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Carotenoid:β-cyclodextrin stability is independent of pigment structure.

Elisabet Fernández-García[a,b,*] and Antonio Pérez-Gálvez[a]

[a]Food Phytochemistry Department, Instituto de la Grasa (CSIC), Campus Universitario Pablo de Olavide – Ctra. de Utrera, km 1, Edificio 46, 41013 Sevilla, Spain.

[b]Andalusian Center of Molecular Biology and Regenerative Medicine (CABIMER), University of Seville, Avd. Americo Vespucio s/n, 41092 Sevilla, Spain.

*Corresponding author:

Telf: +34 954 467 789

Fax: +34 954 461 664

Email address: efernandez@cica.es; elisabet.fernandez@cabimer.es

ABSTRACT

Carotenoids refer to a wide class of lipophilic pigments synthesized by plants. Some carotenoids exert photoprotective and antioxidant properties that are lost upon carotenoid degradation. Thus, the inclusion of carotenoids into hydrophilic host-molecules could improve their stability. Cyclodextrins (CDs) provide a hydrophobic cavity in the core of their structure while the outer configuration is suitable with aqueous environments. Carotenoids can accommodate into the hydrophobic core of CDs and therefore they are protected from exogenous stress. Literature reports that carotenoid structure could modulated stability of the complexes, however no conclusions can be drawn as the studies performed so far were not completely analogous. We describe the synthesis of several carotenoids/β-CDs inclusion complexes and provide experimental evidences that β-CDs inclusion renders these compounds more stability towards the oxidizing agents 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH) and hydrogen peroxide (H_2O_2). Esterified carotenoids were also used in this work to screen the influence of this particular structural configuration of xanthophylls against oxidation.

KEYWORDS

Carotenoid, cyclodextrin, stability, oxidant agents

1. Introduction

Carotenoids are a group of bioactive compounds that have appealed interest of food industry because of their positive impacts on human health. Beneficial activities include their potential to function as vitamin A precursors (mainly β-carotene and β-cryptoxanthin), to display antioxidant activities, and to enhance the immune system (Stahl & Sies, 2005). Mammals rely on diet to incorporate these compounds, but only ca. 10% of the ingested carotenoids are assimilated by the body (Boileau, Moore & Erdman, 1999). To improve cellular carotenoid uptake, it is conceivable that the inclusion of carotenoids in processed foods or new food matrices could increase carotenoid bioavailability. Alternative food formulations based on hydrophilic matrices improve solubility of hydrophobic compounds through dispersion, emulsion or encapsulation (Pérez-Gálvez & Mínguez-Mosquera, 2004). Thus, such hydrophilic matrices could provide an approach to improve cellular uptake of hydrophobic carotenoids.

Cyclodextrins (CDs) are cyclic oligomaltosaccharides obtained from enzymatic digestion of starch. The particular structure of cyclodextrin has led to a wide range of applications in several fields such as agriculture, analytical chemistry, food and pharmaceutical industry (Singh, Sharma & Banerjee, 2002). Within the array group of CDs (α-, β-, γ-, and δ-CDs), β-CD is the most commonly used form in pharmaceuticals and its behavior has been frequently studied in humans (Martin del Valle, 2004). The most important property of CDs is their ability to increase assimilation of hydrophobic drugs by acting as transporters to carry them properly into cellular membranes of different tissues (skin, mucosal membrane, cornea…) and by increasing the availability of the complexed drug (Rajewski & Stella, 1996).

Carotenoids are compounds extremely sensitive to light, temperature and other oxidizing agents such as reactive oxygen species (ROS). Their degradation involves the loss of their properties, so that the application of strategies to preserve their stability is frequently required. Furthermore, the hydrophobic character of the carotenoids may be limiting. A hydrophilic matrix requires the use of pigment in emulsion or encapsulation. The combination of carotenoids with CDs to form inclusion complexes can solve deficiencies in carotenoid stability while allowing incorporation into aqueous solutions as well. In fact, there are already some patent applications in the pharmaceutical, food and cosmetic industries related to inclusion complexes between CDs and carotenoids (Schwartz, Shklar & Sikorski, 1995). Several studies have observed an increase of carotenoid stability when they are incorporated into CDs, an outcome with relevance to ensure the effectiveness of the several of the carotenoid functions mentioned above. Lyng, Passos & Fontana (2005) analyzed bixin:α-CD stability against oxidizing agents such as air, ozone, heat and light. The results showed that the inclusion complex is more stable than free bixin in an oxidative environment. The carotenoid inclusion into CDs also protects the pigments from breakdown caused for temperatures not exceeding 50ºC. Chen, Chen, Guo, Li & Li (2007) have demonstrated the increase of astaxanthin and lycopene stability with β-cyclodextrin against physical and chemical oxidizing agents. Thus, experimental evidence indicates that the carotenoid stability increases when they are complexed with CDs considering that CDs protect those areas of the carotenoid structure more sensitive to the oxidation. Indeed, some reports indicate that carotenoid structure is a modulating factor of carotenoid stability into the complexes(CITAS), however no conclusions can be drawn as the studies performed until now were not directly comparable.

The aim of this work was to synthesize carotenoids/β-CD inclusion and analyze their stability against the oxidizing agents 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH) and hydrogen peroxide (H_20_2). Different dietary carotenoids were selected for the study: lycopene, lutein, capsanthin and capsorubin, so that the influence of the structure in the chemical stability could be drawn. Moreover, the esterified forms of the xanthophylls lutein, capsanthin and capsorubin were also included in the study to analyze the influence of this particular structural configuration in carotenoid/CD oxidation.

2. Experimental

2.1. Raw materials

Tomato oleoresin was supplied by LycoRed (Beer-Sheva, Israel). Marigold oleoresin was provided by Kemin Foods (Des Moines, Iowa) and paprika oleoresin were kindly supplied by EVESA (La Línea de la Concepción, Spain).

2.2. Chemicals and reagents

High-performance liquid chromatography (HPLC) grade acetone, metanol, hexane and methylene chloride were supplied by Teknokroma (Barcelona, Spain). 2, 2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH), hydrogen peroxide (H_20_2) and β-cyclodextrin (98% pure) were provided by Sigma (St. Louis, MO).

2.3 Carotenoid stock solutions

The method used to isolate and purify lycopene, lutein and its esters, capsanthin and its esters, and capsorubin and its esters carotenoids from the corresponding oleoresins was the same as described by Fernández-García, Carvajal-Lérida, Rincón, Ríos & Pérez-Gálvez (2010).

Lutein, capsanthin and capsorubin esters: 0.05 g of marigold (for lutein esters), and paprika (for capsanthin and capsorubin esters) oleoresins were dissolved in 25 mL of

acetone. The isolation of the free and ester forms was accomplished by thin layer chromatography on silicagel $60GF_{254}$ using the developing mixture hexane/ethyl acetate/ethanol/acetone (95:3:2:2). Bands for esterified lutein, capsanthin and capsorubin were scraped off, eluted in acetone (lutein) or benzene (capsanthin and capsorubin). Once separated and purified carotenoids, their concentration was determined. The ester lutein concentration in the solution was measured at λ_{max} = 446 nm with an extinction coefficient of $E_{1cm}^{1\%}$ = 2340. The capsanthin and capsorubin esters concentration were determined spectrophotometrically at λ_{max} = 483 and 489 nm with an extinction coefficient of $E_{1cm}^{1\%}$ = 2072 and 2200, respectively (Davies, 1976).

Lycopene, and free lutein, capsanthin and capsorubin : 0.02 g of tomato oleoresin (for lycopene) or an aliquot of esterified lutein, capsanthin or capsorubin (0.05 g) were dissolved in 25 mL of ethyl ether. The dilutions were placed in a separating funnel, and 10 mL of KOH in methanol (10%, w/v). The saponification reaction lasted 1h to eliminate the oily matter present in the oleoresins or the fatty acids bounded to the xanthophylls. Once reactions were completed, 200 mL of aqueous NaCl solution (10%, w/v) was added. The organic and aqueous phases were allowed to separated, and the aqueous layer discarded. The organic phase was washed with distilled water until neutral pH, and then washed twice with 200 mL of aqueous Na_2SO_4 solution (2%, w/v). The organic phase was filtered through a solid bed of Na_2SO_4 and then evaporated in a vacuum rotatory evaporator. The residue was dissolved in 25 mL of light petroleum ether. Lycopene concentration in the solution was measured at λ_{max} = 470 nm with an extinction coefficient of $E_{1cm}^{1\%}$ = 3450 (Davies, 1976). Lutein, capsanthin and capsorubin concentrations were determined as mentioned above.

2.4. Preparation of carotenoid/β-cyclodextrin inclusion complexes

To obtain the carotenoid/β-cyclodextrin inclusion complexes, a modified version of the methodology described by Pfitzner, Istvan, Francz & Biesalski, (2000) was applied. This procedure was also described by Fernández-García, Carvajal-Lérida, Rincón, Ríos & Pérez-Gálvez, (2010). First, an aliquot of the stock carotenoid solution was evaporated (400 µg), and the residue was dissolved in 2 mL of methylene chloride. Next, 48 mL of ethanol and β-cyclodextrin (1 g) were added. The molar ratio of β-cyclodextrin to the carotenoid was kept at 20. The resulting mixture was shaken at 36.7×g in a mixing/heating bath at 37 ºC for 24 h. Once the process was completed the solution was evaporated in a vacuum rotatory evaporator and dried using a stream of N_2 gas. The solid residue was homogenized and stored at -30 ºC until use. The final carotenoid concentration in the inclusion complex was analyzed using the following procedure: 50 mg of encapsulated carotenoid was dissolved in water (3 mL), and mixed with 8 mL of N, N-dimethylformamide/hexane (1:1, v/v). To promote phase separation, 5 mL of aqueous NaCl solution (10%, w/v) was added. The mixture was shaken for 1 min in a vortex mixer and then centrifuged at 1467×g for 5 min. The organic phase was placed in a test tube, and the remaining aqueous phase was extracted again with the same procedure. Both organic phases were combined and the solvent was evaporated. The residue was dissolved in the suitable solvent to analyze the concentration at the corresponding λ_{max} (see above).

2.5. Stability of carotenoid:β-CD inclusion complexes

Stability carotenoid:β-CD inclusion complexes was analyzed inducing the degradation of the complex through two oxidants agents; AAPH and H_2O_2. A solution of encapsulated carotenoid (2 µg carotenoid:β-CD/mL) was mixed with AAPH or H_20_2 (1.5 mM). The reaction was carried out in a test tube immersed in a thermostatic bath at 40 ºC and shaken at 36.7×g. Samples were drawn at 2 min. intervals for

157 spectrophotometric measurement of the absorbance until the reaction was considered
158 finished (absorbance value lower than 0.2 units).

159 **2.6. Kinetic study**

160 The kinetic parameters (rate constant and reaction order) were obtained from the
161 representation of carotenoid concentration vs. time using the integral method (Eq. 1). If
162 the reaction order is correct, the representation of concentration *vs.* time must result a
163 linear regression (Fogler, 1992).

164 $$-\frac{d(C_{car})}{dt} = k \times C_{car} \qquad (1)$$

165 **2.7. Computational methods**

166 Analysis of carotenoid:β-CD structures was performed with HyperNewton (Hyperchem
167 release 6.03) by the self-consistent field (SCF) method. Electronic charge densities as
168 well as final optimized structure were obtained by energy minimization of the optimized
169 structure with the HyperNDO (Hyperchem release 6.03) using the AMI Hamiltonian
170 (Dewar, Zoebisch, Healy & Stewart, 1985). Once the geometry was optimized, a
171 structure of the carotenoid:β-CD complex was built. In the case of lutein and capsanthin
172 the position of the different end groups (β- and ε-rings in the case of lutein, β- and κ-
173 rings in the case of capsanthin) with different alignments was also considered.
174 Afterwards, the energy of each inclusion complex was obtained. The difference between
175 the total energy of the complex (which is the sum of Van der Waals forces, electrostatic
176 interactions and hydrogen bonds) and the sum of the energies of the individual
177 components represents the gain in potential energy due to inclusion complex formation.

178 **2.8. Statistical Analysis**

179 Each one of the inclusion complexes synthesized (7 in total) were exposed to oxidative
180 degradation by AAPH or H_20_2. Experiments were done in quadruplicate, representing a
181 total of 56 experiments. The Scheffé test was applied for estimation of significant

differences between mean values of the rate constants for the samples, and differences between mean values of the rate constant for the groups of samples, so that homogenous sets can be established. (STATISTICA computer software (version 5.5 for Windows, 1999; Statsoft, Inc., Tulsa), and the level of statistical significance was set at $P<0.05$.

3. Results and Discussion

Figure 1 shows progress of the oxidation of carotenoid:β-CD complexes by AAPH or H_2O_2, considering pigment content decay with time. Several carotenoids were used in the study with different structural features: lycopene, and lutein, capsanthin, capsorubin and their esterified forms (see Figure 2). A continuous loss of carotenoid content *vs.* time was observed in all cases. Tables 1 and 2 show the values of reaction rate constant *k*, for each carotenoid:β-CD inclusion complex studied, including the values for the oxidation reaction promoted by AAPH (Table 1) or H_2O_2 (Table 2). In both cases, the first-order model fits with the experimental data. Higher reaction rate constant values were obtained for the reaction with AAPH. The higher constant rate value, the faster pigment degradation, and consequently an earlier loss in carotenoid concentration results from oxidation. Certainly, AAPH oxidation shows a wide range of constant reaction rates (2.08-11.47, minimum and maximum values) although only significant differences are observed for lutein ester:β-CD and capsanthin ester:β-CD that present the highest rate constant values (see Table 1), while oxidation with H_2O_2 follows closely related rate constant reaction values. Indeed, no significant differences were found when the oxidative reaction was promoted with H_2O_2 for the 7 carotenoid inclusion complexes, with range of rate constants between 2.10 and 3.38 (see Table 2).

Statistical analysis for significant differences of rate constant values between both oxidative process for each carotenoid: β-CD complex establishes that the oxidative process mediated by AAPH resulted in higher pigment degradation rate than H_2O_2 for

lutein:β-CD (k_{AAPH}=5.12×10^{-2} *vs.* k_{H2O2}=3.38×10^{-2}, P=0.024), lutein ester:β-CD (k_{AAPH}=11.47×10^{-2} *vs.* k_{H2O2}=2.32×10^{-2}, P=0.045) and capsanthin ester:β-CD (k_{AAPH}=8.07×10^{-2} *vs.* k_{H2O2}=2.10×10^{-2}, P=0.047), while no significant differences (P>0.05) are observed for rate constant values of the rest of carotenoid:β-CD complexes, between both oxidative agents. Therefore, pigment degradation follows the same trend independently of the oxidative agent that promotes degradation, and structural features of each pigment do not play a key role in the rate constant values, with the above-mentioned exceptions (β-CD inclusion complexes of lutein, lutein ester and capsanthin ester, for oxidative degradation promoted by AAPH).

One of the factors that may have promoted such exceptions is the polar/apolar characteristics of the end-groups located at both sides of the polyenoic chain, as they could affect insertion of the pigment into the β-CD. Menard, Dedhiya & Rhodes (1990) observed that incorporation of apolar compounds to β-CD is higher than polar ones, and the measurement of the increase on potential energy due to inclusion complex formation is a reference to establish such differences. Thus, lycopene with two ψ-type end groups and eight allylic hydrogens very prone to oxidation (see Figure 2), shows a 47.9 eV gain on potential energy, a lower value in comparison with the 279.3 eV increment for capsorubin:β-CD. However, no significant differences were observed in the rate constant values for both carotenoid:β-CD complexes (independently of the oxidative agent). Consequently, although the incorporation of lycopene to β-CD is most favored than in the case of capsorubin, considering their keto/hydroxylated κ-rings that may even suppose a structural hindrance for the inclusion of capsorubin, this is not a significant constraint for subsequent stability of the molecule once the complex has been obtained, as capsorubin:β-CD complex is stable as much as lycopene:β-CD. Capsanthin:β-CD complex behave in the same fashion as capsorubin:β-CD with

increments on potential energy from 90.4 eV (when the β-type ring enters in the core of β-CD) and 223 eV (when the κ-ring is complexed by β-CD). Finally, lutein:β-CD displays similar increases on potential energy (90.4 eV for the β-type ring inclusion, and 105.9 eV for the complex in the ε-ring). Consequently, although energy of the carotenoid:β-CD complex increases with the inclusion of polar cyclic groups of the carotenoids (particularly κ- and ε-ring) this factor does not promote oxidation of the complex.

As differences on rate constant values were specifically higher in the case of inclusion complexes with carotenoid esters (lutein and capsanthin), influence of fatty acids in the oxidative process promoted by AAPH was considered. Unsaturated fatty acids may promote an increase on the rate constant values following the lipid peroxidation chain reaction. However, nature of fatty acids participating in the esterification of lutein is mainly saturated. This was shown by Breithaupt, Wirt & Bamedi (2002), who determined that lutein is esterified by palmitic, myristic and stearic acid in marigold oleoresins. These authors also mention that paprika oleoresins follow the same behavior, and fatty acids bound to the characteristic *capsicum* xanthophylls (capsanthin and capsorubin) are also saturated ones. Thus, nature of esterification is not a source for rate constant increases of lutein ester:β-CD and capsanthin:β-CD complexes. Therefore, some other plausible explanation(s) may arise for the intriguing differences observed in this study.

The rate constant values for each inclusion complex in aqueous solution were lower than the results obtained for the carotenoids in organic solvent solution (Woodall, Lee, Weesie, Jackson & Britton, 1997; Pérez-Gálvez & Mínguez-Mosquera, 2002), indicating a higher stability of the carotenoids into the β-CD, independently of their structure. Consequently, inclusion complex generation is a convenient strategy not only

to allow solubility of carotenoid pigments in hydrophilic environments but also to increase their stability towards oxidative agents.

4. Conclusions

Carotenoids are sensitive to light, temperature and other oxidizing agents such as reactive oxygen species. Their application in food industry requires solutions to preserve carotenoid structure and function. Furthermore, the hydrophobic character of the carotenoids limits their inclusion in aqueous formulations, and also their subsequent absorption and thus the significance of their beneficial impacts on health. The assembly of hydrophilic carotenoid inclusion complexes into CDs could improve both carotenoid stability and absorption. We describe here the procedure for isolation of dietary carotenoids:β-CD inclusion complexes (lycopene, lutein, capsanthin and capsorubin) and provide experimental evidences that inclusion into β-CD renders carotenoids (lycopene, lutein, capsanthin and capsorubin) more stable against oxidating agents as AAPH and H_2O_2, and in comparison with the oxidation of those pigments promoted in a hydrophobic environment. However, special attention should be pointed when inclusion complexes are obtained from the esterified pigments as this structural configuration promotes in some cases the oxidation process.

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326

Figure captions

Figure 1. Oxidation of carotenoid:β-cyclodextrin complexes by AAPH (circles) or H_2O_2 (squares). Lycopene (a); Lutein (b); Capsanthin (c); Capsorubin (d).

Figure 2. Chemical structures of capsanthin, capsorubin, lycopene, lutein and β-cyclodextrin.

Table 1. Kinetic constant for the oxidation reaction by AAPH 1.5 mM for each inclusion complexes studied.

Table 2. Kinetic constant for the oxidation reaction by H_2O_2 1.5 mM for each inclusion complexes studied.

Table 1.

Rate constant values for the oxidation reaction of carotenoid:β-cyclodextrin inclusion complexes by 1.5 mM AAPH.

Carotenoid in β-cyclodextrin	**(k ± S.E.) × 10^{-2}**	**R^2_{adj}**
Lutein	5.12 ± 0.42[b]	0.954
Lycopene	3.56 ± 0.16[b]	0.936
Capsanthin	3.27 ± 0.35[b]	0.945
Capsorubin	3.54 ± 0.99[b]	0.951
Esterified lutein	11.47 ± 0.40[a]	0.938
Esterified capsanthin	8.07 ± 0.24[a]	0.959
Esterified capsorubin	2.08 ± 0.18[b]	0.971

First-order model: $\ln(\%ret) = 4.605 - k \times t$ (min^{-1}). S.E.: Standard error in the determination ($P < 0.05$). Marked values with the same letter are not significantly different (Scheffé test, $P < 0.05$).

Table 2.

Rate constant values for the oxidation reaction of carotenoid:β-cyclodextrin inclusion complexes by 1.5 mM H_2O_2.

Carotenoid in β-cyclodextrin	**(k ± S.E.) × 10^{-2}**	**R^2_{adj}**
Lutein	3.38 ± 0.61	0.963
Lycopene	3.21 ± 0.16	0.956
Capsanthin	2.91 ± 0.73	0.978
Capsorubin	3.24 ± 0.54	0.939
Esterified lutein	2.32 ± 0.53	0.952
Esterified capsanthin	2.10 ± 0.18	0.971
Esterified capsorubin	2.20 ± 0.17	0.948

First-order model: $\ln(\%ret) = 4.605 - k \times t$ (min^{-1}). S.E.: Standard error in the determination ($P < 0.05$). Rate constant values are not significantly different (Scheffé test, $P = 0.716$).

Figure 1

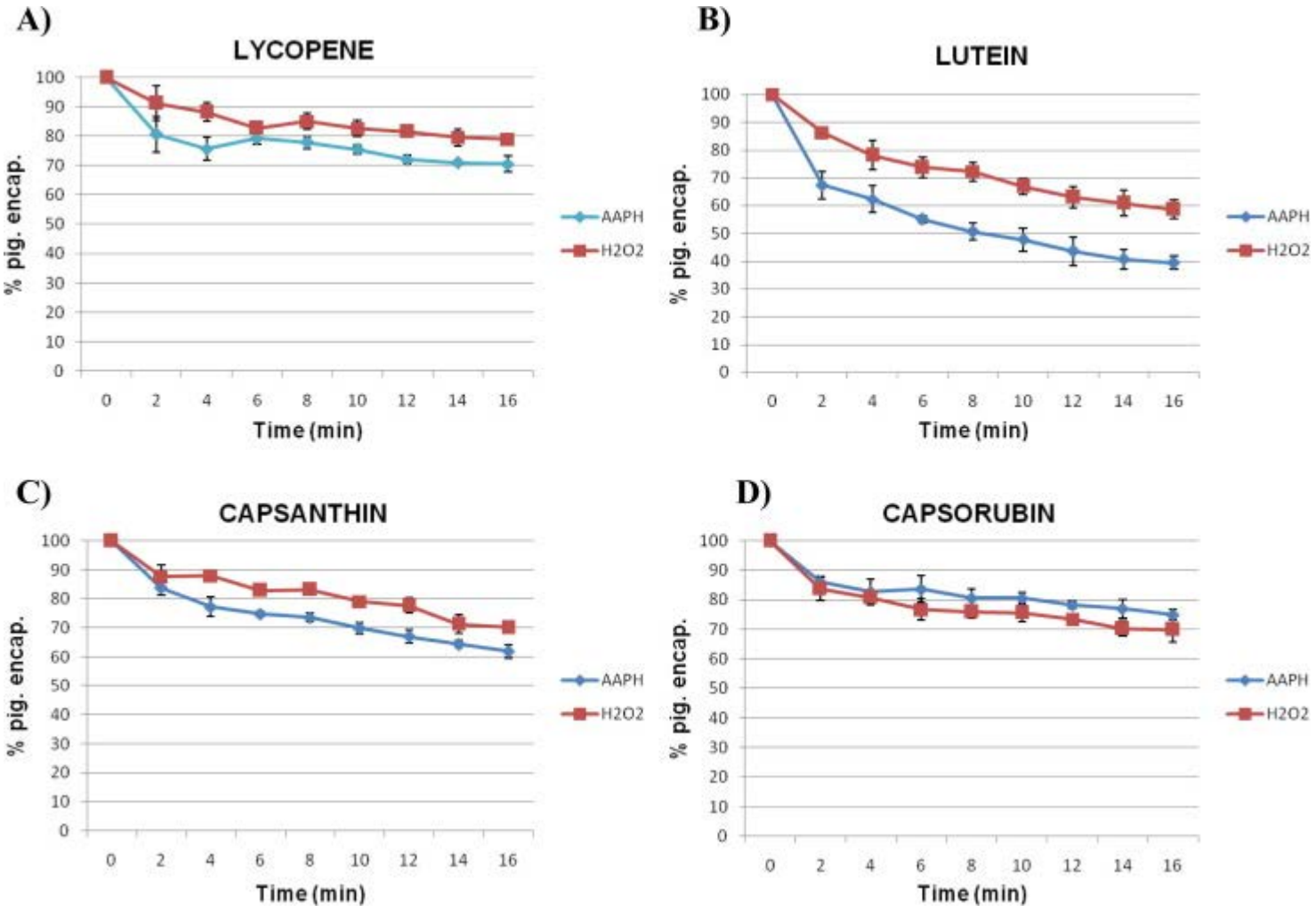

Fig. 1.
Oxidation of carotenoid:β-cyclodextrin complexes by AAPH (circles) or H2O2 (squares). Lycopene (a); lutein (b); capsanthin (c); capsorubin (d).

Figure 2

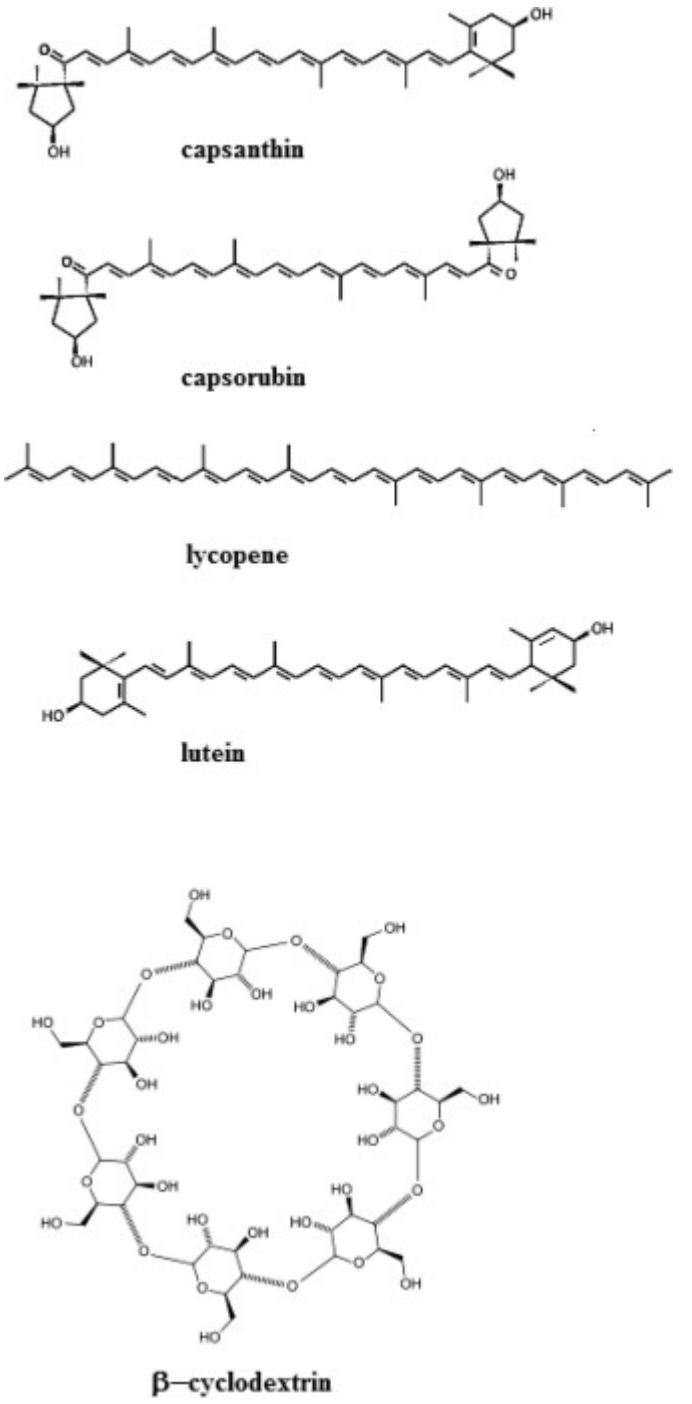

Fig. 2.
Chemical structures of capsanthin, capsorubin, lycopene, lutein and β-cyclodextrin.