Quantitative assessment of antioxidant properties of natural colorants and phytochemicals: carotenoids, flavonoids, phenols and indigoids. The role of β-carotene in antioxidant functions†

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Abstract: Reactive oxygen species are potentially damaging molecules. An important function of antioxidants is to intercept harmful triplet states, in order to prevent the formation of singlet oxygen, or to quench singlet oxygen directly. However, antioxidants are also reactive towards other active oxygen species such as the hydroxyl radical, the superoxide anion and the non-excited oxygen ground state in the presence of radical initiators. It is well known that flavonoids and carotenoids show strong antioxidant properties. Polyenes and carotenoids are the best known among the compounds that quench singlet oxygen by efficient energy transfer. A large number of modified, synthetic analogues and derivatives have been synthesised to prepare even better quenchers than the natural carotenoids. Phenols are also excellent chain-breaking antioxidants. Recently, many indigoid dyes (including bacterial indigoids) were studied, with the remarkable result that most, but not all, members of this class of chromophores quench singlet oxygen at the diffusion limit and some of them are excellent radical traps. It has been shown in this study that a quantitative assessment of antioxidant properties of flavonoids, carotenoids, phenols and natural indigoids can be achieved using the following three assays: (1) oxygen pressure dependence; (2) peroxide formation; (3) singlet oxygen quenching. Reactivities towards both excited states and ground state radicals can be properly described by these assays. The remarkable role of β-carotene as an 'unusual antioxidant' (Burton GW and Ingold KU, *Science* 224: 569–573 (1984)) in reactions using various oxygen pressures becomes clearer. The so-called 'pro-oxidant effects' concern primarily the antioxidant itself and its degradation, since no or very little damage to the substrate occurs in this type of experiment. Three main categories of antioxidants may be classified: (1) excellent antioxidants that perfectly quench excited states as well as ground state radicals (eg actinioerythrol, astaxanthin); (2) good antioxidants that strongly inhibit peroxide formation but are less efficient in quenching excited states (eg flavonols, tocopherols) or lead to considerable degradation of the antioxidant itself (eg β-carotene, lycopene); (3) moderate antioxidants that fail to excel in both reactivities (eg ζ-carotene, flavone).

Keywords: antioxidants; singlet oxygen; oxygen radicals; oxygen pressure; carotenoids; flavonoids; indigoids

INTRODUCTION

More than 600 known carotenoids are widely distributed in nature, particularly in the plant kingdom.[1] Carotenoids exert many important functions,[2] among which are the outstanding antioxidant effects in lipid phases by free radical scavenging or singlet oxygen quenching.[3,4] With regard to antioxidant activity in biological systems, carotenoids appear to be involved

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in protection against both singlet oxygen and triplet oxygen (as radical chain-breaking antioxidants). Carotenoids are most effective biological quenchers of $^1O_2$.[5] Singlet oxygen is known to be capable of damaging DNA[6] and of being mutagenic.[7] Furthermore, singlet oxygen can damage lipids.[8,9] Animal and cell culture studies demonstrate anticarcinogenic and antimutagenic properties of these compounds.[10,11] The underlying mechanisms are yet unknown, but the importance of the carotenoids is at least partially discussed in terms of their antioxidant activity.[12]

Polyenes and carotenoids are the best known among the compounds that quench $^1O_2$ by efficient energy transfer:[13] $^1O_2 + Q \rightarrow {}^3O_2 + {}^3Q$. A large number of modified, synthetic analogues and derivatives have been synthesised in order to prepare even better quenchers than the natural carotenoids β-carotene, canthaxanthin and astaxanthin. Although in these cases the triplet energy of the carotenoid is the determinant, colour can give an indication of the quenching efficiency. The deeply coloured magenta, purple and blue carotenoids and polyenes turn out to be excellent quenchers at the diffusion limit.

Flavonoids constitute a major subclass of antioxidants.[14–16] The quenching rate constants for singlet oxygen of flavonoids and phenols are of the order 10^6–$10^8\,M^{-1}s^{-1}$. They are about one to three orders of magnitude lower than those of carotenoids which can reach the diffusion limit.[17,18] However, the inhibition of radical-induced oxidation of substrates such as cumene or methyl linoleate can be most efficient. Time-resolved studies of $^1O_2$ quenching by phenols in aqueous solution at different pH values show that the reaction occurs via a charge transfer from the phenolate PhO^-.[19] Phenols are also excellent chain-breaking antioxidants and good $^1O_2$ quenchers.[17]

Many indigoid dyes were included in these studies, with the remarkable result that most, but not all, members of this class of chromophores quench $^1O_2$ at the diffusion limit.

EXPERIMENTAL

Singlet oxygen quenching

Synthesis, characterisation and properties of the carotenoids are described in several published articles.[13,20–22] β-Carotene **1b**, canthaxanthin **2b**, astaxanthin **3b** and lycopene **14b** were provided by BASF AG. Rhodoxanthin **15** was generously supplied by Dr A Rüttimann, Hoffmann-La Roche. Methyl linoleate (99%) was obtained from Aldrich, cumene from Merck, DL-α-tocopherol (98%) from Fluka, 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN) from Wako, 3-hydroxyflavone and 7-hydroxyflavone from Aldrich, 5-hydroxyflavone, flavone, apigenin and genistein from Lancaster, and quercetin from Riedel-deHaën. Syntheses of indigoids were described in a previous publication.[23]

All chemicals were stored at −20 °C. Pure solvents (pa) were used as received. The solution of disodium 3,3′-naphthalene-1,4-diyl-dipropionate endoperoxide ($NDPO_2$) in D_2O was stored at −70 °C before use. Singlet oxygen ($^1O_2$) was generated chemically by thermal dissociation of $NDPO_2$. Photosensitised generation of $^1O_2$ was disadvantageous, since considerable photodecomposition of some compounds was observed. In addition, reactions of sensitisers with the quenchers may occur. The rate of phosphorescence at 1270 nm was measured using a germanium diode photodetector (Model EO-817, North Coast Scientific Co, Santa Rosa, CA, USA) cooled with liquid nitrogen. The diode signal was amplified (Model 5205, EG&G, Brookdeal Electronics Princeton Applied Research, Bracknell, Berkshire, UK) and documented with a recorder (BBC Goerz-Servigor 22). At 37 °C, 3 ml ethanol/chloroform (50:50 v/v) was placed in a thermostatted cuvette. Reactions were started by addition of 5 mmol $NDPO_2$ dissolved in D_2O, leading to a solvent mixture containing ethanol/chloroform/D_2O (50:50:1 v/v/v). The photoemission at 1270 nm was followed until a maximum (S^0) was reached; immediately thereafter a solution of the sample (up to 10 µl) in $CHCl_3$ was added and the resulting level of photoemission (S) was recorded. Stern–Volmer plots of $S^0/S = 1 + k_q\tau(^1O_2)[Q]$ were used to determine the rate constants k_q. [Q] is the concentration of the quencher and τ ($^1O_2$) is the lifetime of $^1O_2$. Under the conditions used here, the latter was 33 µs. The measurements were carried out for quencher concentrations [Q] that led to S^0/S ratios between 1.2 and 2.0. Otherwise, deviations of the Stern–Volmer plot are likely to occur. Each compound was investigated using at least five different concentrations, typically not higher than 6 µM, and the mean of several recordings was taken. The mean quenching rate constants determined in this way showed standard deviations less than 5%. The method used is especially suited for effectively quenching compounds. For moderate quenchers the measurements require higher concentrated solutions or large injection volumes in order to get reasonably high S^0/S ratios. This may lead to unpredictable errors.

Standard deviations depend on the solvent and on the compound used for singlet oxygen generation. With $CHCl_3$ as solvent and 1,4-dimethyl-1,4-naphthalene endoperoxide ($DMNO_2$) as singlet oxygen-generating compound, β-carotene showed a second-order rate constant for quenching $^1O_2$ ($k = 8.1 \times 10^9\,M^{-1}s^{-1}$) with standard deviation 13% (these measurements were based on eight different solutions, each recorded twice for five different concentrations).

Radical-induced oxidations (method 1)

This method (similar to that described by Terao[24]) uses radical-induced formation of methyl linoleate hydroperoxides and monitors the increase via HPLC/DAD detection at 235 nm. Carotenoids were monitored at their corresponding λ_{max} wavelengths. The reaction was carried out in a mixture of *n*-hexane,

propan-2-ol and chloroform (6:5:2 v/v/v, 6.5 ml sample volume) at 37 °C and followed by HPLC analysis (solvent: *n*-hexane/propanol (99:1 v/v), flow rate 2.0 ml min^{-1}; column: YMC SIL-ASP, 150 mm × 6 mm, 5 μm, 60 A) at 20–30 min intervals (diode array UV-vis: HP 1040 m Series II; pumping and injection system: HP 1050).

Compounds were used at concentrations of 7.7×10^{-4} M. The concentration of the initiator AMVN was 7.7×10^{-3} M and that of methyl linoleate was 7.7×10^{-2} M. Antioxidants were dissolved in chloroform. In order to get reproducible results, a large excess of oxygen should be available in the reaction vessel. A closed system avoids loss of solvent (high vapour pressures of solvents at 37 °C). The internal equilibrium pressure after 10–20 min is *ca* 1250 mbar. Reference measurements showed that the original content of peroxide (0.1–0.5%) does not affect the results. The data points obtained were linearly interpolated for 30 min intervals. Each graph represents the average of eight 6 h measurements.

For determination of the half-life of antioxidants, compounds are recorded at λ_{max}, simultaneously with the analysis of the substrate. Plotting of area (%) against time yields curves which are fitted by an exponential function or by a third-degree polynomial. The half-life is then calculated using the fit.

Radical-induced oxidations (method 2)

This method (related to that of Burton and Ingold[3]) was carried out using a pressure transducer (MKS Baratron 223B) to monitor the uptake of oxygen pressure in a sealed reaction vessel and, with it, the consumption of oxygen during the radical-induced oxidation (AMVN, 4.5×10^{-2} M) at 30 °C in chlorobenzene. The system was capable of being filled with different mixtures of O_2 and N_2 containing 20, 200 and 1013 mbar (15, 150 and 760 Torr) of oxygen partial pressure. The samples were injected into the reaction vessel via a septum. The following experiments were performed: the radical-induced autoxidation of pure compounds (carotenoids, flavonoids, indigoids) and the effect of these antioxidants on the radical-induced oxidation of cumene (3.57 M). Experiments were carried out at concentrations of 5×10^{-5}, 1×10^{-4}, 5×10^{-4}, 1×10^{-3} and 5×10^{-3} M (indigoids at 2×10^{-5}, 4×10^{-5}, 2×10^{-4}, 4×10^{-4} and 2×10^{-3} M).

RESULTS AND DISCUSSION

Carotenoids and singlet oxygen

Carotenoids and observed quenching rate constants are shown in Fig 1 and Table 1.

As mentioned above, quenching of singlet oxygen $^1O_2$ can be assumed to occur mainly via energy transfer. Singlet oxygen $^1O_2$ as donor transfers its energy onto the acceptor carotenoid. Recently, the lowest triplet energy level of all-*trans*-β-carotene has been directly measured as 88 ± 3 kJ mol^{-1} from the

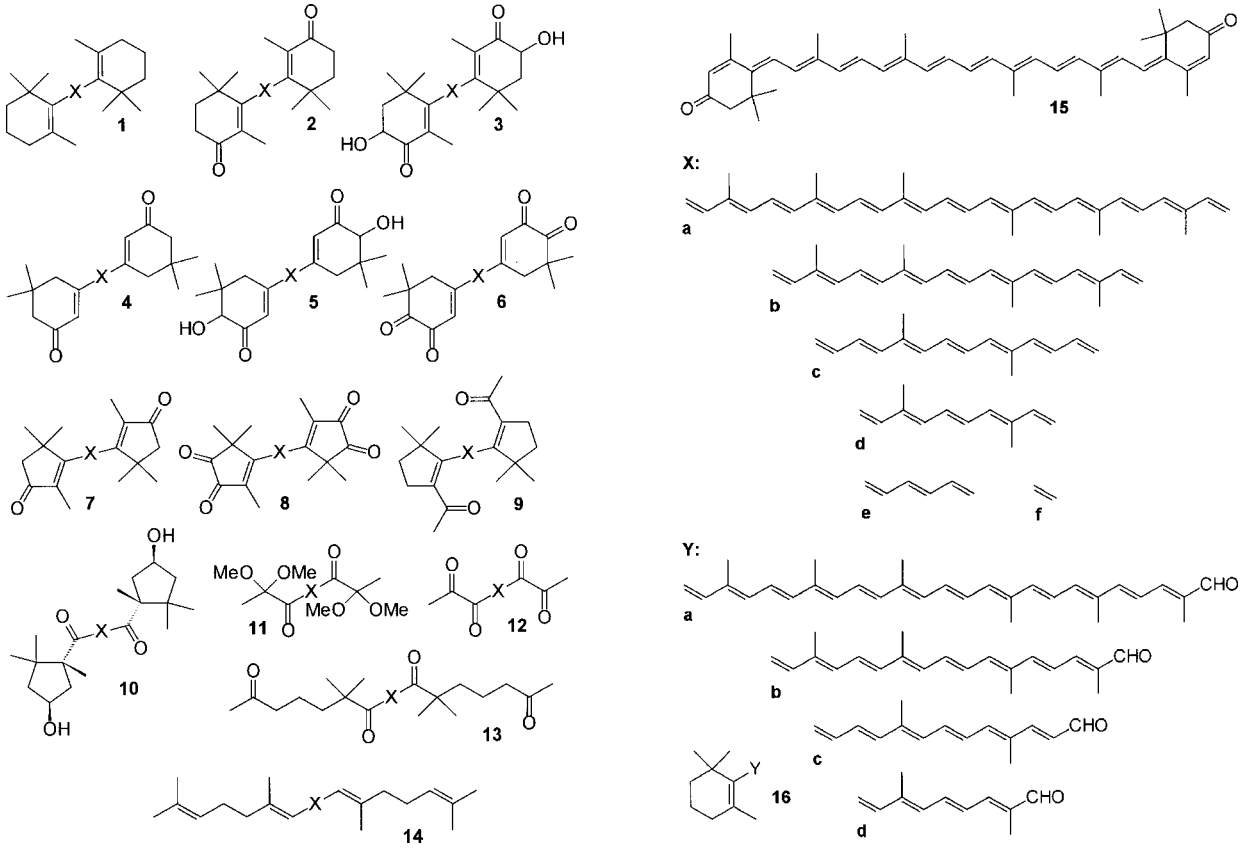

Figure 1. Structures of investigated carotenoids.

Compound	$k_q(10^9 M^{-1} s^{-1})$	$\log(k_q)$	λ_{max} (nm)	E(S) ($10^3 cm^{-1}$)
4a	13.8	10.1	550	18.18
1a	13.3	10.1	516	19.38
3a	13.0	10.1	528	18.94
6b	12.7	10.1	552	18.12
7b	12.7	10.1	505	19.80
5b	12.6	10.1	519	19.27
5a	12.3	10.1	554	18.05
8b	12.1	10.1	584	17.12
4b	12.0	10.1	512	19.53
12b	11.7	10.1	532	18.80
9b	11.1	10.0	505	19.80
11b	11.1	10.0	499	20.04
15	11.0	10.0	510	19.61
2b	10.2	10.0	486	20.57
12c	10.2	10.0	488	20.49
3b	9.0	10.0	488	20.49
10b	9.0	10.0	491	20.37
14b	8.8	9.9	484	20.66
1b	8.4	9.9	460	21.74
13b	8.4	9.9	487	20.53
11c	3.0	9.5	447	22.37
10c	1.6	9.2	449	22.27
4d	0.5	8.7	440	22.73
12d	0.1	8.0	442	22.62
16a	12.9	10.1	522	19.16
16b	12.4	10.1	503	19.88
16c	8.1	9.9	473	21.14
16d	0.2	8.3	438	22.83

Table 1. Second-order rate constants k_q for quenching of singlet oxygen in $C_2H_5OH/CHCl_3/D_2O$ (50:50:1 v/v/v) at 37 °C, and $\pi\pi^*$ absorptions ($CHCl_3$; maximum and second vibronic transition)

weak phosphorescence observed using a sensitive Fourier transformation-based interferometer.[25] The assignment places the lowest triplet energy of β-carotene just below that of singlet oxygen $^1O_2(^1\Delta_g)$ ($94\,kJ\,mol^{-1}$). The plot of $\log(k_q)$ versus the excitation energy E(S) of the longest-wavelength $\pi\pi^*$ excitation shows a functional dependence that is very common for bimolecular deactivation processes (Fig 2).

The graph in Fig 2 can be roughly divided into three areas.

1 At higher excitation energies (*ca* $(22.0–23.0)\times10^3\,cm^{-1}$) the functional dependence is linear, having a slope $-1/2.303\,RT$ that is indicative of the thermal activation energy necessary for quenching. In this range the donor energy of singlet oxygen $^1O_2$ lies below the triplet energy of the carotenoid acceptors.
2 Around $(21.0–22.0)\times10^3\,cm^{-1}$ the dependence exhibits the most conspicuous curvature. It is remarkable that β-carotene is being found here.
3 From *ca* $21.0\times10^3\,cm^{-1}$ down to lower excitation energies the dependence converges to a limit which is given by the diffusion-controlled rate constant in this solvent. At these lower singlet excitation energies ($<21.5\times10^3\,cm^{-1}$) the carotenoid triplet level is below the singlet oxygen donor energy.

The remarkable fact is that a recent density

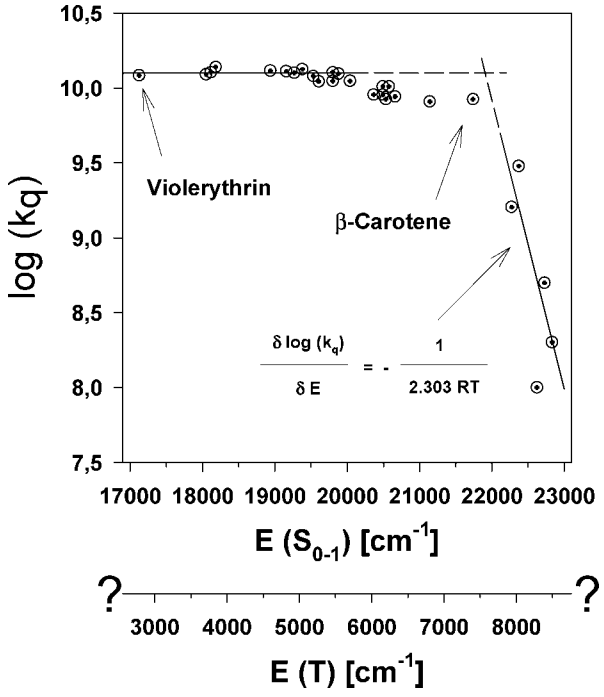

Figure 2. Plot of singlet oxygen-quenching constant $\log(k_q)$ versus singlet excitation energy for the $S_0 \rightarrow S_2$ transition of carotenoids. A tentative abscissa is given for the predominantly unknown triplet energies; the E(T) value of β-carotene[25] was used for adjustment. From the slope of the onset, $\Delta\log(k_q)/\Delta E = -1/2.303RT$, it can be concluded that there is a proportionality of E(T) to E(S).

functional theory (DFT) approach to the singlet oxygen–carotene interaction[26] unveils some surprising similarities to the otherwise different disulphides.[27] It was found that the main energy pathway is determined by an almost barrierless energy transfer $^1O_2 + {}^1Q \rightarrow {}^3O_2 + {}^3Q$ and $^3Q \rightarrow {}^1Q + \text{heat}$. In addition to the very fast energy transfer, there are concomitant low-energy-barrier reactions leading to diradicals that can undergo ring closure to 1,2-dioxetane products. Otherwise they lead, after intersystem crossing, to a regeneration of the carotenoid via dissociation. This addition–dissociation pathway of singlet oxygen quenching seems to be at least competitive with oxidation and therefore constitutes a second mechanism of physical quenching in analogy to disulphides.[27]

Carotenoids and radicals

The thermal oxidation of β-carotene can be investigated in the presence and absence of radical starters such as AIBN or AMVN. The self-initiated autoxidation of β-carotene and the induced oxidation are both autocatalytic and inhibited by tocopherol. There are two possible pathways for the non-radical-induced interaction of $^3O_2$ with β-carotene.

1 The intermediate in the thermal *cis–trans* isomerisation, 'Doering's diradical',[28] captures oxygen and leads to a plethora of products such as epoxides, aldehydes, ketones, peroxides and other minor side-products.[20]
2 The addition of oxygen takes a reaction channel where the addition to an undisturbed carotene was calculated to require about $18\,\text{kcal mol}^{-1}$. The experimental value of $E_a = 16\,\text{kcal mol}^{-1}$ is in good agreement.[29]

The mechanism of substrate (lipid, cumene, tetralin, etc) peroxidation is a chain reaction that provides a steady supply of free radicals. It may be represented as follows.

- Initiation:

  starter (eg AMVN) + RH $\rightarrow$ R$^\bullet$

- Propagation:

  R$^\bullet$ + O_2 $\rightarrow$ ROO$^\bullet$

  ROO$^\bullet$ + RH $\rightarrow$ ROOH + R$^\bullet$

- Termination: ROO$^\bullet$ + ROO$^\bullet$ $\rightarrow$ non-radical products[12]

Since the first propagation step is reversible, ie ROO$^\bullet$ $\rightarrow$ R$^\bullet$ + O_2, in particular for resonance-stabilised radicals, oxygen partial pressure is of importance. If the rate of oxidation ($-\text{d}[O_2]/\text{d}t$) of a substrate is plotted against the concentration of carotenoid using varying oxygen partial pressures, the diagrams in Fig 3 are obtained.[22]

The investigated carotenoids can be divided into three classes (Fig 4(b)). The first group (class I) contains molecules with very little antioxidative capability and these are therefore not of interest here. The second group (class II) comprises compounds with good antioxidative but also 'pro-oxidative' properties. They may be defined here by an elevated minimum value and a positive slope of the graph at 200 mbar (150 Torr) (eg β-carotene; see Fig 3(a)). The third group (class III) consists of carotenoids which react as strong antioxidants and without any 'pro-oxidative' nature. All those investigated here contain conjugated oxo functions within their end groups (eg astaxanthin; see Fig 3(b)).

These results can be compared satisfactorily with the antioxidative effect on the autoxidation of methyl linoleate in Fig 4(a).

The above results lead to the suggestion that the different chemical anti- and pro-oxidant behaviour of the carotenoids is caused by the different structure of their end groups, their chain length (minor importance) and the number and position of methyl groups.

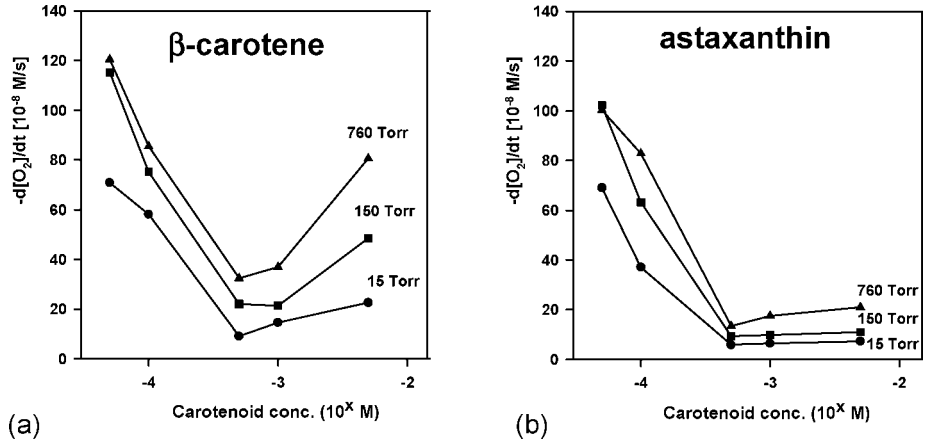

Figure 3. Dependence of rate of oxygen consumption on carotenoid concentration and partial pressure of oxygen for two selected carotenoids, (a) β-carotene **1b** and (b) astaxanthin **3b**.

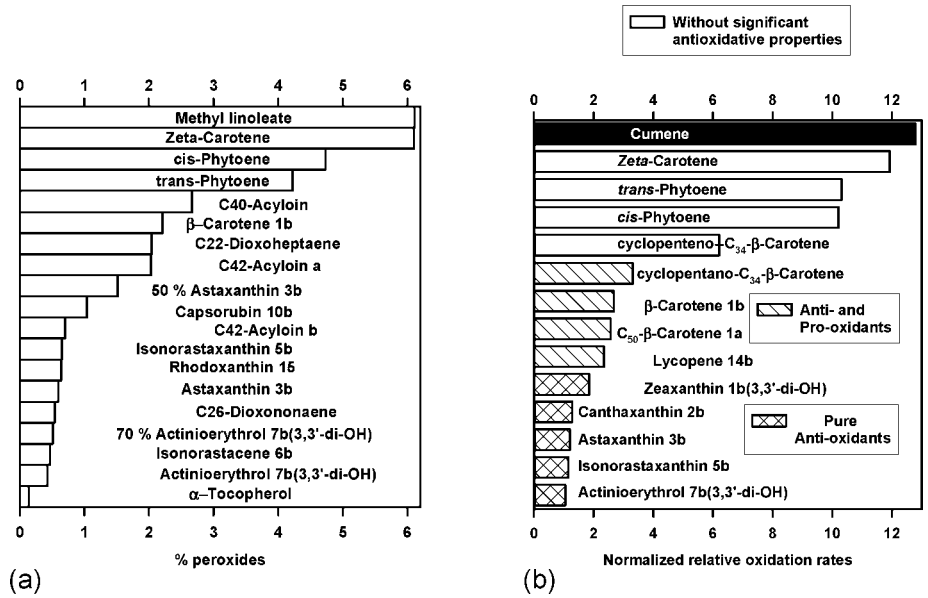

Figure 4. (a) Peroxide formation of pure methyl linoleate and in the presence of antioxidants (50% astaxanthin means that the standard concentration 7.7×10^{-4} M is lowered by 50%). (b) Normalised (with respect to actinioerythrol=1) rates of oxidation at $p(O_2)$=200 mbar (150 Torr). The rates of oxidation are extracted from the experimental 200 mbar graphs by taking the intersection point with a vertical line at 7.7×10^{-4} M.

β-Carotene reacts as a hydrocarbon with active allylic hydrogen atoms that can be removed by radicals. On the other hand, β-carotene also binds to peroxyl radicals. The two processes combine to produce reactions with the concomitant formation of epoxides and carbonyl compounds. It is possible to develop a sequence of radical abstraction and oxygen addition reactions as well as cleavage reactions resulting in a radical chain reaction. Obviously the ketocarotenoids such as astaxanthin **3b**, isonorastacene **6b** and actinioerythrol (the 2,2′-dihydroxy derivative of **7b**) do not enter these pathways or do so much more slowly.

CarH	+	ROO•	→	Car•	+	ROOH
Car•	+	O_2	→	Car-O_2•		
Car-O_2•	+	CarH	→	Car-O_2-CarH•		
Car-O_2-CarH•			→	Car-O•	+	Epoxide
Car-O•	+	CarH	→	Car-O-CarH•		
Car-O-CarH•	+	O_2	→	Car-O-Car H-O_2•		
Car-O-CarH-O $_2$•	+	CarH	→	Car-O-Car H-O_2-CarH•		
Car-O-CarH-O $_2$-CarH•			→	Car-O-CarH-O•	+	Epoxide
Car-O-CarH-O•			→	Car•	+	2 Carbonyls

Figure 5. Reaction sequence used not only to explain the formation of the various products (epoxides, carbonyl compounds) but also to obtain an already quite reasonable fit to our experimental results (see Fig 6).

To find a model that is able to explain the pressure dependences, we have started to simulate the autoxidation of β-carotene (using the program Acuchem, kindly provided by Dr Kahaner and Dr Herron, National Institute of Standards and Technology, Gaithersburg, MD, USA). In this simulation the aforesaid reaction sequence (Fig 5) is used. It is not only capable of explaining the formation of the various products (epoxides, carbonyl compounds), but also gives an already quite reasonable fit to our experimental results (see Fig 6).

The chain reaction (Car$^{\bullet}$) provides for the efficient consumption of carotenoids in the case of class I and II carotenoids. The proposal that the oxo function is able to resonance-stabilise carbon-centred radicals might explain the powerful antioxidative properties of all class III carotenoids without pro-oxidative contributions, particularly the remarkable efficacy of isonorastacene **6b** and actinioerythrol (the 2,2′-dihydroxy derivative of **7b**).

Since these ketocarotenoids, lacking any indication of pro-oxidative behaviour, turn out to be more

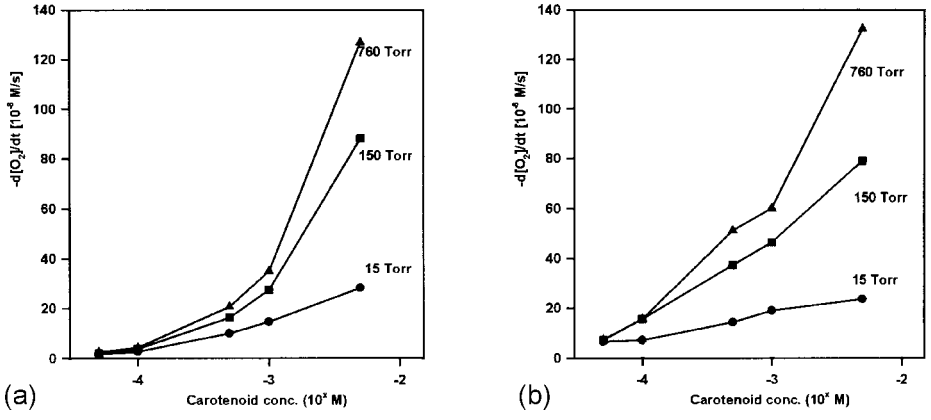

Figure 6. Simulated oxidation rates of β-carotene for different oxygen partial pressures and different carotenoid concentrations using the reaction sequence in Fig 5. These simulations may be compared with (b) the experimental oxidation rates of pure β-carotene in chlorobenzene/AMVN.

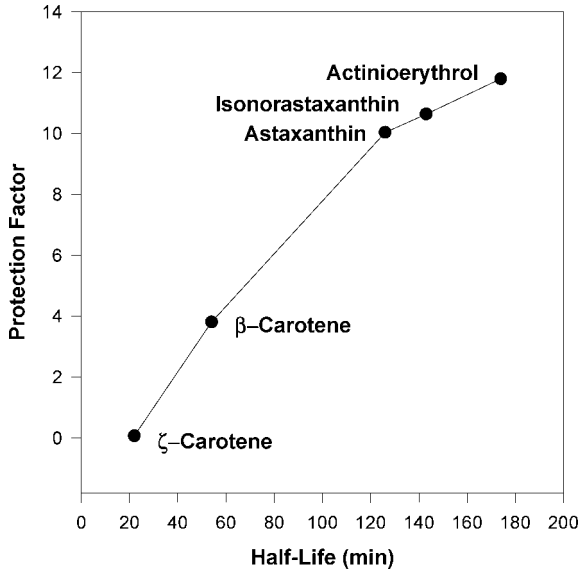

Figure 7. Correlation of half-life of carotenoids against oxidation rate expressed as a protection factor PF=[(oxidation rate without inhibitor)/(oxidation rate with inhibitor)] −1. Half-life is determined as described in method 1.

resistant towards oxidation than compounds such as lycopene or β-carotene, an attempt was made to find out which experimental quantity might give a useful prediction for the non-appearance of a positive slope in the graphs at 200 mbar (150 Torr). Indeed, a correlation between the half-life of the inhibitor and the oxidation rate (expressed as a protection factor PF_{300}) displayed a satisfactory relationship (Fig 7). PF_{300} is defined as

$$PF_{300} = \frac{[\text{cumene hydroperoxide without inhibitor}]}{[\text{cumene hydroperoxide with inhibitor}]} - 1$$

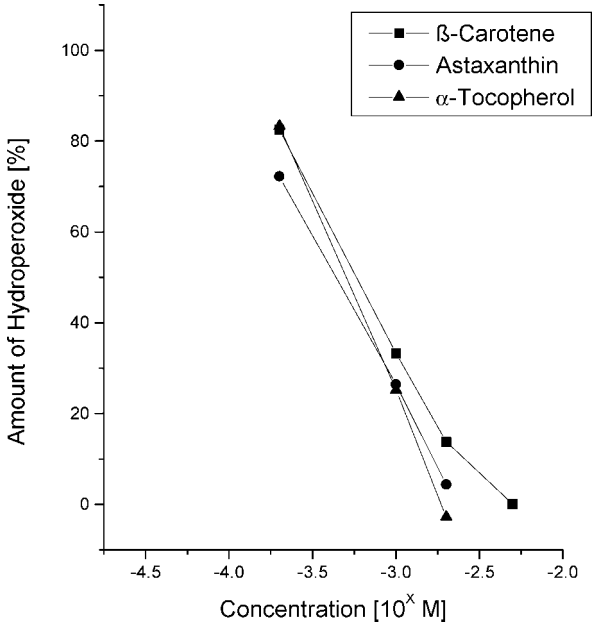

Figure 8. Amount of hydroperoxide formed plotted against concentration of antioxidants. Conditions: method 1, 200 mbar oxygen pressure.

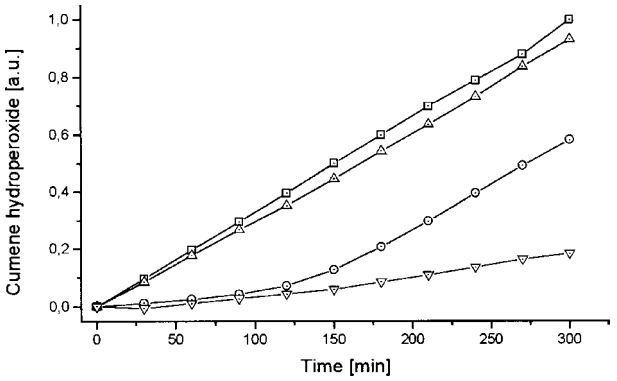

Figure 9. Formation of cumene hydroperoxide without inhibitor (squares) and in the presence of flavonoids **17a** (triangles), **17d** (circles) and **18** (inverted triangles).

after a reaction time of 300 min. A half-life of *ca* 100 min may be the decisive limit that separates class II from class III carotenoids.

Even more surprising and unexpected, however is the finding that during the 'so-called pro-oxidative' process in the graphs of class II carotenoids at concentrations $>7.7\times10^{-4}$ M no oxidation product of the substrate itself (cumene, methyl linoleate) can be detected. In Fig 8 the amount of hydroperoxide is plotted against the concentration of antioxidant. No indication of damage to the substrate is observed. Thus the oxygen consumption is caused solely by the degradation of the antioxidant itself. This might lead to interesting speculations and discussions about the 'putative harm' that is possibly inflicted on important biological tissues by class II antioxidative carotenoids.

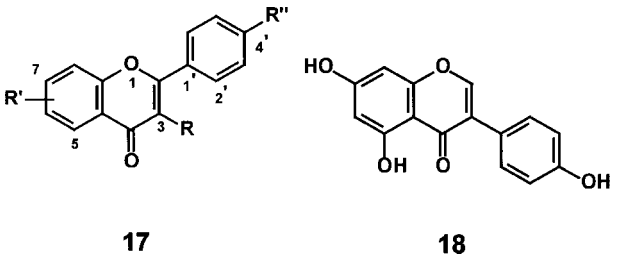

Figure 10. Structures of flavonoids.

Table 2. Protective factors PF_{300} of flavonoids in chlorobenzene, except 17f which was recorded in chlorobenzene/propan-2-ol (80:20 v/v)

Flavonoid	*PF_{300}*
17a Flavone	<0.1
17b 5-Hydroxyflavone	<0.1
17c 7-Hydroxyflavone	0.2
17d 3-Hydroxyflavone	0.7
17e 3,3′,4′,5,7-Pentahydroxyflavone (quercetin)	6.4
17f 4′,5,7-Trihydroxyflavone (apigenin)	1.0
18 4′,5,7-Trihydroxyisoflavone (genistein)	4.4

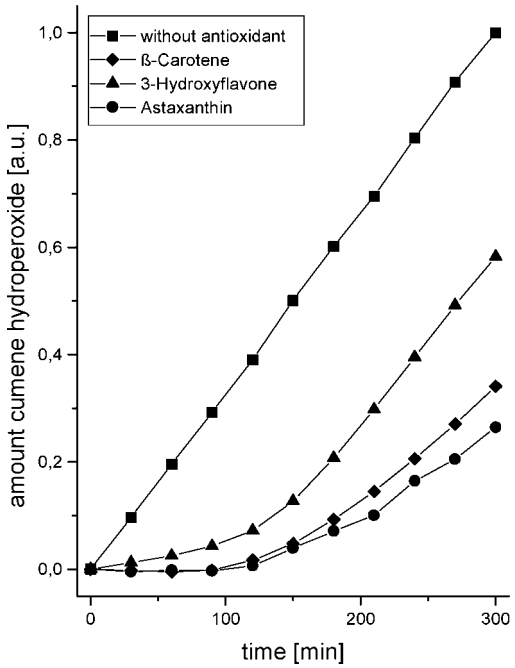

Figure 11. Formation of cumene hydroperoxide without inhibitor and in the presence of 3-hydroxyflavone, β-carotene and astaxanthin. Conditions: method 1, but with cumene (3.57 M) in chlorobenzene, AMVN (4.5×10^{-2} M), inhibitor (10^{-3} M).

Flavonoids

The quenching rate constants for singlet oxygen of flavonoids are of the order of $10^6\,M^{-1}s^{-1}$. They are about three orders of magnitude lower than those of carotenoids which can reach the diffusion limit.[18] However, the inhibition of radical-induced oxidation of substrates such as cumene or methyl linoleate can be most efficient (Fig 9). The antioxidative effect depends on the flavonoid (Fig 10) and is best described in terms of the protective factor PF_{300} (Table 2).

A comparison of carotenoids with flavonoids in Fig 11 shows the comparable efficiency of these two groups of antioxidants. It is remarkable that genistein **18** displays strong antioxidative properties, conspicuously more efficient than the isomeric apigenin **17f** (Fig 9).

Indigoids

Indigoids are special chromophores, called H-chromophores, which contain an efficient combination of two merocyanine moieties. The parent compound, though not the smallest member of this family, is indigo **19a**; more elaborate examples are **20**–**22** (Fig 12).

The indigoidine or 'bacterial indigo' chromophore **21** ($n = 0$, R $\equiv$ H) is found in some bacteria, eg in *Pseudomonas indigofera*. The chromophores **20**–**22** may be reduced to the structure **23**, which indicates the special nature of these indigoid π systems.

Since indigo **19a** is a sparingly soluble compound with a high tendency to form aggregates, the determination of quenching constants is subject to considerable experimental error, and k_q is likely to be higher than $1.6 \times 10^8\,M^{-1}s^{-1}$ in $CHCl_3$. These problems can be avoided by measuring the readily soluble derivative **19b**: $k_q = 7.9 \times 10^9\,M^{-1}s^{-1}$ in $CHCl_3$. Thioindigo **19c**, on the other hand, is a very inefficient quencher with $k_q < 10^6\,M^{-1}s^{-1}$, which concurs with the low efficiencies of other sulphur-containing compounds. The indigoids **19**–**22** seem to follow regularities similar to carotenoids: the deeper the colour and the more bathochromic the absorption wavelength, the more efficient is the quenching ability up to the diffusion limit (Table 3 and Fig 13). This might indicate an

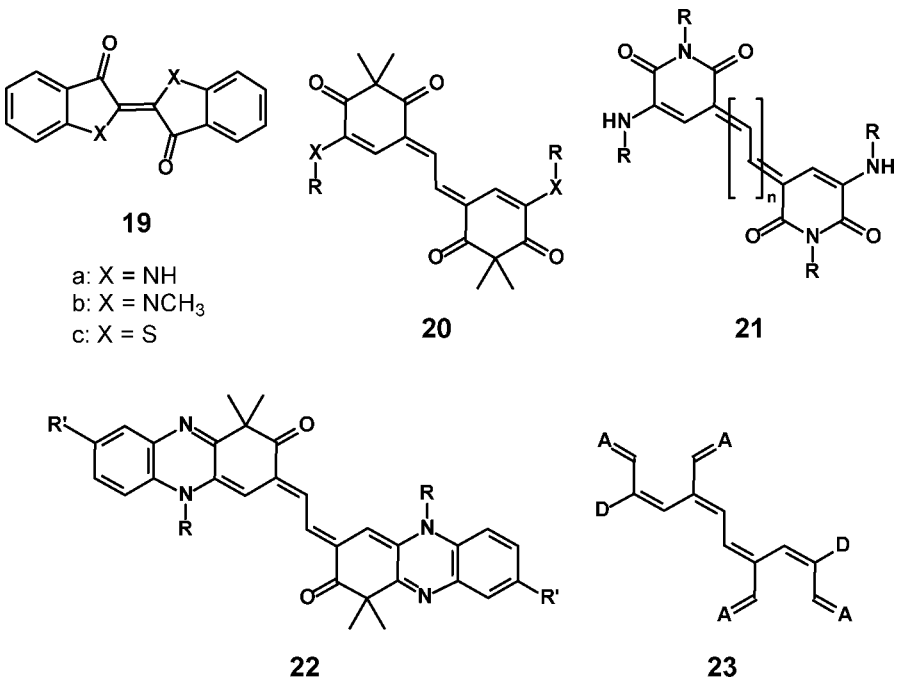

Figure 12. Structures of indigoid dyes.

Compound	k_q ($10^9 M^{-1} s^{-1}$)	log(k_q)	λ_{max} (nm)	E(S) ($10^3 cm^{-1}$)
21e, $n = 0$, R ≡ *n*-Bu	10.77	10.0	633	15.80
22b, R′ ≡ H, R ≡ Ph	10.77	10.0	648	15.43
20e, X ≡ NH, R ≡ Ph	9.74	10.0	562	17.79
21a, $n = 1$, R ≡ *n*-Bu	9.74	10.0	596	16.78
21c, $n = 0$, R ≡ *n*-Pr	9.74	10.0	631	15.85
21d, $n = 0$, R ≡ *i*-Bu	9.74	10.0	633	15.80
22a, R′ ≡ H, R ≡ Me	9.74	10.0	642	15.58
22c, R′ ≡ H, R ≡ *p*-MeO-C_6H_4	8.82	9.9	650	15.39
22e, R′ ≡ OMe, R ≡ Et	8.82	9.9	682	14.66
20f, X ≡ NH, R ≡ *n*-Bu	7.98	9.9	538	18.59
22d, R′ ≡ *n*-Bu, R ≡ Et	7.98	9.9	664	15.06
19b	7.90	9.9	628	15.92
20g, X ≡ NH, R ≡ *m*-CF_3-C_6H_4	7.22	9.9	546	18.32
21b, $n = 1$, R ≡ *i*-Bu	7.22	9.9	599	16.69
20a, X ≡ O, R ≡ H	0.29	8.5	439	22.78
20d, X ≡ O, R ≡ Me	0.01	7.0	431	23.20
20b, X ≡ O, R ≡ Ac	<0.01	<6.5	387	25.84
20c, X ≡ O, R ≡ Bz	<0.01	<6.5	390	25.64
19a	~0.16	~8.2[a]	610	16.39
19c	≪0.01	≪6.5	546	18.32

[a] Value uncertain (see text).

Table 3. Second-order rate constants k_q for quenching of singlet oxygen in $CHCl_3$ at 37 °C, and $\pi\pi^*$ absorptions ($CHCl_3$)

energy transfer mechanism and compares well with the behaviour of carotenoids (Fig 2).

In order to examine the antioxidative ability of indigoids towards radical-induced oxidations, the dye **22b** was subjected to method 2. It turned out to be an excellent antioxidant at all oxygen pressures (Fig 14).

CONCLUSION

The *in vitro* antioxidant ability of all compounds investigated in this study can be properly described by three assays: (1) singlet oxygen quenching; (2) inhibition of peroxide formation; (3) dependence on oxygen partial pressures. This allows the assessment of antioxidants and their classification into groups. Many of these compounds turn out to be excellent antioxidants in both ground state radical-induced reactions and excited state quenching processes. The so-called 'pro-oxidative and unusual' behaviour ascribed to β-carotene is a common feature of class II carotenoids. However, no oxidation product of the substrate itself (cumene, methyl linoleate) can be detected using these carotenoids, and no indication of damage to the substrate is observed. Thus the oxygen consumption is caused solely by the degradation of the antioxidant itself. Expressed along these lines, one may say that β-carotene, like other congeners of class II, is an unusual antioxidant to the extent that it is

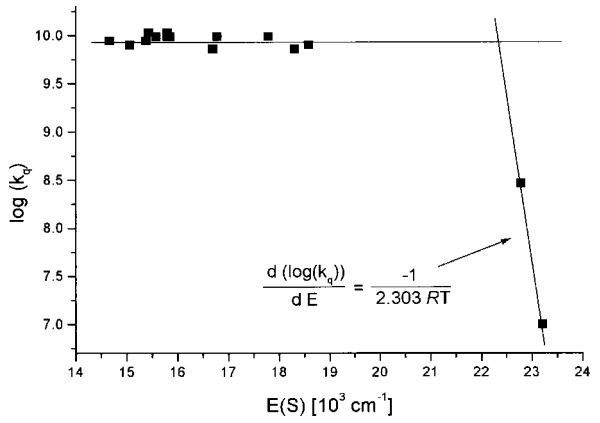

Figure 13. Plot of singlet oxygen-quenching constant log(k_q) versus singlet excitation energy of indigoids. From the slope of the onset, $\Delta\log(k_q)/\Delta E = -1/2.303RT$, it can be concluded that there is a proportionality of E(T) to E(S).

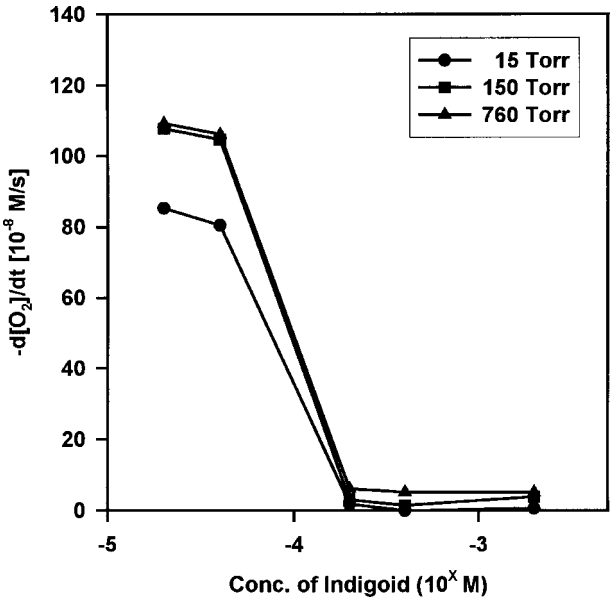

Figure 14. Dependence of rate of oxygen consumption on indigoid **22b** concentration and partial pressure of oxygen according to method 2.

more labile and short-lived than the representatives of class III.

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