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Reactivity of β-carotene towards peroxyl radicals studied by laser flash and steady-state photolysis

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Abstract Peroxyl radicals, as model for peroxyl radicals formed during autoxidation of lipids, have been generated in three solvent systems (cyclohexane, tetrahydrofuran and *tert*-butanol/water) by steady-state and laser flash photolysis, and their reaction with β-carotene studied. Steady-state photolysis experiments showed that alkyl, alkoxyl and alkylperoxyl radicals all react with β-carotene. However, laser flash photolysis experiments indicated that the reaction with peroxyl radicals (second-order rate constant estimated to be less than 10^6 M^{-1} s^{-1}) is slower than with alkyl and alkoxyl radicals, and that β-carotene is hence a poor direct scavenger of peroxyl radicals. Scavenging of peroxyl radicals by β-carotene is suggested not to proceed via electron transfer but rather by adduct formation and/or hydrogen abstraction. For different phenoxyl radicals, differences in reactivity towards β-carotene seem to be correlated with standard reduction potential.

Key words: β-carotene; Peroxyl radical; Photolysis; Laser flash photolysis

1. Introduction

During the course of autoxidation of lipids, lipid peroxyl radicals are generated as relatively short-lived intermediates [1]. Lipid peroxyl radicals, LOO·, may decay by self-reaction to generate an unstable tetroxide, LOOOOL, or by abstraction of an allylic or bisallylic hydrogen from another lipid. While the self-reaction terminates the free radical chain reaction, the reaction with another lipid is chain-carrying as it generates even shorter-lived carbon-centered radicals which further react with oxygen. The lipid peroxyl radical present in a steady-state concentration during lipid peroxidation may also react with an antioxidant. Vitamin E is the most important lipid-soluble scavenger of peroxyl radicals reacting with most alkylperoxyl radicals with a second-order rate constant on the order of 10^6–10^7 M^{-1} s^{-1} [2]. Carotenoids are another important class of lipid-soluble antioxidants. Many studies have shown that carotenoids inhibit autoxidation of lipid systems, though prooxidative behavior of carotenoids has been observed as well. It has been proposed that carotenoids scavenge peroxyl radicals by forming a chain-carrying peroxyl-carotenoid adduct [3], and that this adduct may react with oxygen like the carbon-centered lipid radical. This chain-carrying carotenoid-peroxyl adduct may be the reason why carotenoids can act as prooxidants at elevated oxygen pressures [3], while at lower oxygen pressures the carbon-centered carotenoid radical undergoes reaction with other radicals to form non-radical products. Mechanistic studies of scavenging peroxyl radicals by carotenoids are scarce: so far only the reaction with the trichloromethylperoxyl radical has been examined in detail [4,5]. One of these studies [5] seemed to indicate that carotenoids scavenged the trichloromethylperoxyl radical both by forming an adduct and by electron transfer to generate a peroxide anion and a carotenoid radical cation. The pattern of parallel electron transfer and adduct formation has also been observed for the reaction between phenoxyl radicals and β-carotene [6].

We have undertaken a study of the ability of β-carotene to scavenge alkylperoxyl radicals in homogenous solutions ranging from apolar, lipid-like (cyclohexane), moderately polar (tetrahydrofuran) to strongly polar (*tert*-butanol/water) by employing laser flash and steady-state photolysis. The rationale behind varying the solvent is that there are many conflicting reports on the peroxyl radical scavenging ability of carotenoids, and we wanted to test whether differences in solvent polarity could be an explanation.

2. Materials and methods

β-Carotene (98%) was supplied by Roche A/S (Hvidovre, Denmark) sealed in ampoules under argon. Cyclohexane (LiChrosolv), benzene (p.a.), phenol (p.a.) and *tert*-butanol from Merck (Darmstadt, Germany), di-*tert*-butylperoxide, *o*-cresol and *p*-cresol from Merck-Schuchardt (Hohenbrunn bei München, Germany), and tetrahydrofuran (HPLC grade) from Sigma-Aldrich (Steinheim, Germany) were all used as received.

Solutions were made up of 0.10 M di-*tert*-butylperoxide in either cyclohexane, tetrahydrofuran or *tert*-butanol/water (70:30, v/v) and with or without 10 μM β-carotene. In the case of the *tert*-butanol/water solvent system, β-carotene was dissolved in benzene (1.00 mM) and added to the solvent (1%). Oxygen was removed from some of the samples by three freeze-pump-thaw cycles.

Laser flash photolysis experiments were carried out with an LKS.50 laser flash photolysis spectrometer from Applied Photophysics Ltd. (Leatherhead, UK). The fourth harmonic at 266 nm or the third harmonic at 355 nm of a pulsed Q-switched Nd-YAG laser, Spectron Laser Systems (Rugby, UK), was used for excitation. The intensity of the laser pulse was approximately 25 mJ (266 nm) and 55 mJ (355 nm) and the duration of the pulse was around 8 ns. A 1P28 photomultiplier tube from Hamamatsu (Hamamatsu City, Japan) was used to detect transient absorption. UV cut-off filters were used when appropriate to minimize degradation of the sample by the monitoring light. Spectral slit widths were typically 4–5 nm. The samples were excited in 1 cm × 1 cm fluorescence cells from Hellma (Müllheim, Germany). All samples were thermostated at 20.0 ± 0.5°C. Solutions were used the same day they were prepared.

Steady-state photolysis was performed by exciting the samples in 1 cm × 1 cm fluorescence cells with 266 nm light from a 150 W xenon lamp selected by a monochromator. Decay of β-carotene was followed by UV-VIS absorption spectroscopy with an HP 8452 diode array spectrometer (Hewlett Packard, Palo Alto, CA) on an optical axis perpendicular to the excitation axis in the photochemical unit previously described [7]. The samples were kept at 10 ± 1°C during photolysis in order to minimize the extent of thermal degradation. The

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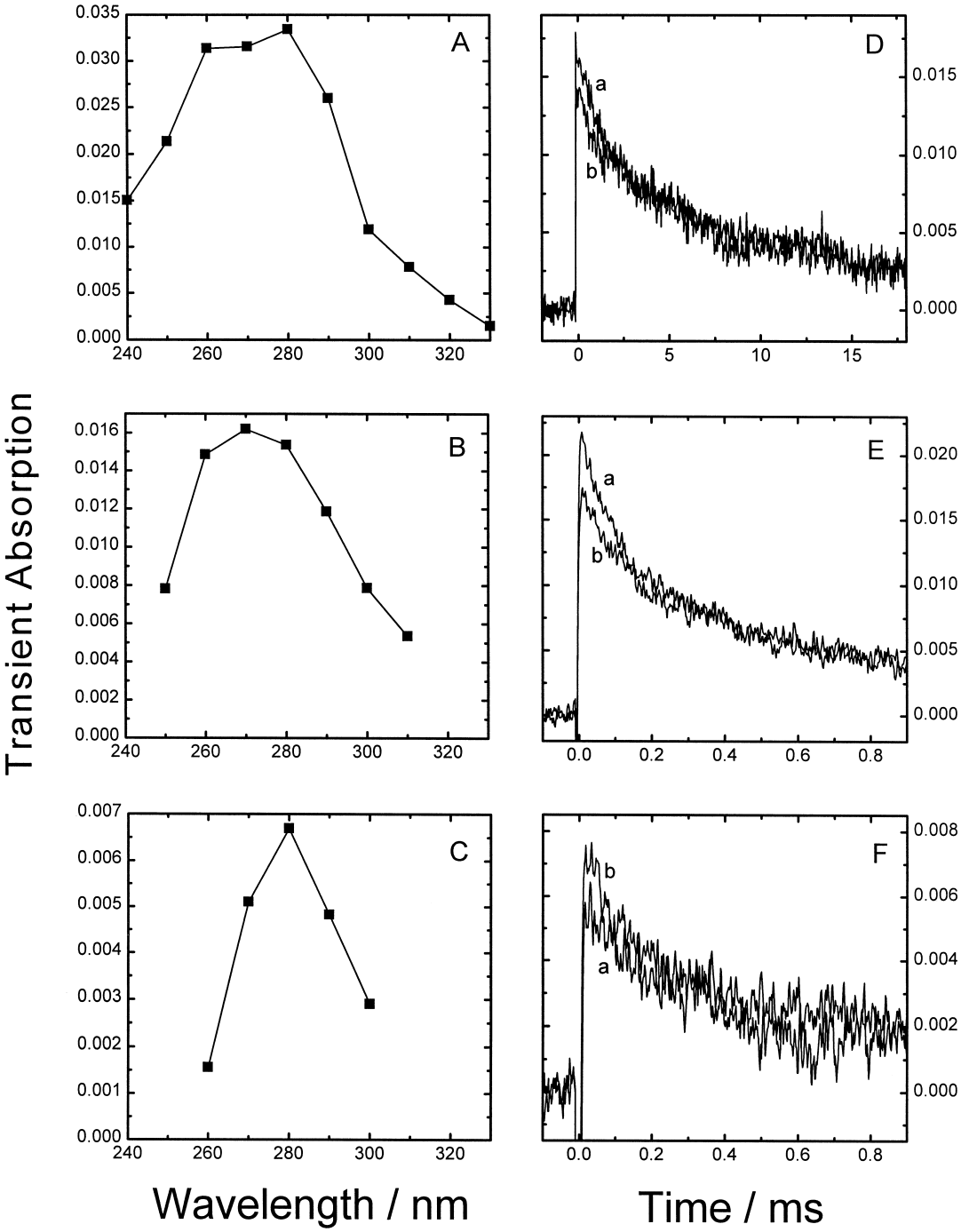

Fig. 1. Transient absorption spectra of (A) the cyclohexylperoxyl, (B) tetrahydrofuranperoxyl, and (C) the *tert*-butanolperoxyl radicals 9 μs (A) and 40 μs (B and C) after the laser pulse obtained by laser flash photolysis at 266 nm of 0.1 M di-*tert*-butylperoxide in cyclohexane (A), tetrahydrofuran (B), and *tert*-butanol/water (7:3, v/v). Time traces of decay of (D) the cyclohexylperoxyl (300 nm), (E) tetrahydrofuranperoxyl (280 nm), and (F) the *tert*-butanolperoxyl (280 nm) radicals in the presence (a) or absence (b) of 10 μM β-carotene.

samples were in contact with the atmosphere through small holes in the lid of the cuvette or were degassed by three freeze-pump-thaw cycles and kept free of oxygen during photolysis.

3. Results

Photolysis of di-*tert*-butylperoxide at 266 nm generates the *tert*-butoxyl radical

$$(CH_3)_3COOC(CH_3)_3 \rightarrow 2(CH_3)_3CO\cdot \quad (1)$$

The *tert*-butoxyl radical abstracts hydrogen from the solvent (cyclohexane, tetrahydrofuran or *tert*-butanol)

$$(CH_3)_3CO\cdot + RH \rightarrow (CH_3)_3COH + R\cdot \quad (2)$$

The rate constant of this reaction is typically 10^6–10^7 M^{-1} s^{-1} for aliphatic compounds [8], and the *tert*-butoxyl radical

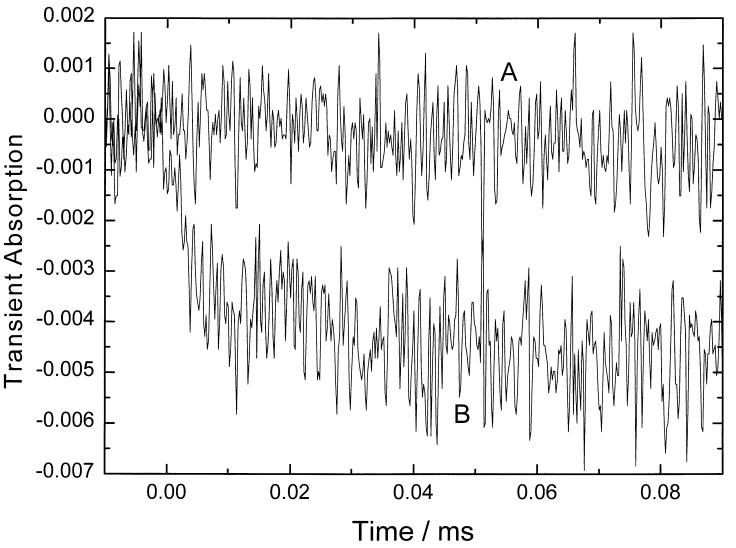

Fig. 2. Time traces of 10 μM β-carotene and 0.1 M di-*tert*-butylperoxide in (A) aerated cyclohexane or (B) aerated *tert*-butanol/water (7:3, v/v) obtained by laser flash photolysis at 266 nm.

is hence completely quenched in less than 1 μs by the solvent.

In the presence of oxygen, peroxyl radicals will be formed at a close to diffusion-controlled rate ($k = 10^9$ M^{-1} s^{-1}) [9,10]

$$R^{\cdot} + O_2 \rightarrow ROO^{\cdot}. \quad (3)$$

The cyclohexylperoxyl, tetrahydrofuranperoxyl and *tert*-butanolperoxyl radicals are thus the dominant radical species during a nanosecond laser flash photolysis experiment in air-saturated solvents.

3.1. Laser flash photolysis experiments generating peroxyl radicals

In Fig. 1 are shown the transient spectra of the cyclohexylperoxyl, tetrahydrofuranperoxyl and *tert*-butanolperoxyl radicals. The spectrum of the cyclohexylperoxyl radical is in good agreement with a published spectrum [11]. The peroxyl radicals decay by second-order kinetics

$$2ROO^{\cdot} \rightarrow \text{products}. \quad (4)$$

Reaction between alkylperoxyl radicals and saturated compounds is usually very slow (the second-order rate constant of reaction between the highly reactive trichloromethylperoxyl radical and cyclohexane is 10^3 M^{-1} s^{-1} [2]) and may thus completely be neglected compared to the reaction in Eq. 4.

In the presence of β-carotene, the decay of the peroxyl radical is expected to be faster if it reacts with β-carotene

$$ROO^{\cdot} + \beta - car \rightarrow \text{intermediate} \quad (5)$$

and bleaching of β-carotene would take place. However, as the time traces in Fig. 1 show, the decay of the peroxyl radicals is unaffected by the presence of β-carotene in the three solvents. In accordance with this, no bleaching of β-carotene is observed except in the case of the *tert*-butanol/water mixture (Fig. 2). The bleaching, however, is on a much shorter time scale (μs) than the decay of the peroxyl radical (ms) and must hence be due to another free radical, possibly a carbon-centered solvent radical or the *tert*-butoxyl radical. This bleaching is also observed in the absence of oxygen, further showing that it is not due to peroxyl radicals. The alkyl and *tert*-butoxyl radicals formed in the three solvents in the absence of oxygen decayed on a microsecond time scale and were found not to react with β-carotene, except in the *tert*-butanol/water mixture.

3.2. Steady-state photolysis experiments

Steady-state photolysis at 266 nm of β-carotene in aerated cyclohexane for several hours leads only to very modest bleaching (Fig. 3). The decay is zero-order for the steady-state photolysis conditions used with a rate constant of 64 ± 1 nM h^{-1} at 10°C, at least for the initial photoconversion. In the presence of 0.10 M di-*tert*-butylperoxide, β-carotene is bleached much faster during photolysis (Fig. 3). The bleaching of β-carotene follows first-order kinetics with a rate constant of 0.203 ± 0.003 h^{-1} at 10°C. In the presence of di-*tert*-butylperoxide but in the absence of oxygen, the bleaching of β-carotene also follows first-order kinetics with a rate constant of 0.125 ± 0.001 h^{-1} for otherwise identical conditions. These rate constants were obtained employing the same light intensity, but should only be discussed relative to each other as they will depend on the light intensity.

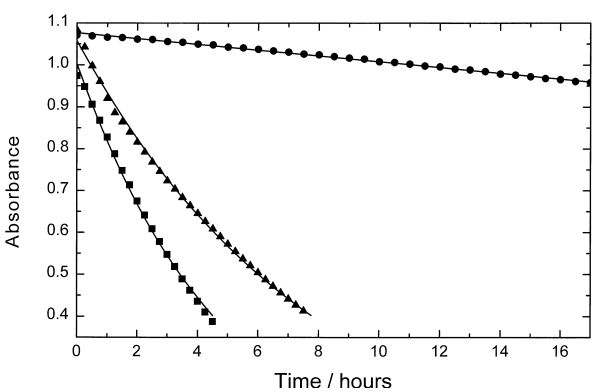

Fig. 3. Bleaching of 10 μM β-carotene in aerated (■ and ●) or deaerated (▲) cyclohexane irradiated at 266 nm in the presence (■ and ▲) or absence (●) of 0.1 M di-*tert*-butylperoxide. The solid curves are first-order (■ and ▲) and zero-order (●) fits, respectively, to the kinetic data.

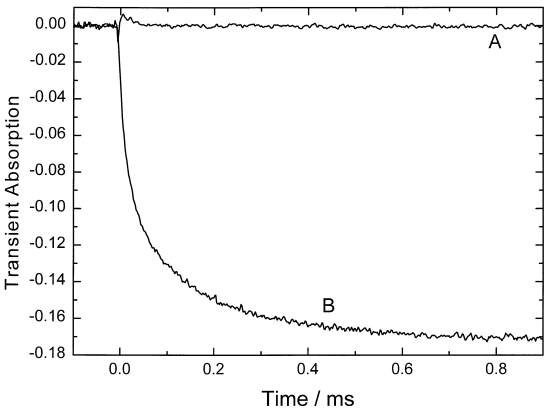

Fig. 4. Time traces of bleaching at 505 nm of 30 μM β-carotene and 1.75 M *o*-cresol (A) or 1.75 M phenol (B) in di-*tert*-butylperoxide/benzene (7:3, v/v) after laser flash photolysis at 355 nm.

3.3. β-Carotene reactivity towards aryloxyl radicals

Laser flash photolysis of di-*tert*-butylperoxide in the presence of a large excess (1.75 M) of a phenol leads to rapid formation of aryloxyl radicals [6]

$$(CH_3)_3CO^{\cdot} + PhOH \rightarrow (CH_3)_3COH + PhO^{\cdot} \quad (6)$$

The phenoxyl radicals can decay by self-reaction or by reacting with β-carotene which leads to rapid bleaching as has been previously demonstrated [6]. However, *o*- and *p*-cresoxyl radicals do not react with β-carotene under similar conditions as shown in Fig. 4. Only a weak positive transient absorption at short times due to the cresoxyl radical is observed (Fig. 4).

4. Discussion

The laser flash photolysis results seem to indicate that β-carotene does not react with peroxyl radicals (Fig. 1). On the other hand, steady-state photolysis generating peroxyl radicals clearly shows an increased bleaching of β-carotene compared to photolysis under anaerobic conditions where peroxyl radicals are not formed (Fig. 3). There are two possible explanations to this apparent discrepancy. It could be that peroxyl radicals do react with β-carotene but at such a slow rate compared to the reaction in Equation 4 that the reaction cannot be observed by laser flash photolysis. However, steady-state photolysis would generate a high number of peroxyl radicals whereof a small proportion would react with β-carotene giving rise to slow bleaching (Fig. 3). Alternatively, the presence of other radicals, i.e. solvent-derived alkyl and *tert*-butoxyl, could be the reason why β-carotene is bleached. Fig. 3 clearly shows that in the absence of oxygen where only cyclohexyl and *tert*-butoxyl radicals are generated, β-carotene in cyclohexane is bleached. In aerated solutions, these radicals would be at a very low concentration a few microseconds after the laser flash, and would hence not be expected to give an appreciable bleaching of β-carotene by a single flash. However, during prolonged photolysis they may be due to the high total number of radicals generated. The higher rate of bleaching in aerated compared to deaerated solutions (Fig. 3) indicates that β-carotene also does react with peroxyl radicals. However, the higher rate does not imply that β-carotene reacts faster with the peroxyl radicals than with the more reactive alkyl and *tert*-butoxyl radicals. Alkyl and *tert*-butoxyl radicals disappear by self-reaction in a few microseconds (diffusion-controlled) after a laser flash whereas peroxyl radicals disappear in a few milliseconds (Fig. 1). Thus, a smaller proportion of alkyl and *tert*-butoxyl radicals than peroxyl radicals are available and react with β-carotene leading to an apparently higher rate of bleaching in the presence of oxygen. The reason why bleaching of β-carotene in *tert*-butanol/water but not in cyclohexane or tetrahydrofuran is observed by laser flash photolysis (Fig. 2) could be the higher viscosity of the former solvent which would give a slower rate of formation of peroxyl radicals, and hence a longer lifetime of the more reactive alkyl and *tert*-butoxyl radicals.

The extinction coefficient of the cyclohexylperoxyl radical is $1–2\times10^3$ M^{-1} cm^{-1} [11]. The concentration produced by one laser flash is thus less than 30 μM (Fig. 1). The second-order rate constant of decay of the cyclohexylperoxyl radical is $2k = 10^7$ M^{-1} s^{-1} [11]. This implies that if the rate constant of reaction of peroxyl radicals and β-carotene is less than roughly 10^6 M^{-1} s^{-1}, reaction between peroxyl radicals and β-carotene could not be observed by laser flash photolysis. The rate constant of reaction between β-carotene and the trichloromethylperoxyl radical is 1.5×10^9 M^{-1} s^{-1} [4]. However, this radical is much more reactive than simple alkylperoxyl radicals: the reduction potential of $CCl_3OO^{\cdot}$ is 1.44 V whereas that of alkylperoxyl radicals is 0.7–0.9 V [12] in aqueous solution, and the trichloromethylperoxyl radical is thus not a good model compound for a lipid peroxyl radical. The reduction potential of the phenoxyl radical is 0.79 V whereas the *p*-cresoxyl radical has a reduction potential of 0.68 V [13] (the reduction potential of the *o*-cresoxyl radical is probably similar to that of the *p*-cresoxyl radical). By analogy, these results thus indicate that β-carotene does not react with alkylperoxyl radicals by electron transfer due to a rather low reduction potential of the peroxyl radicals. However, β-carotene may possibly scavenge peroxyl radicals by two other mechanisms: by forming an adduct, as originally suggested [3], or by hydrogen atom transfer. Based on product analysis studies it has thus been suggested that β-carotene indeed does scavenge alkyl, alkoxyl, and alkylperoxyl radicals by both of these mechanisms [14]. The bond dissociation energy of the most labile hydrogen in β-carotene can be estimated as 309 kJ/mol [15] whereas the bond dissociation energy of ROO-H is around 370–380 kJ/mol. Hydrogen atom transfer from β-carotene to peroxyl radicals is thus thermodynamically feasible. It may be that electron transfer is inherently faster than the other two quenching mechanisms. β-Carotene may thus scavenge radicals with a high reduction potential like the phenoxyl and trichloromethylperoxyl radicals rapidly by electron transfer whereas radicals, like alkylperoxyl radicals, with a lower reduction potential are scavenged more slowly by other mechanisms (hydrogen atom transfer and/or adduct formation). This would explain why we see no reaction between peroxyl radicals and β-carotene and between cresoxyl radicals and β-carotene in our laser flash photolysis experiments.

Our results clearly demonstrate that β-carotene is not an efficient scavenger of alkylperoxyl radicals (like lipid peroxyl radicals): the second-order rate constant is estimated to be less than 10^6 M^{-1} s^{-1}. This should be compared to the reactivity of α-tocopherol towards alkylperoxyl radicals ($k = 10^6$–10^7 M^{-1} s^{-1}, [2]). The role of carotenoids as a lipid peroxyl radical scavenger in lipid systems where both tocopherols and carotenoids are present may thus be expected to be minimal

considering the fact that α-tocopherol scavenges peroxyl radicals faster than β-carotene and the fact that α-tocopherol is usually present in a much higher concentration than β-carotene in most lipid systems. However, carotenoids may still have an antioxidant effect in a synergistic action with tocopherols [16–18]. β-Carotene is thus a prooxidant in oil and an oil-in-water emulsion [19,20] whereas a combination of β-carotene and tocopherol is more efficient in retarding lipid peroxidation than tocopherol alone [19,20].

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