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Theoretical and experimental study of the inclusion complexes of ferulic acid with cyclodextrins

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The inclusion complexation behaviour of ferulic acid (FA) with β-cyclodextrin (β-CD) and hydroxypropyl-β-cyclodextrin (HP-β-CD) was investigated by UV–vis, fluorescence and ^{1}H NMR spectroscopy. Since the guest may exist in either anionic or neutral form, the experiments were performed at different pH values. The stoichiometry and association constants of the complexes were determined by nonlinear regression analysis. The phase-solubility studies indicated that the water solubility of FA was improved through complexation with β-CD and HP-β-CD. An increase in the antioxidant reactivity was observed when inclusion complexes that FA formed with CDs were studied. Based on the NMR data, the spatial configurations of FA/β-CD and FA/HP-β-CD complexes were proposed, which suggested that FA entered into the cavity of β-CD from the narrow side, with the lipophilic aromatic ring and ethylenic moieties inside the CD cavity, and the —COOH group was close to the wider rim and exposed outside the cavity. A theoretical study of the complexes using molecular modelling gives the results in good agreement with the NMR data.

Keywords: FA; cyclodextrin; fluorescence; NMR; molecular modelling studies

1. Introduction

Cyclodextrins (CDs) are doughnut-shaped cyclic oligosaccharides composed of six, seven or eight glucopyranose units (α-, β- and γ-cyclodextrins, respectively) (*1–4*). Due to their special structure, characterised by a hydrophobic guest exterior and an apolar cavity, the CDs are able to form inclusion complexes in aqueous solutions with a wide variety of molecules (*5*).

CDs have been used for various analytical purposes. In pharmaceutical product development, CDs have been widely used to enhance solubilities, chemical stabilities and bioavailabilities of a number of drugs, reduce toxicity or control the release rate, etc. (*6–9*). Moreover, CDs can be used as models to probe proteins and enzymes (*7*).

Ferulic acid (FA; Figure 1), namely 3-methoxy-4-hydroxy cinnamic acid, can readily form resonance-stabilised phenoxy radical due to its phenolic nucleus and an extended side chain conjugation, which account for its strong antioxidant potential (*10*). The ability to form stable radicals and to show a radical-scavenging activity (*10*, *11*) makes FA an interesting candidate for many industrial applications. As a well-known antioxidant of natural source with promising properties as photoprotective agent, its derivatives are under screening for the prevention of photo-induced skin tumours. Besides the various physiological protections against several diseases, FA may constitute the active ingredient in many skin lotions and sunscreens designed for photoprotection (*10*, *12*, *13*).

Due to its emission properties and its water solubility, FA is also suitable to be used as a protein marker. As a first step in this direction, we are interested in the sensitivity of its properties to the presence of a hydrophobic cavity in an aqueous medium. In this paper, we report the UV–vis, fluorescence and NMR spectroscopic characterisation of FA complexed with β-CD and HP-β-CD. The stoichiometry and the association constants of the complexes were determined and the spatial configurations of FA/β-CD and FA/HP-β-CD complexes were also proposed.

2. Experimental and theoretical methods

2.1 UV and fluorescence spectroscopy

Absorption and fluorescence measurements were performed with UV-757 CRT spectrophotometer (Shanghai Precise and Scientific Instrument Co. Ltd, Shanghai, P.R. China) and a F-4500 FL spectrofluorometer, respectively. Both excitation and emission bandwidths were at 10 nm. All experiments were carried out at room temperature.

The stock solution of 1×10^{-3} M FA was prepared by dissolving FA in water. The concentrations of β-CD and HP-β-CD were 1×10^{-2} M. Phosphate buffer solution was used to control the pH value of the media. All other reagents were of analytical-reagent grade and were used without purification. Doubly distilled water was used throughout.

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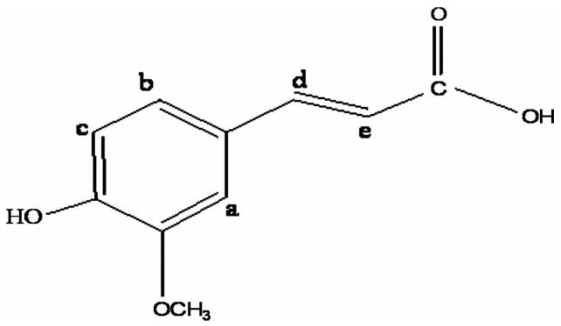

Figure 1. Chemical structure of FA.

2.2 Phase-solubility studies

Solubility studies were performed as described by Higuchi and Connors (*14*). With a standard procedure, excess amount of FA was added to a series of solutions that contained increasing amounts of β-CD and HP-β-CD. The mixtures were subjected to ultrasonic radiation for 1 h and equilibrated for another 7 days and then filtered. Sample absorbances were then measured. The phase-solubility profiles were obtained by plotting the solubility of FA vs. the concentration of CDs.

2.3 DPPH· radical-scavenging method

The antioxidant activities (AA) of free FA and FA/CDs were measured in terms of hydrogen-donating or radical-scavenging ability, using the stable radical DPPH·. The method, proposed by Brand-Williams et al. (*15*), was modified, depending on the strength and solubility of measured antioxidant. About 1 mg of FA was weighed and dissolved in 50 ml of water. For the determination of antioxidant activity, 0.5 ml aliquot of the solution was taken and vigorously mixed with 3 ml of ethanolic solution of DPPH· (10^{-5} M). After exactly 60 min of the reaction, the absorbance at 520 nm was measured in a 10 mm cuvette.

Blank sample was prepared in the following manner: 0.5 ml of distilled water was combined with 3 ml of DPPH· solution. The AA of FA/CDs complexes were measured in the same way except that FA/CDs complexes were used instead of FA, which was prepared by adding 3 ml of CDs solution (1×10^{-2} M) to 1 ml of the above-mentioned FA solution and then allowing equilibration for 30 min at 20 ± 1°C.

The results were expressed as percentage of DPPH· elimination calculated according to the following equation: $AU = [1 - A_s/A_0] \times 100\%$, where AU is the radical-scavenging activity, A_s is the absorbance of sample (FA and complexes) and A_0 is the absorbance of blank sample.

2.4 NMR measurements

The ^{1}H NMR spectra of FA and CDs as well as their inclusion complexes were recorded on a Bruker Avance DRX 300 MHz superconducting NMR spectrometer. The inclusion complexes were prepared by mixing 1×10^{-4} mol/l FA and 1×10^{-4} mol/l CDs solutions with a volume ratio of 1:1.

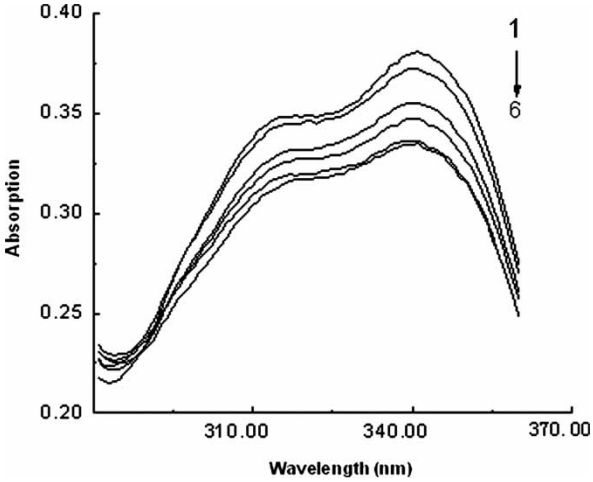

Figure 2. Absorption spectra of 1×10^{-5} mol/l FA in the presence of β-CD. The concentration of β-CD (M): $0–5 \times 10^{-3}$.

2.5 Molecular modelling of the inclusion complexes

The host–guest interaction was simulated by molecular modelling, using the consistent valence force field. The geometry of β-CD for our simulations was taken from neutron diffraction studies (*16*). *In vacuo* optimisations were performed using the Polak–Ribiere method and a gradient of 0.01 kcal/mol per Å. The salvation process was modelled using a cubic water box with dimensions of $(35)^3$ Å^3. For the solvent-dependent optimisations, a larger gradient value was used, namely 1 kcal/mol per Å. All the calculations were performed with the insight II 2000 program.

3. Results and discussion

3.1 UV spectroscopy

As illustrated in Figure 2, FA alone in water exhibits two absorption peaks at 283 and 310 nm in water. The absorption intensity of FA gradually increased with the stepwise addition of β-CD. This change might be partly attributed to the shielding of chromophore groups in the FA molecule due to the complex formation between FA and β-CD. With the increase in HP-β-CD concentration, the absorbance of FA also increased. However, the absorption peak red shifted to 290 and 315 nm. These results might also suggest the possibility of an interaction between FA and HP-β-CD as a result of a partial shielding of the chromophore electrons in the HP-β-CD cavity.

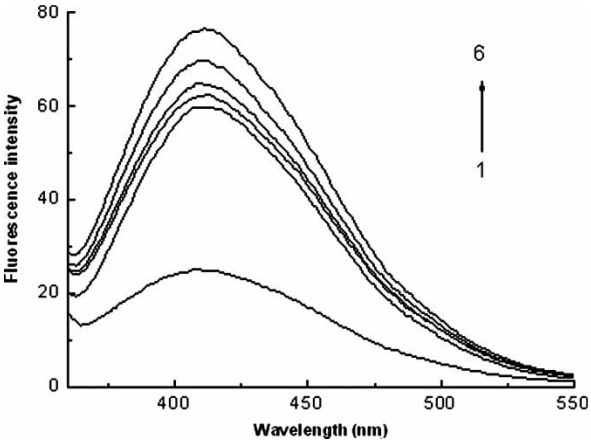

Figure 3. Fluorescence emission spectra of 1×10^{-5} M FA in the presence of β-CD. The concentration of β-CD (M): $0–5 \times 10^{-3}$. Excitation and emission wavelengths were at 310 and 330 nm, respectively.

3.2 *Fluorescence study*

As shown in Figure 3, the fluorescence intensity of FA increased with the increase of β-CD concentration with no noticeable peak shift. With an increase in the HP-β-CD concentration, however, the fluorescence intensity of FA was enhanced accompanied by a slight hypsochromic shift of the emission peak. These findings indicated the formation of FA–CDs inclusion complexes. The fluorescence enhancement of FA encapsulated by the CD cavity is a result of better protection from quenching and other processes occurring in the bulk solvent.

The formation constants (K) and the ratio of the complex were calculated from fluorescence data using the modified Benesi–Hildebrand equation:

$$\frac{1}{F - F_0} = \frac{1}{(Kk[P]_0[\mathrm{CD}]_0)} + \frac{1}{KQ[P]_0}$$

where F and F_0 represent the fluorescence intensities of FA in the presence and absence of CDs, respectively; $[P]_0$ and $[\mathrm{CD}]_0$ represent the initial concentrations of FA and CDs, respectively; k is an instrumental constant; K is the formation constant of the complex and Q is the quantum yield of the inclusion complex. The double reciprocal plots $1/(F - F_0)$ vs. 1/[CDs] for FA complexed with β-CD and HP-β-CD (shown in Figure 4) exhibit good linearity, implying that the inclusion complexes have a stoichiometric ratio of 1:1. The formation constants of FA/β-CD and FA/HP-β-CD complexes are 87 and 98 M^{-1}, respectively. Thus, the capability of HP-β-CD to form an inclusion complex with FA is higher than that of β-CD, probably due to its larger cavity size and higher hydrophobicity than that of β-CD.

Table 1. Binding constants (K) of the inclusion complexes at different pH values.

		pH		
Cyclodextrin	K	3.05	7.5	10.53
β-CD	87	102	205	–
HP-β-CD	98	128	590	93

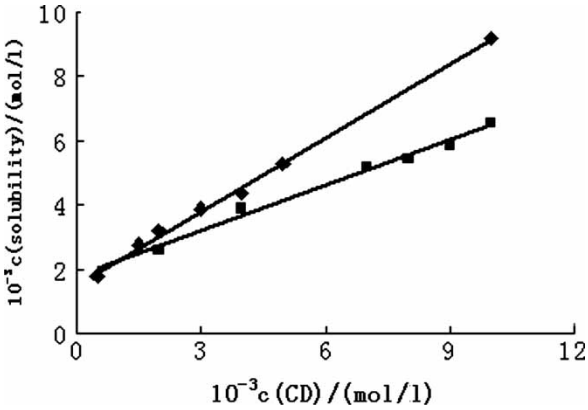

Figure 5. Phase-solubility profiles for FA/β-CD (◆) and FA/HP-β-CD (■).

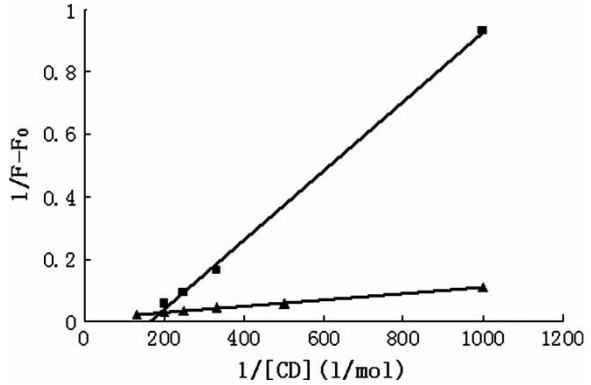

Figure 4. Double reciprocal plots for FA in the presence of β-CD (■) and HP-β-CD (▲).

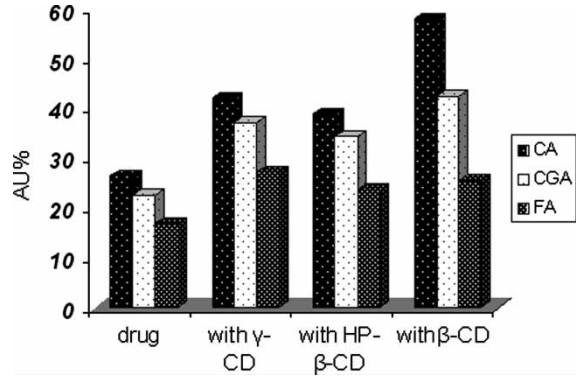

Figure 6. Scavenging of DPPH by free or complexed FA and its derivatives.

Table 2. Chemical shifts changes of β-CD and HP-β-CD before and after forming the inclusion complexes.

^{1}H assignment	δ β-CD free	δ β-CD/FA	Δδ	δ HP-β-CD free	δ HP-β-CD/FA	Δδ
H-3	3.728	3.683	−0.045	3.761	3.758	−0.003
H-5	3.633	3.565	−0.068	3.607	3.562	−0.045
H-6	3.633	3.626	−0.007	3.607	3.620	0.013

As different forms of FA are known to exist as a function of pH, the effect of pH on the formation constants of the FA–CD complexes was examined. As shown in Table 1, the formation constant values were very sensitive to pH and increased in the order $K_{7.5} > K_{3.05} > K_{10.5}$, revealing a selective interaction of CDs with different species of FA.

3.3 *Phase-solubility studies*

The phase-solubility diagrams of FA in β-CD and HP-β-CD solutions are shown in Figure 5. The solubility of FA was found to increase with increasing concentrations of β-CD and HP-β-CD. Both phase-solubility diagrams of FA with β-CD and HP-β-CD within the concentration range studied displayed a typical A_L-type diagram (*17*) ($r^2 = 0.9887$ and 0.9974), indicating a 1:1 molecular complex formation for both CDs (Figure 3).

HP-β-CD gave higher enhancement in the solubility of FA compared with β-CD. In the presence of 1×10^{-2} mol/l CD, a sixfold increase in the solubility of FA was observed with FA–HP-β-CD complex, whereas a threefold increase was observed for the FA–β-CD complex.

3.4 *Determination of antioxidant activity*

The antioxidant capacity of phenols is generally tested by the reaction with oxidants, where resonance-stabilised phenoxyl radicals occur. Antioxidant activity of phenolic compounds depends on the position and degree of hydroxylation, as well as the nature of radicals of the ring structure (*17*). It has been reported that the presence of the —CH═CH—COOH group increased the antioxidant activity of FA by participating in stabilisation of the phenoxyl radicals by resonance (*18*).

As illustrated in Figure 6, the antioxidant activity decreased in the order of caffeic acid > chlorogenic acid > ferulic acid, which was in good agreement with the results reported by Saskia (*19*). The AA of the tested substances were all increased by the formation of inclusion complexes with β-CD and HP-β-CD, which is probably related to the formation of intermolecular hydrogen bonds between the guest and CDs. The distance between the secondary hydroxyl group of CDs and the hydroxyl group on the aromatic ring of the compounds tested is approximately the same as the distance between the hydroxyl groups of the caffeic acid molecule (< 3.315 Å) (*20*), which makes the formation of an intramolecular hydrogen bond of the inclusion complexes possible.

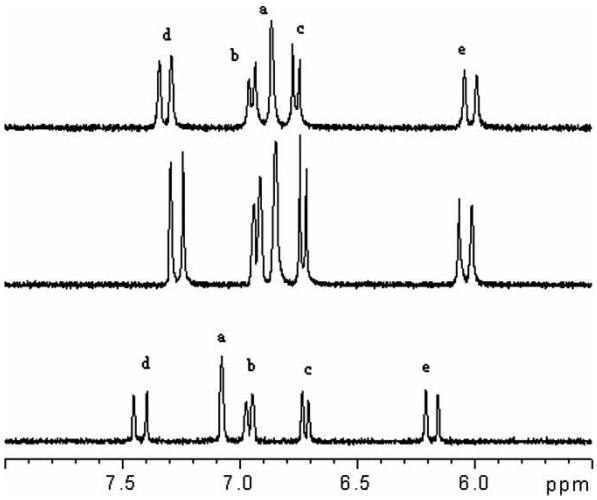

Figure 7. ^{1}H NMR spectra of samples: (a) FA/HP-β-CD, (b) FA/β-CD and (c) FA.

3.5 *^{1}H NMR*

It is well known that the insertion of a guest molecule into the hydrophobic cavity of cyclodextrin can affect chemical shifts of both the guest and host molecule protons. In fact, NMR spectroscopy has become a routine tool for the study

Table 3. Chemical shifts changes of FA before and after forming the inclusion complexes.

^{1}H assignment	δ FA free	δ FA/β-CD	Δδ	δ FA free	δ FA/HP-β-CD	Δδ
H-a	7.076	6.8490	−0.227	7.076	6.865	−0.211
H-b	6.947	6.914	−0.033	6.947	6.936	−0.011
H-c	6.734	6.745	0.011	6.734	6.774	0.040
H-d	7.398	7.214	−0.157	7.398	7.291	−0.107
H-e	6.208	6.068	−0.114	6.208	6.044	−0.164

of the CDs complexes that can provide not only quantitative information but also detailed information on the geometry of the complex (*21*). The interaction between FA and CDs was investigated by NMR spectroscopy. The effect of complexation on the chemical shifts of the internal and external protons of CDs in the presence of FA was observed. As shown in Table 2, the chemical shifts of H-3 and H-5, located inside the CD cavity, displayed a larger change in the presence of FA. These protons show a downfield shift due to the shielding effect exerted by the guest molecule. By contrast, the other CD protons located outside the cavity, namely H-1, H-2 and H-4, are relatively unaffected. Since H-3 protons are near the wide side of CD cavity while H-5 protons are near the narrow side, we can deduce that FA should penetrate into the CD cavity from the narrow side.

The resonances of the FA protons are also affected by forming inclusion complexes with CDs. As shown in Figure 7, a modification of the chemical shift values of the anisotropically shielded atoms were observed. The ^{1}H NMR spectrum of FA displayed five different types of proton signals, namely a-H, b-H, c-H, d-H and e-H. The hydrogen chemical shift values of free FA and those of the complex are listed in Table 3. When complexed with β-CD, high delta values ($\Delta\delta$) were observed for an aromatic proton a ($\Delta\delta = -0.227$) and protons d and e ($\Delta\delta = -0.157$ and -0.114) belonging to the adjacent —CH═CH— group. Hence, it is reasonable to postulate that the phenyl ring and ethylene side chain of the molecule, which is highly hydrophobic, must be deeply buried inside the lipophilic core of CDs. The most polar groups of the molecule, the —COOH, the phenol and methoxy residues are exposed outside the cavity. Similar results were obtained in the case of HP-β-CD.

3.6 Molecular modelling of the inclusion complexes

A molecular modelling study was performed to find out the most probable conformation of the FA/β-CD complex and to give a meaningful 3D visualisation of the complex. Four starting geometries of the complex were generated by docking the guest in the cavity. In model I, the hydrophobic part of FA was placed within the cavity with the —COOH group pointing towards the secondary rim of β-CD. In model II, the —COOH group was in an opposite orientation, pointing towards the primary rim of β-CD. In the other two models (III and VI), the orientation of β-CD was changed. In all approaches, an equatorial orientation of the guest was maintained (*22*), the axial orientation being disregarded as the fluorophore dimension overrides the cavity diameter. The most stable structure of the complex was searched by modifying the host–guest distance. The distance was varied in the range of -8 to 8 Å, with a step of 1 Å. In such a way, the guest molecule penetrates partially, totally and then goes over the cavity of the host. The first step of the optimisation process was performed imposing this distance as a restriction. Second, the restriction was removed and the complex was totally optimised.

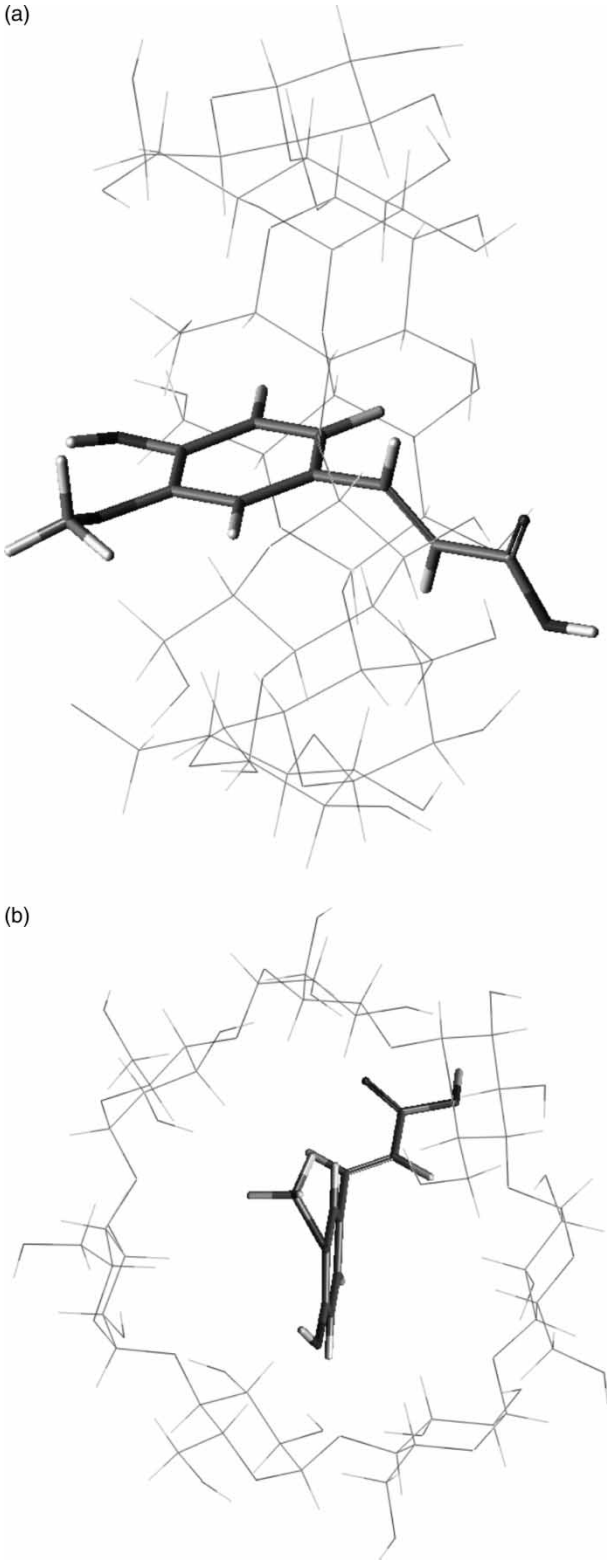

Figure 8. The most stable configuration of the 1:1 complexes of FA/β-CD.

The energy minimised structure (Figure 8) shows that FA penetrates into the CD cavity from the narrow side with the benzene ring and the portion of ethylene deeply embedded inside the lipophilic core of CDs, leaving the —COOH group close to the wider rim and exposed outside the CD cavity. This is in agreement with the NMR data.

4. Conclusion

The results from this study clearly demonstrated that both β-CD and HP-β-CD can form stable inclusion complexes with FA and therefore can be used for the encapsulation/release of this potent antioxidant. The association constants of the complexes, calculated from fluorescence data, revealed that the capability of HP-β-CD to form an inclusion complex with FA is higher than that of β-CD, probably due to its larger cavity size and higher hydrophobicity than that of β-CD. NMR analysis and molecular modelling demonstrated that the aromatic ring and the ethylene side chain of FA were embedded inside the cavity of CDs, leaving the more polar groups exposed outside the cavity.

Acknowledgements

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