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Antioxidant Synergy and Regeneration Effect of Quercetin, (−)-Epicatechin, and (+)-Catechin on α-Tocopherol in Homogeneous Solutions of Peroxidating Methyl Linoleate

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Antioxidant interactions between flavonoids and α-tocopherol have been demonstrated by oximetry (oxygen concentration measured by ESR signal line width). In *tert*-butyl alcohol, a solvent in which flavonoids are weak retarders of peroxidation of methyl linoleate when initiated by α,α′-azoisobutyronitrile, quercetin and (−)-epicatechin were found to act synergistically with the chain-breaking antioxidant α-tocopherol. In chlorobenzene, a solvent in which flavonoids are chain-breaking antioxidants, quercetin and (+)-catechin each regenerated α-tocopherol, resulting in a co-antioxidant effect. The stoichiometric factor of the flavonoids as chain-breaking antioxidants in 1:1 mixtures with α-tocopherol was measured to be close to 1 for quercetin and slightly smaller for the catechins. The apparent inhibition rate constant, k_{inh}, for the mixture quercetin/α-tocopherol was measured to be 4.1 × 10^5 and 2.6 × 10^6 M^{-1} s^{-1} in *tert*-butyl alcohol and chlorobenzene, respectively, at 50 °C. A k_{inh} of 4.4 × 10^5 M^{-1} s^{-1} was measured for (+)-catechin alone in chlorobenzene at 50 °C.

KEYWORDS: Quercetin; (−)-epicatechin; (+)-catechin; α-tocopherol; regeneration; synergism; antioxidants; solvent effect

INTRODUCTION

Flavonoids are polyphenolic compounds that are widespread in foods and beverages such as fruits, vegetables, chocolate, teas, and wines, and the average intake of flavonoids in Western countries has been estimated to be 23 mg/day (*1*, *2*). The antioxidant properties of flavonoids depend on both metal-chelating properties (*3*) and free radical scavenging of reactive oxygen species (*4*, *5*). The *relative* antioxidant capacity of flavonoids as chain-breaking inhibitors in food systems has also been studied, although the ability of flavonoids to inhibit lipid peroxidation has been evaluated mainly by antioxidant indices relative to the effect of vitamin E. Thus, these indices are dependent on the type of method used, and they do not provide information to establish absolute antioxidant hierarchies or to clarify reaction mechanisms when comparing different flavonoids. The *intrinsic* antioxidant activity of flavonoids as chain-breaking antioxidants, that is, the rate constant of the H-transfer reaction from the flavonoid to peroxyl radicals, the chain-carrying species in the lipid peroxidation, has recently been measured for a few flavonoids and was found to be smaller than that of α-tocopherol (αTOH) (*6*, *7*). Also, as has been found for tocopherols and simple phenols, their activity is reduced if the solvent in which the lipid peroxidation takes place has a high hydrogen-bond-accepting ability. As a consequence of H-bonding interactions with the solvent, flavonoids are weak retarders in polar solvents but good chain-breaking antioxidants in apolar media. Such a kinetic solvent effect has been found to be larger for flavonoids than for αTOH (*7*, *8*).

To achieve a complete understanding of the antioxidant mechanism of flavonoids in food and living organisms (*9*), their interaction with other antioxidants must also be explored. There has been some evidence of a higher antioxidant effect when combinations of flavonoids and αTOH were used (*10*, *11*). However, the mechanism of such interactions is not well understood, and even the evidence presented for the order of antioxidant regeneration is rather controversial. From an experimental investigation (*12*) and a theoretical study (*13*), regeneration of αTOH from its one-electron-oxidized form by some flavonoids was suggested in analogy to the well-known synergism between αTOH and ascorbate (*14*−*16*), whereas a recent electrochemical study combined with ESR spectroscopy showed that flavonoids were regenerated by αTOH under certain conditions (*17*).

In the present work, we have studied the antioxidant effect of quercetin (QC), a flavon-3-ol, and of two catechins, (−)-epicatechin [(−)-EC] and (+)-catechin [(+)-C], when combined with the chain-breaking inhibitor αTOH. Oximetry, a direct method for measurement of oxygen consumption by means of electron spin resonance (ESR) spectroscopy, was used (*18*). The effect of these antioxidant mixtures on the nature of the inhibition reaction and its rate was investigated at 50 °C in a homogeneous solution of peroxidating methyl linoleate. Two

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solvents with different hydrogen-bond-accepting ability, *tert*-butyl alcohol and chlorobenzene, where chosen in order to test if solvent effects affect the mechanism of antioxidant interaction. In both solvents, lipid peroxidation was initiated by thermal decomposition of α,α′-azoisobutyronitrile (AIBN). The rates of oxygen consumption in the presence of either a flavonoid or αTOH, alone or in combination, were compared and then converted to second-order rate constants, k_{inh}, for the inhibiting reaction of the antioxidant mixtures. Synergistic (SE%) or regeneration (RE%) effects and stoichiometric factors (*n*) for flavonoids as antioxidants in the antioxidant mixtures at different concentration ratios were calculated from the oxygen depletion curves. Finally, a mechanism of antioxidant interaction between flavonoids and αTOH in homogeneous solutions is suggested.

MATERIALS AND METHODS

Materials. All chemicals were commercially available, chosen of the highest purity and used as received. Methyl linoleate, chlorobenzene, quercetin, (−)-epicatechin, (+)-catechin, and 2,2,6,6-tetramethyl-1-piperidinyloxyl (TEMPO) were purchased from Aldrich-Sigma (St. Louis, MO), α,α′-azoisobutyronitrile was from Fluka (Buchs, Switzerland), and *all-rac*-α-tocopherol and *tert*-butyl alcohol were from Merck (Darmstadt, Germany).

Apparatus. The ESR spectra were recorded on a Bruker ECS 106 spectrometer (Bruker, Rheinstetten, Germany) by using the following settings: microwave frequency, 9.42 GHz; power, 0.80−2 mW; modulation amplitude, 0.5 G; center field, 3360 G; sweep time, 81 s; time constant, 81 ms.

Samples. ESR samples from homogeneous solutions of peroxidating methyl linoleate were air-saturated at room temperature and introduced (∼100 μL) into a capillary tube with an internal diameter of ∼2 mm. A second capillary tube, having a thinner diameter and sealed at one end, was introduced into the sample tube so as to leave very little dead volume space. The tube was then introduced into the ESR cavity kept at 50.0 ± 0.1 °C, and the first spectrum was recorded after ∼1 min to allow the temperature to equilibrate. The ESR spectra of a solution of 3.5×10^{-5} M TEMPO in *tert*-butyl alcohol and in chlorobenzene were recorded at 50 °C in air atmosphere and in nitrogen atmosphere and used as standards for measuring the line width of the spin probe TEMPO under air and in the absence of oxygen. The oxygen solubility under air atmosphere is 1.73×10^{-3} M in *tert*-butyl alcohol (*19*) and 1.61×10^{-3} M in chlorobenzene at 50 °C (*20*).

RESULTS AND DISCUSSION

Antioxidant Interaction of Flavonoids and α-Tocopherol in *tert*-Butyl Alcohol. The autoxidation of 0.24 M methyl linoleate (ML) in *tert*-butyl alcohol was initiated by 0.034 M AIBN at 50 °C in the presence of quercetin (QC) or (−)-epicatechin [(−)-EC] alone or in combination with α-tocopherol (αTOH). The rate of oxygen consumption was monitored by means of ESR spectroscopy in a closed system with no headspace over the time required for oxygen depletion. The ESR oximetry method is based on the fact that the line width of the ESR signal of a nitroxide stable radical, which was added to the peroxidating mixture as an oxygen spin probe, varies linearly with the oxygen concentration in solution (*7*, *18*, *19*). The time traces of the oxygen consumption observed during the peroxidation of ML in the absence of inhibitor, in the presence of QC or αTOH, and in the presence of a combination of QC and αTOH are shown in **Figure 1**.

The rate of oxygen consumption during lipid peroxidation was weakly reduced in the presence of QC, in agreement with the recent findings that flavonoids behave as retarders of the autoxidation in *tert*-butyl alcohol (*7*), whereas a definite

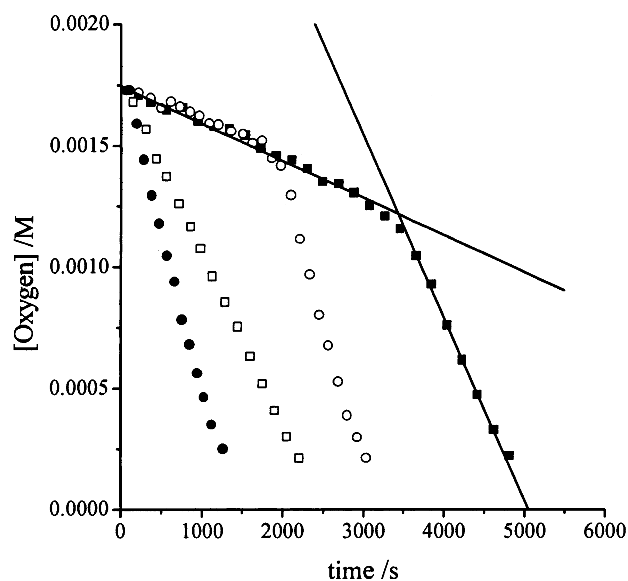


Figure 1. Oxygen depletion during autoxidation of methyl linoleate (0.24 M) in *tert*-butyl alcohol initiated by AIBN (0.034 M) at 50 °C in the absence (●) or presence of quercetin (7.7×10^{-5} M) (□), in the presence of α-tocopherol (7.7×10^{-5} M) (○), or in the presence of α-tocopherol (7.7×10^{-5} M) and quercetin (7.7×10^{-5} M) (■) in a closed system with no headspace.

Table 1. Synergistic Effects (SE%) and Stoichiometric Factors (*n*) of Flavonoids in Antioxidant Mixtures with α-Tocopherol during an AIBN-Initiated Autoxidation of Methyl Linoleate in *tert*-Butyl Alcohol at 50 °C[a]

antioxidant mixture	[αTOH]/ 10^{-5} M	[FlH_2]/ 10^{-5} M	SE%	*n*
α-tocopherol/ quercetin	7.7	4.0	30[b]	1.15[b]
	7.7	7.7	55 ± 5	1.09 ± 0.09
	7.7	15.2	100[b]	1.00[b]
α-tocopherol/ (−)-epicatechin	7.7	4.0	19[b]	0.76[b]
	7.7	7.7	41 ± 3	0.83 ± 0.06
	7.7	15.2	55[b]	0.56[b]

[a] [AIBN] = 0.034 M; [linoleate] = 0.24 M; [TEMPO] = 3.5×10^{-5} M; R_i = $(7.5 \pm 0.3) \times 10^{-8}$ M s^{-1} (average from seven independent measurements); SE% and *n* for αTOH/FlH_2 1:1 are averages from at least three independent measurements (±standard deviation). [b] Only single detection.

induction period, τ, was observed in the presence of αTOH. Moreover, the results reported in **Figure 1** clearly show antioxidant interaction. The combination of QC and αTOH produced a longer induction period than the one measured when αTOH was used alone; that is, a clear synergistic effect is the result of the interaction. Further, the rate of oxidation throughout the induction period with both antioxidants present was the same as that during the inhibited period observed when αTOH was used alone, an observation providing evidence for regeneration of αTOH by the flavonoid QC. In the presence of the flavonoid, αTOH thus produces a longer and more effective inhibition of the lipid peroxidation. Synergistic effects were also obtained (oxygen depletion curves not shown) when αTOH was combined with (−)-EC, which is known to be a poor inhibitor when used alone (*7*).

The lengths of the induction periods (τ) when QC or (−)-EC was combined with αTOH have allowed calculation of the synergistic effects, SE%, for antioxidant mixtures FlH_2/αTOH (FlH_2 = flavonoid) in different concentration ratios by

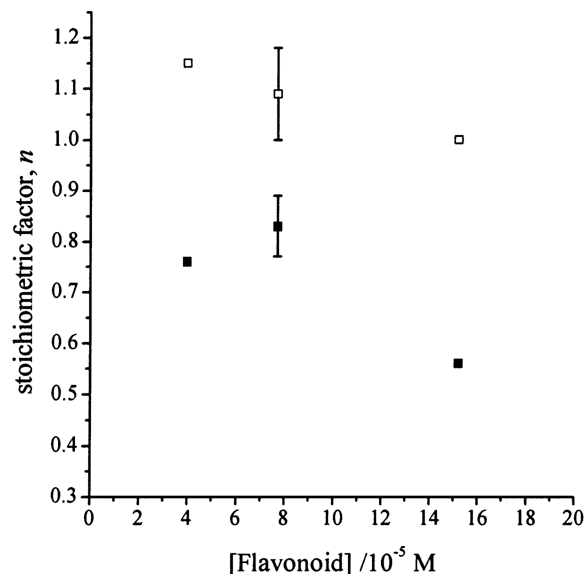


Figure 2. Stoichiometric factor of quercetin (□) and (–)-epicatechin (■) for increasing flavonoid concentration in antioxidant mixture with α-tocopherol (7.7 × 10^{-5} M) during AIBN-initiated lipid peroxidation of methyl linoleate in *tert*-butyl alcohol at 50 °C.

eq 1 (see **Table 1**). The induction period τ_{FlH_2} is zero for *tert*-butyl alcohol as solvent.

$$SE\% = \frac{\tau_{\alpha TOH + FlH_2} - (\tau_{\alpha TOH} + \tau_{FlH_2})}{\tau_{\alpha TOH} + \tau_{FlH_2}} \times 100\% \qquad (1)$$

The data in **Table 1** show that at constant αTOH concentration, a larger ratio FlH_2/αTOH leads to a higher SE% effect. This result is in agreement with the fact that, if flavonoids regenerate αTOH, then the length of the extended τ should be dependent on the flavonoid concentration.

In the presence of a single chain-breaking antioxidant, the length of the induction period depends on the antioxidant concentration, [AH], and provides the number of radical chains interrupted (stoichiometric factor, n) by a single molecule of antioxidant. The value of n is given by eq 2, where R_i is the rate of formation of the initiating radicals (*21*). For αTOH, the

$$n = R_i\tau/[AH] \qquad (2)$$

stoichiometric factor has the value $n = 2$, according to the well-known mechanism where each molecule of αTOH inactivates two peroxyl radicals (eqs 3 and 4).

$$\alpha TOH + ROO^\bullet \rightarrow \alpha TO^\bullet + ROOH \qquad (3)$$

$$\alpha TO^\bullet + ROO^\bullet \rightarrow \text{adducts} \qquad (4)$$

Therefore, in the presence of an antioxidant mixture αTOH and FlH_2, an extended induction period provides information of the amount of αTOH regenerated for a particular R_i and thus on the amount of radicals trapped by the flavonoid. Therefore, when calculated per mole of flavonoid (see eq 5), a measure of the stoichiometric factor, n, is obtained for the flavonoid in the antioxidant mixture with αTOH.

$$n = R_i[\tau_{\alpha TOH + FlH_2} - \tau_{\alpha TOH}]/[FlH_2] \qquad (5)$$

The values of n calculated from antioxidant mixtures αTOH/FlH_2 at different concentration ratios (see **Table 1** and **Figure 2**) were found to decrease slightly in the presence of a higher amount of flavonoid; this was especially clear for (−)-EC. This drop in n might be due to loss of polyphenol in the oxidation process (autoxidation of the flavonoid), which happens slowly during the antioxidant interaction (vide infra). This is also indicated by the fact that the SE% values did not increase proportionally with the flavonoid concentration.

Because there are many competing reactions involved in the system under investigation (vide infra), the observed differences in the values of the stoichiometric factor n for antioxidant mixtures αTOH/FlH_2 at different concentration ratios are understandable, and relatively large experimental errors (see **Figure 2**) expected. Therefore, no attempt was made to determine the absolute value of n by linear regression through zero of the variation of $R_i[\tau_{\alpha TOH + FlH_2} - \tau_{\alpha TOH}]$ with $[FlH_2]$.

The numbers of equivalent oxidation chains terminated by QC or (−)-EC, when added in the concentration ratio 1:1 with αTOH during an AIBN-initiated peroxidation of methyl linoleate in *tert*-butyl alcohol at 50 °C, were calculated to be 1.1 and 0.8, respectively. In other words, the addition of a flavonoid to αTOH produces an increment of the inhibition effect close to 50% of the effect resulting from addition of a similar amount of αTOH ($n = 2$).

So far, no studies have attempted a quantification of the synergistic effect of QC when combined with αTOH either in homogeneous solutions or in micelles. However, regeneration of αTOH by QC has been suggested as a possibility based on the lower reduction potential of the flavonoid ($E = 0.33$ V) compared to that of Trolox C ($E = 0.48$ V, both values at pH 7) (*13*). On the other hand, a synergistic effect of 34% has been reported for the mixture αTOH/(−)-EC in the concentration ratio 1:2 in *tert*-butyl alcohol−water (3:2 v/v) during the inhibition of an AAPH-initiated peroxidation of linoleic acid at 37 °C (*12*).

Our data indicate that QC and (−)-EC have similar αTOH regeneration ability, with QC being slightly more effective. Since QC and (−)-EC both have been reported (*7*) to possess a similar reactivity toward peroxyl radicals (competing self-oxidation), these results might indicate a slightly larger reactivity of QC toward tocopheroxyl radicals than of (−)-EC in *tert*-butyl alcohol. Mukai et al. have found a higher rate constant for the regeneration of 5,7-diisopropyltocopheroxyl by quercetin, 2.98 × 10^2 M^{-1} s^{-1} (*22*), than by epicatechin, 1.52 × 10^2 M^{-1} s^{-1} (*23*), at 25 °C in ethanol. Similar findings have been recently reported by Niki et al. (*24*): a higher reactivity of QC toward α-tocopheroxyl radicals (second-order rate constant of 2.3 × 10^3 M^{-1} s^{-1}) than of epigallocatechin gallate (5.9 × 10^2 M^{-1} s^{-1}) at 37 °C in ethanol.

The rate constant for the chain-breaking reaction, that is, the rate constant for the reaction of an antioxidant with peroxyl radicals from the oxidizing substrate, can be determined from the slope of the inhibited oxygen consumption, $(-d[O_2]/dt)_{inh}$, when a clear induction period is observed using eq 6 (*21*). The comparison of this rate with the rate measured in the presence of αTOH provides the value for k_{inh} for the other antioxidant when k_{inh} for αTOH is known under the same experimental conditions. This equation has been derived under the assumption

$$(-d[O_2]/dt)_{inh} = (k_p[RH]R_i/nk_{inh}[AH]) + R_i \qquad (6)$$

that all peroxyl radicals are quenched by the antioxidant, that is, in the presence of a chain-breaking inhibitor. Both in the presence of αTOH alone and in the presence of the antioxidant mixture αTOH/FlH_2 (see **Figure 1**) such conditions exist, and k_{inh} of the mixture αTOH/QC was calculated from eq 6. Moreover, experimental conditions were selected in order to ensure a radical chain reaction with a relatively long oxidation

Table 2. Apparent Inhibition Rate Constants (k_{inh}) for Antioxidant Mixtures of α-Tocopherol with a Flavonoid in *tert*-Butyl Alcohol and Chlorobenzene at 50 °C during AIBN-Initiated Peroxidation of Methyl Linoleate

antioxidant mixture	k_{inh}/10^5 M^{-1} s^{-1}	
	*t*BuOH	PhCl
α-tocopherol/quercetin	4.1 ± 0.2	25.6 ± 1.5
α-tocopherol/(−)-epicatechin[a]	2.8	
α-tocopherol[b]	6.28	35.5

[a] Reference *12*. [b] These values were calculated from the measured value of 5.1×10^5 M^{-1} s^{-1} at 37 °C in *t*BuOH (*16*) and 2.7×10^6 M^{-1} s^{-1} at 25 °C in chlorobenzene (*25*) by assuming a log *A* factor of 8 in the Arrhenius equation (*26*).

chain length ($\nu \geq 4$); that is, an AIBN-initiated peroxidation for a relatively high concentration of methyl linoleate in the presence of low amounts of the combined antioxidants was used.

In the presence of both αTOH and QC, the oxidation was suppressed quite efficiently, and the apparent inhibition rate constant in *tert*-butyl alcohol was measured to be (4.1 ± 0.2) × 10^5 M^{-1} s^{-1} at 50 °C. This value for the reaction between αTOH/QC and the peroxyl radicals should be compared with the rate constant for reaction of QC with methyl linoleate peroxyl radicals, which has been measured to be 2.1×10^4 M^{-1} s^{-1} in *tert*-butyl alcohol at 50 °C (*7*). That is, QC when used alone is 30 times less active than αTOH, which has a hydrogen-transfer rate constant of 6.28×10^5 M^{-1} s^{-1} at 50 °C (see **Table 2**). Therefore, the inhibition rate constant of the antioxidant mixture αTOH/QC is much larger than that for the flavonoid alone and close to that observed for αTOH. A similar antioxidant activity of a flavonoid was also recognized by Jia et al. (*12*) for the mixture αTOH/(−)-EC at 37 °C in *tert*-butyl alcohol−water (3:2 v/v) (see **Table 2**).

A careful examination of the trace of oxygen consumption recorded in the presence of a combination of αTOH and QC (**Figure 1**) shows that, when the induction period is over, the oxygen uptake is weakly retarded with respect to the rate of uninhibited oxidation. A similar behavior was obtained with (−)-EC (data not shown). This secondary inhibition was confirmed by the length of the oxidation chain, ν, which was calculated to be shorter than the one measured in the absence of antioxidant. This late inhibition might be indicative of antioxidant properties of the oxidized flavonoid. Evidence of the antioxidant ability of oxidized flavonoids has previously been reported. It was suggested that either the hydroxyl groups still available in ring A (*27*), or the quinone formed in ring B (*7*), or an even more polar phenolic oxidation product might be responsible for this late inhibition of oxidation (*28*). However, because in some cases the flavonoid is only partly consumed to regenerate αTOH ($n = 0.8$ for (−)-EC), the contribution of the flavonoid to this late inhibition of the autoxidation should not be ruled out.

Antioxidant Interaction of Flavonoids and α-Tocopherol in Chlorobenzene. Recent findings have demonstrated that in non-hydrogen-bonding solvents, flavonoids behave as chain-breaking inhibitors (*6*, *7*). Like αTOH, they inhibit the lipid peroxidation over a clear induction period, the length of which corresponds to a stoichiometric factor of about 2. The H-transfer rate constants measured for QC and (−)-EC toward lipid peroxyl radicals in chlorobenzene at 50 °C are 4.3×10^5 and 4.2×10^5 M^{-1} s^{-1}, respectively (*7*). Thus, in chlorobenzene these flavonoids are only 8 times less reactive than αTOH, which has an inhibition rate constant of 3.55×10^6 M^{-1} s^{-1} (see **Table 2**).

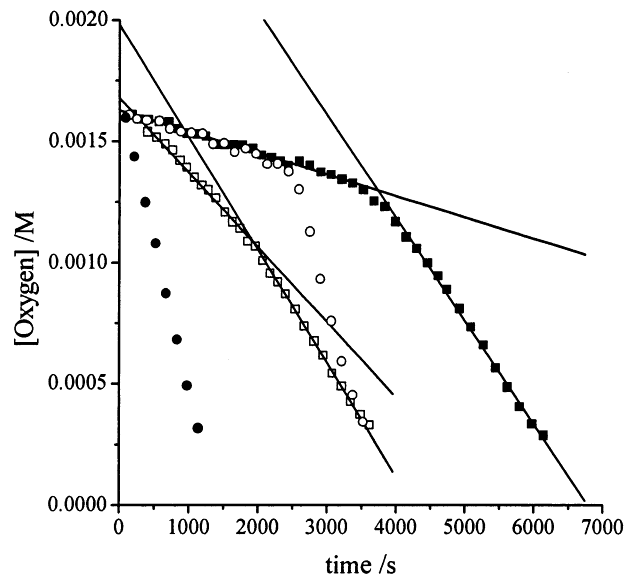


Figure 3. Oxygen depletion during autoxidation of methyl linoleate (0.24 M) in chlorobenzene initiated by AIBN (0.017 M) at 50 °C in the absence (●) and in the presence of (+)-catechin (8.0×10^{-5} M) (□), in the presence of α-tocopherol (8.0×10^{-5} M) (○), or in the presence of α-tocopherol (8.0×10^{-5} M) and (+)-catechin (8.0×10^{-5} M) (■) in a closed system with no headspace.

The efficiency of the antioxidant interaction between flavonoids and αTOH was accordingly also investigated in chlorobenzene. The autoxidation of 0.24 M ML was initiated by 0.017 M AIBN at 50 °C in the presence of a flavonoid used alone or in combination with αTOH. The time traces of the oxygen consumption were again monitored by ESR oximetry. The scarce solubility of polyphenols in chlorobenzene was circumvented by the use of highly concentrated flavonoids solutions in *tert*-butyl alcohol (10% v/v in chlorobenzene).

The inhibited peroxidation of ML in the presence of (+)-catechin ((+)-C), or of αTOH, and in the presence of a combination of (+)-C and αTOH is shown in **Figure 3**. Similar results were found for oxygen uptakes in the presence of the antioxidant mixture αTOH/QC (oxygen depletion curves not shown).

The antioxidant activity of (+)-C in chlorobenzene was unknown, and the ability of (+)-C to inhibit AIBN-initiated peroxidation of ML at 50 °C was accordingly also studied. As **Figure 3** illustrates, (+)-C behaved as a chain-breaking inhibitor in chlorobenzene. The length of the induction was found to be dependent on the flavonoid concentration, and a stoichiometric factor, *n*, of 1.5 was calculated by eq 2. However, this value is probably not very accurate compared to the value $n \approx 2$ for (−)-EC and QC (*7*) because of the difficulty in evaluating the induction period with accuracy under the present experimental conditions [see (+)-C oxygen depletion curve in **Figure 3**]. The rate constant, k_{inh}, for the hydrogen-transfer reaction from (+)-C to the lipid peroxyl radicals was calculated using eq 6 to have the value (4.4 ± 0.5) × 10^5 M^{-1} s^{-1} at 50 °C. This value may be compared to the one for (−)-EC, (4.2 ± 0.4) × 10^5 M^{-1} s^{-1}, measured under the same conditions (*7*). This result confirms that the two catechins have similar antioxidant activity in chlorobenzene, as has previously been demonstrated for *tert*-butyl alcohol (*29*).

Figure 3 also shows that the combination of (+)-C and αTOH in chlorobenzene gave a prolonged induction period, τ, compared to the induction period observed in the presence of each antioxidant. Because of the difficulties in determining the

Table 3. Regeneration Effects (RE%) and Stoichiometric Factors (n) of Flavonoids in Antioxidant Mixtures with α-Tocopherol during AIBN-Initiated Autoxidation of Methyl Linoleate in Chlorobenzene at 50 °C[a]

antioxidant mixture	[αTOH]/ 10^{-5} M	[FlH_2]/ 10^{-5} M	RE%	n
α-tocopherol/ quercetin	8.0	4.0	21[b]	0.84[b]
	8.0	8.0	45 ± 2	0.90 ± 0.04
	8.0	16.0	64[b]	0.64[b]
	3.9	7.9	81[b]	0.81[b]
α-tocopherol/ (+)-catechin	8.0	4.0	18[b]	0.72[b]
	8.0	8.0	31 ± 1	0.63 ± 0.02
	8.0	16.0	54[b]	0.54[b]
	3.9	7.9	75[b]	0.75[b]

[a] [Linoleate] = 0.24 M, [TEMPO] = 3.5×10^{-5} M, [AIBN] = 0.017 M, R_i = 5.5×10^{-8} M s^{-1} (average from seven independent measurements); RE% and n for αTOH/FlH_2 1:1 are averages from at least three independent measurements (±standard deviation). [b] Only single detection.

induction period for the flavonoid alone under these experimental conditions, the total length of the induction period produced by the antioxidant mixture, τ, was not directly compared with the sum of the induction periods obtained for the two antioxidants in the separate experiments. Nonetheless, the extended lengths of the inhibited periods pointed toward an additive or co-antioxidant effect rather than a synergism effect. Further, because the induction period in the presence of the antioxidant mixture exhibited the same efficient inhibition of the autoxidation observed for αTOH alone for a shorter induction period, the existence of an antioxidant interaction was indicated also for chlorobenzene as solvent. αTOH is regenerated by the flavonoid in chlorobenzene as in *tert*-butyl alcohol.

The lengths of the induction periods have allowed the estimation of the regeneration effect, RE%, by eq 7 for both QC and (+)-C, when combined with αTOH. The lengths of

$$\text{RE\%} = \frac{[\tau_{\alpha\text{TOH} + \text{FlH}_2} - \tau_{\alpha\text{TOH}}]}{\tau_{\alpha\text{TOH}}} \times 100\% \qquad (7)$$

the extended induction periods were dependent on the flavonoid concentration, in agreement with the mechanism of regeneration of α-tocopheroxyl radicals by the flavonoid (see **Table 3**).

The stoichiometric factor, n, of the flavonoid in the antioxidant mixture, that is, the number of radicals trapped by each molecule of flavonoid (see **Table 3**), was calculated by eq 5. The data showed a decrease of n for ratios FlH_2/αTOH with high concentrations of flavonoids. This trend of n is similar to the one observed in *tert*-butyl alcohol and is due to flavonoid autoxidation (vide infra). This effect was more evident in chlorobenzene than in *tert*-butyl alcohol, in agreement with the fact that flavonoids are 8 times less reactive than αTOH toward peroxyl radicals in chlorobenzene, whereas they are ca. 30−40 times less reactive in *tert*-butyl alcohol. As further evidence for the importance of the flavonoid autoxidation on the regeneration ability, higher RE% values were found when experiments were repeated for the ratio αTOH/FlH_2 1:2 at lower antioxidant concentrations (**Table 3**).

The stoichiometric factors, n, of QC and (+)-C in antioxidant mixtures with αTOH in the concentration ratio 1:1 were calculated during the AIBN-initiated peroxidation of methyl linoleate at 50 °C in chlorobenzene to be 0.9 and 0.6, respectively. In chlorobenzene, as in *tert*-butyl alcohol, QC possesses a slightly stronger ability to regenerate αTOH than the catechin.

For both flavonoids, the values of n determined for chlorobenzene are very close to the respective values measured in *tert*-butyl alcohol. This fact might indicate that the kinetic solvent effect, which is responsible for a reduced flavonoid activity in *tert*-butyl alcohol compared to that in chlorobenzene [ca. 20-fold, (*7*)], does not affect their ability to regenerate αTOH.

The antioxidant activity expressed in the second-order rate constant, k_{inh}, of the mixture αTOH/FlH_2 toward peroxyl radicals in chlorobenzene was also calculated using eq 6. High concentrations of methyl linoleate, which were required to achieve long oxidation chain lengths ($\nu > 3$), were used. The inhibition rate constant, k_{inh}, of the mixture αTOH/QC (1:1) during AIBN-initiated peroxidation of methyl linoleate was measured to be $(2.56 \pm 0.15) \times 10^6$ M^{-1} s^{-1} in chlorobenzene at 50 °C. This value is larger than the one measured in the presence of the QC alone, and close to the one measured in the presence of αTOH alone (**Table 2**).

Further, as reported in **Table 2**, a 6-fold decrease in the antioxidant activity of the αTOH/QC mixture is noted when passing from chlorobenzene to *tert*-butyl alcohol. This change is in agreement with the kinetic solvent effect recently measured for the αTOH activity toward peroxyl radicals (*25*). This result further confirms that αTOH is regenerated by the flavonoid, and that αTOH is responsible of the inhibition produced by the antioxidant mixture.

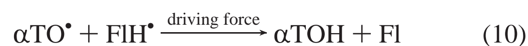
Mechanisms of Antioxidant Regeneration. Inhibited oxygen uptakes measured in the presence of a combination of αTOH and flavonoids confirmed the existence of an antioxidant interaction which produced a more effective inhibition of lipid peroxidation. The extended inhibition periods in *tert*-butyl alcohol and in chlorobenzene clearly indicated the existence of synergistic and co-antioxidant effects, respectively. The similarity of the inhibition efficiency observed in the presence of the antioxidant mixture to that observed in the presence of αTOH alone strongly suggested that flavonoids react with α-tocopheroxyl radicals to regenerate αTOH.

These results appear to be in analogy to the well-known synergism between vitamin E and vitamin C. An extended induction period and an inhibited oxidation rate similar to that provided by vitamin E when used alone were observed when both vitamins E and C were present to inhibit lipid peroxidation in homogeneous solution (*16*) and in micelles (*14*). These observations have been used to demonstrate that ascorbate is capable of regenerating αTOH from its one-electron-oxidized form.

Our ESR results strongly suggest that αTOH scavenges peroxyl radicals more quickly than flavonoids, but also that the α-tocopheroxyl radicals formed react with the flavonoid to regenerate αTOH (eqs 8−10).

$$\alpha\text{TOH} + \text{ROO}^\bullet \xrightarrow{k_8} \alpha\text{TO}^\bullet + \text{ROOH} \qquad (8)$$

$$\alpha\text{TO}^\bullet + \text{FlH}_2 \underset{k_{-\text{H}}}{\overset{k_\text{H}}{\rightleftharpoons}} \alpha\text{TOH} + \text{FlH}^\bullet \qquad (9)$$



$$\alpha\text{TO}^\bullet + \text{FlH}^\bullet \xrightarrow{\text{driving force}} \alpha\text{TOH} + \text{Fl} \qquad (10)$$

The H-abstraction reaction between $\alpha TO^\bullet$ radicals and flavonoids, which was thought until now to be rather endothermic, should rather be described as an equilibrium (eq 9). This new conclusion is based on the fact that the bond dissociation energy of 3,5-di-*tert*-butylcatechol, a molecule which resembles the catechol group in ring B of the flavonoids, recently has been measured by means of ESR spectroscopy to

be relatively low [331.8 kJ/mol in benzene, (*30*)] and therefore close to the value for αTOH (327.3 kJ/mol).

Further, because in our system $FlH^•$ radicals are formed together with $αTO^•$ radicals, reaction 10 may also occur. This mechanism was for the first time suggested for the regeneration of vitamin E by a ubiquinol radical (*31*), where reaction 10 was predicted to be exergonic by ca. 40−65 kJ/mol and thus to be thermodynamically feasible. Furthermore, although $[FlH^•] \ll [FlH_2]$, (10) being a radical−radical reaction, its rate constant is expected to approach the diffusion limit and thus to be faster than the reaction of eq 9. Therefore, we suggest that, in homogeneous solutions of peroxidating methyl linoleate, the reaction of eq 10 might act as a strong *driving force* for the reaction of eq 9, being in effect responsible for the regeneration of αTOH.

Experimental results obtained for *tert*-butyl alcohol and chlorobenzene as solvent have shown that, at high flavonoid concentrations, the oxidation of flavonoids (eqs 11 and 12) competes with αTOH regeneration (eqs 9 and 10).

$$FlH_2 + ROO^• \xrightarrow{k_{11}} FlH^• + ROOH \qquad (11)$$

$$FlH^• + ROO^• \xrightarrow{k_{12}} Fl + ROOH \qquad (12)$$

Therefore, the regenerating capability of the flavonoids, that is, the magnitude of the synergistic effect in *tert*-butyl alcohol and of the co-antioxidant effect in chlorobenzene, is expected to depend on the favorable competition of the flavonoid and its aroxyl radical for the α-tocopheroxyl radical (eqs 9 and 10) vs the chain-carrying peroxyl radical (eqs 11−13). The rate

$$αTO^• + ROO^• \xrightarrow{k_{13}} \text{products} \qquad (13)$$

constant, k_{13}, for the addition of peroxyl radicals to the α-tocopheroxyl radical is estimated to be approximately 7.3×10^8 (*14*) and 4.3×10^8 M^{-1} s^{-1} for 1-cyano-1,3-dimethylbutylperoxyl radical $[(CH_3)_2CHCH_2C(CH_3)(CN)O_2]$ in chlorobenzene at 45 °C (*32*). Values for the rate constant k_{12} were not found in the literature, but k_{12} is reasonably expected to be of a similar magnitude. Even though reactions 12 and 13 are fast, the higher concentrations of $αTO^•$ and $FlH^•$ radicals compared to the concentration of the $ROO^•$ radicals at the steady-state condition might explain the favorable competition of reaction 10 vs reactions 12 and 13. This also indicates that the extension of a successful competition of the flavonoid for the α-tocopheroxyl radical might further be affected by the rate of initiating radicals, R_i. Thus, the stoichiometric factor of flavonoids in mixture with αTOH, *n*, must accordingly depend on the actual rate of initiation of oxidation, R_i, as already recognized for the case of antioxidant interaction between ascorbate and αTOH (*14*).

The suggested mechanism of interaction by H-transfer reaction between flavonoids and αTOH in organic solvents is considered to be more likely than an electron-transfer mechanism because flavonoids are in their nondissociated form. However, the possibility of more than one type of mechanism being responsible for the antioxidant interaction, such as formation of complexes between antioxidants or interaction through H-bonding, even though likely not to be the main mechanism, cannot be ruled out on the basis of the results achieved by the ESR oximetry method.

In conclusion, the existence of an antioxidant interaction between flavonoids and αTOH in homogeneous solution of peroxidating methyl linoleate in *tert*-butyl alcohol and chlorobenzene was demonstrated by means of ESR oximetry. The combination of the two types of antioxidants resulted in longer induction periods (synergistic or co-antioxidant effects) and an efficient inhibition ($k_{mixture}$ close to $k_{αTOH}$), experimental results which all point toward a regeneration of αTOH by the flavonoid. Further, kinetic solvent effects, which are known to drastically reduce the antioxidant effect of phenols and polyphenols, have been shown not to affect the regeneration of αTOH by the flavonoid, but to decrease the activity of the antioxidant mixture by the same amount by which the activity of αTOH is reduced when used alone. The present results support a positive effect of antioxidant interaction, where even weak retarders, as the flavonoids are in *tert*-butyl alcohol, can produce a synergistic effect in combination with αTOH. Therefore, the apparent inhibition rate constants of antioxidant mixtures are of relevance in order to draw hierarchies of antioxidant activity and thus to design better protective systems, in particular in food systems which are characterized by compartments with different water contents.

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