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# Analytical characterization of a ferulic acid/γ-cyclodextrin inclusion complex

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**Abstract**

Ferulic acid (FA) is a well-known antioxidant of natural source with promising properties as photoprotective agent (approved in Japan as sunscreen) and its derivatives (alkyl ferulates) are under screening for the prevention of photoinduced skin tumours.

In the present work we describe the preparation of a solid inclusion complex between ferulic acid and γ-cyclodextrin (γ-CD) and its characterization by different analytical techniques: differential scanning calorimetry (DSC), X-ray diffractometry (XRD), nuclear magnetic resonance spectroscopy ($^1$H NMR) and by supporting information of molecular modelling. All these approaches indicate that ferulic acid is able to form an association complex with γ-CD but only $^1$H NMR and molecular modelling studies give an unequivocal evidence that the antioxidant molecule is embedded into the γ-CD cavity to form an inclusion complex. In detail it is entrapped inside the hydrophobic core of γ-CD with the lipophilic aromatic ring and the ethylenic moieties, leaving the more polar functional groups close to wider rim or outside the cavity.
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*Keywords:* Ferulic acid; γ-CD inclusion complex; DSC; XRD analyses; $^1$H NMR; Molecular modelling studies

## 1. Introduction

Cyclodextrins are untoxic cyclic oligosaccharides, consisting of (α-1,4)-linked α-D-glucopyranose units, with a hydrophilic outer surface and hollow hydrophobic interior. The most abundant natural cyclodextrins are α-cyclodextrin (α-CD), β-cyclodextrin (β-CD) and γ-cyclodextrin (γ-CD) containing six, seven and eight glucopyranose units, respectively [1–4]. Cyclodextrins are able to form water-soluble inclusion complexes with many lipophilic water-insoluble drugs and for this reason are reported as complexing agents able to improve the physico-chemical properties of chemicals. In the case of cosmetic ingredients, aqueous solubility, chemical stability and skin delivery are greatly improved [4–7]. The most probable mode of binding involves the insertion of the lipophilic portion of the guest molecule into the cavity of the host and displacement of the water molecules located inside the cavity [2]. It has been demonstrated for a well-known sunscreen (ethylhexyl methoxycinnamate) that the cyclodextrin inclusion complex ensures a long-lasting protection of the skin against solar radiation well greater than that of conventional formulations. This since solar irradiation could induce photodegradation of the sunscreen agents and decreases their photoprotective power [8]. In this context it has been proposed that cyclodextrins act as sunscreen reservoirs on skin surface [9].

Ferulic acid (FA) is a well-established antioxidant molecule prepared in large amount from rice bran [10] and widely used in cosmetic formulations for cutaneous protection as photoprotectant and for delaying the premature skin aging process. Other biological activities in addition to its antioxidant and anti-inflammatory properties have been reported. Ferulic acid is now claimed as antiproliferative agent (due to COX-2 inhibition) and

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some of its lipophilic esters are now under screening in several tumor cell lines for the prevention of photoinduced skin tumours [11].

Hence its topical application may be useful in the prevention of different types of photoinduced skin cancer (squamous cell carcinoma, SCC; basal cell carcinoma, BCC; cutaneous melanoma CM). Since ferulic acid has been recently demonstrated to be, at least in part, susceptible to light exposure with formation of some decomposition products [12], in this work we have prepared a ferulic acid/γ-CD association complex with the aim to improve the physico-chemical stability of the antioxidant in the light of its use as sunscreen. In a previous paper [13] we have studied the stability of ferulic acid/γ-CD complex through a detailed analysis of the protonation thermodynamics of the ferulic acid/γ-CD compounds. Thermodynamic data were consistent with the hypothesis of the inclusion complexation obtained by the penetration of the neutralized guest FA into the γ-CD host cavity.

As an extension of this work, in the present investigation, we have carried out an analytical study on the FA/γ-CD complex in order to better define the mode of binding of ferulic acid to the hydrophobic core of γ-CD. The study was performed using physical and diffractometric techniques (differential scanning calorimetry, DSC; X-ray diffractometry, XRD) and also nuclear magnetic resonance spectroscopy (NMR) and molecular modelling analyses. These two last showed by us [14] to be very promising in understanding the mechanism of interaction of ferulic acid derivatives with biological membranes.

## 2. Experimental

### 2.1. Materials

All the organic solvents used were of analytical reagent grade purity (Aldrich, Milan, Italy). Ferulic acid was kindly supplied by Tsuno Rice Fine Chemicals (Wakayama, Japan) and was of pharmaceutical grade; γ-cyclodextrin was purchased from Aldrich. The $^1$H NMR measurements were carried out in deuterium oxide (Aldrich) solutions.

### 2.2. Preparation of ferulic acid/γ-CD complex

The complex was prepared by mixing (at 1:1 molar ratio) ferulic acid and γ-CD according to the coprecipitation procedure reported by Chikuno and Terao [15]. In detail, a mixture of γ-cyclodextrin (0.038 mol) and ferulic acid (0.038 mol) was diluted in 500 ml of water and left under stirring for 1 h in the dark and at room temperature. After reaction completeness, the precipitate was collected by filtration and washed twice with 100 ml of water to remove the residual of γ-cyclodextrin and ferulic acid.

After drying in vacuum, a white powdered product of the complex was obtained. The physical mixture of ferulic acid and γ-CD (at 1:1 molar ratio) was prepared by suitable mixing of the pulverized powders. This was used for analytical comparison with the coprecipitate.

### 2.3. HPLC analysis

HPLC analysis was performed using a Shimadzu Chromatograph LC-10 ADVP equipped with a Shimadzu SPD-M10 AVP photodiode array detector (Shimadzu, Milan, Italy). The chromatographic determination was carried out using a Supelcosil LC-18 column (250 mm × 4.6 mm, 5 μm particle size) from Supelco (Milan, Italy). The solutions were injected in a manual injector (Rheodyne Model 7725) using a 20 μl sample loop.

The mobile phase was an isocratic mixture of acetonitrile:water (2% acetic acid) (22:78, v/v) with a flow rate of 1 ml/min. The detector wavelength was set at 320 nm. Responses were recorded and integrated using Shimadzu CLASS VP Chromatography Data System 4.2 version. Stock solution of ferulic acid (1.0 mg/ml) was prepared in dimethylformamide (DMF) and stored at −20 °C. The working standards (10 and 100 μg/ml) were freshly prepared from the stock solution by dilution with an appropriate volume of DMF, to build up the calibration curve ($y = 356{,}942x + 108{,}760{,}156r^2 = 0.999$) according to the method previously proposed by Li and Bi [16]. The ferulic acid association complex with γ-CD was dissolved in DMF (1mg/ml) and filtered through a 0.45 μm filter and the concentration of ferulic acid determined as described. In these conditions the retention time of ferulic acid was 10.5 min and no interference from γ-CD was observed.

### 2.4. Differential scanning calorimetry

DSC analysis was carried out with a Mettler-Toledo DSC821 differential calorimeter calibrated with indium (Mettler-Toledo S.P.A., Milan, Italy). The detection system was a Mettler PT 100 ceramic sensor, with a calorimetric resolution <0.7 μW and noise level <1 μW. All scans were performed in the range of 40–300 °C with a gradient of 5 °C/min on the powder previously dried for 24 h at 110 °C. All samples were prepared weighing 3–5 mg of dried powder in a 40 μl-holed lid aluminium pan.

Analyses were performed in duplicate on pure γ-cyclodextrin, ferulic acid, association complex and γ-cyclodextrin/ferulic acid physical mixture.

### 2.5. XRD study

Powder X-ray diffraction (PXRD) patterns were obtained with a PW 1710 Philips diffractometer (Lelyweg, The Netherlands) using the Ni-filtered Cu Kα radiation (λ = 1.5418 Å) over the interval 2°–45° 2$\theta$, in the following conditions: target, Cu; filter, Ni; voltage, 40 kV; current, 20 mA; time constant, 4 s; angular speed 1° (2$\theta$) min; 1°, −0.1° and −1° slit; angular range $2° < 2\theta < 45°$. Analyses were performed on the same samples prepared for DSC studies.

### 2.6. $^1$H NMR analysis

NMR analysis was performed on a Bruker DRX 600 spectrometer (Bruker Biospin, Milan, Italy) operating at 600 MHz equipped with an "Ultrashielded" Magnex magnet, three channels for acquisition, gradients on *X*-, *Y*-, *Z*-axis (50 G/cm). A

Silicon Graphics $O_2$ workstation with the XWin NMR software was used for calculations.

Samples were dissolved in deuterium oxide at a concentration of $5 \times 10^{-3}$ mol/l. The chemical shift ($\delta$) at 4.7 ppm due to traces of water present in the solvent was used as internal reference. Typical parameters for $^1$H NMR spectra were 32 scans, 10 s relaxation delay and 90° pulse angle.

### 2.7. Molecular modelling

Molecular modelling was carried out using AMBER force field and AMBER software [17] running on a Linux workstation equipped with a Pentium CPU. Modelling was performed by docking the optimized structure of the ferulic acid into the γ-cyclodextrin cavity and allowing for full-geometry optimization. The dielectric constant for electrostatic interactions was set at 1, the non-bonded cut-off distances set to 20 Å. A C8 symmetrical γ-cyclodextrin molecule was built from γ-D-glucopyranose units. A starting geometry for ferulic acid was generated by building the molecule from scratch and then running a 5000 steps minimization. The final structure was manually docked in the γ-CD cavity according to the NMR observations to obtain a starting geometry for the complex. To maintain the correct guest molecule orientation intermolecular restraints between γ-CD and ferulic acid protons were established; then a simulation was carried out using a simulated annealing protocol: cooling from 300 to 50 K in 50 K steps, 15 ps simulation for each step, 0.5 fs time step, energy minimization at the end of each step.

## 3. Results and discussion

### 3.1. Differential scanning calorimetry studies

DSC is a powerful analytical tool for the physico-chemical characterization of association complexes of drugs with cyclodextrins, in particular when coupled with a complementary technique such as PXRD [18]. In the present work DSC was applied to the analysis of ferulic acid, γ-CD, γ-cyclodextrin/ferulic acid physical mixture (1:1) and γ-CD/ferulic acid association complex. DSC thermograms revealed marked structural differences between pure components, the physical mixture and the association complex. The thermal profile of ferulic acid alone (Fig. 1A) showed endothermic $T_{max}$ at 175.2 °C, corresponding to the melting point of the crystalline form of the drug followed by an exothermic effect due to decomposition phenomena at higher temperatures. A similar behaviour was observed for ferulic acid in the physical mixtures with γ-CD (Fig. 1C). In fact, the thermal profile showed besides unchanged cyclodextrin broad bands at 288.3 and 93.7 °C due to the γ-CD dehydration and decomposition, a well-distinct melting peak, which appeared substantially unaffected in its shape and melting temperature. This indicated that ferulic acid maintained its original crystalline structure in the mixture. A different pattern was observed in the thermogram of the association complex (Fig. 1D). The disappearance of the melting peak of ferulic acid at 175.2 °C together with the shifting and broadening of the two characteristic γ-CD bands (from 288.3 to 269.7 °C and from 93.7 to 85.7 °C) is indicative of a change in the substrate structure and of a tight interaction between ferulic acid and γ-CD.

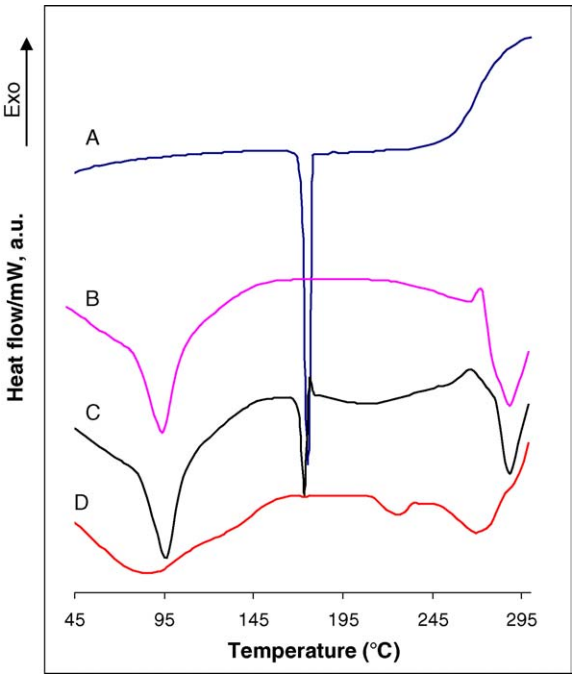


Fig. 1. DSC curves: ferulic acid (A), γ-CD (B), physical mixture (ferulic acid/γ-CD; 1:1) (C) and ferulic acid/γ-CD complex (D).

These findings show that no association takes place when the two powders are simply mixed together and that conversely coprecipitation gives rise to an association compound.

### 3.2. Powder X-ray diffraction studies

The X-ray diffractometric pattern of a 1:1 molar ratio physical mixture of γ-cyclodextrin and ferulic acid (Fig. 2c) is basically the combination of the signal profile relative to ferulic acid (Fig. 2a) and to γ-cyclodextrin (Fig. 2b). By contrast, the coprecipitate showed a completely different profile, sharply distinct from that of the physical mixture, which indicates the formation of a new crystalline phase (Fig. 2d), suggesting the association of ferulic acid with γ-cyclodextrin. In particular, the differences in the interplanar spacing, relative diffraction peak intensities and diffraction angles confirm the formation of an association complex, which has different crystalline structure from that of the pure constituents and of the physical mixture.

### 3.3. $^1$H NMR studies

The interaction between ferulic acid and γ-cyclodextrin was investigated in water/solvent by nuclear magnetic resonance spectroscopy, since this technique provides direct and detailed information on the physico-chemical interactions indicative of the formation of an inclusion complex [19–23].

Ferulic acid and γ-cyclodextrin structures are shown in Fig. 3 with the numbering systems used to $^1$H assignment. The $^1$H

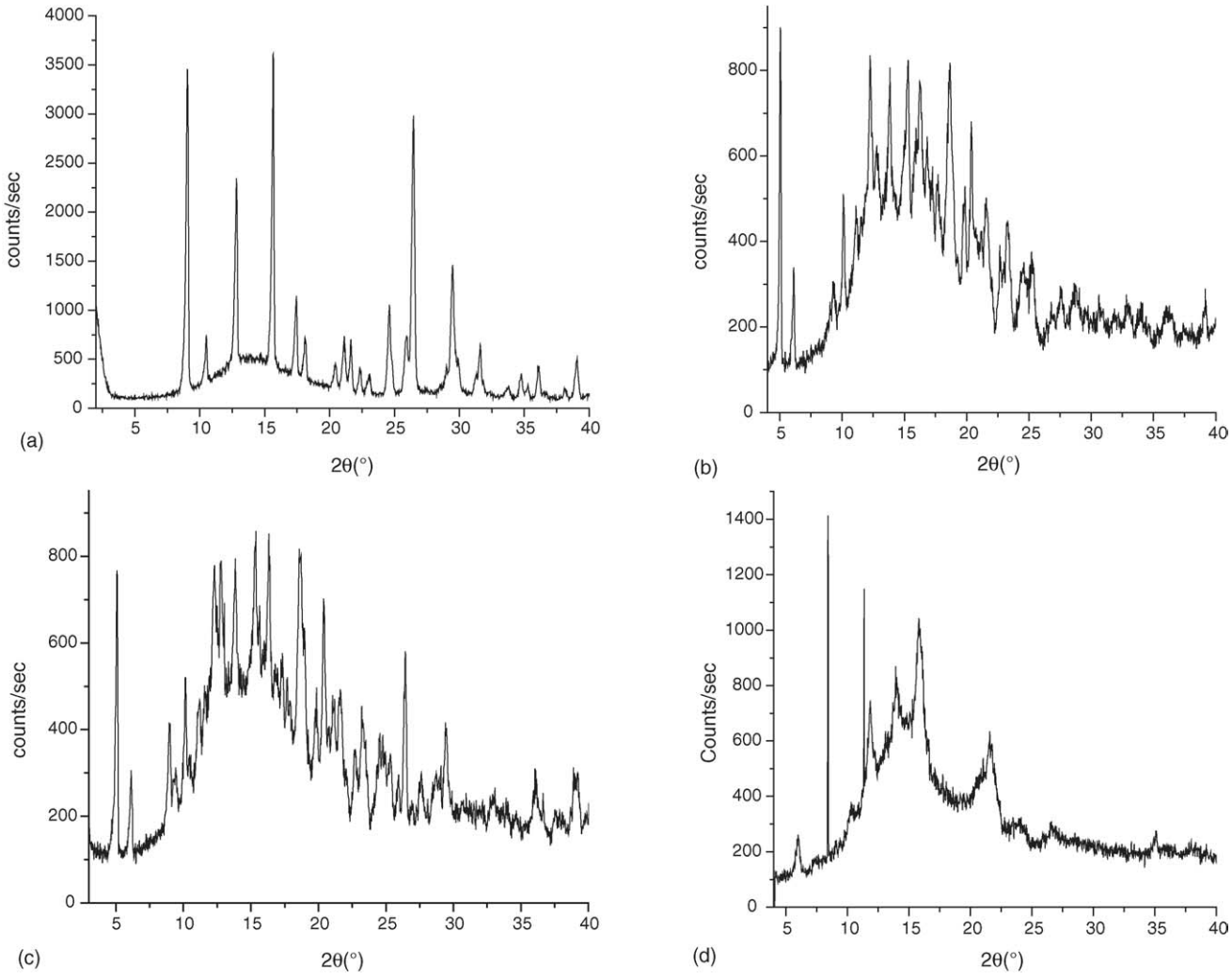


Fig. 2. PXRD analyses: ferulic acid (a), γ-CD (b), physical mixture (ferulic acid/γ-CD; 1:1) (c) and ferulic acid/γ-CD complex (d).

NMR chemical shifts of the protons of native γ-cyclodextrin or when complexed with ferulic acid are reported in Table 1. These data evidence that the presence of ferulic acid induces upfield changes in the $^{1}$H NMR chemical shift values for both the protons (H3 and H5) which are located inside the cyclodextrin cavity (Fig. 4A and B). These protons show an upfield shift due to the shielding effect exerted by the guest molecule. This is consistent with the insertion of ferulic acid inside the lipophilic core of γ-CD, i.e. with the formation of an inclusion complex.

The ferulic acid resonances are also affected by the inclusion: we can observe in the NMR spectrum a modification of the chemical shift values of the anisotropically shielded atoms (Fig. 5A and B). The $^{1}$H chemical shift values of free ferulic acid and those of the complex are reported in Table 2.

We observed high delta values ($\Delta$) for an aromatic proton d ($\Delta = 0.47$, Fig. 3) and for protons c ($\Delta = 0.25$) and g ($\Delta = 0.30$) belonging to the adjacent–CH=CH– group. Hence it is reasonable to postulate that this part of the molecule, which is highly

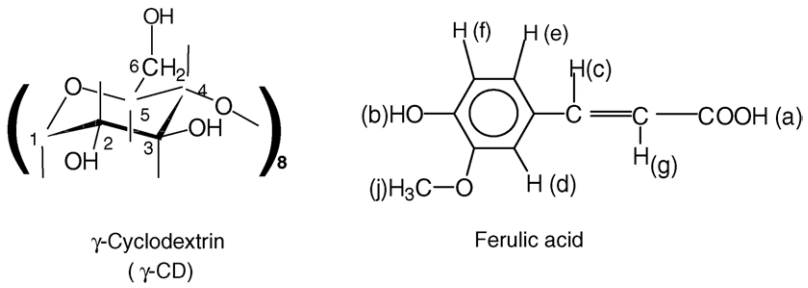


Fig. 3. Structures of γ-CD and ferulic acid.

Table 1
$^1$H NMR chemical shifts of the protons of γ-CD free or complexed with ferulic acid in $D_2O$

| $^1$H assignment | $\delta$ γ-CD$_{free}$ (ppm) | $\delta$ γ-CD$_{complexed}$ (ppm) | $\Delta\delta$ (γ-CD$_{complexed}$ − γ-CD$_{free}$) |
|---|---|---|---|
| H1 | 5.01 (d) | 5.02 (d) | 0.01 |
| H3 | 3.83 (t) | 3.78 (t) | −0.05 |
| H6 | 3.76 (s) | 3.77 (s) | 0.01 |
| H5 | 3.75 (d) | 3.73 (d) | −0.02 |
| H2 | 3.54 (dd) | 3.56 (dd) | 0.02 |
| H4 | 3.48 (t) | 3.51 (t) | 0.03 |

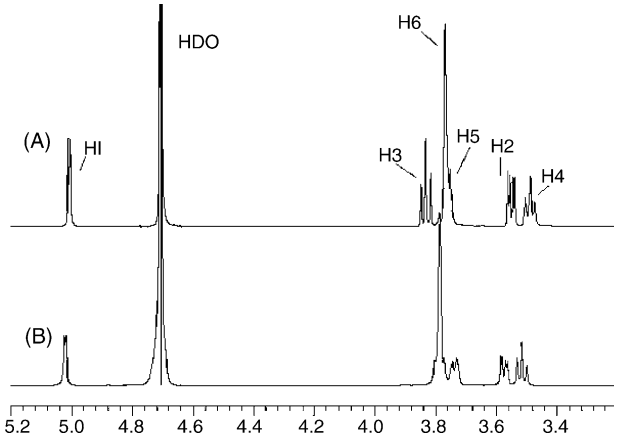


Fig. 4. $^1$H NMR spectra (600 MHz, $D_2O$, 300 K) of the inclusion complex (A) and of γ-CD (B).

Table 2
$^1$H NMR chemical shifts of the protons of ferulic acid (FA) free or complexed with γ-CD in $D_2O$

| $^1$H assignment | $\delta$ FA$_{free}$ (ppm) | $\delta$ FA$_{complexed}$ (ppm) | $\Delta\delta$ (FA$_{complexed}$ − FA$_{free}$) |
|---|---|---|---|
| H (c) | 7.56 (d) | 7.31 | −0.25 |
| H (d) | 7.22 (s) | 6.85 | −0.47 |
| H (e) | 7.11 (d) | 6.93 | −0.18 |
| H (f) | 6.87 (d) | 6.84 | −0.03 |
| H (g) | 6.33 (d) | 6.03 | −0.30 |
| H (j) | 3.83 (s) | 3.78 | −0.05 |

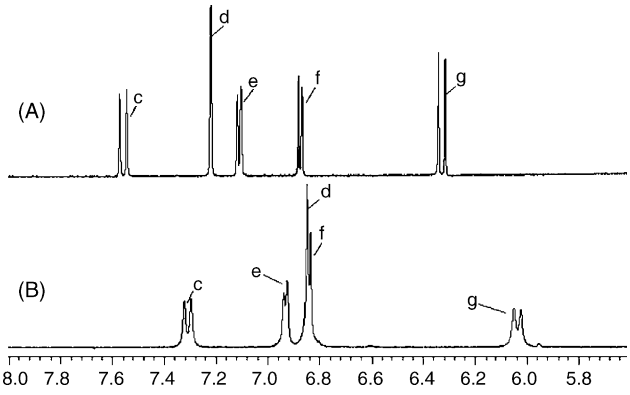


Fig. 5. $^1$H NMR spectra (600 MHz, $D_2O$, 300 K) of the inclusion complex (A) and of ferulic acid (B).

hydrophobic, must be deeply embedded inside the lipophilic core of γ-cyclodextrin.

Therefore the results of the NMR experiments provide a clear demonstration that ferulic acid is entrapped inside the γ-cyclodextrin cavity through the hydrophobic portion of the molecule, corresponding to the benzene ring and ethylene moiety. Very likely these last are completely embedded inside the lipophilic core. The most polar groups of the molecule, the −COOH, the phenol and methoxy residues are close to wider rim of γ-CD or exposed outside the cavity.

### 3.4. Molecular modelling study

Computational studies on host–guest interactions are carried out, in general, to find out the most probable conformation of the complex and to give a meaningful three-dimensional visualization of the complex. The most probable structure of the ferulic acid/γ-CD inclusion complex was further investigated using the molecular modelling program AMBER taking into account the constraints provided by the NMR study (Figs. 6 and 7). The full-geometry optimization of the complex showed that the inclusion of the benzene ring and ethylene side chain of the guest in the lipophilic core of the cavity was the most energetically favoured (data not shown) while the −COOH, the methoxy and

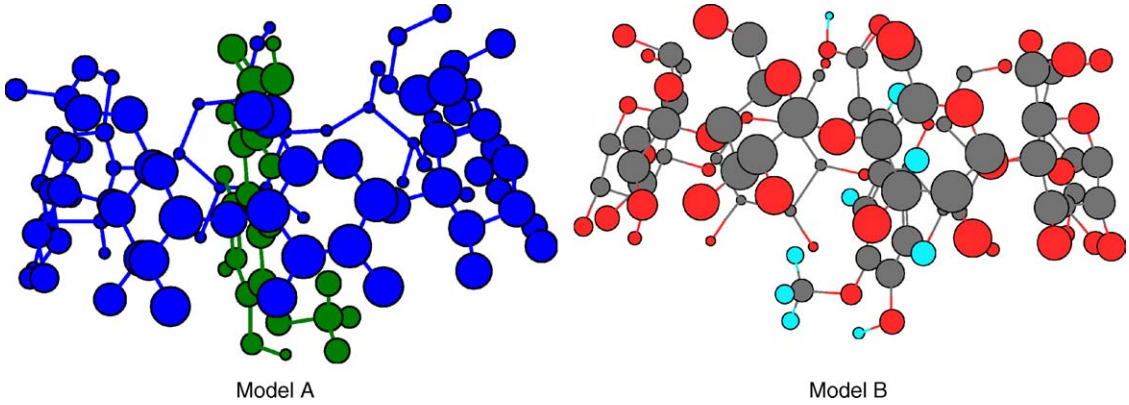


Fig. 6. Molecular modelling of ferulic acid/γ-CD complex (front view). Model A: the host molecule and the guest molecule are shown with different colours. Model B: the H, C and O atoms of the inclusion complex are displayed with standard colours (blue H, grey C and red O). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

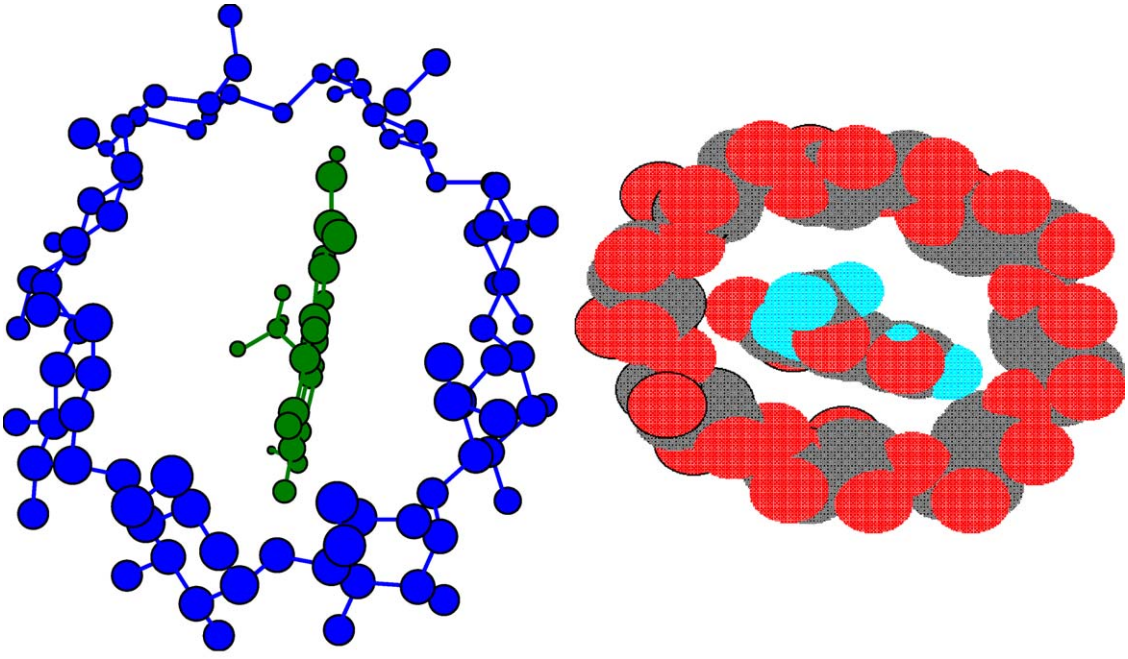


Fig. 7. Molecular modelling of ferulic acid/γ-CD complex (upper view). Model A: the host molecule and the guest molecule are shown with different colours. Model B: the H, C and O atoms of the inclusion complex are displayed with standard colours (blue H, grey C and red O).

the hydroxyl residues were close to wider rim of γ-CD. This is in agreement with the NMR data.

### 3.5. HPLC analysis

The results of the quantitative analysis carried out on the ferulic acid/γ-CD complex showed that the percentage of the ferulic acid included is: 14.99 ± 0.06 (mean value ± S.D. of five determinations, data not shown).

## 4. Conclusions

The results of this study clearly evidence that ferulic acid can be efficiently complexed with γ-cyclodextrin in a relatively high proportion forming an inclusion complex.

DSC and X-ray diffractometric experiments give strong support for the formation of an association complex, while NMR analyses and modelling data unequivocally demonstrate that ferulic acid is embedded inside the cavity of γ-cyclodextrin with the aromatic and ethylene moieties, and the more polar groups arranged close to the wider rim. Hence NMR provides good description regarding structural information and dynamic characteristic of the inclusion phenomena, and docking results evidence that γ-CD void pocket is viable for ferulic acid and orientations of the guest molecule are considerably restricted. The complementary information obtained from NMR and docking data demonstrate that γ-CD is a powerful pharmaceutical/cosmetic tool for the encapsulation/release of this potent antioxidant, which, to exert its biological activity, needs to be saved on the skin from physical and environmental stresses.

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