J Incl Phenom Macrocycl Chem (2011) 71:25–34
DOI 10.1007/s10847-011-9930-9

REVIEW ARTICLE

Spectroscopic studies on the inclusion behavior between caffenic acid and γ-cyclodextrin

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Received: 16 July 2010 / Accepted: 20 January 2011 / Published online: 11 February 2011

Abstract To investigate the inclusion ability of γ-cyclodextrin (γ-CD) for caffenic acid (CA). The conditions for the formation of inclusion complex and the binding constant between γ-CD and CA were determined by fluorescent and ultraviolet spectroscopic methods. The behavior of CA as a free radical scavenger before and after its inclusion was investigated. In addition, solid samples of the inclusion complex, prepared through the co-precipitation and grinding methods, were characterized via IR spectroscopy and differential scanning calorimetry. The inclusion complex was further characterized with ^{1}H NMR spectroscopy. By using fluorescent and ultraviolet spectroscopy, the conditions for the formation of inclusion complex between γ-CD and CA were optimized and the binding constant determined. It was observed that the guest molecule behaves as a better anti-oxidant after its inclusion into γ-CD.

Keywords Caffenic acid · Cyclodextrins · Inclusion compounds

Introduction

Molecular recognition is among the most active research areas in the chemistry of supramolecules [1]. Similar to the "key and lock" binding principle of biological molecules, molecular recognition is the driving force for the formation of supramolecular structures and also forms the basis for the understanding of the selective binding between the host and the guest molecules. In this respect, there exist three types of the most attractive host molecules: cyclodextrins (CDs), crown, and calixarenes. All three types of molecules have been used as molecular models for biomolecule. Therefore, the investigation of the interaction of these host molecules with small organic molecules as well as the physical chemical properties of these interactions has become an important research area. Among them, CDs are by far the most important host molecules.

As host molecules, CDs have many interesting and useful properties [2]. With a lipophilic interior and a hydrophilic exterior, CDs can form various inclusion complexes with many organic and inorganic molecules. As the microenvironment inside the CD cavity differs from that of an aqueous solution, the guest molecule often displays rather different photochemical and photophysical properties. CDs have been extensively used for the formation of supramolecular complexes by selective binding to their substrates (or guests) and research interests in this area can be seen in various fields of chemistry and biology. CDs are oligomers of glucose (Fig. 1a). Depending on their size, CDs can be α, β, or γ. The researches associated with γ-cyclodextrin (γ-CD), which has eight glucose units, are relatively fewer than those of its α and β analogues. On the other hand, γ-CD has a larger cavity and should in principle form inclusion complexes with a bigger variety of guest molecule [3].

CA, shown in Fig. 1b, is a naturally occurring phenylpropanoid and is found in many Chinese herbs. CA has been shown to be possessed general antibiotic, antiviral, and hemostatic properties. It is also found to be able to increase the level of human leukocytes.

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Experimental section

Reagents

CA was dissolved in water to prepare a 1.0×10^{-3} mol/L solution, and γ-CD were prepared for the solutions of 1×10^{-2} mol/L. All the other reagents were of analytical-reagent grade and were used as received. 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Tokyo, Japan Chemical Industry Co., Ltd.) was used in clearing free radicals experiment and need prepared for the concentration with the same of CA solutions. Phosphate buffer solutions of NaH_2PO_4/H_3PO_4 were used to control the pH = n (n = 3.05, 5.0, 6.5, and 8.96) of the working media. Doubly distilled water was used throughout. All experiments were carried out at room temperature.

Apparatus

The UV absorption experiments were carried out on a UV-757CRT spectrophotometer (Shanghai Precision & Scientific Instrument Co., Shanghai, China). Fluorescence measurements were conducted on a Hitachi F-2500 FL spectrofluorimeter (Tokyo, Japan) using an excitation wavelength of 285 nm and emission wavelength of 445 nm. Excitation and emission slit width were both set at 10 nm. IR spectra were obtained with FT-1370 IR spectrometer using KBr pellets. NMR spectra were recorded on a DRX-300 spectrometer (Fällenden, Switzerland) using D_2O as solvent. Differential scanning calorimetry (DSC) analysis was carried out on DSC-60 thermal analyzer (Shimadzu, Japan).

Procedure

Analysis of the conditions of inclusion complex formation

1 mL of the CA stock solution was transferred into a 10 mL volumetric flask, and then an appropriate volume of 1.0×10^{-2}mol/L γ-CD solution was added. The mixed solution was diluted to 10 mL final volume with distilled water and shaken thoroughly, and ultrasonic treatment was applied to the mixture at 20 ± 1 °C for 30 min. On the one hand, effect of the concentration of the CD was investigated by fluorescence spectroscopy. On the other hand, the temperature was measured by the UV absorption at 25, 30, 37, and 45 °C, respectively. Finally, pH effects on the formation of the inclusion complex were investigated by fluorescence spectroscopy, varying the pH from 3.05 to 8.96.

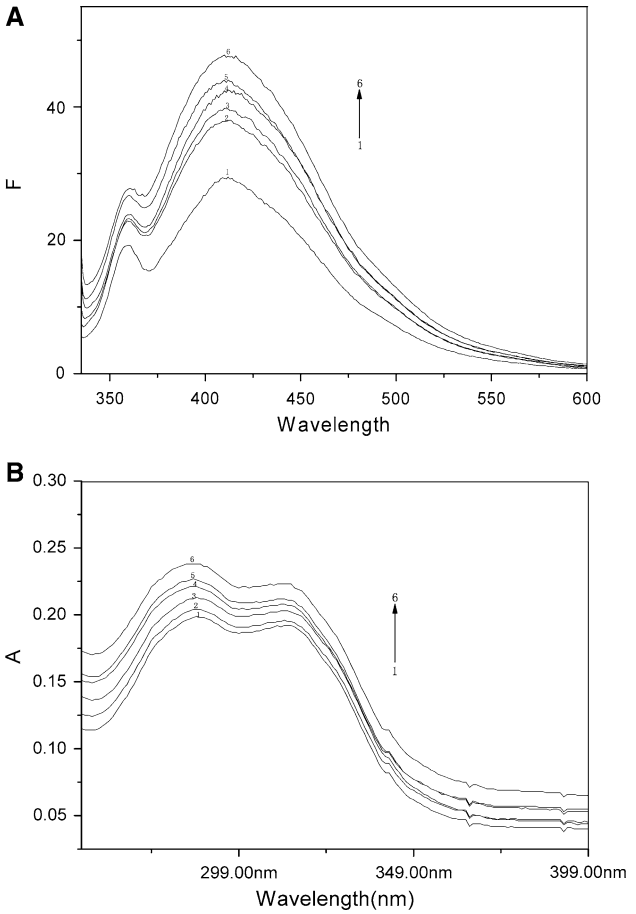
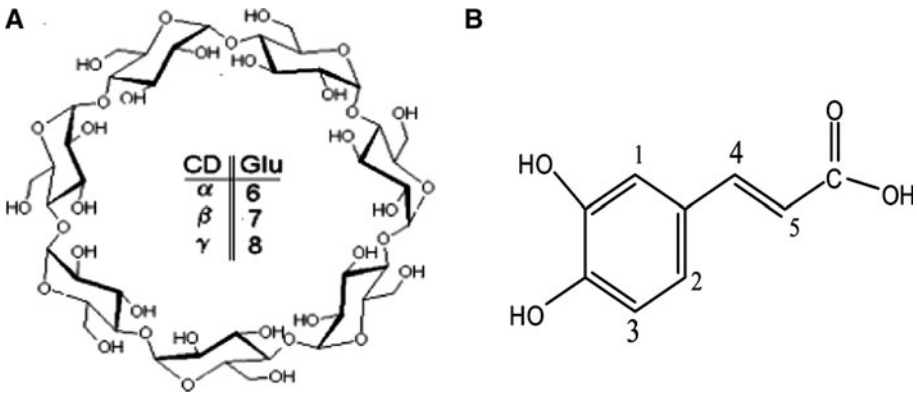


Fig. 2 The spetras of CA in the presence of various amounts of γ-CD. **a** Fluorescence spectra, **b** UV spectra

Fig. 1 Structure of CD and CA



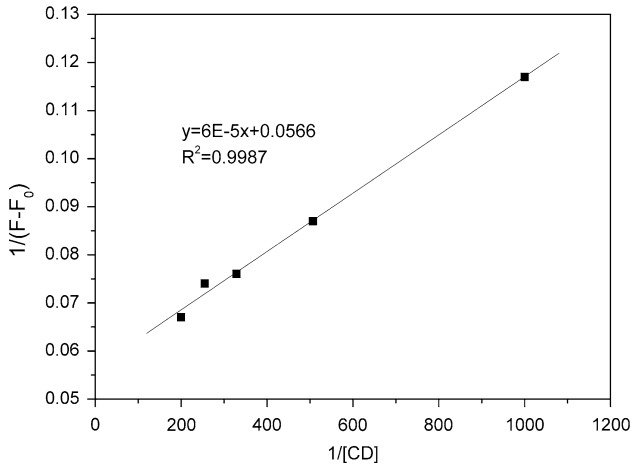


Fig. 3 The reciprocal plot (1/[CD] Vs. 1/[F−F_0]) for the inclusion complex between CA and γ-CD

DPPH radical scavenging test

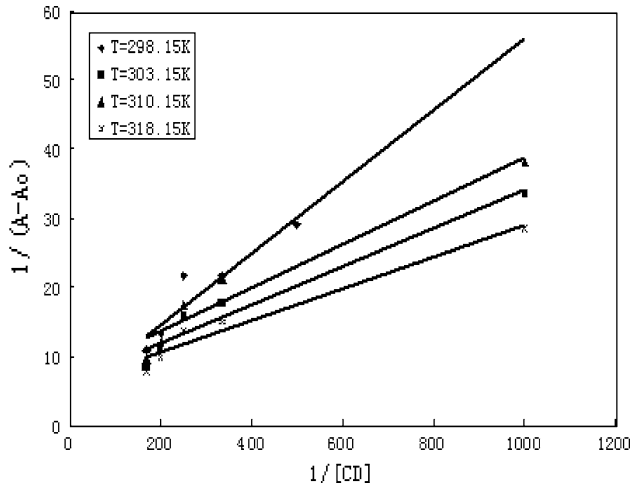


Fig. 4 Effect of temperature

Clearing of free radicals was used to determine the efficiency of CA. We also examined thermal and photolysis stability of CA. The same concentration of γ-CD and CA solutions of identical concentration were mixed at 80 °C and were kept for 2 h, then UV absorbance was measured every half hour and residual rate was calculated.[4] The results were expressed as percentage of DPPH radical elimination calculated according to the following equation:

$$AU = \left[1 - \left(A_i - A_j\right)/A_c\right] \times 100\% \quad (1)$$

To alcohol: water = 9:1 as a reference solution, A_i, A_c, and A_j were measured by using UV absorbance spectroscopy. Where AU is the radical-scavenging activity, A_i was considered as the absorbance of the mixture of 3 mL DPPH and 0.5 mL complex solution, A_c represented the absorbance of the mixture of 3 mL DPPH and 0.5 mL contrast solution, and A_j was the absorbance of the mixture of 3 mL complex solution and 0.5 mL contrast solution. By this way, the rate of clearing free radical could be obtained.

Preparation of CA/γ-CD solid complex

Solid complex of CA with γ-CD was prepared with the co-precipitation method or the grinding method [5].CD was made of saturated aqueous solution, then 40% ethanol solution of caffeic acid was dropped slowly into it, stired constantly for a long time and cooled gradually to room temperature. The solid precipitation of inclusion complex was filtered, washed with a small amount of 40% ethanol, and then dry to stable inclusion complex.

NMR measurements

Equal volumes of γ-CD and CA solutions, both at concentrations of 1.0×10^{-4} mol/L, were mixed until complete reaction, and NMR measurements were taken and contrasted with γ-CD and CA by Bruker Avancev DRX300 MHz. All the spectra were recorded in 99.96% D_2O at 298 ± 1 °C.

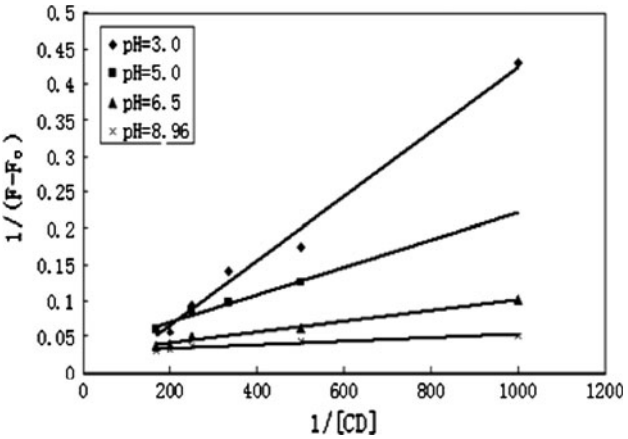


Fig. 5 Plots of 1/[CD] ~ 1/[F−F_0] at different pH values

Table 1 The thermodynamic data for the inclusion of caffenic acid in γ-CD

T	K	ΔG	ΔH	ΔS
298.2	52.4	−9.8	−80.5	−360.3
303.2	113.8	−11.9	−80.5	−360.3
310.2	208.5	−13.8	−80.5	−360.3
318.2	84.3	−11.7	−80.5	−360.3

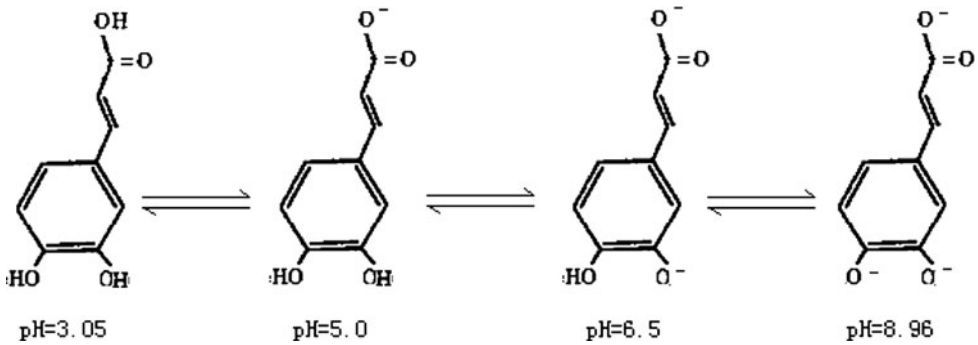


Fig. 6 Ionisation of CA from pH 3.05 to pH 8.96

Results and discussions

Conditions for inclusion complex formation

Effect of the CD concentration

Figure 2 gives the fluorescent spectra (a) and UV spectra (b) of CA in the presence of various amounts of γ-CD. It can been seen from these spectra, the intensity of both the fluorescence and the UV absorption increases with increase of the γ-CD concentration, indicating that the inclusion of CA into the cyclodextin has occurred and the concentration of the cyclodextin has an influence in the inclusion. Specifically, the inclusion interaction between the host and the guest molecules increases with the increase in the concentration of γ-CD.

According to the modified Benesi–Hildebrand equation [6]:

$$1/(F - F_0) = 1/([CD]k\alpha) + 1/\alpha$$

$$or \quad 1/(A - A_0) = 1/([CD]k\alpha) + 1/\alpha \qquad (2)$$

where F and F_0 represent the fluorescent intensity of CA in the presence and in the absence of γ-CD, respectively; and A and A_0 represent the intensity of its UV absorption in the presence and in the absence of γ-CD, respectively; [CD] is the total concentration of γ-CD, whereas k and α are constants. A plot of the reciprocals 1/[CD] versus $1/[F-F_0]$ gives a straight line as shown in Fig. 3, from which the binding constant can be calculated by dividing its intercept with its slope.

Table 2 The maxima of fluorescence and UV absorption of the CA–γ-CD complex

pH	K	UV absorption λex (nm)	Fluorescence λex (nm)
3.05	57.5	320	280
5.0	168.5	286	280
6.5	377.1	310	279
8.96	1430.0	285	269

It is apparent from Fig. 2 that the maximum absorption underwent a red shift, which is also indicative of the formation of an inclusion complex between CA and γ-CD. Based on the good linear correlation revealed in Fig. 3, it can be concluded that the inclusion complex has a 1:1 stoichiometry with the binding constant being 943.

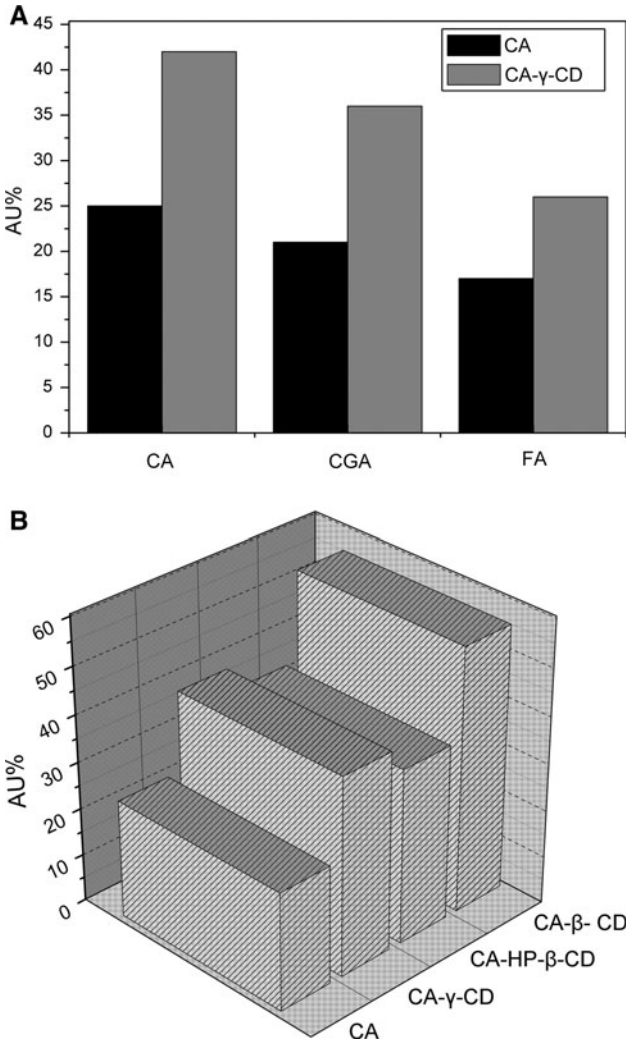


Fig. 7 DPPH removal rates (Figs 7a and b should be skipped and replaced by a table). **a** A comparison on the DPPH removal rate between CA, CGA, FA and their inclusion complexes **b** The DPPH removal rate of CA and its inclusion complexes with different CDs

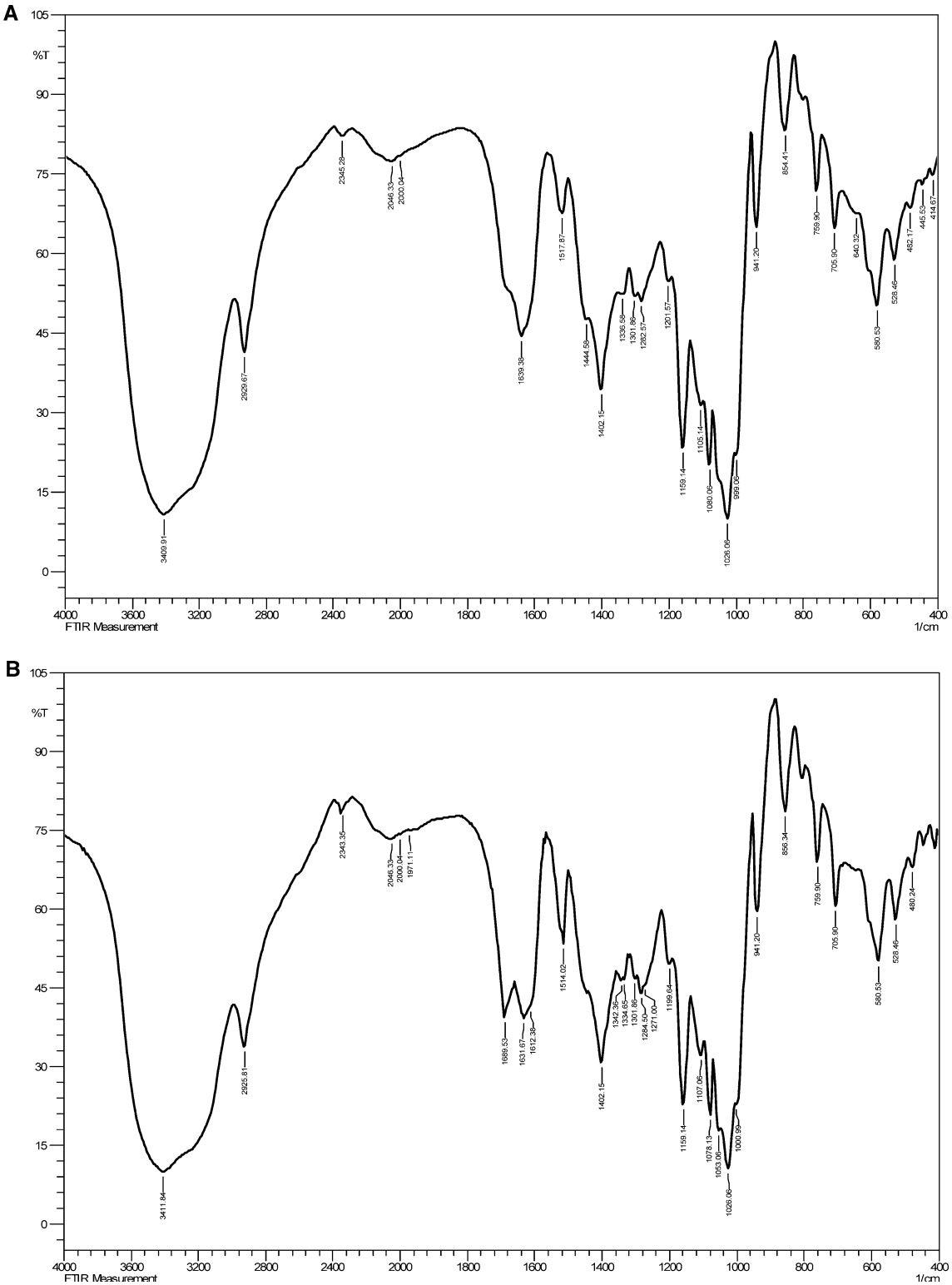


Fig. 8 IR spectra. **a** CA–γ-CD inclusion complex prepared by co-precipitation, **b** CA–γ-CD inclusion complex prepared by grinding, **c** γ-CD, **d** CA

Effect of the temperature

The UV absorption of CA in the presence of different levels of γ-CD was measured at 25, 30, 37, and 45 °C, respectively. Linear regression analysis on the $1/[CD] \sim 1/[A-A_0]$ plots gave the binding constants at these temperatures. The results were shown in Fig. 4.

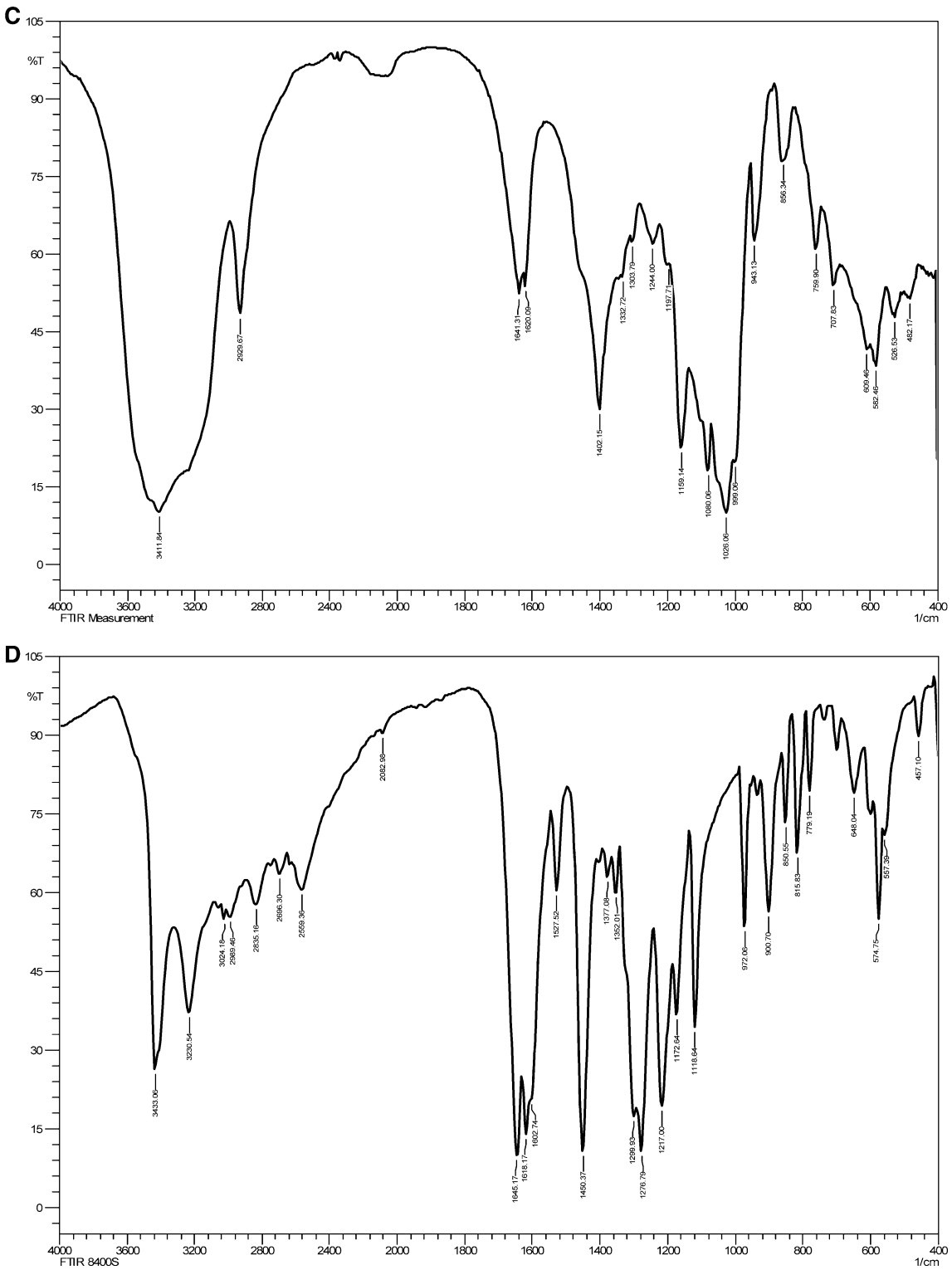


Fig. 8 continued

The change in Gibbs free energy at different temperatures can be calculated from $\Delta G = 2RT\ln K_T$. According to the Van't Hoff equation [7]:

$$\ln K_T = -\Delta H/RT + \Delta S/R \quad (3)$$

A plot of $\ln K_T$ versus 1/T followed by linear regression analysis yielded the values of ΔH and ΔS [7], which were tabulated in Table 1.

The negative value of ΔG as shown in Table 1 is consistant with the expectation that the formation of the inclusion complex is an energetically favored and spontaneous process. It is also obvious from the negative ΔS that the order of the system has decreased upon formation of the complex. The process is driven by its favorable negative enthalpy change that overcompensates the unfavorable negative entropy change.

Effect of pH on the formation of the inclusion complex

We measured UV spectra of aqueous solutions of CA in the presence of γ-CD at different pH values. The maximum of UV absorption varies with pH undergoing a blue shift with increasing pH value. At when the pH of the solution changes from 3.05 to 8.96, the maximum absorption undergoes a blue shift. A possible reason for this could be that as the pH increases, there is a higher degree of ionization for the carboxyl group of CA. The effect of cross-conjugation results (Cross-conjugation is a special type of conjugation in a molecule, when in a set of three p-i bonds only two pi-bonds interact with each other by conjugation, the third one is excluded from interaction) from structure of CA that plays a major role in the formation of the inclusion complex. The ionization of the carboxyl group may cause an overall reduction in the dipole moment of the molecule and bring about a diminishment of the conjugation effect, ultimately leading to the observed blue shift of its UV spectrum.

Figure 5 shows the $1/[CD] \sim 1/[F-F_0]$ plots measured at different pH values. All of the plots displayed a good linear relationship and indicated the formation of a 1:1 inclusion complex. The binding constants can be obtained by linear regression analysis on the plots and listed in Table 2. It can be seen from the data in Table 2 that the binding constant K of the CA–γ-CDDE complex increases as the pH increases. This trend is related to the structure of CA, whose dissociation equilibration is shown in Fig. 6 [8].

As the pH value increases, CA will be ionized into different anions and these species may form inclusion complexes with CD more easily. However, CA may undergo changes in its properties under alkaline conditions. Therefore, the optimal acidity for the formation of inclusion complex should be at neutral pH as Fig. 6 [9].

DPPH removal rate

Figure 7a shows the comparison of the anti-oxidizing property for CA and its derivatives before and after being included in γ-CD. It can be seen from Fig. 7 that DPPH removal rate for CA is larger than the removal rates of chlorogenic acid (CGA) and ferulic acid (FA), that is, the order of DPPH removal rate is CA > CGA > FA. It is also apparent that the DPPH removal rate increases significantly when the inclusion complexes are formed and follows the same trend, CA > CGA > FA. These results are consistent with what has been proposed by Min Zhu and Fangquan Wang[11]. It shows that these drugs underwent some changes in their properties, resulting in an improvement in their anti-oxidizing ability. It may be caused by an increased activity once CA is included in the cavity of the CD.

Figure (7b) shows the DPPH removal rates of CA and of CA complexes with γ-CD, hydroxypropyl-β-cyclodextrin (HP-β-CD) and β-CD. Again, it can be seen that the removal rate has increased after the formation of the inclusion complex and the rate is higher for the HP-β-CD complex than that of the γ-CD complex, and the rate is relatively small for the γ-CD complex. Nevertheless, the γ-CD complex can still improve the activity and should be of certain value to the study of inclusion compounds.

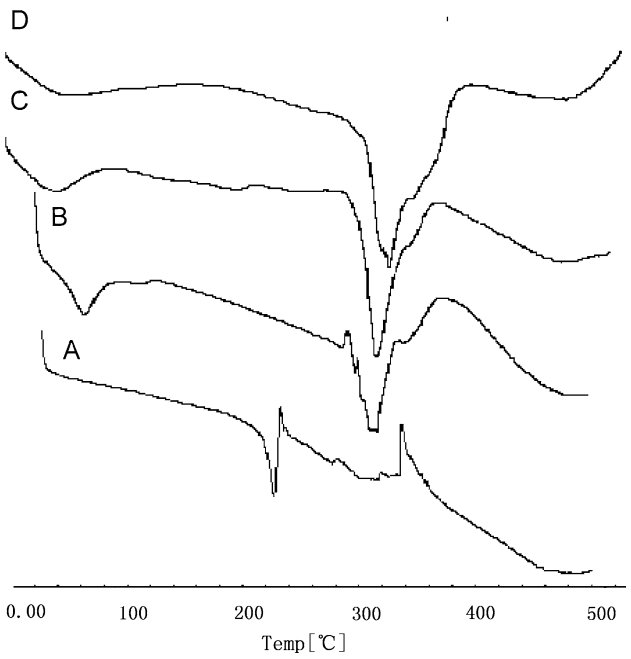


Fig. 9 DSC melting curves *A* CA, *B* γ-CD, *C* CA–γ-CD inclusion complex prepared by grinding, *D* CA–γ-CD inclusion complex prepared by co-precipitation

Characterization of the solid form of the CA–CD complex

The IR spectra of CA, γ-CD and the inclusion complex are shown in Fig. 8.

Figure 8 shows IR spectrum, and It can be seen from these spectra that there are significant changes in the peaks at 2,500–3,450 cm^{-1} between CA itself and the inclusion complexes prepared either with the co-precipitation method or the grinding method, with the big peak at 3,411 cm^{-1} remaining identical to that of the γ-CD; there wasn't any bigger change between γ-CD and CA-γ-CD at 400–1,602 cm^{-1}, but the intensity of the peaks at 1,645–1,602 cm^{-1} corresponding to the benzene ring significantly decreased, indicating that both the benzene ring and its OH groups are included in the cavity of γ-CD and both methods have been successful in preparing the inclusion complex [10].

DSC Characterization of the solid CD inclusion complex

The results of the DSC study are shown in Fig. 9. It can be seen that CA melts at around 225 °C as indicated by the

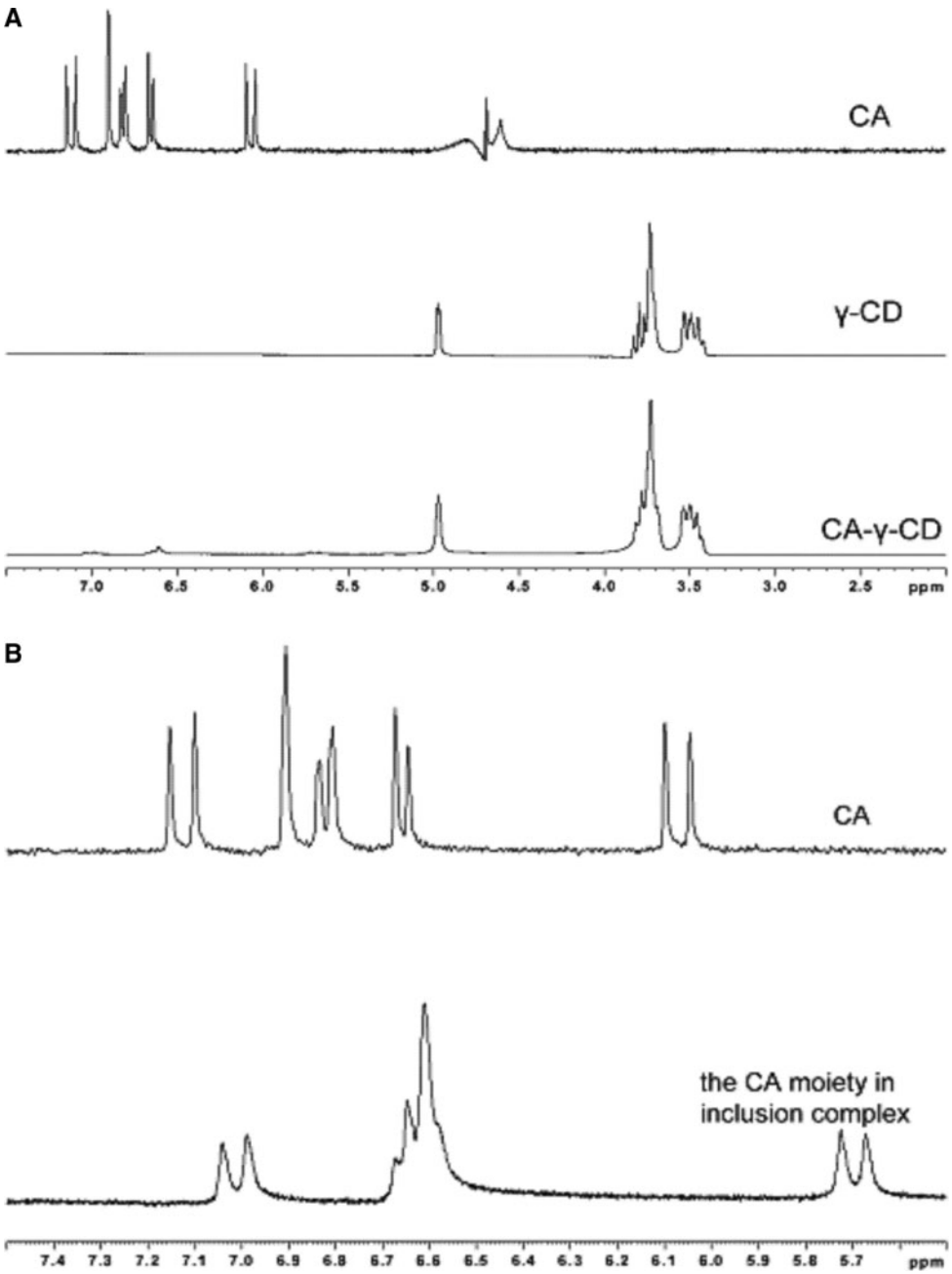


Fig. 10 ^{1}H NMR spectra of CA, γ-CD and the CA–γ-CD inclusion complex **(a)** and CA moiety of the inclusion complex **(b)** The spectrum of the CA moiety is enlarged

Table 3 Changes in the chemical shifts

(1) ^{1}H chemical shifts of free and complexed γ-CD			
^{1}H	$\delta\gamma$-CD free (ppm)	$\delta\gamma$-CD complexed (ppm)	$^{\Delta}\delta$(γ-CD complexed-γ-CD free)
H1	4.969	4.971	0.002
H3	3.800	3.815	0.015
H6	3.768	3.784	0.016
H5	3.736	3.730	−0.006
H2	3.530	3.535	0.005
H4	3.496	3.462	0.034
(2) ^{1}H chemical shifts of free and complexed CA			
^{1}H	ΔCA free (ppm)	ΔCA complexed (ppm)	$^{\Delta}\delta$(CA complexed-CA free)
H4	7.100	7.042	−0.058
H1	6.907	6.649	−0.258
H2	6.807	6.611	−0.196
H3	6.674	5.725	−0.949
H5	6.100	5.674	−0.426

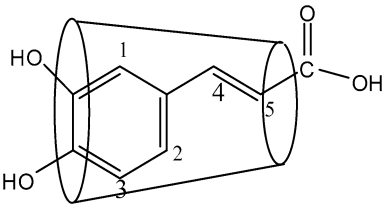


Fig. 11 Proposed structure of the inclusion complex

exothermic peak at this temperature(Fig. 9A); for γ-CD itself (Fig. 9B), several melting peaks that vary in their intensity appear between 270 and 320 °C; however, for the inclusion complex (Fig. 9C and D), there is only one endothermic peak at 320 °C. These results indicate that the interaction between CA and γ-CD is accompanied by the appearance of a new phase that indicates the formation of an inclusion complex [11, 12].

^{1}H NMR characterization of the CD inclusion complex

The NMR spectra are shown in Fig. 10. Figures 10A and B are those of CA and γ-CD, respectively. For convenience, the numbering of these molecules are also indicated [13].

Changes of the ^{1}H chemical shifts of CA and γ-CD by inclusion complex formation. The ^{1}H chemical shifts of CA all undergo notable changes, especially for H1, H3, and H5, indicating that these protons reside in the cavity of the CD.

We conclude from the NMR data given in Table 3 that CA enters the cavity of γ-CA and forms an inclusion complex.The results of the IR and DSC spectroscopic studies suggest that the benzene ring and the olefinic bond of CA are included in the CD cavity, whereas the COOH group is located either near the rim or outside the cavity of the CD. A possible structure for the inclusion complex is proposed as in Fig. 11.

Conclusion

CA has been examined for its ability to form a complex with γ-CD. The results clearly demonstrated that γ-CD can form a stable complex with CA and therefore can be used for the encapsulation/release of this potent antioxidant. The DPPH scavenging ability of CA-γ-CD inclusion complex is larger as that of free CA. IR, DSC, and NMR spectroscopic studies show that the aromatic ring and the ethylene side chain of CA were deeply included inside the CD cavity. This study would be helpful to promote the application of caffenic acid.

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