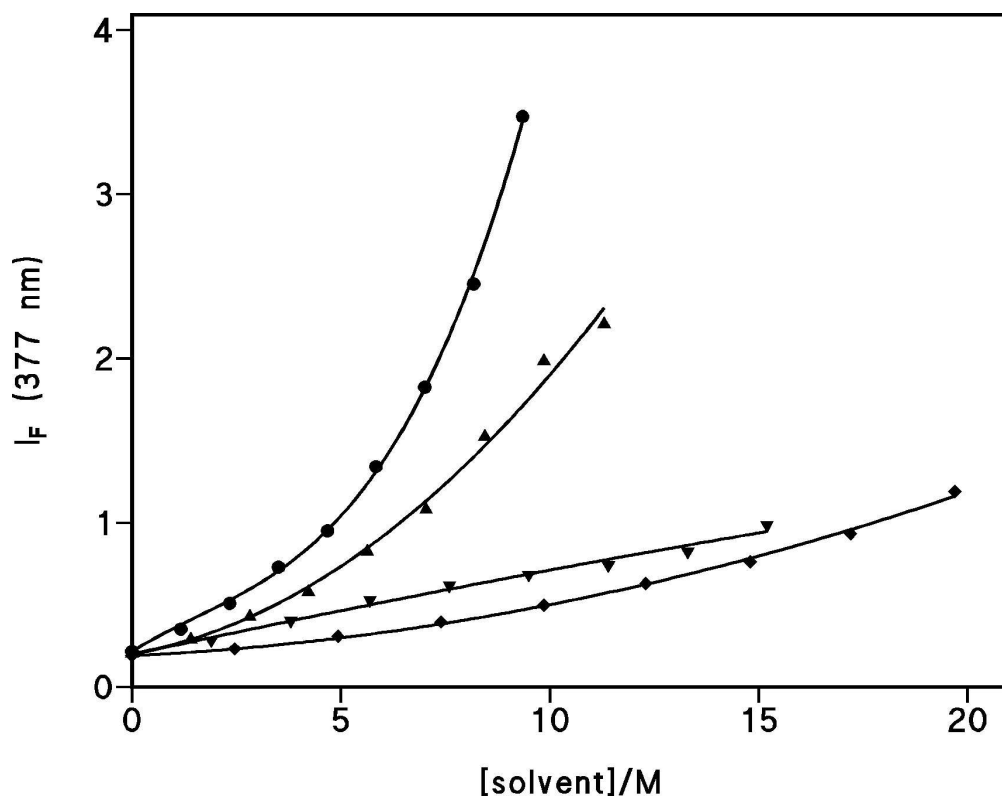


Figure 2. Plot of fluorescence intensities read at (b)377 nm as a function of the solvent concentration in the water-solvent mixtures; symbols correspond to (□)dioxane, (△)dimethylsulphoxide, DMSO; (●)acetonitrile, AN, and (◊)methanol, MeOH.
144x113mm (300 x 300 DPI)

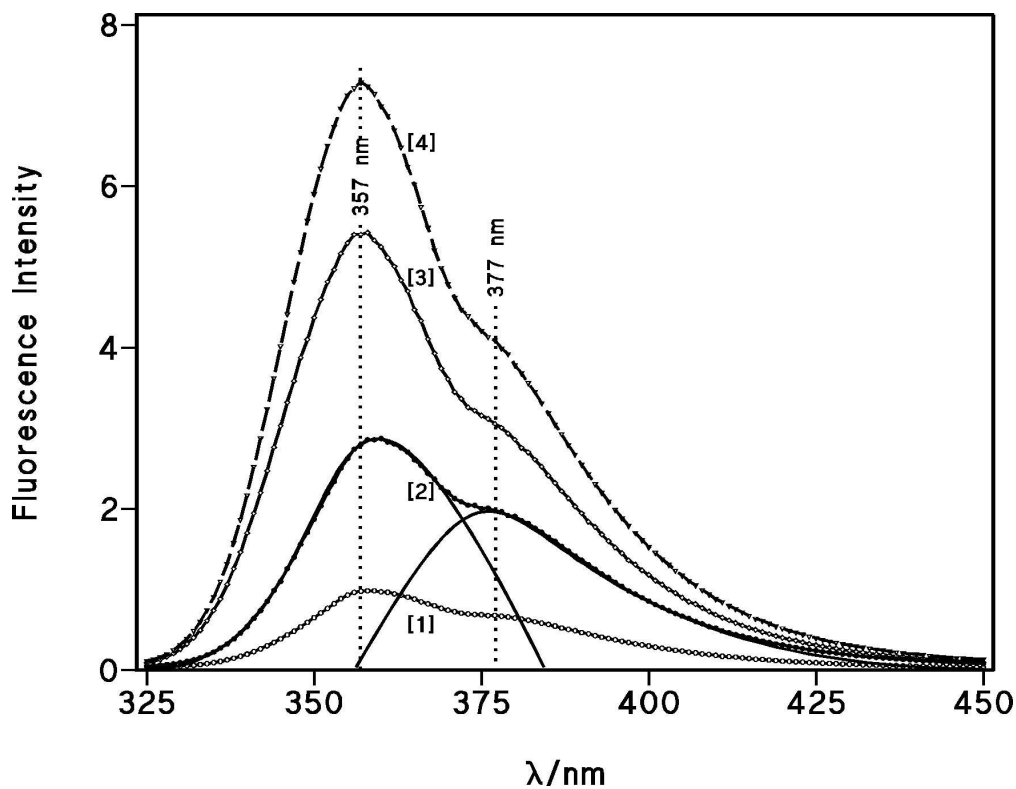


Figure 3. Emission spectra of TCA 10.4 μM in (a) aqueous- β -cyclodextrin (1.29 mM) solutions of [1] hydrochloric acid 0.033 M; [2] acetic acid-acetate buffer 0.066 M pH 4.78; [3] alkaline medium of $[\text{OH}^-]=0.048$ M, and [4] carbonate-bicarbonate buffer 0.033 M pH 10.2. $\lambda_{\text{ex}}=315$ nm. 148x114mm (300 x 300 DPI)

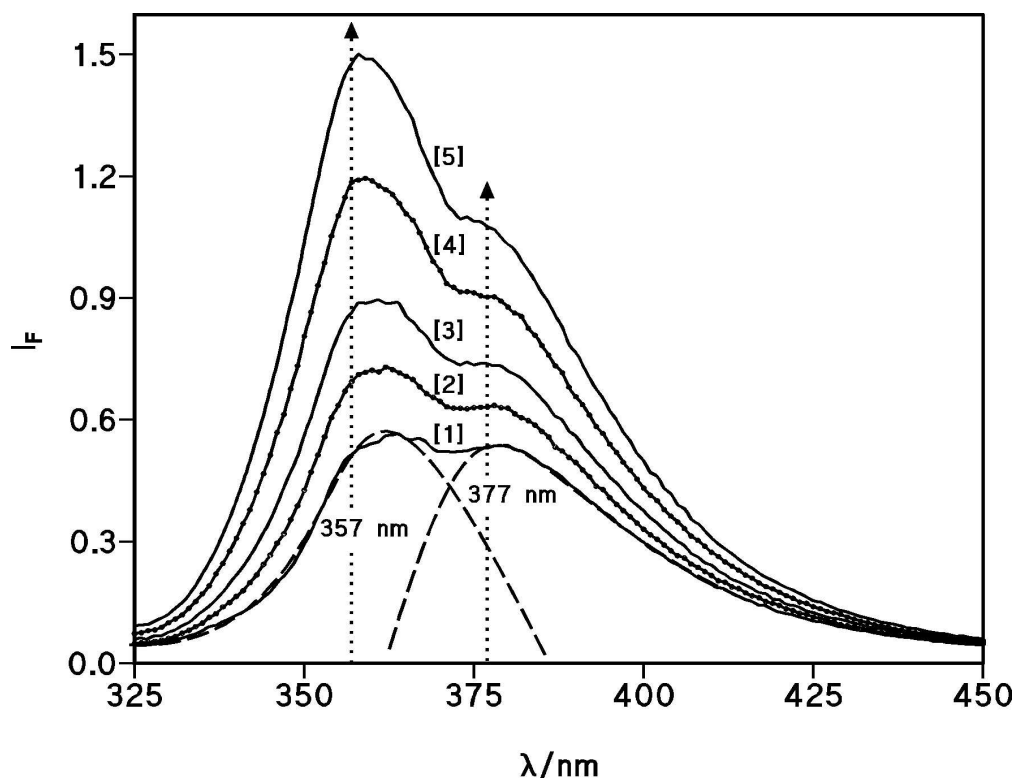


Figure 3. Emission spectra of TCA 10.4 μM in aqueous sodium acetate 0.067M as a function of β -cyclodextrin concentration equal to [1] 0; [2] 0.026, [3] 0.052; [4] 0.10, [5] 0.16 mM. $\lambda_{\text{ex}}=315$ nm.
149x114mm (300 x 300 DPI)

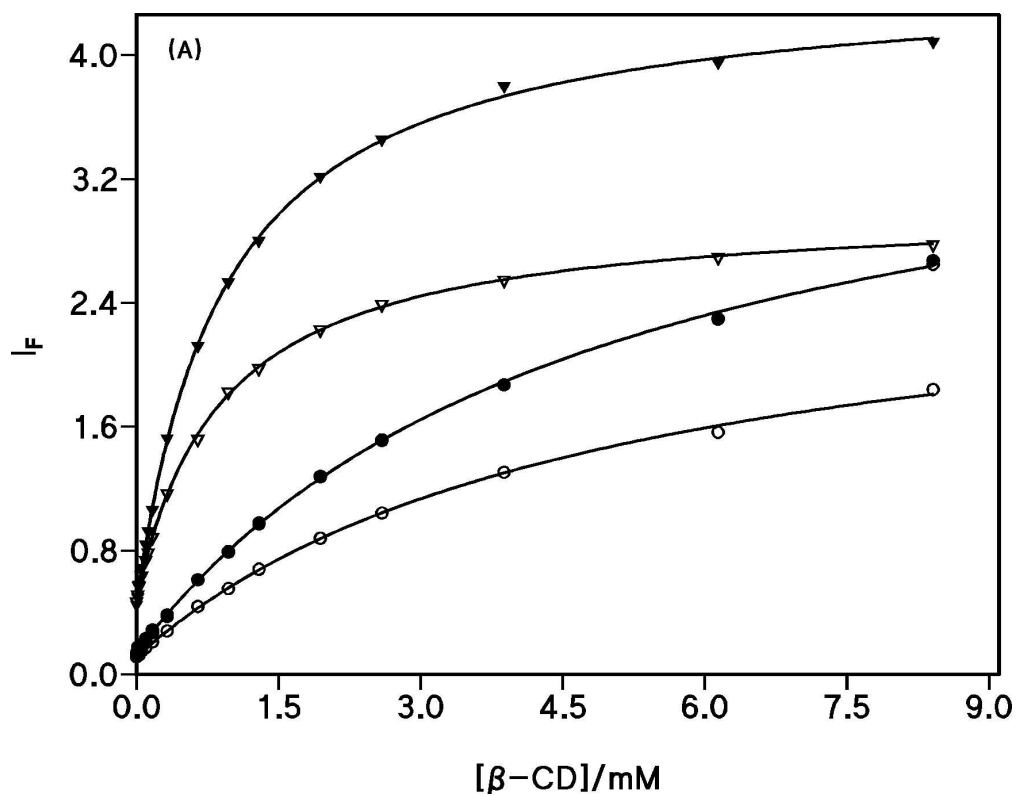
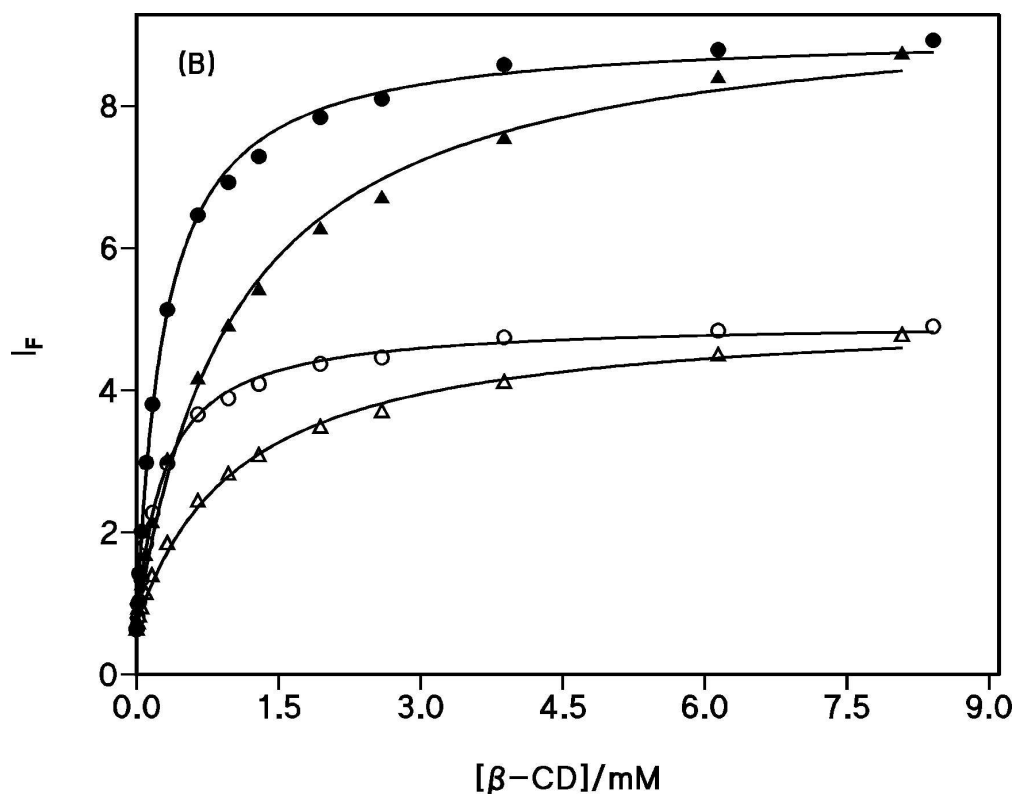


Figure 4. Variation of the fluorescence emission intensities of 10.4 μM tetracaine dissolved in (a) (\square, \square) aqueous HCl 0.033M, and in (θ, σ) aqueous 0.067 M acetic acid-acetate (1:1) 147x114mm (300 x 300 DPI)



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Figure 4. Variation of the fluorescence emission intensities of 10.4 μ M tetracaine dissolved in (b) (π, ρ)aqueous 0.048 M of NaOH, and in (\square, \square)aqueous 0.033 M carbonate-bicarbonate (1:1) buffer, as a function of β -cyclodextrin concentration: (open) symbols correspond to 377 nm or ICT emission state, (solid) symbols correspond to 357 nm or LE emission state.
147x114mm (300 x 300 DPI)

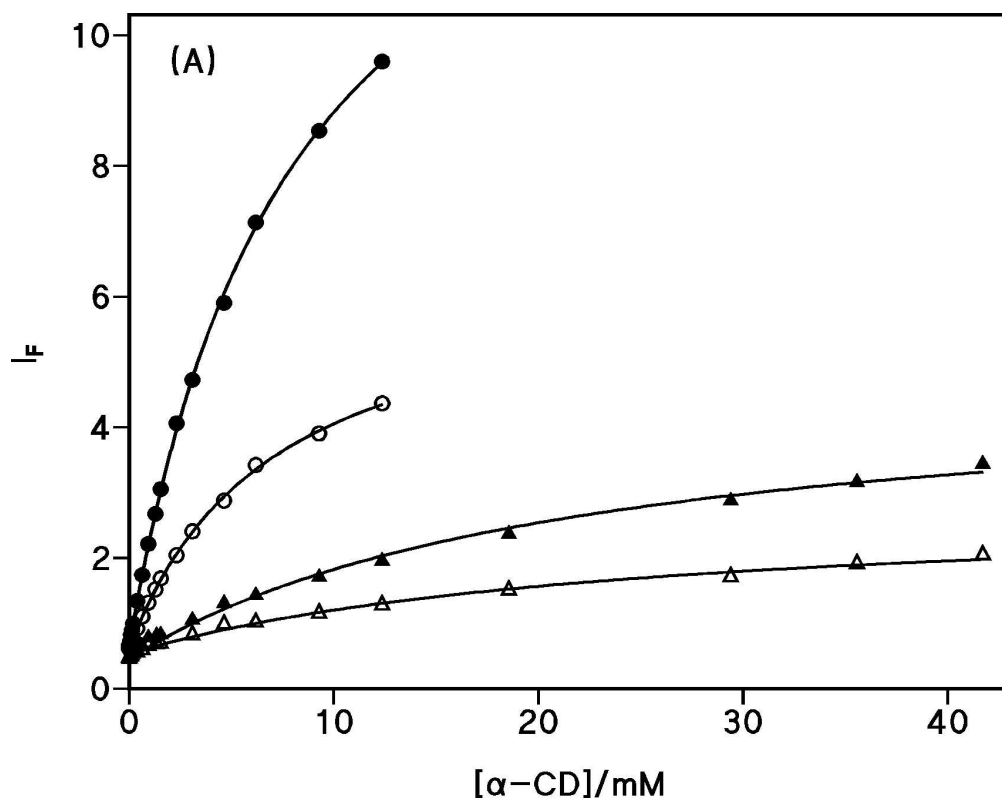
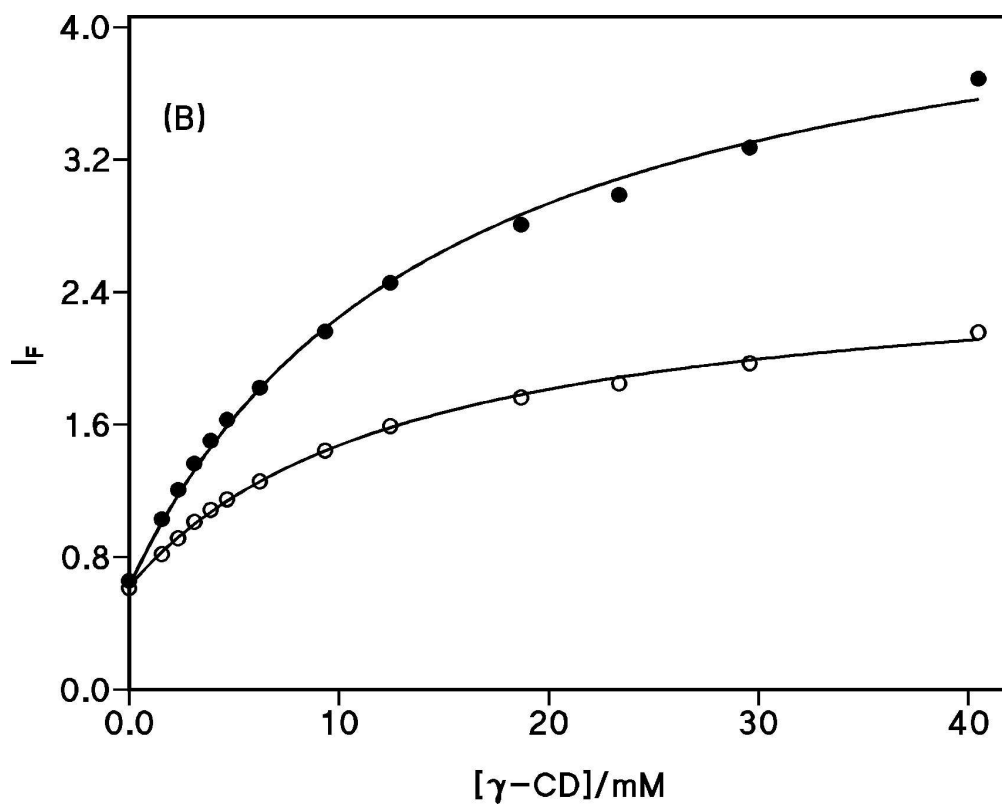


Figure 5. Variation of the fluorescence emission intensities of 10.4 μM TCA dissolved in water (a) (\square, \square) at 0.033 M carbonate-bicarbonate (1:1) buffer and (θ, σ) at 0.067 M acetic acid-acetate (1:1) as a function of α -CD concentration;
144x113mm (300 x 300 DPI)



35 Figure 5. Variation of the fluorescence emission intensities of 10.4 μ M TCA dissolved in water (b)
36 (\square, \square) at 0.033 M carbonate-bicarbonate (1:1) buffer, as a function of γ -CD concentration; (open)
37 symbols correspond to 377 nm or ICT emission state, (solid) symbols correspond to 357 nm or LE
38 emission state.

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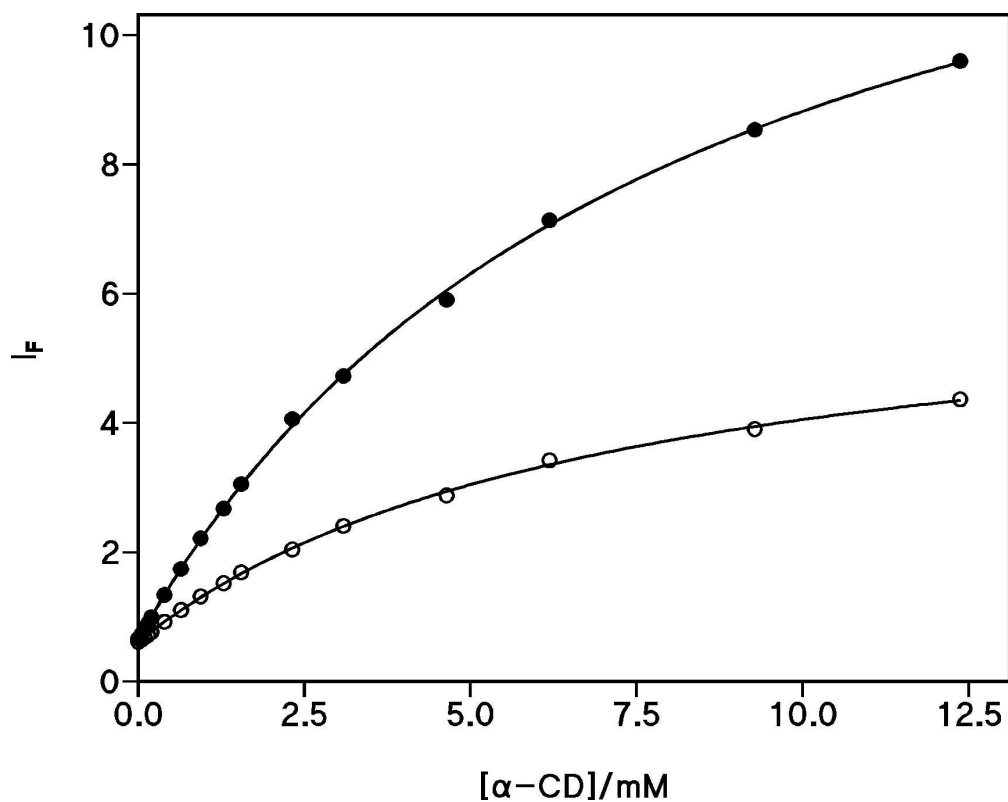


Figure 5. Variation of the fluorescence emission intensities of 10.4 μ M TCA dissolved in water (a) (\square, \square) at 0.033 M carbonate-bicarbonate (1:1) buffer and (θ, σ) at 0.067 M acetic acid-acetate (1:1) as a function of α -CD concentration; the inset shows the indicated expanded plot, 145x114mm (300 x 300 DPI)

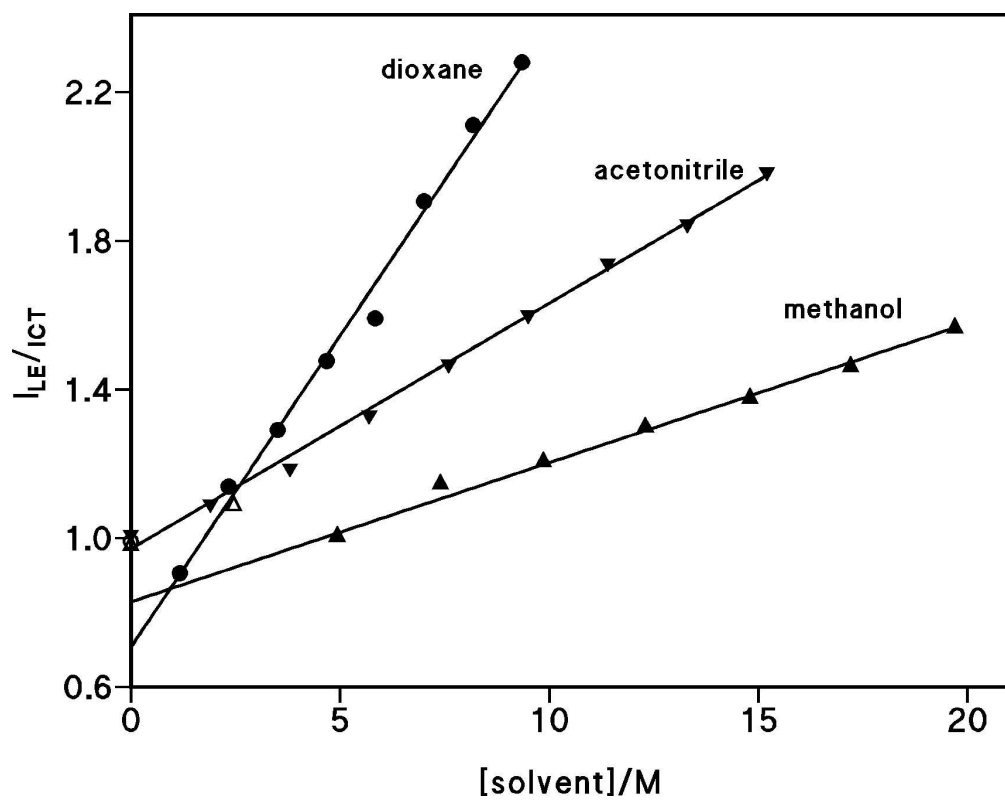


Figure 6. Plot of the ratio I_{LE}/I_{ICT} as a function of (a) solvent concentration in the aqueous mixture
(□) dioxane, (θ) acetonitrile, (π) methanol
144x113mm (300 x 300 DPI)

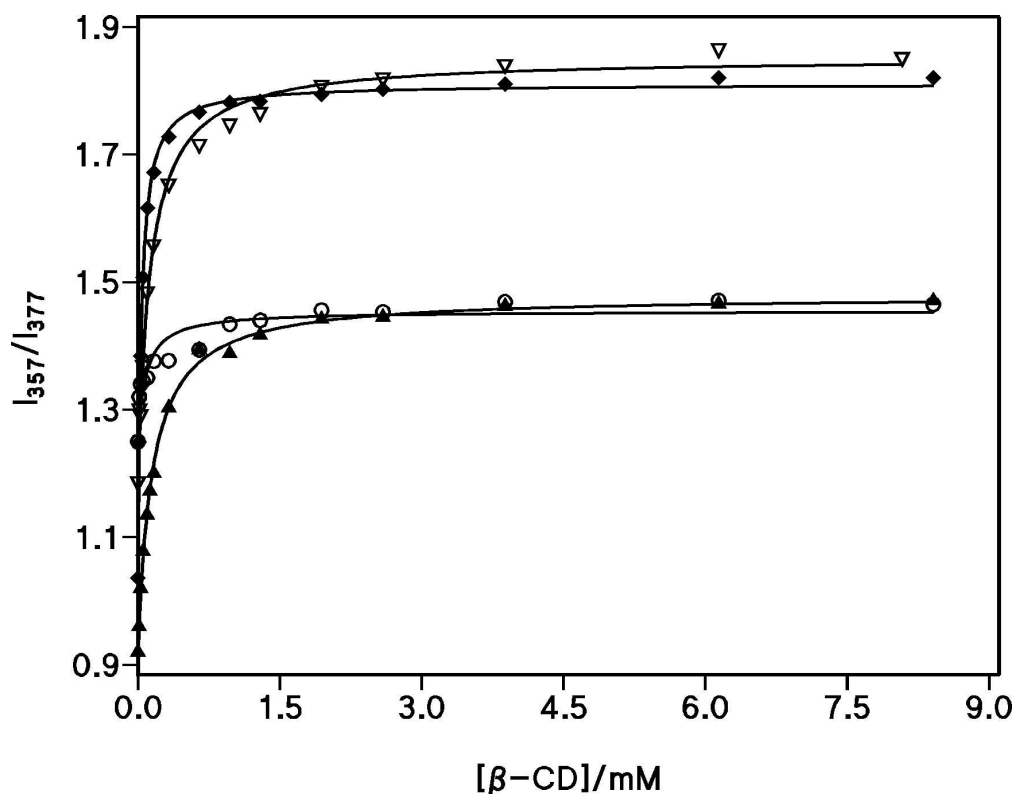


Figure 6. Plot of the ratio ILE/I₃₇₇ as a function of (b) $\beta\text{-CD}$ concentration in (π) strong acid, HCl; (\square) mild acid, acetic acid-acetate; (σ) mild base, carbonate-bicarbonate, and (\blacktriangledown) alkaline medium.
147x114mm (300 x 300 DPI)