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Spectrofluorimetric study of estrogen-cyclodextrin inclusion complexes in aqueous systems

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For the first time, the spectrofluorimetric properties of estrone (E1), 17β-estradiol (E2), estriol (E3), and 17α-ethinylestradiol (EE2) are studied in aqueous solutions after the addition of native and derivative cyclodextrins. In contrast to previous reports, the behavior of the systems is analysed in the absence of organic solvents able to modify the guest-host interaction. The significant differences between the obtained association constants with those reported in solvent mixtures are shown and discussed. In order to evaluate the influence of both the estrogen structure and the presence of cyclodextrin substituents in the inclusion phenomena, fluorescent and acid-base behaviors of the systems are compared and discussed. The interaction of estrogens with micellar media formed by selected surfactants is also studied. It is demonstrated that estrogen-cyclodextrin complexes in aqueous solution are useful for improving fluorimetric detection limits and, since cyclodextrins are non-toxic and mitigate most of the solubility problems which require the use of organic solvents, the studied complexes are excellent candidates for extraction, separation, pre-concentration and removal processes maintaining the principles of the green analytical chemistry.

Introduction

Estrogens are a type of steroid hormones which play a widespread role in human physiology, including reproductive female functions, modulation of growth of different tissues, bone integrity, influence on cardiovascular apparatus, immune and nervous systems, and regulation of male physiology.[1] As adverse effects, these hormones are also implicated in the development or progression of various types of cancer, osteoporosis, neurodegenerative and cardiovascular diseases, insulin resistance, lupus erythematosus, endometriosis, and obesity.[1]

The three major naturally occurring estrogens are 17β-estradiol (E2), estriol (E3), estrone (E1), being E2 the most potent and dominant in humans (Fig. 1). On the other hand, 17α-ethynylestradiol (EE2, Fig. 1) is a synthetic estrogen derivative of E2 used in almost all modern formulations of combined oral contraceptive pills.

The chemical structure of estrogens, like all steroid hormones, is characterized by a cyclopentan-*o*-perhydrophenanthrene ring which is composed of three six member rings and one five member ring, labeled A, B, C, and D respectively (Fig. 1). Estrogens are of low polarity and, thus, they are capable to interact, in case of geometric compatibility, with the hydrophobic cavity of some cyclodextrins (CDs) to form inclusion complexes.[2] Estrogen-CD complexes have been used in multiple chemical areas with different purposes. CDs have been included in pharmaceutical formulations as solubilizer and absorption enhancer of estrogens,[3] and in chromatographic and electrophoretic methods for their separation and determination.[4–9] Solid-phase extractions based on complex formation with CDs have been applied for removing these endocrine disrupting compounds from environmental samples,[10] and for sample purification in mass spectrometric analysis.[11] CDs

were also used for suppressing the harmful effect of estrogens in environmental water through complex formation.[12]

So far, the association constants for estrogen-CD complexes have been measured, through different methods, in mixtures of water and miscible solvents.[9,13–17] However, the presence of an organic solvent (*e.g.* alcohols) could alter the equilibrium constants between CDs and guest molecules.[18] It is known that at low concentrations (*e.g.* 0.05–2 mol L^{-1}), alcohols can either form a ternary complex (analyte-CD-alcohol) or act as a competing guest compound, since alcohols themselves are able to interact with the CD cavity.[19–22] At higher concentrations (> 7 mol L^{-1}), dissociation of the binary analyte-CD complex can occur by changes in bulk solvent hydrophobicity (non-specific solvent effects).[23]

The objective of this work was to study, for the first time, the influence of organized media in the fluorescence properties of estrogenic compounds in the absence of additional organic molecules able to interact with the analysed systems. Fluorescence spectroscopy, applied in the present experiments, possesses high sensitivity and allows to work with aqueous diluted estrogen solutions, without any addition of organic solvent.

By comparing the behavior of native CDs, we were interested in determining the relationship between estrogen structure and cavity size. The rationale for employing derivative CDs was to determine the effect of substituents at the CD entrances on estrogen complexation. Notable differences between the results here obtained with those reported in water/co-solvent mixtures were found and discussed.

Influence on the estrogen fluorescence properties of other factors such as the presence of micelles formed by surfactants, temperature and pH were also evaluated.

Experimental

Reagents and solutions

All reagents were of high-purity grade and used as received. Estrone, 17β-estradiol, estriol, 17α-ethynylestradiol, methyl-β-cyclodextrin (M-β-CD), sulfate-β-cyclodextrin sodium salt (S-β-CD), Brij 35, Tween 80, heptakis(2,6-di-*o*-methyl)-β-cyclodextrin (DM-β-CD), and hexadecyltrimethylammonium bromide (HTAB) were purchased from Sigma-Aldrich (Milwaukee, WI, USA). Hexadecyltrimethylammonium chloride (HTAC), and (2-hydroxyethyl)-β-cyclodextrin (HE-β-CD) were provided by Fluka (Buchs, Switzerland). Sodium dodecylsulfate (SDS), and methanol were obtained from Merck (Darmstadt, Germany). α-, β- and γ-cyclodextrins (α-CD, β-CD and γ-CD), and (2-hydroxypropyl)-β-cyclodextrin (HP-β-CD) were acquired from Cyclolab (Budapest, Hungary).

Methanol stock solutions of EE2, E2, E3 and E1 of about 2500 μg mL^{-1} were prepared and stored in dark flasks at 4 ^{o}C. From these solutions, more diluted methanol solutions (ranging from 10 to 500 μg mL^{-1}) were obtained. Working aqueous solutions were prepared immediately before their use by taking appropriate aliquots of methanol solutions, evaporating the organic solvent by use of dry nitrogen and diluting with ultrapurified water from a Millipore system (Massachusetts, USA) to the desired concentrations. Stock solutions of CDs and surfactants were prepared in ultrapurified water.

Apparatus

Fluorescence measurements were done on an Aminco Bowman (Rochester, NY, USA) Series 2 luminescence spectrometer equipped with a 150W xenon lamp and using 1.00 cm quartz cell and slit widths of 4 nm. The excitation and emission wavelengths were 270 and 310 nm, respectively. The temperature of the cell holder was regulated using a Lauda

(Frankfurt, Germany) RM6T thermostatic bath. The pH of solutions was measured with a Metrohm (Herisau, Switzerland) 713 pHmeter equipped with glass and Ag/AgCl reference electrodes.

Influence of cyclodextrin and surfactant concentrations

The effect of the concentration of CDs and surfactants in the fluorescence spectra of the estrogens was studied through the following procedure:[24] 2.00 mL of solutions in water of each analyte (C_{EE2} = C_{E2} = C_{E3} = 420 ng mL^{-1}, C_{E1} = 1300 ng mL^{-1}) were spiked with increasing volumes of each CD or surfactant solutions containing the same concentration of the corresponding estrogen, in order to avoid analyte dilution. The E1 concentration was about three times larger than those of the remaining estrogens, because its fluorescence signal is not detected at lower concentrations. The complete dissolution of E1 at this concentration was corroborated by UV-visible absorption measurements. After each addition, the fluorescence spectrum was read. This procedure could be applied even with β-CD, the less soluble CD, because the concentrations of the CD titrating solutions were about $6 – 8\times10^{-3}$ mol L^{-1}, below the β-CD aqueous solubility (0.01 mol L^{-1}). It is necessary to remark that inclusion complexes are rapidly formed, as was verified by the constancy of the obtained fluorescence signal after each addition of CD solution.

With the purpose of obtaining the blank signals, a similar procedure was also performed in the absence of analyte. Finally, blank signals were subtracted from the corresponding spectra and the plot of the corrected fluorescence intensity at a maximum as a function of either CD or surfactant concentration was obtained.

Influence of the pH

The changes in the fluorescence intensity of EE2, E2, E3 and E1, and their β- and DM-β-CD complexes as a function of pH were studied by the following procedure: to 50 mL stirred hydrochloric acid solution (C_{HCl} *ca.* 0.01 mol L^{-1}) of the estrogens or their CD complexes, NaOH solution (0.05–1 mol L^{-1}) was added in small increments (0.01–0.05 mL). For each pH point a known aliquot of solution was extracted and the fluorescence spectrum was read. The initial concentrations in the reaction vessel of EE2, E2 and E3 were 420 ng mL^{-1}, and the initial concentration of E1 was 1300 ng mL^{-1}. In the experiments with CDs, their concentrations were kept at 5×10^{-4} mol L^{-1}. The temperature was maintained at 20°C. The measurements were performed in duplicate. The profiles of fluorescence at the wavelength of the emission maxima *vs.* pH were used to calculate the deprotonation constant values of the estrogens in the excited state, both in the presence and in the absence of the selected CDs. These calculations were performed with the aid of the PKFIT program,[25] which is based on a least-squares procedure and can be obtained from the authors on request.

Results and discussion

Fluorescence spectra

Fig. 2A shows the excitation fluorescence spectra for the studied estrogens, with maxima at about 220 and 278 nm, and the emission spectra with maxima at about 310 nm. Although E2, E3 and EE2 are relatively more fluorescent than E1, all signals have low intensity. Indeed, Raman bands from the solvent, which appear near those of the analytes signals, are clearly visualized. This fact represents a problem when selecting excitation and emission

wavelengths for analytical purposes. In fact, in order to avoid the Raman band, 270 nm was selected as excitation wavelength in the present work. The signal intensities of compounds with low native fluorescence could be increased, for example, through the use of organized media. The addition of selected CDs and micelles to estrogens water solutions results in an increase in fluorescence intensity of different magnitude depending on the evaluated system (Figs. 2B-D).

Cyclodextrins effect in aqueous solution

These experiments were conducted at neutral pH, where the fluorescence intensity of each system is the highest and estrogens remain in their neutral structures (see below), which are optimal for inclusion complex formation with CDs.[2]

The investigated CDs involved the three major cyclodextrins: α-, β-, and γ-CDs (constituted by six, seven, and eight glucose units, respectively), and the β-CD derivatives S-β-, HE-β-, HP-β-, M-β- and DM-β-CDs (Fig. 1).

Fig. 3 displays the intensities of the fluorescence emissions of each studied estrogen at different concentrations of selected CDs. Changes in fluorescence with the CD concentration can be attributed to the formation of inclusion complexes between the analytes and CD. Assuming 1:1 complex stoichiometries, equal extinction coefficients of the free and complexed substrate (the absorption spectrum of estrogens was not significantly modified by CDs) and free CD concentration approximately equal to its analytical concentration (CD is in large excess with respect to the guest), the following equations can be written:

$$F = b(\text{Ø}_E[E] + \text{Ø}_C[C]) \quad (1)$$

$$F_0 = b\,Ø_E C_E \quad (2)$$

where F and F_0 are the observed fluorescence intensity in the presence and absence of CD, b is a constant depending on the experimental conditions, $Ø_E$ and $Ø_C$ are the fluorescence quantum yields of the free and complexed estrogens, respectively, and [E] and [C] are the free concentrations of estrogen and complex, respectively. From these equations, the following expression is obtained:[26,27]

$$\frac{F}{F_0} = \frac{(1 + (Ø_C / Ø_E)KC_{CD})}{(1 + KC_{CD})} \quad (3)$$

where K is the corresponding association constant. The K values obtained by a non-linear lest-square procedure from Eq. 3 are reported in Table 1. From the same calculation, fluorescence quantum yield ratios ($Ø_C$/ $Ø_E$) are also derived (Table 2). The good fit of each plot (Fig. 3) supports the existence of complexes with 1:1 stoichiometric ratio. The same stoichiometry was previously reported for estrogen-CD complexes in water/co-solvent samples.[13–17]

For comparison, association constants were also estimated through a relation analogous to the Benesi-Hildebrand equation:[16]

$$\frac{F_0}{\Delta F} = \frac{Ø_E}{(\varepsilon_C / \varepsilon_E)Ø_C - Ø_E} + \frac{Ø_E}{[(\varepsilon_C / \varepsilon_E)Ø_C - Ø_E]} \frac{1}{C_{CD}K} \quad (4)$$

where ΔF is the difference between the fluorescence intensity in the presence and in the absence of CD, and ε_C and ε_E are the molar absorptivities with and without CD. The good fit of this linear regression (Fig. 4 shows selected examples) does also suggest the existence of 1:1 complexes. Table 1 summarizes the calculated association constants applying both non-linear and linear regressions. As can be appreciated, both procedures are in very good agreement. Further, a rapid inspection of Table 1 allows concluding that the interaction

between the studied estrogens and some CDs in aqueous medium is very strong. This fact could be explained on the basis of the high hydrophobicity of estrogens, which also justifies the differences found with the values reported in the presence of variable percentages of organic solvents (see below).

Among the three native CDs, α-CD does not appreciably modify the fluorescence properties of any of the studied estrogens, a slight increase of fluorescence is verified in the presence of γ-CD in most systems, and β-CD appears to have the best cavity for inclusion complex formation. These results suggest that the cavity size of β-CD (which is larger than α-CD and smaller than γ-CD) produces a tighter complex structure, efficient for protection towards non-radiative decay processes occurring in the bulk solution.

It can be noted in Table 1 that the complex formed by γ-CD and E1 could not be detected using the present methodology, possibly because the changes in fluorescence intensity with the addition of this CD are not sensitive enough to detect it and, therefore, the related association constant could not calculated.

In addition to the cavity size, the presence of substituents in the CD molecule can contribute to the complex formation and, therefore, different functionalised β-CDs were investigated. Among the β-CD derivatives, S-β-CD does not significantly modify the fluorescence intensity of the estrogen systems. Apparently, the polarity of this host molecule would prevent the association with guests of low polarity such as the analised estrogens. Both 2-HE and 2-HP-β-CDs display a positive effect toward the complex formation and *o*-methyl substituents, such as those present in M- and DM-β-CDs, showed to be the most efficient. The analysis of molecular models of these complexes suggests that oxygen atoms in the substituent chains of 2-HE, 2-HP, M-, DM-β-CDs are approximately at the same position. Therefore, the difference in the stability of the corresponding

inclusion complexes could be explained on the basis of a possible inductive effect of methyl groups in the *o*-methyl derivatives on the oxygen lone electron pairs, with the concomitant formation of stronger hydrogen bonds with the guest molecule.

O-methyl β-CD derivatives, with relative quantum yields around 2 (Table 2), produce the best fluorescence enhancement and, consequently, would be the most adequate for quantitative analysis.

Comparing the relative stability of the formed complexes, the following order e justified focusing on the hydrophobicity of the guest molecule since, in general, the more hydrophobic the analyte, the more stable is its CD complex in aqueous solution.[28] However, the fact that E3 (with the lowest octanol-water partition coefficient, Table 3)[29] forms complexes nearly as stable as those of E2, and more stable than those of E1, cannot be explained on the basis of its hydrophobicity. Certainly, in addition to the van der Walls and hydrophobic forces which favor the inclusion in the CD cavity of the virtually planar cyclopentan-*o*-perhydrophenanthrene nucleus (Fig. 1), the two OH groups in the D-ring of E3 are able to form hydrogen bonds with the host molecule and, therefore, play a key role in the complex formation process. In accordance with the conclusion of Yañez et al,[17] the differences among the association constant values suggest that inclusion may take place through the insertion of the D-ring of these estrogens into the CD cavities.

On the other hand, the structural feature of the A-ring of the steroid molecule has also a great influence on the stability of the inclusion complexes. Moon et al., in their work about solid-phase extraction of steroids with an entrapped-β-CD polymer, demonstrated that hydroxylated estrogens were more effectively captured by the β-CD polymer than other tested steroids, possibly due to the hydrogen bonding between the estrogen phenolic hydroxyl and the external hydroxyl groups of β-CD.[11] In our case, all evaluated estrogens

have the same A-ring structure. Hence, the higher stability of the complexes formed by some derivative β-CDs would be given by the link of the phenolic hydroxyl with the substituent groups, as will be demonstrated through the acid-base properties of the four estrogens in the presence of CDs (see below).

Acid-base behaviour in the absence and in the presence of CDs

Estrogens are very weak acids with p*K*a values in the range 10–10.5 (Table 3).[30–32] Here, the acid-base behaviour of EE2, E2, E3 and E1 in the excited-state was investigated by spectrofluorimetric titrations. Fig. 5A shows the experimental profiles of each estrogen as a function of pH at the emission maximum. In all systems, fluorescence intensity is highest and remains almost constant in the pH range 2–9.5 and decreases at larger pH values. From these profiles, values of deprotonation constants for the phenolic groups in the excited state could be obtained (Table 3). As can be seen, the estimated values are similar to those reported in the ground state. In order to conclude from these results, information for the Förster cycle was analysed assuming equal entropies of protonation in the ground and electronically excited states.[27,33] The relevant equation is:

$$pKa - pKa^{*} = k\ (\bar{\nu}_a - \bar{\nu}_b) \qquad (5)$$

where k is a constant which includes the Avogadro´s number, the Planck´s constant, the speed of light, the universal gas constant, the absolute temperature and the logarithm factor conversion, and $\bar{\nu}$ are the wavenumbers corresponding to the transitions from the acid to the excited acid form ($\bar{\nu}_a$) and from the conjugate base to the excited conjugate base form ($\bar{\nu}_b$). In the studied systems, no differences between $\bar{\nu}_a$ and $\bar{\nu}_b$ were verified, suggesting that the process probed by the fluorescence-pH study does also correspond to the deprotonation in the ground-state.

By comparing the p*K*a values of estrogens in both the presence and absence of CDs, conclusions about the interaction of the phenolic hydroxyl of the A ring with the CD molecule can be inferred. Fluorimetric titrations were performed with both β- and DM-β-CDs (Fig. 5B and 5C), which are the most efficient native and derivative CDs, respectively.

The high constant values of the complexes formed by these CDs provide a high percentage of complexed analyte ensuring that the measured p*K*a values correspond to the complexed analyte rather than to the free analyte. For example, for the E2-β-CD and E2-DM-β-CD systems, using the association constants given in Table 1, and considering that in a typical experiment analytical concentrations of CD and E2 are equal to 5×10^{-4} and 1.5×10^{-6} mol L^{-1}, the percentages of formed complex for β- and DM-β-CDs are 95 and 99 %, respectively. Thus, the measured deprotonation constants involve a very significant contribution from these complexes, and safer conclusions from the acid–base behavior can be elaborated.

The p*K*a values in the presence of β- and DM-β-CDs (Table 3) are slightly larger than those obtained in their absence, and the differences are more marked with the latter CD. Consequently, an interaction between the acidic group of each estrogen molecule and an external hydroxyl group in β-CD and the methyl moiety in DM-β-CD is verified. These results strongly suggest that the contribution of the phenolic hydroxyl of the estrogen on the stability of the inclusion CD complexes is significant. The increase in the p*K*a value when the analyte is included in the cavity of the CD has been observed in similar systems formed by indole, naphthol and phenol derivatives.[27]

CD inclusion complexes in water/co-solvent mixtures

Due to the low water solubility of estrogens (Table 3),[34–36] all previously reported association constants for estrogen-CD complexes correspond to values measured in the presence of variable amounts of organic solvents, such as methanol, ethanol and acetonitrile (Table 4). As indicated above, under fluorimetric conditions the studied compounds were found to be soluble in the analysed range of pH (2-12) and, therefore, in the present work determinations were performed in aqueous solution.

Nevertheless, the association constant of the model E2-β-CD complex was fluorimetrically calculated in different methanol-water mixtures for comparison purposes (Table 4). It should be noticed that, in contrast to Tables 1 and 3, Table 4 shows the uncertainties expressed as standard deviations rather than confidence intervals for better comparison with literature values. The obtained constant values (4.3×10^4, 2.3×10^4, 1.6×10^4 and 4.8×10^3 mol^{-1} L for 0, 5, 10 and 20 % of methanol) decrease with increasing methanol content. A *ca*. five-fold decrease in the association constant in going from pure water to 20 % methanol is corroborated. The presence of the organic solvent certainly modifies the affinity of the estrogen by the CD cavity. Reasonable agreement is observed between our results in the presence of 20 % of methanol ($K = 4.8\times10^3$ $mol^{-1}L$) and the previously reported values for this system under the same solvent composition: 6.8×10^3 $mol^{-1}L$ (ref. 15) and 3.7×10^3 $mol^{-1}L$ (ref. 16).

Table 4 also includes reported association constants for some estrogen-CD complexes in other experimental conditions and using various determination methods. In all reported water/co-solvent systems, the constant values are lower than those obtained in water. The relative order of the association constants in water/co-solvent systems at low co-solvent concentrations is EE2>E2>E3>E1, similar to the order found in aqueous systems.[15,16] However, in β-CD systems and at higher amounts of organic solvent (*e.g.* 30 and 45 %),

more hydrophobic subtrates tend to leave the CD cavity and, in those cases, EE2 complexes are less stable than the corresponding E2 ones.

Another difference between aqueous and water/co-solvent systems is the relative stability of the complexes formed by β- and γ-CDs. In the former cases, β-CD complexes are more stable than γ-CD ones. A similar conclusion was obtained by Oishi et al. in 10 % methanol solutions.[12] Nevertheless, at higher methanol ratios the order is reversed (Table 4) possibly due to the better affinity of the alcohol for the β-CD cavity, displacing the analyte from the host molecule. Indeed, Wu et al. demonstrated that the association constants of γ-CD with alcohols are about one order of magnitude smaller than those of β-CD.[37]

Estrogen-CD association constants determined by capillary electrophoresis using SDS in the run buffer (Table 4) cannot be compared with those obtained here. In this case, the presence of micelles in the run buffer is necessary for differentiating the mobility of analytes while adding neutral CDs, but the estrogen-micelle interaction is strong and competes with the CD inclusion processes. Thus, the obtained constant values are significantly lower than those obtained in water, and the affinity of the estrogens towards a particular CD type also changes.

Micellar-enhanced fluorescence

The effect produced by micelles on the fluorescence intensity of estrogens was studied by keeping constant the concentration of each analyte and increasing the concentrations of selected surfactants (Fig. 6). For ensuring the micelle formation, the maximum concentrations of the evaluated surfactants were higher than the corresponding critical micelle concentrations (CMC).[38] Those surfactants with large methylene chains showed to be adequate in terms of fluorescence enhancement, probably due to the better interaction of

this group with the analytes. The maximum intensities reached, even with the best systems formed by HTAC, were lower than that obtained with several studied CDs. Apparently, CDs offer a better shielding environment to the excited estrogen molecules from non-radiative pathways, in accordance with the high stability constant values calculated for the corresponding inclusion complexes.

Temperature effect

The influence of the temperature on the fluorescence emission of E2 in the presence of DM-β-CD, taken as a model system, was evaluated. The experiment showed that a temperature decrease does not significantly improve the fluorescence intensity. For instance, if the experiment is conducted at 5 °C, the fluorescence increases only about 3 % of the original value at 20 °C. Therefore, no efforts were made to keep a low working temperature.

Analytical Parameters

The linear dependence between the fluorescence response and the estrogens concentration was investigated by building calibration curves under optimum conditions. According to the above results, the construction of the curves was carried out in the presence of 5×10^{-4} mol L^{-1} DM-β-CD at 20 °C and neutral pH. In order to evaluate the effect of the selected λ_{exc} on the calibration parameters, two curves using either 222 or 270 nm as excitation wavelength were constructed. While the concentration of DM-β-CD was kept constant in the calibration samples, the concentrations of E2, E3 and EE2 were varied from 0 to 200 ng mL^{-1}, and the concentration of E1 was varied from 0 to 1000 ng mL^{-1}. These samples were

prepared by suitable dilution of a stock water solution of each estrogen and CD. It is necessary to point out that although no attempts were made to establish the upper concentration of the linear range, linear relationship between signal and concentration was verified at least until 1500 ng mL^{-1} in all systems by applying the *F* test recommended by IUPAC[39]. However, we decided to work with a more restricted concentration range, since the goal is to detect low concentrations of these compounds.

Table 5 summarizes the obtained results. For comparison, statistical parameters obtained in the absence of the selected CD were also included in this table.

In comparing the results using different maximum excitation wavelengths, one may note that the difference is not significant. The use of the selected organized medium allows to obtain detection limits in the range 2–4 ng mL^{-1} for E2, E3 and EE2, with relative standard deviations (n = 5) of 1.0–1.8 % for 50 ng mL^{-1}. As expected, for the less fluorescent E1 system poorer values were obtained. When the calibration in the absence of CD is carried out, the limits of detection and quantification increase and the analytical sensitivities decrease, highlighting the advantage of using DM-β-CD in the determinations.

Taken into account that the evaluated procedure involves direct measurements without using organic solvents and employing unsophisticated instrumental, the attained limits of detection in the presence of DM-β-CD are very suitable. Limits of detection at part-per-billion levels, as those here determined, for different estrogens in environmental and fishery samples have been found, for example, using excitation-emission fluorescence matrices coupled to second-order calibration,[40] molecularly imprinted solid-phase extraction with HPLC and fluorescence detection,[41] and through a flow-injection chemiluminescence method.[42] Limits of detection at part-per-trillion levels or lower have been reported for the

determination of these hormones, but using different pretreatments involving extraction and pre-concentration and, sometimes, derivatization steps.[43–46]

Conclusions

The results of this study contribute to the understanding of the interaction of organized media and estrogens without the influence of third parties.

The poor fluorescence intensity of estrogens in water is significantly enhanced by β-cyclodextrin and its hydroxyethyl, hydroxypropyl and *o*-methyl derivatives. The increases in fluorescence intensity with increasing concentrations of cyclodextrin were used to evaluate both the stability and stoichiometry of the formed complexes. The high constant association values for the 1:1 complexes, in the range 5×10^3–5×10^5 mol^{-1} L, demonstrate that the interaction of these hydrophobic molecules with the cyclodextrin nanocavity is extremely strong. The relative stability of the formed complexes could be explained on the basis of both hydrophobicity and specific hydrogen bonds of the guest molecule with the cyclodextrin, allowing to conclude about structural issues.

The acidity constants of estrogens in both the presence and absence of cyclodextrins allows inferring the interaction of the phenolic hydroxyl of the A ring of the estrogens and the external groups of the CDs.

The presence of organic solvents in the reaction medium decreases the affinity of the estrogen by the cyclodextrin cavity in a magnitude that depends on the percentage of organic solvent present. Micellar systems formed by surfactants with large methylene chains also increase the fluorescence of the studied estrogen, but not as significantly as in some cyclodextrin systems.

The use of heptakis(2,6-di-*o*-methyl)-β-cyclodextrin could be a suitable strategy to determine poorly fluorescent estrogens at part-per-billion levels, without using either derivatization reactions or toxic organic solvents.

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Table 1 Association constant values for estrogen-cyclodextrin complexes obtained from non-linear regression (K) and from the Benesi-Hildebrand type equation ($K_{B\text{-}H}$)[a]

	EE2		E2		E3		E1	
	log K	log $K_{B\text{-}H}$	log K	log $K_{B\text{-}H}$	log K	log $K_{B\text{-}H}$	log K	log $K_{B\text{-}H}$
γ-CD	4.3 ± 0.1	4.3 ± 0.1	4.2 ± 0.1	4.26 ± 0.05	4.00 ± 0.07	4.00 ± 0.08	[b]	[b]
β-CD	4.95 ± 0.01	4.9 ± 0.1	4.63 ± 0.07	4.63 ± 0.02	4.52 ± 0.06	4.51 ± 0.03	3.9 ± 0.3	3.6 ± 0.3
HE-β-CD	4.97 ± 0.09	4.96 ± 0.06	4.68 ± 0.1	4.65 ± 0.06	4.5 ± 0.1	4.48 ± 0.08	3.7 ± 0.2	3.88 ± 0.07
HP-β-CD	5.05 ± 0.06	5.05 ± 0.03	4.83 ± 0.06	4.83 ± 0.04	4.67 ± 0.06	4.66 ± 0.02	3.70 ± 0.08	3.7 ± 0.1
M-β-CD	5.4 ± 0.1	5.4 ± 0.1	4.99 ± 0.08	4.99 ± 0.06	4.70 ± 0.04	4.68 ± 0.04	3.9 ± 0.2	4.0 ± 0.2
DM-β-CD	5.7 ± 0.2	5.7 ± 0.2	5.2 ± 0.2	5.2 ± 0.2	5.11 ± 0.01	5.09 ± 0.09	4.1 ± 0.3	4.2 ± 0.2

[a] Confidence intervals were calculated as: SD×Student t value, where SD is the standard deviation and t is taken at 95 % of confidence and (n–2) degrees of freedom. The number of data points (n) in each experiment varied between 7 and 14. [b] See text.

Table 2 Fluorescent quantum yield ratios between complexed ($Ø_C$) and free ($Ø_E$) analyte[a]

	EE2	E2	E3	E1
	$Ø_C/ Ø_E$	$Ø_C/ Ø_E$	$Ø_C/ Ø_E$	$Ø_C/ Ø_E$
γ-CD	1.36(1)	1.56(1)	1.43(1)	[b]
β-CD	1.78(1)	1.84(1)	1.74(1)	1.77(4)
HE-β-CD	1.73(1)	1.83(1)	1.81(1)	2.17(5)
HP-β-CD	1.74(1)	1.95(1)	1.84(1)	2.47(4)
M-β-CD	1.80(1)	1.91(1)	1.91(1)	2.02(5)
DM-β-CD	2.07(1)	1.87(1)	2.04(1)	1.96(5)

[a] Standard deviations in the last significant figure are given between parentheses. [b] See text.

Table 3 Octanol-water partition coefficient (K_{OW}), deprotonation constant (pKa), and aqueous solubility (S) for selected estrogens[a]

	EE2	E2	E3	E1	Ref.
log K_{OW}	4.15	3.94	2.81	3.43	29
pKa	10.21	10.23	10.05		30
	10.40	10.46		10.34	31
			10.4		32
	10.3 ± 0.1	10.1 ± 0.1	10.4 ± 0.1	10.3 ± 0.1	This work
	10.4 ± 0.1[b]	10.80 ± 0.06[b]	10.6 ± 0.1[b]	10.60 ± 0.04[b]	This work
	10.87 ± 0.06[c]	11.16 ± 0.06[c]	11.30 ± 0.04[c]	10.9 ± 0.1[c]	This work
S (mg L^{-1})	4.8	13	13	13	29
	9.7	3.9		0.8	31
	19.1	3.85	30.2		34
	9.20[d]	1.51[d]		1.30[d]	35
	3.1[e]	3.1[e]		2.1[e]	36

[a] Confidence intervals were calculated as: SD×Student t value, where SD is the standard deviation and t is taken at 95 % of confidence and (n–2) degrees of freedom. The number of experimental pH values (n) for each pKa determination varied between 16 and 23. [b] In the presence of β-CD, 20 °C. [c] In the presence of DM-β-CD, 20 °C. [d] 25 °C, pH 7. [e] 23 °C.

Table 4 Association constant values for complexes formed by estrogens and cyclodextrins in different experimental conditions[a]

Experimental conditions	Method	EE2	E2	E3	E1	Ref.
		β-CD				
W, 20 °C	F	$9.0(1)\times10^4$	$4.3(3)\times10^4$	$3.3(2)\times10^4$		This work
MeOH/W(5/95 v/v), 20 °C	F		$2.3(1)\times10^4$			This work
MeOH/W(10/90 v/v), 20 °C	F		$1.60(4)\times10^4$			This work
MeOH/W(20/80 v/v), 20 °C	F		$4.8(3)\times10^3$			This work
MeOH/W(20/80 v/v), 35 °C	F	$5.6(5)\times10^3$	$3.7(7)\times10^3$	$3.2(4)\times10^3$		16
MeOH/W(20/80 v/v), 35 °C	HPLC	$7.6(8)\times10^3$	$6.8(7)\times10^3$	$4.7(4)\times10^3$	$3.1(6)\times10^3$	15
MeOH/W(45/55 v/v), 35 °C	HPLC	$2.71(9)\times10^2$	$4.0(1)\times10^2$	$2.23(2)\times10^2$	$2.38(9)\times10^2$	13
MeOH/W(45/55 v/v), 30 °C	H-D[b]	364	465	300	267	14
ACN/W(30/70 v/v), 30°C	H-D[b]	125	191	152	92	14
ACN/phosphate buffer (35/65 v/v, pH 5), 25 °C	DPV		$1.1(1)\times10^2$		$6.0(8)\times10^1$	17
ACN/phosphate buffer (35:65 v/v, pH 5), 25 °C	HPLC		$2.67(6)\times10^2$		$8.9(2)\times10^1$	17
EtOH/phosphate buffer (25/75 v/v, pH 5), 25 °C	DPV		$2.0(3)\times10^2$		$1.4(2)\times10^2$	17
Na_2PO_4H/SDS, 20 °C	CE	$1.3(8)\times10^2$	$7(7)\times10^1$	$4(1)\times10^1$		9
		γ-CD				
W, 20 °C	F	$2.2(3)\times10^4$	$1.6(2)\times10^4$	$1.01(7)\times10^4$		This work
MeOH/W(20/80 v/v), 35 °C	F	$9(1)\times10^3$	$6.3(3)\times10^3$	$3.2(6)\times10^3$		16
MeOH/W(20:80 v/v), 35 °C	HPLC	$1.1(1)\times10^4$	$7.1(4)\times10^3$	$3.2(4)\times10^3$	$2.6(2)\times10^3$	15
MeOH/W(45/55 v/v), 30 °C	H-D[b]	910	882	523	352	14
ACN/W(30/70 v/v), 30°C	H-D[b]	414	384	313	182	14
Na_2PO_4H/SDS, 20 °C	CE	$1.87(3)\times10^3$	$1.23(2)\times10^3$	$1.28(1)\times10^3$	$6.4(2)\times10^2$	9
		HP-β-CD				
W, 20 °C	F	$7.1(2)\times10^4$	$3.9(2)\times10^4$	$3.7(2)\times10^4$	$4.9(4)\times10^3$	This work
Na_2PO_4H/SDS, 20 °C	CE	$1.0(3)\times10^2$	$1.0(3)\times10^2$	$1.1(2)\times10^2$	$6(3)\times10^1$	9

[a] Standard deviations in the last significant figure of equilibrium constants are given between parentheses. [b] Hummel-Dreyer method.[47] Abbreviations: ACN, acetonitrile; CE, capillary electrophoresis; DPV, differential pulse voltammetry; EtOH, ethanol; F, fluorimetric; HPLC, high-performance liquid chromatography; MeOH, methanol; SDS, sodium dodecylsulfate; W, water.

Table 5 Calibration results for estrogens in the absence and in the presence of DM-β-CD[a]

	EE2		E2		E3		E1	
	λ_{exc}		λ_{exc}		λ_{exc}		λ_{exc}	
	222 nm	270 nm	222 nm	270 nm	222 nm	270 nm	222 nm	270 nm
	Without DM-β-CD							
CR[b] (ng mL^{-1})	0–200	0–200	0–200	0–200	0–200	0–200	0–1000	0–1000
R^c	0.9997	0.9989	0.9989	0.9985	0.9991	0.9981	0.9993	0.9990
a^d	2.9(5)	4.3(6)	4.3(7)	3.5(8)	3.7(9)	3.5(5)	1.2(2)	1.6(2)
b^e	0.542(5)	0.405(5)	0.689(7)	0.432(7)	0.574(8)	0.367(4)	0.0154(3)	0.0120(3)
γ^f (ng^{-1} mL)	0.36	0.25	0.35	0.19	0.22	0.27	0.03	0.03
LOD[g] (ng mL^{-1})	6.5	9.3	6.8	13	11	8.7	89	88
LOQ[h] (ng mL^{-1})	19	27	19	36	31	25	258	255
RSD[i] (%)	2.5	4.3	3.5	6.2	5.1	4.3	13	13
	With DM-β-CD							
CR (ng mL^{-1})	0–200	0–200	0–200	0–200	0–200	0–200	0–1000	0–1000
R	0.9998	0.9996	0.9999	0.9998	0.9998	0.9997	0.9979	0.9988
A	12.8(4)	6.8(2)	13.7(3)	7.5(2)	14.6(5)	7.7(2)	12.0(3)	7.7(1)
B	0.761(4)	0.324(2)	0.984(3)	0.422(2)	0.823(4)	0.348(2)	0.0316(5)	0.013(1)
γ (ng^{-1} mL)	0.64	0.53	1.0	0.78	0.67	0.64	0.04	0.05
LOD (ng mL^{-1})	3.7	4.4	2.3	3.0	3.5	3.7	59	44
LOQ (ng mL^{-1})	11	13	6.6	8.8	10	11	170	128
RSD (%)	1.4	1.8	1.0	1.4	1.6	1.6	14	13

[a] The number of data for each calibration curve corresponds to seven different concentration levels, with three replicates for each level (n = 21). [b] Calibration range. [c] Correlation coefficient. [d] Intercept (standard deviation within parentheses). [e] Slope (standard deviation within parentheses). This value is the calibration sensitivity according to IUPAC.[39] [f] Analytical sensitivity: $\gamma = b/S_S$, where S_S is the standard deviation of the regression residuals.[48]. [g] Limit of detection calculated according to ref. 49 using 0.05 as assurance probabilities. [h] Limit of quantification calculated as LOD × (10/3.3). [i] Relative standard deviation. In all cases five replicates were measured. $C_{DM\text{-}\beta\text{-}CD}$ = 5×10^{-4} mol L^{-1}, $C_{EE2} = C_{E2} = C_{E3}$ =/50 ng mL^{-1}; C_{E1} = 100 ng mL^{-1}).

Figure captions

Fig. 1 Estrogen structures and schematic representation of derivative β-cyclodextrins.

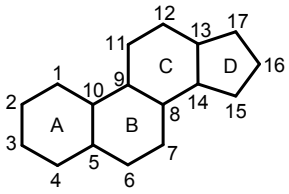
Fig. 2 Excitation and emission fluorescence spectra for E2 (black), E3 (red), EE2 (blue) and E1 (green) in aqueous solution (A), in the presence of β-CD (B), in the presence of DM-β-CD (C), and in micellar medium given by HTAC. The gray lines in all plots are the corresponding blanks and asterisks mark the Raman signals. $C_{E2} = C_{E3} = C_{EE2} = 420$ ng mL^{-1}, $C_{E1} = 1300$ ng mL^{-1}; $C_{\beta\text{-CD}} = C_{\text{DM-}\beta\text{-CD}} = 5\times10^{-4}$ mol L^{-1}; $C_{HTAC} = 7\times10^{-3}$ mol L^{-1}.

Fig. 3 Effect of cyclodextrins, as indicated, in the fluorescence emission of E2 (black), E3 (red), EE2 (blue) and E1 (green). λexc = 270 nm; $C_{E2} = C_{E3} = C_{EE2} = 420$ ng mL^{-1}; $C_{E1} = 1300$ ng mL^{-1}; t = 20 °C. The left vertical axis corresponds to E2, E3 and EE2; the rigth vertical axis to E1. Additional data (not shown) at higher CD concentrations were measured. Each point was background-corrected. Solid lines are the non-linear fit to the data.

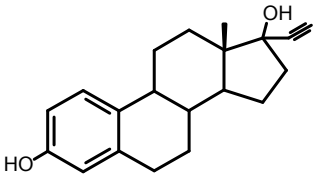
Fig. 4 Benesi-Hildebrand type plots for E2 (black) and E3 (red) –β-CD complexes. Solid lines are the linear fit to the data.

Fig. 5 Experimental fluorescence values vs. pH for E2 (black), E3 (red), EE2 (blue) and E1 (green) in the absence of organized media (A), in the presence of β-CD (B), and in the presence of DM-β-CD (C). λexc = 270 nm; $C_{E2} = C_{E3} = C_{EE2} = 420$ ng mL^{-1}; $C_{E1} = 1300$ ng mL^{-1}; $C_{\beta\text{-CD}} = C_{\text{DM-}\beta\text{-CD}} = 5\times10^{-4}$ mol L^{-1}; t = 20 °C. Solid lines are the non-linear fit to the data.

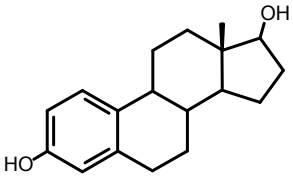
Fig. 6 Effect of surfactants, as indicated, in the fluorescence emission of E2 (black line), E3 (red line), EE2 (blue line) and E1 (green line). Critical micellar concentration (CMC) corresponding to each system are indicated (ref. 37). λexc = 270 nm; $C_{E2} = C_{E3} = C_{EE2} = 420$ ng mL^{-1}; $C_{E1} = 1300$ ng mL^{-1}; t = 20 °C. Each point was corrected with the corresponding background.



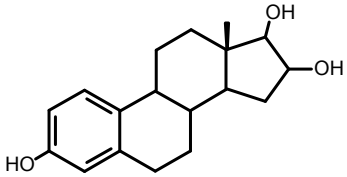
Cyclopentan-*o*-perhydrophenanthrene



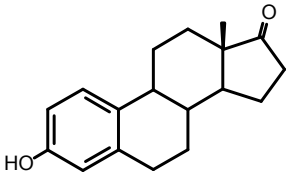
Ethynylestradiol (EE2)



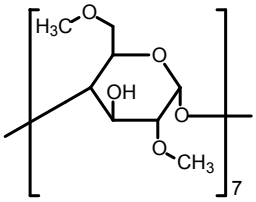
Estradiol (E2)



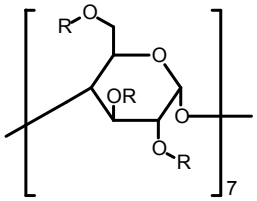
Estriol (E3)



Estrone (E1)



Heptakis(2,6-di-*O*-methyl)-β-cyclodextrin



2-Hydroxyethyl-β-cyclodextrin (R = H or CH_2CH_2OH)
2-Hydroxypropyl-β-cyclodextrin (R = H or $CH_2CH(OH)CH_3$)
Methyl-β-cyclodextrin (R = H or CH_3)
β-cyclodextrin sulfated sodium salt (R = H or SO_3Na)

FIGURE 1

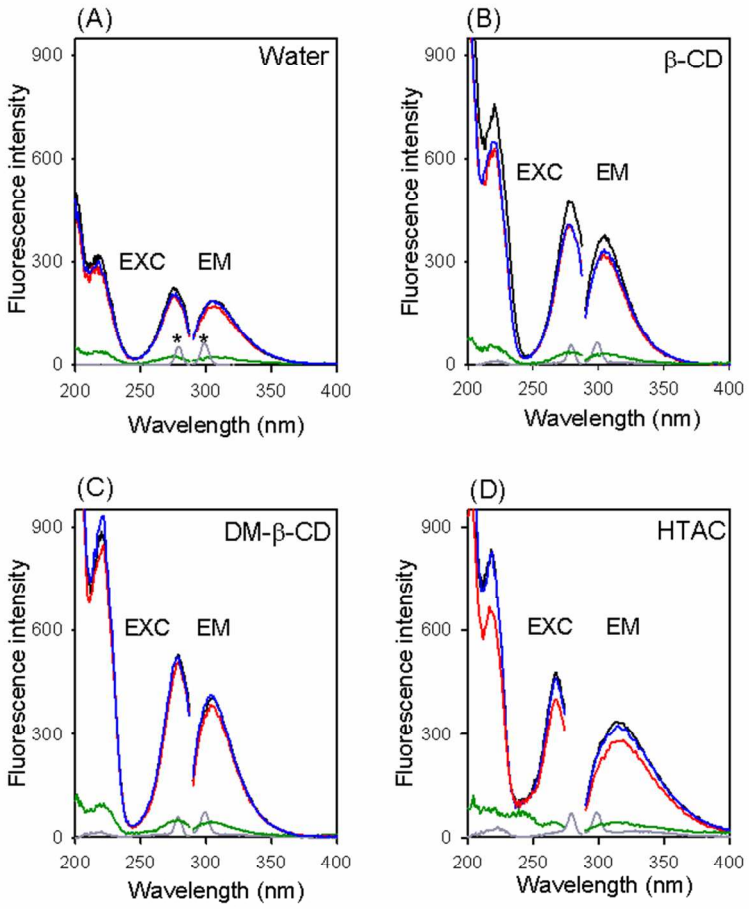


FIGURE 2

122x183mm (150 x 150 DPI)

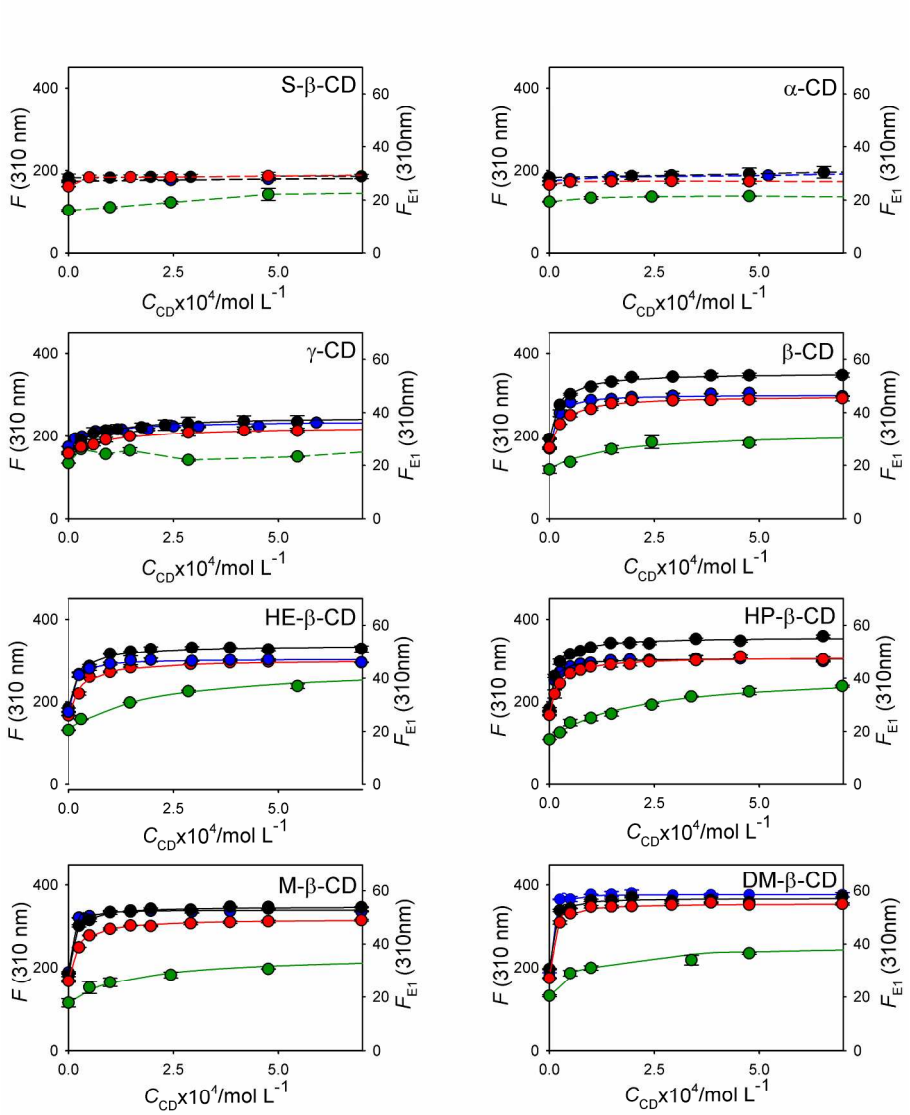


FIGURE 3

225x298mm (300 x 300 DPI)

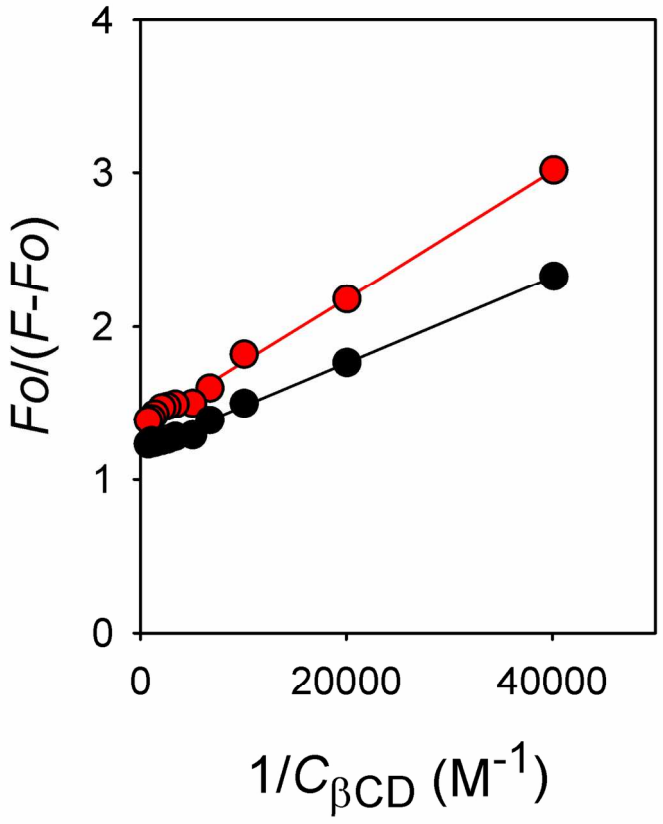


FIGURE 4

117x192mm (300 x 300 DPI)

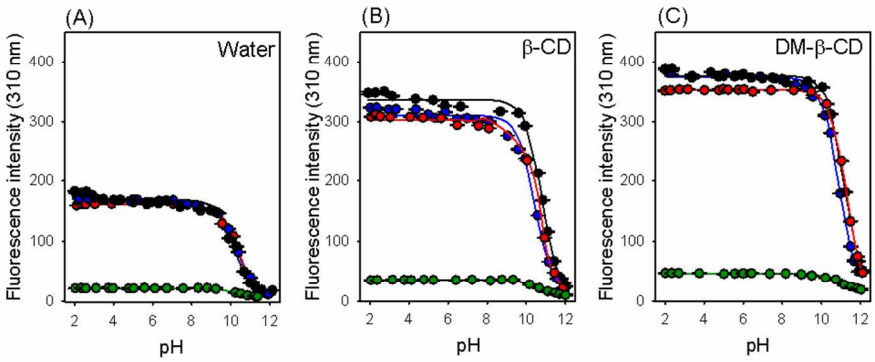


FIGURE 5

168x223mm (150 x 150 DPI)

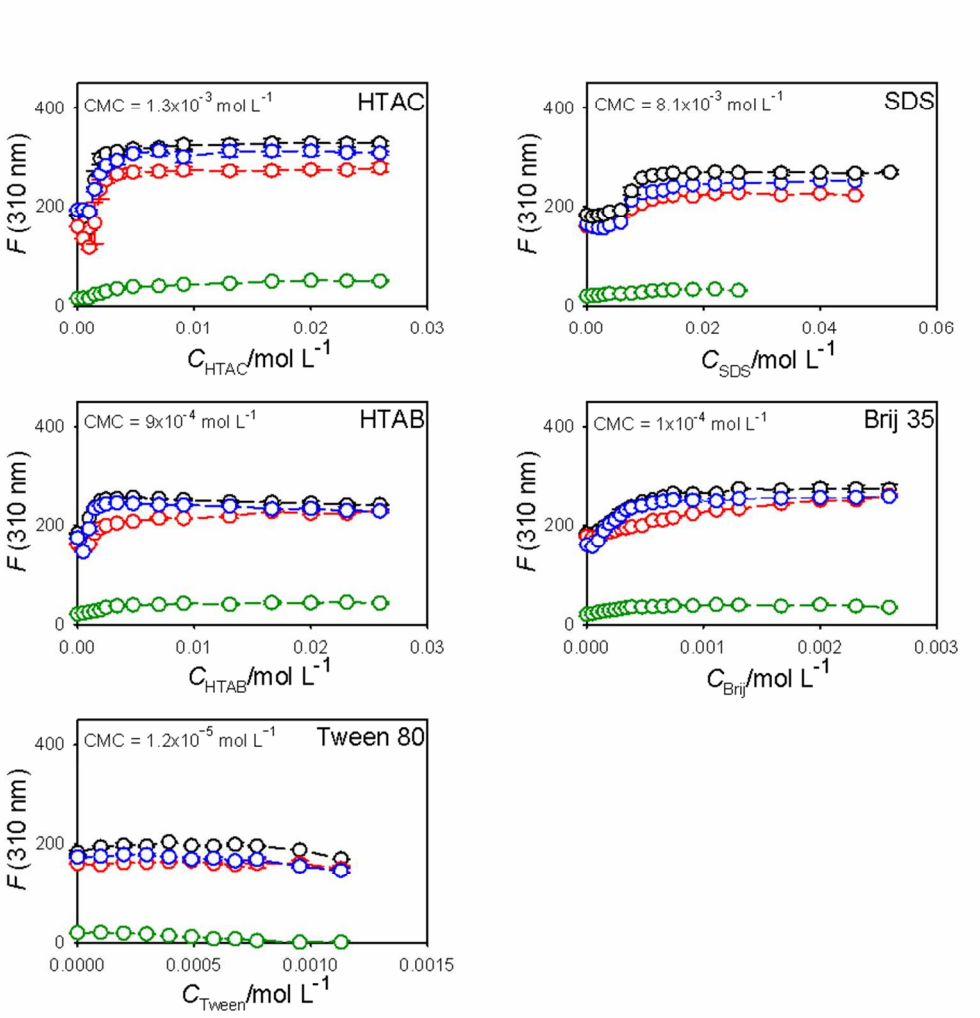


FIGURE 6

153x188mm (150 x 150 DPI)

The strong interaction between estrogens and selected cyclodextrins in the absence of organic solvents is demonstrated through a spectrofluorimetric study. The formed complexes would be of interest in analytical areas based on green chemicals.