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Efficiency of Natural Phenolic Compounds Regenerating α-Tocopherol from α-Tocopheroxyl Radical

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Benzoic acid-derived phenolics (*p*-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, vanillic acid, syringic acid, and gallic acid) and the polyphenols epicatechin and epigallocatechin gallate (EGCG) were evaluated for their efficiency in regenerating α-tocopherol from α-tocopheroxyl radical in comparison with ascorbyl palmitate, which is known to regenerate α-tocopherol. Ethanolic solutions of phenolic compounds were added to a homogeneous hexane medium containing α-tocopheroxyl radical generated by reaction of α-tocopherol in molar excess with 1,1-diphenyl-2-picrylhydrazyl radical, and the α-tocopheroxyl radical was monitored by electron spin resonance spectroscopy. *p*-Hydroxybenzoic acid, vanillic acid, and syringic acid (400 μM) did not exhibit a significant effect on α-tocopheroxyl radical concentration (0.6−0.7 μM). In contrast, 3,4-dihydroxybenzoic acid and gallic acid were able to reduce the concentration of α-tocopheroxyl radical by 16 and 64%, respectively. Epicatechin showed a reduction of α-tocopheroxyl radical similar to gallic acid, and EGCG and ascorbyl palmitate were the most effective, reducing α-tocopheroxyl radical completely at a much lower phenolic concentration (66.7 μM). The moles of α-tocopheroxyl radical reduced per mole of ascorbyl palmitate (0.93), EGCG (0.066), gallic acid (4.3×10^{-4}), and epicatechin (4.5×10^{-4}) were determined, and the logarithm of these stoichoimetric ratios showed a negative linear relationship with the bond dissociation enthalpies of the O−H bond of the phenolics. The relative capacity to reduce α-tocopheroxyl radical was found to be ascorbyl palmitate (2142) > EGCG (151) > gallic acid (1) ≈ epicatechin (1).

KEYWORDS: α-Tocopherol regeneration; α-tocopheroxyl radical; synergism; benzoic acid-derived phenolics; polyphenols

INTRODUCTION

It is generally recognized that lipid oxidation during processing and storage has a major effect on the shelf life of fish and other muscle-based foods rich in polyunsaturated fatty acids (*1*, *2*). Oxidative reactions have also been implicated in a variety of pathological processes, including inflammation, atherosclerosis, cancer, and many degenerative diseases (*3*, *4*). During the past decade, the application of antioxidant compounds has become an attractive strategy to minimize the negative consequences of oxidation in both the human body and food products (*5*, *6*).

Many of the antioxidant treatments described in literature are based on application of natural phenolic compounds, widespread in foods and beverages such as fruits, vegetables, cocoa, tea, and wine, and well-known for their potential antioxidant capacity (*7*). Free radical-scavenging and metal-chelating properties are the best understood antioxidant mechanisms for phenolic compounds. Phenolic compounds are efficient radical chain-breaking agents since the phenolic structure possesses the ability of donating an electron or a hydrogen atom to a reactive free radical and of stabilizing the resulting phenoxyl radical by delocalizing the unpaired electron. Metal-chelating capacity, mainly associated with the presence of at least two hydroxyl groups in the ortho position in the phenolic structure, is of importance for the deactivation of catalytic transition metal ions (*2*).

α-Tocopherol (or vitamin E) is a lipophilic component of the membranes in muscle tissues. Several investigations have found that lipid oxidation does not occur substantially in postmortem flesh until α-tocopherol is depleted below critical levels, implying that maintenance of a high α-tocopherol level may be critical for protecting flesh against oxidation (*8*, *9*). It is well-documented that the α-tocopheroxyl radical formed during antioxidant actions of α-tocopherol can be regenerated by ascorbic acid in human platelets, in low-density lipoproteins, and in solution (*10*, *11*). Such cooperative activity seems to explain the antioxidant synergism observed when α-tocopherol

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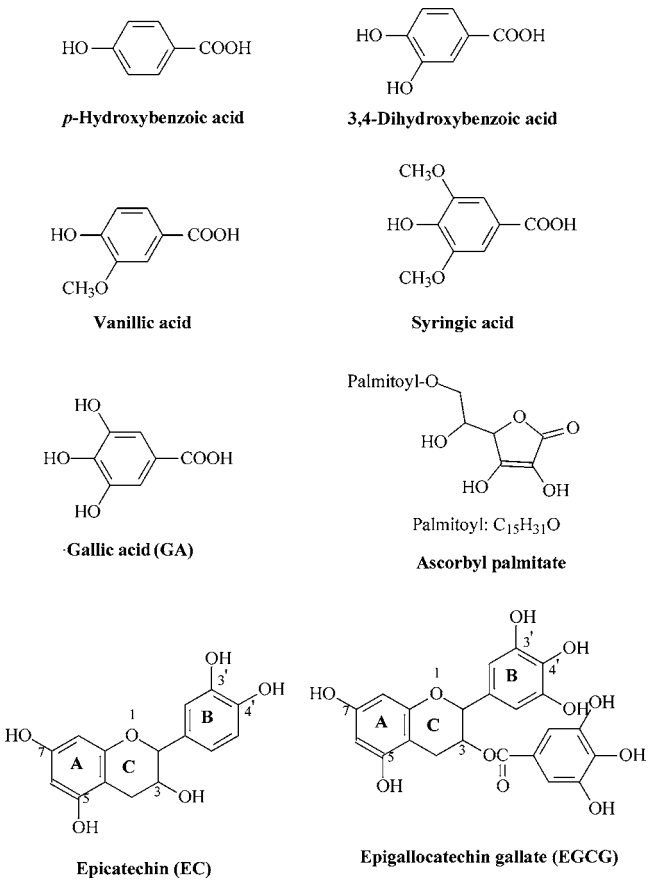


Figure 1. Chemical structures of the benzoic acid-derived and polyphenols studied.

and ascorbic acid are used in combination (*2*). Ubiquinol and other reduced biological hydroquinones have also been found to regenerate α-tocopherol (*12*). Recent investigations have demonstrated a protective effect of several phenolics (grape procyanidins, hydroxytyrosol, and caffeic acid) on α-tocopherol in fish muscle (*8*, *13*) and an antioxidant synergism between α-tocopherol and some phenolics (green tea catechins and quercetin) (*14*, *15*). These findings suggested that phenolic compounds may also repair α-tocopherol, and such regeneration activity has recently been demonstrated for green tea catechins in low-density lipoproteins (*16*), sodium dodecyl sulfate (SDS) micelles (*17*, *18*), and homogeneous solutions (*18*).

The aim of the present work was to investigate the capacity of benzoic acid-derived phenolics and several polyphenols (**Figure 1**) for regenerating α-tocopherol from the α-tocopheroxyl radical. To this purpose, the α-tocopheroxyl radical was generated by a chemical reaction between α-tocopherol and 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) radical in hexane, and subsequently, the phenolic compounds were added in ethanolic solution. α-Tocopheroxyl radical was quantified by electronic spin resonance (ESR) spectroscopy in both samples with and without phenolics, in order to evaluate the α-tocopherol-regenerative properties of phenolics. The capacity of phenolic compounds to reduce α-tocopheroxyl radical was investigated and discussed in terms of the redox potentials and the bond dissociation enthalpies (BDEs) of the O−H bond of the phenolic compounds.

MATERIALS AND METHODS

Chemicals. DL-all-*rac*-α-Tocopherol, DPPH, *p*-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, vanillic acid, syringic acid, gallic acid (GA), and epigallocatechin gallate (EGCG) were purchased from Sigma

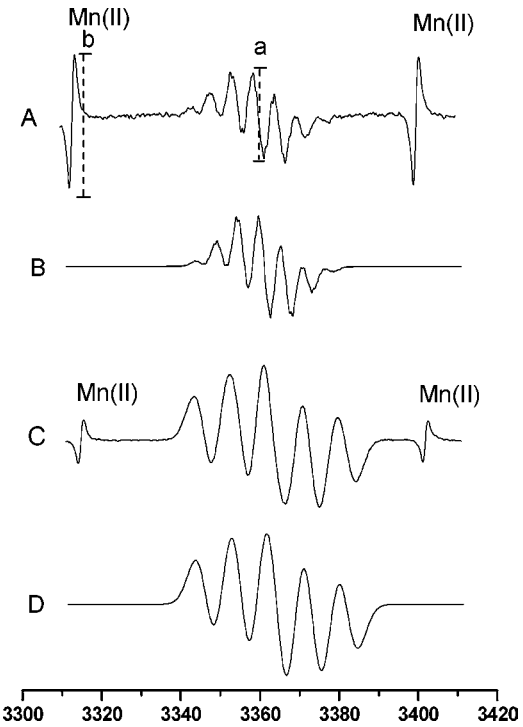


Figure 2. ESR spectrum of α-tocopheroxyl obtained 20 s after mixing α-tocopherol (2 mM) and DPPH (0.013 mM) in N_2-saturated hexane:ethanol (93:7) (**A**) and computer simulation of the spectrum (**B**). ESR spectrum of DPPH radical in N_2-saturated hexane:ethanol (93:7) (**C**) and computer simulation of the spectrum (**D**).

(Steinheim, Germany). 2,2,6,6-Tetramethylpiperidine-1-oxyl (TEMPO) (98%), epicatechin (EC), and 6-*O*-palmitoyl-L-ascorbic acid (ascorbyl palmitate) were obtained from Fluka (Buchs, Swizerland). All chemicals were of analytical grade, and the water was purified using a Milli-Q system (Millipore, Billerica, MA).

Regeneration of α-Tocopherol by Phenolics. The α-tocopheroxyl radical was generated directly in an ESR quartz capillary tube with an internal diameter of 4.2 mm (Wilmad, Buena, NJ) by mixing 1.3 mL of a N_2-saturated α-tocopherol in hexane solution with 0.1 mL of a DPPH radical in hexane. After 20 s, 0.1 mL of an ethanolic solution of the phenolics (**Figure 1**) was added. The phenolic solution was substituted by absolute ethanol in control samples. The final concentrations of α-tocopherol and DPPH radical in the reaction mixtures were 2.0 and 0.013 mM, respectively. The reaction mixture was bubbled with N_2 until 20 s before the start of recording ESR spectra on a JEOL Jes-FR30 ESR spectrometer (JEOL Ltd., Tokyo, Japan). The settings used were as follows: microwave power, 4 mW; sweep width, 50 G; sweep time, 2 min; modulation amplitude, 3.2 G; and time constant, 0.3 s. Spectra were recorded at room temperature after 1 min of DPPH addition, except when other conditions were indicated.

The ratio between peak-to-peak amplitude of α-tocopheroxyl radical (a) and Mn(II) marker attached to the cavity of the spectrometer (b) was used as a relative signal intensity of α-tocopheroxyl radical, as shown in **Figure 2**. The efficiency of phenolics in decreasing the levels of α-tocopheroxyl radical was determined as the percentage of the signal amplitude reduced in the presence of phenolics (A_{sample}) as compared to the signal amplitude in control samples without phenolics ($A_{control}$):

$$\text{decrease in } \alpha\text{-tocopheroxyl radical intensity (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

The slopes of the linear regressions between concentrations of reduced α-tocopheroxyl radical and phenolic concentrations were used to estimate the number of moles of α-tocopheroxyl radical reduced per mole of phenolic. The concentration of α-tocopheroxyl radical was calculated relating the total double integrated area of the α-tocopheroxyl

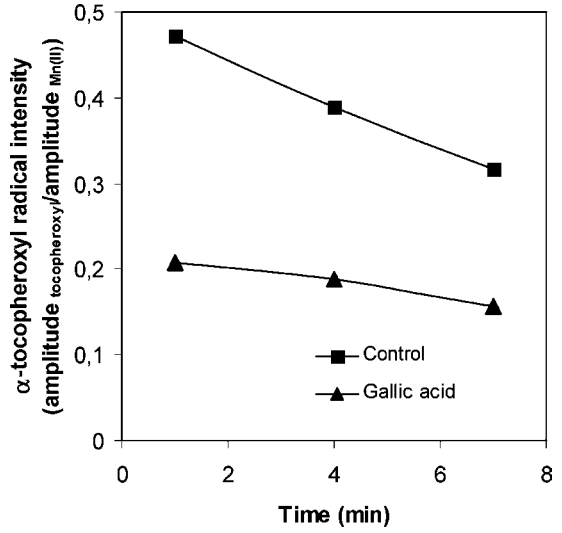


Figure 3. Time profile of α-tocopheroxyl radical intensity in samples with and without GA (400 μM).

radical signal to the total signal area corresponding to a known concentration of TEMPO radical. The area of signals was integrated by using the Bruker WinEPR software, whereas the simulation and fitting of the ESR spectra were performed using the PEST WinSIM program (*19*).

Statistical Analysis. The experiments were performed at least twice, and data were reported as means ± standard deviations of three replicates. The data were analyzed by one-way analysis of variance and the least-squares difference method (*20*). Statistical analyses were performed with the software Statistica 6.0 (*21*).

RESULTS

Generation and Detection of α-Tocopheroxyl Radicals. α-Tocopheroxyl radicals were generated by reacting α-tocopherol and DPPH radical with α-tocopherol in molar excess. This reaction was carried out in N_2-saturated hexane/ethanol (93:7) at room temperature (≈20 °C). Nitrogen gas was introduced to accelerate the homogenization of the reaction system and also to improve the sensibility in the α-tocopheroxyl radical detection, since α-tocopheroxyl radical was not detected without saturation with nitrogen (data not shown). A combination of narrowing of the ESR signal of organic radicals in N_2-saturated solvents due to the absence of the Heisenberg spin exchanges under anaerobic conditions and a potentially higher stability of α-tocopheroxyl radicals as consequence of oxygen removal could explain the higher sensibility for α-tocopheroxyl radical in N_2-saturated conditions. The ESR spectrum recorded 20 s after the addition of DPPH showed that DPPH was totally consumed, and the reaction between α-tocopherol and DPPH was completed (**Figure 2A**). The ESR signal obtained from this reaction had seven well-resolved peaks, which were successfully simulated using four different hyperfine coupling constants, $a_H = 5.63$ G, $a_H = 4.73$ G, $a_H = 0.88$ G, and $a_H = 1.47$ G (**Figure 2B**). These hyperfine coupling constants were similar to values previously reported for α-tocopheroxyl radical (*22*) and differed from the ESR spectrum obtained for DPPH radical (**Figure 2C**). The DPPH ESR signal was efficiently simulated using two different hyperfine coupling constants, $a_N = 9.72$ G and $a_N = 8.01$ G, which were similar to those previously reported (*23*) (**Figure 2D**).

The α-tocopheroxyl radical intensity decayed after being generated by DPPH, which may be a result of mainly disproportionation reactions (*24*) (**Figure 3**). A concentration of 0.6−0.7 μM for α-tocopheroxyl radical was found 1 min after adding 13 μM DPPH radical in control samples (without phenolics). The addition of GA (400 μM) at the time when DPPH radical had been consumed by reaction with α-tocopherol (20 s after mixing α-tocopherol and DPPH) produced a significant reduction of α-tocopheroxyl radical measured after 1 min, followed by a continuous slow decay during (**Figure 3**). These results suggested that GA was able to react with α-tocopheroxyl radicals regenerating α-tocopherol. However, a precise determination of this regenerative effect depended on a strict control of time of adding the phenolics and time of recording ESR spectra. In the final experiments in which the regeneration of α-tocopherol by several phenolics was evaluated, the times that elapsed between generating α-tocopheroxyl radical by adding DPPH radical and the addition of the phenol and recording the ESR spectra were 20 and 60 s, respectively.

Reduction of α-Tocopheroxyl Radical by Benzoic Acid-Derived Phenolics and Polyphenols. Five benzoic acid-derived phenolics (*p*-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, vanillic acid, syringic acid, and GA) and two polyphenols (EC and EGCG) were separately added to α-tocopheroxyl radical solutions, and their effect on α-tocopheroxyl radical concentration was studied by ESR spectroscopy under the conditions described above. The reduction of α-tocopheroxyl radicals was also investigated for ascorbyl palmitate as a standard compound with well-documented regeneration of α-tocopherol. The addition of 3-hydroxybenzoic acid, vanillic acid, and syringic acid to yield a concentration of 400 μM did not significantly affect ($p > 0.05$) the α-tocopheroxyl radical concentration (**Figure 4A**). In contrast, the benzoic acid-derived phenolics 3,4-dihydroxybenzoic acid and GA reduced the levels of α-tocopheroxyl radical corresponding to 16 and 64%, respectively. EC showed a regeneration capacity on α-tocopherol similar to GA, decreasing the α-tocopheroxyl radical by 57%. In the presence of EGCG and ascorbyl palmitate at the same concentration (400 μM), α-tocopheroxyl radical disappeared completely (**Figure 4A**). The regeneration of α-tocopherol was also studied for lower phenolic concentration (66.7 μM) (**Figure 4B**) and a reduction of 24−30% of α-tocopheroxyl radicals by GA and EC was found for these conditions. EGCG and ascorbyl palmitate were also found to reduce α-tocopheroxyl radicals completely for this lower concentration (66.7 μM).

With the purpose of studying the molecular mechanisms behind regeneration of α-tocopherol by phenolics, the extent of α-tocopheroxyl radical reduction by GA, EC, EGCG, and ascorbyl palmitate was studied. In agreement with the experiments described above, higher concentrations of GA and EC were needed to reduce α-tocopheroxyl radicals as compared to EGCG and ascorbyl palmitate (**Table 1**). For the concentration range studied, the amount of reduced α-tocopheroxyl radical exhibited a linear relationship ($R^2 > 0.91$) with the phenolic concentration. The slopes of the regression lines were used to estimate the amount of regenerated α-tocopheroxyl radical per mole of phenolic compound. One mole of GA or EC reduced approximately 4.5×10^{-4} mol of α-tocopheroxyl radical, while 1 mol of EGCG reduced 0.066 mol of the α-tocopheroxyl radical. Ascorbyl palmitate was significantly more efficient than the phenolics studied; it was able to reduce 0.93 mol of α-tocopheroxyl radical/mol. The relative capacity to reduce α-tocopherol from α-tocopheroxyl radical was found to be ascorbyl palmitate (2142) > EGCG (151) > GA (1) ≈ EC (1) (**Table 1**). Moreover, the BDEs of the O−H bond of those phenolics exhibited a negative linear relationship with the logarithm of the mol of α-tocopheroxyl radical reduced/mol of phenolic (**Figure 5**).

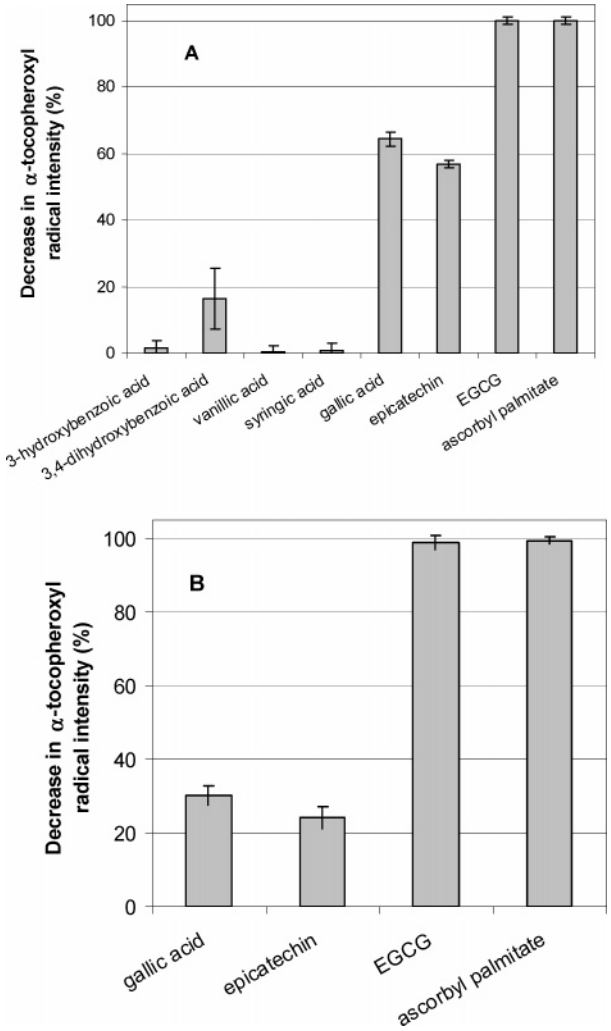


Figure 4. Effect of phenolics on α-tocopheroxyl radical intensity at phenolic concentrations of 400 (**A**) and 66.7 μM (**B**).

DISCUSSION

α-Tocopherol is able to donate either two electrons or two hydrogens to alkyl, peroxyl, and alkoxyl radicals (*25*), scavenging these radicals and stopping the chain initiation and chain propagation reactions in lipid oxidation. α-Tocopheroxyl radical is the one-electron-oxidized form produced as a consequence of the antioxidant action of α-tocopherol. Tocopheroxyl radicals interact with other compounds to form a variety of products, which are dependent on oxidation rates, oxidation species, physical location in bulk or membrane lipids, and tocopherol concentration. For example, tocopheroxyl radicals are converted to tocopherylquinone in fish (*8*) and beef (*9*) flesh under oxidative stress. Tocopherylquinone was also found to be generated by oxidation of α-tocopherol by peroxynitrite (*26*). In another, more uncommon pathway, tocopheroxyl radical can interact with peroxyl radical to yield epoxytocopherolquinone as a final product (*8*, *9*, *27*). Additionally, two tocopherxyl radicals can react to form tocopherol dimers via the coupling of α-tocopherol-derived radicals (*28*). Considering these mechanistic aspects of the oxidative depletion of α-tocopherol, there are two feasible routes by which α-tocopherol eventually may be recycled by reducing species such as ascorbate and phenolics. These routes involve reduction of α-tocopheroxyl radical into the original α-tocopherol or reduction of α-tocopherylquinone into α-tocopherylhydroquinone, which has been shown to have antioxidant capacity in model experiments inhibiting methyl linoleate oxidation (*29*).

In the present investigation, the efficiency of phenolics to regenerate α-tocopherol through reduction of α-tocopheroxyl radical has been studied in a homogeneous system consisting of hexane/ethanol (93:7), which provides a complete dissolution of the reagents (α-tocopherol, DPPH radical, and phenols). Electron transfer and H-atom transfer are considered the two major mechanisms by which the reduction of radical species by phenolics (ArOH) can take place. Both the H-atom transfer and the single-electron transfer mechanisms give the same net result, that is, $\alpha\text{-TO}^{\bullet} + \text{ArOH} \rightarrow \alpha\text{-TOH} + \text{ArO}^{\bullet}$, but the H-atom transfer is an one-step process whereas the electron transfer occurs via an initial electron transfer, in which a radical phenolic cation is formed, followed by rapid and reversible deprotonation of the radical phenolic cation in solution. The capacity of the phenolic compounds for regenerating α-tocopherol from α-tocopheroxyl radical could be related with the one-electron redox potentials and the BDEs of the O−H bond of the phenolic compounds, since these thermodynamic parameters quantify the thermodynamic control of the electron transfer and the H-atom transfer processes, respectively. However, we will mainly discuss our results based on the BDEs given that nonpolar solvents such as hexane favor H-atom transfer mechanisms, and redox potentials of phenolics are only commonly available for protic mediums (aqueous solutions).

The *p*-monohydroxy benzoic acids (*p*-hydroxybenzoic acid, vanillic acid, and syringic acid) did not reduce α-tocopheroxyl radicals, even when used at a very high molar ratio (phenolic: α-tocopheroxyl radical ≈ 400:0.6) (**Figure 4A**). This finding is in agreement with the BDEs of the O−H bond reported for monohydroxyphenolics and α-tocopherol. The BDEs of the O−H bond for hydroxyphenol (88.3 kcal/mol) and *o*-methoxyhydroxyphenol (83.2 kcal/mol) are notably much higher than that for α-tocopherol (78.2 kcal/mol) (*30*). The presence of a second hydroxyl group as in the 3,4-dihydroxybenzoic acid, and even more for the presence of a third hydroxyl group as in GA, enhanced the tendency of the phenolics to reduce α-tocopheroxyl radicals. GA and EC showed similar efficiencies in diminishing the α-tocopheroxyl radical concentrations, as GA and EC were found to reduce $\approx 4.5 \times 10^{-4}$ mol of α-tocopheroxyl radical/mol of phenolic (**Table 1**). This similar capacity for regenerating α-tocopherol is consistent with the BDEs of the O−H bond for GA and EC (**Table 1**). However, the reduction of α-tocopheroxyl radical to α-tocopherol (BDE O−H bond = 78.2 kcal/mol) by these phenolics is not thermodynamically feasible as concluded from the BDE of the O−H bond. Recently, the effectiveness of GA and EC reducing α-tocopheroxyl radical has also been reported for SDS micelles in water, in which both phenolics exhibited an analogous behavior regenerating α-tocopherol (*17*). The tocopherol regeneration by these phenolics is also not thermodynamically feasible taking into consideration the redox potentials, since GA ($E = 0.56$ V vs NHE) and EC ($E = 0.57$ V vs NHE) have higher redox potentials than Trolox ($E = 0.48$ V vs NHE), the water-soluble analogue of α-tocopherol, which is assumed to have an identical redox potential as α-tocopherol. Amorati et al. (*31*) suggested that the phenolic semiquinone radical formed during the regeneration of α-tocopherol from α-tocopheroxyl radical could also reduce another α-tocopheroxyl radical to give α-tocopherol and an *ortho*-quinone phenolic derivate (**Scheme 1**). The removal of the phenolic semiquinone radical could be the driving force for the reductive capacity of GA and EC on α-tocopheroxyl radicals, making it an energetically feasible reaction.

EGCG reduced α-tocopheroxyl radical approximately 150 times more effectively than GA and EC, and it was 14 times

Table 1. Moles of α-Tocopheroxyl Radical Reduced Per Mole of Phenolic, Determined as the Slope of Plots of Concentration of Reduced α-Tocopheroxyl Radical vs Phenolic Concentration, Reduction Potentials of Phenoxyl Radicals, and BDEs of O–H Bond of Phenolics

	tocopheroxyl radical regenerated (μM)	phenolic concentration (μM)	R^2	slope (mol tocopheroxyl radical reduced/ mol phenolic)	relative regenerative capacity	*E* (V vs NHE)[a]	BDE O–H bond (kcal/mol)[b]
GA	0.13–0.35	67–533	0.913	4.3×10^{-4}	1	0.56	82.0
EC	0.15–0.42	67–667	0.992	4.5×10^{-4}	1	0.57	81.2
EGCG	0.13–0.35	0.33–3.3	0.922	0.066	151	0.43	77.8
ascorbyl palmitate	0.21–0.50	0.17–0.50	0.929	0.93	2142	0.28	73.2

[a] The reduction potentials for aqueous solution at pH ≈ 7. Reduction potentials of GA, EC, and EGCG radicals were obtained from ref *33*. For ascorbyl palmitate, the reduction potential of ascorbate is shown (*34*). [b] The BDEs of the O–H bond of GA and ascorbyl palmitate are for 1,2,3-trihydroxyphenol and ascorbate (*31*, *35*). BDEs for EC and EGCG were obtained from ref *36*.

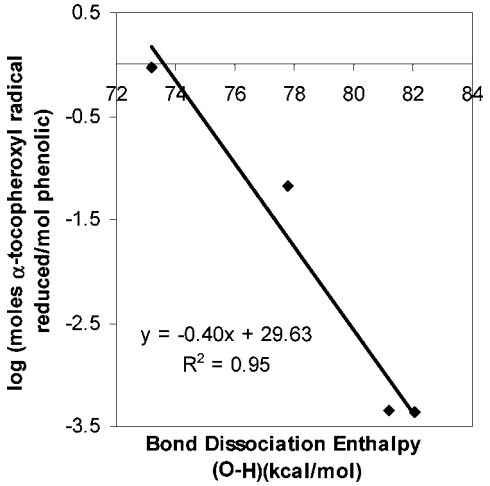
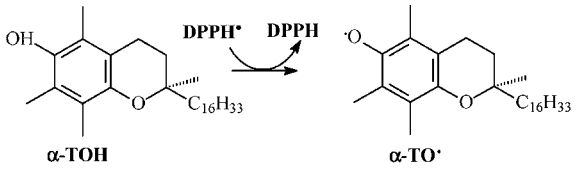


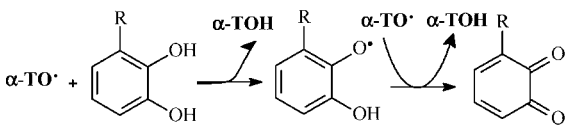
Figure 5. Correlation between the BDEs of the O–H bond for GA, EC, EGCG, and ascorbyl palmitate and their efficiency at reducing α-tocopheroxyl radical.

Scheme 1. Reactions Involved in the Production of α-Tocopheroxyl Radical by DPPH Radical and in the Regeneration of α-Tocopherol from α-Tocopheroxyl Radical by Phenolic Compounds (Catechol and Pyrogallol Derivates)

Step I: Generation of α-tocopheroxyl radical



Step II: Reduction of α-tocopheroxyl radical by phenolic compounds



less active than palmitoyl ascorbate. These extents of the reaction are consistent with the rate constants reported in aqueous SDS micelles for the reaction of α-tocopheroxyl radicals with EC, GA, EGC, ECG, and EGCG , found to be 0.45, 0.43, 1.11, 1.31, and 1.91×10^2 M^{-1} s^{-1}, respectively, (*17*). Mukai et al. (*18*) also verified a much higher rate constant for the reaction of the α-tocopheroxyl radical with EGCG in ethanol, 2-propanol/water, and TritonX-100 micelles, as compared to other catechins. The capacity of the phenolic compounds and ascorbyl palmitate exhibited in the present paper for repairing α-tocopherol from α-tocopheroxyl radical, that is, ascorbyl palmitate > EGCG > EC ≈ GA, is in agreement with the much lower BDE of the O−H bond for ascorbic and EGCG (**Table 1**). In fact, the BDE of the O−H bond of phenolics showed an inverse linear relationship with the logarithm of the mol of α-tocopheroxyl radical reduced by mol of phenolic (**Figure 5**). Additionally, it should be mentioned that the α-tocopherol-regenerative capacity of phenolics in our hexane/ethanol medium had also an inverse linear correlation with the redox potential of phenolics reported in protic medium (**Table 1**). The higher α-tocopherol-regenerative ability of phenolic compounds with lower redox potentials has also been described in protic mediums (*17*, *18*).

In conclusion, it was demonstrated that phenols were potentially active in the regeneration of α-tocopherol via reduction of α-tocopheroxyl radical and that capacity was found to be directly proportional to the ability of phenolic compounds to transfer a single H-atom. Our ESR-based procedure developed for homogeneous hexane/ethanol was shown to provide results in agreement with previous thermodynamic and kinetic data. This investigation demonstrates the potential utility of ESR spectroscopy to determine potential synergism between α-tocopherol and novel antioxidants in more complex food systems.

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