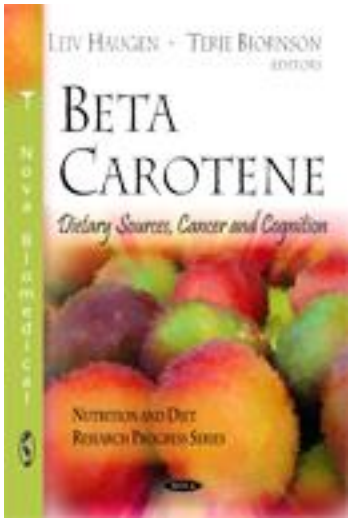

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Water Soluble Supramolecular Complexes of β-Carotene and Other Carotenoids.

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ABSTRACT

It is well known, that the wide practical application of β-carotene and other carotenoids as antioxidants or food colorants is substantially hampered by their hydrophobic properties, instability in the presence of oxygen and metal ions, and high photosensitivity. The majority of carotenoids are lipophilic molecules with near zero inherent aqueous solubility. Moving carotenoids into a pharmaceutical application requires a chemical delivery system that overcomes the problems with parenteral administration of a highly lipophilic, low molecular weight compound. Many different methods have been developed to make the carotenoids "water dispersible", as true water solubility has not been described. Most of the attempts to increase in solubility of carotenoids are related to the preparation of cyclodextrin inclusion complexes. Application of inclusion complexes was first related to an attempt to minimize the aforementioned disadvantages of carotenoids when these compounds are used in food processing (colors and antioxidant capacity) as well as for production of therapeutic formulations considering the better solubility and consequently higher bioavailability. In this chapter we present three examples of supramolecular complexes of carotenoids with natural oligosaccharides and polysaccharides: cyclodextrin, glycyrrhizin and arabinogalactan. It was demonstrated that incorporation of carotenoids into the “host” macromolecule results in significant change in their properties. In particular, we present the first example of water soluble complexes of carotenoids with natural polysaccharide arabinogalactan, a branched polymer with molecular mass 15000-20000. Compared to pure carotenoids, polysaccharide complexes show enhanced photostability in water solutions. A significant decrease in the reactivity towards metal ions (Fe^{3+}) and reactive oxygen species in solution was also detected.

INTRODUCTION

Carotenoids are a class of pigments widely found in nature. These essential nutrients are synthesized by plants and microorganisms and exist in many foods including vegetables, fruits, and fish. About 600 various carotenoids are known. However, only a few (about 20) have been found in human tissues. These include β-carotene, canthaxanthin, zeaxanthin, etc. The presence of a polyene chain and various terminal substituents in carotenoid molecules determines their color, redox properties and location inside the lipid layers in biological media.

Recently much attention has been focused on the reactions between carotenoids and free radicals [1-7] because of the ability of carotenoids to prevent the development of diseases caused by toxic free radicals [8]. One factor contributing to the development of various diseases, including infarction, cerebral thrombosis, and tumors has been attributed to the action of free radicals and the toxic forms of oxygen [9]. Carotenoids are assumed to protect cells by scavenging either free radicals or excited oxygen that have a severe impact on cells. Of no less significance are the membrane-stabilizing and immunostimulating functions of carotenoids as well as their provitamin A activity. Vitamin A and carotenoids favor normal metabolism, enhance the resistance of an organism against infections, provide normal operation of the organ of vision, exert beneficial effect on the performance of skin and mucous membranes and are involved in redox processes. At the same time, wide practical application of carotenoids as antioxidants or food colorants is substantially hampered by their hydrophobic properties, instability in the presence of oxygen and high photosensitivity.

One of the promising way to overcome there problems is the preparation of supramolecular inclusion complexes. Complex formation is widely used in medicine to improve the solubility of preparations, to deliver drugs, and to decrease their toxicity (see Figure 1) [10-12].

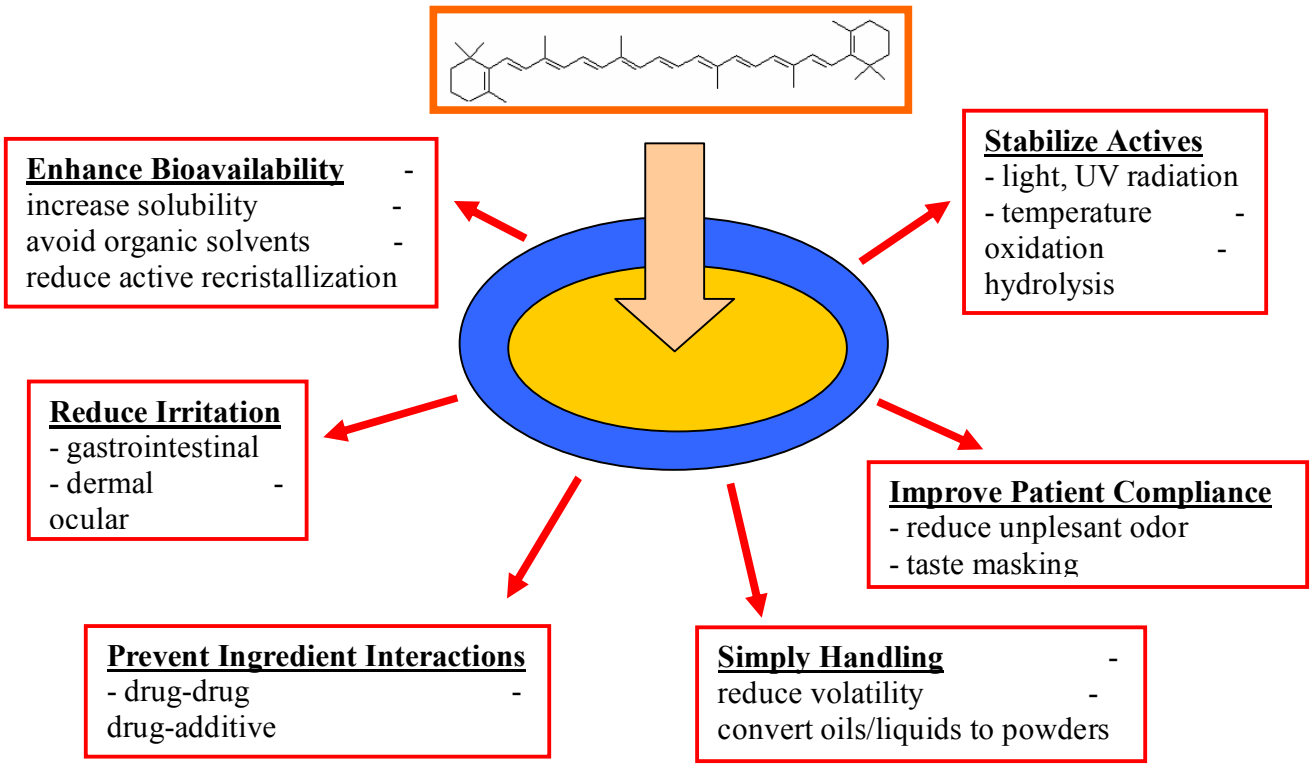
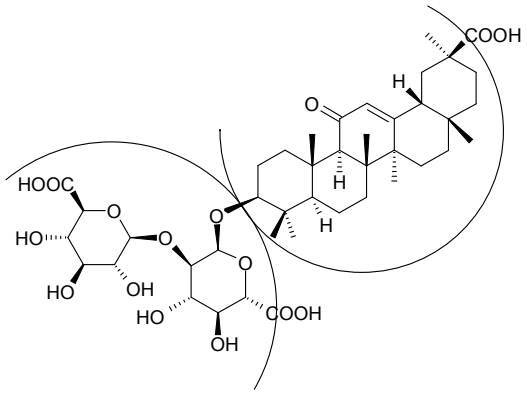


Figure 1. *Application spectrum of the inclusion complexes in medicine.*

Fundamental and applied research has recently been devoted to the inclusion complexes of carotenoids with natural compounds, specifically the cyclodextrins (CD) [13-21], which are assumed to possess protective properties and to decrease the hydrophobic behavior of the included molecules. Application of inclusion complexes was first related to an attempt to minimize the aforementioned disadvantages of carotenoids when used in the food industry, cosmetology, and medicine.

At present, it can be stated with confidence that it is the chemical and physicochemical studies of the reactivity of carotenoids in redox processes in organized media that are most promising. In particular, this refers to a study of supramolecular inclusion complexes of active compounds that can be of interest in medicine, pharmacology, artifical light-harvesting, and other areas. As it was mentioned above, most studies concern the inclusion complexes of cyclodextrins that are widely used in practice as agents for transporting and conserving drugs. The main difficulty in practical applications of "host-guest" complexes of cyclodextrins is that the rigidly fixed volume of the cyclodextrin cavity prevents binding of either very small or very large molecules including many compounds that are of interest in medicine and pharmacology. Therefore, the search is being continued for complexing agents devoid of these disadvantages. One of the compounds that appear to be promising is glycyrrhizic acid (or glycyrrhizin). β-glycyrrhizic acid (GA) belongs to the triterpene glycosides and contains both

hydrophilic (glucuronic acid) and hydrophobic (glycyrrhetic acid) regions. GA is extracted from the Ural licorice root (*Glyzyrrhiza glabra L*).

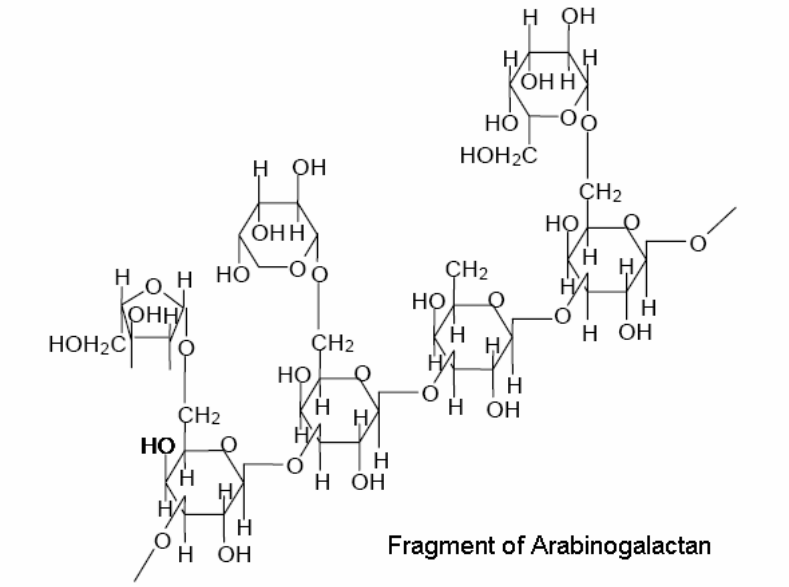


β-glycyrrhizic acid (GA)

It was suggested in many studies that glycyrrhizic acid in solution can create cyclic structures that can form inclusion complexes with various organic compounds [22, 23]. GA is of considerable interest to pharmacologists because of its unique physiological activity. In particular, its preparations are very popular in connection with AIDS treatment [24]. This compound is particularly attractive for three main reasons. First, GA in contrast to the cyclodextrins has an open chain structure and thus, for complex formation, there are no rigorous restrictions on the size of a "guest" molecule. Second, all authors indicate unusual stability of GA complexes [22, 23, 25, 26]. The stability constants of GA complexes are in the range of 10^5 M^{-1}, which are two orders of magnitude higher than a mean stability constant of cyclodextrin complexes [27]. And third, it was demonstrated that application of glycyrrhizic acid together with other medicines strengthens their therapeutic efficiency by orders of magnitude and reduces side effects, e.g., the toxic action on the alimentary canal [28-30].

Another imperfection of carotenoid-cyclodextrin complexes is their poor solubility. In reality, these complexes form water dispersions, rather than solutions. According to the studies of Mele with coauthors [16-17], carotenoid-cyclodextrin complexes in water form large aggregates with size 100-200 nm. This results in weakly colored opalescent solution [20]. The reduced color intensity significantly decreases the application area of carotenoids, in particular as food colorants.

Now we can present the first example of water soluble complex of β-carotene and some other carotenoids. The present chapter describes the complex formation between carotenoids and natural polysaccharide arabinogalactan (AG), a branched polymer with molecular mass 15000-20000.



Arabinogalactans are found in a variety of plants but are more abundant in the Larix genus, primarily Western and Siberian Larch [31,32]. Larch arabinogalactan is approved by the U.S. Food and Drug Administration (FDA) as a source of dietary fiber, but also has potential therapeutic benefits as an immune stimulating agent and cancer protocol adjunct. The immune-enhancing herb echinacea also contains AG, as do leeks, carrots, radishes, pears, wheat, red wine, and tomatoes. AG is an important source of dietary fiber. It is known that AG increases the production of short-chain fatty acids, principally butyrate and proprionate, which are essential for the health of the colon. AG also acts as a food supply for "friendly" bacteria, such as bifidobacteria and lactobacillus, while eliminating "bad" bacteria. AG has a beneficial effect upon the immune system as it increases the activity of natural killer cells and other immune system components, thus helping the body to fight infection [32]. The increased aqueous solubility of a number of carotenoids will likely find utility in their introduction into mammalian cell culture systems that have previously been dependent upon liposomes, or toxic organic solvents, for the introduction of carotenoids into aqueous solution [21]. Also water soluble carotenoids displays several technological applications that could be used in food processing to enhance color and antioxidant capacity as well as for the production of therapeutic formulations considering the better solubility and consequently higher bioavailability [13-15, 33]. It is worth noting that at present, progress in developing novel forms of medicines has been related not only to a search for new active substances but also to regulating the effect of already available preparations. Complexation is one method for regulating this effect.

The present chapter describes our studies of the inclusion complexes of a number of natural and synthetic carotenoids with natural oligosaccharides and polysaccharides, namely with cyclodextrins,

glycyrrhizin and arabinogalactan. We have studied the solubility of these complexes of carotenoids in water and their reactivity in redox processes. The reactivity was studied using important electron transfer reactions with metal ions and quinones as well as in the reactions with free radicals. These processes have been previously studied in detail in homogeneous solutions [34-35].

RESULTS AND DISCUSSION

1. Inclusion Complexes of Carotenoids with Cyclodextrins (CD) [20].

Since carotenoids are highly hydrophobic, air- and light-sensitive compounds, developing methods for increasing their bioavailability and stability towards irradiation and reactive oxygen species is essential. One can find several examples of practical application of carotenoid-cyclodextrin complexes in the food, cosmetics and pharmaceutical industry [13-15]. In the food industry, carotenoids are mainly used as food colorants and antioxidants. The application of their CD complexes instead of pure carotenoids results in increasing stability of colorants under storage and simplicity in using without preliminary solubilization in organic solvents. In cosmetics, carotenoids are used as antioxidants, but limited in application by the intense color of carotenoids. Incorporation of carotenoids into cyclodextrin cavity reduces significantly their color intensity. In particular, the cosmetic cream with β-carotene-cyclodextrin complex has a nice pink color instead of the saturated red color for pure carotene [15]. However, in spite of this practical application, there is still no strong evidence of real inclusion complex formation, and only a few attempts of structural studies of such complexes have been reported [16-17]. Previous studies of short-chain analogues of carotenoids, β-ionone [36], and retinoids [37], demonstrated the formation of stable inclusion complexes of these substrates with different cyclodextrins (β-CD), 2-hydroxypropyl-β-cyclodextrin (HP-β-CD), 2-hydroxypropyl-γ-cyclodextrin (HP-γ-CD). It was shown that the terminal cyclohexene fragment of β-ionone, which is present in most carotenoids (Figure 2), has the requisite size for incorporation into the CD cavity. It seemed likely that carotenoids, which are even more hydrophobic, should also form inclusion complexes with CDs.

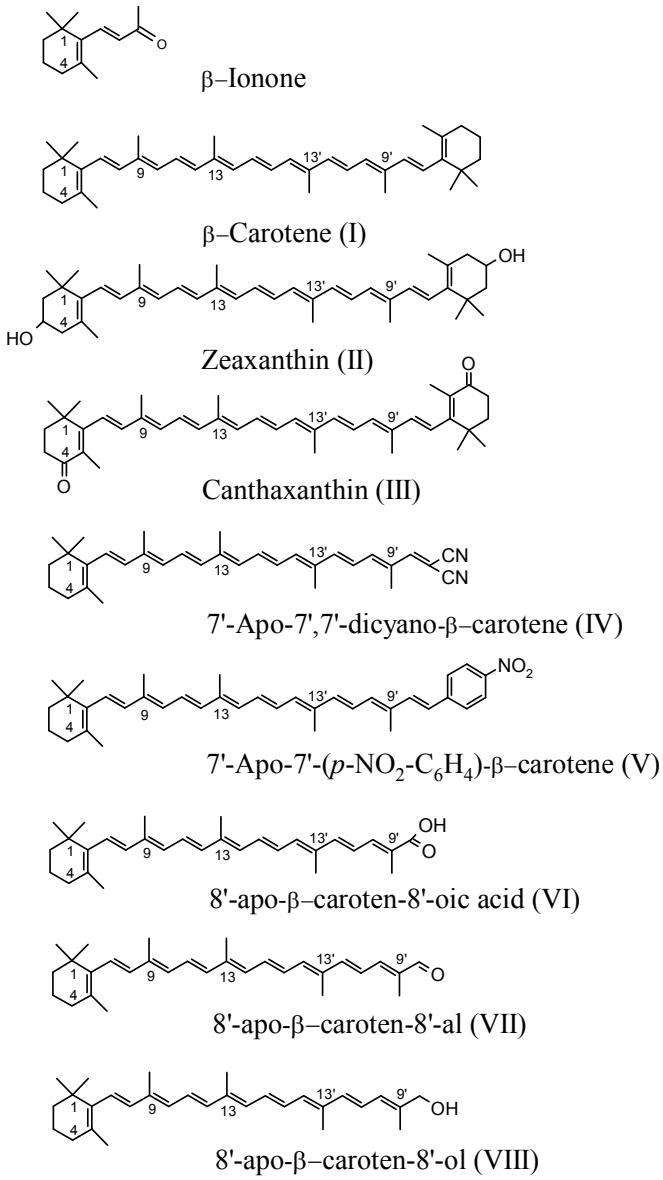


Figure 2. *The structures of β-ionone and some natural and synthetic carotenoids.*

In our study we have focused on two aspects. The first aim was to obtain direct evidence of inclusion complex formation. For this purpose we applied standard approaches using ^{1}H- NMR and UV-Vis absorption spectroscopy. The second aim was the investigation of the reactivity of carotenoid-CD inclusion complexes towards peroxyl radicals (antioxidant activity). It was earlier suggested that β-carotene and other carotenoids react with peroxyl radicals primarily at the 4-C position of the cyclohexene ring (see Figure 2) [2, 6, 7]. One can expect that the reactivity of carotenoids towards free radicals may decrease if the cyclohexene ring is embedded in the CD cavity. This investigation was

beneficial for the purpose of application of the carotenoid-CD complexes to protect carotenoids against damage caused by O_2 and free radicals.

In our study we used two methods of complex preparation, described in details in the literature [13-16]. In the first method, "solid mixture" (SM), solid carotenoid and requisite amounts of CD (1 or 2 equiv) were ground together until a homogeneous powder was obtained. Grinding was continued after adding a small amount of deionized water to give a paste, which was then stored overnight under nitrogen, treated with water to obtain a final carotenoid concentration of 1 mM, and the suspension was stirred for several hours. In the second method, "liquid mixture" (LM), the solution of carotenoid in methanol (or other organic solvent) was added to the aqueous CD solution.

UV-Vis absorption study. All carotenoid-CD complexes prepared in water by the SM method show a considerable change in color compared to carotenoid solutions in organic solvents. Note that with the exception of **VI**, all carotenoids are completely insoluble in water. For example, **I**-CD complex has opalescent intensely pink-orange in aqueous solution. The **IV**-CD complex is black, while the MeOH or CH_2Cl_2 solutions of **IV** are violet. All complexes have a very broad absorption band up to 1100 nm with reduced intensity (about one order of magnitude). We suggest that the broadening of the absorption band is due to aggregation of complexes in aqueous solution. Such aggregate formation was previously detected by light scattering spectroscopy [16, 17]. Assuming that only a cyclohexene ring of the carotenoid can be embedded in the CD cavity, we suggest that aggregates of the complexes have a micelle-like structure in the aqueous media.

Aggregate formation could be observed by changes occurring in the UV-Vis spectra when mixing separately prepared solutions of CD in H_2O and carotenoid in ethanol. For the highly water soluble HP-β-CD and HP-γ-CD used in UV-Vis experiments, similar effects were observed. The maximum absorption of Car-CD complexes in water is at a much shorter wavelength than that of the pure carotenoids in ethanol. The most significant blue shifts were observed for carotenoids with polar terminal groups: -CN, -Ph-NO_2, -COOH. For example, carotenoid **V** shows the blue shift of the absorption maximum from 485 nm in ethanol to 315 nm in water with simultaneous considerable decrease of extinction coefficient. The spectrum of the Car-CD mixture gradually changed with time due to aggregate formation. With large excess (50 eq.) of HP-β-CD, the changes with time were greatly attenuated (see Figure 3). We suggest that this is due to the existence of an equilibrium between individual complexes and aggregates of the complexes. Compounds with other polar groups (**IV** and **VI**) showed similar behavior, an increase of complex solubility with increase of CD concentration.

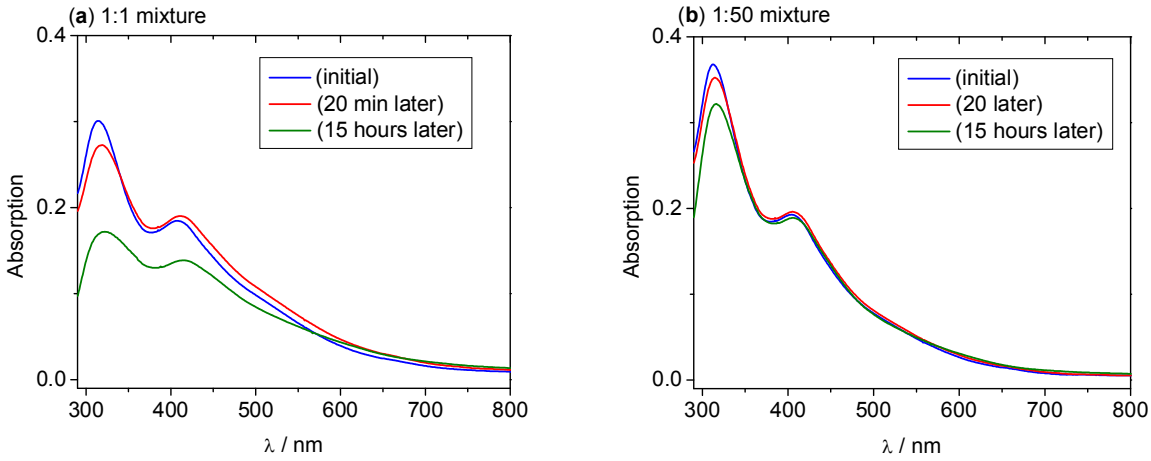


Figure 3. *Transformation of UV-Vis. absorption spectrum of* ***V****-HP-β-CD (a) 1:1 mixture and (b) 1:50 mixture in water-ethanol solution as a function of time (adopted from [20], Fig. 1).*

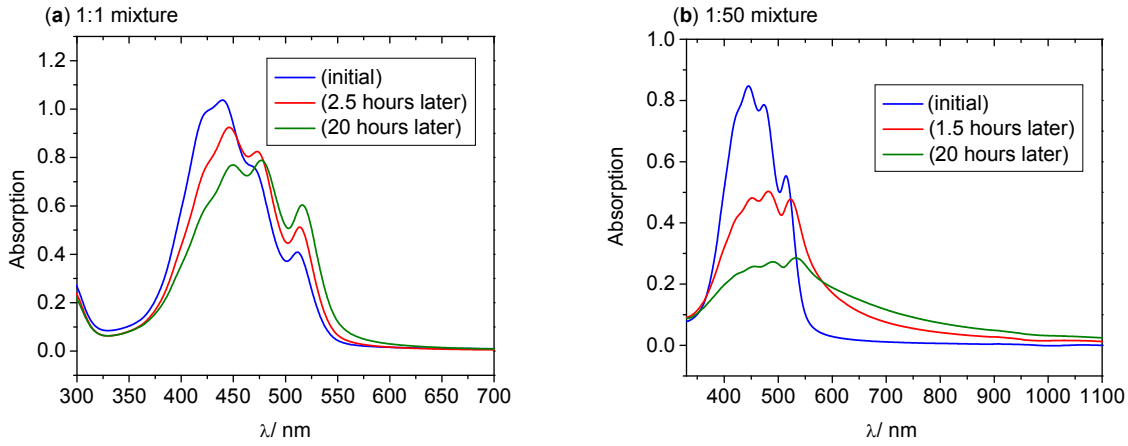


Figure 4. *Transformation of UV-Vis. absorption spectrum of β-carotene-HP-β-CD (a) 1:1mixture and (b) 1:50 mixture in water-ethanol solution as a function of time (adopted from [20], Fig. 2).*

However, β-carotene (**I**), which does not contain polar groups, displayed divergent spectral changes (Figure 4). No large blue shift was observed in this case, and the presumed equilibrium favors the formation of complex aggregates. According to published phase-solubility diagrams [38], two types of complex can exist. The first type demonstrates the increase of solubility with increased CD concentration, and the second type shows the opposite effect. Figures 3 and 4 show that both types of complexes take place for the carotenoids under study. Different behavior of various carotenoids could

be explained by the difference in their structure, namely the structure of terminal groups. Assuming that the Car-CD complex has a hydrophilic CD head and hydrophobic carotenoid tail, one can suggest the formation of more stable aggregates for complexes with non-polar carotenoids.

The presence of aggregation makes elucidation of the structure of CD complexes difficult for most of carotenoids. For this purpose we used the partly water soluble carotenoid **VI** with acid terminal group. It is known that the solubility of CD complexes with organic acids can be increased dramatically by changing the pH of the solution [39]. Indeed, the presence of 4 mM NaOH in 2 mM aqueous complex solution resulted in increased complex solubility so that the monomers of the **VI**-CD complex could be observed by UV-Vis as well as NMR techniques. The absorption maximum of **VI** in water shows a strong blue shift (λ_{max} = 285 nm) compared to that in ethanol (λ_{max} = 400 nm). The spectrum of the **VI**-CD complex in the presence of NaOH is nearly identical to that of **VI** in ethanol (see Figure 5). This observation might be considered as proof of incorporation of carotenoid within the CD cavity, since it is known that the polarity of the CD interior is similar to that of an ethanolic solution [40]. No changes were detected in the absorption spectrum after addition of NaOH in the absence of CD.

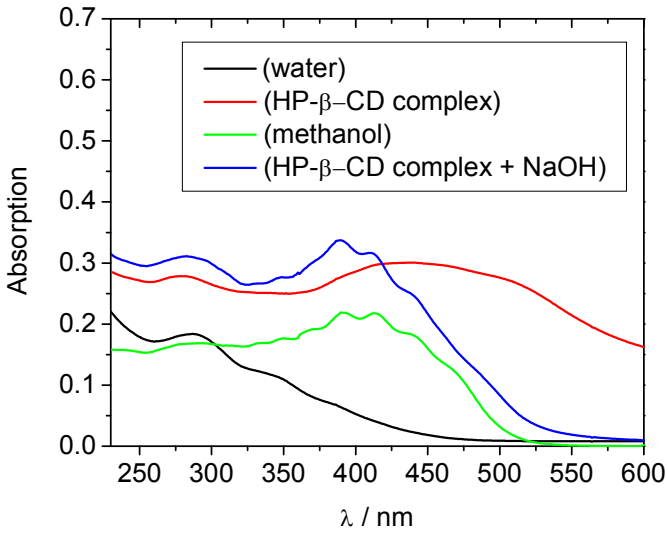


Figure 5. *UV-Vis. absorption spectra of **VI** in different media. The concentrations of carotenoid were different in each case (adopted from [20], Fig. 3).*

^{1}H-NMR study of CD complex. Usually ^{1}H-NMR experiments can provide information about the stoichiometry, stability, and the structure of CD complexes [27, 40, 41]. In particular, Job's plot, which

correlates the chemical shift and the host/guest ratio, has been widely used to determine complex stoichiometry [42, 43]. The possibility of detecting inclusion complexes by NMR spectroscopy is based on the expectation that if a guest molecule is incorporated into the CD cavity, the screening constants of the CD protons inside the cavity (H_3 and H_5) should be sensitive to the changed environment, but the outside protons (H_1, H_2, and H_4) should not (Figure 6).

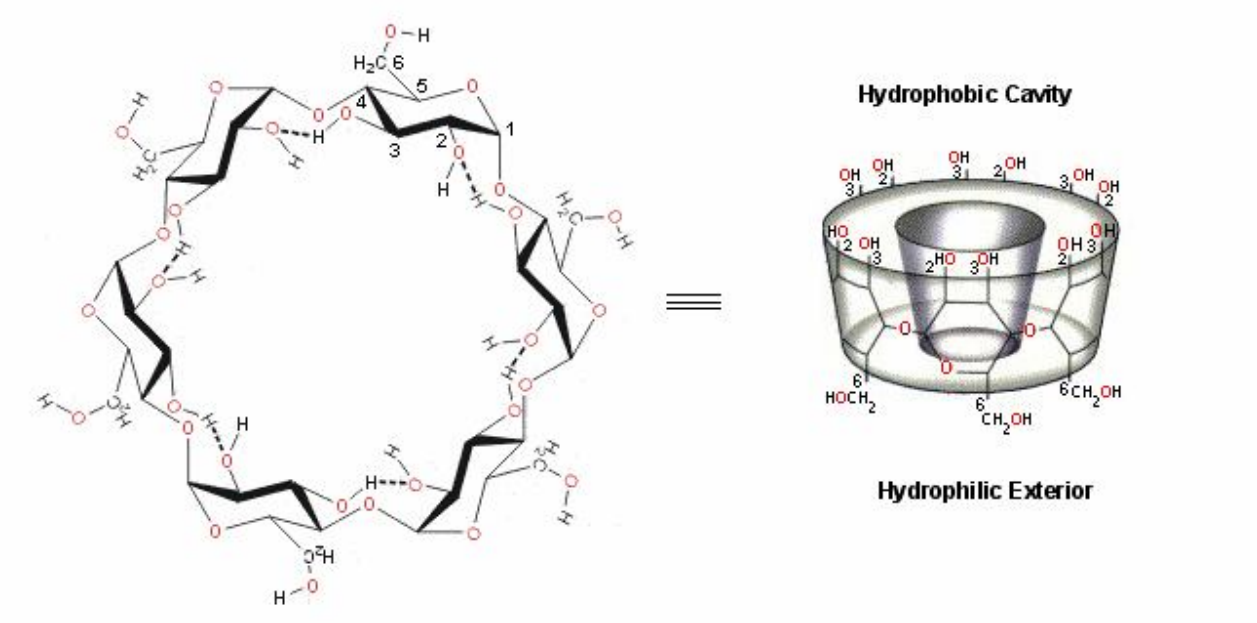


Figure 6*. Schematic presentation of the structure of cyclodextrin.*

As stated above, sufficiently high concentrations of carotenoid-CD complex (1 mM) needed for NMR measurements could be obtained only for carotenoid **VI** in the presence of NaOH. As it was earlier detected for β-ionone-CD complex [36], the shift of the internal protons of the CD cavity was detected in the presence of carotenoid **VI** (see Figure 7).

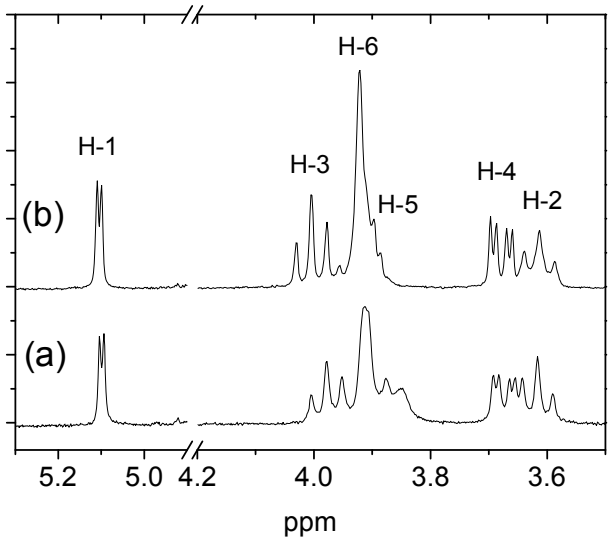


***Figure** 7. [1]H-NMR (360 MHz) spectra of aqueous solution of β-CD with addition of 4 mM NaOH in the presence (a) and absence (b) of **VI**. See Figure 6 for identification of CD protons. Concentrations of CD and carotenoid are 2 mM (adopted from [20], Fig. 4).*

For calculation of stoichiometry of this inclusion complex and its association constant, the variation of NMR chemical shifts of CD protons was measured with changes of carotenoid concentration. This approach is widely used by many authors for analysis of an inclusion complex [44, 45]. Let's consider the equilibrium process of complex (C_{nm}) formation between **n** cyclodextrin (CD) molecules and **m** guest (G) molecules (1):

$$mG + nCD \rightleftharpoons C_{nm} \quad (1)$$

The association constant of this complex is described as:

$$K_{nm} = \frac{[C_{nm}]}{[G]^m[CD]^n} \quad (2)$$

Since the association-dissociation process is rapid relative to the NMR time scale (in the microsecond to millisecond range), the chemical shift of CD protons can be determined as follows:

$$\delta_{obs} = f_{CD}\,\delta_{CD} + f_{Cnm}\delta_{Cnm} \quad (3)$$

where δ_{CD} and δ_{Cnm} are the chemical shifts of the free and complexed CD, and f_{CD} and f_{Cnm} are their molar fractions. Substituting $\Delta\delta_{obs} = \delta_{obs} - \delta_{CD}$ and $\Delta\delta_{Cnm} = \delta_{Cnm} - \delta_{CD}$ we obtain:

$$\Delta\delta_{obs} = n\Delta\delta_{Cnm} \frac{[C_{nm}]}{[CD]_o} \quad (4)$$

From the mass balance, the initial concentrations of carotinoid $[G]_0 = [G] + m[C_{nm}]$ and cyclodextrin $[CD]_0 = [CD] + n[C_{nm}]$. If m = n = 1, from eq. (2) the following equation for concentration of a 1:1 complex can be obtained:

$$[C_{11}]^2 - ([G]_0 + [CD]_0 + 1/K_{11})[C_{11}] + ([G]_0 [CD]_0 = 0 \quad (5)$$

Combination of eqs (4) and (5) results in the expression (6) from which the value K_{11} can be obtained from the G_0 concentration dependence of $\Delta\delta_{obs}$ (CD).

$$\Delta\delta_{obs} = \frac{n\Delta\delta_{Cnm}}{2[CD]_0} \left\{[G]_0 + [CD]_0 + 1/K_{11} - \left(\left([G]_0 + [CD]_0 + 1/K_{11}\right)^2 - 4[G]_0[CD]_0\right)^{1/2}\right\} \quad (6)$$

The observed values of $\Delta\delta_{obs}$(CD) were measured at varying carotenoid concentration and constant concentration of CD. To increase the accuracy of the measurements, a small amount of CH_3OH (~ 1 mM) was added to all samples as reference. It is known that methanol is a good internal reference due to its low association constant with cyclodextrins [46]. The stoichiometry of this complex was obtained by the continuous variation technique (Job's plot) [41]. The position of the maximum at R = 0.5 on the Job's plot (Figure 8) indicates that carotenoid **VI** forms a 1:1 complex with β-CD. The value of the association constant (K_{11} = 1536 ± 75 M^{-1}) was extracted using equation (3) from the dependence of the chemical shifts of CD protons on carotenoid concentration (Figure 9). Taking into account that the sodium salt of **IV** itself is water soluble, one can expect even higher complex stability for carotenoid **IV** in the absence of NaOH. This example is the first direct evidence of inclusion complex formation of a carotenoid.

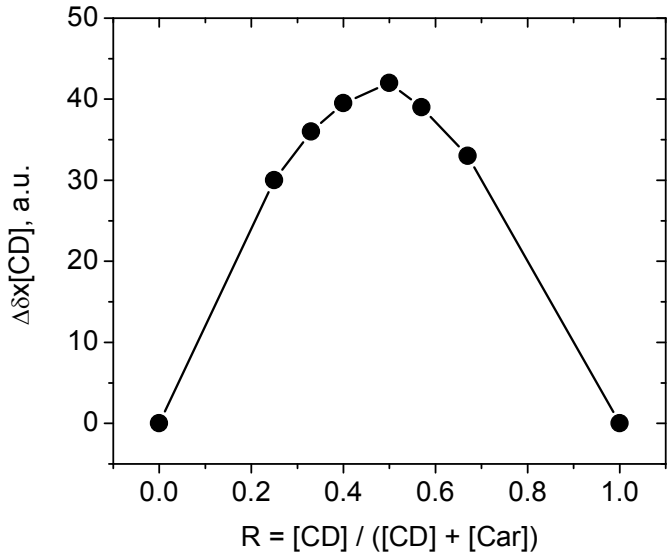


Figure 8. *Job's plot corresponding to the chemical shift displacement of 3-H protons of β-CD in the presence of* ***VI****. The total concentration of CD plus carotenoid was 2 mM in this experiment (adopted from [20], Fig. 5).*

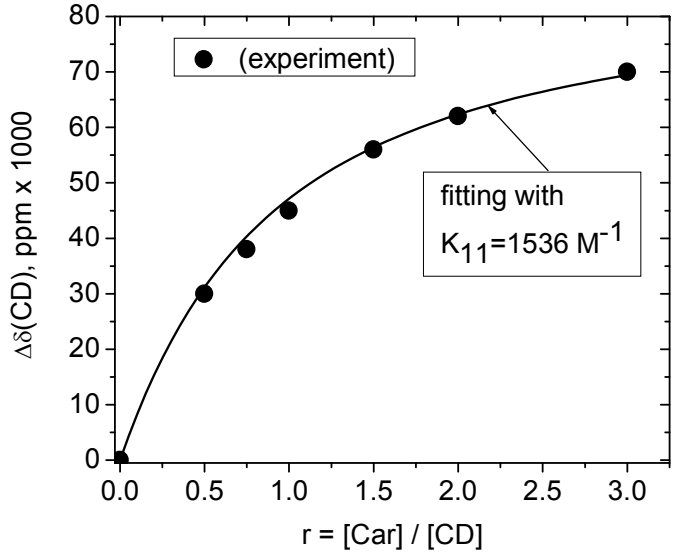
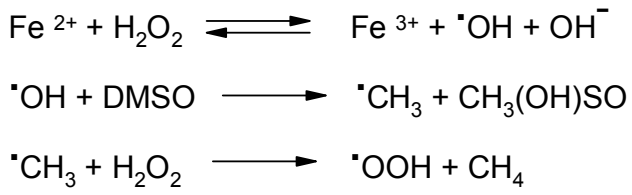


Figure 9. *Dependence of chemical shift of 3-H β-CD protons on* ***VI:****CD ratio in D_2O: experimental points and calculated curves for association constant K_{11} = 1536 M^{-1}. [β-CD] = 2 mM, and [NaOH] = 4 mM (adopted from [20], Fig. 6).*

EPR study of scavenging ability of carotenoids towards peroxyl radicals. For investigation of the scavenging ability of carotenoid IV and its complex with CD we used the well known Fenton reaction for generation of free peroxyl radicals [47-49].



At low H_2O_2 concentration (H_2O_2] ~ [$FeCl_2$] = 1 mM) only one spin adduct PBN-CH_3 was detected with ESR parameters a(H) = 3.4 G and a(N) = 14.9 G. However, at higher H_2O_2 concentration (0.5 M) the reaction of CH_3 radicals with H_2O_2 results in disappearance of the PBN-CH_3 adduct, and appearance of another adduct with higher yield which was assigned to the PBN-OOH spin adduct (a(H) = 2.3 G and a(N) = 13.9 G) [7]. On the other hand, it is known that the ·OOR spin adducts are relatively unstable especially in the presence of transition metal ions which can reduce ·OOR radicals yielding the ·OR spin adduct [50, 51]. However, these facts are mainly related to alkyl peroxyl radicals. We have found several examples in the literature of the observation of the PBN-OOH adducts at normal conditions [52-54]. The additional confirmation of the PBN-OOH adduct formation was obtained using the superoxide dismutase (SOD) test [55]. Both the PBN-OOH and the PBN-CH_3 adducts were observe in the absence of SOD at concentration of hydrogen peroxide 200 mM (Figure 10, bottom). The addition of SOD (200 U/ml) completely suppressed the PBN-OOH signal, but had no influence on the PBN-CH_3 adduct (Fig. 10, top).

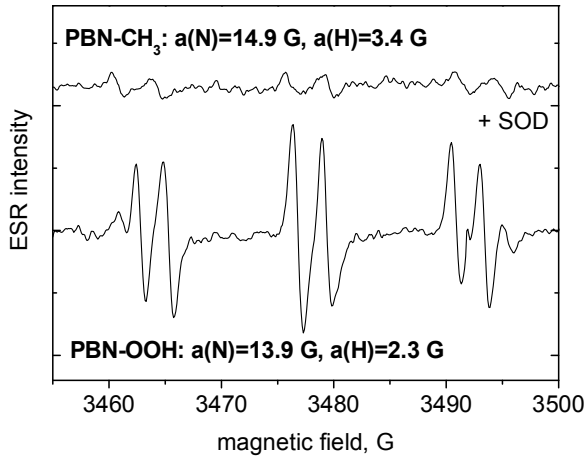


***Figure 10**. ESR spectra of PBN–OOH and PBN–CH_3 adducts detected in the presence (at the top), and in the absence of 200 U/ml SOD in DMSO (contained 10% H_2O). Experimental conditions: [PBN] = 5 mM, [$FeSO_4$] = 1 mM, [H_2O_2] = 0.2 M. Experiments with SOD (Sigma, EC 1.15.1.1) were carried out using ER-200D SRC (X-band, 9.5 GHz) ESR spectrometer (from [55], Fig. 3).*

Note that the Fenton reaction has been suggested as one of the possible sources of reactive species in living cells [56, 57].

The ability of carotenoid **VI** to scavenge peroxyl radicals was compared in the absence and presence of CD. In previous studies, the EPR spin trapping technique was applied to measure the scavenging rates of carotenoids towards free radicals [7, 58]. The scavenging ability was measured as a relative scavenging rate of carotenoid (Car) and spin trap (ST). These values were determined from concentration dependence of spin adduct yield (A) by using the equation (7):

$$\frac{A_0}{A} = \frac{k_{ST}[ST] + k_{Car}[Car]}{k_{ST}[ST]} \qquad (7)$$

Here k_{Car} and k_{ST} are the reaction rate constants of carotenoid and spin trap with a free radical, and A_0 is spin adduct yield at zero carotenoid concentration. It was observed that k_{Car} values depend on the redox properties of carotenoid and increase with increasing of their oxidation potentials [7]. According to our results, β-carotene shows the worst antioxidant ability among the carotenoids under study. The values of k_{Car}/k_{ST} change from 0.6 for **I** to 24 for **IV**. Figure 11 demonstrates the decrease of PBN-OOH spin adduct yield with increase carotenoid **VI** concentration as a result of scavenging process.

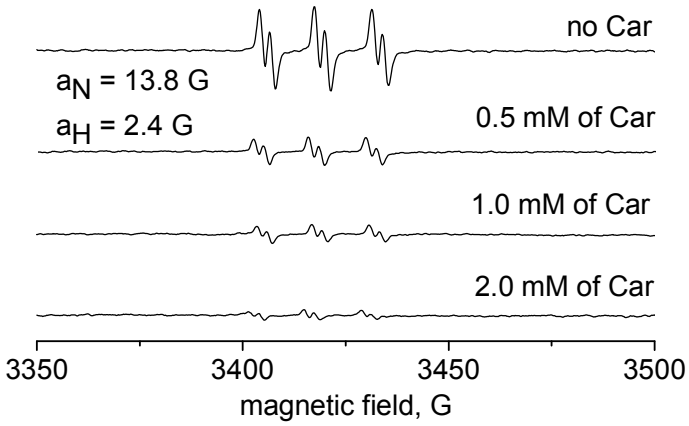


Figure 11. *Variation of PBN-OOH spin adduct EPR spectrum in the presence of* ***VI****. Concentration of PBN = 10 mM;* Fe^{2+} *= 1 mM;* H_2O_2 *= 500 mM in DMSO (from [20], Fig. 7).*

Plot ($A_0/A - 1$) vs. [Car]x[CD] shows linear dependence. The value $k_{Car}/k_{ST} = 40$ was calculated from this plot. This is the highest value from all carotenoids previously studied.

The same technique was applied to study the scavenging ability of the inclusion complex of **VI** in water. The highly water soluble hydroxypropyl substituted CDs were used for these EPR experiments. In contrast with Figure 11, no decrease in spin adduct yield was observed for CD complex of this carotenoid (see Figure 12). Moreover, one can see the appearance of the pro-oxidant effect (increase of spin adduct yield) in the presence of carotenoid.

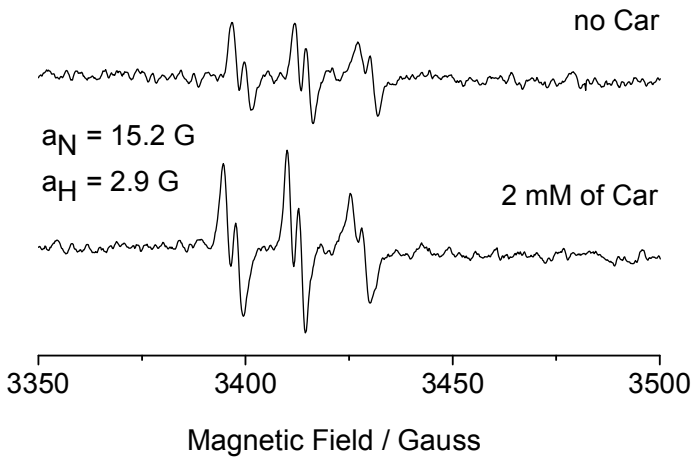
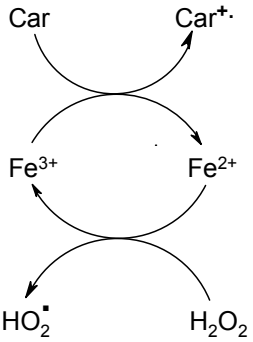


Figure 12. *Variation of PBN/OOH spin adduct EPR spectrum in the presence of* ***VI-****HP-β-CD complex. Concentration of PBN = 10 mM;* Fe^{2+} *= 1 mM;* H_2O_2 *= 500 mM; HP-β-CD = 4 mM in* H_2O *(from [20], Fig. 9).*

To confirm that this observation is not due to encapsulation of the spin trap by CD the same measurement was made in the absence of carotenoid. The presence of CD does not result in a change of spin adduct yield in this case. We suggested that the absence of the antioxidant effect is due to protection of the radical sensitive site of the carotenoid (cyclohexene ring) by the CD. This result confirms our suggestion that peroxyl radicals attack mainly the cyclohexene ring of carotenoid. The occurrence of the pro-oxidant effect for the Car-CD complex was attributed to chain elongation by reaction of carotenoid with Fe^{3+} ions. Fe^{3+} ions, present in solution as the product of the Fenton reaction, oxidize the carotenoid and regenerate Fe^{2+}:

$$Car + Fe^{3+} \rightarrow Car^{+\bullet} + Fe^{2+}$$

The radical cation of carotenoid, $Car^{+\bullet}$, was detected as a product of this reaction. In the presence of excess of H_2O_2 this reaction will results in repetition of the redox cycle of the Fenton process and production of additional portion of free radicals.



Due to their biological importance for living systems, the reaction of electron transfer between carotenoids and other metal ions such as Cu, Ti, and Ni have been intensively studied [59-62]. In particular, transition-metal complexes of carotenoids were detected in these studies. The role of Fenton-like processes in *in vivo* generation of toxic free radicals is now being widely discussed [56, 57, 63-68].

The results of our study [20] show that complexation with CD protects the carotenoid during transportation to the target, but to be an effective antioxidant, the carotenoid should be extracted from the CD cavity after delivery to the membrane. It is an important consideration in using carotenoid-CD complexes in medical practice. Recent *in vivo* and *in vitro* experimental data demonstrated that cyclodextrins can be used as carriers for the incorporation of dietary carotenoids into plasma and mitochondrial and microsomal cell membranes. Cyclodextrins, in contrast to dimethylsulfoxide, stabilize carotenoids and allow efficient cellular uptake [18, 19, 69]. At the same time, carotenoids encapsulated in the CD cavity show no photoprotection of human skin fibroblasts against UV irradiation [70].

2. Host-Guest Complexes of Carotenoids with β-Glycyrrhizic Acid [55, 71].

This section deals with the complexation of carotenoids with β-glycyrrhizic acid (GA). Glycyrrhizic acid is an unique natural compound of considerable interest to pharmacologists not only due to its physiological activity, but also due to its ability to enhance the activity of some drugs by non-covalent complex formation. The purpose of our study was the investigation of the structure of these complexes as well as the influence of GA on radical processes involving carotenoids. Special attention was paid to the antioxidant activity of carotenoids in the complexes.

Measurement of the stability and stoichiometry of GA complexes in aqueous solutions [71]. First of all we have have shown that complex formation occurs between the carotenoid and glycyrrhizic acid, and the structure of this complex has been estimated. The high extinction coefficients of carotenoids (~10^5

$M^{-1}cm^{-1}$) make it convenient to use optical methods and thus allow us to work with very low concentrations. Note that the using of optical methods for inclusion complex analysis is more convenient compared to NMR techniques in the case of carotenoids. This is due to two reasons. First is the very low solubility of carotenoids even in water-alcohol solutions. Since carotenoids are insoluble in water, in these experiments we used water-ethanol mixtures. From our experience, the addition of small amounts of alcohol can slightly decrease complex stability but has no effect on its stoichiometry. Second, the sensitivity of NMR spectra to complex formation decreases considerably in the presence of alcohol or other organic solvents. NMR techniques are widely used for studying the stability and stoichiometry of cyclodextrin inclusion complexes, but in the case of GA complexes the changes in chemical shifts are much lower even in aqueous solutions.

To calculate the stability constant and stoichiometry of GA complexes, we used an approach similar to the analysis of CD complexes. In this case it was used to analyze the concentration dependence of the absorption and fluorescence spectra of the carotenoids [40, 72]. In our experiments all carotenoids showed nearly the same change in extinction coefficient at a fixed wave length in the presence of GA (~10%). The stoichiometry of the complexes was calculated using Job's plot of the dependence of the optical density of the solution with mole fraction of carotenoid. The measurements were performed with only two carotenoids **VII** and **VIII** that displayed the best solubility in alcohols. Both carotenoids have the same Job's plot. The position of a maximum of the curve at R = [Car] / ([Car] + [GA]) = 0.33 corresponds to the 1:2 ratio between carotenoid and GA molecules in the complex. It was earlier suggested that in aqueous solutions GA molecules form cyclic dimers of either torus [22] or podant [23] type.

To estimate the complex stability constant, the changes in optical density of the solution were measured at a fixed concentration of carotenoids with varying GA concentration (Fig. 13).

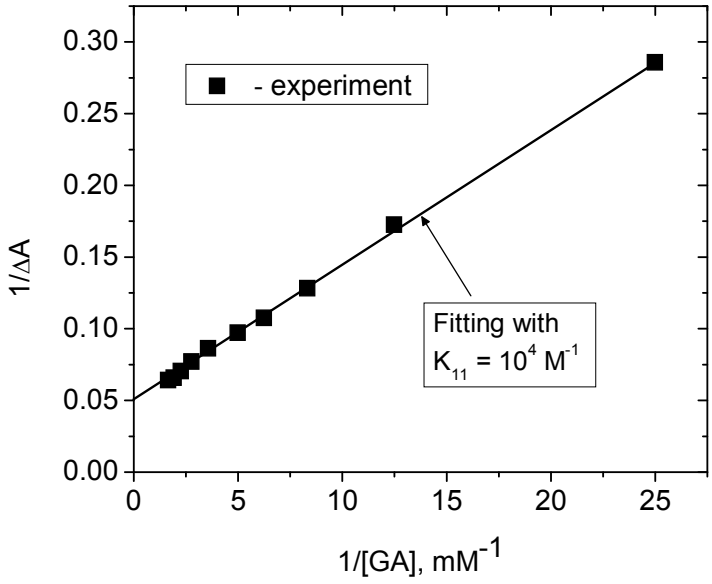


Figure 13. *Benesi-Hildebrand plot of optical density changes (ΔA) of carotenoid* ***I*** *(2.5 μM) at 440 nm vs. GA concentration in 20% aqueous ethanol solution (adopted from [71], Fig. 2).*

The Benesi-Hildebrand plot (8) allows one to estimate both the complex stability constant and the order of complexation from a single experiment:

$$A/\Delta A - 1 = 1/[GA]^n \times 1/K \qquad (8)$$

Here $\Delta A = \Delta\varepsilon \times [Car]$, and K is the stability constant of the complex for the reaction:

$$Car + nGA \overset{K}{\rightleftharpoons} CarGa_n$$

$$K = \frac{[CarGA_n]}{[Car] \times [GA]^n}$$

In our experiments, in all cases, the plot of $A/\Delta A$ versus $1/[GA]^n$ provides the linear dependence only for n = 1. Taking into account the result of the analysis of Job's plot, it was concluded that the reaction of complexation is second order between one carotenoid molecule and one dimer of glycyrrhizic acid.

$$Car + GA_2 \overset{K}{\rightleftharpoons} CarGa_2$$

The complex structure is suggested to consist of a carotenoid molecule located within a torus formed by the glycyrrhizic acid dimer (Fig.14).

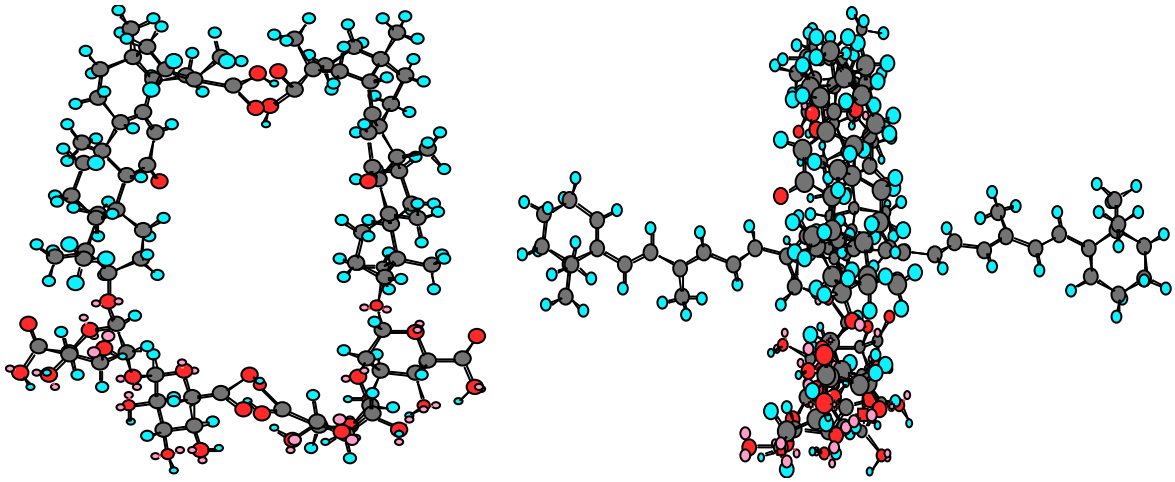


Figure 14. *Schematic Chem3D Pro (Cambridge Software, Cambridge, MA) presentation of the suggested structures of the GA dimer and their inclusion complex with carotenoid (from [71], Fig. 9).*

Computer simulation of the experimental concentration dependence in Fig. 13 provide the value K = 10^4 ($\pm 10^3$) M^{-1} for the β-carotene-GA complex. Carotenoids **VII** and **VIII**, whose solubility in water-alcohol solutions is greater than that of β-carotene, form less stable complexes (K~10^3 M^{-1}), as expected from the hypothesis that hydrophobic interactions are essential for complex formation with GA. To elucidate the role of hydrophobic interactions in the formation of the carotenoid-GA complex, a thermodynamics study was carried out for carotenoid **VII** in the temperature interval 293 – 306 K°. Thermodynamic parameters were estimated using the relevant equations (9-11):

$$-\Delta G = RT\ln K \qquad (9)$$

$$\Delta G = \Delta H - T\Delta S \qquad (10)$$

$$\ln(K_2/K_1) = -(\Delta H/R)\cdot(1/T_2 - 1/T_1) \qquad (11)$$

Here $K_i = K(T_i)$ are the values of the stability constants at different temperatures. Enthalpy of this complex formation was determined equals +42.9 kJ/mol, ΔG = 19.9 kJ/mol and ΔS = +211 J/(mol·K) at T = 293 K°. Experimental error in this study was about 25%. Positive values of both enthalpy and entropy contributions point out that hydrophobic interactions are an important factor in the binding, and that complex formation follows considerable desolvation of the carotenoid molecules. This feature is characteristic of inclusion complexes. We have found several examples of thermodynamic measurements of molecular complexes of β-glycyrrhizic acid with various organic molecules [23, 25]. It should be noted the heat of formation of molecular complexes is generally dependent on the electronegativity of the donor group in the 'host' molecule. A linear correlation was found between the

formation enthalpies of GA complexes and the Hammett constants of the substituents in the nitro derivatives [23]. A thermodynamic study of the GA complex with two drugs, 8-hydroxy-5-nitroquinoline and nitroglycerin shows the presence of both enthalpy and entropy contribution to the complex stability [25]. Indeed, the presence of a number of carboxy and hydroxy groups in the GA and guests molecules is favorable for intermolecular H-bond formation in these cases.

Stability and stoichiometry of carotenoid-GA complexes in non-aqueous solutions [71]. A very important question for understanding the behavior of these complexes in living systems is their stability in non-aqueous media. For this purpose we investigated the possibility of complex formation between carotenoids and GA in several organic solvents, namely in alcohols, acetonitrile and DMSO. Since the optical absorption spectra of carotenoids are insensitive to the presence of GA in non-aqueous media, an attempt was made to study the influence of GA on the fluorescence spectrum of carotenoids. Due to high sensitivity of fluorescence intensity to the media properties, this approach is widely used to study inclusion complexes of the "guest-host" type [73-75]. The luminescence of molecules imbedded in the cavity of cyclodextrin is strengthened by the protection against quenching and other processes occurring in solution. Carotenoid canthaxanthin (**III**) was used in this investigation. The increase of fluorescence intensity of canthaxanthin in the presence of 1 mM GA in pure DMSO was about 15% from the value in the absence of GA. The addition of a small amount of water (5%) to DMSO had no influence on the fluorescence intensity in the absence of GA, but leads to an increase of the effect up to 50% in the presence of 1 mM of GA. The complex stability constant in DMSO was estimated by the methods of analysis applied to aqueous solutions. Over this concentration range (0-1 mM of GA), the calculation provides the value $K = 0.3\text{-}1\times10^4$ M^{-1}. The large error in the calculation of the stability constant is due to superposition of two processes. One can see that in the plot of the concentration dependence, the fluorescence intensity does not reach the plateau but starts to increase linearly when the GA concentration exceeds 1 mM (Fig. 15).

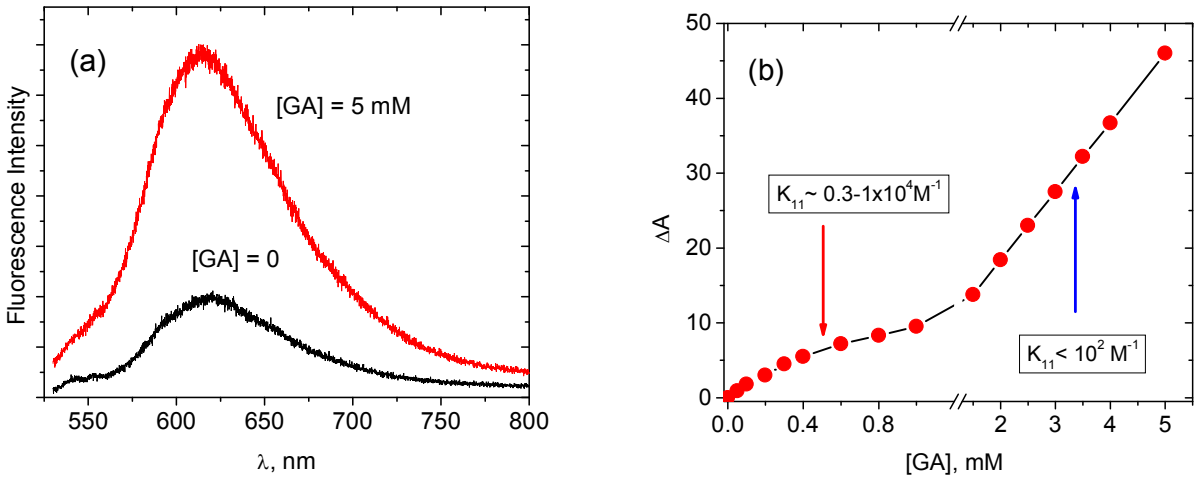


Figure 15. *(a) Fluorescence spectrum of canthaxanthin (**III**) solution, 0.02 mM with and without GA in DMSO containing 5% of water. The excitation wave length is 470 nm, the detection wave length is 620 nm. (b) Dependence of fluorescence intensity of carotenoid **III** on GA concentration in solution (adopted from [71], Fig.3).*

The changes in the properties of GA solutions at the same concentration point (1 mM) have been observed in several studies, and this effect was explained by GA micelles formation [76, 77]. This hypothesis for micelle formation in water solution was confirmed by studying the processes of micelle formation of water-soluble GA derivatives, in particular, their sodium sulfates[24]. There is no evidence of micelle formation in other solvents at this time.

Interaction of carotenoids and their GA complexes with Fe ions and quinones [71]. An important question we tried to answer in our work was the influence of complex formation on carotenoid reactivity. The reactivity of carotenoids towards metal ions, quinones and free radicals is closely related to their antioxidant activity as well as stability in living systems, food and medical preparations. One of the most important natural processes involving carotenoids is electron transfer from carotenoids to acceptors. The mechanism of the reaction between carotenoids and Fe^{3+} ions has been studied in detail in our previous work [35]. As it was mentioned above, the reaction with Fe ions is considered by a number of authors to be one of the possible mechanisms of the pro-oxidant activity of β-carotene. The first step of this reaction is the electron transfer from carotenoid to acceptor resulting in formation of the carotenoid radical cation. The latter also can react with Fe^{3+}:

$$Car^{+\cdot} + Fe^{3+} \rightleftharpoons Car^{2+} + Fe^{2+}$$

Carotenoid radical cation and dication can undergo *cis-trans* isomerization. In the presence of oxygen, the oxidation of carotenoids leads to a stable product, a 5,8-epoxide. In acetonitrile, all carotenoids form relatively stable radical cations according to their absorption spectra, but are not detectable in aqueous solutions. The strongest radical cation signal was observed for β-carotene that has the lowest redox potential, $E^{ox}_{1/2}$ = 0.54 V vs. SCE [78], (Fig. 16). For other carotenoids, the signal intensity at room temperature was about 10% of that recorded for β-carotene.

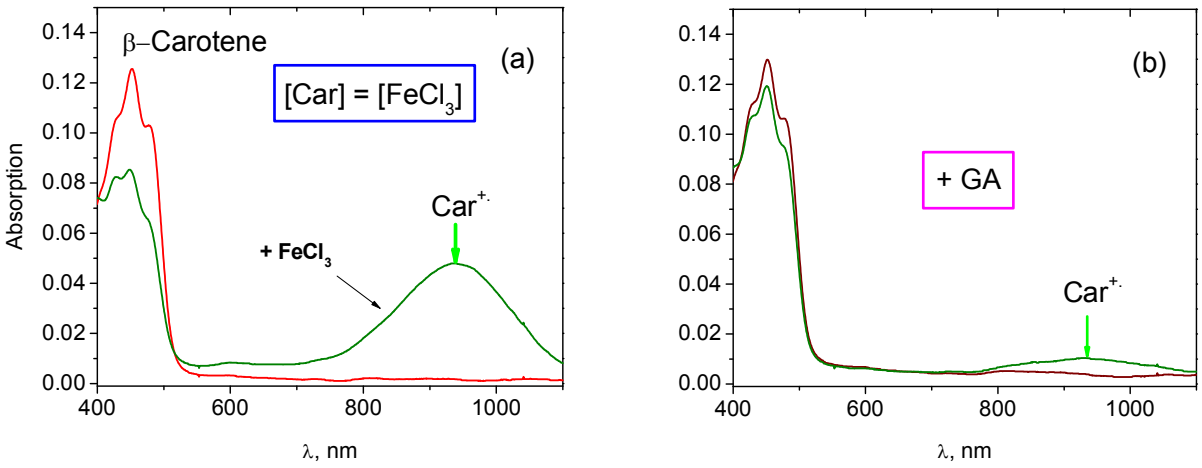


Figure 16. *Absorption spectra of β-carotene, 1.3 μM, recorded before and after mixing with $FeCl_3$, 1.3 μM, in acetonitrile at room temperature. (a) in the absence of GA; (b) in the presence of 0.1 mM GA (adopted from [71], Fig.4).*

Figure 16b shows the absorption spectra of the β-carotene-GA complex recorded before and after mixing with $FeCl_3$. The absorption of radical cation resulting from the reaction is observed at 935 nm. A considerable decrease in the yield of β-carotene radical cation was observed in the presence of GA. It can be attributed to a decrease in the electron transfer rate in the case of complexation. With excess $FeCl_3$, all carotenoid is relatively rapidly transformed into the radical cation both with and without GA (Fig. 17).

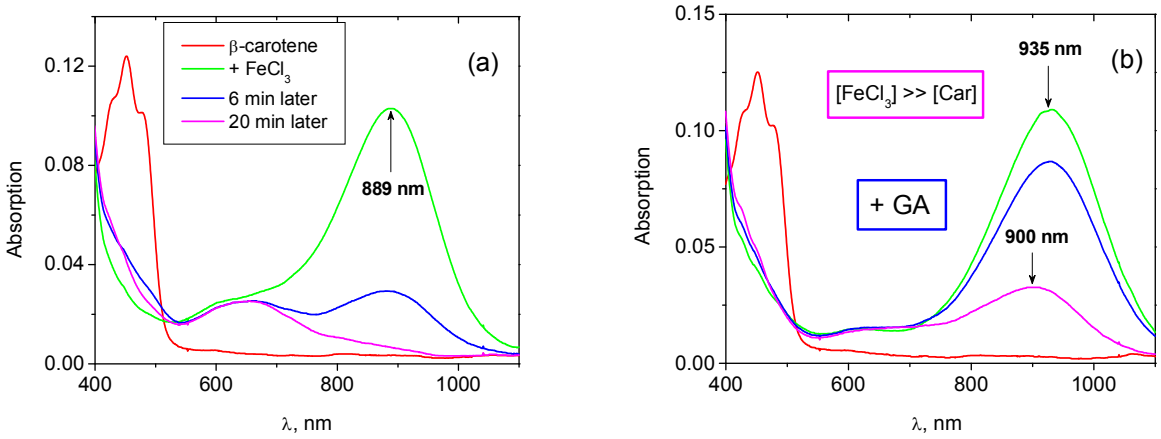


Figure 17*. Absorption spectra of β-carotene, 1.3 μM, recorded before and after the mixing with $FeCl_3$, 7 μM, in acetonitrile at room temperature. (a) in the absence of GA; (b) in the presence of 0.1 mM GA. Signal at 935 nm is due to β-carotene radical cation, that at 889 nm – dication of β-carotene (adopted from [71], Fig.6).*

One can see that the behavior of β-carotene radical cation is quite different in the absence or presence of GA (Fig. 17). Since the first and second redox potentials of β-carotene nearly coincide [78], both radical cation and dication are present when oxidation occurs and are in comproportionation equilibrium.

$$2Car^{+\cdot} \rightleftharpoons Car^{2+} + Car$$

Whereas a free radical cation transforms rapidly into dication (absorption at 889 nm), in the complex the radical cation is stabilized, i.e., it can be observed for a much longer period (tens of minutes). Since the rates of electron transfer (both the first and the second) decrease significantly, it means that the charged forms of carotenoids (cations and dications) do not leave the complex after the reaction and are also stabilized inside the cavity.

Control measurements (the addition of acetic acid with $pK_a \sim 4.6$ to a carotenoid solution which is close to that of GA) indicate that none of the observations can be attributed to changes in medium acidity.

Another example of the influence of GA on the reactivity of carotenoids is the electron transfer from carotenoid to quinone. Quinones are known to be important natural electron acceptors in photosynthetic centers [79, 80]. As a model system for studying the reactivity of inclusion compounds, we used dichloro-dicyano-benzoquinone (DDQ), which, owing to its low reduction potential, reacts

with carotenoids without additional initiation by either light or temperature. Formation of a radical ion pair (RIP) in this reaction occurs via an intermediate charge transfer complex (CTC) [34]:

$$\text{Car} + \text{Q} \rightleftharpoons \text{CTC} \rightleftharpoons \text{Car}^{+\cdot} + \text{Q}^{-\cdot} \longrightarrow \textit{cis}\text{-isomers} + \text{Car-Q}$$

The product of RIP recombination is the quinone–carotenoid adduct, whereas the radical cations escaping recombination result in the formation of *cis*-isomers [81]. Since the radical cations of β-carotene are stable in acetonitrile solution, we used optical absorption spectroscopy for monitoring their behavior in the reaction with quinone. Figure 18a demonstrates the difference in the form of the kinetic curves describing the carotenoid radical cation decay with and without GA. Whereas for a free carotenoid the radical cation signal decays exponentially with a half-life τ of ~20 s, in the presence of GA biexponential decay was observed. The fast process exhibits exponential decay with τ ~20 s, and the slow process is described by eq.: $I = a/(1 + t/\tau)$, with τ ~1000 s.

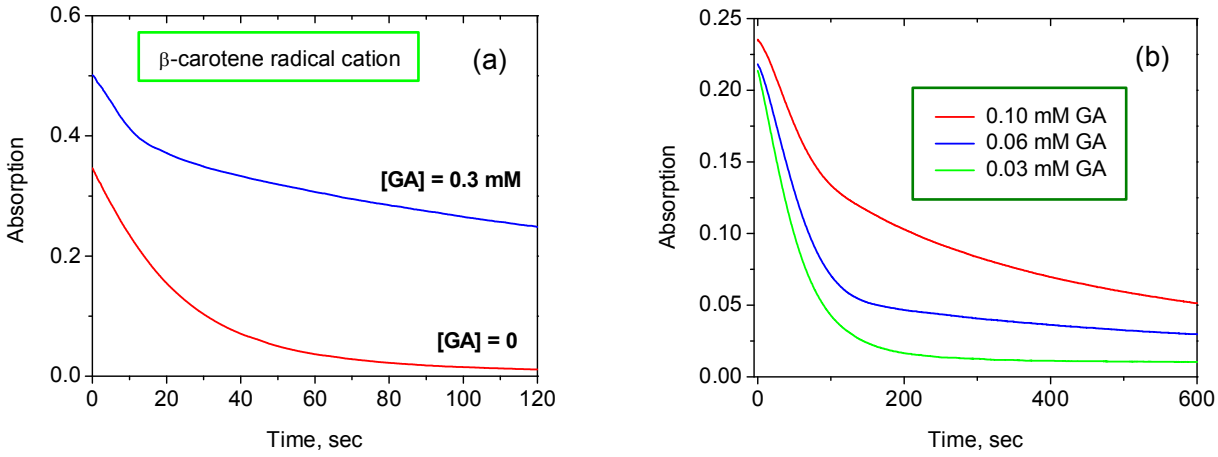


Figure 18. *Kinetics of the decay of β-carotene radical cation at 935 nm. (a) 4 μM β-carotene + 4 μM DDQ with and without GA; (b) 2 μM β-carotene + 5 μM DDQ for various GA concentrations (adopted from [71], Fig.7).*

The contribution of a slow component of the kinetic increases nonlinear with increase of GA concentration (Fig. 18b). It was suggested that the slow decay component is due to the radical cation in the complex. The observed ratio of fast and slow components is proportional to the square GA concentration, so we can estimate the stability constant of GA complex with radical cation in acetonitrile from the ratio of slow and fast kinetics components using equation (12).

$$K_{12} \times [\text{GA}]^2 = \frac{[\text{CarGA}_2^{+\bullet}]}{[\text{Car}^{+\bullet}]} \qquad (12)$$

The estimated stability constant in acetonitrile is near 10^8 M^{-2}. The two-component kinetics of the radical cation signal decay indicates the absence of a fast exchange between complexed and free radical cations.

How will the increase in the lifetime of the radical cation influence the yield of main reaction products? We propose that the changes in the ratio of the reaction products depend on whether only radical cation or CTC is imbedded in GA complex. To elucidate this question, we compared the yields of the reaction products for two carotenoids (**III, IV**) using HPLC method. As a result, a substantial increase in the yield of carotenoid-quinone adduct was observed for these carotenoids in the presence of GA (Fig. 19).

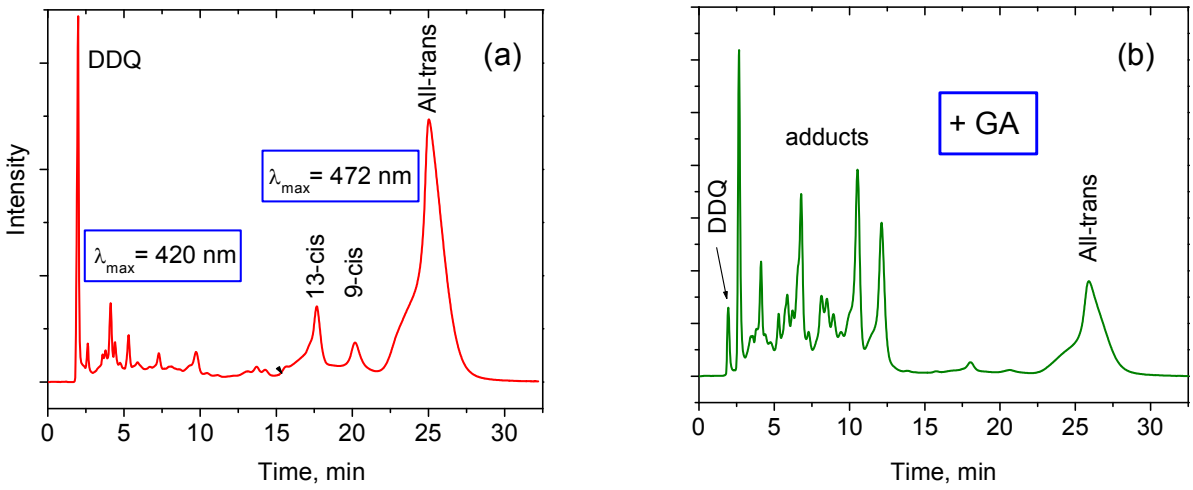


Figure 19. *HPLC spectrum detected at 420 nm in acetonitrile of the products of the reaction between carotenoid **III** and dichloro-dicyano-benzoquinone (concentrations of 0.06 mM) (a) without and (b) with 0.2 mM GA (adopted from [71], Fig.8).*

As an example, Figure 19 demonstrates HPLC spectra of the products of the reaction between carotenoid **III** and DDQ with and without 0.2 mM GA. One can see the decrease of the amount of initial compounds (quinone and carotenoid) in the presence of GA as well as carotenoid isomers (17 – 27 min). Simultaneously an increase of the yield of adducts (3 -10 min) was detected. Several peaks of adducts appear due to addition of quinone at the various double bonds of the conjugated canthaxanthin chain. This observation allowed us to conclude that GA can form stable complexes not only with individual compounds and their ions but also with charge transfer complexes. In the case of carotenoids, this leads to a change in both the reaction direction and the ratio of products. The

important point is also the possibility to control the life time of carotenoid radical cations by the formation of "host-guest" complexes.

Antioxidant and redox properties of supramolecular complexes of carotenoids with glycyrrhizic acid [55]. Antioxidant activity is known to be one of the most important biological properties of carotenoids, because they react with toxic free radicals and thus prevent damage to living organism [2-4]. From a practical point of view, it is interesting to know how the complexation of carotenoids with glycyrrhizic acid will affect their ability to scavenge free radicals. As it was stated above, the complexes of carotenoids with cyclodextrin is used to improve their solubility, and to increase bioavailability, however, it has failed to improve their antioxidant properties. As it was shown in the first part of this chapter, measuring the scavenging rate of peroxide radicals by carotenoids in solutions indicates that the reaction can be almost totally inhibited by cyclodextrin due to embedding the cyclohexene fragment in the cyclodextrin cavity [20].

The antioxidant activity of carotenoids and their complexes with GA was studied by the same EPR spin-trapping technique. EPR spectra of the spin adduct of OOH radical with the spin trap *N-tert*-butyl-α-phenylnitrone (PBN) were recorded using an ESR Varian E-12 (X-band, 9.5 GHz) spectrometer. Note: the method involves measuring the yield of the stable spin adduct of peroxyl radicals as a function of carotenoid concentration. Because of the two competing processes, i.e., reactions of the radical with carotenoid and spin trap, the yield of the spin adduct is proportional to the carotenoid concentration (see Fig. 20 and Eq. 7).

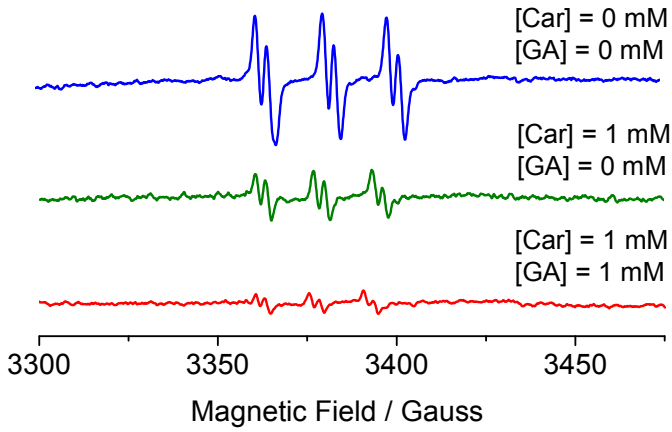


Figure 20. *ESR spectra of PBN–OOH adduct detected without carotenoid (top), and in the presence of canthaxanthin or its complex with GA in DMSO. Experimental conditions: [PBN] = 5 mM, [$FeCl_2$] = 1 mM, [H_2O_2] = 0.5 M (adopted from [55], Fig.2).*

The relative rates of radical scavenging (k_{Car}/k_{ST}) by carotenoids were calculated from the dependence of the spin adduct yield with carotenoid concentration. The absolute value of the rate constant can be estimated using the available kinetic database (Spin Trap Data Base: http://epr.niehs.nih.gov) which provides the value of the rate constant k_{ST} measured in water ($k_{ST} \leq 10^6$ $M^{-1}s^{-1}$ for the PBN spin trap). Using the same approach we have measured the reaction rates of the OOH radical with GA complexes of carotenoids. Our experiments show that GA itself displays a substantial antioxidant activity with $k/k_{ST} = 12$ (Table 1). Note that the rate constant of the reaction between GA and peroxyl radicals is independent of GA concentration. On the other hand, comparison of the scavenging rates of peroxyl radicals by free carotenoids and their complexes in DMSO shows a strong dependence of the rate constants on the concentration of GA.

Table 1. *Relative rate constants of OOH radicals scavenging by glycyrrhizic acid, carotenoids, and their complexes (k/k_{ST}) in DMSO. Experimental error ~10%. $E_{1/2}$ are the redox potentials of carotenoids (in V vs. SCE) [78].*

[GA] mM	**GA**	**II** ($E_{1/2}$ = 0.56 V)	**III** ($E_{1/2}$ = 0.68 V)	**IV** ($E_{1/2}$ = 0.72 V)
0		4	2	7
0.5	12	4	59	133
1	12	4	46	116
2	12	4	6	38

The k_{Car}/k_{ST} values given in Table 1 were obtained by subtracting the GA contribution from the total rate constant measured in the presence of glycyrrhizic acid. From these values, it is possible to determine whether a synergetic effect of GA on the scavenging ability of carotenoids **III** and **IV** occurs. Of importance is the absence of this effect for zeaxanthin (**II**). Analyzing the oxidation potentials of these three carotenoids (see Table 1) and the dependence of the carotenoids scavenging rate [7] on their $E_{1/2}$, points out that GA can affect the oxidation potential of the carotenoids. This hypothesis was verified by CV measurement of the oxidation potential of two carotenoids **II** and **III** in

the presence of GA. In both cases, we have observed an increase in $E_{1/2}$: by 0.05 V for canthaxanthin (Fig. 21) and by 0.03 V for zeaxanthin.

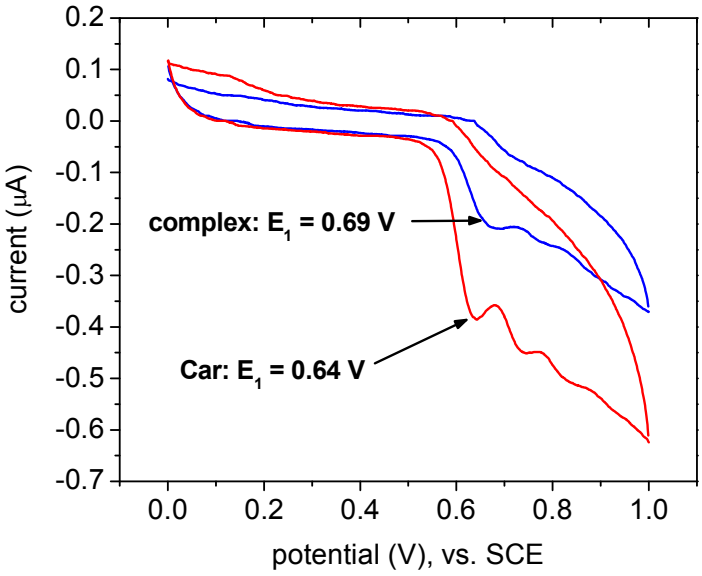


***Figure 21**. Cyclic voltammetry (CV) plot of canthaxathin (0.1 mM) and its complex with GA (0.2 mM of GA) in acetonitrile. Scan rate is 10 mV/s (adopted from [55], Fig.4).*

Using this result and the diagram in Fig. 22 we can explain the different behavior of carotenoids **II - IV** in the presence of GA. As one can see in Figure 22, the dependence of the rate constant of the reaction of peroxyl radicals with oxidation potential of carotenoids is nonlinear. A negligible change in the oxidation potential for beta-carotene and zeaxanthin (<0.05 V) should cause no changes in their antioxidant activity. At the same time, this diagram allows us to predict a substantial increase in the reaction rate for carotenoids with $E_{1/2}$ ~ 0.7 V when their oxidation potential increases due to complexation.

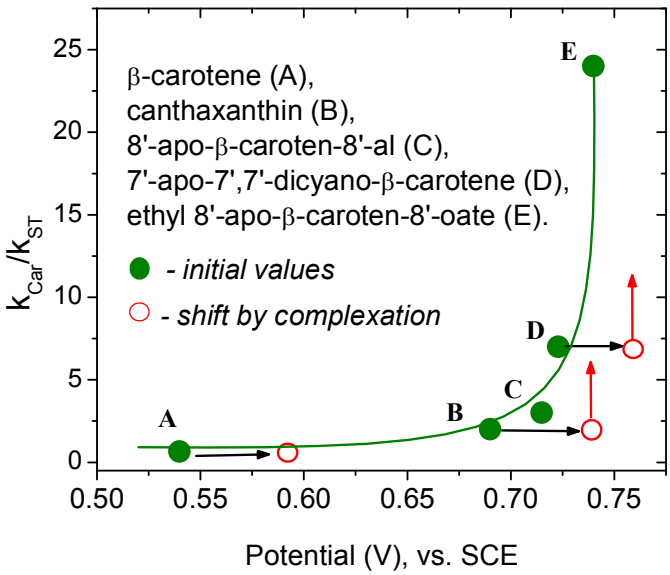


***Figure 22**. Diagram of the dependence of the carotenoid scavenging rate toward peroxyl radicals on the oxidation potential of carotenoids [7]. Arrows denote the shifts in oxidation potentials due to complexation (adopted from [55], Fig.5).*

These experimental results verify the hypothesis for the role of electron transfer in the scavenging of free radicals by carotenoids. A very important observation is determining the effect of GA on the oxidation potential of carotenoids [55]. An increase in the oxidation potential of carotenoids can serve the main reason for a decrease in their oxidation rate in reactions with electron acceptors. Note also, that the rate constants of peroxyl radical scavenging by carotenoids (Table 1) are different at various GA concentrations. The scavenging rates measured at low GA concentrations (0.5 мМ) considerably exceed those measured at high concentrations (2 мМ). This fact confirms the hypothesis for the dependence of the structure and properties of GA complexes on its concentration. It was shown that in aqueous solutions, at a concentration of above 1 мМ, GA forms micelle type aggregates [76, 77]. However, the data on the micelle formation in non-aqueous solvents are unavailable in the literature. As it was described in the previous paragraph, the stability constant of GA complex with canthaxanthin in DMSO estimated from fluorescence measurements is about 10^4 M^{-1} for low GA concentrations and $<10^2$ M^{-1} for high concentrations. Below GA concentration of 1 mM, the GA-carotenoid complex stoichiometry was determined to be 2:1. The differences in the scavenging rates of peroxyl radicals for various GA concentrations indicate that the properties of 2:1 complexes really differ from those for carotenoids in the assumed micellar solution. The reason for these differences is still unknown.

3. Water soluble complexes of carotenoids with arabinogalactan [82].

The majority of carotenoids are lipophilic molecules with near zero inherent aqueous solubility. Moving carotenoids into a pharmaceutical application requires a chemical delivery system that overcomes the problems with parenteral administration of a highly lipophilic, low molecular weight compound. Increase water solubility of carotenoids opens several technological applications that could be provided in food processing (colors and antioxidant capacity) as well as for production of therapeutic formulations considering the better solubility and consequently higher bioavailability.

In this chapter we present the first example of water soluble complexes of carotenoids. The stability and reactivity of carotenoids in the complexes with natural polysaccharide arabinogalactan (AG) were investigated by different physicochemical techniques: optical absorption, HPLC, and pulsed EPR spectroscopy. Polysaccharide complexes of carotenoids showed enhanced photostability compared to pure carotenoids as well as reduced reactivity towards metal ions (Fe^{3+}) and reactive oxygen species. On the other hand, the yield and stability of carotenoid radical cations produced on titanium dioxide nanoparticles were greatly increased in the solid state complex of arabinogalactan. Canthaxanthin radical cations was stable for 10 days at room temperature in this system. We suggest that these results are important for a variety of carotenoid applications.

Arabinogalactan was extracted from *Larix sibirica* [83]. Arabinogalactans are long, highly branched polysaccharides composed of galactose and arabinose molecules in a 6:1 ratio. Pharmaceutical-grade larch arabinogalactan is a fine, dry, off-white powder with a slightly sweet taste and mild pine-like odor. It is low in viscosity, dissolves completely in water or juice, and therefore easy to administer, even to children.

Solubility of carotenoids in water as a criterion of complex formation. It is well known that carotenoids are highly hydrophobic, air- and light-sensitive compounds. Earlier there were several attempts to prepare water soluble complexes of carotenoids using water-oil emulsion or cyclodextrin solutions. However, this products when placed in water forms an obvious dispersion, with particles visible to the naked eye and the β-carotene separates from the water within a few days. While water dispersible β-carotenes are suitable for some applications (e.g., beverages) they are not suitable for others (e.g., fat substitutes) because the beta-carotene itself is still oil soluble. Also, the solutions of all carotenoid-cyclodextrin complexes show a considerable decrease in color intensity as compared to carotenoid solutions in organic solvents [20]. This fact significantly constricts the application of these complexes as food colorants.

The estimated solubility of the complexes of carotenoids **I**, **III** and **IV** with arabinogalactan prepared mechanochemically in solid state with molar stoichiometry 1:1, is 2-5 mM in water solution. The carotenoid-arabinogalactan complexes maintain their original color and show insignificant changes in absorption spectra. UV-Vis spectrum of aqueous solutions of canthaxanthin-AG complex has the same absorption maximum as the spectrum of canthaxanthin solution in 30% ethanol (Figure 23).

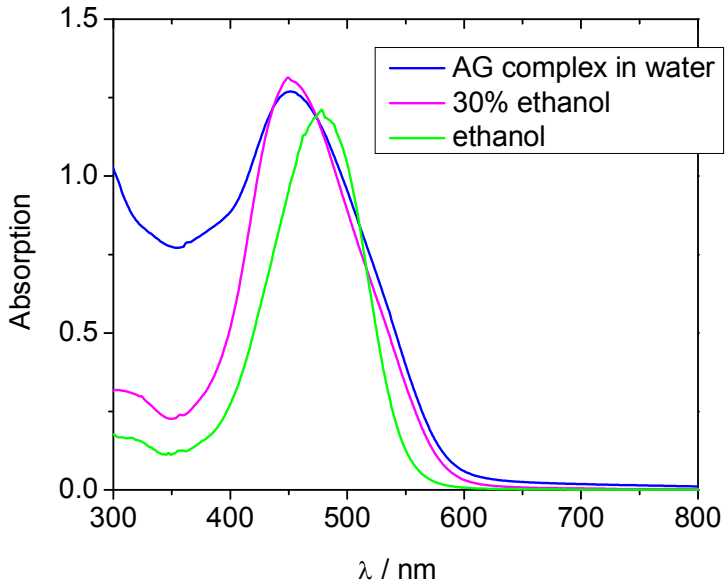


Figure 23. *Absorption spectra of carotenoid **III** in different solvents. The increase of optical density at λ < 400 nm is due to arabinogalactan absorbance (adopted from [82], Fig.1)..*

The formation of carotenoid complexes of arabinogalactan were monitored by X-ray diffraction phase analysis and differential scanning calorimetry (DSC) techniques (Figure 24).

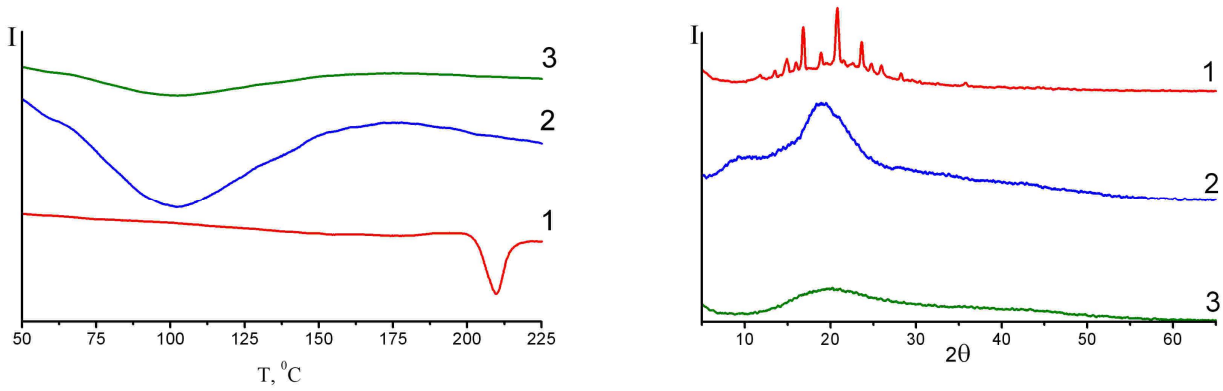


Figure 24. *X-ray diffraction phase analysis (right) and differential scanning calorimetry analysis (left) of solid canthaxanthin-arabinogalactan complex prepared mechanochemically. 1 - canthaxanthin; 2 - arabinogalactan; 3 - complex (from [82], Fig.2).*

In the mixture of initial compounds before treatment one can see the characteristic peaks of the crystal structure of carotenoid (plot 1), which disappears during complex formation (plot 3). We suggested that the absence of crystal structure in this case is due to molecular penetration of carotenoid into arabinogalactan polymer matrix. It means that complex formation occurs in the solid state without using any toxic organic solvents. Further solubilization in water results in preservation of the complex structure. This is confirmed by significant increase in solubility of the complex as compared with traditional solvent-mediated methods.

Photostability of carotenoids and their AG complexes in water solution. One of the main problems in the practical application of carotenoids is their photosensitivity and instability, especially in the presence of oxygen and water. The photostability of drugs and vitamins attracts increasing attention since serious toxic reactions produced by many pharmacologically important chemicals occur under sunlight irradiation [84]. Photoallergic and photomutagenic effects are also of current concern. Photogenerated intermediates can interact with cell components and lead to cell degeneration or death. Control of the drug photostability and preparation of protective strategies against the light-induced damage requires understanding of the structural and environmental factors determining their photoreactivity.

We have studied the relative photodegradation rate of carotenoids and their complex with AG using canthaxanthin as an example. The photolysis of pure canthaxanthin and its complex in aerated water-ethanol mixture shows a significant increase in the photostability of canthaxanthin when incorporated into an AG complex (Figure 25). The estimated decrease in the photodegradation rate is ten times for this system.

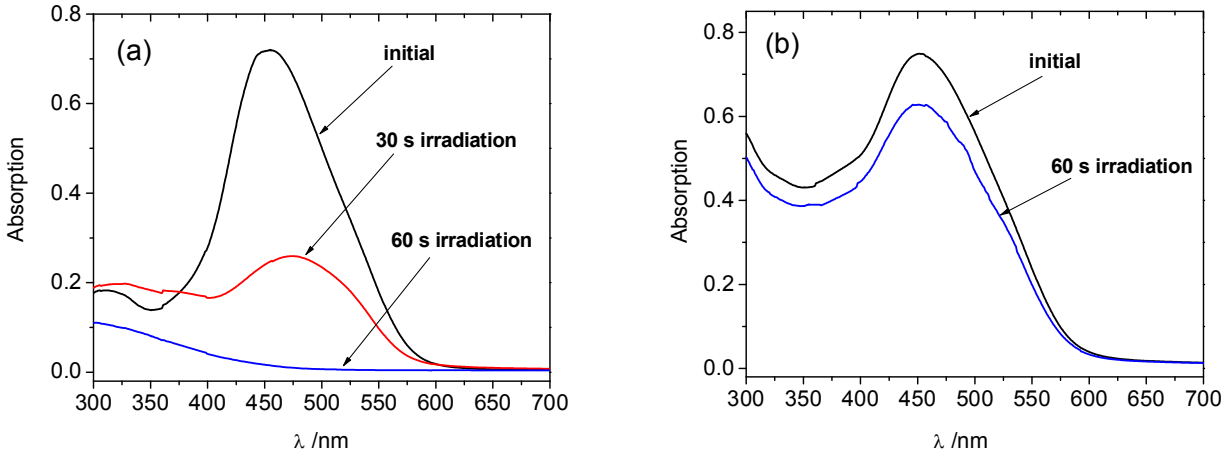


Figure 25. *Photodegradation of canthaxanthin. Absorption spectra were recorded after different irradiation times in aerated 30% ethanol solution by the full light of a xenon lamp. (a) Pure canthaxanthin; (b) Canthaxanthin-AG complex (adopted from [82], Fig.3).*

It is thought but not proven that the decrease in stability of the carotenoids when redox processes occur in the presence of water is due to deprotonation of their radical cations and dications with formation of neutral radicals. Earlier we have demonstrated that the carotenoid neutral radicals are formed from the corresponding radical cations generated electrochemically or photochemically by proton loss [85-87]. Electrochemical measurements showed that the radical cations of a majority of carotenoids have pK's ranging between 4-7 and, therefore, can deprotonate spontaneously even without photoexcitation [81, 88]. We propose that incorporation of carotenoids into the hydrophobic polymer environment reduces their interaction with water molecules. It was confirmed by the absence of a considerable blue shift in the absorption spectra of the AG complex of carotenoid containing a polar group (canthaxanthin, for example) observed earlier in the presence of water in homogeneous solutions [20].

Reactivity of the carotenoid complexes of AG in solution. One of the most important natural processes involving carotenoids is the electron transfer from carotenoids to a variety of acceptors. In the previous section, we have shown that complexation with natural oligosaccharide glycyrrhizin (GA) has a noticeable effect on the reactivity of carotenoids, such as a decrease in the electron transfer rates in the reaction with ferric chloride, and an increase in the lifetime of the complexing radical cations [71]. To study a single electron transfer reaction of carotenoids in aqueous solution, we have chosen a ferric citrate complex (Fe:cit) as an electron acceptor [89]. The first step of this reaction is the electron transfer with formation of the carotenoid radical cation. In the presence of water carotenoid radical

cations can undergo deprotonation reaction resulting in neutral carotenoid radicals with further dimerization:

$$Car^{+\cdot} \rightleftharpoons \dot{C}ar + H^{+}$$

$$2\dot{C}ar \longrightarrow Car_2$$

The formation of carotenoid dimers was detected from the broadening of the carotenoid absorption spectra with the shift of absorption maxima to higher wavelength (see Figure 26, for example). In the absence of water, in pure ethanol, reaction of β-carotene with Fe:cit results in its degradation without dimer formation (Fig. 27).

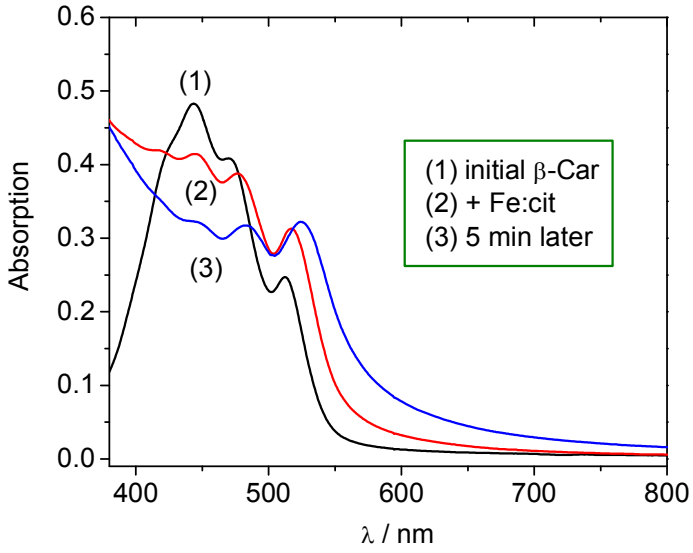


Figure 26. *Transformation of the absorption spectra of β-carotene during the reaction with ferric citrate (0.5 mM) in 30% ethanol (adopted from [82], Fig.4).*

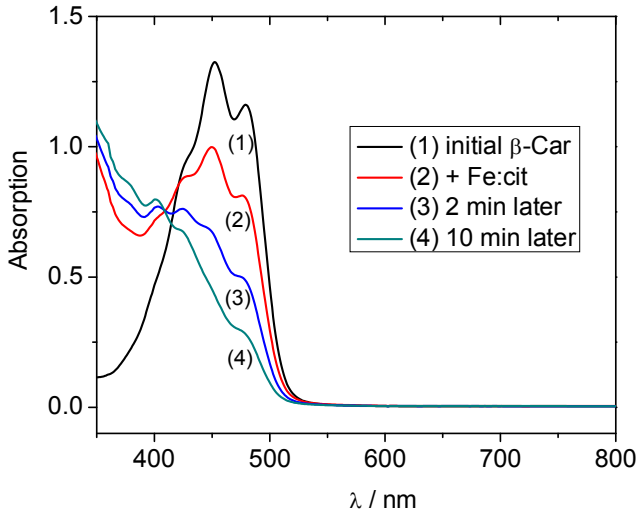


Figure 27. *Absorption spectra of β-carotene in the presence of ferric citrate (0.5 mM) in ethanol solution (adopted from [82], Fig.4).*

In contrast to a homogeneous solution, AG complexes of carotenoids under study (**I**, **III** and **IV**) show no change in the absorption spectra in the presence of ferric citrate during a 20 minute observation period in 30% aqueous ethanol solution, as well as in water solution. We suggest that two factors play an important role in the stabilization of carotenoids in AG complexes, namely, isolation from water molecules and isolation from metal ions. The important conclusion that be can made from this result, is that the Fe:cit complex does not penetrate into the polysaccharide matrix.

Another important reaction of carotenoids which was investigated in the presence of AG is the interaction with active oxygen species. This reaction attracts great attention in relation to antioxidant and pro-oxidant activities of carotenoids. In the present study we used the photo-Fenton reaction [89, 90] listed below and Fe(III):cit for generation of free radicals.

$$Fe^{3+} + H_2O \xrightarrow{h\nu} Fe^{2+} + H^+ + \dot{O}H$$

$$H_2O_2 + Fe^{2+} \longrightarrow Fe^{3+} + OH^- + \dot{O}H$$

UV irradiation of a water solution of Fe(III):cit shows only a decrease of the Fe^{3+} absorption spectrum band due to a low extinction coefficient of Fe^{2+}. Irradiation of the same solution in the presence of hydrogen peroxide results in the appearance of a new absorption peak near 500 nm (Fig. 28). A modern interpretation of the Fenton (and photo-Fenton) mechanism [90] assumes that other

oxidizing intermediates such as highly valent iron complexes (Fe^{4+}) are formed during oxidation of Fe^{2+} to Fe^{3+}.

$$H_2O_2 + Fe^{2+}(aq) \longrightarrow [H_2O_2 + Fe^{2+}] \longrightarrow Fe^{4+}(aq) \longrightarrow Fe^{3+} + OH^- + OH^{\bullet}$$

It is suggested that the long lived species observed at 500 nm (life time more than one hour) might be a citrate complex with highly valent iron ion.

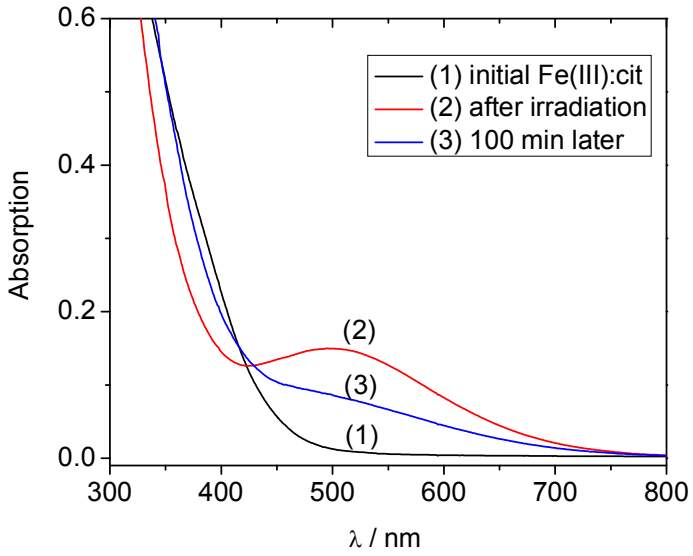


Figure 28. *Absorption spectra of Fe:cit complex (0.5 mM) irradiated 1 min in the presence of H_2O_2 (0.25 M) in 30% ethanol(adopted from [82], Fig.5).*

The Fenton reaction is widely used for generation of free radicals in solution for model experiments. According EPR spin-trapping study, only peroxyl radicals were detected in this system at high H_2O_2 concentration (more then 0.1 M) [7]. The interaction of OOH radicals with the carotenoid polyene chain results in fast bleaching of the solution. As an example, Figure 29 shows a rapid decrease of the β-carotene absorption immediately after addition of H_2O_2 to the mixture of β-carotene with Fe(II):cit.

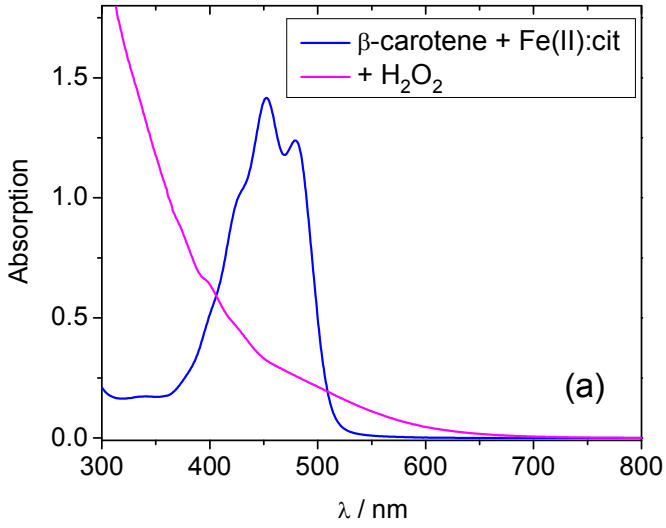


Figure 29. *Absorption spectra of β-carotene in the presence of Fe(II):cit (0.5 mM) and H_2O_2 (0.25 M) in ethanol (adopted from [82], Fig.6).*

A big difference was observed in the behavior of β-carotene when it is incorporated into the AG complex. Figure 30 shows the significant effect of complexation, namely the stability of β-carotene in the AG complex in the presence of Fe^{3+} ions (plot (2)) as well as in the presence of peroxyl radicals generated by irradiation of the reaction mixture (plots (3) and (4)).

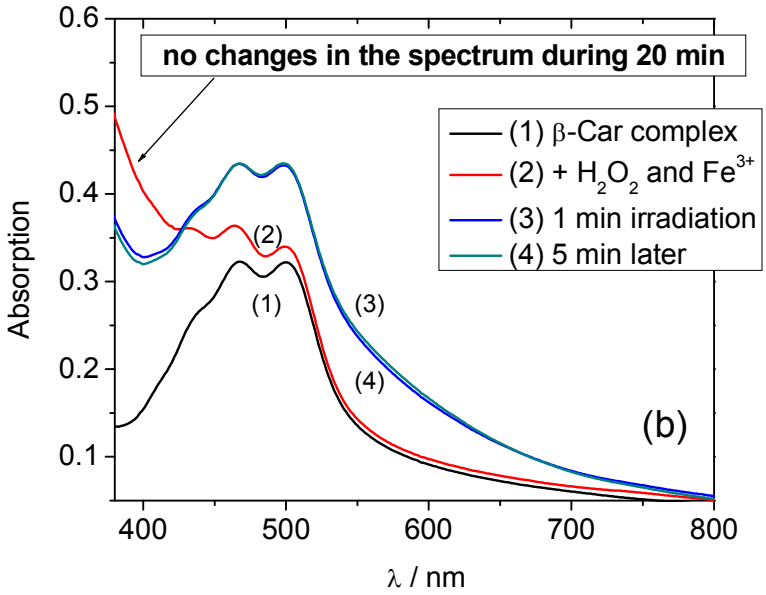


Figure 30. *Absorption spectra of β-carotene in the presence of Fe(III):cit (0.5 mM) and H_2O_2 (0.25 M) in AG complex in 30% ethanol solution. The observed increase in optical density after irradiation of the sample is due to formation of Fe^{4+} (adopted from [82], Fig.6).*

A decrease in absorption at 300-450 nm and an increase of absorption at 450-700 nm after irradiation is due to formation of Fe^{4+} from Fe^{3+} during the reaction (see Fig. 28). Similar effect of increasing stability was also observed for the other two carotenoids, **III** and **IV**. Figures 31a and 31b show the difference in decay rates for carotenoid **III** and its AG complex respectively in thr presence of the photo-Fenton reaction. The estimated ratio of decay rates equals 20 for this system. Ethanol was chosen as a solvent for the homogeneous reaction to decrease the contribution of the carotenoid – Fe^{3+} interaction to the decay rate.

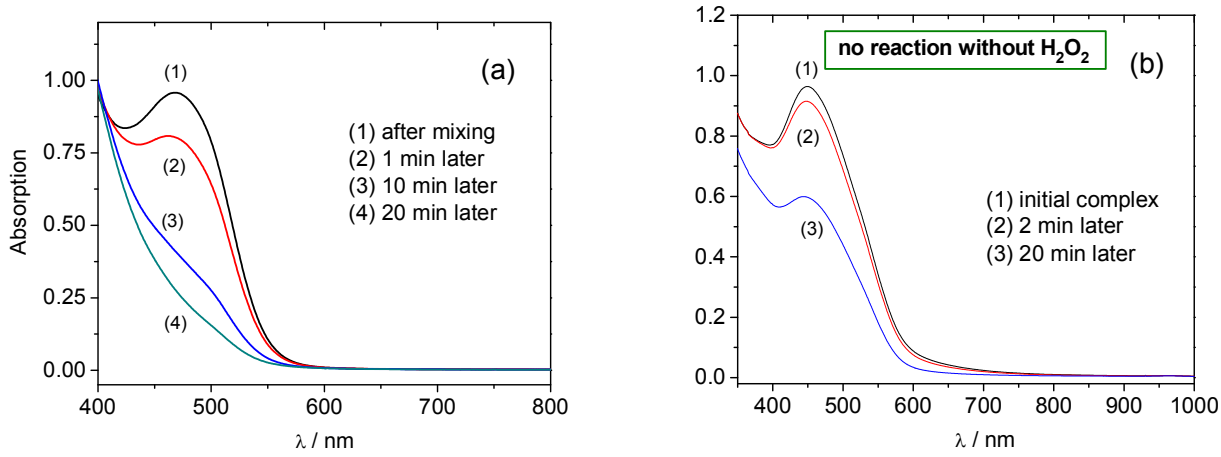


Figure 31*. Absorption spectra of Canthaxanthin in the presence of Fe(II):cit (0.5 mM) and H_2O_2 (0.25 M) (a) in ethanol; (b) in AG complex in water solution (adopted from [82], Fig.7).*

It is proposed that the stability of the carotenoids incorporated into the AG macromolecule might have wide practical application. A decrease in reaction rate towards free radicals does not mean a decrease in antioxidant activity of a complex in living systems since polysaccharides are easily assimilated by living media.

Photo-induced electron transfer from carotenoid in the solid state [82]. The next study was devoted to the electron transfer processes from carotenoids to acceptors in the solid state. The main question of interest concerns the properties of the carotenoid radical cations in the AG complex in the solid state. The properties of carotenoid radical cations in organized media have been intensively investigated [91-

93] because of the importance of carotenoids in photosynthesis and their possible use in artificial solar cells. In this study we have succeeded in detecting the canthaxanthin radical cation incorporated in a AG macromolecule during photoirradiation on the surface of TiO_2 nanoparticles. Among the semiconductors, titanium dioxide is the most suitable for many environmental applications. Due to its ability to absorb light, TiO_2 is widely used in photocatalysis and in artificial solar cells [94-98]. Figure 32 provides the schematic illustrations of the photocatalytic reactions of Carotenoid (Car) adsorbed on the surfaces of TiO_2 nanoparticles.

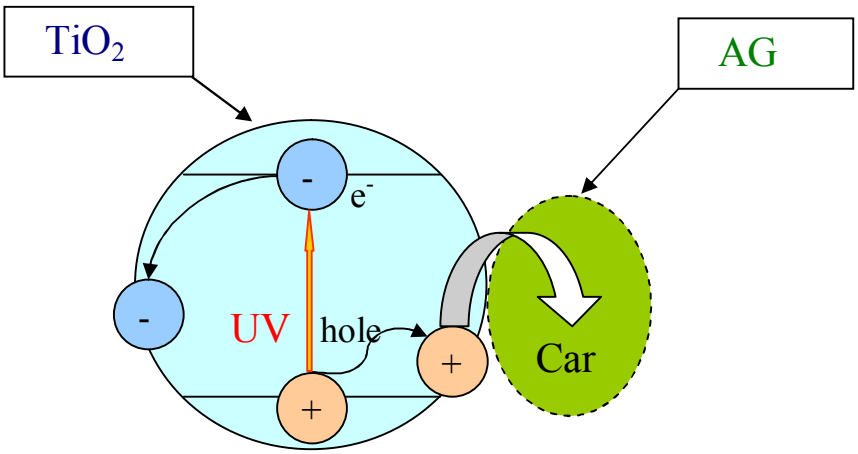


Figure 32. *Schematic illustrations of the photocatalytic processes on the surfaces of the TiO_2 nanoparticles in the presence of carotenoid (Car) (adopted from [82], Fig.8).*

Irradiation of TiO_2 nanoparticles (T = 77° K, $\lambda > 350$ nm) in the absence of carotenoid results in the appearance of an EPR signal which we attributed to Ti^{3+} (Fig. 33a). Irradiation of pure canthaxanthin at the same conditions (without AG) adsorbed on the TiO_2 surface shows only a weak signal with g = 2.0028 ± 0.0002 and ΔH_{pp} = 13.0 ± 0.5 G, which is characteristic of carotenoid radical cations [99-101] (Fig. 33b). In contrast, carotenoid-AG complex irradiated on TiO_2 shows a significant increase in the intensity of EPR signal (Fig. 33c) compared to that of the pure carotenoid.

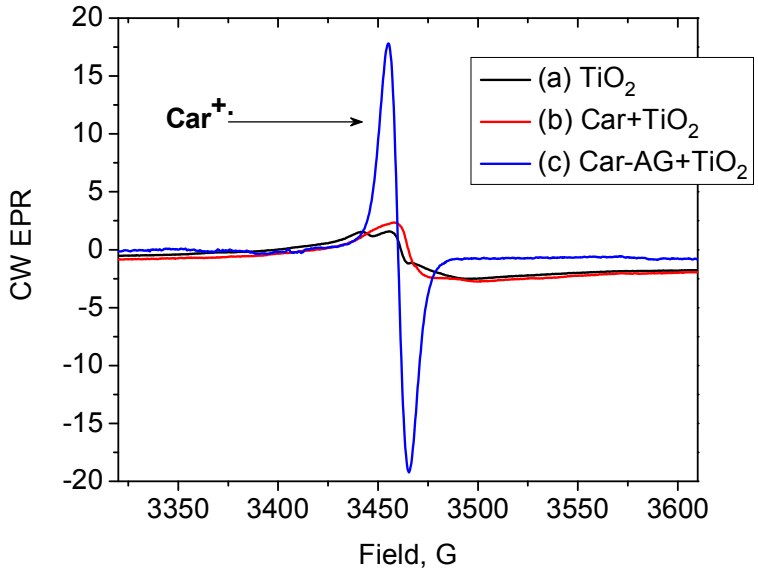


Figure 33. *EPR spectra detected after irradiation of TiO_2 powder (a); TiO_2 in the presence of canthaxanthin (b); and TiO_2 in the presence of canthaxanthin-AG complex in solid state at 20 K (c); (adopted from [82], Fig.9).*

It is suggested that the low yield of the charge separated state in the absence of AG might be due to efficient back electron transfer on semiconductor materials. The "redox cycling", where a product of the hole transfer acts, in turn, as scavenger for the photogenerated electrons, appears as a frequent cause of weak photocurrents [102,103]. The isolation of the carotenoid radical cation from the TiO_2 surface by incorporation into the polysaccharide matrix allows more efficient charge separation, reducing the rate of back electron transfer. A number of authors used the same approach for design of the "donor-bridge-acceptor" molecular triads as a model of the light harvesting complex [104-106]. In these studies porphyrin was chosen as the initial electron donor, and quinone (or fullerene) as an electron acceptor. Initial charge separation in such triads was accomplished by photoinduced electron transfer from the excited porphyrin to the attached quinone to generate the charge-separated species $CP^{\cdot+}\ Q^{\cdot-}$. In a second step, an electron is donated by the attached carotenoid moiety to the porphyrin to form the species $C^{\cdot+}P\ Q^{\cdot-}$. This species lives long enough to transfer an electron to a freely diffusing secondary quinone which act as a proton shuttle [104, 105]. Apparently, such a way of light energy transformation is similar to the mechanism used by plants for utilisation of solar energy in photosynthesis. The life time of the model molecular triads has an order of tens nanoseconds, and is mainly restricted by back electron transfer reaction [106]. Previous studies on the use of a carotenoid-sensitized TiO_2 nanocrystalline mesoporous electrode in the preparation of a photovoltaic cell,

indicated that the cell efficiency also was partially limited by the recombination of the injected electron with the oxidized form of the carotenoid radical cation. The current results suggest the use of the polysaccharide complex in the preparation of a more efficient cell. Stabilization of the carotenoid radical cation would reduce the recombination rate. Simultaneously AG matrix provides the defense of the carotenoid from degradation decay.

According to earlier published results [107], carotenoid radical cations generated on the surface of TiO_2 are stable at 77° K, but disappear when the temperature is increased above 250° K. The most important design feature of the carotenoid-AG complex is the significant increase in stability of the carotenoid radical cation. Increasing the temperature up to room temperature does not lead to disappearance of the spectrum (Figure 34).

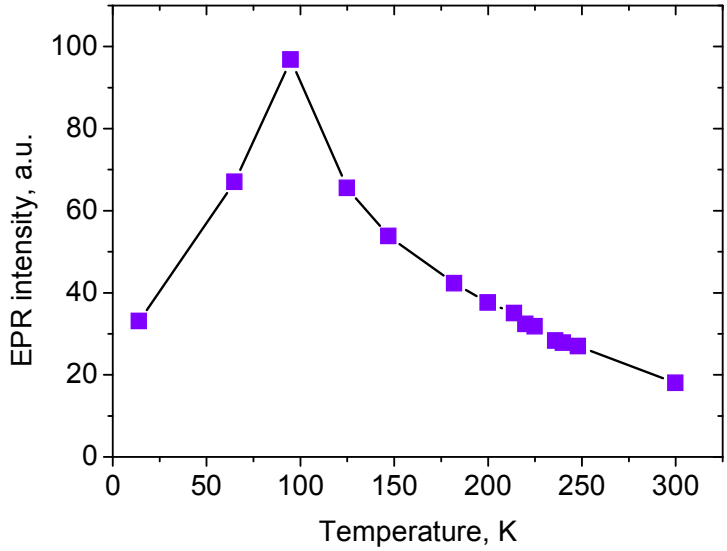


Figure 34. *Temperature dependence of EPR intensity of Canthaxanthin radical cation in solid state complex of arabinogalactan on TiO_2 surface.*

The estimated life time of the canthaxanthin radical cation in the solid state complex of arabinogalactan on TiO_2 surface is approximately 10 days at room temperature. The increase in lifetime of such molecular devices opens up wide possibilities for their use in molecular electronics as the nanosized means of communication and data processing as well as in sensors.

CONCLUSION

In conclusion we can summarize that incorporation of carotenoids into the oligosaccharide or polysaccharide macromolecules result in significant change in their physical and chemical properties. This result opens new possibilities to control the reactivity of carotenoids in living systems as well as for wide practical application in various fields. In addition to cyclodextrins inclusion complexes which are already used in pharmacology, cosmetics and food industry, we propose two new compounds, glycyrrhizin and arabinogalactan which form more stable complexes with carotenoids and demonstrate some unique properties useful for many applications. In particular, complexes of glycyrrhizin with some carotenoids show enhanced ability to scavenge free radicals. In practice it means a significant reduction in the required dosage of antioxidants in medical preparations and enriched drinks. Complexes with arabinogalactan are unique water soluble compositions which provide enhanced stability of carotenoids at room temperature in the presence of sunlight and water, and significantly reduce their interaction with different additives including metal ions.

Another important field of carotenoid application is photosynthesis and artificial solar cells. The increased stability of carotenoid radical cations in solution (in GA complex) and in the solid state (in AG complex) at room temperature may results in new discoveries in design of artificial light-harvesting, photoredox and catalytic systems.

It was demonstrated that the mechanochemical method of solid state complex preparation has significant advantages as compared with traditional techniques. Primarily, the interest in solvent-free conditions stems from the possibility of obtaining the same product as that from solution *without solvent* because the process is cheaper, less time consuming and often more environmentally friendly. In the case of carotenoid chemistry the solvent-free conditions open the possibility of obtaining products not otherwise accessible from solvents.

Finally, it is worth noting that the creation of novel, more effective compositions based on the complexes of tested medicines and vitamins with natural compounds is now an intensively developed field of medicinal chemistry, cosmetology, and food industry. Fundamental studies substantially lag behind the nature of the complexation and physicochemical properties of complexes. At the same time, these studies are highly important from the point of view of their predictive potential. In medicine, screening of new drugs is, as a rule, performed using animals. Therefore, the possibility to control the reactivity of chemical compounds by complexation and equally important, to predict the range of either an increase or decrease in therapeutic activity, would allow one to substantially reduce the number of *in vivo* experiments.

REFERENCES

1. Jørgensen, K.; Skibsted, L. H. *Z.* Lebensm. Unters. Forsch. 1993, 196, 423-429.
2. Edge, R.; McGarvey, D. J.; Truscott, T. G. J. Photochem. Photobiol., B: Biol. 1997, 41, 189-200.
3. Woodall, A. A.; Lee, S. W.-M.; Weesie, R. J.; Jackson, M. J.; Britton, G. Biochim. Biophys. Acta 1997, 1336, 33-42.
4. Al-Agamey, A.; Lowe, G. M.; McGarvey, D. J.; Mortensen, A.; Phillip, D. M.; Truscott, T. G.; Young, A.J. Arch. Biochim. Biophys. 2004, 430, 37-48.
5. Palozza, P.; Calviello, G.; Serini, S.; Maggiano, N.; Lanza, P.; Ranelletti, F. O.; Bartoli, G. M. Free Rad. Biol. Med. 2001, 30, 1000-1007.
6. Hill, T. J.; Land, E. J.; McGarvey, D. J.; Schalch, W.; Tinkler, J. H.; Truscott, T. G. J. Am. Chem. Soc. 1995, 117, 8322-8326.
7. Polyakov, N.E.; Kruppa, A. I.; Leshina, T. V.; Konovalova, T. A.; Kispert, L. D. Free Rad. Biol. Med. 2001, 31, 43-52.
8. Cross, C. E.; Halliwell, B.; Borish, E.T. Annals Int. Med. 1987, 107, 526-545.
9. Halliwell, B. Nutr. Rev. 1997, 55, 44–52.
10. Loftsson, T.; Brewster, M. E. J. Pharm. Sci. 1996, 85, 1017–1025.
11. Buschmann, H.-J.; Schollmayer, E. J. Cosmet. Sci. 2002, 53, 185–191.
12. Szejtli, J. Cyclodextrin technology; Kluwer Academic: Dordrecht, 1988.
13. Hasebe, K.; Ando, Y.; Chikamatsu, Y.; Hayashi, K. Patent JP 62267261, 1987.
14. Murao, T.; Maruyama, T.; Yamamoto, Y. Patent JP 04244059, 1992.
15. Schwartz, J. L.; Shklar, G.; Sikorski, C. Patent WO 9513047, 1995.
16. Mele, A.; Mendichi, R.; Selva, A. Carbohydr. Res. 1998, 310, 261-267.
17. Mele, A.; Mendichi, R.; Selva, A.; Molnar, P.; Toth, G. Carbohydr. Res. 2002, 337, 1129-1136.
18. Szente, L.; Mikuni, K.; Hashimoto, H.; Szejtli, J. J. Inclusion Phenom. Mol. Recognit. Chem. 1998, 32, 81–89.
19. Lancrajan, I.; Diehl, H. A.; Socaciu, C.; Engelke, M.; Zorn-Kruppa, M. Chem. Phys. Lipids 2001, 112, 1–10.
20. Polyakov, N.E.; Leshina, T.V.; Konovalova, T.A.; Hand, E. O.; Kispert, L.D. Free Rad. Biol. Med. 2004, 36, 872-880.
21. Lockwood, S.F.; O'Malley, S.; Mosher G.L. J. Pharm. Sci. 2003, 92, 922-926.
22. Sangalov, E. Yu. Russ. J. Gen. Chem. (Engl. Transl.) 1999, 64, 641-644.

23. Gusakov, V. N.; Maistrenko, V. N.; Safiullin, P. P. Russ. J. Gen. Chem. (Engl. Transl.) 2001, 71, 1307-1316.

24. Saito, S.; Furumoto, T.; Ochiai, M.; Hosono, A.; Hoshino, H.; Haraguchi, U.; Ikeda, R.; Shimada, N. Eur. J. Med. Chem. 1996, 31, 365-381.

25. Maistrenko, V. N.; Gusakov, V. N.; Rusakov, I. A.; Murinov, Yu. I.; Tolstikov, G. A. Dokl. Akad. Nauk 1994, 335, 329-331.

26. Polyakov, N. E.; Khan, V. K.; Taraban, M. B.; Leshina, T. V.; Salakhutdinov, N. F.; Tolstikov, G. A. J. Phys. Chem. B. 2005, 109, 24526-24530.

27. Connors, K. A. Chem. Rev. 1996, 97, 1325-1357.

28. Kondratenko, R. M.; Baltina, L. A.; Mustafina, S. R.; Ismagilova, A. F.; Zarudii, F. S.; Davydova, V. A.; Basekin, G. V.; Suleimanova, G. F.; Tolstikov, G. A. Pharm. Chem. J. 2003, 37, 485-488.

29. Tolstikova, T. G.; Bryzgalov, A. O.; Sorokina, I. V.; Hvostov, M. V.; Ratushnyak, A. S.; Zapara, T. A.; Simonova, O. G. Lett. Drug Design Discovery 2007, 4, 168-170.

30. Sorokina I. V.; Tolstikova T. G.; Dolgikh M. P.; Shul'ts E. E.; Dushkin A. V.; Karnatovskaya L. M.; Chabueva E. N.; Boldyrev V. V. Pharm. Chem. J. 2002, 36, 11-13.

31. Odonmazig, P.; Ebringerova, A.; Machova, E.; Alfoldi, J. Carbohydr. Res. 1994, 252, 317-324.
32. D'Adamo, P. J. Naturopath. Med. 1996, 6, 33-37.
33. Fortier, N. E. US Patent 5532009, 1996.

34. Polyakov, N. E.; Konovalov, V. V.; Leshina, T. V.; Luzina, O. A.; Salakhutdinov, N. F.; Konovalova, T. A.; Kispert, L. D. J. Photochem. Photobiol., A 2001, 141, 117-126.

35. Gao, Y.; Kispert, L. D. J. Phys. Chem. B 2003, 107, 5333-5338.

36. Polyakov, N. E.; Leshina, T. V.; Petrenko, A.; Hand, E.; Kispert, L. D. J. Photochem. Photobiol. A: Chem. 2004, 161, 261–267.

37. Munoz-Botella, S.; Martin, M. A.; del Castillo, B.; Lerner, D. A.; Menendez, J. C. Anal. Chim. Acta 2002, 468, 161–170.

38. Higuchi, T.; Connors, K. A. Adv. Anal. Chem. Instrum. 1965, 4, 117–212.

39. Redenti, E.; Szente, L.; Szejtli, J. J. Pharm. Sci. 2001, 90, 979–986.

40. Szejtli, J.; Osa, T. In Comprehensive Supramolecular Chemistry; Atwood, J.L.; Davies, J.E.D.; MacNicol, D.D.; Vögtle, F.; Ed.; Elsevier Sci. Ltd.: Oxford, UK, 1996. Vol. 3, pp 5-41.

41. Yamamoto, Y.; Inoue, Y. J. Carbohydr. Chem. 1989, 8, 29–46.

42. Greatbanks, D.; Pickford, R. Magn. Res. Chem. 1987, 25, 208–215.

43. Casy, A. R.; Cooper, A. D.; Jefferies, T. M.; Gaskell, R. M.; Greatbanks, D.; Pickford, R. J. Pharm. Biomed. Anal. 1991, 9, 787–792.

44. Schneider, H.-J.; Hacket, F.; Rudiger, V. Chem. Rev. 1998, 98, 1755–1785.

45. Cabrer, P. R.; Alvares-Parrilla, E.; Meijide, F.; Seijas, J. A.; Nunez, E. R.; Tato, J. V. Langmuir. 1999, 15, 5489–5495.

46. Matsui, Y.; Tokunada, S. Bull. Chem. Soc. Jpn. 1996, 69, 2477–2480.

47. Lai, C.; Piette, L. H. Tetrahedron Lett. 1979, 9, 775–778.

48. Saprin, A. N.; Piette, L. H. Arch. Biochem. Biophys. 1977, 180, 480–492.

49. Walling, C. Acc. Chem. Res. 1998, 31, 55–157.

50. Buetter, G. R. In Handbook of methods of oxygen radical research; Greenwald, R. A.; Ed.; CRC Press: Boca Raton, FL, 1986, pp 151–155.

51. Dicalov, S. I.; Mason, R. P. Free Radic. Biol. Med. 1999, 27, 864–872.

52. Buettner, G. R. Free Radic. Biol. Med. 1987, 3, 259–303.

53. Harbour, J. R.; Chow, V.; Bolton, J. R. Can. J. Chem. 1974, 52, 3549–3554.

54. Yoshimura, Y.; Inomata, T.; Nakazawa, H. J. Liq. Chromatogr. Rel. Technol. 1999, 22, 419–428.

55. Polyakov, N. E.; Leshina, T. V.; Salakhutdinov, N. F.; Konovalova, T.A.; Kispert, L. D. Free Rad. Biol. Med. 2006, 40, 1804–1809.

56. Welch, K. D.; Davis, T. Z.; Van Eden, M. E.; Aust, S. D. Free Rad. Biol. Med. 2001, 32, 577–583.

57. Barbouti, A.; Doulias, P.-T.; Zhu, B.-Z.; Frei, B.; Galaris, D. Free Rad. Biol. Med. 2002, 32, 93–101.

58. Polyakov, N. E.; Leshina, T. V.; Konovalova, T. A.; Kispert, L. D. Free Rad. Biol. Med. 2001, 31, 398–404.

59. Konovalova, T. A.; Gao, Y.; Kispert, L. D.; van Tol, J.; Brunel, L.-C. J. Phys. Chem. B 2003, 107, 1006–1011.

60. Konovalova, T. A.; Gao, Y.; Schad, R.; Kispert, L. D. J. Phys. Chem. B 2001, 105, 7459–7464.

61. Gao, Y.; Konovalova, T. A.; Xu, T.; Kispert, L. D. J. Phys. Chem. B 2002, 106, 10808–10815.

62. Gao, Y.; Konovalova, T. A.; Lawrence, J. N.; Smitha, M. A.; Nunley, J.; Schad, R.; Kispert, L. D. J. Phys. Chem. B 2003, 107, 2459–2465.

63. Persson, H. L.; Yu, Z.; Tirosh, O.; Eaton, J. W.; Brunk, U. T. Free Rad. Biol. Med. 2003, 34, 1295–1305.

64. Suh, J.; Zhu, B.-Z.; Frei, B. Free Rad. Biol. Med. 2003, 34, 1306–1314.

65. Celander, D. W.; Cech, T. R. Biochem. 1990, 29, 1355–1361.

66. Li, S.; Nguyen, T. H.; Schoneich, C.; Borchardt, R. T. Biochem. 1995, 34, 5762–5772.

67. Platis, I. E.; Ermacora, M. R.; Fox, R. O. Biochem. 1993, 32, 12761–12767.

68. Rana, T. M.; Meares, C. F. J. Am. Chem. Soc. 1990, 112, 2457–2458.

69. Francz, P. I.; Biesalski, H. K.; Pfitzner, I. Biochim. Biophys. Acta 2000, 1474, 163–168.

70. Offord, E. A.; Gautier, J.-C.; Avanti, O.; Scaletta, C.; Runge, F.; Kramer, K.; Applegate, L. A. Free Rad. Biol. Med. 2002, 32, 1293–1303.

71. Polyakov, N. E.; Leshina, T. V.; Salakhutdinov, N. F.; Kispert, L. D. J. Phys. Chem. B. 2006, 110, 6991-6998.

72. Martı´n, L.; Leo´n, A.; Olives, A. I.; del Castillo, B.; Martı´n, M. A. Talanta 2003, 60, 493-503.

73. Lopez, E. A.; Bosque-Sendra, J. M.; Rodriguez, L. C.; Campana, A. M. G.; Aaron, J. J. Anal. Bioanal. Chem. 2003, 375, 414-423.

74. Rekharsky, M. V.; Inoue, Y. Chem. Rev. 1998, 98, 1875-1917.

75. Maafi, M.; Laassis, B.; Aaron, J. J.; Mahedero, M. C.; Munoz de la Pena, A.; Salinas, F. J. Inclusion Phenom. Mol. Recogn. 1995, 22, 235-247.

76. Kornievskaya, V. S.; Kruppa, A. I.; Polyakov, N. E.; Leshina T. V. J. Phys. Chem. B 2007, 111, 11447-11452.

77. Polyakov, N. E.; Khan, V. K.; Taraban, M. B.; Leshina, T. V. J. Phys. Chem. B 2008, 112, 4435-4440.

78. Liu, D.; Kispert, L. D. In Recent research developments in electrochemistry; Pandalai, S. G.; Ed.; Transworld Research Network: Trivandrum, India, 1999, pp 139–157.

79. Lawlor, D. W. Photosynthesis: Metabolism, Control and Physiology; Wiley: New York, 1987.

80. Kohl, D. H. In Biological Applications of Electron Spin Resonance; Swartz, H. M.; Bolton, J. R.; Berg D. C.; Eds.; Wiley: New York, 1972.

81. Kispert, L.D.; Konovalova, T.A.; Gao, Y. Arch. Biochim. Biophys. 2004, 430, 49-60.

82. Polyakov, N. E.; Leshina, T. V.; Meteleva, E. S., Dushkin, A. V.; Konovalova, T. A.; Kispert, L. D. J. Phys. Chem. B, 2008, in press.

83. Babkin, V.A.; Kolzunova, L.G.; Medvedeva, E.N.; Malkov, Yu.A.; Ostroukhova L.A. Russian Patent 2256668, 2005.

84. Tonnesen, H.H.; Ed.; Photostability Of Drugs And Drug Formulations; CRC Press: Orlando, FL, 2004.

85. Wu, Y.; Piekara-Sady, L.; Kispert, L. D. Chem. Phys. Lett. 1991, 180, 573-577.

86. Piekara-Sady, L.; Khaled, M. M.; Bradford, E.; Kispert, L. D.; Plato, M. Chem. Phys. Lett. 1991, 186, 143-148.

87. Gao, Y.; Webb, S.; Kispert, L.D. J. Phys. Chem. B. 2003, 107, 13237-13240.

88. Liu, D.; Gao, Y.; Kispert, L.D. J. Electroanal.Chem. 2000, 488, 140-150.

89. Silva, M.R.A.; Trovó, A.G.; Nogueira, R.F.P. J. Photochem. Photobiol., A: Chem. 2007, 191, 187-192.

90. Bacardit, J.; Stötzner, J. ; Chamarro, E.; Esplugas S. Ind. Eng. Chem. Res., 2007, 46, 7615-7619.

91. Konovalova, T. A.; Krzystek, J.; Bratt, P. J.; van Tol, J.; Brunel, L.-C.; Kispert, L. D. J. Phys. Chem. B 1999, 103, 5782-5786.

92. Konovalova, T. A.; Dikanov, S. A.; Bowman, M. K.; Kispert, L. D. J. Phys. Chem. B 2001, 105, 8361-8368.

93. Focsan A.L.; Bowman, M.K.; Konovalova, T.A.; Molnár, P.; Deli, J.; Dixon, D.A.; Kispert L.D. J. Phys. Chem. B 2008, 112, 1806-1819.

94. Hoffmann, M. R.; Martin, S. T.; Choi, W.; Bahnemann, D. W. Chem. Rev. 1995, 95, 69-96.

95. Chen, L. X.; Rajh, T.; Wang, Z.; Thurnauer, M. C. J. Phys. Chem. B 1997, 101, 10688-10697.

96. Mills, A.; Hunte, S. L. J. Photochem. Photobiol. A 1997, 108, 1-35.

97. Hagfeld, A.; Grätzel, M. Chem. Rev. 1995, 95, 49-68.

98. Bard, A. J. J. Phys. Chem. 1982, 86, 172-177.

99. Jeevarajan, A. S.; Kispert, L. D.; Piekara-Sady, L. Chem. Phys. Lett. 1993, 209, 269-274.

100. Konovalova, T. A.;. Kispert, L. D.; Konovalov, V. V. J. Phys. Chem. B 1997, 101, 7858-7862.

101. Konovalova, T. A.; Kispert, L. D. J. Chem. Soc., Faraday Trans. 1998, 94, 1465-1468.

102. Solarska, R.; Rutkowska, I.; Morand, R.; Augustynski, J. Electrochim. Acta, 2006, 51, 2230-2236.

103. Nakade, S.; Saito, Y.; Kubo, W.; Kanzaki, T.; Kitamura, T.; Wada, Y.; Yanagida, S. Electrochem. Commun. 2003, 5, 804-808.

104. Kuciauskas, D.; Liddell, P.A.; Hung, S.-C.; Lin, S.; Stone, S.; Seely, G.R.; Moore, A.L.; Moore, T.A.; Gust D. J. Phys. Chem. B, 1997, 101, 429-440.

105. Liddell, P.A.; Kuciauskas, D.; Simuda, J.P.; Nash, B.; Nguyen, D.; Moore, A.L.; Moore, T.A.; Gust D. J. Am. Chem. Soc. 1997, 119, 1400-1405.

106. Kodis, G.; Liddell, P. A.; Moore, A. L.; Moore, T. A.; Gust, D. J. Phys. Org. Chem. 2004, 17, 724-734.
107. Konovalova T.A.; Kispert L.D. J. Phys. Chem. B, 1999, 103, 4672-4677.