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Modified Radical Scavenging and Antioxidant Activity Measurement of β-Carotene with β-Cyclodextrins Complexation in Aqueous Medium

Saliha Esin ÇELİK,* Burcu BEKDEŞER,*, Ayşe Nur TUFAN,*,** and Reşat APAK*,**†**

**Department of Chemistry, Faculty of Engineering, Istanbul University, Avcilar 34320, Istanbul, Turkey*
***Istanbul University—Application & Research Center for the Measurement of Food Antioxidants, Istanbul University, Avcilar 34320, Istanbul, Turkey*

In order to evaluate the antioxidant capacity/activity of β-carotene (BC) in aqueous media, we investigated the inclusion complexes of BC with methyl-β-cyclodextrin (Me-β-CD), 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) and 2-hydroxyethyl-β-cyclodextrin (HE-β-CD) that enhance water solubility and chemical stability. The inclusion complexes (monitored by FTIR) exhibited higher solubility than free BC, and phase solubility studies showed a linear increase in the solubility with the Me-β-CD concentration. Cupric ion-reducing antioxidant capacity (CUPRAC), ABTS-persulfate, peroxyl and hydroxyl radical scavenging assays were applied. CD-complexed β-carotene exhibited less effective antioxidative and radical scavenging than free BC dissolved in acetone. β-Carotene showed the highest antioxidant capacity in the presence of HE-β-CD, and the lowest with Me-β-CD, probably due to the deeper and more hydrophobic cavity of the latter. We believe that this is the first report on devising simple spectrophotometric methods for the wholistic assessment of antioxidant activity/capacity, hydroxyl and peroxyl radical scavenging activity of β-carotene in aqueous solution with CDs.

Keywords β-Carotene, β-cyclodextrin, inclusion complexes, antioxidant capacity, radical scavenging activity

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Introduction

Carotenoids are an important class of bioactive compounds mostly abundant in yellow, orange, and red-colored fruits and vegetables, such as apricot, carrot, pepper, tomato and dark-green leafy vegetables, such as spinach and kale. In this class, β-carotene is a naturally occurring orange-colored carbon-hydrogen carotenoid in our diet, which has antioxidant, antimutagenic, anticardiovascular, antiinflammatory, and anticancer activities.[1] In several studies, the role of BC as an antioxidant against serious disorders, such as lung cancer[2,3] and cardiovascular diseases,[4-6] was investigated. Many published studies were performed for determining the antioxidant efficiency of carotenoids in different solvent media. The most widely used methods for the determination of carotenoid antioxidant activity were ABTS (2,2′-azinobis[3-ethylbenzo-thiazoline-6-sulfonate])/TEAC (trolox equivalent antioxidant capacity)[7-13] CUPRAC (cupric ion reducing antioxidant capacity)[13-16] and DPPH (2,2-diphenyl-1-picrylhydrazyl) assays.[12,17] In these studies, pure acetone, dichloromethane, hexane and acetone-water solutions containing Me-β-CD were used. However, some total antioxidant capacity (TAC) tests (such as Folin–Ciocalteu phenolics assay) were shown to give high blank values with acetone as a solvent.[18]

It is well known that BC is insoluble in water and slightly soluble in vegetable oils. It is also quite sensitive to the temperature, pH, and presence of light and oxygen.[19] To overcome these drawbacks, complexation with oligosaccharide-type host macromolecules, such as CDs, can be applied as a convenient technique to enhance the solubility, bioavailability and stability of hydrophobic guest molecules, such as BC. CDs have hydrophobic inner cavity and hydrophilic external surface responsible for their ability to solubilize several non-polar guest molecules.[20] Due to the polarity of hydrophilic exterior walls, CDs are able to generate inclusion complexes with hydrophobic guest molecules incorporated into the inner cavity by displacing water in an aqueous medium.[21] α-, β-, γ-CDs consisting of six to eight α-(1,4)-linked D-glucopyranose units are native CDs that are inexpensive and friendly molecules capable of improving the biological, chemical and physical properties of bioactive molecules.[22] The most common CD used for complexation is β-CD; however, it is less soluble in water. Therefore, synthetic β-CD derivatives have been used to increase their solubility in water, molecular selectivity and binding ability, such as methyl-β-cyclodextrin, 2-hydroxyethyl-β-cyclodextrin, 2-hydroxypropyl-β-cyclodextrin, heptakis (2,6-di-*o*-methyl)-β-cyclodextrin, and sulfobutylether-β-cyclodextrin.[23] Randomly methylated β-CD (7% (v:v)) was also used as a solubility enhancer in lipophilic ORAC (oxygen radical absorbance capacity)[24] and HORAC (hydroxyl radical averting capacity) assays[25] for non-hydrophilic products. However, in the ORAC test incorporating a high amount of CD (7%) in the reaction medium, it should always be remembered that the tested antioxidant may not get out of the relatively stabilized inclusion complex to perform the test.

† To whom correspondence should be addressed.
E-mail: rapak@istanbul.edu.tr

The encapsulation of carotenoids with various cyclodextrins has been reported in the literature.[26-29] Oliviera *et al.*[26] investigated the inclusion processes of β-carotene, astaxanthin, lycopene, and norbixin into the β-CD cavity by means of Raman spectroscopy and quantum-mechanical calculations. Complexes of various carotenoids with different CDs were studied by proton nuclear magnetic resonance (^{1}H NMR) and UV-Vis absorption spectroscopy by Polyakov and coworkers.[27] The authors investigated the antioxidative reactivity of carotenoid-CD inclusion complexes toward peroxyl radicals by an electron paramagnetic resonance (EPR) trapping technique. The inclusion complex of β-carotene with β-cyclodextrin was characterized by Fourier transform infrared spectroscopy (FTIR) and NMR spectroscopy, and monitored by TEM.[28] Zaibunnisa *et al.*[29] studied the characterization of a β-carotene-γ-cyclodextrin inclusion complex performed by FTIR and field emmision scanning electron microscopy (FESEM) using different complex preparation techniques. The co-precipitation technique was found to be the best method that may increase the solubility of β-carotene.[29] Many studies have emphasized the characterisation of carotenoid inclusion complexes, such as water solubility, bioavailability, and stability. However, very scarce information on the antioxidant capacity and radical scavenging activity measurements of carotenoid-cyclodextrin complexes could be obtained,[30-32] probably due to the non-compatibility of the test medium with the solvent requirement for carotenoid dissolution. The antioxidant capacities of gum arabic and maltodextrin microcapsules containing antioxidant molecules (trolox, α-tocopherol, β-carotene, apo-80-carotenal and apo-120-carotenal) against reactive oxygen and nitrogen species (ROS/RNS) were evaluated by Rodrigues *et al.*[30] Unfortunately, the micro-encapsulation material (gum arabic), which should stay inert toward TAC assays, showed antioxidant properties itself.[33] The antioxidant activities of inclusion complexes of some xanthophyll carotenoid pigments such as astaxanthin[31] and cantaxanthin[32] with HP-β-CD were assayed along with their water solubility and storage stability.

The objective of this study is to determine the total antioxidant capacity and radical scavenging activity of BC complexed within Me-β-CD, HP-β-CD and HE-β-CD in an aqueous medium. Due to its hydrophobicity, there is no specific study concerning the overall antioxidative characteristics (comprising a whole bunch of antioxidant activity and capacity tests) of BC in aqueous media. Especially ROS scavenging tests in organic solvents are very difficult to perform for BC, because Fenton-type reactions used for generating ROS also produce reactive species from organic solvents (such as acetone peroxides), thereby interfering with the selected assay. Therefore, additional measures, such as the complexation of BC with β-CDs, should be taken for carrying out the mentioned conventional tests in aqueous media (without a need for organic solvents) to reliably and precisely measure the scavenging ability of BC toward reactive species. This is the first time that the total antioxidant capacity and radical quenching ability of BC (in complexed form) against ROS, such as hydroxyl and peroxyl radicals in aqueous medium, were reported. The present results can be applied for the complexation and encapsulation of such carotenoids with antioxidant functionality.

Experimental

Reagents and chemicals

Chemical substances of analytical reagent grade were provided from the sources given below: Neocuproine (2,9-dimethyl-1,10-phenanthroline (Nc)), AAPH (2,2′-azobis(2-amidinopropane) dihydrochloride), absolute ethanol, acetone, dichloromethane (DCM) and hexane: Sigma (Steinheim, Germany); L-ascorbic acid: Aldrich (Steinheim, Germany); copper(II) chloride dihydrate, hydrogen peroxide (35 wt%), potassium mono- and di-hydrogen phosphate, disodium terephthalate, potassium persulphate: Merck (Darmstadt, Germany); ammonium acetate, iron(III) chloride: Riedel-de Haen (Steinheim, Germany); ethylenediamine tetraaceticacid disodium salt (Na_2-EDTA), fluorescein (FL), 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) diammonium salt, trolox (TR), β-carotene, methyl β-cyclodextrin, 2-hydroxyethyl-β-cyclodextrin and 2-hydroxypropyl-β-cyclodextrin: Fluka (Buchs, Switzerland).

Apparatus

The fluorescence intensity and molecular absorption spectral measurements were made using an Agilent (Mulgrave, Victoria, Australia) Cary Eclipse spectrofluorometer and Perkin Elmer Lambda 35 UV-Vis spectrophotometer (Norwalk, USA), respectively, in quartz cuvettes. Infrared characterization analyses of complexes were conducted using an Agilent Cary 630 FTIR spectrometer equipped with a single-reflection diamond ATR and Agilent MicroLab FTIR method-driven software. The organic solvent in the complex and extracts was removed with a Buchi Rotavapor (R-210, Switzerland) coupled with a heating bath (B-491) and a vacuum pump (V-100). Food samples were dried in a freeze-drier (Telstar LyoQuest, Terrassa, Spain). Extractions were carried out using a Bandelin Sonorex (Germany) ultrasonic bath. Other related equipment were a Memmert water bath (Germany) and a Millipore Simpak1 Synergy 185 (France) ultrapure water system.

Preparation of inclusion complexes of β-carotene in synthetic solutions and real samples with β-cyclodextrin derivatives

The β-carotene stock solution was prepared in dichloromethane at 2.5 mg mL^{-1} concentration. β-Cyclodextrin derivatives were prepared in ethanol at 20 mg mL^{-1} concentration. Inclusion complexes of β-carotene were prepared by using Me-β-CD, HP-β-CD, and HE-β-CD. β-Carotene stock solution (2.5 mg mL^{-1}) (x = 1 mL) was added to (50 – x)mL ethanolic solution of CD (20 mg mL^{-1}). The mixture in a total volume of 50 mL was incubated for 24 h in a shaking water bath kept at 37°C. At the end of this period, DCM and ethanol solvent mixture was evaporated to dryness under vacuum at 30°C using a rotary evaporator. The dried residue was redissolved in 4 mL of water and filtered through a 0.45-μm membrane filter before analysis to remove free β-carotene.[34]

Carrots and apricots were supplied from the local market and chopped into small pieces with a ceramic knife and freeze-dried at –40°C for 24 h prior to use. Two grams of sample were taken and extracted for 10 min with hexane/ethanol/acetone (2:1:1, v/v/v) at room temperature in an ultrasonic bath protected from light. The extraction procedure was applied thrice, and the upper phases were combined and completed to 50 mL.[13] At the end, the solvent mixture was removed with the aid of a rotary evaporator and the dried residue was redissolved in 2 mL of DCM. The inclusion complexes of real sample extracts were prepared according to the procedure mentioned above.

Determination of β-carotene content complexed in β-cyclodextrin derivatives

The BC content in the inclusion complexes was determined so as to calculate the complex stabilities and correlate to antioxidant capacity/activity data. For this purpose, inclusion complexes (BC-Me-β-CD; BC-HP-β-CD; BC-HE-β-CD) were

disrupted by adding 2 mL of ethanol and 3 mL of hexane to 1 mL of an aqueous complex, according to the method of Kaur *et al.*[28] β-Carotene was disposed to dissolve in the hexane phase, followed by the evaporation of hexane under a N_2 atmosphere and dilution with 3 mL of acetone. This procedure was repeated twice and the intrinsic absorbance of BC was measured at 450 nm. The concentration of BC retained in the complexes was then determined using a calibration curve constructed as absorbance (A_{450}) *versus* concentration using standard solutions of free BC in acetone in the range of 0.005 – 0.03 mg mL^{-1}.

Phase solubility measurement

A phase solubility measurement was performed according to a method defined by Higuchi and Connors.[35] To each dark-colored erlenmeyer flask, an excess amount of BC (5 mg) was added to ethanolic cyclodextrin solutions in various concentrations, varied from 0 to 20 mM. The solutions were equilibrated at 37°C for 24 h in a shaking water bath (Memmert, Germany), and then centrifuged at 3000 rpm for 10 min. The supernatants were filtered through a 0.45-μm membrane filter to remove undissolved solid particles and the absorbance values were recorded at 450 nm by using a Perkin Elmer Lambda 35 UV-Vis spectrophotometer (Norwalk, USA). The stability constants (K_c) were estimated from the slope and the intercept of phase solubility curve by using

$$K_c = \frac{\text{Slope}}{S_0(1-\text{slope})}, \quad (1)$$

where S_0, solubility of β-carotene in ethanol at 37°C; slope, corresponding slope of the phase solubility curve.

Fourier transform infrared (FTIR) spectroscopy

FTIR spectroscopy was used to prove the complexation of β-carotene with β-CDs. Inclusion complexes of β-carotene were prepared by using Me-β-CD, HP-β-CD, and HE-β-CD. A β-carotene stock solution (2.5 mg mL^{-1}) (x = 1 mL) was added to a (50 – x) mL ethanolic solution of CD (20 mg mL^{-1}). The mixture in a total volume of 50 mL was incubated for 24 h in a shaking water bath kept at 37°C. At the end of this period, a DCM and ethanol solvent mixture was evaporated to dryness under a vacuum at 30°C using a rotary evaporator. The dried residues were powdered in a mortar. A small amount of each ground-sample powder was placed on the diamond ATR crystal and pressed against the diamond crystal using the attached pressure clamp. FTIR spectra were recorded in the range of 650 to 4000 cm^{-1} using an Agilent Cary 630 FTIR spectrophotometer.

CUPRAC total antioxidant capacity assay

The antioxidant capacity of the CD inclusion complexes of β-carotene was determined using the CUPRAC method, as described by Apak *et al.*[36] in the following manner:

1 mL $CuCl_2$ (10 mM) + 1 mL Nc (7.5 mM) + 1 mL NH_4Ac buffer (1.0 M) + x mL BC-CD complex (diluted with water at an appropriate volume ratio) + (1.1 – x)mL H_2O.

The absorbance at 450 nm of the final solution was recorded 30 min later against a reagent blank. The tested CD inclusion complexes exhibited no absorbance at 450 nm. The trolox equivalent antioxidant capacity (TEAC) coefficients were calculated by dividing the absorption coefficient (ε) of each inclusion complex to the absorption coefficient of trolox under the specified conditions. The total antioxidant capacity of carrot and apricot extracts as mg TR per gram sample could be found by dividing the observed absorbance to the absorption coefficient of trolox. The assays were carried out in triplicate and the results expressed as (mean ± standard deviation).

ABTS-persulfate total antioxidant capacity assay

An ABTS chromogenic reagent solution at 7 mM concentration was converted to the colored $ABTS^{\bullet+}$ radical cation solution by adding $K_2S_2O_8$ containing 2.45 mM final persulfate concentration. This radical solution was kept in the dark at room temperature for 12 – 16 h and diluted with ethanol at a ratio of 1:10 before use in real measurements.[7] To a test tube were added an (x)mL CD inclusion complex, 1 mL final $ABTS^{\bullet+}$ solution, and (4 – x)mL of EtOH; the decrease in the absorbance at the end of 6th min was recorded at 734 nm. The absorbance difference (ΔA) was calculated by subtracting the absorbance measured in the presence and absence of inclusion complex. The TEAC coefficients of the inclusion complexes and the TAC values of real samples were calculated from the absorption coefficient of trolox obtained by the ABTS method as specified in the previous section.

Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of CD inclusion complexes was determined by using a terephthalate (TP) probe that produces a fluorescent product, hydroxyterephthalate (hTP), upon hydroxylation with $^{\bullet}OH$ radicals.[37] The hydroxyl radical was generated by the Fenton reaction system. To a test tube were added 0.6 mL of 200 mM phosphate buffer (pH 7.4), 0.6 mL of 1 mM TP probe, 0.3 mL of 1 mM Na_2EDTA, 0.6 mL of 0.5 mM iron(III) chloride solution, 0.3 mL of 1 mM ascorbic acid, 0.3 mL of a CD inclusion complex at various concentrations, and 0.3 mL of 10 mM hydrogen peroxide rapidly in this order. The mixture in a total volume of 3 mL was incubated for 30 min in a water bath kept at 37°C. At the end of this period the fluorescence intensity (λ_{ex} = 315 nm, λ_{em} = 450 nm) of the reaction mixture was recorded.

The inhibition ratio of scavengers (%) was calculated using the following formula:

$$\text{Inhibition (\%)} = 100\frac{(A_0 - A)}{A_0}, \quad (2)$$

where A_0 and A are the absorbances of the system in the absence and presence of a CD inclusion complex, respectively. The IC_{50} values (the half maximal inhibitory concentration) were determined by means of a linear plot of the inhibition percentage as a function of BC concentration retained in methyl-, hydroxypropyl-, and hydroxyethyl-β-CDs.

Peroxyl radical scavenging activity

The peroxyl radical scavenging activity of the CD inclusion complexes of β-carotene was determined using the ORAC method (with the use of a non-automated fluorescence spectrometer), as described by Ou *et al.*[38] in the following manner:

2.0 mL of 78 nM FL + 2.0 mL of CD inclusion complex (at different concentrations) + 1.0 mL of 221 μM AAPH

The mixture in a total volume of 5 mL was incubated for 10 min in a water bath kept at 37°C. At the end of this period the fluorescence intensity (λ_{ex} = 493 nm, λ_{em} = 515 nm) of the reaction mixture was recorded. The $ROO^{\bullet}$ scavenging activity

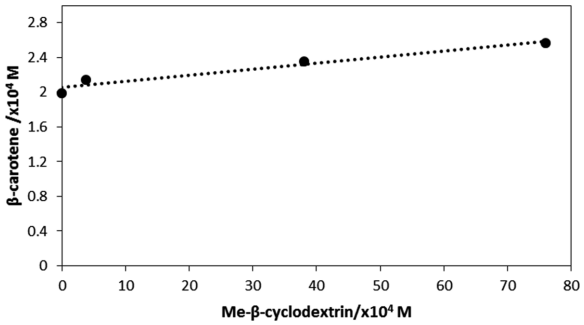


Fig. 1 Phase solubility diagram of the inclusion complex, indicating increased β-carotene dissolution as a function of the Me-β-CD concentration.

was calculated using the following equation:

$$\text{Inhibition}\ (\%) = 100\frac{(I_2 - I_1)}{(I_0 - I_1)}. \quad (3)$$

Here, I_0 is the initial fluorescence intensity of the fluorescein probe, and I_1 and I_2 are the fluorescence intensities of FL probe subjected to peroxyl radical attack in the absence and presence of a scavenger compound, respectively. The IC_{50} values inclusion complexes were calculated by linear regression of the BC concentration retained in methyl-, hydroxypropyl-, and hydroxyethyl- β-CDs *versus* inhibition (%).

Results and Discussion

Phase solubility

A phase solubility study was performed by mixing an excess amount of BC with ethanolic solutions containing increasing amounts of derivatized β-CDs. A phase solubility diagram of BC with Me-β-CD at 37°C was drawn as a function of the CD concentration by plotting the changes in the solubility of BC measured spectrophotometrically (Fig. 1). According to the results, the complex exhibited higher solubility than did free BC, and a linear increase in the solubility was observed with an increase in the Me-β-CD concentration. The stability constants (K_c) were estimated from the slope and intercept of the phase solubility curve ($y = 0.007x + 0.2059$ ($r = 0.9754$), where x is the molar concentration of Me-β-CD and y is the molar concentration of BC solubilized in solution) by using Eq. (1). The calculated stability constant of BC-Me-β-CD was 35 M^{-1}. The slope of the calibration curve was calculated to be lower than 1, corresponding to a complex stoichiometry of 1:1, as stated by Zaibunnisa *et al.*[29] The calculated complex stability constant indicated a weak interaction between the guest and host molecules, because of the molecular structure of the guest molecule BC, in accordance with the work of Zaibunnisa *et al.*,[29] who reported a similar weak association between BC and γ-CD (K_c = 43 M^{-1}).

Fourier-transform infrared spectroscopy

This technique was applied to characterize solid inclusion complexes. The FTIR spectra of BC-β-CD complexes were compared to those of the pure derivatized β-CDs and free BC by utilizing the difference of the frequency and intensity of the IR-absorption bands, where some significant differences were noticeable (Fig. 2). The FTIR spectra of the BC showed

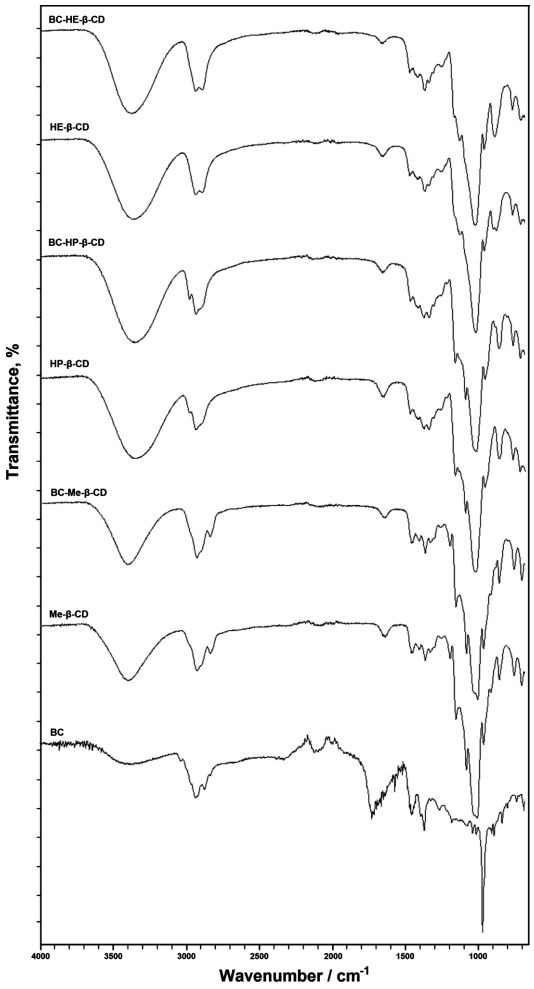


Fig. 2 (From bottom to top) FTIR spectra of solid β-carotene (BC) alone; Me-β-CD alone; BC-Me-β-CD complex; HP-β-CD alone; BC-HP-β-CD complex; HE-β-CD alone; BC-HE-β-CD complex.

characteristic absorption peaks at 2928 cm^{-1} (C–H stretching), 1479 cm^{-1} (C–C stretching), 1381 cm^{-1} (CH_3 bands) and 965 cm^{-1} ((RH)–C=C–(RH) vibration).[39] Carbonyl stretching bands were registered at 1020, 1078 and 1180 cm^{-1} for β-CDs. The characteristic OH bands were observed around 3350 cm^{-1} in the IR spectra of all CD types. These broad peaks belonging to the multiple O–H functional groups of CDs were observed for the inclusion complexes, as compared to the pure BC, which has no hydroxyl group. The relatively sharp absorption band of BC alone at 965 cm^{-1} was basically not identified in the FTIR spectra of complexes due to the host-guest interaction. This shows the penetration of pure BC into the cyclodextrin cavity. The absorption peak of free Me-β-CD at around 955 cm^{-1} was shifted to a higher wavenumber in the BC-Me-β-CD complex, because of the vibrations of (RH)–C=C–(RH) groups present in the β-carotene structure. A significant decrease in the intensity of the carbonyl stretching band at 1020 cm^{-1} was monitored for the complexes, as compared to free CDs. In the same manner, the BC-Me-β-CD complex showed a shoulder at 1020 cm^{-1} in contrast to free Me-β-CD, which may be an indication of complexation. C–H aliphatic band appeared around at 2928 cm^{-1} in all spectra, though this band of BC-complexes of HP-β-CD and Me-β-CD showed a few cm^{-1} lower wavenumber shifts than those of free CDs in this region, indicating that C–H stretching

Table 1 Total antioxidant capacities, peroxyl and hydroxyl radical scavenging activities of inclusion complexes

Inclusion complex	TEAC		IC_{50}/μg mL^{-1}		Sample	TAC/mg TR g^{-1} sample		Inhibition, %[a]	
	CUPRAC	ABTS	ROO$^{\bullet}$	$^{\bullet}$OH		CUPRAC	ABTS	ROO$^{\bullet}$	$^{\bullet}$OH
BC-Me-β-CD	1.02	0.26	9.89	0.20	CE-Me-β-CD	0.22 ± 0.01	0.21 ± 0.02	71.3 ± 1.4	90.1 ± 0.5
BC-HP-β-CD	1.47	0.54	6.08	0.14	AE-Me-β-CD	0.21 ± 0.01	0.16 ± 0.02	68.3 ± 0.9	75.7 ±1.2
BC-HE-β-CD	1.49	1.28	2.71	0.07					

Data presented as (mean ± SD), where $N = 3$. AE, apricot extract; CE, carrot extract. a. The inhibition percentage of hydroxyl and peroxyl radicals was calculated for 1:50 and 1:10 diluted extract, respectively.

vibrations are rather restricted due to complex formation.[28] Due to the weak association of CDs to BC (with a stability constant at the order of 35 M^{-1}), this aliphatic band seemed to be least affected by complexation. The intensity of this peak slightly increased in the BC-Me-β-CD complex compared to Me-β-CD. In the literature, an evaluation of the FTIR bands of the γ-CD-β-carotene complex obtained by coprecipitation revealed that the shift of the C–H aliphatic band (2928 cm^{-1}) and C = 0 band (1718 cm^{-1}) to higher wavenumber indicated that the C–H and C = 0 groups were used for the formation of the inclusion complex.[29] The peak at 1650 cm^{-1} in Me-β-CD, showing O–H bending of water molecules within the cyclodextrin cavity, was shifted to a lower wavenumber in the BC-Me-β-CD complex due to the removal of water molecules from the CD cavity, as mentioned by Kaur *et al.*[28]

Quantification of β-carotene in inclusion complexes

The concentration of BC in β-CD inclusion complexes were determined according to the method determined by Kaur *et al.*[28] The content of BC entrapped in methyl-, hydroxypropyl-, and hydroxyethyl-β-CDs was calculated using the calibration equation of BC constructed as intrinsic absorbance (A_{450}) *versus* concentration, where c_{BC} was in mg mL^{-1} ($A_{450} = 25.44c_{BC} + 0.007$ ($r = 0.9996$)) and found as 0.283, 0.184, 0.088 mg mL^{-1}, respectively.

Me-β-CD exhibited a stronger inclusion ability than did HP- and HE-β-CDs due to its deeper and more hydrophobic cavity compared to those of other CDs. This is because the methyl substituents enlarge the opening of Me-β-CD and break the strong intramolecular hydrogen bond, which let more hydrophobic molecules to access more easily to the modified CD cavity.[40]

Antioxidant capacity of inclusion complexes

The antioxidant capacities of BC in the absence and presence of CDs were measured using two electron transfer-based TAC assays, namely CUPRAC assay and ABTS-persulfate assay. Both CUPRAC and ABTS assays were successively applicable to hydrophilic and lipophilic antioxidants in polar and nonpolar solvent media for measuring the antioxidant capacity because they involve univalent-charged chromophore species (*i.e.*, ABTS$^{\bullet+}$ and $Cu(Nc)_2^{+}$) capable of being solvated by both water and alcohols as well as by less polar solvent mixtures. The CUPRAC assay is based on 450 nm absorbance measurements of the chromophore, Cu(I)-neocuproine (Nc) chelate, formed as a result of the redox reaction of antioxidants with the CUPRAC reagent, Cu(II)-Nc. The degree of color change is correlated to the concentration of antioxidants in the sample.[41] The CUPRAC-DCM extractive-photometric method[14] was developed for measuring the lipophilic antioxidant capacity of synthetic or real samples responsive toward carotenoids. Simultaneous measurements of lipophilic and hydrophilic antioxidants in the same acetone–water solution containing 2% Me-β-CD using the modified CUPRAC method have been reported. The obtained results were found to be close to each other (1.78 for CUPRAC assay, 2.14 for ABTS assay).[15] The $TEAC_{CUPRAC}$ and $TEAC_{ABTS}$ values for β-carotene were reported earlier as being 3.10 and 1.60 in pure acetone, respectively,[13] whereas the same were 3.35[14] and 2.57[16] in DCM, respectively. Depending on the solvent polarity, β-carotene may show slow kinetics with the assay reagent. On the other hand, Folin–Ciocalteu and FRAP (ferric reducing antioxidant power) methods were not preferred, because the tetravalent-charged Folin–Ciocalteu and the divalent FRAP chromophores exhibited a higher affinity to the aqueous phase due to ion-dipole interactions of the chromophore with the solvent water molecules.[42] The presence of the polyene chain and bicyclic structure of BC makes it difficult for steric-demanding oxidants to interact with the carotenoid. Thus, BC could not respond to the FRAP assay.[12]

The trolox equivalent antioxidant capacity coefficients and peroxyl and hydroxyl radical scavenging activities (IC_{50} (μg/mL)) of the inclusion complexes of BC with derivatized β-CDs were assayed (Table 1). The more BC was associated with the hydrophobic cavity of CD derivative, the less antioxidant activity it would exhibit, and thus the antioxidant activity order of Me-β-CD < HP-β-CD < HE-β-CD was experimentally observed. The TEAC coefficients were calculated by dividing the absorption coefficient of each inclusion complex to the absorption coefficient of trolox under the specified conditions ($\varepsilon = 66.7$ mL mg^{-1} cm^{-1} for CUPRAC assay; $\varepsilon = 104$ mL mg^{-1} cm^{-1} for ABTS assay). For example, the TEAC coefficient of BC-Me-β-CD complex with respect to the CUPRAC assay was calculated from the calibration equation constructed as the CUPRAC absorbance (A_{450}) *versus* concentration, where c_{BC} was in mg mL^{-1} ($A_{450} = 68.4c_{BC} + 0.0086$ and $r = 0.9990$).

Complexation with β-CDs could affect the antioxidant capacity of β-carotene. For example, Moore *et al.*[43] noticed that randomly-methylated β-cyclodextrin could reduce the sensitivity of hydroxyl radical scavenging capacity (HOSC) measurements, since their experimental findings indicated the possibility of the $^{\bullet}$OH quenching of Me-β-CD.[43] García-Padial *et al.*[44] pointed out that cyclodextrins may yield high blank values in the ORAC-FL antioxidant activity assay, and that intermolecular H-bonding between the cyclodextrin and the antioxidant it includes may attenuate the measured antioxidant activity.[44] It was found in this work that the complexed BC with β-CDs was a less effective antioxidant than free BC, considering the TEAC value of β-carotene in acetonated medium previously given as 3.10.[13] Inclusion complexation phenomenon is competitive to redox behavior, and is expected to cause a decrease in antioxidant capacity due to the difficult release of β-carotene from the complex. Among the tested inclusion complexes, β-carotene showed the highest antioxidant capacity in the presence of

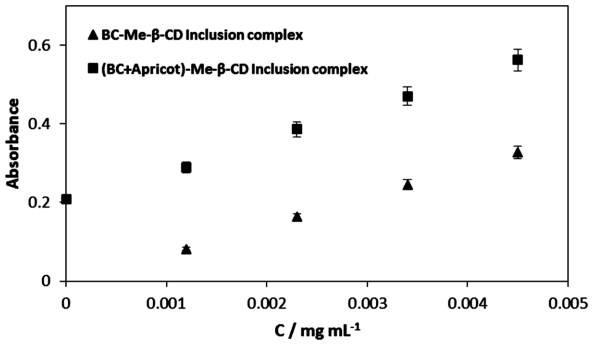


Fig. 3 Standard calibration curves of BC-Me-β-CD inclusion complex alone and in the presence of apricot extract (AE) with respect to the CUPRAC assay.

HE-β-CD and the lowest capacity with Me-β-CD, in accordance with the higher complexation ability of the latter. Polyakov *et al.* mentioned that complexation with CD derivatives results in a considerable decrease in the antioxidant ability of the carotenoid.[27] The total antioxidant capacities (mg trolox per g sample) of the complexed carrot extract (CE) and apricot extract (AE) with Me-β-CD were evaluated using the CUPRAC and ABTS methods (Table 1). The TAC values of the complexed food samples assayed with the CUPRAC assay were basically in accordance with those of the ABTS assay, though it has been well established in literature that no two assays may yield the same result for a given sample.

Standard calibration curves of BC-Me-β-CD inclusion complexes in the absence and presence of apricot extract obtained by CUPRAC assay are given in Fig. 3. For this purpose, increasing amounts of standard BC solution were added to a fixed volume of apricot extract (1:5 diluted with water), and then complexed with Me-β-CD. The initial CUPRAC absorbance of AE-Me-β-CD was recorded to be 0.42 at 450 nm. The antioxidant capacities of the tested inclusion complexes were found to be additive (results not shown). The parallellism of the linear calibration curves (with and without a real sample) effectively demonstrated that there was no chemical interactions of the interferent nature (*i.e.* that may give rise to chemical deviations from Beer's law) among the BC-Me-β-CD complex and the real matrix (apricot extract) constituents.

Radical scavenging activity of BC-β-CD inclusion complexes

Carotenoids are known to be potent quenchers of ROS and RNS. The radical scavenging activity of xanthophylls (which contain oxygen) soluble in ethanol can be measured by conventional methods applicable to water-ethanol mixtures. For example, Yuan *et al.*[31] measured that 83.16% of hydroxyl radicals (generated by a Fenton system) were quenched by astaxanthin at 4.55 µmol/L. A hydroxyl radical scavenging activity of 50% was obtained for canthaxanthin at a concentration of 20 µmol/L according to a study of Gharibzahedi *et al.*[32] However these methods could not be applied to carotenes, which are purely carbon-hydrogen compounds not containing oxygen. When we applied these methods, turbidity was observed due to the low solubility of β-carotene in aqueous buffers or alcoholic solutions. Therefore, the radical scavenging activity of free β-carotene could not be determined. The complexation of lipophilic compounds with cyclodextrins makes it possible to measure ROS scavenging in aqueous systems. With similar reasoning, Rodrigues *et al.*[30] mentioned that lipophilic antioxidants could not be determined in free forms by utilizing conventional methods. Consequently, it was not possible to compare the scavenging activity of complexed carotenoids in free forms due to the non-availability of a reference method. Faria *et al.*[45] evaluated the quenching of photochemically generated singlet molecular oxygen ($^1O_2$) by free and biopolymer-encapsulated β-carotene using the time-resolved phosphorescence detection of $^1O_2$ in an acetonitrile solvent medium.

The hydroxyl and peroxyl radical scavenging activities (IC_{50}) of BC-Me-β-CD were measured as 0.20 and 9.89 µg mL^{-1}, respectively (Table 1). Among the tested inclusion complexes, BC-HE-β-CD was found to be the most effective hydroxyl and peroxyl radical scavenger, possibly due to the least entrapment of free BC (see *Quantification of β-carotene in inclusion complexes*) and to the presence of hydroxyl groups in the mentioned CD. By analogy to phenolic antioxidants containing adjacent –OH groups, Bors and Michel hypothesized from pulse radiolysis transient spectra and the decay rate constants of •OH-absorbed species that catechol and/or pyrogallol groups are the sites of a radical attack, attributing the high •OH scavenging rate of epigallocatechin gallate to the number of adjacent aromatic hydroxyl groups in the molecule.[46] When the hydroxyl and peroxyl radical scavenging activities (inhibition %) of complexed carrot and apricot extracts with Me-β-CD were evaluated, complexed carrot extract was found to be a more effective •OH and ROO• scavenger than apricot extract (Table 1). Shibata *et al.* hypothesized that the recommended daily intake of β-carotene was contained in half a carrot or three apricots.[47]

Conclusions

In this study, the antioxidant capacity and ROS scavenging activity of water-soluble CD-inclusion complexes of β-carotene were evaluated. The complexed form of BC was more soluble and stable, and showed 1:1 stoichiometry with derivatized β-CDs. The CD-complexed β-carotene exhibited a less effective antioxidant capacity than did free BC dissolved in pure acetone; however, the outcomes of certain radical scavenging tests could not be compared, because there is no reliable data regarding the ROS scavenging ability of BC. The simple reason is that BC is not soluble in aqueous solution, and if the test is alternatively carried out in a non-aqueous medium, ROS scavenging tests may give rise to the formation of reactive intermediates from organic solvents, such as acetone and alcohols during Fenton-type reactions used to generate these reactive species. Thus, hydroxyl and peroxyl radical scavenging activity testing for BC was carried out for the first time in an aqueous medium using derivatized β-CDs, which may fill in an important literature gap. Among inclusion complexes, β-carotene showed the highest and lowest total antioxidant capacity in the presence of HE-β-CD and Me-β-CD, respectively, probably due to the deeper and more hydrophobic cavity of the latter releasing the associated BC more difficultly. The present study also demonstrates the possibility for β-carotene to be protected from oxidative degradation by complexation or encapsulation to the Me-β-CD cavity; this finding may be important to estimate the shelf-life of BC-containing foodstuffs.

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