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ARTICLES

Study of the Complexation of Fisetin with Cyclodextrins

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In this work, the interaction between fisetin (3,3′,4′,7-tetrahydroxyflavone) (Fis) and cyclodextrins (CDs) (α and *β*) was studied through UV−vis absorption, steady-state fluorescence, induced circular dichroism, and ^{1}H NMR experiments with dependence on temperature and pH. Some experimental data were compared with quantum-mechanics studies based on the SAM1 (AMPAC) semiempirical model, as well as with the B3LYP and MPW1PW91 functional models from density functional theory using the 6-311G* and 3-21G* basis sets. The spectroscopic measurements show that Fis does not form stable complexes with α-CD. On the other hand, at pH 4.0 and 6.5, the complex Fis−*β*-CD is formed in a Fis:*β*-CD 1:1 stoichiometry and an equilibrium constant (*K*) of 900 ± 100 M^{-1}. In basic medium (pH 11.5), *K* decreases to 240 ± 90 M^{-1} because Fis deprotonation leads to its better solubilization in water. Molecular modeling points out that Fis is not totally inserted into the inner cavity of *β*-CD. The formation of the inclusion complex renders an environment that enhances intramolecular excited state proton transfer. The inclusion complex is formed preferentially via entry of the Fis phenyl group into *β*-CD.

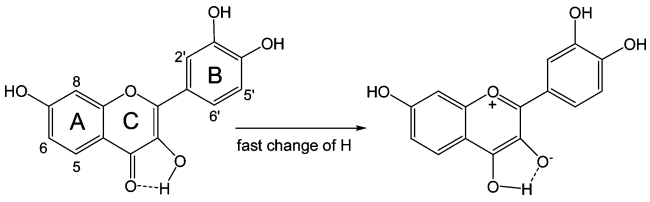
1. Introduction

Fisetin (3,3′,4′,7-tetrahydroxyflavone) (Fis) has very interesting spectroscopic and pharmacological properties. For instance, it exhibits a dual fluorescence behavior that is very dependent on solvent polarity,[1,2] which enables its use as a laser dye.[3] Besides that, like other flavones and related compounds, Fis has antioxidant properties and a great biological potential for therapeutic applications. Although some published papers have reported on the interaction between Fis and some drug delivery systems,[2,4,5] there are no papers describing its complexation with cyclodextrins (CDs).

The fluorescence behavior of Fis in media of different polarities has been compared with that of 3-hydroxyflavone (3-HF).[1] Both molecules present a higher dipole moment in the singlet excited state than in their ground state. In addition, in the singlet excited state, 3-HF and Fis can undergo an intramolecular excited state proton transfer (IESPT) from the hydroxyl group in position 3 to the carbonyl oxygen,[6] thus forming a tautomer (Scheme 1). The presence of the latter justifies the dual fluorescence emission spectra of these compounds.

The structures of cyclodextrins (CDs), cyclic oligosaccharides produced from starch and cellulose, consist of an external hydrophilic surface and a hydrophobic cavity. The widely known α-, *β*-, and *γ*-CDs are crystalline, homogeneous, and nonhygroscopic, and they contain macrocycles of six, seven, and eight glucopyranose units, respectively.[7] They easily form inclusion complexes with a wide variety of nonpolar molecules, which greatly enhance their solubility in water, thus favoring their industrial application. When the guest is a drug, the formation of inclusion complexes improves drug bioavailability and stability. In pharmaceutical formulations, the toxic effects of the drugs are decreased and they are more easily dispensed from the body.[8]

SCHEME 1: Intramolecular Excited State Proton Transfer Producing Two Fluorescent Species[6]



In the present work, the Fis complexation with CD was evaluated by UV−vis absorption, steady-state fluorescence, induced circular dichroism, and ^{1}H NMR measurements. The experimental data show that Fis is able to complex with *β*-CD, but not with α-CD. The nature of the Fis−*β*-CD complexes is dependent on pH and on the diameter of the CD inner cavity. The equilibrium constant (*K*) and the thermodynamic parameters

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for the formation of the inclusion complexes Fis−β-CD were also determined. Theoretical data for single molecules were obtained from quantum-mechanics calculations. A comparison between the theoretical data and the experimental results is reported in this paper.

2. Experimental Section

2.1. Materials. Fisetin (Lot No. 05612DO), β-CD (99%, Lot No. A0057301501), and DMSO-d_6 (100.0 atom % D) were purchased from Aldrich. α-CD was a sample gift from Wacker Chemie GmbH. D_2O and MeOD (99.8 atom % D) were acquired from Acros Organics; sodium phosphate, monobasic dihydrate and dibasic, anhydrous (P. A.), and methanol (P. A.) were obtained from J. T. Baker. All reagents were used as received. Ultrapure water from a Millipore filtration system was used to prepare all aqueous solutions.

2.2. Equipment. The temperature of all the experiments was set to 25 °C, unless otherwise stated. Absorption spectra were measured on a Hitachi U-3000 spectrometer, and baseline corrections were always carried out. Steady-state fluorescence spectra were recorded with a Hitachi F4500 fluorimeter (λ_{exc} = 340 nm, λ_{em} = 360−660 nm) at room temperature (23 °C). The excitation and emission slits were set to a bandwidth of 10 nm. Induced circular dichroism (ICD) spectra were recorded with a Jasco J-180 spectropolarimeter equipped with a PTC-423S coupled device for temperature control. An average of two spectra was recorded for each sample. The baseline consisted of an aqueous solution of Fis, an achiral molecule, and this solution contained a maximum concentration of methanol of 0.5% (v/v). The spectra of the aqueous solution of either α- or β-CD without Fis was subtracted from those measured for CD in the presence of the flavonoid. The NMR spectra were performed on a Bruker Avance DRX 500 spectrometer equipped with a 2.5 mm direct probe with field *z*-gradient. For ^{1}H NMR analysis, the 90° pulse was 8.35, 11.0, or 12.0 μs with power of 0, −2.50, or −3.50 dB. For the NOESY spectra, the same parameters were used with a mixing time of 0.75 s.

2.3. Methods. *Preparation of Fis−CD Complexes.* Due to the low Fis solubility in water, a Fis stock solution (1 mM) in methanol was prepared. Aliquots of this solution were used to prepare the aqueous and buffer solvents for the CD complexes containing both Fis (5 or 25 μM) and a maximum concentration of methanol of 0.5% (v/v). A more concentrated CD aqueous solution (α-CD, 30 mM; β-CD, 12 mM) containing Fis/methanol was prepared and left under stirring for 4 h. Then, more diluted CD solutions were prepared by using the same aqueous solvent. All the solutions were left under stirring and were protected from light for at least 24 h before any experimental measurements.

CD Complexes in Buffer Solutions. Two buffer conditions were used: an acid one and a basic one. For the basic buffer, equal volumes of aqueous solutions of 0.2 M sodium phosphate dibasic and 0.1 M sodium hydroxide were used to obtain a pH value of ca. 12. For the acid buffer, ca. pH 4.6 solutions of NaH_2PO_4 and Na_2HPO_4 were added at a volume ratio of 400:1, respectively. A certain aliquot of the Fis stock solution in methanol was added to each of these buffer solutions to obtain the desired final concentration of the flavonoid (5 or 25 μM) and a maximum concentration of methanol of 0.5% (v/v).

Basic Solution in D_2O for ^{1}H NMR Measurements. Two procedures were adopted. The first consisted of using gaseous ammonia to basify the deuterated water. This ammonia was produced from the reaction of a solution of 2 M NaOH over solid NH_4Cl, under stirring and slight heating, in a semiclosed system. A pH control experiment was carried out by means of a pHmeter placed in a beaker containing only ultrapure water. The second procedure consisted of using a commercially available solution of 30% NaOD in D_2O.

Determination of Equilibrium Constants. The Benesi−Hildebrand equation[9] (eq 1) was used to determine the ground-state equilibrium constant (K) through UV−vis absorption and fluorescence experiments.

$$\Delta I = \frac{[\mathrm{Fis}]_T \Delta x K[\mathrm{CD}]}{1 + K[\mathrm{CD}]} \tag{1}$$

It was possible to apply this equation because β-CD, [CD], was present in excess when compared to Fis, $[\mathrm{Fis}]_T$, and because there was a change in the intensity measurement, ΔI, with increasing β-CD concentration. Δx represents the difference between the emission quantum yields or molar absorptivities of the free and complexed Fis in the fluorescence and UV−vis absorption experiments, respectively. It was possible to fit the plot of ΔI versus CD concentration through eq 1, which provides K straightforwardly. These experiments were carried out at room temperature (23 °C). A linear relationship, eq 2, was obtained for the double-reciprocal plot of eq 1:

$$\frac{1}{\Delta I} = \frac{1}{[\mathrm{Fis}]_T} + \frac{1}{[\mathrm{Fis}]_T \Delta x K[\mathrm{CD}]} \tag{2}$$

Equation 2 is valid if the complex formed follows the 1:1 stoichiometry.

Solubility Studies of Fis in β-CD. The method described by Higuchi and Connors[10] was employed to determine Fis solubility in β-CD. Briefly, excess Fis (ca. 1 mg) was added to a predefined β-CD amount (0−0.012 M) and the solutions were left to stir over 24 h at room temperature. Then, the solutions were placed in a thermostatized bath for approximately 2 h at 25 °C. Afterward, the absorbance of the filtered solutions were registered at 248, 318, and 360 nm to measure Fis concentration. The molar absorptivities at these wavelengths had been previously determined experimentally. The same procedure was repeated at 15, 35, and 45 °C. The experiment was carried out twice.

The obtained data enabled the plot of Fis concentrations as a function of β-CD concentration. In the linear portion of the solubility diagram, the equilibrium constant for complex formation was determined by the Higuchi and Connors equation:[10]

$$K = \frac{\text{slope}}{\text{intercept}(1 - \text{slope})} \tag{3}$$

From this study, the thermodynamic parameters for the formation of the Fis−CD complex was determined through the integrated van't Hoff equation:

$$\ln K = -\frac{\Delta G}{RT} = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \tag{4}$$

The changes in enthalpy and entropy were calculated from the dependence of ln K on the inverse of the temperature.

3. Results and Discussion

3.1. Fis Intramolecular Excited State Proton Transfer (IESPT). Experimentally, Fis fluorescence emission spectra in ethanol display two fluorescence bands, at 463 and 530 nm. The latter is attributed to the occurrence of IESPT (Scheme 1)

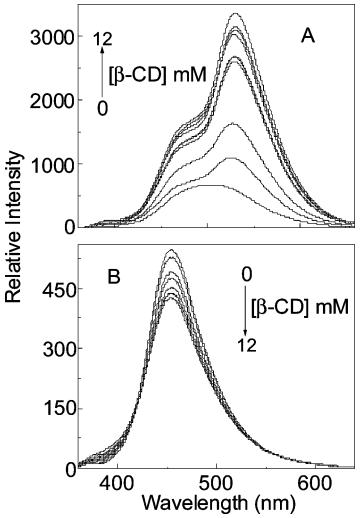

Figure 1. Fis fluorescence spectra with increasing β-CD concentration (from 0 to 12 mM) at (A) pH 4.0 and (B) pH 11.5, $\lambda_{exc} = 340$ nm.

due to the tautomer emission.[1,2] The Fis dual fluorescence behavior is observed in both hydrogen bond acceptor (HBA) solvents, such as dioxane, ethyl acetate, and acetonitrile, and hydrogen bond acceptor−donor (HBA−D) solvents, such as methanol, ethanol, and 1-propanol.[2] In addition, the fluorescence of normal Fis is strongly solvent-dependent, in full agreement with the sharp dipole moment changes between the ground (S_0) and excited singlet (S_1) states.[2] Indeed, the optimized Fis molecules in the ground (S_0) and excited singlet (S_1) states exhibit very similar structures, with a considerable difference in the dipole moments (μ). Estimated μ values are 2.3890 and 5.7630 D for S_0 and S_1, respectively. This suggests the presence of an $S_0 \rightarrow S_1$ transition, which is typically π,π^*, as confirmed by the analysis of the involved highest occupied and lowest unoccupied molecular orbitals and by the oscillator strength calculated by time-dependent density functional theory employing the hybrid functional B3LYP and the 6-31G** basis set.[11]

The theoretical calculations also showed that Fis molecule at the ground state is totally planar,[12] but there is a high energetic barrier that prevents the equilibrium between Fis and its tautomer. However, for the excited state there is a change in the Fis electronic density. For the tautomer, there is a small deviation of the planarity,[12] and the estimated dipole moment decreases to 1.8806 D. For this reason, in the excited state, the equilibrium between the Fis normal structure and its tautomer becomes feasible due to the lowering of the energetic barrier.

3.2. Evidence of Formation of the Fis−β-CD Complex. The preparation of solutions to study the complexation of the flavonoid with CDs adopted herein is that described by Barra and co-workers.[13] This method ensures the necessary elapsed time to achieve equilibrium during formation of the inclusion complexes.

Since Fis is a polyhydroxyl molecule, it is important to evaluate its behavior as the pH changes. The pK_a of Fis has already been described in the literature, indicating that the protons in the 7, 3′, and 4′ positions have pK_a values of 8.87, 10.31, and 13.20, respectively.[14] On the basis of this information, two conditions were chosen: an acid one with pH 4.0, which led to results similar to those obtained in neutral media (pH 6.5), and a basic environment with pH 12, which ensured deprotonation of OH 3′ and OH 4′.

Figure 1 shows how the intensity of Fis (5 μM) fluorescence behaves as function of β-CD concentration (from 0 to 12 mM) at neutral, acid (Figure 1A), and alkaline (Figure 1B) pH. The

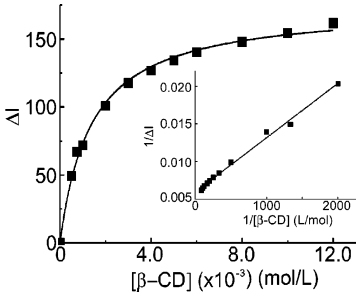

Figure 2. Representative plot of the variation in fluorescence intensity as a function of β-CD concentration as analyzed by the Benesi−Hildebrand equation[9] at 530 nm. Insert: the double-reciprocal plot.

aqueous solution of Fis and β-CD at pH 6.5 exhibits the same behavior as the solution at pH 4.0. In water at pH 6.5 or 4.0, Figure 1A, the Fis fluorescence spectrum displays a single broad band attributed to the strong overlap of the normal and tautomer emissions[2] (Scheme 1). At neutral or acid pH, Figure 1A, the addition of β-CD enhances the dual fluorescence emission, suggesting the formation of a host−guest inclusion complex. The fluorescence emission spectra of Figure 1A can be explained by the dual emission process, where normal Fis (470 nm) is in equilibrium with the tautomer (530 nm) (Scheme 1). The study of the dependence of the Fis fluorescence on the solvent shows two opposite effects when the polarity is decreased. The fluorescence band of normal Fis shifts to higher energy (from 482 nm in methanol to 408 nm in dioxane), while the tautomer emission exhibits only a moderate shift to lower energy (from 527 nm in MeOH to 543 nm in dioxane).[2] Moreover, in neutral or acid solutions (Figure 1A), Fis fluorescence is enhanced with increasing β-CD concentration. The CD inner cavity, a hydrophobic environment, can act in several ways. On one hand, it may promote the dual fluorescence emission behavior, since its polarity is different from that of the aqueous solution and similar to that of an alcoholic solution.[15] On the other hand, it can also protect the Fis molecule against the excited state deactivation promoted by internal conversion and collisions with the water molecules from the bulk solution.

At pH 11.5 (Figure 1B), as the concentration of the host increases, the intensity of Fis fluorescence decreases and only one band is seen with a maximum at 455 nm. Because the medium is basic, IESPT cannot occur and, thus, this band can be attributed to deprotonated Fis. Also, Fis fluorescence is much more intense under acid and neutral conditions than in alkaline medium. In basic solutions (Figure 1B), the interaction between deprotonated Fis and the solvent molecules is stronger, thus leading to a higher vibronic state density. This process enhances the rate constant for internal conversion, making the latter the main deactivation route for the excited state. Besides that, in basic conditions, the intensity of Fis fluorescence decreases with increasing β-CD concentration. The theoretical calculation for the inclusion complex of deprotonated Fis with β-CD, simulating a condition of ca. pH 12, shows a distortion in the β-CD structure and strong interactions between the phenoxide and OH groups present in the entrance of the β-CD cavity, due to the formation of many hydrogen bonds, one of them resulting from the sharing of a proton.[16] This interaction decreases Fis electronic density, and thus the fluorescence for deprotonated Fis is lower in the presence of CD.

From the experimental data of Figure 1, when a certain wavelength is considered, the difference in the intensity of the probe's fluorescence in the absence and presence of several amounts of CD (ΔI) was plotted versus the β-CD concentration; see Figure 2. The experimental data were fitted using eq 1, and

TABLE 1: Equilibrium Constant (*K*) for the Fis−*β*-CD Complex in Several Media, at Different Temperatures

medium	temp (°C)	K (M^{-1})
neutral	15	1000 ± 120
acid/neutral	23	900 ± 100
basic	23	240 ± 90
neutral	25	860 ± 70
neutral	35	510 ± 30
neutral	45	360 ± 30

K for complex formation was obtained at several wavelengths, from 420 to 530 nm, at different pH values. All the investigated wavelengths furnished similar trends as shown in Figure 2. The insert in Figure 2 is the double-reciprocal plot of the variation in the fluorescence intensity with increasing *β*-CD concentration at 530 nm. The obtained linear correlations for all the fits were higher than 0.9935, indicating that the presumed stoichiometry of the complex is 1:1 (Fis:*β*-CD) in acid, neutral, and basic environments. The plot of $1/\Delta I$ as a function of $1/[\beta\text{-CD}]^2$ was also analyzed, because this plot should provide information about the presence of higher order complexes, especially at high *β*-CD concentration. However, none of the experimental data gave a good linear fit in these plots, ruling out this possibility. The plot of the difference in UV−visible absorption intensities of Fis in the absence and presence of various concentrations of *β*-CD was similar to that obtained in Figure 2, in both acid/neutral and basic conditions.

Table 1 reports the complexation constant (*K*) for the Fis−*β*-CD complex obtained from fluorescence and UV−vis absorption measurements. In acid conditions, at 23 °C, $K = 900 \pm 100\ M^{-1}$. In basic conditions, it is $240 \pm 90\ M^{-1}$. The efficiency of the complexation between aromatic ketones and CD is determined by the size of the guest molecules and the CD inner cavity.[8] In acid medium, Fis has a *K* value that is similar to that observed for the inclusion of xanthone[17] ($1100 \pm 200\ M^{-1}$) and flavone[18] ($1090 \pm 80\ M^{-1}$) into CD. Chromone, a smaller molecule, has a lower equilibrium constant[18] ($240 \pm 40\ M^{-1}$). Rutin,[19] which contains glucose in its structure, does not efficiently complex with *β*-CD ($265\ M^{-1}$). In the case of Fis in basic solutions, the lower *K* value is due to the presence of negative charges in its molecule, which induce better Fis solubilization in the bulk solution rather than in the hydrophobic part of the CD.

The ICD spectrum (Figure 3) also confirms formation of the Fis−*β*-CD complex. The Fis molecule is achiral and does not display a circular dichroism signal. Nevertheless, a positive Cotton effect appears[8] when Fis is included in the *β*-CD inner cavity, as shown in Figure 3. The signal and intensity of the Cotton effect reveals the adopted orientation of the guest molecules when inserted into the CD inner cavity.[8] A positive signal, as shown in Figure 3, depicts that the Fis electric dipole moment coincides with the *β*-CD symmetry axis. In this work, the Fis orientation inside the *β*-CD cavity is similar to that of axially included 2-substituted naphthalenes.[8,20]

It is worth mentioning that Bohne and co-workers[18] studied the dynamic aspects of the complexation of flavone with *β*-CD. On the basis of experimental results only, they were not able to establish in which the way the flavonoid was complexed: whether it was via chromone ring or through the phenyl part of the flavone molecule. For this reason, this work gives the opportunity for one to theoretically discuss[16] both ways of entry of the flavonoid into *β*-CD: either through chromone (A) or via the phenyl group (B) of the Fis molecule (Scheme 1), and to compare some of the theoretical results with experimental values.

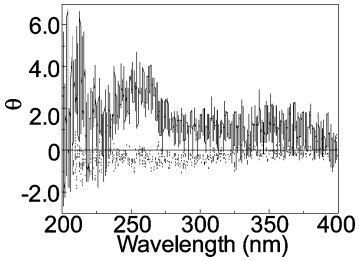

Figure 3. ICD signal of Fis in the presence of 12 mM *β*-CD (—) and in water (···).

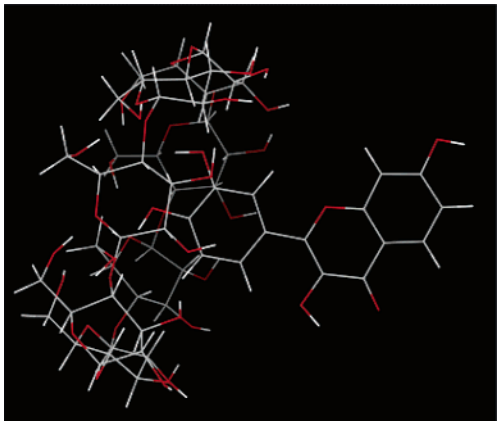

Figure 4. Data derived from the application of the SAM1 semiempirical model[16] to the study of the incorporation of Fis into *β*-CD through the Fis phenyl group.

The calculations showed that when Fis is inserted through the phenyl group, the hydroxyl protons 3′ and 4′ form two intermolecular bonds with the O atom in position 6 of the *β*-CD molecule. The hydroxyl oxygen in position 3′ is also capable of forming an intermolecular hydrogen bond with *β*-CD (Figure 4). On the other hand, the complexation via chromone entry gives rise to the formation of only one intermolecular hydrogen bond between the hydrogen of the Fis OH 7 and the OH group in position 6 of *β*-CD. Therefore, the preferential orientation adopted by Fis in the inclusion complexes (Figure 4) coincides with the ones determined experimentally by ICD spectra (Figure 3).

Theoretically, the Benesi−Hildebrand equation[9] could be applied to calculate the complexation constant through the ICD data. However, the ICD signal was too noisy, making this kind of analysis difficult. In addition, the experimental evidence from fluorescence and UV−vis absorption spectroscopic measurements confirmed our data.

3.3. Studies with α-CD. The same experiments described above were carried out to study whether complexation of Fis with α-CD was feasible. However, none of the UV−vis, fluorescence, or ICD measurements gave evidence of the characteristics mentioned above for complexation of Fis with *β*-CD. This leads to the conclusion that Fis is not able to form stable complexes with α-CD.

3.4. Solubility and Thermodynamic Parameters of the Fis−*β*-CD Complex. Figure 5A shows a representative solubility diagram for Fis in the presence of *β*-CD at 25 °C. The temperatures 15, 35, and 45 °C gave plots similar to those in Figure 5A, which suggests that neutral Fis is preferred to incorporate into the *β*-CD. For all solubility diagrams of Fis in *β*-CD for all investigated temperatures, it was observed that Fis solubility in *β*-CD aqueous solution is improved as the temperature increases.

From these plots, and by using eq 3, the *K* value for the Fis−*β*-CD complex was determined at three different wavelengths

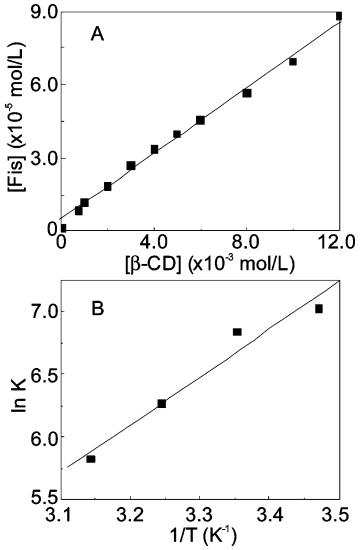

Figure 5. (A) Representative solubility diagram and (B) van't Hoff plot of Fis in β-CD at 25 °C.

TABLE 2: Thermodynamic Parameters for the Complexation of Fis with β-CD

thermodyn param	expt
ΔH (kJ·mol^{-1})	-27 ± 4
ΔS (J·mol^{-1}·K^{-1})	-40 ± 10
ΔG_{25} (kJ·mol^{-1})	-16.7 ± 0.8

(248, 318, and 360 nm). Results vary very slightly and are independent of the wavelength used for analysis. The average values of duplicate experiments are shown in Table 1. The previously determined K value, at a room temperature of 23 °C, agrees well with the ones described in this table. It can be seen that K decreases as the temperature increases (Table 1). Thus, the temperature provides a negative contribution to the stability of the complex.[8]

From the results depicted in Table 1, the van't Hoff plot (ln K vs the inverse of temperature) was constructed (Figure 5B). Through eq 4, the enthalpy (ΔH) and entropy (ΔS) changes for the complexation of Fis with β-CD were evaluated from the slope and intercept, respectively. Table 2 presents these experimental thermodynamic parameters.

It is well-known that much more reliable thermodynamic data are obtained from calorimetric determinations.[8] Even so, the experimental variation of enthalpy shows a negative value (Table 2), denoting that the interaction between the host and the guest is exothermic. The complex dissociates as the temperature increases. This enthalpy variation could be a consequence of many factors related to the bond formation or breakage. For instance, the water molecules initially surrounding the organic compound are released and lose their ordering. The number of hydrogen bonds in the system decreases, resulting in an exothermic process. On the other hand, the water molecules that come out from the CD inner cavity could combine with the other water molecules present in the bulk of the solution. The number of hydrogen bonds between solvent−solvent molecules increases in an endothermic process. One factor can compensate another one.[8]

The experimental changes in entropy are also negative (Table 2), corresponding to a decrease in the rotational and translational freedom degrees of the free guest and host in solution. The entropy is not high since there are two opposite effects in action. Loss of rotational and translational degrees of freedom of the free guest and host in solution occur upon complex formation. At the same time, the highly ordered water molecules surrounding the flavonoid are released, thus increasing the system entropy. As these opposed effects are nearly equal, the variation in entropy is very small or equal to zero.[8]

The variation in the experimental free energy was calculated through the Gibbs equation, considering the standard temperature of 298 K. The negative ΔG value suggests that the process is spontaneous. The plot of ΔH versus ΔS for different guest molecules is seen as a linear relationship.[8] ΔH of the Fis−β-CD complex is large and ΔS is negative, indicating that complexation gives higher order to the system.

The theoretically obtained thermodynamic parameters[16] tend to confirm the trends evidenced experimentally (Table 2). For example, the calculated entropy values for the formation of the inclusion complexes are all negative, even though they are systematically higher than the experimentally determined ones. The reasons for these differences are due to the fact that the computational work did not take the interaction of the solvent molecules into account.

3.5. NMR Measurements. A series of ^{1}H NMR spectra in different solvents and NOESY experiments were performed, to aid the characterization of both Fis and the inclusion complex.[21] The assignment of the Fis ^{1}H NMR spectra has already been presented in $(CD_3)_2SO$,[22] in a CD_3OD/D_2O mixture,[23] and in pure CD_3OD,[24] and the data collected in this work[21] in this last solvent are fully consistent with the previously described ones. However, to our knowledge, this is the first report of Fis ^{1}H NMR in basic aqueous solution.

The purpose for recording ^{1}H NMR data in D_2O was to compare the chemical shift values of free and included Fis. Actually, in neutral aqueous media, the very poor Fis solubility precludes the acquisition of good NMR data. To overcome this problem, the NMR measurements were carried out in basic media, obtained by dissolving the samples in 30% $NaOD/D_2O$ solution or in D_2O after bubbling $NH_{3(g)}$ (pD ~11.5). Control experiments were done to probe the effects of the basicity on the ^{1}H spectra of β-CD.

In D_2O/30% NaOD, a drastic broadening of the CD signals was observed, thus preventing a satisfactory spectral assignment. This is in agreement with the theoretical result,[16,21] which showed that the structure of β-CD is highly distorted and an inadequate medium to study the interaction of the species under investigation.

In D_2O bubbled with $NH_{3(g)}$, the Fis ^{1}H NMR spectral profile is consistent with an ABX spin system, with two sets of signals corresponding to rings A and B (Scheme 1). In this case, two different samples were prepared for ^{1}H NMR measurements. In both cases, H5 (H5′) appears as a doublet, split by the coupling with H6 (H6′), which, in turn, appears as a double doublet, split by the strong coupling mentioned above and a long-range coupling with H8 (H2′). The absence of hydroxyl signals, foreseen in the theoretical spectra,[21] is due to the fast exchange of these protons with deuterium in solution. The comparison between the CD ^{1}H spectra in pure D_2O and in D_2O bubbled with $NH_{3(g)}$ shows that the spectra do not differ significantly.

Nevertheless, we observe that the Fis samples evolve with time. From preparation of the sample until acquisition of the NMR spectra there was a difference of 4 h between samples 1 and 2. It was observed that most δ values for sample 2 are shifted upfield when compared with the values reported for sample 1. It is known that the hydroxyl groups may undergo oxidation in a pH-dependent fashion, yielding the semiquinone

and quinone forms of the flavonoid, especially those containing a cathecol-like 3′,4′-dihydroxy substituent pattern on ring B (such as quercetin and Fis).[14,23,25] Thus, one possible explanation for the differences in the chemical shift values observed in samples 1 and 2 could be the presence of different species in solution: in the former case the deprotonated form of Fis is observed, whereas in the latter case, its semiquinone/quinone forms should be observed.

Another important feature is a significant variation in the integration values (*I*) of the signals of H8, H6′, and H2′ (lower than the expected value of 1) and in the pattern of multiplicity of the signals of H6′ and H2′, which coalesce into a multiplet in sample 2. The controlled deuteration of the aromatic rings of some flavonoids in $D_2O/D_3PO_4{\cdot}BF_3$ solutions has been described by Rasku and Wähälä;[22] in the particular case of Fis, the order of proton exchange was determined as H8 > H6′, H2′, H5′ ≫ H6, H5. They also comment on the possible occurrence of the deuteration process in basic media, once the excess base is able to deprotonate the aromatic rings, although in lower yields. Assuming that deuteration of some positions in rings A and B may take place under our experimental conditions, a decrease in the signal intensity of the partially substituted protons would be expected. In fact, the *I* values for protons H6, H5, and H5′ signals are 1.0, 1.0, and 0.96, respectively, but for protons H8 and H6′ + H2′ signals they are equal to 0.25 and 1.2, suggesting that the substitution of the latter set of protons occurs to some extent and is coherent with the previously reported order of reactivity toward deuteration.[22] Obviously, the relative deuteration of positions 6′ and 2′ would affect the coupling of the remaining protons, modifying the multiplicity of the observable signal.

Attempts to probe the presence of the inclusion compound of Fis and β-CD in $D_2O/NH_{3(g)}$ solution were made. In the presence of Fis, the spectrum of β-CD does not vary widely in terms of the chemical shift values for the internal protons H3 and H5. As a matter of fact, all the signals[21] presented values of $\Delta\delta$ in the range 0.02−0.04 ppm for β-CD. It is worth noting that these low $\Delta\delta$ values are not accompanied by any kind of change in the spectral profile, such as that observed for the signals of the internal protons when there was formation of an inclusion compound.[26,27] Similarly, the signals related to Fis presented average $\Delta\delta$ values of 0.02 ppm.

Finally, NOESY experiments for mixtures of both CDs and Fis were performed, and we were not able to observe the expected correlations between the internal protons H3 and H5 and the guest's protons in any of the cases. These findings suggest that the formation of the inclusion compound between Fis and β-CD does not occur to a significant extent so as to be detected under the NMR experimental conditions. In basic media (pD ∼11.5), taking the pK_a values of Fis[14] into account, at least two hydroxyl groups are deprotonated, yielding a charged Fis molecule. In such a case, the inclusion into the CD cavity would be an unfavorable interaction from the viewpoint of substituting the water solvation of the charged species by its interaction with a nonpolar medium. This is consistent with the lower formation constants determined in basic media when compared to the ones obtained in neutral/acid media (Table 1). In such a complex, Fis could be not totally included in the CD inner cavity, thus not interacting to the internal protons H3 and H5. Another supposition is that the dynamic of the complexation of Fis could be very fast under the time scale of the NMR technique, leading to no correlation between the CD and Fis protons. The last assumption is that, at the concentrations of Fis and CD employed, the concentration of the complex would be too low to be detected by NMR.

4. Conclusions

Fis and CD are known to be a potential therapeutic drug and a good drug delivery system, respectively. The former efficiency is related to its ability to reach and interact with a biological target through either a covalent bond or an ionic interaction, among others, depending on the structure of both the drug and the target. This work showed that the β-CD inner cavity provides an environment that enhances the Fis intramolecular excited state proton transfer forming its tautomer, a charged species. For this reason, in pharmaceutical formulations or in photodynamic therapy studies, it should be very important to determine preliminarily which form of Fis, the normal and uncharged structure or its tautomer, has more biological potential for therapeutic applications, since the latter will be induced in the presence of β-CD and irradiation of light. At neutral or acid pH, the preferential insertion mode of Fis into β-CD is via the phenyl group, although inclusion through the chromone part of the molecule is also possible as shown by the theoretical data.

This work is also the first report to describe Fis in alkaline conditions by the NMR technique. These measurements showed that Fis evolves with time, producing degradation species. Also, in basic media, the complexation of Fis with β-CD is unfavorable, because the flavonoid molecule is totally or partially deprotonated, making this pH totally inappropriate for biological applications.

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Supporting Information Available: A detailed description of the results derived from quantum-mechanical methods, and the assignment of the Fis and Fis−β-CD complex ^{1}H NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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(11) Details of the theoretical calculations are provided in the Supporting Information.

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