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Non-covalent inclusion of ferulic acid with α-cyclodextrin improves photo-stability and delivery: NMR and modeling studies

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Abstract

Ferulic acid (FA) is a highly effective antioxidant and photo-protective agent, already approved in Japan as a sunscreen, but it is poorly suited for cosmetic application because of its low physicochemical stability. We prepared the inclusion complex of FA with α-cyclodextrin by co-precipitation from an aqueous solution, and used ^{1}H NMR and molecular dynamics to investigate the most probable structure of the inclusion complex. In rotating frame nuclear Overhouser effect spectroscopy (ROESY) experiments FA penetrated the α-CD hydrophobic cavity with the α,β-unsaturated part of the molecule and some of its aromatic skeleton. In proton chemical shift measurements of FA and α-cyclodextrins we determined the stoichiometry of the association complex (1:1) by Job's method, and its stability constant ($K_{1:1}$ $1162 \pm 140\,M^{-1}$) and described the molecular dynamics of the complex on the basis of theoretical studies. Encapsulation with α-cyclodextrin improves (i) the chemical stability of FA against UVB stress (10 MED [Minimal Erythemal Dose: 1 MED = 25 mJ/cm^2 for skin phototype II: 30]), since no degradation products are formed after irradiation, and (ii) the bioavailability of FA on the skin, slowing its delivery (Strainer cell model).

Keywords: Ferulic acid; α-Cyclodextrin; Inclusion complex; NMR; Molecular modeling; Photo-stability; Antioxidant activity; Delivery

1. Introduction

Ferulic acid (FA, 4-hydroxy-3-methoxycinnamic acid) is a phenolic acid ubiquitous in the plant kingdom, where it is one of the most abundant compounds in plants and plant-derived foods [1–3]. It has received much attention in Chinese medicine since it exerts several biological effects: cholesterol-lowering activity in animals (rats and hamsters) [4,5], antimicrobial and anti-inflammatory activities (COX-2 inhibition) [6], and plays a role in the prevention of thrombosis and atherosclerosis [7,8]. In addition it showed chemopreventive action against colon and rectal cancer and is now under investigation as an anti-proliferative agent [9]. Some of its lipophilic esters are being screened for the prevention of UV light-induced skin tumors [10].

As free radicals play an important role in the development of cancer, the anticancer effect of FA is partly attributed to its free-radical scavenging activity since it forms a resonance-stabilized phenoxyl radical; it does in fact quench a large array of free radicals: superoxide ion, alcoxyl, peroxyl and nitrogen dioxide radicals and prevents peroxynitrite-mediated attack of protein-bound and free tyrosine [11].

In view of all these promising properties, and the high degree of conjugated unsaturation (strong UV-absorption) and permeability on the skin, FA has been approved in Japan as a sunscreen [12].

UV light is an exogenous factor that can cause skin damage, resulting in both precancerous and cancerous skin lesions, and

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accelerating skin aging, through a mechanism involving free-radical formation. Therefore topical application of FA may be useful for preventing the different types of light-induced skin cancer, but its application on the skin is limited by the poor stability of the molecule, which under physical and thermal stresses can break down into inactive products [2]. The problem may be overcome by the use of cyclodextrins (CDs), which in the pharmaceutical field have been shown to form stable inclusion complexes with a variety of small lipophilic molecules through non-covalent interactions. For ingredients used in cosmetics, the formation of an inclusion complex can increase the stability of the active principle, and improve its solubility, bioavailability and delivery on the skin.

In a previous paper we reported the analytical characterization of the inclusion complex between FA and γ-CD by different analytical techniques, and tentatively indicated by ^{1}H NMR that FA can penetrate the γ-CD cavity [13]. Similar results were obtained by Qi et al. who, using spectrofluorometry, showed that FA binds to the cavity of γ-CD, but with a weak binding constant (707 M^{-1}) [14], so this cannot ensure a long lasting photoprotection against UVB stress (unpublished data).

Since α-CD has a hydrophobic cavity smaller in diameter than γ-CD (0.45 Å vs. 0.85 Å), and in the light of the fact that the size/shape fitted relationship and hydrogen bond interactions are vital for stability of the guest/host inclusion complex, this study carried our earlier work forward by assessing whether complexation of FA with α-CD improved the physicochemical stability of the inclusion complex.

In the first part of the study we obtained a detailed description of the geometry of the complex (obtained through ROESY experiments) and determined the complex stoichiometry and binding constant by investigating the H-f chemical shift of FA. We also did some theoretical investigations on the complex molecular dynamics (MD).

In the second part, we evaluated the suitability of α-CD for encapsulating FA in order to improve its light-stability and slow-down the delivery in cosmetic sunscreen preparations.

2. Experimental

2.1. Materials

All the organic solvents used were of analytical grade (Aldrich, Milan, Italy). FA was kindly supplied by Tsuno Rice Fine Chemicals (Wakayama, Japan). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and α-cyclodextrin (α-CD) were purchased from Aldrich (Aldrich, Milan, Italy). 2,2′-Azobis(2-amino-propane)-dihydrochloride (AAPH), *R*-phycoerythrin (*R*-PE) and 2′,7′-dichloro-dihydrofluorescein diacetate (DCFH-DA) were purchased from Molecular Probes (Molecular Probes, Rome, Italy). Deuterium oxide (D_2O), sodium 2,2,3,3-$^2H_4$-trimethyl-silan-propionate (TSP) and deuterium chloride (DCl) for ^{1}H NMR experiments were purchased from Sigma–Aldrich (Milan, Italy). The excipients for the emulsion were obtained as follows: tri-$C_{12–13}$ alkyl citrate (Cosmacol ECI) from Sasol Italy S.p.A. (Milan, Italy); cetearyl glucoside, cetearyl alcohol (Montanov 68) from Seppic S.A. (Paris, France); potassium cetyl phosphate (Amphysol K) from ResPharma (Trezzo sull'Adda, Milan, Italy); disodium ethylenediaminotetra-acetic acid salt (disodium EDTA) and triethanolamine (TEA) from BASF Italia Spa (Cesano Maderno, Milan, Italy); methylchloroisothiazolinone, methylisothiazolinone (Kathon CG) from ROHM and HAAS Italia S.r.l. (Mozzate, Como, Italy); imidazolydinyl urea (Gram 1) from Sinerga S.p.A. (Pero, Milan, Italy).

2.2. Preparation of the ferulic acid–α-CD complex

FA and α-CD were co-precipitated at different molar ratios (2:1 and 1:1) according to Chikuno and Terao [15]. A methanol solution of FA was added to CD dissolved in water and stirred for 24 h in the dark at room temperature. Once the reaction was complete, the precipitate was collected by filtration, dried in vacuum and washed with ether to remove residual FA.

The equimolecular mixture of FA and α-CD (1:1 molar ratio) was prepared by thoroughly mixing the two powders. Some analyses were done on the equimolecular mixture in parallel with the co-precipitate.

2.3. HPLC analysis

HPLC analysis was done as previously reported [13,16]. In these conditions the retention time of FA was 10.5 min and there was no interference from α-CD.

2.4. X-ray diffraction (XRD) analysis

Powder X-ray diffraction (PXRD) patterns were obtained with a PW 1710 Philips diffractometer (Lelyweg, The Netherlands) using Ni-filtered Cu Kα radiation ($\lambda = 1.5418$ Å) over the interval 2–45°/2θ, in the following conditions: target Cu; filter Ni; voltage 40 kV; current 20 mA; time constant 4 s; angular speed 1°/min (2θ); 1°, −0.1° and −1° slit; angular range $2° < 2\theta < 45°$. Analyses were run in duplicate on pure α-CD, FA, the association complex and the α-CD/FA physical mixture.

2.5. ^{1}H NMR analysis and determination of complex stoichiometry and stability constant ($K_{1:1}$)

Spectra were recorded using a Bruker DRX Avance 600 spectrometer operating at 14.1 T. The NMR data were processed with a SGI O2 R5000 workstation and the Bruker XWinNMR v2.1 software. Chemical shifts were measured from TSP used as external standard (to avoid TSP signal shifting upon interaction with α-CD) with resonance set at 0 ppm. All the spectra were recorded in 99.96% D_2O at 298 ± 0.1 °C.

^{1}H NMR spectra were acquired with a spectral width of 6 kHz and 32,768 data-points (acquisition time 2.730 s), a 90° pulse of 9.0 μs, relaxation delay of 2.0 s and 256 transients. The residual water signal was suppressed by pre-saturation during the recycling delay. FA and α-CD resonances were assigned using 2D NMR correlation spectroscopy (COSY) spectra. The COSY spectrum was recorded in the phase-sensitive mode, over a spectral width of 6000 Hz, relaxation delay 1.0 s, 1024 points in F2

dimension and 256 points in F1 dimension which was zero-filled once before processing. The spectrum was acquired with 8 transients for each of the 256 increments. Spin-lattice relaxation rates were measured by inversion recovery pulse sequences. The same sequence was used to measure single-selective relaxation rates with suitably shaped π-pulses instead of the usual non-selective π-pulse. All rates were calculated by regression analysis of the initial recovery curves of longitudinal magnetization components leading to errors not greater than ±2%.

For stoichiometric analysis, all the 1D proton spectra were acquired with a spectral width of 6000 Hz and 16,384 datapoints. Depending on the sample concentrations, the number of scans ranged from 8 to 128 with 3 s relaxation delay.

All samples were adjusted to pH 4.0 using a minimal amount of DCl and pH was measured with an Orion 720A pH-meter (Orion, Cambridge, England) coupled to a glass microelectrode calibrated on a standard buffer. The pH was not corrected for isotopic effects and was considered within ±0.05 confidence.

To determine the complex stoichiometry, the continuous variation method (Job plot) [17] was employed, based on the differences in the chemical shift of FA in the presence of α-CD, $\Delta\delta_{obs} = \delta - \delta_0$, where δ represents the shift in the presence of α-CD, and δ_0 the shift attributed to the free FA in the absence of the CD. FA solution (3 mM) and α-CD solutions (3 mM) were prepared in D_2O and mixed, keeping the total molar concentrations of FA and α-CD at 3 mM. Chemical shifts were then recorded at molar fractions of FA from 0.2 to 0.8 (0.1 increments), and chemical shift changes were plotted against r. $r = m/(m+n)$ is the molar FA fraction and m and n the stoichiometric coefficients of FA and α-CD in the complex. An r value of 0.5 indicates a 1:1 inclusion complex stoichiometry (i.e. $m = n = 1$). For any other ratio of the components, r is less than 0.5. For the determination of the association constant ($K_{1:1}$), the concentration of FA was kept at 0.2 mM and that of α-CD was raised from 0 to 2.4 mM. The change in the chemical shift of the H-f proton of FA was monitored during the titration.

Data analyses for stoichiometry and binding constant determination were done using GRACE software running on a Linux workstation.

2.6. Molecular modeling

FA and α-CD structures were prepared in their optimal geometric conformation (*in vacuo*) using a molecular mechanics approach, running the conjugate gradient algorithm up to better than 10^{-3} kcal/mol Å. The FA fragment was then placed inside the α-CD cavity and the geometry of the complex was minimized with the assumption of an effective dielectric medium, setting the dielectric constant at $\varepsilon = 78$, in order to simulate the correct solvent value (water). The MD of the complex was then simulated in water (1416 TIP3P molecules), using periodic boundary conditions (30 Å × 30 Å × 30 Å periodic box) and with 1 atm pressure and temperature 300 K, controlled by a Berendsen thermostat. The dynamic equations were integrated with the Verlet algorithm with a constant time step of 1.0 fs. The MD run lasted 1.0 ns and the instantaneous coordinates (or frames) were periodically saved for further analysis or geometry optimization. Within these runs, the total and potential energy initially decreased, then fluctuated around a constant value, indicating that a state of equilibrium had been reached. Each frame collected during the MD run was then minimized up to a gradient of less than 1×10^{-3} kcal mol^{-1}.

All simulation steps, including data analysis, were done with the NAMD package (developed by the Theoretical and Computational Biophysics Group in the Beckman Institute for Advanced Science and Technology at the University of Illinois at Urbana-Champaign [18]), using a modified version of the AMBER force field [19–21] on a dual-PIII workstation, working under LINUX RedHat 7.3 (SGI-SMP kernel version 2.4.18-4SGI-XFS-1.1).

2.7. Photo-stability

Photo-stability was evaluated using an oil/water emulsion containing 3% of FA or an equal amount of the antioxidant included in α-CD. The emulsion composition was tri-$C_{12–13}$alkyl citrate (15%), cetearyl glucoside, cetearyl alcohol (5%), potassium cetyl phosphate (0.5%), methylchloroisothiazolinone, methylisothiazolinone (0.02%), imidazolydinyl urea (0.3%), disodium EDTA (0.15%), distilled water, FA or FA/α-CD complex. FA dissolved in water/TEA (150 mM), or its CD complex dispersed in water, was added to the cooling phase of the emulsion preparation at about 40 °C. A portion (10–15 mg) of the emulsion containing free or complexed FA was layered onto a quartz lamina.

Samples were irradiated using a solar simulator (Universal Arc Lamp Housing model 66000 and Arc Lamp Power Supply model 68805, LOT ORIEL Italia, Milan, Italy) with a xenon arc lamp calibrated with a radiometer (Goldilux Smart Meter model 70234, LOT ORIEL Italia, Milan, Italy) equipped with a UVB probe. The lamp was calibrated before each determination. Samples were kept 40 cm from the lamp, irradiated with 300 mJ/cm^2, 10 MED (Minimal Erythemal Dose), and analyzed by HPLC as described.

2.8. Antioxidant activity: ORAC assay

The ORAC assay was carried out as described by Cao et al. [22]. Peroxyl radicals were generated by thermal decomposition of the radical initiator AAPH, and *R*-PE (16.7 nM in acetate buffer 75 mM adjusted to pH 5.5) was used as fluorescent oxidation probe. Trolox 20 μM was used as control standard. All fluorescence measurements were made using a Victor^2 multiwell reader (Perkin Elmer Life Sciences, Milan, Italy) thermostated at 37 °C. The excitation filter was set at 485 nm and emission filter at 535 nm. The analyzer was programmed to record the fluorescence of *R*-PE every 5 min for 3 h after addition of AAPH (3 mg/mL). All compounds were dissolved in acetate buffer then added to the *R*-PE. Blanks were all the reagents except the test compound.

The results were calculated as described by Cao et al. and expressed as Trolox equivalents (μM) [22]. FA and the FA/α-CD complex were tested for antioxidant activity in the concentration range from 2.5 to 10 μM.

2.9. FA release

The release of FA alone or complexed with α-CD from an oil/water (o/w) emulsion was evaluated using a modification of the method proposed by Casolaro et al. [23]. Briefly, 2.0 g of the o/w emulsion containing FA (5%) free or complexed with CD were layered on the bottom of a Strainer cell, and placed in close contact with the surface of 20 mL of phosphate buffer at pH 5.5. The buffer was gently stirred (300 rpm) and 0.5 mL were taken at each time point (0, 5, 15, 30, 60, 120, 240, and 420 min), diluted to 5 mL and analyzed by HPLC. After each withdrawal 0.5 mL of fresh buffer was added to keep the volume constant. Analyses were run in triplicate and the results were corrected for the dilution effect.

2.10. Statistical analysis

The unpaired Student's *t*-test was used (Instat, Graphpad Software, San Diego, CA). *P*-values <0.05 were considered significant.

3. Results and discussion

3.1. X-ray diffractometry

Before the XRD study, we ran a set of differential scanning calorimetry (DSC) experiments which showed there was a tight interaction between FA and α-CD, confirming that co-precipitation gives an association complex (data not shown).

The XRD pattern of FA showed intense, sharp peaks that prove the crystalline nature of the compound. The inclusion complex of FA with α-CD gave a large, broad background under the crystalline peaks indicating the formation of a significant amount of amorphous material (Fig. 1B). The peaks corresponding to 10.5°, 12.5°, and 14.5° (2θ) in α-CD (Fig. 1A) were less intense in the inclusion complex, proof for the intimate changes at lattice level during inter-penetration of the two substances. There was a new peak at 7.9° in the diffractogram of the inclusion complex.

Since DSC and XRD data provide only limited information on the supra-molecular structure, and no unequivocal proof of the inclusion of FA in α-CD, we focused on the ^{1}H NMR analysis of the association complex, in order to gain further information on its solution structure.

3.2. ^{1}H NMR studies

α-CD cavity protons, in the presence of FA, were assigned with the help of the COSY spectral data. The α-CD cavity protons, namely H-3 and H-5, showed significant changes in their resonance frequencies in the presence of FA, which moved upfield with increasing concentration of FA, while other α-CD protons showed negligible downfield frequency shifts, with the exception of H-4 (Table 1 and Fig. 2), very likely due to the local anisotropic effect induced by the aromatic conjugated electronic system of FA (a similar effect was previously observed for the FA/γ-CD complex [13]). The resonance shift data for all the α-CD protons, in the presence of FA, are given in Table 1. The

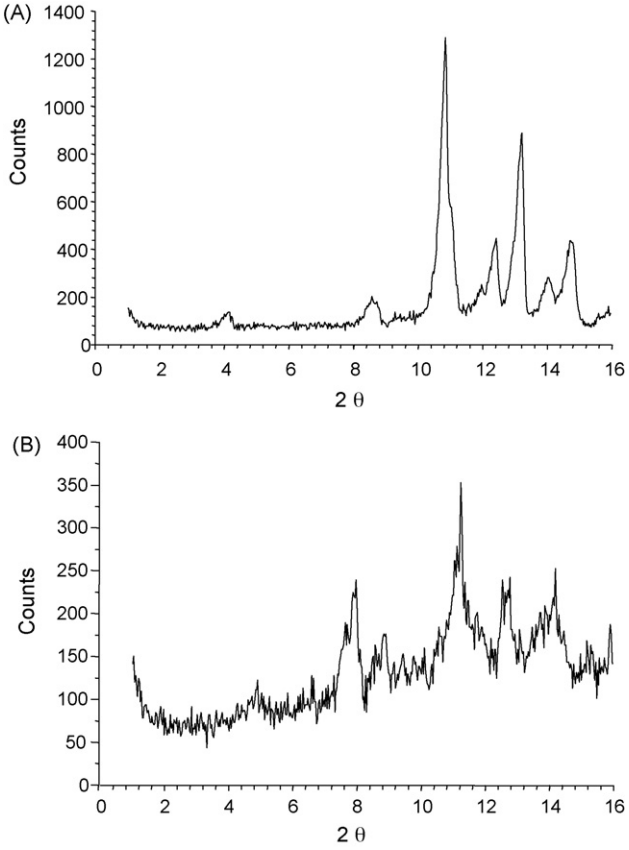
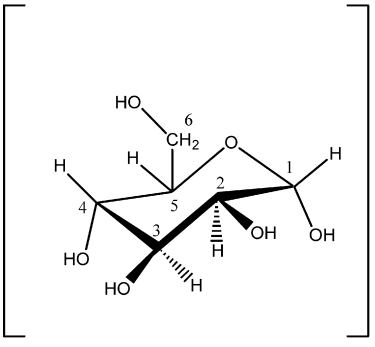


Fig. 1. Powder XRD analyses: α-CD (A) and ferulic acid/α-CD complex (B).

Table 1
Chemical structure of α-CD and ^{1}H NMR chemical shifts of the protons of α-CD, free and after complexation with FA (3.0 mM, 1:1 complex), in D_2O at 298 K



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Proton	δ_{free} (ppm)	δ_{bound} (ppm)	$\Delta\delta = \delta_{bound} - \delta_{free}$ (ppm)
H-1	5.01	5.02	0.01
H-2	3.58	3.60	0.02
H-3	3.92	3.86	−0.06
H-4	3.55	3.59	0.04
H-5	3.83	3.84	0.01
CH_2-6	3.86	3.85	−0.01

Chemical shifts are referenced to internal TSP.

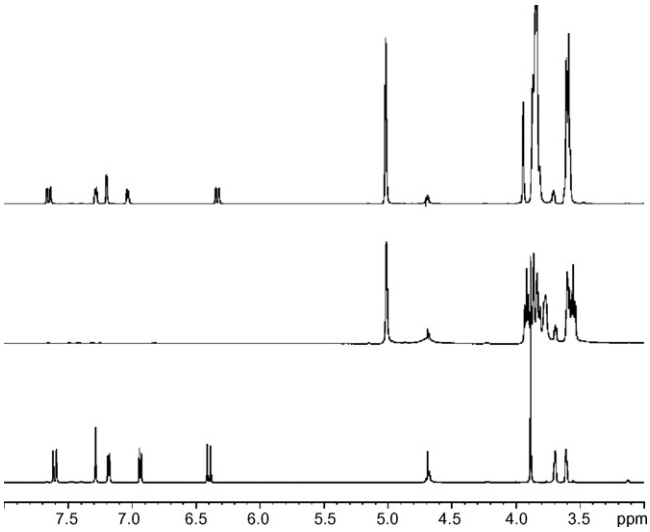


Fig. 2. ^{1}H NMR spectra of FA (lower), α-CD (middle) and ferulic acid/α-CD complex (upper) in D_2O.

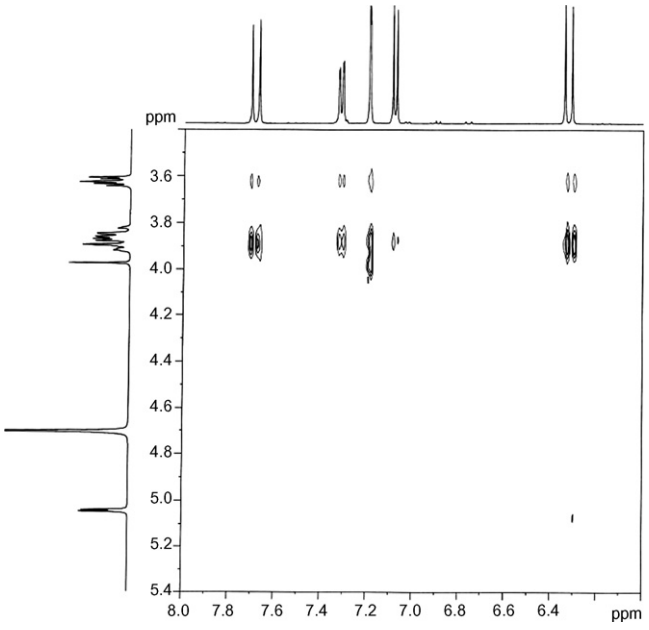


Fig. 3. ROESY spectrum of a solution containing the 1:1 FA/α-CD inclusion complex (7.0 mM, D_2O, pH 4.5).

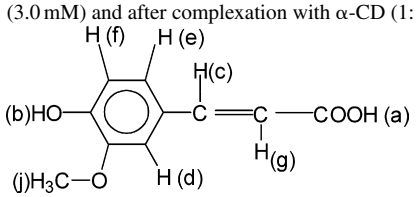
upfield frequency shift of the H-3 and H-6 protons and downfield shifts of H-5 in the α-CD cavity is an unequivocal indicator that FA fits deeply inside α-CD, resulting in the formation of the FA/α-CD inclusion complex, in accordance with previous reports [24].

All the FA protons showed significant frequency changes in the presence of α-CD (Table 2). The signals of H-d and H-g displayed upfield frequency changes, while the rest of the proton resonances moved downfield. Regarding the magnitude of the chemical shift changes, there was an important shift at the level of H-f that was not present in the previous FA/γ-CD inclusion complex studied [13]. These different interactions suggest a different type of inclusion of the molecule inside the cavity or, as reported elsewhere, the inclusion of the guest might cause distortion of the host [25].

The movements of these proton signals suggested that the penetration of FA involves insertion of the α,β-unsaturated portion and part of the aromatic skeleton of FA inside the cavity, with the phenol and methoxyl groups projected outside the wider rim. This interpretation is supported by the strong H-c, H-d, H-f and H-g cross-peaks with H-3, H-5 and H-6 protons of the annular interior of α-CD in the rotating frame nuclear Overhouser effect spectroscopy (^{1}H ROESY) spectrum (mixing time 0.5 ms) of the inclusion complex (Fig. 3), which indicates that these protons are located inside the α-CD cavity. No cross-peaks were observed for the methoxyl protons and the α-CD protons and this is consistent with the location of the methoxyl group in proximity to either the primary or secondary rim of α-CD.

The other approach was to check FA binding to α-CD by estimating the selective and non-selective proton spin-lattice relaxation rates of the ligand [26]. The ^{1}H spin-lattice relaxation rates of FA and FA/α-CD are summarized in Table 3. FA protons experienced interacted differently with the macromolecule. The data suggest a strong hydrophobic interaction in the formation of the inclusion. *F* ratios suggest a slowing of the re-orientational dynamics of the ligand FA when going from the free to the bound state, as in the inclusion with α-CD.

Table 2
Chemical structure of FA and ^{1}H NMR chemical shifts of the protons of FA, free (3.0 mM) and after complexation with α-CD (1:1 complex) in D_2O at 298 K



Proton	δ_{free} (ppm)	δ_{bound} (ppm)	$\Delta\delta = \delta_{bound} - \delta_{free}$ (ppm)
H (c)	7.60 (d)	7.65	0.05
H (d)	7.28 (s)	7.20	−0.08
H (e)	7.18 (d)	7.28	0.01
H (f)	6.93 (d)	7.03	0.10
H (g)	6.40 (d)	6.33	−0.07
CH_3 (j)	3.88 (s)	3.94	0.06

Chemical shifts are referenced to internal TSP.

Table 3
^{1}H NMR non-selective (R_1^{nsel}) and selective (R_1^{sel}) spin-lattice relaxation rates and *F* ratios for selected protons of FA free and bound to α-CD in D_2O at 298 K

	R_1^{nsel} (s^{-1})		R_1^{sel} (s^{-1})		$F = R_1^{nsel}/R_1^{sel}$	
	Fer	Fer + α-CD	Fer	Fer + α-CD	Fer	Fer + α-CD
H_a	–	–	–	–	–	–
H_b	–	–	–	–	–	–
H_c	0.84	0.98	0.80	1.47	1.05	0.67
H_d	0.85	1.03	0.77	1.34	1.10	0.77
H_e	0.64	0.94	0.68	1.31	0.94	0.72
H_f	0.52	0.69	0.55	0.72	0.95	0.96
H_g	0.50	0.82	0.54	0.97	0.93	0.85
H_j	1.27	–[a]	1.21	–[a]	1.05	–

[a] In the bound state, the peak of CH_3 in *j* position is overlapped, with some α-CD resonances.

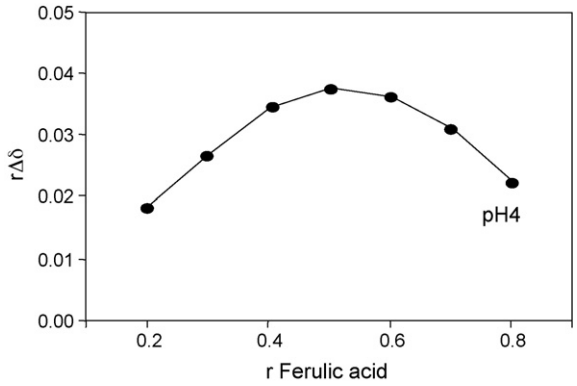


Fig. 4. Job plot of proton H-f at pH 4.0 at 298 K.

3.3. Stoichiometry and binding constant

An NMR study was done to define the stoichiometry and the association constant of the FA/α-CD complex. Stoichiometry was investigated at 298 K and pH 4.0. The proton spectra gave only one set of signals, whose chemical shifts were significantly different from those of FA and α-CD. This indicates that the FA/α-CD association/dissociation equilibrium is faster than the ^{1}H NMR time-scale. Hence the stoichiometry of the complex can be calculated by Job's continuous variation analysis [17], following the changes in the chemical shift of the H-f of FA (Fig. 4).

At pH 4.0 the change in chemical shift reached maximum at an r value of 0.5 (Fig. 5); $r = m/(m+n)$, m and n being the portions of FA and α-CD in the complex, indicating 1:1 stoichiometry. From the data fitting in the ^{1}H NMR titration experiments, we calculated a $K_{1:1}$ using the following equation [27]:

$$\Delta\delta_{obs} = \left\{ \frac{K_{1:1}(\Delta\delta_c C_{\alpha\text{-CD}} - \Delta\delta_{obs} C_f)}{[\Delta\delta_c + K_{1:1}(\Delta\delta_c C_{\alpha\text{-CD}} - \Delta\delta_{obs} C_P)]} \right\} \Delta\delta \qquad (1)$$

where $\Delta\delta_{obs}$ is the change in chemical shift of a FA proton observed on varying the concentration of α-CD, $C_{\alpha\text{-CD}}$; C_f is the concentration of FA, which is maintained constant during the titration experiment; $\Delta\delta_c$ is the chemical shift difference of a FA proton in its free and bound states. The concentration of FA was kept at 0.2 mM during titration while that of α-CD was raised from 0 to 2.4 mM. Nine different measurements were made, which were then used to derive $K_{1:1}$ by non-linear regression analysis of $\Delta\delta_{obs}$ and $C_{\alpha\text{-CD}}$ using Eq. (1).

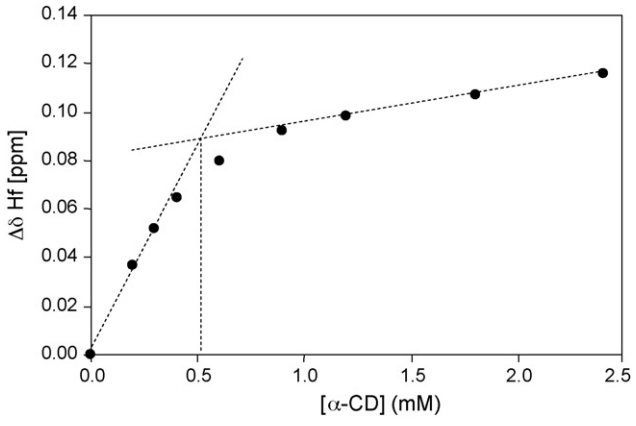


Fig. 5. Non-linear regression fitting of experimental data for H-f FA proton at pH 4.0 and 298 K.

The graphic data fitting for the H-f proton of FA is shown in Fig. 5. Once $K_{1:1}$ was calculated ($1162 \pm 140\ M^{-1}$), the fraction of complexed FA was determined following the assumption [28,29] that

$$\left[\frac{FA}{\alpha\text{-CD}}\right] = (D - (D^2 - 4K^2 C_{FA} C_{\alpha\text{-CD}})^{1/2})(2K)^{-1} \qquad (2)$$

where $D = KC_{FA} + KC_{\alpha\text{-CD}} + 1$. By substituting $C_{FA} = C_{\alpha\text{-CD}} = 1.5$ mM, the midpoint in the Job plot, n, the fraction of complexed FA, can be obtained, as $n = [FA/\alpha\text{-CD}]/C_{FA}$ [3]. The n value was 0.48.

3.4. Molecular modeling

To find the most probable conformation of the inclusion complex, we studied its solution structure by MD simulation in water. There was a significant initial adsorption of the guest, accompanied by conformational rearrangements. The lowest energy minima, corresponding to the most stable state found by this procedure, is set as zero for the FA/α-CD complex, and in this condition the interaction energy was −1634.21 kcal/mol taking into account the following equation: $E_{int} = (E_{FA} + E_{\alpha\text{-CD}}) - E_{tot}$, where E_{tot} is the energy of the FA/α-CD complex, E_{FA} is the energy of the free, isolated FA, $E_{\alpha\text{-CD}}$ is the energy of the free α-CD and $E_{int} > 0$ is the energy released by the acid upon adsorption inside the α-CD cavity.

The MD trajectory of the complex (not shown) confirms that FA binds to the α-CD cavity with the aromatic ring, since this interaction is the most favored one from the energy point of view. In particular, the accommodation of the guest inside the cavity forces the α,β-unsaturated carboxyl moiety and part of the aromatic skeleton of FA in the α-CD interior near the narrow rim, while the phenol and methoxyl groups are left outside the wider rim (Fig. 6), according to the NMR data.

3.5. HPLC analysis

Quantitative analysis on the FA/α-CD complex showed that 15.1 ± 0.25% of FA was included.

3.6. Antioxidant activity

The results reported in Fig. 7 indicate that at all concentrations tested, the FA/α-CD complex had less antioxidant potency than the equimolecular mixture of FA and α-CD or FA alone. These results confirm that in the inclusion complex FA is firmly encapsulated within the α-CD cavity, and that its phenol group, responsible for quenching peroxyl radicals, although close to the surface of the wider rim of α-CD (Fig. 6), as indicated by our molecular modeling studies, is free to interact by hydrogen bonding with the neighbouring hydroxyl groups of α-CD, and is consequently less available for the interaction with peroxyl radicals.

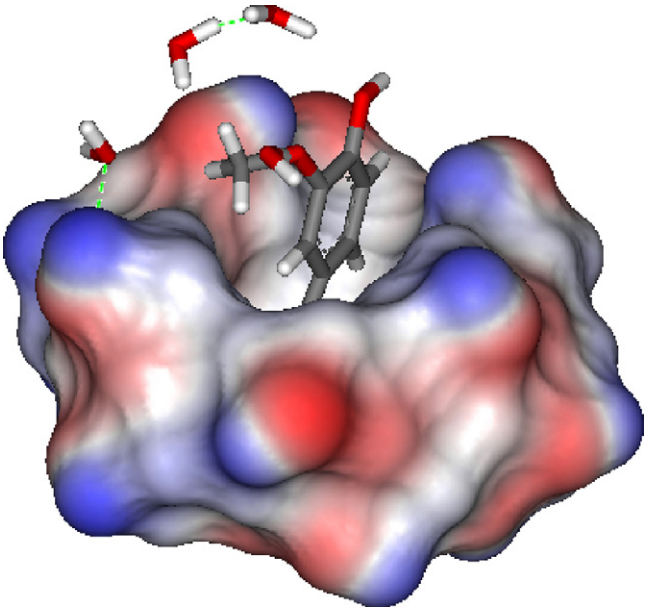


Fig. 6. Molecular modeling of ferulic acid/α-CD inclusion complex.

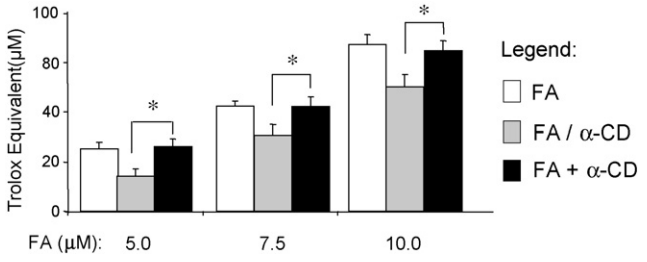


Fig. 7. Antioxidant protective effect of FA, FA/α-CD complex and of their physical mixture at different concentrations against free-radical stress. Data are mean ± S.D. of four experiments, expressed as Trolox equivalent (TE, μM). $^{*}P < 0.05$.

3.7. Photo-stability

As indicated by the qualitative profile and the HPLC data (Fig. 8 and Table 4), the exposure of an o/w emulsion containing FA alone to 10 MED irradiation (1 MED = 25 mJ/cm^2 for skin phototype II [30]), results in its partial conversion to the *cis*-isomer. This degradative pathway does not take place when FA is included in α-CD, where there is only one sharp, distinct peak with the retention time of native FA (10.033). This indicates that inclusion makes FA insensitive to this level of UVB stress. By contrast, complexation with γ-CD does not ensure the same degree of FA protection (data not shown). The improvement in stability of the sunscreen in complexed α-CD is extremely important for long lasting skin protection and ensures safer exposure of the consumer to UV rays.

Table 4
FA content (free or complexed with α-CD) in an o/w emulsion before and after 10 MED irradiation

Sample	% FA before irradiation	% FA after irradiation
Oil/water emulsion containing free FA	2.73 ± 0.25	1.90 ± 0.27
Oil/water emulsion containing the FA/α-CD complex	2.73 ± 0.21	2.82 ± 0.11

Table 5
Release of free and α-CD complexed FA from an o/w emulsion: Strainer cell model

Time (min)	Release (%)	
	Free FA	FA/α-CD
0	0	0
5	5.6 ± 0.5	1.2 ± 0.2
15	12.2 ± 1.4	2.6 ± 0.62
30	16.9 ± 1.3	4.6 ± 1.2
60	22.3 ± 1.6	7.0 ± 0.5
120	31.7 ± 5.3	10.4 ± 0.4
240	42.7 ± 4.4	16.0 ± 3.2
420	53.1 ± 6.2	20.6 ± 4.5

3.8. Delivery

The results reported in Table 5 indicate a time-dependent linear release of FA, alone or complexed with α-CD, from the o/w emulsion but to a different extent on account of the tight insertion of FA inside the α-CD cavity, in accordance with previous

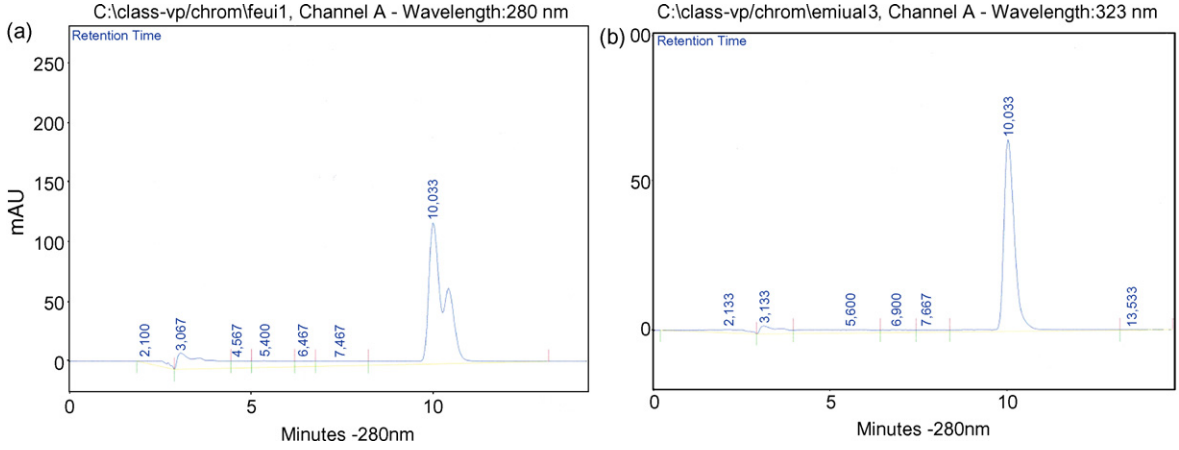


Fig. 8. HPLC profile after 10 MED UVB irradiation of an o/w emulsion containing (a) 3% of free FA and (b) FA complexed with α-CD.

observations for the antioxidant activity. The FA released from the complex was practically negligible within the first observation times (5–30 min), slightly increasing thereafter. By contrast, the release of FA alone was already appreciable within 0–5 min, and after 7 h it reached more than 50% (compared to 20% from the complex). This indicates that the formulation, used on the skin, will release FA more slowly as it comes into contact with the skin's biological fluids.

4. Conclusions

The FA/α-CD complex, its stoichiometry and association strength were assessed by NMR. On the basis of the findings of the ROESY experiments and subsequent dynamic simulations we propose that the insertion of the guest into the lipophilic interior of α-CD involves basically the COOH and α,β-unsaturated groups and part of its aromatic moiety. The phenol and methoxyl groups of FA lie on the plane of the wider rim.

The complexation of FA with α-CD results in a 1:1 inclusion complex with satisfactory stability ($K_{1:1}$ $1162 \pm 140\,M^{-1}$), a value close to that previously found by Qi et al. using a fluorescence spectroscopy approach (Ks $1113\,M^{-1}$) [14].

From the applicational viewpoint, this encapsulation increased the photo-stability of FA and consequently of an o/w emulsion containing the complex. In fact there was no formation of the *cis*-isomer of FA (which is less active as a sunscreen agent than *trans*-isomer) and other degradation products [2]. In addition it slowed FA release from the same emulsion in the Strainer cell model (20% release from the complex compared to 50% from free FA after 420 min).

These findings, if extrapolated to *an vivo* situation, suggest that the topical application of a cosmetic formulation containing the FA sunscreen complexed with α-CD, to prevent photodamage, will ensure safer and longer-lasting protection of the skin against solar radiation.

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