Free Radical Research, May 2005; 39(5): 547–563

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Inhibition of the metmyoglobin-induced peroxidation of linoleic acid by dietary antioxidants: Action in the aqueous vs. lipid phase

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Accepted by Professor E. Niki

(Received 26 October 2004; in revised form 21 January 2005)

Abstract

The gastric digestion of food containing oxidizable lipids and iron catalysts for peroxide decomposition such as (met)myoglobin from muscle meat can be accompanied by an extensive formation of potentially toxic lipid hydroperoxides. An early protective action by dietary antioxidants in the gastro-intestinal tract is plausible, especially for poorly bioavailable antioxidants such as polyphenols. Hence, the ability of antioxidants to inhibit lipid peroxidation initiated by dietary iron in mildly acidic emulsions is a valuable and general model. In this work, the ability of some ubiquitous dietary antioxidants representative of the main antioxidant classes (α-tocopherol, the flavonol quercetin, β-carotene) to inhibit the metmyoglobin-induced peroxidation of linoleic acid is investigated by UV–visible spectroscopy and HPLC in mildly acidic emulsions. The phenolic antioxidants quercetin and α-tocopherol come up as the most efficient peroxidation inhibitors. Inhibition by quercetin essentially proceeds in the aqueous phase via a fast reduction of an unidentified activated iron species (with a partially degraded heme) produced by reaction of metmyoglobin with the lipid hydroperoxides. This reaction is faster by, at least, a factor 40 than the reduction of ferrylmyoglobin (independently prepared by reacting metmyoglobin with hydrogen peroxide) by quercetin. By contrast, α-tocopherol mainly acts in the lipid phase by reducing the propagating lipid peroxyl radicals. The poorer inhibition afforded by β-carotene may be related to both its slower reaction with the lipid peroxyl radicals and its competitive degradation by autoxidation and/or photo-oxidation.

Keywords: *Metmyoglobin, linoleic acid, lipid peroxidation, quercetin, α-tocopherol, β-carotene*

Introduction

Lipid peroxidation can be detrimental to foods and several biological assemblies that include polyunsaturated lipids such as biomembranes and plasma lipoproteins [1–5]. As such, lipid peroxidation is likely involved in the development of degenerative diseases like atherosclerosis, cancer and neuron impairment. Consequently, inhibition of lipid peroxidation is the most popular model for comparing the efficiency of antioxidants.

Naturally occurring antioxidants can be used as additives in the preservation of lipid-containing foods. Once ingested, they may also participate in the beneficial effects of diets rich in plant products [6–8]. However, in this nutritional perspective, the bioavailability of dietary antioxidants comes up as a crucial criterion. Regarding polyphenols for instance, only low concentrations of heavily conjugated forms (less antioxidant than the native forms) are detected in the plasma of volunteers after a meal rich in plant products [9,10]. In consequence, alternative biological mechanisms, more specific than the antioxidant activity, are currently favoured to account for the possible beneficial health effects of polyphenols. On the other hand, it has been proposed that antioxidant mechanisms prior to intestinal absorption could be biologically relevant [11–14]. Indeed,

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2005 Taylor & Francis Group Ltd
DOI: 10.1080/10715760500073865

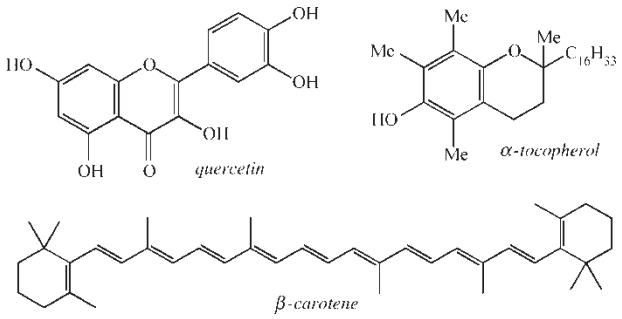


Chart 1.

dietary antioxidants can accumulate in large concentrations in the gastro-intestinal tract where lipid peroxidation induced by dietary iron is quite fast, especially in the acidic gastric conditions. Hence, the scavenging by dietary antioxidants of highly oxidizing species formed during heme iron-induced lipid peroxidation (hypervalent iron species, lipid oxyl and peroxyl radicals) may be a general and biologically significant model [11]. On the other hand, the scavenging of reactive oxygen species formed in the reaction between dietary iron and hydrogen peroxide is also of interest since H_2O_2 is present in meat in low concentrations and may be produced in the intestine by microflora activity [12].

In this work, three compounds abundant in the human diet and representative of the main classes of plant antioxidants are investigated for their ability to inhibit the peroxidation of linoleic acid in mildly acidic emulsions. Peroxidation is initiated in the aqueous phase by metmyoglobin [15], one of the main forms of dietary iron (red meat). For comparison, initiation by the hydrosoluble diazo compound AAPH is also considered. The three antioxidants selected are β-carotene, the flavonoid quercetin (polyphenol) and α-tocopherol (Chart 1). The formation of lipid oxidation products is followed by UV–visible spectroscopy and the products further characterized by HPLC-MS analysis. To gain insight in the possible inhibition mechanisms at work, the reactivity of the selected antioxidants towards the hypervalent iron species formed by reacting metmyoglobin and hydrogen peroxide is also investigated.

Experimental

Materials

Horse heart metmyoglobin ($MbFe^{III}$, type II, MW *ca.* 17600 g mol^{-1}), hematin (hydroxyprotoporphyrin IX), AAPH (2,2′-azobis(2-amidinopropane) dihydrochloride), linoleic acid, tween 20 (polyethoxysorbitan monolaurate), hydrogen peroxide (30% in water), all-*trans*-β-carotene, (±)-α-tocopherol and quercetin were purchased from Sigma-Aldrich. All these products of the highest quality available, (95–99%) were used without purification. Phosphate buffers (5 mM, pH 5.8 or 6.8), prepared with Millipore Q-Plus water, were passed through a column of Chelex-100 chelating resin (Bio-Rad) to remove contaminating metal ion traces. All solvents used were of analytical grade. Chloroform and THF were further purified by elution on Al_2O_3.

Absorption spectra

Spectra were recorded on a Hewlett-Packard 8453 diode-array spectrometer equipped with a quartz cell (optical pathlength: 1 cm). The temperature in the cell was kept at 37°C by means of a water-thermostatted bath. Magnetic stirring in the cell at a constant rate (1000 rpm) was ensured by a Variomag stirrer (Telemodul 20 C).

Kinetic experiments

All experiments were monitored by UV–visible spectroscopy.

Inhibition of the metmyoglobin-induced peroxidation of linoleic acid. The experimental conditions were adapted from an already published procedure [16]. Briefly, 17.6 mg of metmyoglobin were dissolved in 100 ml of a pH 6.8 phosphate buffer ($MbFe^{III}$ concentration *ca.* 10 μM, standardized spectrophotometrically using $\varepsilon_{525} = 7700\ M^{-1}\ cm^{-1}$ [17]). Given volumes of daily prepared solutions of linoleic acid, tween 20 and antioxidant in $CHCl_3$ or MeOH were mixed and the solvents removed under reduced pressure (to achieve reproducibility in the experiments with β-carotene, the stock solution must be stored under argon in the dark at 4°C between each sampling). The residue was dissolved immediately in 20 ml of pH 5.8 phosphate buffer under gentle stirring. The final concentrations in the solution were: Linoleic acid, 0.7 mM; tween 20, 2 mM; β-carotene, 0.5–5 μM; quercetin and α-tocopherol, 0.2–1 μM. A measure of 2 ml of the freshly prepared solution was transferred to the thermostatted spectrometer cell equipped with a teflon stopper. The sample was in contact with air in the headspace. At time zero, 20 μl of 10 μM $MbFe^{III}$ were added to the sample under constant magnetic stirring and the UV–visible spectra were recorded at regular time intervals (30 or 40 s).

Hematin-induced peroxidation of linoleic acid. The same procedure was used, the $MbFe^{III}$ solution being replaced by a 8 μM hematin solution in MeOH/DMSO (7:3, *v*/*v*).

AAPH-induced peroxidation of linoleic acid. The same procedure was used for the preparation of pH 5.8 buffered solutions of linoleic acid and tween 20. At time zero, 25 μl of a 80 mM solution of AAPH in

MeOH were added to the sample (2 ml) under constant magnetic stirring at 37°C and the UV–visible spectra were recorded at regular time intervals. After 200 s, 25 µl of a concentrated solution of antioxidant (quercetin, α-tocopherol) in MeOH were added to ensure final antioxidant concentrations in the range 0.2–1 µM. Solutions of β-carotene in THF were used to prepared pH 5.8 buffered solutions of linoleic acid, β-carotene (final concentrations in the range 0.5–5 µM) and tween 20. Peroxidation was then started by addition of AAPH. Control experiments without β-carotene were run.

Reduction of ferrylmyoglobin by quercetin. Ferrylmyoglobin ($MbFe^{IV}$=O) was previously formed in the spectrometer cell upon adding small volumes of a concentrated aqueous solution of H_2O_2 (concentration determined spectrophotometrically using $\varepsilon_{240} = 39.4\ M^{-1}\ cm^{-1}$, final concentration in the cell: 30 µM) to a 30 µM $MbFe^{III}$ solution in a pH 5.8 phosphate buffer containing 2 mM tween 20. The spectral changes featuring the conversion of $MbFe^{III}$ into $MbFe^{IV}$=O were recorded in the visible range until stability (2–3 min). Then, 20 µl of a concentrated solution of quercetin in MeOH (final quercetin concentrations in the cell: 5, 10, 15, 20 and 25 µM) was added and the reduction of $MbFe^{IV}$=O back to $MbFe^{III}$ was monitored at 590 nm.

Reduction of perferrylmyoglobin by quercetin. To a 30 µM $MbFe^{III}$ solution in a pH 5.8 phosphate buffer containing 2 mM tween 20 were added 20 µl of a concentrated solution of quercetin in MeOH (final quercetin concentrations in the cell: 5, 10, 15, 20, 25 and 30 µM). Then, a small volume of a concentrated aqueous solution of H_2O_2 was added to give a final concentration in the cell of 30 µM.

Data analysis. The curve-fitting and derivation procedures of the absorbance vs. time plots were carried out on a Pentium 120 PC using the "Scientist" program (MicroMath, Salt Lake City, USA). Beer's law and sets of differential kinetic equations (see text) with initial conditions on concentrations (see "Kinetic experiments") were given as input data. Curve-fittings were achieved through least square regression and yielded optimized values for the parameters (kinetic rate constants, stoichiometries, molar absorption coefficients). Standard deviations are reported.

HPLC-MS experiments

They were performed on a HP 1050 model equipped with a diode-array detector and coupled to a Micromass LCZ 4000 mass spectrometer. A C_{18} column (4.6 × 150 mm, 5 µm particle size) equipped with a pre-column (4.6 × 7.5 mm, 5 µm particle size) and kept at 25°C was used. The mobile phase (flow rate: 1.0 ml min^{-1}) was a linear gradient of acetonitrile and 0.05% aqueous HCO_2H. Lipid oxidation products: $t = 0$–5 min, 50% MeCN; $t = 15$ min, 60% MeCN; $t = 30$ min, 73% MeCN; $t = 40$ min, 100% MeCN. Quercetin oxidation products: $t = 0$ min, 5% MeCN; $t = 21 - 25$ min, 50% MeCN; $t = 30$ min, 60% MeCN; $t = 45$ min, 73% MeCN; $t = 55$ min, 100% MeCN. Mass spectra were recorded in the negative electrospray mode. Parameters, and especially cone voltage, were optimised in order to avoid fragmentation. The Masslynx program was used for data analysis.

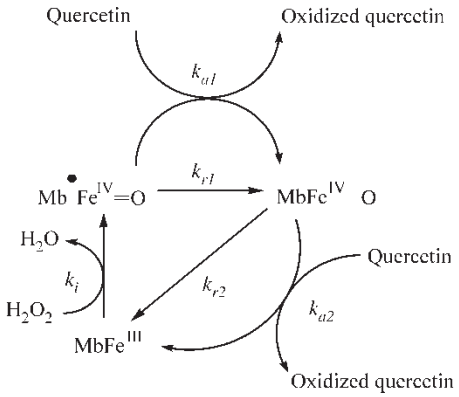
Characterization of lipid oxidation products. At $t = 0$ (just before addition of $MbFe^{III}$), 2, 15 and 30 min, peroxidation samples (10 ml) were taken up, acidified to pH 2 with 5 ml of 0.05 M HCl and extracted twice by 2 × 10 ml of ethylacetate. The combined organic phases were washed with 2 × 20 ml of saturated aqueous NaCl, dried over anhydrous Na_2SO_4 and concentrated to dryness under reduced pressure. The residue was dissolved in 2 ml of acetone and analyzed by HPLC-MS. The LOOH-LOH molar ratio was determined from the total integrated areas of the peaks corresponding to the 4 regio- and stereoisomers for each class of compounds assuming the same molar absorption coefficient for LOOH and LOH.

Characterization of quercetin oxidation products. To a solution of Mb Fe^{III} (3 µM) in the phosphate buffer (pH 5.8) were successively added quercetin (5 µM, from a 0.5 mM solution in MeCN/DMSO 99:1) and H_2O_2 (5 µM, from a 0.5 mM aqueous solution). After 3 min of stirring, a sample (5 ml) was taken up and treated as above for HPLC-MS analysis.

Results

Reduction of hypervalent iron species derived from metmyoglobin

The reaction of metmyoglobin ($MbFe^{III}$) with hydrogen peroxide has been thoroughly studied and the hypervalent iron species that can form have been characterized by UV–visible spectroscopy and ESR [18–20]. Under activation by H_2O_2, two oxidizing equivalents are produced, the one converting the Fe^{III} center into an iron-oxo center (Fe^{IV}=O) and the other causing a one-electron oxidation on a tyrosine or tryptophan residue to form a protein radical detectable by ESR (Scheme 1). This activated form of metmyoglobin is called perferrylmyoglobin and may be written as $Mb^{\bullet}Fe^{IV}$=O ($Mb^{\bullet}$: Globin radical). Perferrylmyoglobin is a short-lived species (half-life in the range 50–280 ms [16]) and, in the absence of external sources of electrons, rapidly evolves toward ferrylmyoglobin ($MbFe^{IV}$=O) upon



Scheme 1. Inhibition of H_2O_2-induced peroxidation by quercetin.

a poorly understood process called auto-reduction. Moreover, reducing agents such as ascorbate, trolox (a water-soluble analog of α-tocopherol) and several polyphenols were demonstrated to quickly reduce $MbFe^{IV}$=O into $MbFe^{III}$ [21–25]. Some anionic antioxidants can also directly interact with the iron center in the heme crevice, as already shown with ascorbate and the polyphenol chlorogenic acid [25].

Investigating the reactivity of the selected antioxidants with perferrylmyoglobin and ferrylmyoglobin can provide information on their possible antioxidant action in the aqueous phase (where metmyoglobin is located) during the inhibition of metmyoglobin-induced lipid peroxidation, i.e when metmyoglobin is activated by the lipid hydroperoxides instead of hydrogen peroxide. The reactions with H_2O_2 and the reactions with linoleic acid were carried out in the same conditions, i.e. in the presence of tween 20.

Reduction of ferrylmyoglobin. When $MbFe^{III}$ is treated by H_2O_2 (1 equiv.) in the absence of antioxidant, formation of $MbFe^{IV}$=O can be followed at 590 nm (Figure 1A). The corresponding A (590)-time curve can be fitted to a simple kinetic model where the rate constants k_i (formation of $MbFe^{IV}$=O) and k_{r2} (slow reduction of $MbFe^{IV}$=O into $MbFe^{III}$) and the molar absorption of $MbFe^{IV}$=O at 590 nm are the adjustable parameters. Then, using the latter ε value, the decay of A (590) following the addition of quercetin (Figure 1B) is analyzed according to second-order kinetics to extract the rate constant k_{a2} for the reduction of $MbFe^{IV}$=O by quercetin (Table I): k_{a2} *ca.* $5 \times 10^3\ M^{-1}\ s^{-1}$.

When the reaction is repeated with α-tocopherol, the decay of A (590) following antioxidant addition is slow and indistinguishable from that observed in the control experiment with MeOH alone (data not shown). This result demonstrates that α-tocopherol does not significantly react with $MbFe^{IV}$=O.

Reduction of perferrylmyoglobin. To investigate the reaction of the antioxidant with perferrylmyoglobin

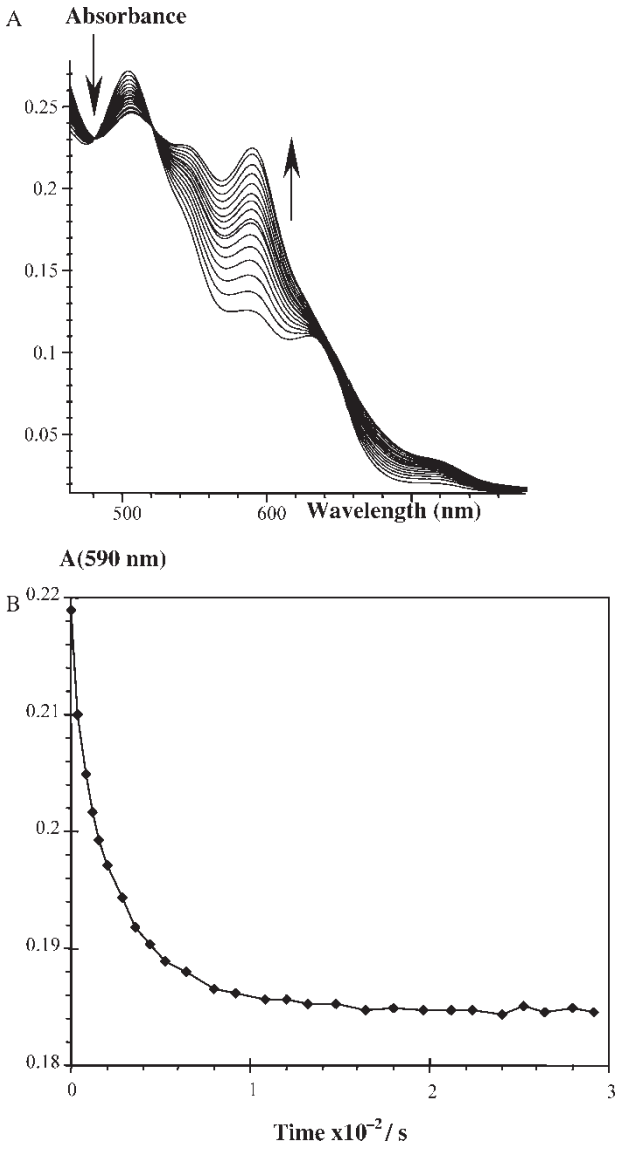


Figure 1. (A) Spectral changes during formation of ferrylmyoglobin. Metmyoglobin (29.8 μM) + H_2O_2 (29.7 μM), pH 5.8 phosphate buffer containing 2 mM tween 20, 37°C. (B) Time dependence of the absorbance at 590 nm during reduction of ferrylmyoglobin by quercetin. Quercetin concentration: 20 μM.

($Mb^{\bullet}Fe^{IV}$=O), the transient precursor of ferrylmyoglobin, H_2O_2 must be added to a solution of $MbFe^{III}$ (1 equiv.) containing the antioxidant. In the case of α-tocopherol and β-carotene, the antioxidant does not inhibit the formation of ferrylmyoglobin as evidenced by the development of the visible absorption at 590 nm (Figure 2A). In fact, α-tocopherol even stabilizes $MbFe^{IV}$=O by preventing its slow decay from the plateau concentration. By contrast, quercetin markedly inhibits the formation of $MbFe^{IV}$=O (Figure 2A). Simultaneously, quercetin is rapidly consumed as evidenced by the decay of its typical visible absorption at 370 nm (Figure 2B). These spectral changes can be analyzed within a kinetic model assuming a steady state for $Mb^{\bullet}Fe^{IV}$=O as a result of

Table I. Rate constants for the formation of ferrylmyoglobin and its reaction with quercetin in 2 mM tween 20 (pH 5.8, 37°C).*

$k_i/M^{-1}s^{-1}$	10^4 (k_{r2}/s^{-1})†	$\varepsilon(MbFe^{IV}{=}O)/M^{-1}cm^{-1}$†	Quercetin/ μM	k_{a2} / $M^{-1}s^{-1}$‡	n‡
716 (± 17)	47.6 (± 1.5)	11170 (± 90)	19.9	5468 (± 63)	0.43 (± 0.01)
693 (± 15)	53.7 (± 1.6)	11290 (± 90)	15.1	5526 (± 64)	0.57 (± 0.01)
973 (± 28)	44.0 (± 1.5)	10570 (± 80)	10.2	4805 (± 60)	0.83 (± 0.01)
839 (± 19)	50.0 (± 1.5)	10870 (± 80)	4.9	5789 (± 115)	1.91 (± 0.01)

* Detection at 590 nm, $MbFe^{III}$ (29.8 μM) + H_2O_2 (29.7 μM), then quercetin.
† Deduced from the curve-fitting of the spectral changes following the addition of H_2O_2 to the $MbFe^{III}$ solution. k_i: Second-order rate constant for the conversion of $MbFe^{III}$ into $MbFe^{IV}{=}O$ by H_2O_2; k_{r2}: First-order rate constant for the reduction of $MbFe^{IV}{=}O$ to $MbFe^{III}$.
‡ Deduced from the curve-fitting of the spectral changes following the addition of quercetin to the $MbFe^{IV}{=}O$ solution. k_{a2}: Second-order rate constant for the reduction of $MbFe^{IV}{=}O$ by quercetin, n: Number of $MbFe^{IV}{=}O$ molecules reduced per quercetin molecule (stoichiometry). The calculated value of the molar absorption coefficient of $MbFe^{IV}{=}O$ was used in the curve-fitting.

its formation from $MbFe^{III}$ (rate constant k_i) and fast consumption via auto-reduction to $MbFe^{IV}{=}O$ (rate constant k_{r1}) or reduction by quercetin (rate constant k_{a1}). Equation (1) expressing the rate R_a of antioxidant (AO) consumption can be used in the curve-fitting procedure with k_i and $r_1 = k_{a1}/k_{r1}$ as the adjustable parameters (Table II).

$$R_a = \frac{k_i(MbFe)(H_2O_2)}{1 + \frac{1}{r_1(AO)}}$$

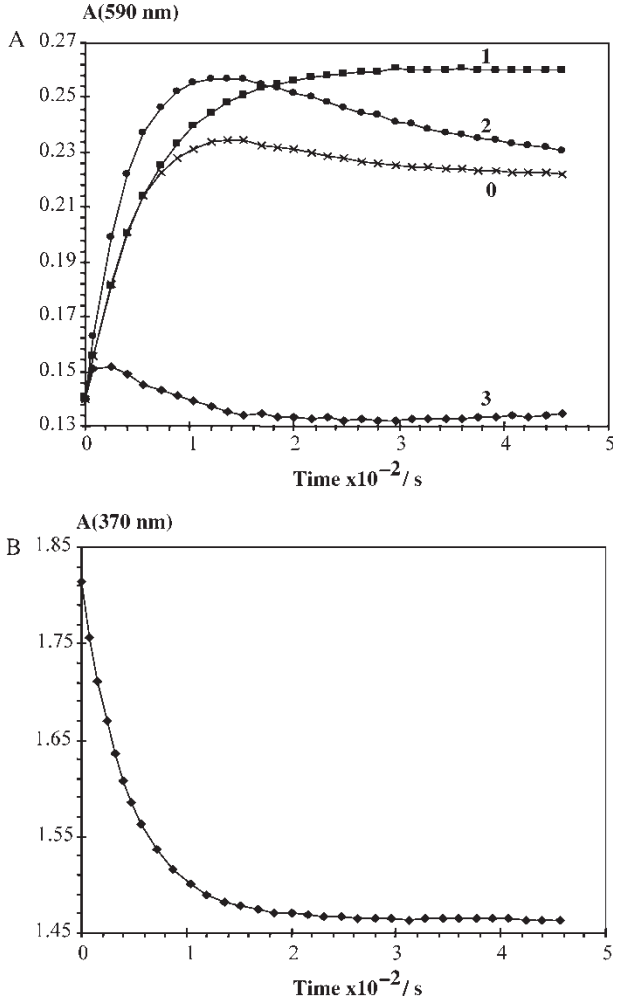


Figure 2. (A) Time dependence of the absorbance at 590 nm following the addition of H_2O_2 (29.3 μM) to a solution of metmyoglobin (30.5 μM) and antioxidant (α-tocopherol 29.5 μM, β-carotene 19.9 μM and quercetin 29.3 μM) in a pH 5.8 phosphate buffer containing 2 mM tween 20, 37°C (control (0), α-tocopherol (1), β-carotene (2) and quercetin (3)). (B) Time dependence of the absorbance at 370 nm during the reaction with quercetin.

The k_i value is consistent with the previous evaluation at 590 nm ($MbFe^{IV}{=}O$ formation, Table I). More importantly, the high r_1 value (*ca.* $10^5\,M^{-1}$) combined with a crude estimation of k_{r1} from the literature [16] (k_{r1} *ca.* $4\,s^{-1}$) points to a fast consumption of quercetin with a rate constant k_{a1} of the order of $2 \times 10^4\,M^{-1}s^{-1}$. The k_{a1} value is 4 times as high as the rate constant for the reduction of $MbFe^{IV}{=}O$ by quercetin (k_{a2}).

Experiments in the absence of tween 20 followed by sample acidification and extraction into ethylacetate allow a partial HPLC-MS analysis of the quercetin oxidation products. Several compounds more polar than quercetin, can thus be evidenced and characterized by their UV absorption and *m/z* value (Table III). These data suggest that the reaction between quercetin and the activated forms of metmyoglobin proceeds via sequences of H-atom abstraction and water addition onto *o*-quinone or *p*-quinonemethide intermediates. Tentative structures are reported on Scheme 2. Some of these compounds (or their analogs formed upon addition of MeOH or EtOH) have already been identified as quercetin oxidation products with various oxidants [26–28].

Since α-tocopherol and β-carotene neither significantly react with $MbFe^{IV}{=}O$ nor inhibit its formation (Figure 2A), it can be stated that these lipophilic antioxidants have no access to the heme crevice for reaction with the hypervalent iron species.

Inhibition of linoleic acid peroxidation

In this work, the peroxidation of linoleic acid (LH, 0.7 mM) is carried out in a mildly acidic (pH 5.8) buffered solution containing the detergent tween 20 (0.2% (*w/v*), *ca.* 2 mM) at 37°C. These conditions allow to investigate the inhibitory properties of antioxidants (0.1–5 μM) with very different lipophilic-hydrophilic balances.

Table II. Kinetic parameters for the reaction of perferrylmyoglobin with quercetin in 2 mM tween 20 (pH 5.8, 37°C).*

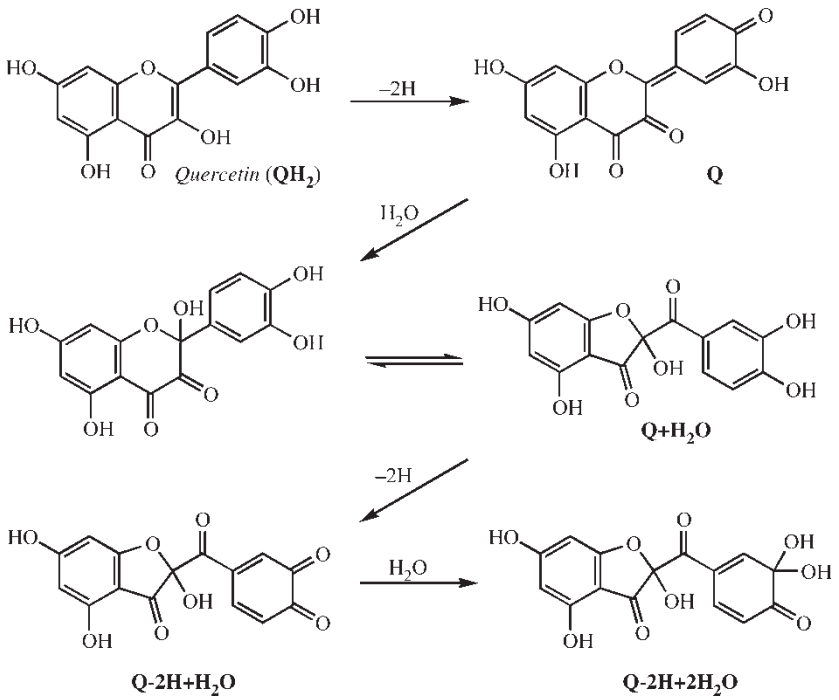
Quercetin / μM	k_i / M^{-1} s^{-1}†	r_1 / 10^3 M^{-1}‡	n¶
29.3 (480 s)	763 (± 3)	92.0 (± 0.5)	0.96 (± 0.01)
25.2 (480 s)	759 (± 3)	98.9 (± 0.4)	0.98 (± 0.01)
19.9 (120 s)	797 (± 13)	118 (± 2)	0.96 (± 0.01)
15.2 (120 s)	736 (± 19)	146 (± 3)	0.95 (± 0.01)

* Detection at 370 nm, $MbFe^{III}$ (30.5 μM) + quercetin, then H_2O_2 (29.3 μM). ε(quercetin) = 21200 M^{-1} cm^{-1}. Between brackets: Duration of the kinetic runs.
† k_i: Second-order rate constant for the conversion of $MbFe^{III}$ into $Mb^{\bullet}Fe^{IV}{=}O$ by H_2O_2.
‡ $r_1 = k_{a1}/k_{r1}$. k_{r1}: First-order rate constant for auto-reduction of $Mb^{\bullet}Fe^{IV}{=}O$, k_{a1}: Second-order rate constant for the reduction of $Mb^{\bullet}Fe^{IV}{=}O$ by quercetin.
¶ n: Number of $Mb^{\bullet}Fe^{IV}{=}O$ molecules reduced per quercetin molecule (stoichiometry).

Table III. HPLC-MS analysis of products formed upon oxidation of quercetin (5 μM) by metmyoglobin (3 μM)/H_2O_2 (5 μM) (pH 5.8, 37°C). QH_2 refers to quercetin. See Scheme 2 for proposed structures.

Compound (t_R / min)	λ_{max} / nm	m/z	Structure
1 (14.2)	324	166 ($z = 2$)	Q-2H + $2H_2O$
2 (16.1)	294	315	Q-2H + H_2O
3 (16.7)	294	317, 273, 229, 209	Q + H_2O
4 (17.7)	322	333, 297, 277, 265 233,211,205	Q-2H + $2H_2O$

AAPH-induced peroxidation. Peroxidation can be initiated by a common water-soluble diazo compound (AAPH, in a large and quasi-constant concentration of 1 mM) which, upon thermal dissociation in the presence of dioxygen, yields initiating peroxyl radicals at a constant rate in the aqueous phase. Consequently, the rate of uninhibited peroxidation (R_p^0) is also constant. Indeed, monitoring the UV absorbance at 234 nm (typical of conjugated dienes) as a function of time yields a straight line. After addition of a typical chain-breaking lipophilic antioxidant such as α-tocopherol, the peroxidation rate is markedly depressed, then resumes as the antioxidant approaches total consumption to finally reach a value that is close to that in the absence of antioxidant (Figure 3A). In other words, a well-defined lag phase is observed whose duration increases linearly as a function of the initial α-tocopherol concentration. When peroxidation is inhibited by the hydrophilic



Scheme 2. Oxidation of quercetin by $MbFe^{III}/H_2O_2$.

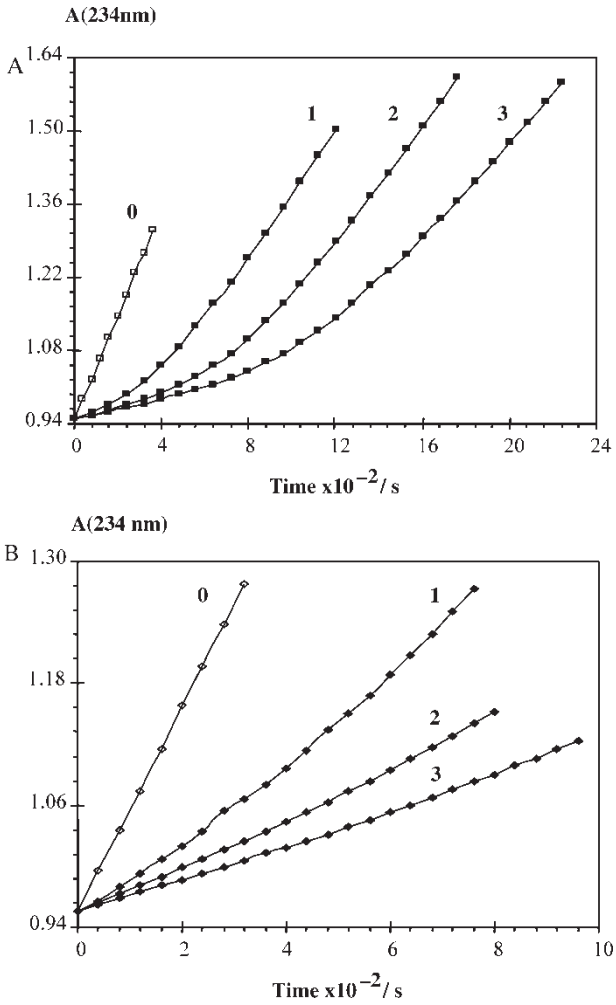


Figure 3. Accumulation of lipid oxidation products (conjugated dienes, UV detection at 234 nm) during the inhibited peroxidation of linoleic acid (0.7 mM) induced by AAPH (1 mM) in a pH 5.8 phosphate buffer containing 2 mM tween 20, 37°C. (A) Inhibition by α-tocopherol. α-Tocopherol concentrations are: 0 (0), 0.2 (1), 0.6 (2) and 1 μM (3). (B) Inhibition by quercetin. Quercetin concentrations are 0 (0), 0.2 (1); 0.6 (2) and 1 μM (3).

antioxidant quercetin, no clear lag phase is observed. The peroxidation is slowed down, then steadily resumes without reaching its rate before antioxidant addition (Figure 3B). These distinct behaviors of α-tocopherol and quercetin can be well accounted for by assuming that the lipophilic antioxidant acts in the lipid phase by reducing the lipid-derived peroxyl radicals ($LOO^{\bullet}$) whereas the hydrophilic antioxidant acts in the aqueous phase by reducing the AAPH-derived peroxyl radicals. Within each model (see "Annex"), kinetic parameters (antioxidant efficiencies AE, antioxidant stoichiometry) can be readily evaluated from the curve-fitting of the A (234)-time curves recorded in the presence of the antioxidant (Table IV). In comparison with the usual procedures for data treatment that only deal with the initial rates of inhibited peroxidation [29], the mathematical treatment developed in the Annex is based on a kinetic analysis of the whole peroxidation curves. In the case of α-tocopherol, the curve-fitting procedure yields the antioxidant efficiency at inhibiting propagation (AE_2) that expresses the competition between the antioxidant (AO) and lipid (LH) for the propagating radicals ($LOO\bullet$): $AE_2 = k(LOO\bullet + AO)/k(LOO\bullet + LH) = k_{a2}/k_2$ *ca.* 120. The stoichiometry n (number of $LOO\bullet$ radicals reduced per antioxidant molecule) is set at 2 for α-tocopherol [30] and is an adjustable parameter for β-carotene. The lower efficiency of β-carotene at inhibiting the AAPH-induced peroxidation of linoleic acid is reflected in both a lower AE_2 value (*ca.* 50) and a lower stoichiometry (Table IV). The low stoichiometry of β-carotene must be due in part to its instability in tween 20 emulsions that is well-evidenced in control experiments (see below). In the case of quercetin, the curve-fitting procedure yields the antioxidant efficiency at inhibiting initiation (AE_1) and the antioxidant stoichiometry (n = number of $ROO\bullet$ radicals reduced per antioxidant molecule). Parameter AE_1 expresses the competition between the antioxidant and lipid for the initiating radicals ($ROO\bullet$): $AE_1 = k(ROO\bullet + AO)/k(ROO\bullet + LH) = k_{a1}/k_1$. Since the nk_{a1} product is in fact the rate constant for the first reduction of the initiating radicals by quercetin, the nAE_1 product is expected to be constant, which is reasonably verified: nAE_1 *ca.* 3×10^4. The latter value points to a very fast scavenging of peroxyl radicals by quercetin in the aqueous phase in agreement with investigations by pulse radiolysis in aqueous solutions [31].

Metmyoglobin-induced peroxidation. Peroxidation can be more efficiently initiated by addition of a low concentration (typically, 0.1 μM) of metmyoglobin. In the absence of antioxidant, the accumulation of conjugated dienes proceeds readily with a short lag phase. Concomitantly, low concentrations of carbonyl compounds (typical UV band at 270–280 nm, molar absorption coefficient of 24,000 $M^{-1}\,cm^{-1}$ estimated from the commercially available analog (*E*,*E*)-2,4-hexadienal) can be detected. The antioxidant markedly slows down the accumulation of conjugated dienes which proceeds with a lag phase that is best defined with α-tocopherol and increases with the antioxidant concentration (Figures 4 and 5, Table V). During inhibited peroxidation, the formation of carbonyl compounds is negligible over the typical duration (30–120 min) of the experiment. Simultaneously, the consumption of the antioxidants displaying a strong UV–visible absorption (β-carotene and, to a lesser degree, quercetin) can also be monitored (Figure 5B). Thus, it can be checked that the lag period essentially reflects the period during which most of the antioxidant is consumed. After the lag phase, the peroxidation rate increases to a maximal value (R_p^{max}, propagation phase). The R_p^{max} value remains lower than the corresponding value in the absence of antioxidant (Figures 4 and 5, Table V). Finally, a third phase

Table IV. Kinetic parameters and stoichiometries for the inhibited peroxidation of linoleic acid (0.7 mM) induced by AAPH (1 mM) in 2 mM tween 20 (pH 5.8, 37°C). Correlation coefficients of the curve-fittings >0.999.

AO, C_{AO} / μM α-tocopherol*	$10^{10}(R_p^0/Ms^{-1})$	AE_2	$10^{10}(R_i / M\ s^{-1})$
0.2	304.6	176 ± 2	6.97 ± 0.03
0.4	317.1	151 ± 3	8.61 ± 0.05
0.6	307.9	125 ± 2	10.06 ± 0.05
0.8	310	72 ± 1	10.41 ± 0.06
1	294.2	97 ± 1	10.98 ± 0.04
AO, C_{AO} / μM β-carotene†	$10^{10}(R_p^0/Ms^{-1})$	AE_2	n
1	354.2	43.4 (± 2.0)	2.13 (± 0.07)
2	358.3	56.5 (± 3.0)	1.11 (± 0.04)
3	395.8	42.7 (± 2.6)	1.08 (± 0.05)
4	366.7	48.5 (± 5.6)	0.84 (± 0.08)
5	362.2	68.3 (± 4.0)	0.63 (± 0.03)
AO, C_{AO} / μM Quercetin†	$10^{10}(R_p^0/Ms^{-1})$	$10^{-3}AE_1$, $10^{-3}nAE_1$	n
0.2	382	9.8 (± 0.2), 32.5	3.32 (± 0.03)
0.4	392	13.1 (± 0.2), 29.1	2.22 (± 0.01)
0.6	420	17.9 (± 0.2), 32.2	1.80 (± 0.01)
0.8	399	20.5 (± 0.4), 31.6	1.54 (± 0.02)
1.0	398	15.1 (± 0.1), 27.5	1.82 (± 0.01)

* n set at 2.
† R_i set at 9.7×10^{-10} M s^{-1}.

following the propagation phase can be observed during which the peroxidation rate tends to decrease. This phase was not considered in the kinetic analysis.

In control experiments without linoleic acid or metmyoglobin or both, the consumption of β-carotene and, to a lesser degree, quercetin is still quite important although much less than when both components are present (Table VI). In such conditions, antioxidant degradation could be due to autoxidation [32,33] (catalysed by adventitious metal ion traces) and/or, especially in the case of β-carotene, photo-oxidation. However, $MbFe^{III}$ itself significantly accelerates degradation of both quercetin and β-carotene.

A first quantitative comparison between the antioxidants investigated can be achieved by measuring, for different antioxidant concentrations C, the period T necessary to accumulate a fixed concentration of conjugated dienes, e.g that corresponding to a 0.7 increase in the absorbance of the conjugated dienes with respect to its value at time zero (addition of $MbFe^{III}$). At least for low C values, T is a quasi linear function of C that can be expressed as: $T = T_0(1 + C/IC50)$. IC50: Antioxidant concentration corresponding to a period T twice as large as the control period with no antioxidant. The IC50 parameter can be estimated from the slope of T vs. C lines. The lower the IC50 value, more efficient the antioxidant is. Clearly, quercetin (IC50 = 0.33(±0.01) μM) and α-tocopherol (IC50 = 0.28(±0.02) μM) appear as more efficient antioxidants than β-carotene (IC50 = 1.52(±0.13) μM).

From HPLC-MS analysis (Figure 6), dienylalcohols (LOH, 4 regio- and stereoisomers, $m/z = 295$) appear as the major lipid oxidation products (2–5% of the total LH concentration) in the commercially available samples of linoleic acid. However, because of a weak autoxidation during sample preparation (see "Experimental" Section), close concentrations of dienylhydroperoxides (LOOH, 4 regio- and stereoisomers, $m/z = 311$) and alcohols are usually detected just before the addition of $MbFe^{III}$ (time zero of the kinetic experiments). Finally, HPLC analysis in the course of the $MbFe^{III}$-induced peroxidation ($t = 2$ and 30 min) clearly shows that LOOH are the main products of linoleic acid peroxidation and that LOH do not significantly accumulate. In addition, mass peaks corresponding to dienylketones ($L = O$, $m/z = 293$) and triols ($m/z = 329$) can be detected.

To gain information on changes occurring on the heme moiety, the peroxidation was also carried out in more concentrated solutions of linoleic acid (2.5 mM) and metmyoglobin (10 μM) for detection in the visible range (Figure 7). In such conditions, the accumulation of conjugated dienes (detection at 234 nm) is very fast and rapidly followed by the accumulation of carbonyl compounds (detection at 274 nm). As expected, α-tocopherol (10 μM) delays both events with a very well-defined lag phase. Monitoring the reaction at 410 nm shows that the decay of the Soret band typical of the porphyrin ring is significantly slowed down by α-tocopherol, then resumes once the antioxidant is consumed (i.e. after the lag period). Detection at 590 nm affords no evidence for the accumulation of ferrylmyoglobin ($MbFe^{IV}$=O) during uninhibited peroxidation. By contrast, the presence of α-tocopherol allows the building-up of low quasi-stationary concentrations of ferrylmyoglobin that sharply collapse after consumption of the antioxidant. This result confirms that α-tocopherol does not react

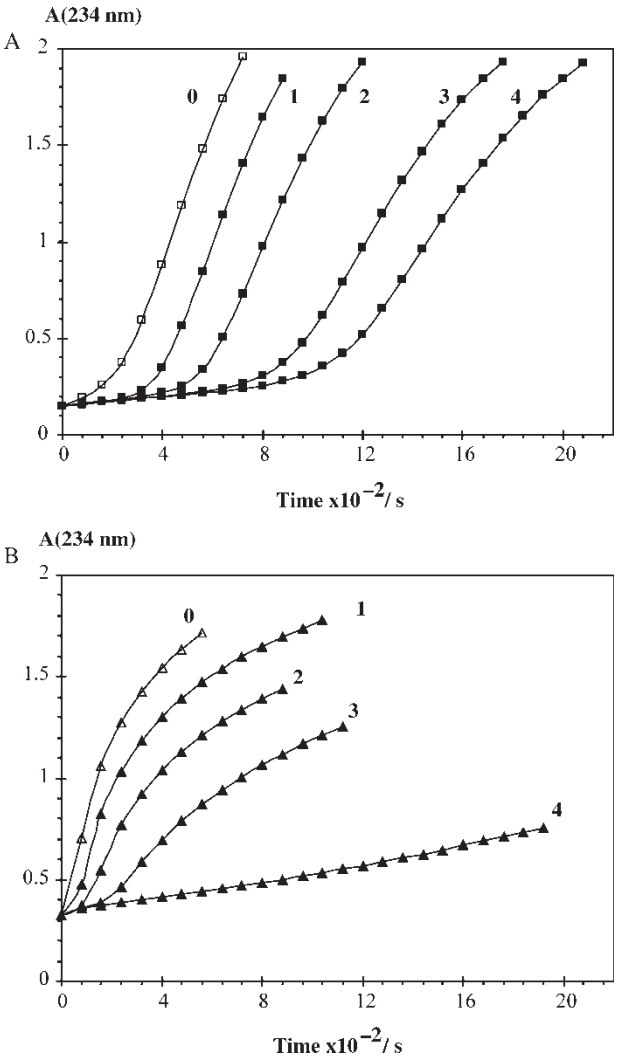


Figure 4. Accumulation of lipid oxidation products (conjugated dienes, UV detection at 234 nm) during α-tocopherol-inhibited peroxidation of linoleic acid induced by metmyoglobin or hematin. Linoleic acid (0.7 mM) + heme (0.1 μM), pH 5.8 phosphate buffer containing 2 mM tween 20, 37°C. (A) Initiation by metmyoglobin. (B) Initiation by hematin. α-Tocopherol concentrations are: 0 (0), 0.2 (1), 0.4 (2), 0.6 (3) and 0.8 μM (4).

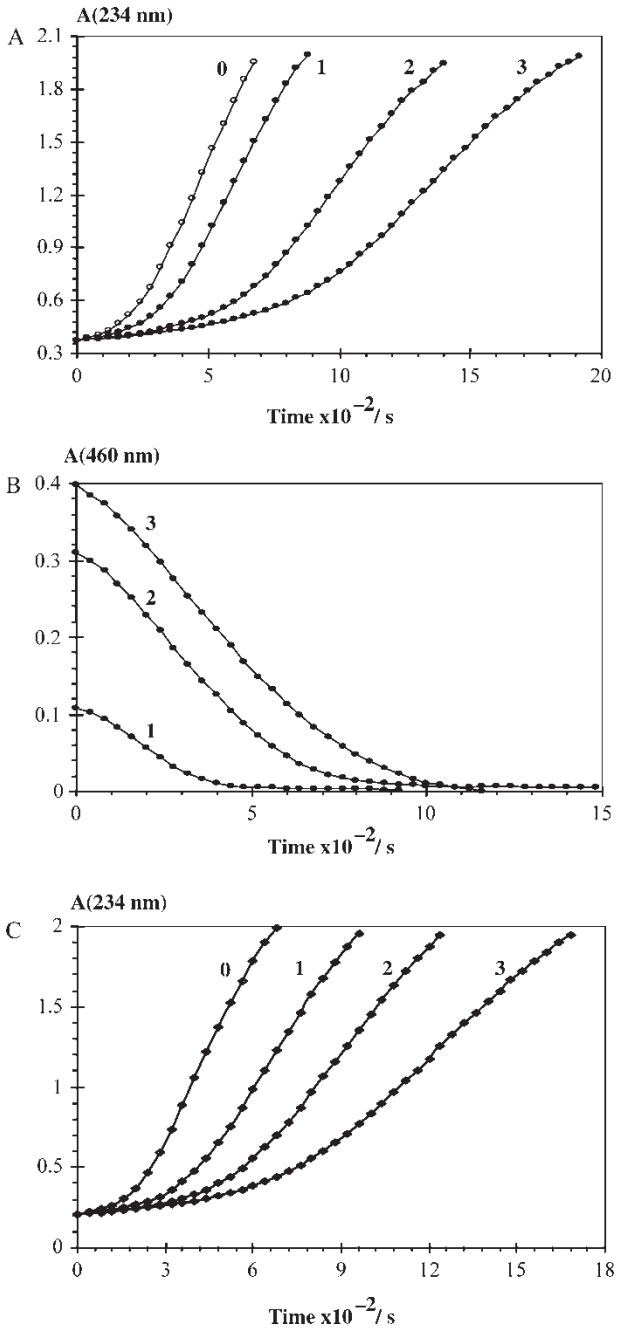


Figure 5. Accumulation of lipid oxidation products (conjugated dienes, UV detection at 234 nm) during the inhibited peroxidation of linoleic acid induced by metmyoglobin. Linoleic acid (0.7 mM) + metmyoglobin (0.1 μM), pH 5.8 phosphate buffer containing 2 mM tween 20, 37°C. (A) Inhibition by β-carotene. β-Carotene concentrations are: 0 (0), 1 (1), 3 (2) and 4 μM (3). (B) Consumption of β-carotene (UV detection at 460 nm). (C) Inhibition by quercetin. Quercetin concentrations are 0 (0), 0.2 (1); 0.4 (2) and 0.6 μM (3).

rapidly with $MbFe^{IV}$=O. In the presence of quercetin, protection of the heme is also evidenced by a slower decay of the Soret band (less clear with β-carotene because of its absorption at 410 nm) (data not shown). However, ferrylmyoglobin remains undetectable with both quercetin and β-carotene. From the decay of the Soret band at 410 nm, the percentage of heme degradation at a fixed time of the peroxidation lag phase (200 s, antioxidant concentration = 10 μM) can be calculated to be 28% for quercetin, 43% for α-tocopherol, and 66% in the control experiment without antioxidant. Hence, quercetin is more efficient than α-tocopherol at protecting the heme during the lag phase. Peroxidation and heme degradation simultaneously resume at the end of the lag phase (Figure 7) which occurs at T_{lag} = 250 s for quercetin and 340 s for α-tocopherol. These T_{lag} values suggest that quercetin consumption is faster than α-tocopherol consumption in those conditions.

Hematin-induced peroxidation. It has been proposed that high fatty acid:$MbFe^{III}$ molar ratios (> 100) result in

Table V. Kinetic parameters for the inhibited peroxidation of linoleic acid (0.7 mM) induced by metmyoglobin (100 nM) in 2 mM tween 20 (pH 5.8, 37°C).

Ref., C_{AO} / μM	$10^9(R_p^{max}/Ms^{-1})^{\star}$	T_{lag} / s†	$10^{10}(R_a$ / M s$^{-1})^{\ddagger}$
β-carotene			
1	127.2	376	27, 28[¶]
2	106.7	536	37, 42[¶]
3	82.4	725	41, 47[¶]
4	60.5	1027	39, 46[¶]
5	48.0	1180	42, 53[¶]
α-tocopherol			
0.2	139.6	448	4.5
0.4	117.3	613	6.5
0.6	102.2	838	7.2
0.8	72.3	1260	6.4
1.0	55.4	1760	5.7
Quercetin			
0.2	125.0	373	5.4
0.4	103.3	547	7.3
0.6	75.6	747	8.0

* $R_p = (1/24000)dA(234)/dt$.
† $R_p = (R_p^{min} + R_p^{max})/2$ at $t = T_{lag}$. R_p^{min} and R_p^{max} are respectively the minimal and maximal values of the peroxidation rate R_p.
‡ $R_a = C/T_{lag}$.
[¶] R_a^{max} deduced from derivation of the A(460)-time curves.

$MbFe^{III}$ denaturation at pH 7.4 and a peroxidation process actually initiated by the sole heme moiety (hematin) [34]. However, under mildly acidic conditions (pH 5.8), hematin-induced peroxidation and metmyoglobin-induced peroxidation display very different kinetic profiles (A (234) vs. time curves), both in the presence and absence of antioxidant (Figure 4). Indeed, whereas hematin-induced peroxidation is as fast as and eventually faster than metmyoglobin-induced peroxidation in a first period, the rate of the latter retains a maximal (almost constant) value in a second period whereas the rate of the former clearly tends to level off. Hence, we assume that the hematin-globin complex is still responsible for the metmyoglobin-induced lipid peroxidation in our conditions, even though part of the heme is degraded in the course of the oxidation as evidenced by the decay of the Soret absorption band (Figure 7). Moreover, when the experiments of hematin-induced lipid peroxidation are repeated at high lipid and heme concentrations (2.5 mM linoleic acid, 10 μM hematin) allowing detection in the visible range, the decay of the Soret band (400 nm) appears faster than in the case of metmyoglobin. However, both quercetin and α-tocopherol afford almost complete protection during the lag phase (data not shown).

Discussion

Myoglobin ($MbFe^{II}$) is the red heme protein responsible for dioxygen transport in the muscles. Its one-electron oxidized form metmyoglobin ($MbFe^{III}$) may represent up to 2–3% of total myoglobin in biological tissues. Myoglobin and metmyoglobin also represent more than 90% of the iron present in red meat. The concentration of the latter tends to increase in muscle food (beef, pork...) because of specific post-mortem conditions (e.g. pH fall) [15]. $MbFe^{III}$ is able to efficiently induce the peroxidation of linoleic acid after activation by

Table VI. Kinetic parameters of antioxidant consumption at pH 5.8 and 37°C (detection at 375 and 460 nm for quercetin and β-carotene, respectively).

AO / μM	LH / mM	$MbFe^{III}$ / nM	$10^5(k_{obs}$ / s$^{-1})^{\star}$	% AO consumed
Quercetin				after 60 min
6.44	0	0	523 (± 21), 23.4 (± 4.1)	19
6.44	0	100	631 (± 34), 26.1 (± 0.5)	36
6.44	0.7	0	–	10
6.44	0.7	100	451 (± 52), 28.6 (± 0.5)	57
β-carotene				after 20 min
4.1	0	0	23 (± 2)	12
4.1	0	100	125 (± 2)	43
4.1	0.7	0	19.6 (± 0.1)	21
4.1	0.7	100	–	98

* Apparent first-order rate constant k_{obs} assuming mono- or biexponential decays.

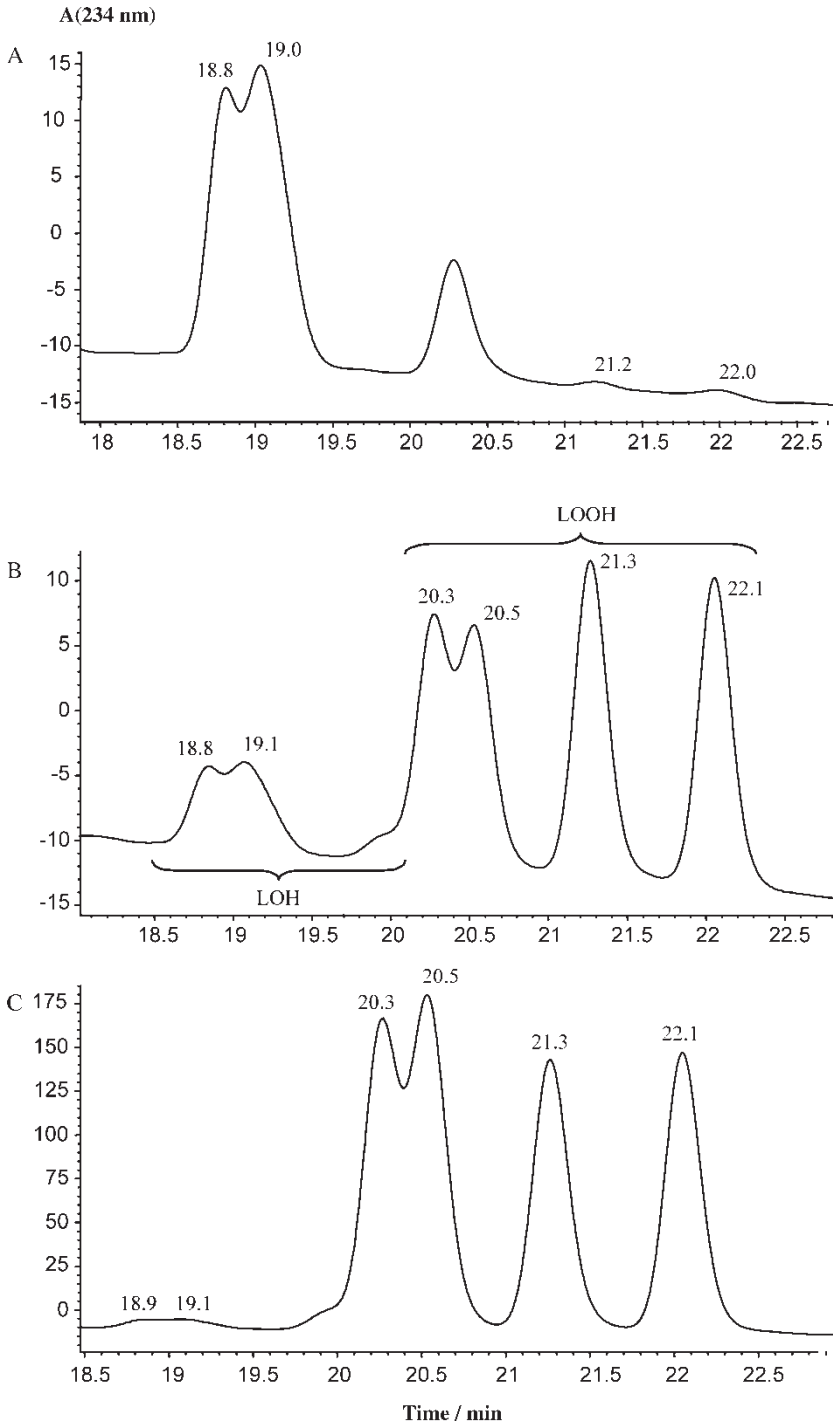


Figure 6. HPLC analysis of the lipid oxidation products formed during the uninhibited peroxidation of linoleic acid induced by metmyoglobin (UV detection at 234 nm). (A) Linoleic acid (11 mM) in acetone. (B) and (C) Linoleic acid (0.7 mM) + metmyoglobin (0.1 μM), pH 5.8 phosphate buffer containing 2 mM tween 20, 25°C. Sample incubated during 2 min (B) and 30 min (C) before extraction for HPLC analysis (see conditions in section "Experimental").

hydrogen peroxide or by the lipid hydroperoxides themselves [15,34–36]. $MbFe^{III}$ could be critical to the peroxidation of dietary lipids not only in the GI tract but also in meat and meat products whose quality and acceptability are clearly limited by lipid oxidation [37], especially since the concentration of lipid hydroperoxides tends to increase during the conversion of muscle to meat.

The peroxidation of linoleic acid induced by metmyoglobin or its activated forms has been investigated in the literature. In mildly acidic linoleic acid emulsions, perferrylmyoglobin, formed *in situ* upon reaction of $MbFe^{III}$ with H_2O_2, is converted into a green pigment (probably, a globin-heme linked Fe^{III} complex) that is unable to catalyse lipid peroxidation [38]. By contrast, ferrylmyoglobin (previously

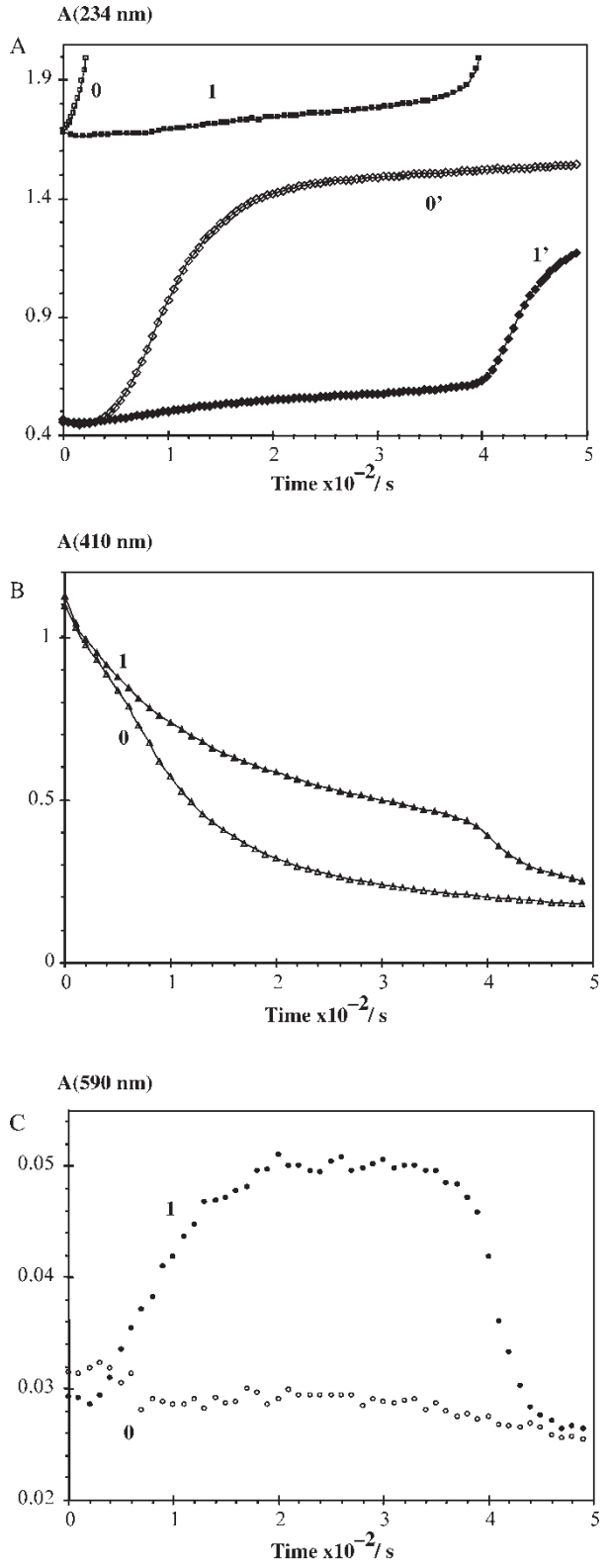


Figure 7. Inhibition of the peroxidation of linoleic acid (2.5 mM) induced by metmyoglobin (10 μM) in the presence or absence of α-tocopherol (10 μM). pH 5.8 phosphate buffer containing 6.75 mM tween 20, 37°C. (A) Conjugated dienes (UV detection at 234 nm, control (0), + α-tocopherol (1)) and carbonyl compounds (UV detection at 274 nm, control (0′), + α-tocopherol (1′)). (B) Soret band (UV detection at 410 nm, control (0), + α-tocopherol (1)). (C) Ferrylmyoglobin (UV detection at 590 nm, control (0), + α-tocopherol (1)).

prepared upon treatment of $MbFe^{III}$ by H_2O_2) is a very efficient initiator of linoleic acid peroxidation in mildly acidic to neutral conditions [34,38,39]. These results are consistent with earlier observations that myoglobin mutants devoid of tyrosine residues are as effective as wild myoglobin at peroxidizing linoleic acid, thus ruling out a peroxidation induced by the protein radical [35]. In neutral emulsions, metmyoglobin-induced peroxidation of linoleic acid proceeds rapidly only at high linoleic acid-$MbFe^{III}$ molar ratios ($\geq$ 200) [34] as if a partial denaturation of the protein with a subsequent increased exposure of the iron-oxo species was necessary for a fast oxidation of the weakly bound linoleic acid molecule [35] (K_d *ca.* 1 mM). At lower linoleic acid-$MbFe^{III}$ molar ratios, binding of linoleate anions results in the formation of the low-spin hemichrome species that does not catalyse lipid peroxidation [34,39].

The mechanism of the $MbFe^{III}$-induced peroxidation of linoleic acid and its inhibition by antioxidants is still uncompletely known. Small initial concentrations of lipid hydroperoxides (formed by autoxidation) are clearly needed to initiate the peroxidation that proceeds without detectable protein radical intermediate [38]. In addition, the observation that $MbFe^{III}$ and $MbFe^{IV}{=}O$ are as effective at initiating peroxidation in mildly acidic linoleic acid emulsions suggests the fast formation of iron-oxo species from $MbFe^{III}$ and linoleic acid hydroperoxides. Similar processes have been studied with a variety of hydroperoxides (ROOH). For instance, ESR investigations suggest that *t*-butylhydroperoxide is homolytically cleaved by metmyoglobin and hematin: [40,41]

$$ROOH + MbFe^{III} \rightarrow MbFe^{III} - O - OR$$

$$\rightarrow MbFe^{IV}{=}O + RO^{\bullet}$$

In addition, accumulation of the ferryl species can be evidenced by UV–visible spectroscopy [42]. Product analysis in the reaction of the linoleic acid hydroperoxide (LOOH) with hematin is also consistent with the homolytic cleavage mechanism. Indeed, the major products are epoxyalcohols and the corresponding triols whose formation involves the rearrangement of the $LO^{\bullet}$ radical [43]. Similar products devoid of dienyl chromophore were also detected in the reaction of LOOH with metmyoglobin at pH 7.4 [44]. With (met)myoglobin, heterolytic hydroperoxide cleavage can also take place and even prevails over the homolytic route:[40,41,45,46]

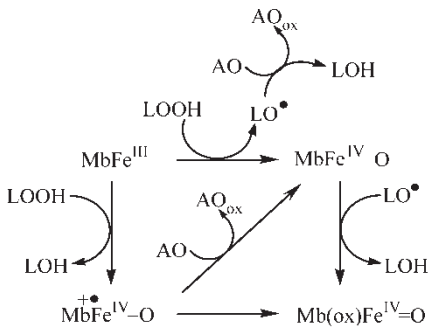
$$MbFe^{III} - O - OR \rightarrow Mb^{\bullet+}Fe^{IV}{=}O + ROH$$

($Mb^{\bullet+}$: Globin moiety bound to a porphyrin radical-cation)

During the metmyoglobin-induced peroxidation of linoleic acid, the heme moiety undergoes a rather fast

degradation (Figure 7B) and ferrylmyoglobin does not accumulate (Figure 7C). In addition, no protein radical can be detected [38]. Hence, it is proposed that the homolytic/heterolytic cleavage of the lipid hydroperoxides leads to reactive oxygen species (iron-oxo species distinct from perferrylmyoglobin, lipid oxyl radicals) that abstract electrons and/or H-atoms either from the lipid or from the heme or from an antioxidant present in the medium (Scheme 3, Mb(ox): Globin moiety bound to a degraded heme). In the heterolytic route, the porphyrin radical-cation is assumed to be the precursor of the degraded heme. In the homolytic route, one of the fates of the highly reactive $LO^{\bullet}$ radical is assumed to be its reaction with the heme. The protection of the heme by the antioxidant, though modest initially, is quite obvious during the lag phase (Figure 7B). Interestingly, once the antioxidant is consumed and the heme essentially degraded, the formation of conjugated dienes and carbonyl compounds resumes very rapidly. This suggests that the degraded ferrylmyoglobin $Mb(ox)Fe^{IV}=O$ is still a very efficient initiator of lipid peroxidation.

α-Tocopherol and quercetin display distinct behaviors when they inhibit the AAPH-induced peroxidation of linoleic acid and when they are confronted to the hypervalent iron species derived from metmyoglobin in tween 20 emulsions. These differences suggest that the partitioning of the antioxidants in the lipid and aqueous phases is a crucial factor for interpreting their antioxidant activities. The results can be interpreted by assuming that quercetin is essentially located in the aqueous phase where it can efficiently trap hydrophilic oxidizing species (AAPH-derived peroxyl radicals, perferrylmyoglobin, ferrylmyoglobin) whereas α-tocopherol is located in the lipid phase where it can reduce the lipid-derived peroxyl radicals that propagate lipid peroxidation. Similar partitioning of antioxidants between aqueous and lipid compartments have already been demonstrated in liposomes, micelles and plasma [47–49]. Typically, polyphenols act in the aqueous phase by reducing hydrophilic peroxyl radicals and regenerating α-tocopherol (from the corresponding aryloxyl radical) at the interface. By contrast, α-tocopherol and carotenoids act in the lipid phase by reducing lipophilic peroxyl radicals.



X = $MbFe^{IV}$=O, $^{+\bullet}MbFe^{IV}$=O, $Mb(ox)Fe^{IV}$=O, $LO^{\bullet}$

$$X + LH + O_2 \xrightarrow{k_1} XH + LOO^{\bullet}$$

$$LOO^{\bullet} + LH + O_2 \xrightarrow{k_2} LOO^{\bullet} + LOOH$$

$$X + AO \xrightarrow{k_{a1}} XH + AOox$$

$$LOO^{\bullet} + AO \xrightarrow{k_{a2}} LOOH + AOox$$

$$2LOO^{\bullet} \xrightarrow{k_t} \text{Non radical products}$$

Scheme 3. Metmyoglobin-induced lipid peroxidation.

Among the three antioxidants investigated, only α-tocopherol allows the detection of low concentrations of ferrylmyoglobin ($MbFe^{IV}=O$) during inhibited peroxidation (Figure 7C). In fact, based on the molar absorption coefficient reported in Table I, up to 20% of total iron may be converted into $MbFe^{IV}=O$ during α-tocopherol-inhibited peroxidation under the conditions of Figure 7. An antioxidant can *a priori* exert two opposite effects on $MbFe^{IV}=O$: it can favour $MbFe^{IV}=O$ accumulation by protecting the heme from degradation. On the other hand, the antioxidant can also quickly reduce $MbFe^{IV}=O$. In the case of α-tocopherol, the former effect must prevail in agreement with α-tocopherol selectively reacting with the lipid radicals rather than with the hypervalent iron species. The detection of $MbFe^{IV}=O$ in the presence of α-tocopherol is also consistent with the ability of this antioxidant to stabilize ferrylmyoglobin prepared from metmyoglobin and hydrogen peroxide (Figure 2A). Despite the better protection it affords to the heme, quercetin does not allow to detect $MbFe^{IV}=O$ during the inhibited peroxidation of linoleic acid. This is consistent with the relatively fast reduction of $MbFe^{IV}=O$ (independently prepared by reaction of $MbFe^{III}$ with H_2O_2) by quercetin (Figure 1, Table I). In the process of heme protection by the antioxidants, it is proposed that quercetin, which can reach the heme to quickly reduce (per)ferrylmyoglobin, essentially repairs the heme from the corresponding radical-cation generated in the heterolytic route, whereas α-tocopherol reduces the lipid oxyl radicals generated in the homolytic route (Scheme 3). The latter mechanism is more efficient with α-tocopherol than with β-carotene (probably too deeply embedded in the lipid phase) so that detection of transient concentrations of $MbFe^{IV}=O$ is observed with α-tocopherol only.

A semi-quantitative analysis of the peroxidation curves allows to gain information on the reactivity of the antioxidants. Beer's law can be expressed as: $A(234) = \varepsilon((LOOH) + (LOH))$, assuming the same molar absorption coefficient for LOH and LOOH ($\varepsilon = 24000\ M^{-1}\ cm^{-1}$ [50]). After repeated controls by HPLC, the dienyl alcohols and hydroperoxides were assumed to be in approximately equal concentrations at time zero, i.e. when metmyoglobin is

added. However, the analysis by HPLC-MS (Figure 6) shows that the dienylhydroperoxides (LOOH) are the main products to be considered during the early steps of lipid oxidation. Consequently, the chemical rate $(1/\varepsilon)dA(234)/dt$ that can be readily extracted upon derivation of the A(234)-time curves is essentially equal to the peroxidation rate $R_p = d(LOOH)/dt$. In Table V are collected the maximal R_p values (R_p^{max}) for different antioxidant concentrations (C) as well as the lag period (T_{lag}) defined as the period needed to reach a peroxidation rate that is half the maximal value. During the lag period, the rate of antioxidant consumption R_a can be roughly expressed as: $R_a = C/T_{lag}$. This relationship assumes that the antioxidant is essentially consumed at the end of the lag period. This assumption is well-verified for β-carotene whose consumption can be directly monitored by the decay of its visible absorption at 460 nm during inhibited peroxidation (Figure 5B). From Table V, it can be noted that the R_a values are essentially independent of the total antioxidant concentration. Constant R_a values during the lag phase can be interpreted by neglecting propagation ($k_2 = 0$) and by assuming that the initiating species ($X = Mb^{\bullet+}Fe^{IV}{=}O$, $MbFe^{IV}{=}O$, $Mb(ox)Fe^{IV}{=}O$, $LO^{\bullet}$, see Scheme 3), and the peroxyl radicals they form after reacting with the lipid, reach a steady-state concentration. Thus, the rate of antioxidant consumption becomes: $R_a = R_i/n = k_i(LOOH)(MbFe^{III})/n$ (R_i: Rate of initiation, n: Antioxidant stoichiometry). During the lag phase, the metmyoglobin concentration is approximately equal to its initial value. This is also true for the lipid hydroperoxides that do not accumulate since only a slight increase of A (234) is observed during the lag phase. In the case of α-tocopherol and quercetin, the R_a values are in the range $4–8 \times 10^{-10}\,M\,s^{-1}$. Typically, α-tocopherol is able to trap two equivalents of oxidizing species before being converted into inert products [30]. Located in the lipid phase, it inhibits peroxidation by reducing the $LOO^{\bullet}$ radicals. However, its ability to partially protect the heme from degradation points to an early action in the initiation step, possibly by reacting with the $LO^{\bullet}$ radicals. If the latter process is neglected (one oxidizing equivalent is released in the initiation step, the other being dissipated within the cofactor), the stoichiometry of α-tocopherol is 2 (two $LOO^{\bullet}$ trapped per antioxidant molecule). On the contrary, if heme protection was total during the lag phase because of α-tocopherol scavenging the second oxidizing equivalent released in the initiation step, the apparent stoichiometry would drop to 1 since only half the α-tocopherol concentration would be available to inhibit lipid peroxidation. Hence, it can be concluded that the stoichiometry of α-tocopherol falls between 1 and 2. Using a n value of 1.5 and estimating the average LOOH concentration during the lag phase to be 5 μM, a rough estimate of the initiation rate constant can be obtained: k_i *ca.* $2 \times 10^3\,M^{-1}\,s^{-1}$. This is also the order of magnitude of the more accurately determined rate constant for the activation of metmyoglobin by H_2O_2 (Table I).

Since α-tocopherol and quercetin have close R_a values, it can be concluded that the stoichiometries of these antioxidants during the lag phase are close. In the case of quercetin, which is proposed to inhibit initiation in the aqueous phase, the rate of antioxidant consumption may be expressed as: $R_a = k_{a1}(AO)(X)/n$, X referring to the initiating iron-oxo species taken collectively (Scheme 3). Taking an average antioxidant concentration of 0.5 μM and assuming that less than 10% of metmyoglobin is under its hypervalent form during the lag phase, we obtain: $k_{a1} > 2 \times 10^5\,M^{-1}\,s^{-1}$. However crude this estimation may be, it clearly suggests that the reaction of quercetin with the transient hypervalent species formed during the metmyoglobin-induced peroxidation of linoleic acid is much faster than the reactions of quercetin with ferrylmyoglobin (k_{a2} *ca.* $5 \times 10^3\,M^{-1}\,s^{-1}$) and perferrylmyoglobin (k_{a1} *ca.* $2 \times 10^4\,M^{-1}\,s^{-1}$). Although a degraded ferrylmyoglobin $Mb(ox)Fe^{IV}{=}O$ could be a stronger oxidant than ferrylmyoglobin because of Mb(ox) being less electron-donating than Mb, this result more probably points to a direct reaction of quercetin with the $Mb^{\bullet+}$ radical-cation in agreement with the partial protection quercetin affords to the heme.

For β-carotene, R_a values are in the range $3–4 \times 10^{-9}\,M\,s^{-1}$. Thus, it can be estimated that the β-carotene stoichiometry during the lag period is roughly 4–8 times as low as that of α-tocopherol and quercetin. Several factors can be put forward to interpret this result: β-carotene may be too embedded in the lipid phase to efficiently react with lipid radicals probably generated at the lipid-water interface. On the other hand, in parallel to their reduction of iron-oxo species and/or lipid radicals (antioxidant action), highly reducing antioxidants such as β-carotene and, to a lesser degree quercetin, undergo competitive oxidative degradation [32,33] as evidenced from control experiments without lipid and/or metmyoglobin (Table VI). Autoxidation (electron tranfer to dioxygen catalysed by unidentified metal traces) is expected to be mainly responsible for quercetin degradation whereas additional photo-oxidation could take place with the light-sensitive β-carotene. Since degradation is particularly fast with β-carotene, it could also be one reason why β-carotene is a less efficient antioxidant than quercetin and α-tocopherol in our model.

Beyond the lag phase, the peroxidation rate reaches a maximal value before slowly decaying probably because of the conversion of the lipid hydroperoxides into secondary oxidation products. However, a residual antioxidant activity is manifested by the observation that the maximal peroxidation rate is typically lower than its value in noninhibited peroxidation (Table V). This phenomenon is strongest with quercetin and suggests that some of its oxidation products retain an ability to

reduce hypervalent iron in agreement with the partial analysis of the products formed upon oxidation of quercetin by $MbFe^{III}/H_2O_2$ (Scheme 2, Table III) that points to successive sequences of two-electron oxidation. Alternatively, covalent coupling between the globin moiety and electrophilic *o*-quinone and *p*-quinone-methide intermediates formed during quercetin oxidation [26] could also take place.

Conclusion

Regardless of their bioavailability (intestinal absorption and distribution to tissues via the blood stream) which may be rather low (e.g. polyphenols), dietary antioxidants could exert their protective action directly in the gastro-intestinal tract. Indeed, the gastric digestion of food containing oxidizable lipids and iron catalysts for peroxide decomposition such as (met)myoglobin may be accompanied by an extensive formation of potentially toxic lipid hydroperoxides [11]. Hence, the ability of antioxidants to inhibit the metmyoglobin-induced peroxidation of linoleic acid in mildly acidic emulsions is proposed as a pertinent and general model of antioxidant activity.

It is shown that quercetin mainly acts in the aqueous phase by quickly reducing the peroxidation initiator, a degraded form of ferrylmyoglobin (produced by reaction of metmyoglobin with the lipid hydroperoxides) and/or a more reactive precursor, e.g., the porphyrin radical-cation. The reaction of quercetin with the activated forms of metmyoglobin is faster by at least a factor 40 than the corresponding reaction with ferrylmyoglobin (independently prepared by reaction of metmyoglobin with hydrogen peroxide). By contrast, α-tocopherol does not significantly react with heme iron and must exert its antioxidant action in the lipid phase by scavenging the lipid peroxyl radicals. Overall, the phenolic antioxidants quercetin and α-tocopherol come up as efficient peroxidation inhibitors with IC50 values lower than 1 μM. The poorer inhibition afforded by β-carotene may be traced to both its slower reaction with the lipid peroxyl radicals and its competitive degradation by autoxidation and/or photo-oxidation.

Annex: Mathematical treatment for the inhibition of AAPH-induced peroxidation

The first step is the formation in the aqueous phase of the initiating species, i.e. the hydrophilic peroxyl radicals (noted $ROO^{\bullet}$) produced by the thermal decomposition of AAPH in the presence of dioxygen. The corresponding rate R_i is expressed as $2ek_d(AAPH)$ where k_d is the dissociation rate constant of the diazo compound and e the molar fraction of AAPH-derived peroxyl radicals that escape recombination in the solvent cage and become available for initiation according to the reaction:

$$ROO\bullet + LH + O_2 \rightarrow ROOH + LOO\bullet, \quad k_1$$

The LOO• radical can then either react with a second LH molecule (propagation) or recombine with a second LOO• to form nonradical products (termination):

$$LOO\bullet + LH + O_2 \rightarrow LOOH + LOO\bullet, \quad k_2$$

$$2LOO\bullet \rightarrow \text{non radical products}, k_t$$

Finally, the antioxidant AO can reduce (electron- and/or H atom transfers) the initiating and/or propagating peroxyl radicals and be simultaneously converted into inert oxidation products AOox:

$$ROO\bullet + AO \rightarrow ROOH + AOox, \quad k_{a1}$$

$$LOO\bullet + AO \rightarrow LOOH + AOox, \quad k_{a2}$$

Assuming a steady-state for the initiating radicals, one gets: $R_i = k_1(LH)(ROO\bullet) + k_{a1}(AO)(ROO\bullet) = k_1(LH)(ROO\bullet)[1 + AE_1(AO)/(LH)]$ with $AE_1 = k_{a1}/k_1$ (antioxidant efficiency at inhibiting initiation). Assuming a steady-state for the lipid peroxyl radicals, one gets:

$$k_1(ROO\bullet)(LH) = k_{a2}(LOO\bullet)(AO) + 2k_t(LOO\bullet)^2 \quad (1)$$

The rate of lipid hydroperoxide formation is: $R_p = d(LOOH)/dt = k_2(LOO\bullet)(LH) + k_{a2}(LOO\bullet)(AO)$. The rate of antioxidant consumption is: $R_a = -d(AO)/dt = k_{a1}(ROO\bullet)(AO) + k_{a2}(LOO\bullet)(AO)$. Solving eqn. (1) for (LOO•) gives:

$$k_{a2}(LOO\bullet)(AO) = k_q(AO)^2 \times \left[\left(1 + \frac{2k_1(LH)(ROO\bullet)^{1/2}}{k_q(AO)^2}\right) - 1\right] \quad (2)$$

$$k_2(LOO\bullet)(LH) = \frac{k_q}{AE_2}(AO)(LH) \times \left[\left(1 + \frac{2k_1(LH)(ROO\bullet)^{1/2}}{k_q(AO)^2}\right) - 1\right] \quad (3)$$

With $k_q = k_{a2}^2/(4kt)$ and $AE_2 = k_{a2}/k_2$ (antioxidant efficiency at inhibiting propagation). In the absence of antioxidant, eqn. (3) becomes eqn. (4):

$$R_p^0 = k_2(LOO\bullet)(LH) = r_2(LH)(k_1(LH(ROO\bullet))^{1/2} \quad (4)$$

With $r_2 = k_2/(2k_t)^{1/2}$

Parameters k_q, r_2 and AE_2 are also bound through the following relationship:

$$k_q = (r_2 AE_2)^2/2 \quad (5)$$

The initial antioxidant concentration is expressed as: $(AO) = nC$, n and C being the antioxidant stoichiometry (number of peroxyl radicals reduced per antioxidant molecule) and analytical antioxidant concentration, respectively. The concentration of linoleic acid (in large excess) is assumed to remain constant throughout the reaction. Finally, Beer's law can be simply expressed as: $A(234) = \varepsilon(LOOH)$ with $\varepsilon = 24000\,M^{-1}\,cm^{-1}$.

Lipophilic antioxidants. Inhibition of initiation is neglected. Therefore, R_i can be identified with $k_1(ROO\bullet)(LH)$. The rates for hydroperoxide formation and antioxidant consumption can be readily expressed from Equations (2)–(4):

$$R_p = k_q(AO)^2\left(1+\frac{(LH)}{AE_2(AO)}\right)\times\left[\left(1+\frac{2R_i}{k_q(AO)^2}\right)^{1/2}-1\right] \quad (6)$$

$$R_p^0 = r_2(LH)R_i^{1/2}$$

$$R_a = k_{a2}(LOO\bullet)(AO) = k_q(AO)^2\left[\left(1+\frac{2R_i}{k_q(AO)^2}\right)^{1/2}-1\right] \quad (7)$$

The uninhibited peroxidation rate R_p^0 is constant and can be estimated from the slope of the A(234) vs. time line prior to antioxidant addition. Then, the A(234) vs. time curves featuring the inhibited peroxidation of linoleic acid can be fitted against Equations (5)–(7) with AE_2, R_i and n as the independent parameters to be optimized. In fact, with α-tocopherol, n can be safely set at 2 (α-tocopherol reduces 2 equiv. of oxidizing species and is simultaneously converted into inert oxidation products [30]), which leaves only AE_2 and R_i as the unknown parameters. The corresponding R_i value (*ca.* $9.7 \times 10^{-10}\,M\,s^{-1}$) is then used in the curve-fittings dealing with β-carotene with AE_2 and n as the adjustable parameters (Table IV).

Hydrophilic antioxidants. They are supposed to act in the aqueous phase so that inhibition of propagation can be neglected. Hence, the peroxidation rate is simply:

$R_p = r_2(LH)[k_1(LH)(ROO\bullet)]^{1/2}$ with $R_i = k_1(LH)(ROO\bullet)[1 + AE_1(AO)/(LH)]$ and $R_p^0 = r_2(LH)R_i^{1/2}$. Since the antioxidant is consumed by reaction with the initiating species only, we also have: $R_a = k_{a1}(ROO\times)(AO)$. Combining these three equations readily gives:

$$R_p = \frac{R_p^0}{\sqrt{1+AE_1(AO)/(LH)}} \quad (8)$$

$$R_a = \frac{R_i}{1+\frac{(LH)}{AE_1(AO)}} \quad (9)$$

Using the R_i value deduced from the α-tocopherol-inhibited experiments and the mean R_p^0 value deduced from the slope of the A(234)-time lines recorded in the absence of antioxidant, eqns (8)–(9) allow satisfactory curve-fittings of the quercetin-inhibited peroxidation with AE_1 and n as the adjustable parameters (Table IV).

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