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ScienceDirect
Food Chemistry 110 (2008) 636–642
FOOD CHEMISTRY
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Effect of β-cyclodextrin on antioxidant activity of coumaric acids ☆

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Received 28 May 2007; received in revised form 26 December 2007; accepted 17 February 2008

Abstract

Inclusion complexes of *o*-, *m*- and *p*-coumaric acid (CA) with β-cyclodextrin (βCD) were prepared in stoichiometric ratios (1:1) and stability constants and antioxidant activity of the complexes were studied. The apparent stability constants in aqueous solution of $0.39 \times 10^3\ M^{-1}$, $2.81 \times 10^3\ M^{-1}$ and $49 \times 10^3\ M^{-1}$ for *o*-, *m*- and *p*-CA complexes, respectively, were determined by phase solubility tests. Different analytical techniques (IR, MS) in combination with different solvent washing procedures, were used for confirmation of the nature of the inclusion complexes. Dioxan was a suitable solvent for removal of only free CA and CA adsorbed on the surface of the βCD, while methanol removed absorbed, included and free CA from the complexes. In the case of *o*- and *m*-CA–βCD complexes, antioxidant activity was significantly increased while, in *p*-CA–βCD, it remained unchanged. The impact of complex structure on antioxidant activity of CA isomers was clarified.

Keywords: Coumaric acids; β-Cyclodextrin; Antioxidant activity; Phase solubility study; IR spectroscopy; Mass spectrometry

1. Introduction

Recently it has become well known that most phenolic compounds are powerful antioxidants. Much attention has been focussed on polyphenolic substances of higher plants, and, it has been established that they may interact with the other mechanisms contributing to anticancerogenic or cardioprotective actions. Minor attention, however, has also been directed to the activity of simple phenolic acids, such as benzoic or cinnamic acids, and their derivatives. All studies have indicated that, even these small monomeric phenols, exhibit considerable antioxidant potential and also display a wide variety of pharmacological properties, e.g., antioxidant, anticancer, antimutagenic and antimalarial activities. Dinkova-Kostova, Abeygunawardana, and Talalay (1998) have found a good correlation between their protection ability against carcinogenesis and mutagenesis and their radical-scavenging capacity. Torres y Torres and Rosazza (2001) reported that antioxidants, such as *p*-CA and other hydroxycinnamic acids, function as chemoprotective agents by quenching carcinogenic nitrosating agents in several biological compartments, including salivary and gastric fluids.

Despite these applicable qualities, biological activities and therefore therapeutic usefulness of these substances are limited because of their unfavourable physicochemical properties, especially very poor water-solubility and low oxidative stability. On the other hand, modern nanochemistry has offered a solution to this problem by incorporation of these substances into cyclodextrins. Cyclodextrins are cyclic compounds with a torous-shaped hydrophobic cavity in which various types of "guest" molecules may be included, either in the solid state or in an aqueous solution. Such host–guest inclusion complexes between appropriate molecules and CDs usually express increased solubility in aqueous solutions, as well as improved stability and bioavailability of the guest molecule.

Some indications of improving antioxidant activities of phenolic substances as the guests of CD have been reported (Ficarra et al., 2002).

☆ This work was supported by the Ministry of Science and Technology of Slovenia.
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doi:10.1016/j.foodchem.2008.02.051

Interactions between the guest and the host molecule in the complexes are stronger than those in physical mixtures. The washing procedure is therefore expected to be more effective in the case of physical mixtures. Park et al. (2002) proved the existence of inclusion complex on the basis of quantitative analysis of conjugated linoleic acid removed by different solvents determined by GC, while Ginés et al. (1996) determined the removed 2,4-dichlorophenoxy acetic acid by spectrophotometry at 284 nm. Szejtli and Bánky-Elöd (1975) eliminated fatty acids from the amylose surface using dioxan which has a weak complex-forming tendency ($K_{a\ (dioxan)} = 0.555$).

The aim of this study was to prepare inclusion complexes of CA isomers (*ortho, meta* and *para*) with βCD by the co-precipitation method in order to improve their water-solubility and their antioxidant activity. Simultaneously, the stoichiometry, the stability constants (K_a) and the inclusion or adsorption nature of these complexes were determined. For these purposes, several techniques, such as solubility determination (tests), mass spectroscopy (MS) and infrared spectroscopy (IR), combined with different washing procedures, as well as radical-scavenging methods for antioxidant activity were used.

2. Materials and methods

2.1. Materials

Standards of phenolic acids, *o*-CA, *m*-CA, *p*-CA, were obtained from Merck (Darmstadt, Germany). Dioxan, ethanol and methanol were used as solvents and were also procured from Merck (Darmstadt, Germany). KBr and βCD were supplied by Aldrich (Steinheim, Germany), while dextrin 20 (D20) was obtained from Serva Feinbiochemica (Heidelberg, Germany).

2.2. Synthesis of CA–βCD and CA–D20 complexes and preparation of physical mixtures of CAs and βCD

The complex CA–βCD was obtained from an aqueous solution of βCD (1.5 g βCD/33.15 ml H_2O) at 76 °C by the addition of CA (217 mg) dissolved in 3 ml of 40 vol.% ethanol. The operating temperature was then lowered to 70 °C and the mixture was stirred at this temperature for 2.5 h. The reaction mixture was then slowly allowed to cool to room temperature, being left in the oil bath. The solvent was evaporated at 30 °C and the sample was left in the oven at 50 °C throughout the night. The complex CA–D20 was prepared in the same manner. Homogeneous physical mixtures were obtained by mixing two pure components, CA and βCD, in a mortar for 30 min at room temperature in a host to guest molar ratio of 1:1.

2.3. Washing procedure

Solid samples were washed with solvents of diverse polarity (dioxan and methanol) by the following steps: 2.6 ml of the solvent were added to 250 mg of the complex or physical mixture in a 15 ml glass tube. The sample was vigorously stirred for two minutes and the filtrate was separated from undissolved residue by filtration, using black-ribbon filter paper. The residue was additionally washed with five portions of 2 ml of solvent; the filtrates were collected in glass tubes and dried at 50 °C to dryness. Dry residue was dissolved in methanol and analyzed by HPLC.

2.4. IR spectroscopy

The IR spectra of pure CA, βCD, their corresponding physical mixtures and inclusion complexes of molar ratio 1:1 were measured as potassium bromide discs. The substance was added to potassium bromide in a ratio of 1:150 (w/w), powdered/pulverised in a mortar and carefully mixed. An IR spectrophotometer, FTIR Perkin–Elmer 720-X interfaced to a Pentium II 350 MHz processor, was used for the analysis. The spectra were obtained in a frequency range between 4000 and 450 cm^{-1}.

2.5. Determination of the CAs by HPLC

An HPLC-method originally described by Mattila and Kumpulainen (2002) was used with some modifications. Chromatographic quantitative determination of CA was achieved with isocratic elution (acetonitrile/phosphoric acid (5 mM, pH 2.5) in a volume ratio of 30:70 (v/v)) on Hypersil® ODS (4.6 × 150 mm, 5 μm; Thermo, USA). Analysis was accomplished within ten minutes at 280 nm. The flow rate was 0.8 ml/min, the column temperature was set at 30 °C and the injection volume was 10 μl. The retention times for *o*-CA, *m*-CA and *p*-CA were approximately 3.85 min, 3.2 and 2.8 min, respectively. Coumaric acids were quantified using the external standard method.

2.6. MS determination of CA–βCD complexes

Four milligrammes of the complex previously washed with dioxan were dissolved in 2 ml of water and samples of 10 μl were directly injected into the MS with a mobile phase (0.1% ammonia in water), the flow rate being 0.5 ml/min. ESI conditions for ionisation were as follows: capillary temperature 230 °C, sheath gas flow (N_2) at 0.74 MPa, auxiliary gas flow (N2) at 0.07 MPa, source voltage −4.5 kV, source current 30.5 μA, APCI vaporiser temperature 450 °C, capillary voltage −9.7 V.

2.7. Phase solubility study

Solubility measurements were performed according to Higuchi and Connors (1965). Excess amounts of CA (70 mg) were added to 3 ml of water or aqueous solution of βCD at different concentrations (2–15 mM) in 4 ml plastic tubes. The samples were vigorously mixed (180 rpm) at

25 °C for 6 days. Before the HPLC analysis was performed, the samples were filtered through a hydrophobic membrane Millipore Millex-HV PVDF ($d = 0.45$ μm) and diluted prior HPLC analysis when necessary. The apparent binding constants of the CA–βCD complexes were calculated from the slopes and intercepts of the straight lines of the phase solubility diagram according to the method of Higuchi and Connors (1965). It was assumed that a single complex between guest (G) and host (H) G_mH_n was responsible for the increase in apparent solubility of G. The complex formation constant for this species is given by

$$K_a = [G_mH_n]/([G]^m \cdot [H]^n). \quad (1)$$

The concentration may be expressed in terms of known quantities:

$$[G] = [G]_0 \quad (2)$$

$$[G_mH_n] = ([G]_t - [G]_0)/m \quad (3)$$

$$[H] = [H]_t - n \cdot [G_mH_n] \quad (4)$$

where $[G]_0$ is the equilibrium solubility of G in the absence of H, $[G]_t$ is the total amount of dissolved G, regardless of molecular state and $[H]_t$ is the total added concentration of H. Eqs. (1)–(4) can be combined to give an expression for K_a in terms of known concentrations, m and n. Assuming that $n = 1$, Eq. (5) can be derived:

$$[G]_t = (m \cdot K_a \cdot [G]_0 \cdot [H]_t)/(1 + K_a \cdot [G]_0) + [G]_0. \quad (5)$$

A plot of $[G]_t$ against $[H]_t$ for the formation of a soluble complex, L_mH should, therefore, yield a straight line. The intercept is equal to $[G]_0$ and the slope is given by

$$b = (m \cdot K_a \cdot [G]_0^m)/(1 + K_a \cdot [G]_0^m). \quad (6)$$

When the $m = 1$, K_a can be obtained from the slope of a phase diagram curve which is constructed by plotting, on the vertical axis, total molar concentration of G ($[G]_t$) found in the solution phase versus the molar concentration of H ($[H]_t$) added to the system:

$$K_a = b/([G]_0 \cdot (1 - b)). \quad (7)$$

2.8. DPPH radical-scavenging method

The antioxidant activities (AA) of free CA and CA–βCD complexes were measured in terms of hydrogen donating or radical-scavenging ability, using the stable radical DPPH· The method, according to Brand-Williams, Cuvelier, and Berset (1995), was modified, depending on strength and solubility of measured antioxidant; 3.06 mg of CA or 24.31 mg of its complex with βCD were weighed into a 4 ml Cryo-tubes. The sample was dissolved in 3.5 ml of water. For determination of antioxidant activity, 1 ml was taken and vigorously mixed with 2 ml of ethanolic solution of DPPH· (0.01 mg/ml). The absorbance at 514 nm was measured in a 12 mm cuvette after exactly 60 min of the reaction vs blank sample. Blank sample was prepared in the following manner: 1 ml of distilled water was combined with 2 ml of DPPH· ethanol solution. The results were expressed as percentage DPPH· elimination calculated according to the following equation:

$$AU = [1 - A_s/A_0] \times 100,$$

where AU is radical-scavenging activity, A_s is absorbance of sample and A_0 absorbance of blank sample.

3. Results and discussion

3.1. Phase solubility study

The stoichiometry of these complexes was determined by the solubility technique (Fig. 2). Phase-solubility diagrams obtained with βCD and CA showed a linear relationship between the amount of CA solubilized and the concentration of βCD in solution (A_L diagram). According to Higuchi and Connors (1965), this may be attributed to the formation of soluble complexes with a CA–βCD stoichiometric ratio of 1:1.

From the slopes and intercepts, the equilibrium association constants ($K_{1:1}$) were calculated:

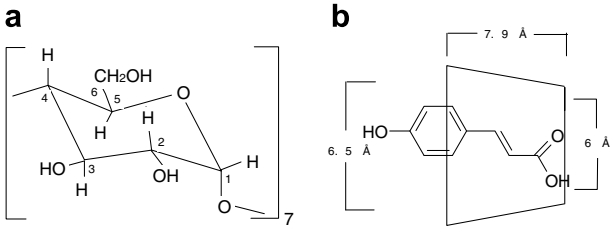

Fig. 1. Structures of βCA, Szejtli (1998), (a) and proposed model of *p*-CA–βCA inclusion (b), based on calculations of crystal structure of *trans*-CA–βCD inclusion complex by Kokkinou et al. (2000).

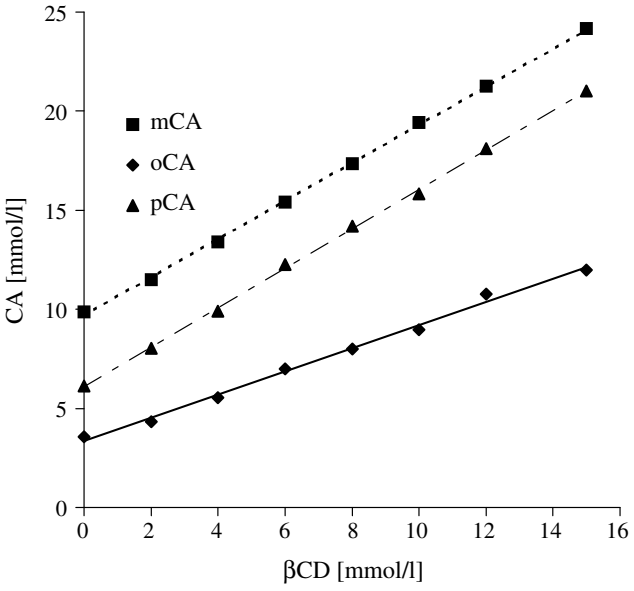

Fig. 2. Phase-solubility diagrams of CA–βCD systems.

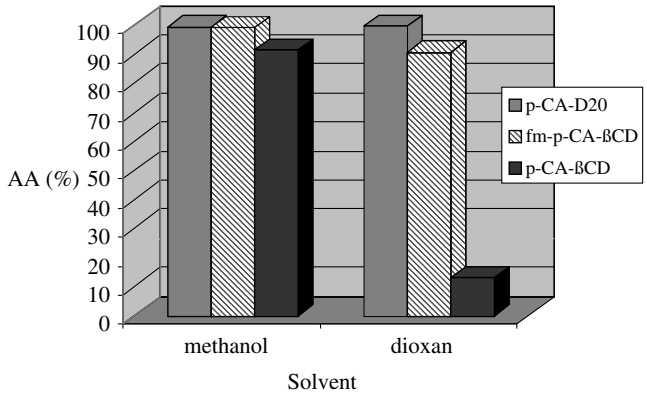

Fig. 3. The percentages of extracted *p*-CA: dioxan removed almost 100% of *p*-CA from CD physical mixture (phm-*p*-CA–βCD) and dextrin complex (*p*-CA–D20) while it extracted only 15% of *p*-CA from the CD complex (*p*-CA–βCD).

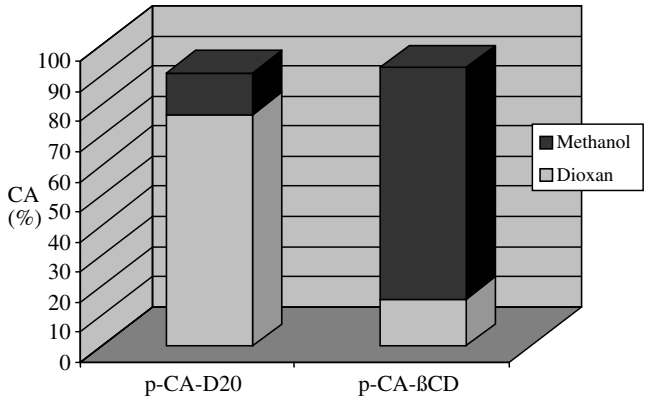

Fig. 4. The efficiency of the washing procedure. Complexes (*p*-CA–D20 and *p*-CA–βCD) were initially washed with dioxan (bright part) and successively with methanol (dark part).

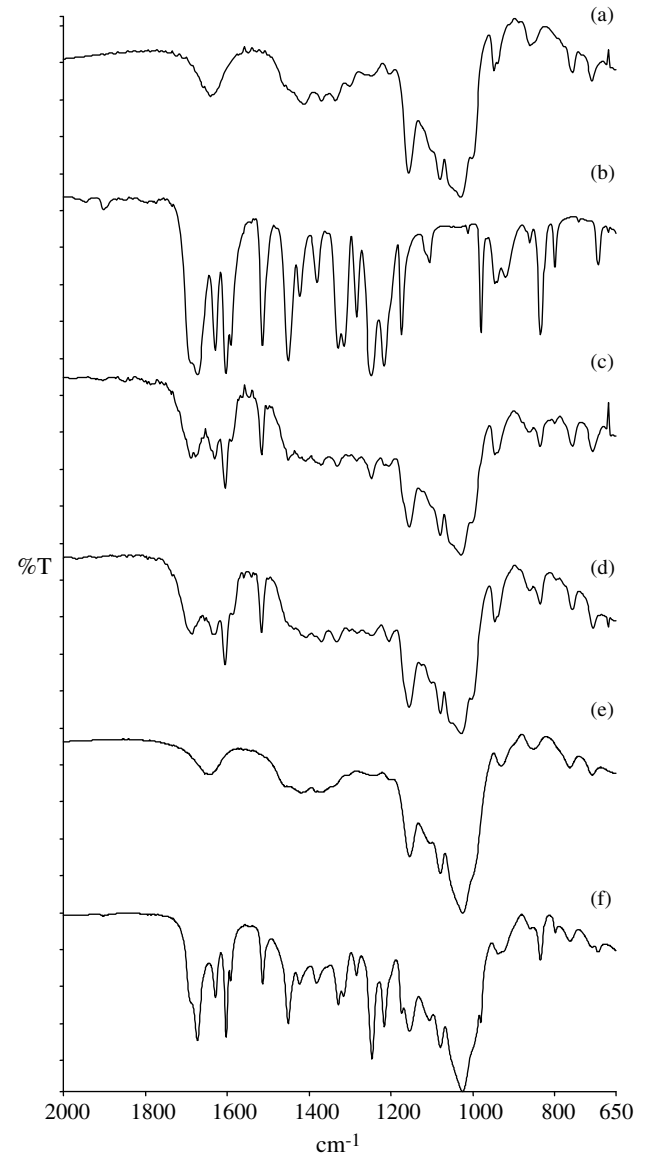

Fig. 5. IR spectra: βCD (a); *p*-CA (b); *p*-CA–βCD physical mixture (c); *p*-CA–βCD complex (d); D20 (e) and *p*-CA–D20 complex (f).

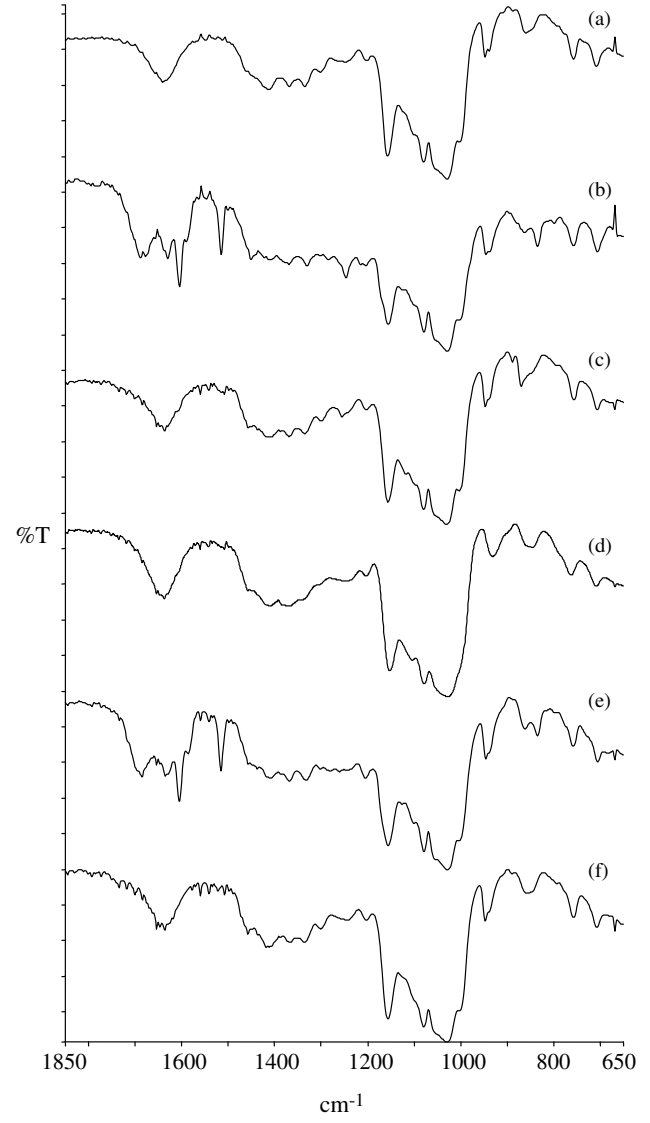

Fig. 6. IR spectra: βCD (a); *p*-CA–βCD physical mixture (b); *p*-CA–βCD physical mixture washed with dioxan (c); *p*-CA–D20 complex washed with dioxan (d); *p*-CA–βCD complex washed with dioxan (e) and *p*-CA–βCD complex washed with methanol (f).

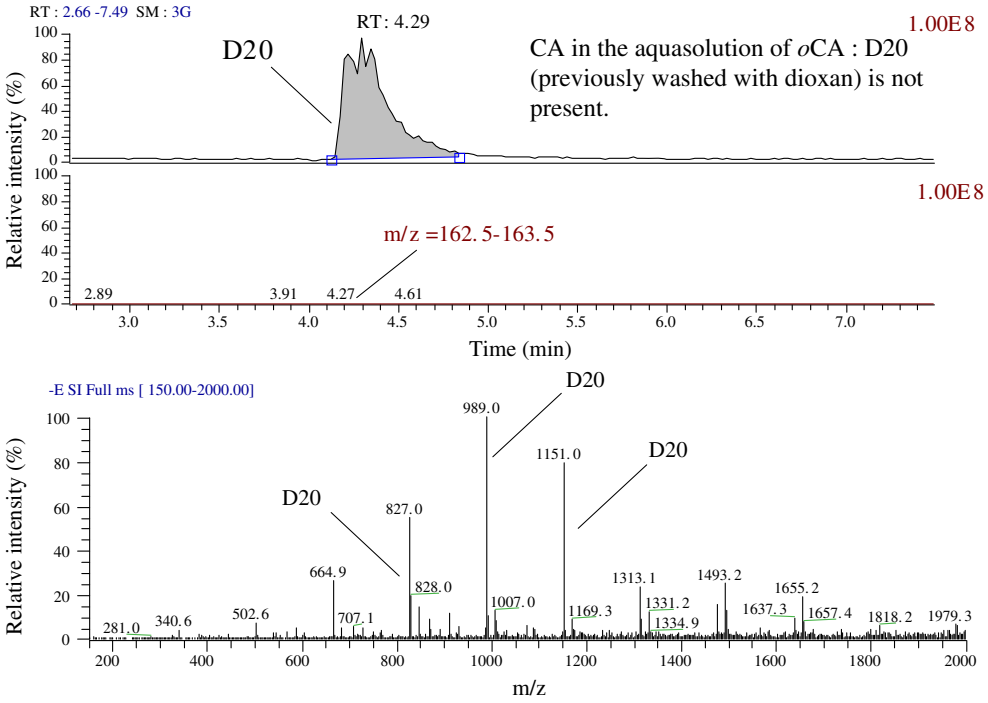

Fig. 7. Mass spectra of *o*-CA–D20 sample preliminary washed with dioxan.

$$K_a(o-\mathrm{CA}) = 0.39 \times 10^3\ \mathrm{M}^{-1},\quad K_a(m-\mathrm{CA}) = 2.81 \times 10^3\ \mathrm{M}^{-1} \text{and } K_a(p-\mathrm{CA}) = 49.25 \times 10^3\ \mathrm{M}^{-1}.$$

3.2. Washing procedure

In order to examine the nature of interactions between *p*-CA and different forms of dextrins, various washing procedures were used. Extracted *p*-CA was quantified by the HPLC-UV system. Figs. 3 and 4 demonstrate the relative amounts of extracted *p*-CA from different systems: CD-physical mixture and CD as well as dextrin-complexes, Fig. 3.

The same functional groups are present on the surface of βCD and D20; however, the D20 molecule has an open structure, while βCD provides a hydrophobic cavity. We explain the difference between the dioxan and methanol washing procedures by the fact that dioxan extracted the *p*-CA from the surface, but not from the βCD cavity (Fig. 1). Methanol, however, removed almost all *p*-CA from all the samples: phm-*p*-CA–βCD, *p*-CA–βCD and *p*-CA–D20, which indicates the removal of both adsorbed *p*-CA and embedded *p*-CA. To confirm these conclusions the consecutive washing procedure was used. It is obvious (Fig. 4) that the extraction of *p*-CA with dioxan is effective only in the case of *p*-CA–D20. On the other hand, in the case of *p*-CA–βCD, dioxan removed a minority of *p*-CA from the surface and he rest of the *p*-CA was successfully washed off by subsequent treatment with methanol.

On this basis, we concluded that methanol successfully penetrated the βCD cavity and totally replaced *p*-CA from the βCD. While indicating the competitive binding, dioxan has little or no affinity for the βCD cavity. Therefore, dioxan can be used as a selective solvent for adsorbed or free *p*-CA in the *p*-CA–βCD system.

3.3. IR spectra

Another method that clarified the nature of the interactions between *p*-CA and βCD was IR-spectroscopy. This technique has previously been used (Pralhad & Rajendrekumar, 2004; Martin, León, Martin, del Castillo & Menéndez, 2003; Lamcharfi, Kunesch, Meyer, & Robert, 1995; Manolikar & Sawant, 2003; Redenti et al., 1996). The investigation of host–guest interactions relies upon the analysis of spectral changes due to atom or group vibrations directly involved in interaction. Changes in the characteristic bands of pure substances confirm the existence of the complex as a new compound with different spectroscopic bands (Ficcara et al., 2002). However, it is not suitable for the determination of inclusion compounds if the resulting spectra present a superposition of host and guest bands (Bratu et al., 1998), which is the major disadvantage of this method.

Fig. 5 presents IR spectra of the pure compounds, βCD, dextrin 20, *p*-CA, and their associations (CD physical mixture as well as CD and dextrin complex). In the regions 1190–930 cm^{-1} and 3690–3000 cm^{-1}, a superposition of βCD and *p*-CA bands is present; therefore the region from 1700 to 1440 cm^{-1} was of current interest.

Our experimental data (Fig. 6) show only the superposition of spectra of *p*-CA, βCD or D20 but no shifting of mayor spectral bands. Therefore the washing procedures with dioxan and methanol were applied on phm-*p*-CA–

βCD, *p*-CA–βCD and *p*-CA–D20 in order to prove the existence of *p*-CA–βCD complex (Fig. 6e). In accordance with spectral data we came to the same conclusion as in Section 3.2, where *p*-CA was retained in the βCD cavity, which is clearly seen by the presence of *p*-CA bands in the region from 1700 to 1440 cm^{-1}(Fig. 6e).

3.4. MS

The formation and stoichiometry of the βCD with CAs complexes were detected and determined by ESI-MS. For all three isomers of CA, the same m/z values were found in all the mass spectra, which indicated that specific inclusion complexes had been formed with certain stoichiometry CA:βCD (1:1 and negligible amount of 1:2).

In order to prove the existence of the inclusion complex of *p*-CA with βCD, *p*-CA–D20 and *p*-CA–βCD washed with dioxan were analysed using mass spectrometry in negative ESI mode. Mass spectra of *p*-CA–D20 washed with dioxan did not reveal *m*/*z* values typical for the *p*-CA–D20 association form (Fig. 7) while, in the mass spectra of *p*-CA–βCD washed with dioxin, two bands, corresponding to different *p*-CA–βCD species, appeared (Fig. 8). This indicates that dioxan could extract all *p*-CA (free and CA from the surface of D20 and βCD), but not from the β-CD cavity.

3.5. Determination of antioxidant activity

The antioxidant capacity of phenols is generally tested by the reaction with oxidants where resonance-stabilized phenoxyl radicals occur. The antioxidant activity of phenolic compounds depends on the position and degree of hydroxylation, as well as the nature of radicals of the ring structure. Antioxidative activity is intensified by the presence of a second hydroxy group, as in caffeic and protocatechuic acids, through the formation of an intramolecular hydrogen bond (Natella, Nardini, Di Felice, & Scaccini, 1999). The presence of the –CH=CH–COOH group ensures increased antioxidative efficiency of CA derivatives participating in

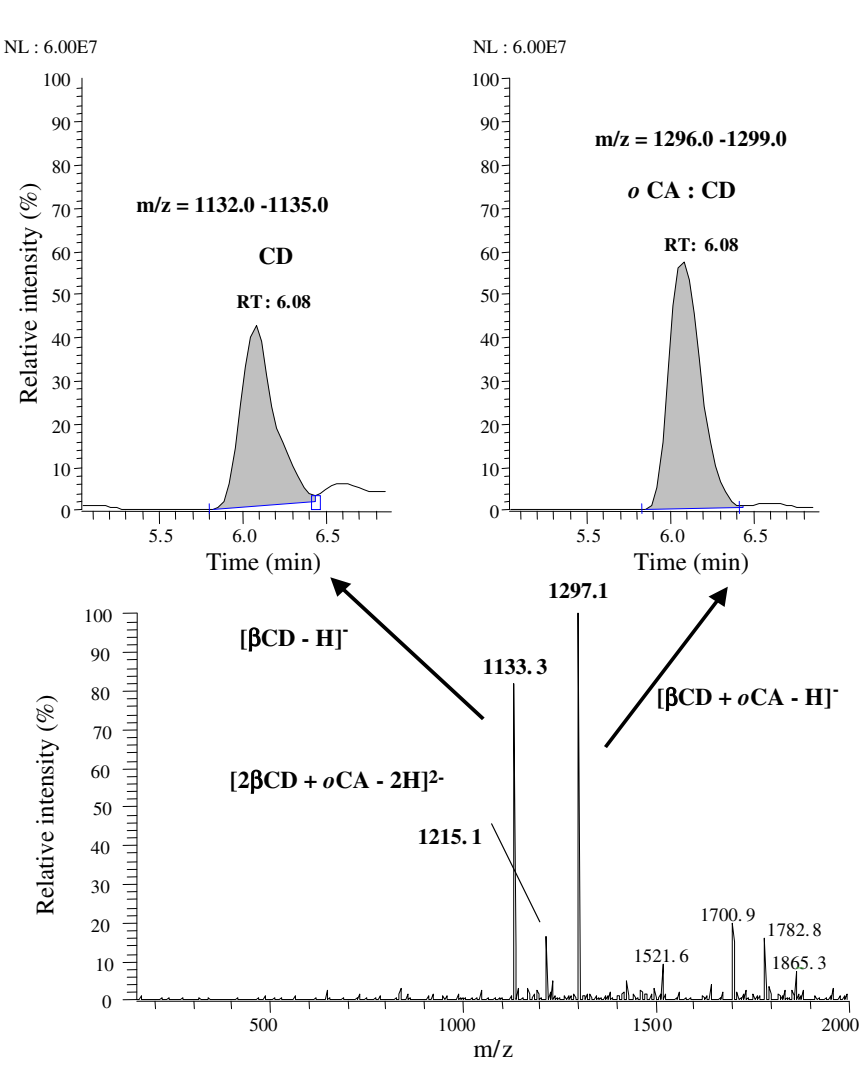

Fig. 8. Mass spectra of *o*-CA–βCD sample preliminary washed with dioxan.

Table 1
Antioxidative activities (%) of free and complexed CAs

	CA	CA–βCD
ortho	14.5	26.4
meta	3.38	8.48
para	29.9	24.1

stabilization of the phenoxyl radicals by resonance (Marinova & Yanishlieva, 2003) in comparison to –COOH and the corresponding hydroxybenzoic acid.

We assume that, in the CD inclusion complexes of *o*-CA and *m*-CA, the distance between secondary hydroxyl groups of βCD and –OH on the aromatic ring of *o*-CA and *m*-CA is approximately the same as the distance of –OH groups in the caffeic acid molecule (<3 Å). Therefore the formation of an "intramolecular" hydrogen bond of the inclusion complex is possible and consequently an increase of antioxidant capacity is expected.

Table 1 shows the decrease in absorbance of DPPH radical after 1 h, due to its reduction by the compounds tested. Antioxidant activity of tested substances, as determined by the DPPH radical-scavenging method, decreased in the order: *p*-CA > *o*-CA–βCD > *p*-CA–βCD > *o*-CA > *m*-CA–βCD > *m*-CA. We confirmed the report of Marinova & Yanishlieva, 2003 that *ortho* and *para* isomers are better antioxidants, due to electron-donating effects of the COOH–CH=CH– group on the aromatic ring, participating in the resonance stabilization of the phenoxyl radicals.

The complex of *o*-CA with βCD showed improvement of the elimination ability of DPPH radical from 14.5% up to 26.4%. In the case of the *meta* isomer, antioxidative activity increased approximately 2.5 times. On the other hand, antioxidative activity of free and complexed *p*-CA did not differ significantly. These differences among *ortho, meta* and *para* isomers can be explained by the distances of CA OH groups from the βCD OH groups in the complex itself. It is evident that the –OH at the *ortho* and *meta* positions of CA molecules is close enough to secondary –OH groups of βCD to form hydrogen bonds and contribute to antioxidant activity (Kokkinou, Makedonopoulou, & Mantzafos, 2000), while the distance of the OH group in the *para* position is too far away from the secondary OH groups of βCD to re-establish the hydrogen bonds (Fig. 1b).

Acknowledgements

We thank J. Kobe for valuable discussions and comments on the manuscript. We also thank A. Golc Wondra for HPLC analysis.

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