

# Antioxidant activities of carotenes and xanthophylls

Nicholas J. Miller<sup>a</sup>, Julia Sampson<sup>a</sup>, Luis P. Candeias<sup>b</sup>, Peter M. Bramley<sup>c</sup>,  
Catherine A. Rice-Evans<sup>a,\*</sup>

<sup>a</sup>Free Radical Research Group, UMDS-Guy's Hospital, St Thomas's Street, London SE1 9RT, UK

<sup>b</sup>Gray Laboratory, Cancer Research Trust, Mount Vernon Hospital, Northwood, Middlesex HA6 2YR, UK

<sup>c</sup>Division of Biochemistry, School of Biological Sciences, Royal Holloway, University of London, Egham, Surrey TW20 0EX, UK

Received 8 March 1996

**Abstract** The purpose of this study was to assess the relative antioxidant activities of a range of carotenes and xanthophylls through the extent of their abilities to scavenge the ABTS<sup>•+</sup> radical cation. The results show that the relative abilities of the carotenoids to scavenge the ABTS<sup>•+</sup> radical cation are influenced by the presence of functional groups with increasing polarities, such as carbonyl and hydroxyl groups, in the terminal rings, as well as by the number of conjugated double bonds.

**Key words:** Lycopene; Carotene; Xanthophyll; ABTS<sup>•+</sup>; Radical cation; Radical scavenger

## 1. Introduction

Many studies have implicated  $\beta$ -carotene as an antioxidant in vivo although there is little direct evidence to support this as yet. In contrast, there is considerable in vitro evidence for interaction of  $\beta$ -carotene with free radicals [1–5], for its properties as a chain-breaking antioxidant [2] and in scavenging and quenching singlet oxygen [6–9].  $\beta$ -Carotene has also been reported to protect against photosensitivity diseases in humans [10]. However, it offers little protection against metal-catalysed LDL oxidation ex vivo [11,12] although the carotenoids within the LDL are consumed on oxidation with Cu<sup>2+</sup>, the sequence of activity being lycopene >  $\beta$ -cryptoxanthin > lutein/zeaxanthin >  $\alpha$ , $\beta$ -carotene, as deduced from rates of consumption during 1 h oxidation [13].

Carotenoids are known to be reactive towards singlet oxygen, lycopene being the most efficient quencher in vitro [14,15], but very few mechanistic studies on their radical scavenging properties have been carried out. The interaction of  $\beta$ -carotene with peroxy radicals generated during lipid peroxidation has been suggested to lead to formation of a radical adduct [ $\beta$ -car...RO<sub>2</sub>]<sup>•</sup> [16]. Mechanisms of free radical scavenging have been investigated [17] by pulse radiolytic generation of nitrogen dioxide radicals (NO<sub>2</sub><sup>•</sup>), thiyl radicals (RS<sup>•</sup>) and sulphonyl radicals (RSO<sub>2</sub><sup>•</sup>), which are rapidly scavenged by  $\beta$ -carotene. The mechanism for the scavenging of the NO<sub>2</sub><sup>•</sup> radical has been described as that of electron donation with radical cation formation [ $\beta$ -carotene]<sup>•+</sup> whereas the scavenging of thiyl radicals involves the generation of a radical adduct [ $\beta$ -car...RS]<sup>•</sup>. The radical cation and adduct radicals are highly resonance stabilised and appear to undergo slow bimolecular decay to non-radical products. Other studies have described the interaction between the trichloromethyl peroxy radical and the carotenoids  $\beta$ -carotene, canthaxanthin, astaxanthin, zeaxanthin and lutein, producing a combination of

carotenoid radical cation and radical adduct, with no distinction between the reactivities of the different carotenoids, except for astaxanthin participating exclusively in radical addition [18].

The purpose of this study was to delineate the differential antioxidant activities of a range of carotenes and xanthophylls of dietary origin through the extent of their abilities to scavenge the ABTS<sup>•+</sup> radical cation. The results show that the relative abilities of the carotenoids to scavenge the ABTS<sup>•+</sup> radical cation are influenced by the presence of functional groups with increasing polarities, such as carbonyl and hydroxyl groups, in the terminal rings as well as by the number of conjugated double bonds. Overall, the carotenes with 11 conjugated double bonds are more active ABTS<sup>•+</sup> radical quenchers than the xanthophylls, with the exception of  $\beta$ -cryptoxanthin.

## 2. Materials and methods

### 2.1. Antioxidant activity

ABTS<sup>•+</sup> radical cation was prepared by passing a 5 mM aqueous stock solution of ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid diammonium salt) (Aldrich Chemical Co.) through manganese dioxide (Sigma-Aldrich) on a Whatman no. 5 filter paper. Excess manganese dioxide was removed from the filtrate by passing it through a 0.2  $\mu$ M Whatman PVDF syringe filter. This solution was then diluted in 5 mM phosphate buffered saline (PBS) pH 7.4 to an absorbance of 0.70 ( $\pm$ 0.02) at 734 nm and pre-incubated at 30°C prior to use. Fresh ABTS<sup>•+</sup> radical cation solution was prepared each day.

Trolox (Hoffman-La Roche) (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (Aldrich Chemical Co.) was used as an antioxidant standard as described previously for the ferryl myoglobin/ABTS assay [19,20]. 2.5 mM Trolox was prepared in PBS for use as a stock standard. At this pH and concentration, the upper limit of the solubility of Trolox in PBS is approached and gentle ultrasonication is required to dissolve the crystals. Fresh working standards were prepared daily by diluting 2.5 mM Trolox with PBS.

All carotenoids were obtained from Hoffman-La Roche except for astaxanthin which was a gift from P.B. Zagalsky. The carotenoids were shown by HPLC to be at least 97% pure except for astaxanthin at 100%. Carotenoids were dissolved in acetone and then diluted to a concentration of ca. 100  $\mu$ M in 90% hexane/10% acetone. The exact concentration of each working solution was derived from the molar absorptivity at the specific absorbance maximum of each carotenoid between 444 nm and 480 nm. After addition of 1.0 ml of ABTS<sup>•+</sup> solution to aliquots of Trolox or the carotenoids (20–300  $\mu$ l, depending on the activity of the particular compound) the solutions were vortex mixed for exactly 30 s; the absorbance at 734 nm was taken exactly 1 min after initiation of mixing in a Beckman spectrophotometer with Peltier electronics set at 30°C. PBS blanks and hexane/acetone blanks were run in each assay. The dose-response curve for Trolox (final concentration 0–25  $\mu$ M) consisted of plotting the absorbance at 734 nm as a percentage of the absorbance of the uninhibited radical cation (blank) and was based on triplicate determinations. The activities of the carotenoids were assessed at a minimum of three different concentrations which had been determined to be within the

\*Corresponding author. Fax: (44) (171) 955 4983.

Table 1

Molar extinction coefficients and absorbance maxima of the carotenes and xanthophylls [21]

Carotenoid	Absorbance maximum (nm)	Molar extinction coefficient (cm <sup>-1</sup> M <sup>-1</sup> )
Astaxanthin	482	106.0 <sup>1</sup>
$\alpha$ -Carotene	448	145.0 <sup>2</sup>
$\beta$ -Carotene	454	134.4 <sup>2</sup>
$\beta$ -Cryptoxanthin	452	131.0 <sup>3</sup>
Canthaxanthin	480	118.2 <sup>1</sup>
Lutein	445	145.1 <sup>4</sup>
Lycopene	474	185.0 <sup>2</sup>
Zeaxanthin	452	133.1 <sup>2</sup>

Solvent key: 1, benzene; 2, acetone; 3, ether; 4, ethanol.

range of the dose-response curve. Each carotenoid was analysed in triplicate at these three concentrations. By reference to the Trolox dose-response curve, the mean Trolox equivalent antioxidant capacity (TEAC) value was derived for each compound. Each compound was assessed on a minimum of 3 separate days (i.e. at least 27 different analyses).

For HPLC analysis, ethyl- $\beta$ -apo-carotenoate was used as the internal standard (Fluka). Samples (50  $\mu$ l aliquots) were chromatographed with an isocratic mobile phase of acetonitrile/acetone/methanol (75:20:5 by volume) (HPLC grade, Rathburn) with a flow rate of 0.7 ml/min. A Beckmann 507 autosampler coupled to a Beckmann photo-diode array detector and a 126 programmable solvent module was used. HPLC analysis was performed with a Nucleosil RP 3 $\mu$  ODS column, 250  $\times$  3 mm (Phenomenex) and the detection wavelength was 450 nm. The amount of carotenoid applied to the HPLC column was calculated using the molar extinction coefficients in Table 1. Recoveries of carotenoids from the column were calculated from carotenoid absorbance at 450 nm in relation to the absorbance at 450 nm of the internal standard. The identification of each carotenoid on HPLC analysis was verified by comparison of the retention time with that of the relative standard compound, as well as by analysis of the spectral profile and with literature values [21]. Carotenoid concentrations in the sample were calculated from calibration curves generated from peak area ratios of the carotenoid to the internal standard.

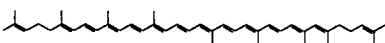
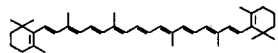

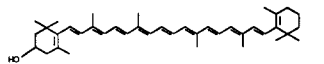
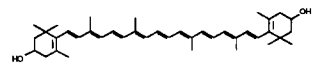
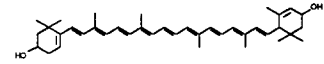
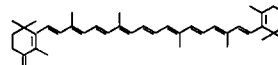
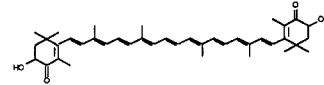
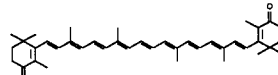
### 3. Results and discussion

The hierarchy of antioxidant activity for the carotenes and xanthophylls in scavenging the ABTS<sup>+</sup> radical cation in comparison with Trolox under the defined conditions is shown in Table 2. The three C-40 carotenes studied show different abilities to scavenge the ABTS<sup>+</sup> radical cation with lycopene ( $\psi,\psi$ -carotene) being more effective than  $\beta$ -carotene ( $\beta,\beta$ -carotene) which in turn was more potent than its isomer  $\alpha$ -carotene ( $\beta,\epsilon$ -carotene). A comparison of their structures shows that both acyclic lycopene (TEAC 2.9 mM) and  $\beta$ -carotene (1.9 mM) have 11 conjugated double bonds, but the latter is bicyclic. However, because of steric hindrance, particularly between the methyl substituent at C-5 of the ring and the hydrogen atom at C-8 of the chain in  $\beta$ -carotene, coplanarity, cannot be achieved and the molecule adopts a preferred conformation in which the rings and the C-5,6 and C-5',6' double bonds are twisted out of plane. Orbital overlap is therefore reduced and the contribution of the ring double bonds to the chromophore is small. In the case of  $\alpha$ -carotene (TEAC 1.3 mM), the double bond of the second ring is not part of the chromophore, leaving 9 conjugated double bonds plus the  $\beta$ -ring. Therefore, it appears that the ability of carotenes to scavenge the ABTS<sup>+</sup> radical cation increases on extension of the chromophore and maximum overlap of the carbon-carbon double bond molecular orbitals.

The presence of functional groups on the terminal rings modulates the radical scavenging activity. The incorporation

of carbonyl groups in the rings has a profound suppressive effect on the ABTS<sup>+</sup> radical scavenging response. For example, echinenone ( $\beta,\beta$ -carotene-4-one) has less than 50% of the antioxidant activity (TEAC 0.7 mM) despite a longer chromophore of 12 conjugated double-bonds. Extension of the chromophore to 13 conjugated double bonds in canthaxanthin ( $\beta,\beta$ -carotene-4,4'-dione) and further hydroxylation as in astaxanthin (3,3'-dihydroxy- $\beta,\beta$ -carotene-4,4'-dione) reduced the ability to scavenge ABTS<sup>+</sup> radicals to TEAC values of 0.02 and 0.03 mM respectively. The electron-withdrawing character of the carbonyl oxygen atoms in echinenone, canthaxanthin and astaxanthin reduces the unpaired electron density in the 11-double-bonded carbon skeleton [22], resulting in a decrease in the reactivity of the hydrogen atoms/elec-

Table 2

Carotenoid	Structure	TEAC mM
Lycopene		2.9 $\pm$ 0.15 [3]
$\beta$ -Carotene		1.9 $\pm$ 0.1 [4]
$\alpha$ -Carotene		1.3 $\pm$ 0.04 [3]
$\beta$ -Cryptoxanthin		2.0 $\pm$ 0.02 [3]
Zeaxanthin		1.4 $\pm$ 0.04 [3]
Lutein		1.5 $\pm$ 0.1 [3]
Echinenone		0.7 $\pm$ 0.2 [3]
Astaxanthin		0.03 $\pm$ 0.03 [3]
Canthaxanthin		0.02 $\pm$ 0.02 [3]

trons on the carbon atoms in carotenoid skeleton with the ABTS<sup>•+</sup> radical cation.

In contrast, the insertion of a single hydroxyl group in the  $\beta$ -ring of the all trans  $\beta$ -carotene, forming  $\beta$ -cryptoxanthin ( $\beta,\beta$ -carotene-3-ol), does not greatly modulate the ability to scavenge the ABTS<sup>•+</sup> radical cation. However, the presence of one hydroxyl group on each of the  $\beta$ -rings as in zeaxanthin ( $\beta,\beta'$ -carotene-3,3'-diol) significantly decreases the antioxidant activity to 1.4 mM. A similar modification of  $\alpha$ -carotene i.e. in lutein ( $\beta,\epsilon$ -carotene-3,3'-diol) had a minor suppressive effect, TEAC 1.4 mM and 1.3 mM for lutein and  $\alpha$ -carotene, respectively. Apparently, the presence of one hydroxyl group at C-3 ( $\beta$ -cryptoxanthin) does not reduce the carotenoid's ability to scavenge ABTS<sup>•+</sup> radical cation but hydroxyls at both C-3 and C-3' (zeaxanthin) results in a significant reduction in activity.

The antioxidant properties of carotenoids have been suggested to reflect not only the rates of free radical scavenging but also the reactivity of the resultant carotenoid-derived radicals. Carotenoid radical-cation or adduct radicals have been shown to be highly resonance stabilised and predicted to be relatively unreactive; they may further undergo bimolecular decay to generate non-radical products or, in the case of carotene radical-adducts, may terminate radical reactions by binding to the attacking free radical [1,17]. In the system described here, the antioxidant activity measured is a marker of the initial reactivity of the carotenoid in its ability to scavenge the ABTS<sup>•+</sup> radical cation and does not take into account the subsequent reactivity or stabilisation of the carotenoid-derived radicals.

The results show that, of the carotenes studied, lycopene scavenges the ABTS<sup>•+</sup> radical cation more extensively than  $\beta$ -carotene, i.e. reduces more radicals at equivalent time points. This is consistent with the observations of others concerning reactivities with singlet oxygen [14,15]. Both overall are more reactive than the xanthophylls (with the exception of  $\beta$ -cryptoxanthin), canthaxanthin and the astaxanthin being relatively unreactive under those conditions. The sequence for relative radical scavenging abilities is lycopene >  $\beta$ -cryptoxanthin  $\approx$   $\beta$ -carotene > lutein  $\approx$  zeaxanthin >  $\alpha$ -carotene > echinenone > canthaxanthin = astaxanthin. The carotenoids studied in this work had antioxidant activities varying from three times that of vitamin E (TEAC = 1.0 mM) [19,23] as for lycopene (TEAC = 2.9 mM) to negligible activity, as in the case of astaxanthin and canthaxanthin.

The properties underlying the activities of carotenoids towards free radicals and their scavenging effects relates particularly to their abilities to donate electrons or hydrogen atoms and to their relative propensities to undergo oxidation. They may also act through forming radical adducts which can undergo bimolecular decay. The findings here demonstrate that,

overall, the carotenes are more efficient quenchers of the ABTS<sup>•+</sup> radical cation than the xanthophylls (with the exception of  $\beta$ -cryptoxanthin) and that this may be influenced by the increasing polarities of the functional groups in the terminal rings.

**Acknowledgements:** Catherine Rice-Evans and Peter Bramley thank the Biotechnology and Biological Sciences Research Council and the Ministry of Agriculture, Fisheries and Food for financial support for their research. Gifts of carotenoids from Roche, Henkel and P.B. Zagalsky are acknowledged with gratitude.

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