



Effect of aggregation behaviour on the antioxidant capacity of carotenoids

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ABSTRACT

Lipophilic substances such as carotenoids show a tendency to aggregate in aqueous medium, that changes the physical and chemical properties of individual molecules. To estimate the antioxidant properties of carotenoids, trolox-equivalent antioxidant capacity (TEAC) values are routinely determined with ABTS⁺ solutions. As this reagent is a salt, it is generally applied in aqueous solutions to which carotenoids are added in various organic solvents. Here we present a systematic investigation on the solvent dependence of TEAC measurements of 8 carotenoids, as well as the relationship between the aggregation and TEAC values. The aggregation of carotenoids was found to have two opposite effects on the antioxidant capacity: the higher the aggregate size, the lower the TEAC value, however, smaller size (10–20 nm) assemblies surpass the individual molecules in antioxidant capacity. The type of aggregation manner (H or J) seems to have less influence on the antioxidant behaviour.

1. Introduction

Carotenoids are ubiquitous isoprenoid dyes, their beautiful red and orange colors are due to the extended conjugated polyene system. They can be hydrocarbon carotenes or oxygenated xanthophylls mostly with hydroxyl, epoxy, keto, and carboxyl functional groups, although almost all of them can be regarded as hydrophobic molecules. This property predestines carotenoids to accumulate in lipid tissues, incorporate into phospholipid membranes and attach to certain proteins [1]. Along with all this, aqueous media induce aggregation of carotenoid molecules that basically alter their physical and chemical properties such as light absorption, solubility, effective size, reactivity, and redox potential [2,3].

The process of the molecular aggregation of carotenoids is mainly driven by the π - π stacking interactions of the polyene backbones and the hydrogen bonding between the hydrophilic groups that may be present in carotenoids [4–6]. The intermolecular interactions of carotenoids are known to provide special features to the aggregates. The characteristic UV–vis absorption is due to these interactions, and they were also shown to improve the energy transfer rate by single exciton fission [7,8], which can have a great importance in harvesting solar energy. Two extreme

kinds of carotenoid aggregates, J- and H-types are generally considered. In H-type aggregates the molecules are stacked parallelly, the assemble has a shape of a card pack, that displays a large blue shift in the absorption spectrum with a complete loss of fine structure, compared to the individual molecules. The J-type aggregates are formed by loosely associated molecules with a head-to-tail orientation, which show a red shift in the UV–vis spectrum with resolved vibrational bands [4]. Nevertheless, in larger complex assemblies molecules can experience both types of packing simultaneously, that results in a red-shifted but sharp, intense peak in the absorption spectrum [9–11]. The optical properties of carotenoid nanoparticles were also explained by assuming some molecular distortion [12]. Carotenoids are able to create optically active aggregates, which can be studied by circular dichroism spectroscopy allowing a deeper view into the structure [6,9].

Besides the structure of carotenoids, there are many factors that have an effect on the aggregation, such as the initial concentration of the carotenoids, the ratio of organic solvent to water, as well as the pH of the solution, and the presence of salts or other additives [1,4,13].

Carotenoid nanoparticles stabilized by a hydrophilic coating are widely used as colorants of soft drinks and other aqueous food products

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[14].

Carotenoids are considered to be valuable natural pigments because they have a crucial role in photosynthesis and photoprotection of plants and algae [15], as well as they act as efficient antioxidants in all living organisms from bacteria to human [1]. The word 'antioxidant' is not always clearly defined, and several mechanisms of action are meant by. The protective role of carotenoids can be attributed to two main processes, which are related to the conjugated polyene structure: i.e. quenching of singlet oxygen and reactions with free radicals. Singlet oxygen is deactivated by physical ($^1\text{O}_2 + ^1\text{Car} \rightarrow ^3\text{O}_2 + ^3\text{Car}^*$, $^3\text{Car}^* \rightarrow ^1\text{Car} + \text{heat}$) and, to a lesser extent, chemical manners (endoperoxide formation). The reactions with radicals ($\text{R}^{\cdot}$ represents all lipid, alkoxy, and peroxy radicals) involve electron transfer ($\text{R}^{\cdot} + \text{Car} \rightarrow \text{R}^- + \text{Car}^{+\cdot}$), addition ($\text{R}^{\cdot} + \text{Car} \rightarrow [\text{R}-\text{Car}]^{\cdot}$), and hydrogen abstraction ($\text{R}^{\cdot} + \text{Car} \rightarrow \text{RH} + \text{Car}(\text{-H})^{\cdot}$) [16]. The formed resonance stabilized carotenoid radicals are more stable and less reactive than the initial $\text{R}^{\cdot}$, this way the radical chain reactions caused by $\text{R}^{\cdot}$ are inhibited. Among these mechanisms, electron transfer leads to the formation of oxidant $\text{Car}^{+\cdot}$ radical cations, that may also be responsible for harmful prooxidant behaviour [17]. The relative reduction potentials of individual carotenoid molecules and carotenoid radical cations have been determined in organic solvents and in Triton-X 100 aqueous system, in which extensive aggregation did not occur [16,18,19].

For the characterization of antioxidant power, a plethora of *in vitro* experimental chemical methods have been developed [20–32]. The simplest procedures use artificially generated radicals as oxidant which may not occur in any biological system and are suitable for the description of the inherent antioxidant character of molecules. Antioxidant activity is generally represented either as inhibition against oxidation of a test compound, or as equivalents of a selected reference antioxidant such as trolox or ascorbic acid [22]. The main advantages of these methods are their quickness, cheapness, and simplicity, but their limitation may be the lack of biological relevance. However, many of them can be performed in aqueous medium at a physiological pH, thus they are used to estimate the antioxidant behaviour of carotenoids by a specific mechanism in living systems.

A number of assays are applied to measure the antioxidant activity by single-electron transfer mechanism. One of the most popular methods is ABTS-TEAC (Trolox Equivalent Antioxidant Capacity), developed by Miller *et al.* [33,34]. In this assay the disodium salt of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) is oxidized to a radical cation ($\text{ABTS}^{+\cdot}$) by a one-electron transfer reaction (note that the terminus 'cation' refers to the removed electron, the actual particle has a net negative charge). The resulting $\text{ABTS}^{+\cdot}$ has absorption maxima at 417, 645, 734, and 815 nm wavelengths, from which the 734 nm is usually applied to follow the reaction between the $\text{ABTS}^{+\cdot}$ and the examined antioxidant. The antioxidant donates an electron to $\text{ABTS}^{+\cdot}$, that results in a drop of absorbance at 734 nm. The antioxidant capacity of any antioxidant is generally compared to that of trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a hydrophilic analog of tocopherol. The trolox equivalent antioxidant capacity (TEAC) is determined by different ways:

A calibration curve is plotted from the drop of absorbance (ΔA_{trolox}) of the $\text{ABTS}^{+\cdot}$ reagent after the reaction with trolox in specific concentrations (standard range of 0–10 μM). The absorbance values are corrected for the solvent. To establish the TEAC for other antioxidant compounds the drop of absorbance (ΔA_{antiox}) caused by the reaction with the antioxidant at a certain concentration ($[\text{antiox}]$) is measured in the same way, and corrected with the blank value. The TEAC of the antioxidant is calculated using the slope (m) of the above calibration curve: $\text{TEAC}_{\text{antiox}} = \Delta A_{\text{antiox}} / (m \cdot [\text{antiox}])$. This method gives how many moles of trolox are equivalent to 1 mmol antioxidant at that specific concentration of the antioxidant. When the ΔA_{antiox} vs. $[\text{antiox}]$ plot is not linear, the TEAC value expressed in this way depends on the applied concentration of the antioxidant [35,36].

The other method to express TEAC uses the percentage inhibitions

of trolox and that of the antioxidant in various concentrations: $(A_0 - A_{\text{antioxidant}})/A_0$, where A_0 stands for the initial absorbance of the $\text{ABTS}^{+\cdot}$ reagent before the addition of the trolox or another antioxidant, and $A_{\text{antioxidant}}$ for the absorbance after the addition (both corrected for the solvent). The percentage inhibitions are plotted against the concentration of the compounds, and the TEAC value is the dimensionless ratio of the slopes of these curves [37]: $\text{TEAC} = m_{\text{antioxidant}} / m_{\text{trolox}}$.

These frequently applied methods change the concentration of trolox and/or antioxidant with a constant concentration of $\text{ABTS}^{+\cdot}$ and perform the photometric measurements after a given incubation time. Depending on the nature of the antioxidant and the incubation time, the reaction with the $\text{ABTS}^{+\cdot}$ may not reach completion, and the determined TEAC value underestimates the actual antioxidant power. To overcome this issue, Arts *et al.* [38] developed a novel approach in which the antioxidant is applied at one specific concentration, and $\text{ABTS}^{+\cdot}$ in a variable concentration was added. To obtain a TEAC value, the maximum amount of the reduced $\text{ABTS}^{+\cdot}$ was determined by extrapolation, which was divided by the concentration of antioxidant and by the value determined similarly with trolox.

Just like in other antioxidant assays, in TEAC determination, ionic reagents are applied in polar solvents, most frequently in ethanolic or aqueous systems. The choice of solvent was found to influence the TEAC values for phenolic compounds [39]. As carotenoids are hydrophobic molecules, they are dissolved in a variety of auxiliary organic solvents for the ABTS-TEAC determinations. These organic solvents are generally water-miscible substances [35,40], but the use of hexane or dichloromethane was also described [41].

Very few studies deal with the solvent dependence of the TEAC values of carotenoids. R. van den Berg *et al.* [35] established that especially in the case of β -carotene the original solvent essentially influenced the measured TEAC in a phosphate buffer, while the values for α -tocopherol or hydrophilic antioxidants (flavonoids, ascorbic acid) were not effected by the initial solvent.

As it is shown above, although being quite popular, there is no standard procedure for TEAC determination even for a specific family of compounds, which results in confusion. We would rather not cite the numerous hair-raising examples where the experimental description does not reveal how the solutions were exactly prepared or how the TEAC values were calculated. Nevertheless, even the TEAC values from the most demanding and elaborated papers cannot be compared if the procedures differ even in a minor way such as the choice for the auxiliary solvent.

In this work, we explore the solvent dependence of the TEAC measurements of carotenoids and the aggregation behaviour behind the data. Also, we suggest standardizing the protocol for carotenoids to allow more relevant comparisons.

2. Experimental section

2.1. Materials

β -Carotene, β -cryptoxanthin, zeaxanthin, lutein and capsanthin (Fig. S1) were isolated from red pepper *Capsicum annuum* [42] or from spinach [43] by standard procedure. Astaxanthin, canthaxanthin, and 8'-apo- β -carotenol were generous donations from CaroteNature GmbH., Switzerland. All carotenoids were of at least 95 % purity by HPLC. The solvents were of HPLC grade, ethanol contained 4.0 v/v% water. Tetrahydrofuran (THF) was free of BHT and other stabilizers. For the buffer solution 9.55 g of DPBS powder (VWR International Kft., Debrecen, Hungary) was dissolved in distilled water forming 1L of solution (composition: 0.138 M NaCl, 0.0027 M KCl, 0.0081 M Na_2HPO_4 , 0.0015 M KH_2PO_4 , pH = 7.40).

2.2. Sample preparation

Carotenoids (Fig. S1) were dissolved in auxiliary solvents dimethyl

sulfoxide (DMSO) or tetrahydrofuran (THF), trolox was dissolved in ethanol to obtain $2.5 \cdot 10^{-4}$ M stock solutions, which were further diluted with 96 % ethanol, distilled water or isotonic phosphate-buffered saline (PBS) to obtain $1.875 \cdot 10^{-4}$, $1.25 \cdot 10^{-4}$, $6.25 \cdot 10^{-5}$, $3.125 \cdot 10^{-5}$, and $1.5625 \cdot 10^{-5}$ M concentrations, respectively. 60 μ L portions of these solutions were used for the experiments.

The reaction mixtures consisted of 60 μ L of the investigated carotenoid or trolox solution and 2940 μ L of ABTS^{•+} solution prepared with ethanol, distilled water, or PBS (main solvents), in each case to obtain final carotenoid or trolox concentrations of 0.3125, 0.625, 1.25, 2.50, 3.75, and 5 μ M, respectively, while the final solutions contained the auxiliary solvent in 0.25–2.0 v/v% (Table 1). In the blank (0 μ M) samples, the solutions of the antioxidants were substituted with ethanol, distilled water, or PBS, respectively. Each sample was incubated at 37 °C for 6 min before the measurements, similarly to those performed by Nagane et al. [37] and Arts et al. [38], because these conditions provide a relatively high temperature and long reaction time facilitating the completion of the reactions.

For recording the UV–Vis and ECD spectra, as well as for the dynamic light scattering and scanning electron microscopy measurements the samples were prepared the same way as the reaction mixtures described above, with the difference that instead of ABTS^{•+} reagent they contained only solvent (96 % ethanol, distilled water, or PBS). Each sample was examined after 6 min incubation time at 37 °C, assuring the same conditions as for the antioxidant measurements.

2.3. ABTS assay and determination of TEAC values

The TEAC values were determined in three different main solvents: 96 % ethanol, distilled water, and phosphate-buffered saline (PBS) of pH 7.40. The ABTS^{•+} reagent was produced freshly each day in aqueous solution by reacting the diammonium salt of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (Tokyo Chemical Industry Co. Ltd., Tokyo, Japan) in 7 mM with potassium persulfate (K₂S₂O₈) (Alfa Aesar, Tewksbury, MA, USA) in 2.45 mM concentrations. The ABTS^{•+} stock solution was prepared 12–16 h before the experiments and stored in dark at room temperature. Right before the experiments, the absorbance of the deep green–blue ABTS^{•+} solution was set to 0.70 ± 0.05 at 734 nm by a ca. 100-fold dilution with 96 % ethanol, distilled water, or phosphate-buffered saline, according to the main solvent of the samples. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (Tokyo Chemical Industry Co. Ltd., Tokyo, Japan) was applied as a general reference. The carotenoid and trolox solutions were prepared freshly as described in section “2.2. Sample preparation”. The reaction mixture (60 μ L of the investigated carotenoid or trolox solution and 2940 μ L of ABTS^{•+} solution) was incubated at 37 °C for 6 min, and the depression in ABTS^{•+} concentration was monitored by measuring the absorbance at 734 nm. The spectrophotometric measurements were implemented on a Jasco spectrophotometer model V-550 UV/Vis (Jasco Corporation, Japan). The percentage inhibition of absorbance at 734 nm was calculated as $(A_0 - A_{\text{antioxidant}})/A_0$, where A_0 is the initial absorbance of the ABTS^{•+} solution and $A_{\text{antioxidant}}$ is the absorbance of the reaction mixture after incubation, both corrected for the solvent. The calculated percentage inhibition values were plotted against the final concentration of the antioxidants. The slopes of the curves were

compared with that for trolox, and the trolox equivalent antioxidant capacity (TEAC) values were calculated as the ratio of the slopes for the antioxidants and for trolox.

2.4. Statistical analysis

All experiments were done in triplicate ($n = 3$). Data were expressed as means (sd). For the comparison of the means one-way ANOVA with Student-Newman-Keuls post-hoc test was calculated, and to analyze the homogeneity of variance Levene statistics was implemented by using SPSS 26.0 (SPSS, Chicago, IL, USA). A difference was considered statistically significant at $p < 0.05$.

2.5. Determination of hydrodynamic diameter

The size of the aggregates in the sample dispersions was determined by dynamic light scattering (DLS) photometric measurements, where the hydrodynamic diameter of particles was measured. The number-weighted size distribution indicated one main peak in most cases, the polydispersity index (PDI) was calculated as $sd^2/\text{mean size}^2$. For the particle size analysis, a Malvern Zetasizer Nano S (Malvern Panalytical Ltd., Great Malvern, Worcestershire, UK) apparatus was used. The measurements were carried out in autocorrelation mode, and the following parameters were kept constant: scattering angle 173°, attenuator 11 and its factor 0.0146, measurement position 4.65 mm. The samples were prepared as described in section “2.2. Sample preparation”, each sample was examined after 6 min incubation time at 37 °C.

2.6. ECD and UV–vis spectrophotometric measurements

The samples were prepared with ethanol, distilled water or PBS as described in section “2.2. Sample preparation” and were incubated at 37 °C for 6 min. The measurements were performed in a 1 cm optical path quartz cuvette with a 1 nm resolution, in the wavelength range of 250–800 nm. Jasco spectrophotometer model V-730 UV/Vis (Jasco Corporation, Japan) was used for recording the UV–Vis spectra of carotenoid solutions/dispersions. Electronic circular dichroism (ECD) spectra were recorded at room temperature with a Jasco J-810 spectropolarimeter (Jasco Corporation, Japan).

2.7. Scanning electron microscopy (SEM)

The aqueous samples described above were freeze-dried with a Christ Alpha 1–2 freeze dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) and the remaining solid particles in the flask were collected by a duct tape. The scanning electron microscope (SEM) images were captured with a Jeol JSM-IT500HR (Jeol, Tokyo, Japan) instrument. Secondary electron mode and a 30 kV accelerating voltage were used after gold coating (Jeol JFC-1300 auto fine coater, Jeol, Tokyo, Japan).

3. Results and discussion

We chose two auxiliary solvents, DMSO and THF, for the preparation of the stock solutions of carotenoids, which were further diluted with the main solvent: 96 % ethanol, water, or PBS. DMSO, being biocompatible at low concentrations, is frequently applied in biological studies. THF, on the other hand, is usually the solvent of choice in the preparation of carotenoid nanoparticles which are used in e.g. food industry [44]. The final DMSO or THF concentration in the reaction mixtures were between 0.25 and 2.0 v/v% (see Sample preparation, Table 1.). In ABTS assays, the use of ethanol as a main solvent was described by many papers, since this solvent overcomes the solubility issues for most carotenoids. Nevertheless, TEAC values determined in aqueous solutions may characterize better the real antioxidant behaviour of the substance in biological systems.

Table 1

Final concentrations for carotenoids and the auxiliary solvents.

final carotenoid concentration (μ M)	final DMSO/THF concentration (v/v%)
0.3125	0.25
0.625	0.5
1.25	0.7
2.50	1.0
3.75	1.5
5.00	2.0

3.1. Behaviour of zeaxanthin: A typical example

In ABTS assays, the plot of percentage inhibition against concentration usually gives a linear line, the slope of which is used to determine the trolox equivalent antioxidant capacity (TEAC). That was the case when the reference compound trolox was used, and the percentage inhibitions, as well as the slope of their concentration dependence showed only a slight dependence of the solvents. Carotenoids, however, in aqueous media did not produce a completely linear graph, but at some specific concentrations the percentage inhibition showed a large negative deviation. This is a rare but not unprecedented phenomenon, some flavonoids were also found to show concentration dependence for TEAC values [35]. A typical example is shown in Fig. 1: the macular pigment zeaxanthin in ethanol behaved as it was expected, the percentage inhibition was directly proportional with the concentration (Fig. 1a and 1b). In solutions prepared with distilled water or PBS, at the same time, the percentage inhibitions at certain concentrations deviated from the linear trendline. Very similar behaviour was observed with all the other studied carotenoids (see Supplement), the typical concentrations at

which deviation occurred were 1.25 μM from THF stock solutions, as well as 2.5 and 3.75 μM from DMSO stock solutions, respectively.

As carotenoids are basically hydrophobic molecules, their assembly was suspected to be responsible for the above findings. The aggregation of carotenoids is a well-studied area [4,14,44–46], nevertheless, in the literature we did not find too much detailed investigation about the effect of aggregation on the antioxidant behaviour. To better understand the background of the above observations, the carotenoid solutions of different concentrations were examined by UV–vis spectrophotometry.

The other examined carotenoids showed similar behaviour to that of zeaxanthin: in ethanolic solution (Fig. 2a and 2b), independently of the auxiliary solvent, the UV–vis spectra did not suggest aggregation, only a simple solvent-induced shift of λ_{max} [47,48] without losing vibrational fine structure was observed. In solutions with water or PBS, however, all the spectra showed a definite alteration compared to those in organic solvents (see Supplement). In the case of zeaxanthin from DMSO stock solution (Fig. 2c and 2e), the large hypsochromic shift of λ_{max} and the complete loss of fine structure clearly indicate the formation of H-type aggregates. Starting from DMSO stock both in water and in PBS the

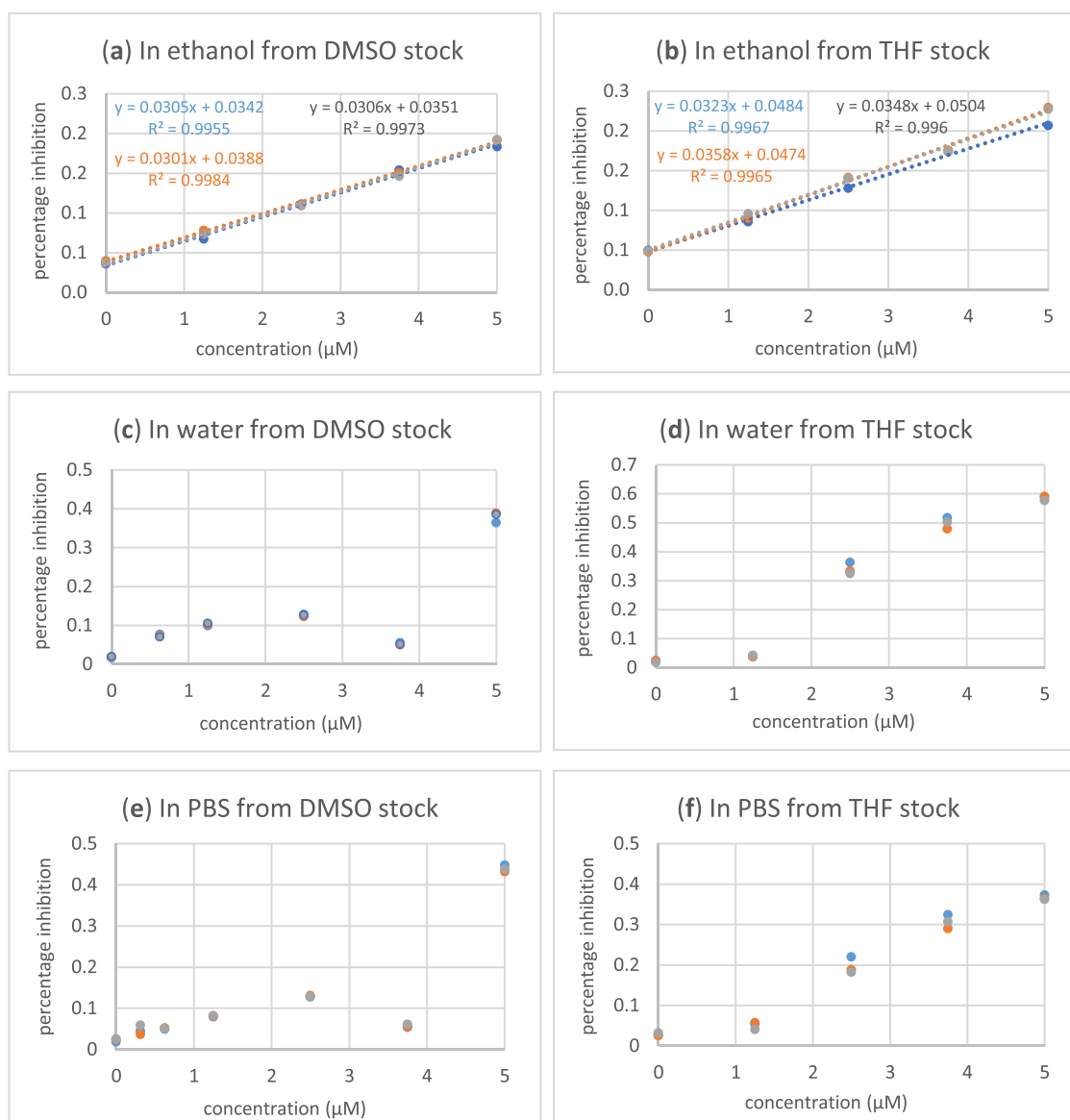


Fig. 1. Concentration dependence of percentage inhibition of zeaxanthin: (a) and (b) in ethanol, (c) and (d) in distilled water, (e) and (f) in PBS. The stock solutions were prepared in DMSO (a, c, e) or in THF (b, d, f). $n = 3$, dots of a given colour belong to the percentage inhibitions of the same series of experiment; in (a) and (b) the linear trendlines are also given together with their equations and regression coefficients.

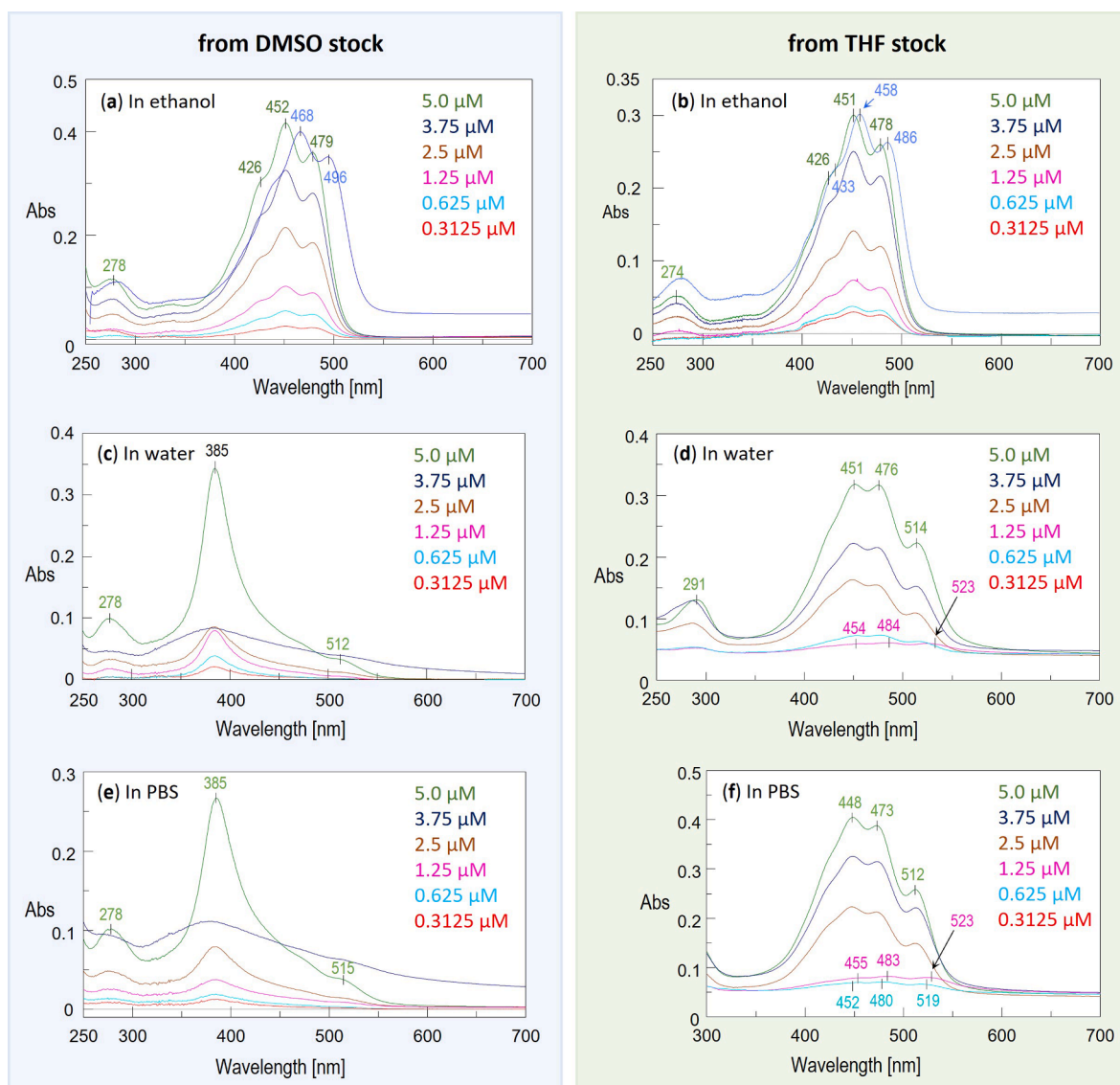


Fig. 2. UV–vis spectra of zeaxanthin solutions at different concentrations: (a) and (b) in ethanol, (c) and (d) in distilled water, (e) and (f) in PBS. The stock solutions were prepared in DMSO (a, c, e) or in THF (b, d, f). In (a) and (b), the upper blue spectra belong to 5.0 μM of zeaxanthin in pure DMSO or THF, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

UV–vis spectra suggest a similar manner of aggregation almost at all studied concentrations, the only difference is in the spectra of 3.75 μM concentration, at which the percentage inhibition with $\text{ABTS}^{+\cdot}$ was out of the linear tendency (Fig. 1c and 1e).

Dilution of the THF stock solution of zeaxanthin with water or PBS also resulted in altered UV–vis spectra (Fig. 2d and 2f), but this time the pattern (bathochromic shift and remained vibrational fine structure) implied J-type aggregation. Based on these spectra, the aggregation seems to be again uniform at different concentrations, with the exception of 1.25 μM , which coincides with the unexpected low value for percentage inhibition with $\text{ABTS}^{+\cdot}$ (Fig. 1d, f).

Electronic circular dichroism (ECD) can be applied to observe aggregation or supramolecular organization of molecules. It has been proven to be a valuable method to predict such behaviour in carotenoid solutions as well, even to differentiate between different type of aggregates [49–51]. The ECD spectra of the above solutions (Fig. 3) confirmed and shaped the conclusions drawn from the UV–vis spectra: Zeaxanthin in water from DMSO stock solution gave a slightly different ECD spectrum at 3.75 μM than in other concentrations, and in 5.0 μM two additional positive peaks appeared at 480 and 520 nm wavelengths (Fig. 3a).

In PBS solution, however, the ECD spectra show an opposite Cotton effect at 0.625 and 1.25 μM compared to the other concentrations.

In PBS solution from THF stock the ECD spectrum of 1.25 and 1.75 μM clearly differs from the other concentrations. The aggregation in pure water from THF stock solution seems to be uniform at different concentrations.

The aggregation of zeaxanthin was also examined by dynamic light scattering photometry (Table 2.). The hydrodynamic diameter of the particles varied between ca. 200–600 nm in most cases, the only exception was the dispersions in water from THF stock solution: here the typical particle size was about 20 nm. In 1.25 μM concentration zeaxanthin formed larger (ca. 120 nm) aggregates, which seems to result in the lower percentage inhibition with $\text{ABTS}^{+\cdot}$. Nevertheless, in the reaction mixtures with distilled water as a main solvent and using THF stock, the percentage inhibitions of zeaxanthin were about 1.6 times higher than in other main/auxiliary solvents (Fig. 1).

The freeze-dried dispersions from water were examined by scanning electron microscopy (SEM). This method has a limitation in the study of the particle size and shape, as particles can agglomerate into larger clumps during the sample preparation. However, because of the low

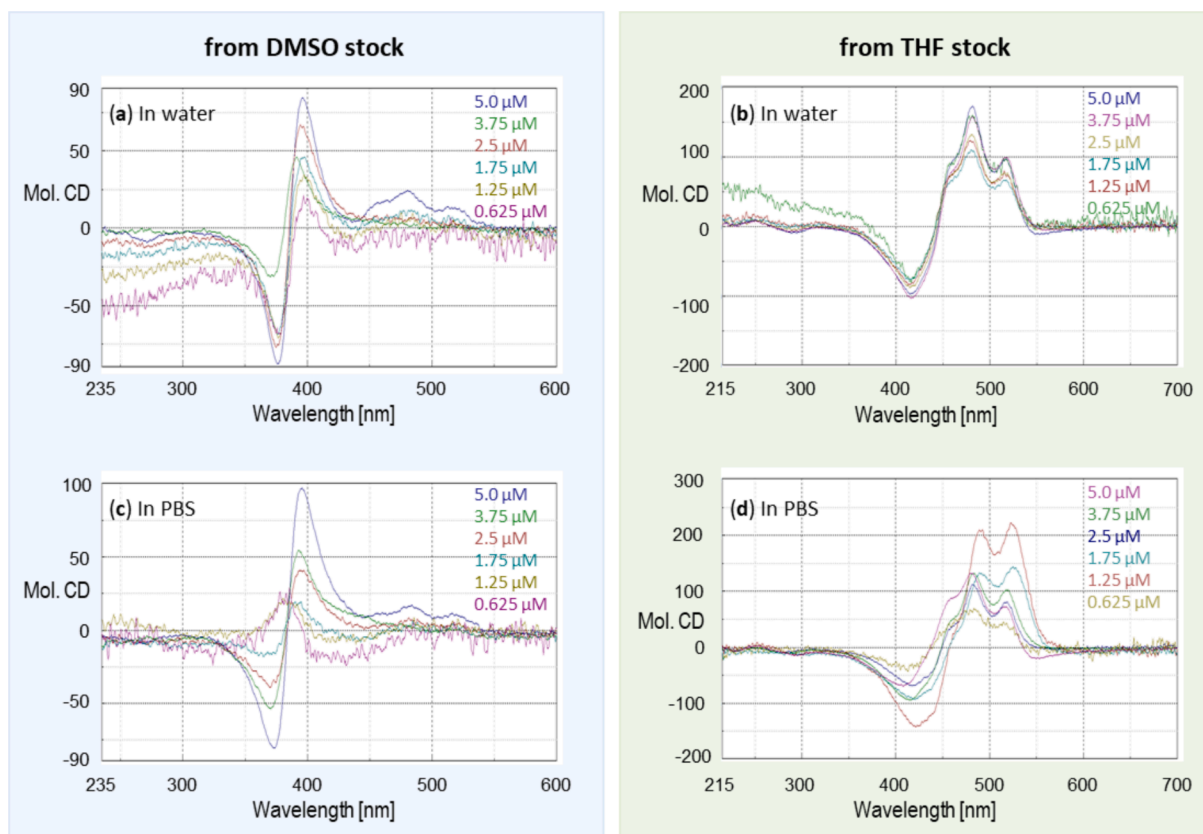


Fig. 3. ECD spectra of zeaxanthin solutions at different concentrations: (a) and (b) in distilled water, (c) and (d) in PBS. The stock solutions were prepared in DMSO (a, c) or in THF (b, d).

Table 2

Hydrodynamic diameter (nm) of zeaxanthin aggregates at different concentrations.

stock solvent: main solvent: concentration (μM)	DMSO water mean size (nm)	sd	PBS mean size (nm)	sd	THF water mean size (nm)	sd	PBS mean size (nm)	sd
0.625	188.3	34.7	79.25	8.2	19.73	6.7	242.6	30.5
1.25	211.7	31.0	296.9	30.8	123.1	12.7	59.09	6.1
2.50	159.4	19.2	325.3	40.9	17.04	2.4	338.3	78.1
3.75	349.5	83.8	494.1	85.7	22.08	6.6	607.7	134.2
5.00	56.72	8.9	530.6	93.3	10.28	3.2	779.1	158.3

concentrations and slow drying, the formed clusters may refer to the particle size of the original dispersions. As it is shown in Fig. 4, in water from THF stock solution fine, grass-like structure of small aggregates formed at 5.0 μM concentration, while at 1.25 μM in the same solvent much larger columns were visible. From DMSO stock the shape of the formed particles was less regular and were similar in different concentrations.

The samples in PBS were not suitable for SEM analysis, since the solid material left after freeze-drying consisted mainly of huge crystals of the salts of the buffer, the carotenoid aggregates were very difficult to find among them.

The above findings suggest that the percentage inhibition is mainly influenced by the size of the aggregates and less by the aggregation manner.

3.2. Behaviour of other carotenoids

Seven other carotenoids, the hydrocarbon β-carotene, the hydroxyl group-bearing β-cryptoxanthin, lutein, the aldehyde 8'-apo-β-carotenal, the keto-derivatives astaxanthin and canthaxanthin, as well as the κ-end

group-containing keto-carotenoid capsanthin were also investigated in a similar fashion (Table 3). The detailed experimental data are given in the [Supplementary material](#). β-Carotene and its diketone derivative, canthaxanthin, were not completely soluble in DMSO and gave micro-crystals that remained visible even in ethanolic solutions. Canthaxanthin gave a hardly reproducible non-linear percentage inhibition curve from DMSO in PBS. Astaxanthin is a diketone derivative of zeaxanthin, from DMSO stock in PBS it showed a very intense and fast precipitation at several concentrations, visible by naked eye. For these compounds TEAC could not be calculated in DMSO-PBS mixture. β-Cryptoxanthin in PBS from THF stock showed an interesting aggregation behaviour, it gave a U-shape concentration vs. size curve with a local minimum size at 2.5 μM concentration.

3.3. Determination of TEAC values

For the TEAC calculation we also determined the percentage inhibitions for trolox. The slope of the calibration curves for trolox was found to depend on the main solvent (Table 4) in accordance with the literature [35,39], however, these differences are virtually negligible

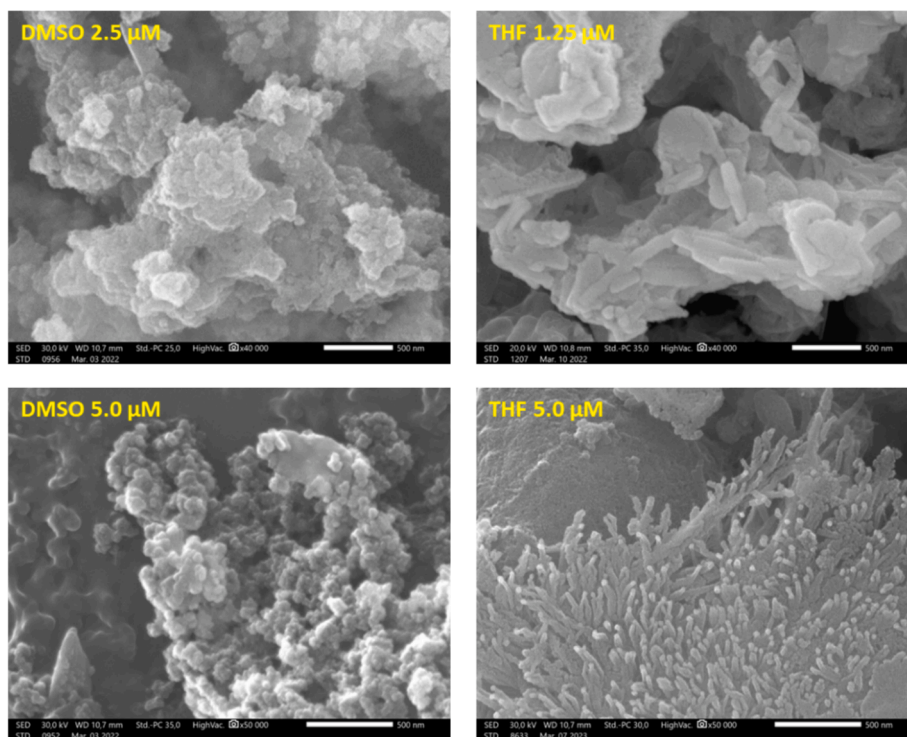


Fig. 4. SEM image of zeaxanthin aggregates at different concentrations from distilled water. The stock solutions were prepared in DMSO or in THF.

compared to those of the carotenoids. The redox potential of ABTS was also postulated to be quite solvent independent [52,53].

From the slope (m) of the percentage inhibition lines the TEAC values ($TEAC = m_{(carotenoid)} / m_{(trolox)}$) were calculated in two ways. When all concentrations for the percentage inhibition curves of carotenoids were considered for TEAC, the regression coefficients (R^2) of the linear trendlines, in most cases, were less than 0.98 because the percentage inhibition usually at one or two concentrations gave outlier values. Based on UV-vis, ECD and DSL measurements a different aggregation behaviour was in the background of these unexpected values compared to that at other concentrations. By the exclusion of the only outlier percentage inhibition value, the R^2 for the line became higher than 0.98, and the slope of the corrected curve was also used to calculate TEAC (corr) (Fig. 5). However, in the case of lutein and zeaxanthin from DMSO stock solutions the spectroscopic data did not indicate unambiguously the outlier values.

In ethanolic solutions correction was not necessary, and the UV spectra did not suggest aggregation. The TEAC values in ethanol, however, slightly differed for the 3-hydroxy- β -end group carotenoids depending on the auxiliary solvent. From DMSO stock we found lower TEAC values for these substances than from THF. Since DMSO is a potent hydrogen bond acceptor, we may assume the formation of stable carotenoid-DMSO associations, which remain even on dilution with ethanol and influence the electron-transfer reaction.

In aqueous systems, the correction for the uneven aggregation resulted in significant differences in TEAC values mainly from DMSO stock. For carotenoids with 3-hydroxy- β -end group the percentage inhibition typically at 2.5 and 3.75 μ M concentrations showed exceptionally low values resulting in $R^2 < 0.90$ in the linear equation of percentage inhibition-concentration plot, for zeaxanthin $R^2 < 0.65$ (Fig. 5). In these cases, the corrected TEAC values considerably differed from the uncorrected ones. Starting from THF stocks, a smaller variance in the data was observed. The corrected TEAC values can characterize the behavior of the compound in general, nevertheless, at the outlier concentrations TEAC should rather be expressed as mmol of trolox equivalent to 1 mmol antioxidant at that specific concentration [35,36].

Carotenoids from DMSO stock solution typically gave H-type aggregates, while from THF stock both extreme kinds and mixed forms could be detected (Fig. 5). Examining the hydrodynamic diameter of the aggregates, in PBS the size of the formed particles was at least twice, in some cases even larger, than in water, independently of the auxiliary solvent (Fig. 6). In PBS, keto-carotenoids from DMSO stock provided inappreciable (non-linear or non-reproducible) percentage inhibition curves (see Supplement). Those carotenoids which could be measured in both systems usually gave smaller size aggregates and higher TEAC values in THF/PBS solvents than in DMSO/PBS (Figs. 5 and 6). That also supports the importance of the aggregate size over the type in the antioxidant behaviour.

3.4. Relationship between TEAC and the size of carotenoid aggregates

Interestingly, despite of the fact that in all aqueous solutions aggregation of carotenoid molecules was clearly observed, the TEAC values were quite similar to those in ethanol, and in many cases even higher. Especially the 3-hydroxy- β -end group carotenoids from THF stock solution displayed considerably higher TEAC values in PBS than in ethanol, and showed enormous TEAC values in water.

The DLS measurements for the hydrodynamic diameter of the aggregates revealed a low (typically < 50 nm) and very uniform particle size in water from THF stock, while in PBS or starting from DMSO stock in both main solvents the formed aggregates were considerably larger (> 100 nm). However, at a concentration of 1.25 μ M, most of the carotenoids from the THF stock yielded particles > 100 nm in water, which coincided with a low percentage inhibition value at this concentration (Fig. 6).

Considering the above, two opposite effects of aggregation were observed: the higher the aggregate size, the lower the percentage inhibition and the TEAC value, nevertheless, the aggregates exhibited similar TEAC values as individual molecules in ethanolic solution. The aggregated carotenoids are obviously less accessible to the $ABTS^{+}$ reagent than the individual molecules, but yet the above findings suggest that the aggregation, to a certain extent, somehow makes the one-

Table 3

Aggregation behaviour of carotenoids in water and PBS starting from DMSO or THF stock solution. (P in the formulas stands for polyene; c_{ag} : concentrations with unexpectedly low percentage inhibition with ABTS^{•+}; UV-vis: the type of aggregate on the basis of UV-vis and ECD spectra; DLS: hydrodynamic diameter determined by dynamic light scattering photometry) See also the Supplement.

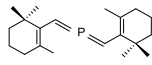
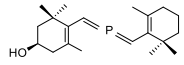
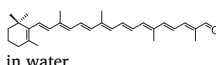
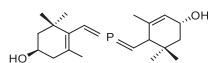
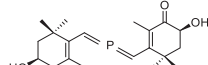
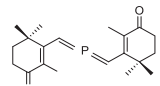
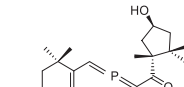
Carotenoid	from DMSO stock	from THF stock
β-carotene	c_{ag} : –	c_{ag} : 1.25 and 5.0 μM
 in water	UV-vis: mixed or H	UV-vis: mixed, at 1.25 μM : J
	DLS: 80–90 nm, at 1.25 μM 230 nm, 2.5 μM 10 nm, but it is not completely soluble in DMSO (microcrystals visible by a magnifier)	DLS: 50–60 nm, at 1.25 μM 250 nm
in PBS	c_{ag} : 3.75 μM (slightly)	c_{ag} : 1.25 μM and 2.5 μM
	UV-vis: H or mixed, at 3.75 μM : J	UV-vis: mixed or J, at 1.25 μM : J
	DLS: 50–220 nm, the lower the concentration, the higher the particle size, but it is not completely soluble in DMSO (microcrystals)	DLS: 40–50 nm, at 1.25 μM 250 nm, 0.625 μM 120 nm
β-cryptoxanthin	c_{ag} : 2.5 and 3.75 μM	c_{ag} : 1.25 μM
 in water	UV-vis: mixed or H	UV-vis: mixed, at 1.25 μM J-type
in PBS	DLS: 20–30 nm, at 3.75 μM 230 nm, at 0.3125 μM 300 nm	DLS: 10–40 nm, at 1.25 μM nd.
	c_{ag} : 2.5 μM	c_{ag} : 1.25 μM
	UV-vis: mixed	UV-vis: mixed or J-type
	DLS: 200–450 nm, at 3.75 μM 140 nm	DLS: 10–350 nm, at 1.25 μM nd.
8'-apo-β-carotenal	c_{ag} : 3.75 μM	c_{ag} : 3.75 μM (high %i)
 in water	UV-vis: H-type	UV-vis: H-type
in PBS	DLS: ~20 nm, at 3.75 μM 200 nm	DLS: 20–50 nm, at 2.5 μM 160 nm
	c_{ag} : 2.5 and 3.75 μM	c_{ag} : 1.25 and 2.5 μM
	UV-vis: H-type	UV-vis: J-type or mixed, at 1.25 μM J-type
	DLS: 140–180 nm, at 5.0 and 3.75 μM ~20 nm	DLS: ~20 nm, at 0.625 μM 40 nm
lutein	c_{ag} : 2.5 and 3.75 μM	c_{ag} : 1.25 μM
 in water	UV-vis: H-type	UV-vis: H-type, at 1.25 μM mixed
in PBS	DLS: 70–190 nm, at 2.5 μM 330 nm, at 3.75 μM nd.	DLS: 15–30 nm, at 1.25 μM 330 nm
	c_{ag} : 3.75 and 5.0 μM	c_{ag} : 1.25 μM
	UV-vis: H-type	UV-vis: H-type or mixed
	DLS: 370–415 nm, at 1.25 μM 210 nm, at 0.625 μM 13 nm	DLS: 200–370 nm, at 2.5 μM 160 nm
astaxanthin	c_{ag} : 2.5 and 3.75 μM	c_{ag} : 1.25 and 5 μM
 in water	UV-vis: H-type, but J or mixed at 2.5 and 3.75 μM	UV-vis: H-type or mixed, but J-type at 1.25 μM
in PBS	DLS: 40–110 nm, at 2.5 μM 140 nm, at 3.75 μM nd.	DLS: 1–35 nm, at 1.25 μM 170 nm
	c_{ag} : *	c_{ag} : 0.625 μM and 1.25 μM
	UV-vis: H-type, but J or mixed at 2.5 and 3.75 μM	UV-vis: mixed, but J-type at 0.625 and 1.25 μM
	DLS: 140–470 nm, at 0.3125, 0.625, 2.5 and 3.75 μM nd. (precipitation)	DLS: 110–130 nm, at 0.625 and 1.25 μM 240–270 nm

Table 3 (continued)

Carotenoid	from DMSO stock	from THF stock
canthaxanthin	c_{ag} : 3.75 μM	c_{ag} : 1.25 and 5.0 μM
 in water	UV-vis: H-type or mixed	UV-vis: H-type or mixed, but J-type at 1.25 μM
	DLS: 6–30 nm, at 3.75 and 0.625 μM 70–95 nm, at 0.3125 μM 500 nm (but it is not completely soluble in DMSO)	DLS: 25–60 nm, at 1.25 μM 300 nm
in PBS	c_{ag} : *	c_{ag} : 1.25 μM
	UV-vis: H or mixed	UV-vis: H-type or mixed, but J-type at 1.25 μM
	DLS: 100–200 nm, at 5.0, μM 20 nm (but it is not completely soluble in DMSO)	DLS: 30–70 nm, at 1.25 μM 320 nm
capsanthin	c_{ag} : 2.5 μM	c_{ag} : 1.25 μM
 in water	UV-vis: H-type	UV-vis: H-type, but mixed at 1.25 μM
in PBS	DLS: 20–50 nm, at 2.5 and 0.625 μM 250–160 nm	DLS: 20–40 nm, at 1.25 μM 315 nm
	c_{ag} : 2.5 μM	c_{ag} : 1.25 and 5.0 μM
	UV-vis: H-type	UV-vis: H or mixed
	DLS: 200–500 nm	DLS: 200–500 nm

nd.: particle size was too large to be determined by DLS.

*: there is no linear tendency, TEAC can not be given as with the other carotenoids.

Table 4

Linear equations ($y = mc + b$) and regression coefficients for trolox calibration curves in different solvents.

solvent	m (sd)	b (sd)	R ² (sd)	n
ethanol	0.0393 (0.0036)	0.0363 (0.0064)	0.9936 (0.0090)	30
water	0.0364 (0.0029)	0.0215 (0.0056)	0.9956 (0.0043)	45
PBS	0.0341 (0.0015)	0.0329 (0.0044)	0.9945 (0.0047)	24

m: slope, c: concentration in μM , b: intercept, R²: regression coefficient, sd: standard deviation, n: number of measurements.

electron oxidation more efficient.

To understand this, we looked for similar findings in the literature, but there are only a few papers that deal with the antioxidant property of carotenoid aggregates. Cantrell *et al.* [54] investigated the singlet oxygen quenching ability of carotenoids in phospholipid membranes, and found that smaller aggregates may still quench singlet oxygen but the large ones would not. The decrease of ¹O₂ quenching was observed at higher concentrations (>20 μM), at lower concentrations the quenching was directly proportional with the concentration.

Carotenoids in much higher (0.5–1.0 mM) concentrations stabilized by carbohydrate derived complexing agents showed lower hydroperoxyl radical scavenging effect in aggregated state, by a hydrogen transfer mechanism [55]. Here we note, that this study used concentrations 2–3 orders of magnitude higher than the usual antioxidant assays, and than that of the human serum, which is typically below 1 μM [56]. A more related study by Mortensen *et al.* [57] established that electron transfer from carotenoids in 10 μM concentration to NO₂ radical resulted in the formation of Car^{•+} radical cations, which decayed bimolecularly (2 Car^{•+} → product). Radical cations of aggregated carotenoids showed faster decay than that of individual molecules. In these studies, the nature of

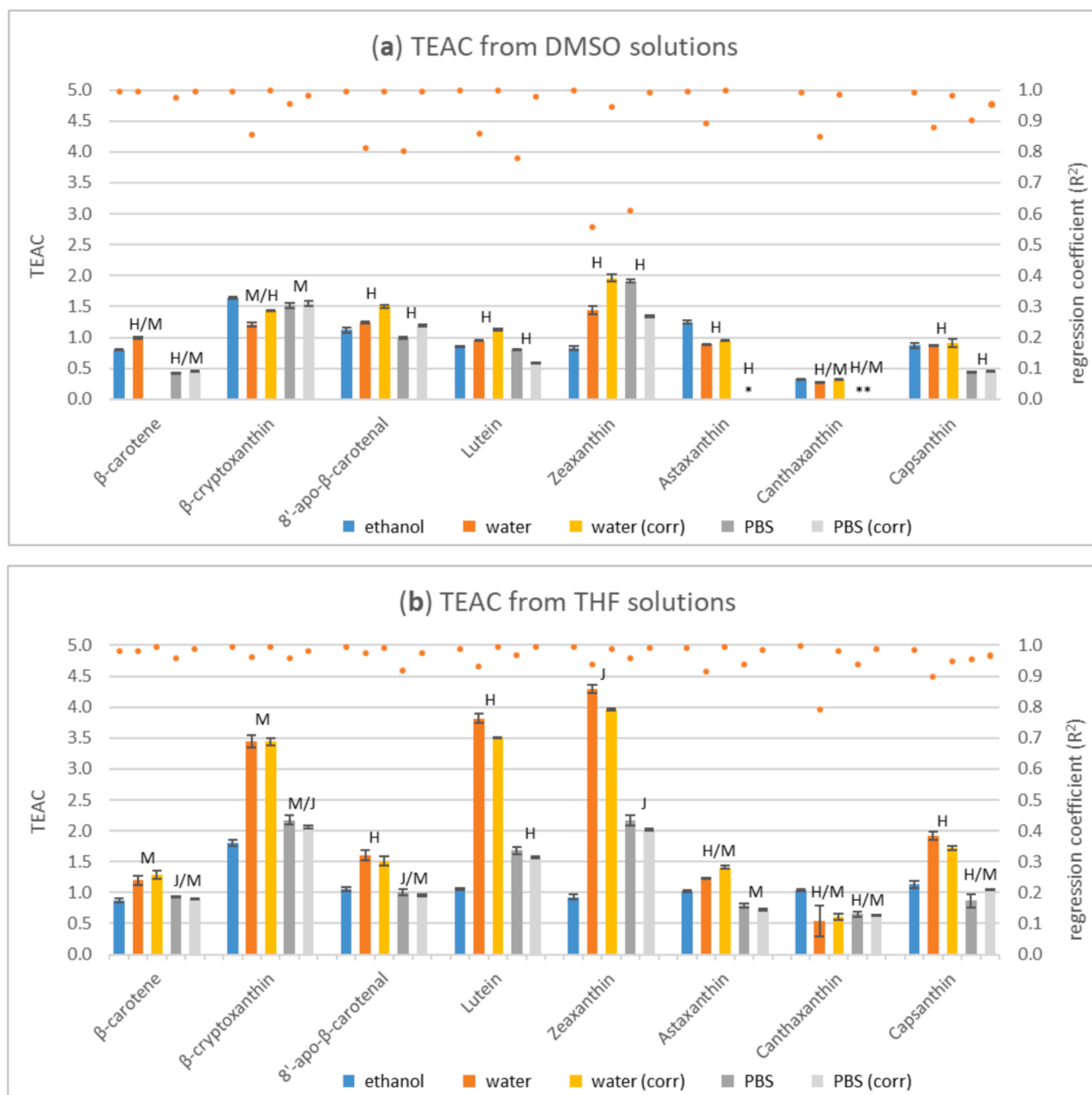


Fig. 5. TEAC values of carotenoids (bars) and regression coefficients (points) for the linear equation of percentage inhibition-concentration plot. (a) from DMSO (b) from THF stock solutions, diluted with ethanol, water, or PBS. (corr): corrected value calculated by neglecting the exceptional concentration giving outlier % inhibition. H: H-type (card-pack), J: J-type (head-to tail), M: mixed aggregate on the basis of UV-vis and ECD spectra. * TEAC can not be determined because of precipitation, or ** non-linear concentration dependence.

intermolecular interactions between the aggregated carotenoids was not considered. However, a study on the photoinduced electron transfer of Langmuir-Blodgett films of 7'-apo-7'-(4-carboxyphenyl)- β -carotene found an efficacious electron transfer both inside and between aggregated carotenoid layers [58].

The energy levels of the frontier molecular orbitals are known to be influenced by the secondary interactions with the surrounding molecules [59]. It is related to the antioxidant behaviour, since the oxidation potential of a carotenoid is associated with its HOMO energy level: the lower it is, the higher the oxidation potential. Polar surroundings, such as water molecules lower the oxidation potential according to theoretical calculations by Gao *et al* [3].

On the basis of our results and the above literature we assume that aggregation of carotenoids influences the energy levels of the frontier

molecular orbitals by reducing the contact between water and carotenoids, as well as by enhancing π - π stacking interactions between carotenoids. Both should result in lower HOMO energy levels and higher oxidation potential. Therefore, the oxidation potential for the $\text{Car}^{+}/(\text{Car})_n$ may be higher at low n values than that for the $\text{Car}^{+}/\text{Car}$ system. The close proximity of molecules in the aggregates may stabilize the formed radical by an intermolecular manner. On the other hand, aggregation does hide the carotenoid molecules, so it has two opposite effects on reactivity. Aggregates of ca. 10–20 nm in size seem to be optimal, which means 5–7 assembled molecules [60], to exhibit high oxidation potential and elevated antioxidant activity by one-electron transfer mechanism.

H-bonding donor ability between the aggregated molecules may be of particular interest, as the enormous elevation in TEAC values were

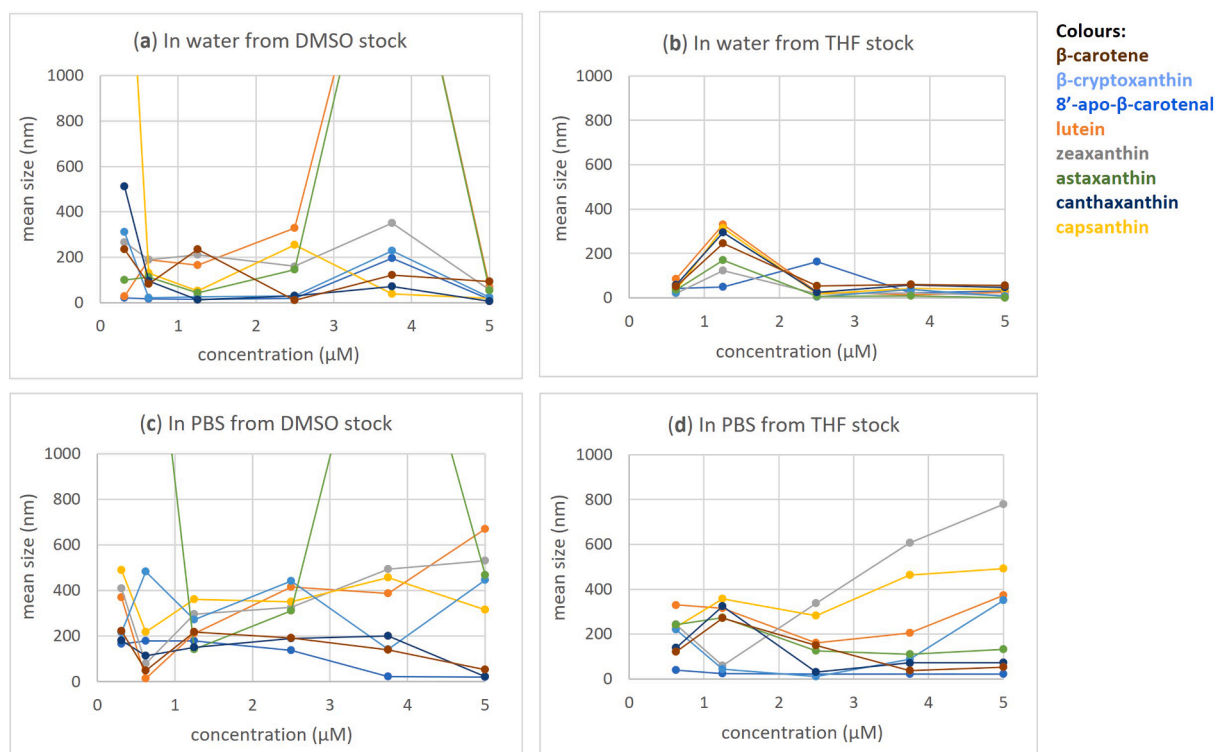


Fig. 6. Hydrodynamic diameter of the aggregates determined by DLS at different concentrations (a) from DMSO stock in water, (b) from THF stock in water, (c) from DMSO stock in PBS, (d) from THF stock in PBS solutions (for sd and PDI values, see Supplement).

observed with the 3-hydroxy- β -end group carotenoids (β -cryptoxanthin, lutein, zeaxanthin, capsanthin).

3.5. Suggestion for standardization of the ABTS-TEAC protocol for carotenoids

The ABTS reagent can be prepared many different ways. We found the method by Re *et al.* simple, appropriate and reproducible [34], i.e. the generation of $\text{ABTS}^{\cdot+}$ radical with the reaction of ABTS and potassium persulfate in aqueous solution of high concentration (see Section 2.3.). The concentrated $\text{ABTS}^{\cdot+}$ solution should be diluted just before use by the chosen main solvent. The use of 96 % ethanol as a main solvent gives information about the inherent one-electron-transfer antioxidant capacity of the individual carotenoid molecules. We suggest using THF (free from stabilizers) as auxiliary solvent, since it dissolves perfectly carotenoids of diverse polarities. The redox reaction between $\text{ABTS}^{\cdot+}$ and antioxidants may follow different kinetics. To promote the completion of the reaction and avoid underestimation of antioxidant power, we suggest using slightly elevated temperature (37 °C) and a relatively long reaction time (6 min). Among these conditions, the percentage inhibitions give linear concentration dependence, and the TEAC values can be calculated from the slopes as $\text{TEAC} = m_{(\text{carotenoid})} / m_{(\text{trolox})}$.

By diluting the $\text{ABTS}^{\cdot+}$ solution with phosphate-buffered saline one can obtain a reaction medium that imitates physiological conditions, such as cytoplasm, however in this solution carotenoids form aggregates having very different antioxidant behaviour than the individual molecules. The aggregation of carotenoids is very sensitive for the applied auxiliary solvent and final concentration and it can not be predicted for actual biological systems, where the presence of other lipids, proteins and carbohydrates certainly has an additional influence. For this reason, we think that using ethanol as a main solvent is more expedient to characterize and compare the antioxidant power of carotenoids. Nevertheless, by a controlled manner of sample preparation, the ABTS-TEAC method in aqueous solutions can be a useful tool for the

characterization of the antioxidant behaviour of carotenoid nanoparticles.

4. Conclusion

The aggregation of carotenoids is a well-studied area, nevertheless, to our best knowledge this is the first systematic investigation on the effect of aggregation on the antioxidant behaviour of carotenoids. In aqueous systems, aggregates are formed not only at concentrations associated with a low percentage inhibition, but at virtually all concentrations. Larger aggregates, up to a few hundred nanometers, can form at the outlier percentage inhibitions, in the other cases the particles are of lower size. The measured TEAC values therefore do not characterize the individual molecules, but the colloidal systems formed from them, whose structure (size) is strongly dependent on the method of production.

The percentage inhibition seems to be primarily depending on the particle size, which is influenced by both the auxiliary and the main solvent. The different auxiliary solvents have also a strong effect on the type of aggregation even in identical main solvents.

Should one intend the TEAC value of the individual carotenoid molecule we suggest performing the measurements in EtOH as main solvent. The auxiliary solvent in this case does not cardinaly alter the results, however, solubility issues do not appear using THF.

The one-electron transfer antioxidant behaviour of carotenoids in biological systems may be more relevantly modelled in PBS. When choosing this main solvent, however, one must be aware of the aggregation of carotenoid molecules. Although DMSO is a frequently used auxiliary solvent in biological studies, in our hands the examined keto-carotenoids gave inappreciable result starting from DMSO stock in PBS (Fig. 5). Using THF auxiliary solvent in PBS such problems did not arise. Canthaxanthin and capsanthin from both auxiliary solvents showed similar aggregate sizes in PBS (Fig. 6), despite the fact that TEAC values could not be determined from DMSO for canthaxanthin. Thus, for these keto-carotenoids the use of THF in PBS seems to be more appropriate in

TEAC determination.

The use of THF as auxiliary solvent in pure water results in the formation of small and uniform carotenoid assemblies with extraordinarily high antioxidant activities. This finding may initiate further studies on these aggregates to reveal the exact mechanism behind of the unusual antioxidant behaviour and broaden the practical application of carotenoid nanoparticles of similar size.

CRedit authorship contribution statement

Dalma Czett: Writing – original draft, Investigation, Formal analysis, Data curation. **Veronika Nagy:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Methodology, Formal analysis, Conceptualization. **Tibor Kurtán:** Funding acquisition, Formal analysis. **Sándor Balázs Király:** Investigation, Formal analysis, Data curation. **Péter Szabó:** Investigation, Data curation. **Attila Agócs:** Writing – review & editing, Funding acquisition. **József Deli:** Funding acquisition. **Katalin Bóddi:** Writing – original draft, Investigation, Formal analysis, Data curation.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Attila Agócs reports financial support was provided by Hungarian Scientific Research Fund. József Deli reports financial support was provided by Hungarian Scientific Research Fund. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.molliq.2025.126870>.

Data availability

We have shared the data in the [Supplementary Material](#).

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