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# Hydrogen-Transfer Reactions from Phenols to TEMPO Prefluorescent Probes in Micellar Systems

**Carolina Aliaga,*,† Juan M. Juárez-Ruiz,‡ J. C. Scaiano,‡ and Alexis Aspée*,†**

*Facultad de Química y Biología, Universidad de Santiago de Chile, Casilla 40 Correo 33, Santiago de Chile, Department of Chemistry, University of Ottawa, Ottawa, Canada K1N 6N5*

*aaspee@usach.cl; caliaga@usach.cl*

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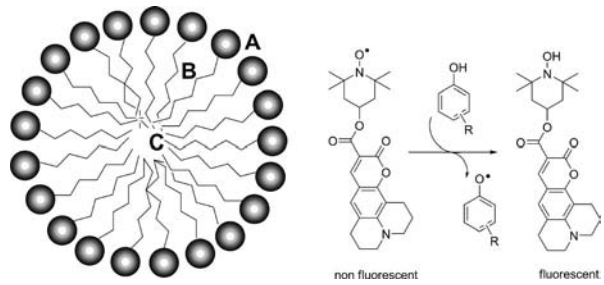
## ABSTRACT




**A nitroxide prefluorescent probe has been used to evaluate local reactivity of antioxidants in micellar systems. An apparent rate constant that directly reflects the relevance of antioxidant hydrophobicity on the reaction toward nitroxide radicals has been defined. Dramatic increases in this parameter for quercetin are shown on moving from methanol to micellar media: 90 and 230 fold enhancements for SDS and Triton X100 micelles, respectively. This is a clear consequence of the favorable partition of reactants in the micelles.**

The antioxidant properties of phenols and polyphenols have been mostly associated to the trapping of radicals involved in the oxidative chain by efficient hydrogen transfer reactions toward free radicals.[1–4] Different methodologies have been developed to evaluate the rate constant for hydrogen transfer from phenols to peroxyl, alkoxyl, nitrogen and carbon-centered radicals. These studies have allowed correlating the reactivity of polyphenols in aqueous and organic solvents with their chemical structure.[5] However, there is limited information related to the participation of free radical processes and the antioxidant mechanism involved in biological membranes. We anticipate that the hydrophobicity of flavonoids will be reflected in different antioxidant activity due to the partition and preferential location of the antioxidant in the membrane.

We have recently proposed the use of nitroxide prefluorescent probes to evaluate hydrogen transfer rate constants from highly reactive polyphenols such as flavonoids in homogeneous media.[6] The methodology reported is based on the intramolecular quenching of the fluorescent chromophore moiety by the tethered TEMPO moiety. The abstraction of a hydrogen atom from the polyphenol by the nitroxide produces the diamagnetic hydroxylamine thereby restoring the fluorescence of the chromophore.[7,8]

† Facultad de Química y Biología, Universidad de Santiago de Chile.
‡ Department of Chemistry, University of Ottawa, Canada.

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In the present work, we extend the potential use of these nitroxide prefluorescent probes to evaluate local reactivity of antioxidants in micellar systems. In this context, it is important to note that not only the polyphenol could be distributed in the micelle but also the probe. As the prefluorescent probe, we employed $C_{343}T$ (see Figure 1 and Supporting Information) an

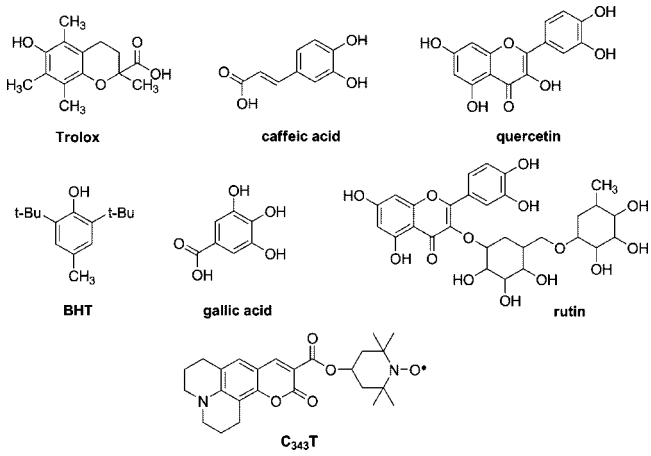



**Figure 1.** Structures of polyphenols employed and $C_{343}T$.

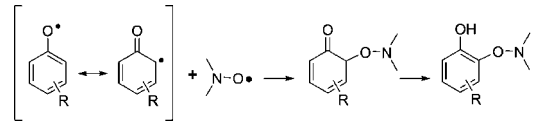
appropriate probe for the study of hydrogen transfer reactions in these systems due to its high hydrophobicity and absorption at long wavelengths, therefore not interfering with the absorbance of the antioxidants.[9]

The hydrogen transfer rate reactions were evaluated employing different phenol antioxidants, including α-tocopherol and Trolox, as models in different solvents and microheterogeneous media (Figure 1). The micellar reactions were carried out in phosphate buffer (20 mM, pH 7.0) pretreated with Chelex100 in order to minimize the concentration of metal ions. The rate constants observed ($k_{obs}$) were obtained by monitoring the growth of the fluorescence according to eq 1, where $I^{\infty}$, $I^0$, and $I^t$ represent the fluorescence intensities in the plateau region; initial and at time "$t$", respectively.

$$\ln\left(\frac{I^{\infty}-I^{0}}{I^{\infty}-I^{t}}\right)=k_{obs}t \qquad (1)$$

The reactions in aqueous or organic solvens were evaluated under pseudo-first-order reaction conditions employing an excess of the phenols (0.5−20 mM) in relation to the nitroxide probe (2.5 $\mu$M). Kinetic analysis leads to the rate constant for hydrogen abstraction directly from the slope of $k_{obs}$ vs the intial antioxidant concentration plot.

We have previously demonstrated that for antioxidants such as Trolox in water or methanol, the reaction with nitroxide shows reversibility in nitrogen-purged solutions. However, at high Trolox concentration the equilibrium is almost completely displaced toward the products allowing an accurate determination of the rate constants.[6] On the other hand, in the case of polyphenol radicals that react rapidly with oxygen in air-saturated solutions in competition with fast self-reaction,[10,11] the initial hydrogen abstraction by the nitroxide is the limiting reaction evaluated in this kinetic treatment. It is important to mention that the rate constant evaluated from fluorescence measurements could be affected at most by a factor of 2, if the antioxidant carbon centered radical is exclusively trapped by the remaining nitroxide probe to produce the fluorescent diamagnetic alcoxyamine, as shown in 2.



(2)

The hydrogen abstraction rate constants toward the nitroxide unit evaluated under these conditions for α-tocopherol in different solvents are shown in Table 1.

**Table 1.** Hydrogen Transfer Rate Constants from α-Tocopherol to $C_{343}T$ in Different Solvents at Room Temperature

| solvent | $k$, $M^{-1}$ $s^{-1}$ |
|---|---|
| hexane | 0.33 |
| benzene | 0.13 |
| acetonitrile | 0.06 |
| water or methanol | 0.21[a] |

[a] Evaluated using Trolox.

These hydrogen-transfer rate constants present a similar solvent dependence to that observed for phenols toward highly reactive free radicals. This solvent effect has been attributed to hydrogen bond formation between the phenols and the solvent. Ingold et al. have discussed in detail how this effect depends on the hydrogen bond accepting (HBA) properties of the solvent and is independent of the nature of the attacking free radical.[12]

Interestingly, the hydrogen-transfer reactions from α-tocopherol to nitroxides in hydroxylic solvents are considerably faster than what could be expected according to the HBA properties of the solvent. This behavior has been attributed to a specific formation of hydrogen bonds between the solvent hydroxyl group and the lone pair on the nitroxide's oxygen. This interaction increases the reactivity of the nitroxide as it has been previously observed in quenching of carbon-centered radicals by TEMPO.[13]

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**Table 2.** Rate Constants for Hydrogen Abstraction from Phenolic Compounds toward $C_{343}T$ Probe

| antioxidant | $k_H$, $M^{-1}$ $s^{-1}$ | $k_D$, $M^{-1}$ $s^{-1}$ | TAR[d] | $k'$ SDS[e] | $k'$ Triton[e] |
|---|---|---|---|---|---|
| Trolox | 0.19[a,c] | $0.5 \times 10^{-3}$[b,c] | 1.0 | 0.83 | 0.52 |
| BHT | $3.3 \times 10^{-3}$[b,c] | $1.3 \times 10^{-3}$[b,c] | | 0.15 | 0.39 |
| caffeic acid | $4.0 \times 10^{-2}$[a,f] $2.2 \times 10^{-3}$[b] | $0.5 \times 10^{-3}$[b] | 1.0 | 0.03 | 0.09 |
| gallic acid | $8.8 \times 10^{-3}$[b,c] | $4.6 \times 10^{-3}$[b,c] | 2.0 | 0.02 | 0.01 |
| quercetin | $22.7 \times 10^{-3}$[a]* $4.0 \times 10^{-3}$[b]* | $0.6 \times 10^{-3}$[b] | 3.3 | 0.35 | 0.93 |
| rutin | unreactive | unreactive | 1.0 | | |

[a] Phosphate buffer, Pi (10 mM, pH 7.0) or in $D_2O$ pD 7.0. [b] MeOH or MeOD. [c] Reference 6. [d] TAR (total antioxidant reactivity) evaluated from competitive experiments of c-phycocyanin protection against peroxyl radicals in Pi (10 mM pH 7.0)[21] [e] Experimental conditions for micelles: 2.5 $\mu$M $C_{343}T$, 20 mM SDS, 15 mM Triton X100, 20 mM phosphate buffer pH 7.0, phenol concentrations from 100 $\mu$M to 5 mM. [f] Pi (100 mM, pH 7.0). * Data obtained by EPR using TEMPO.

In order to make a systematic comparison between the two systems we have defined an apparent rate constant in micelles as

$$k' = \frac{k_{obs}}{[\text{phenol}]_0} \qquad (3)$$

where $[\text{phenol}]_0$ is the initial concentration of the phenol added. This value allows us to have a parameter that directly reflects the importance of the distribution of the antioxidant between the micelle and the dispersing media reflecting the antioxidant hydrophobicity. It is worth mentioning that this apparent rate constant $k'$ depends on the micellar concentration. In the present study, we decided to employ 20 mM SDS and 15 mM Triton X100 after taking into account the critical micelle concentration (CMC) values of the surfactants.[14]

The nitroxide probe reaction with α-tocopherol solubilized in micelles of SDS shows an increase in the apparent rate constant value (17 $M^{-1}s^{-1}$) relative to the rate constant in organic solvents (Table 1). It has been reported that the tocopheryl radical self-reaction rate constant decreases in micelles (showing even longer lifetimes than the millisecond values that are common in SDS micelles);[15] in principle, this could increase the proportion of its cross-reaction with nitroxide prefluorescent probes, however, the reaction with oxygen is still considerably faster (and thus favored), given the very low concentration of probe employed (2.5 $\mu$M).

On the other hand, the reaction of ascorbic acid with the nitroxide probe in SDS micelles was much slower (0.5 $M^{-1}$ $s^{-1}$) than in aqueous media at pH 7.0 (13.1 $M^{-1}$ $s^{-1}$).[16] These results suggest a very efficient micellar compartmentalization of the antioxidants depending on their hydrophobicity; further, it also suggests a preferred location of the nitroxide probe near the micellar interface. The latter is supported by the high partition constant determined for $C_{343}T$ ($K_{SDS} = 500$ $M^{-1}$) and a high polarity microenvironment sensed by the fluorescence emission maximum of the chromophore unit in SDS micelles (489 nm) relative to water or methanol (492 and 481 nm, respectively).

The micellar reaction with Trolox, a predominantly water-soluble antioxidant, showed pseudo-first-order kinetics, but the reaction was faster than in aqueous media (Figure 2).

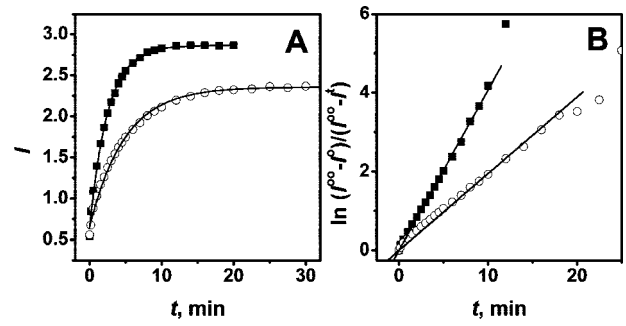


**Figure 2.** (A) Fluorescence−time profile associated to the reduction of $C_{343}T$ to the *N*-hydroxylamine by hydrogen transfer from 10 mM (■) and 5 mM (○) Trolox, in 20 mM SDS−phosphate buffer (20 mM, pH 7). (B) Experimental rate constant evaluated from eq 1. Experimental conditions: 2.5 $\mu$M $C_{343}T$, 20 mM phosphate buffer, pH 7.0. $\lambda_{exc}$ 420nm, $\lambda_{em}$ 470nm.

The same tendency is observed when Triton X100 micelles are employed.

Detailed analysis of the data in Table 2 reveals a remarkable increase of the rate constant for quercetin as we move from water to micelles: 15 and 41 times faster in SDS and Triton, respectively. These dramatic changes can be attributed to the favorable partition of quercetin into micelles due to its hydrophobic character. In fact, the difference is even larger between methanol and micelles (increases of 88- and 230-fold for SDS and Triton, respectively). This is a consequence of the lower rate constant of quercetin in methanol compared with water at pH 7, where the molecule is partially deprotonated ($pK_a$ 7.03),[17,18] thus increasing the reaction rate by an electron transfer mechanism. This argument is supported on the reduction potential of quercetin

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and Tempol evaluated at pH 7.0,[19,20] and the secondary reactions of quercetin radicals, such as crossed reaction with unreacted nitroxides. The difference between the two micelles is due to a less efficient incorporation of this antioxidant in the anionic micelle compared to the neutral one. The negative headgroup of the SDS molecule and the enolate form present at high pH are the subject of repulsive interactions.[15] In fact, in CTAC micelles the kinetics appear almost instantaneous; here the enolate form acts as a micellar counterion, complicating the determination of reliable kinetic parameters (data not shown).

In the case of BHT, the relative rate constants are 1:45:120 for methanol/SDS micelles/Triton micelles, respectively. This is a clear consequence of BHT partition in the micelles while gallic acid shows almost the same reactivity as in methanol.

In methanol, no reaction between the probe and rutin was observed while for quercetin a rate constant of $4.0 \times 10^{-3}$ $M^{-1}$ $s^{-1}$ was obtained. This is a remarkable result in terms of the selectivity of the nitroxyl moiety of the prefluorescent probe, revealing that the more reactive center in quercetin (among the 5 OH groups) is the 3′-hydroxyl group, which in rutin is protected by a sugar residue. This result is in agreement with theoretical results where Fukui indexes of reactivity were employed.[22] Further, the hydrogen transfer selectivity shown by $C_{343}T$ is higher than that of related species, such as nitronyl nitroxide radicals.[23] Nitroxide radicals exhibit a ratio between the rate of hydrogen transfer for ascorbic acid ($13.1$ $M^{-1}$ $s^{-1}$)[16] and quercetin ($22.7 \times 10^{-3}$ $M^{-1}$ $s^{-1}$) of 570, whereas for nitronyl nitroxide radicals the ratio is only 57, with rate constant values of 43 and 0.76 $M^{-1}$ $s^{-1}$ for ascorbic acid and quercetin, respectively.

It is worth mentioning that other methodologies involving peroxyl radicals in competitive experiments in homogeneous systems are not able to discriminate well between two highly reactive phenols. A typical example is the TAR index, which is based on the relative antioxidant protection of the target molecule by the decrease on the stationary concentration of peroxyl radicals (derived from 2,2′-azobis(2-amidinopropane) dihydrochloride, AAPH) caused by the antioxidant. However, as shown in Table 2, with this methodology, antioxidants such as caffeic acid are not distinguishable from the reference compound, Trolox. Whereas the analysis of the rate hydrogen transfer toward the nitroxide probe shows a noticeable higher reactivity for Trolox than caffeic acid (5 times) in phosphate buffer, pH 7. Moreover, there is a clear tendency for higher TAR values with the increase on number of phenol groups in the molecules without relation to their actual reactivity.[21] In fact, we have recently shown that most of the reactivity parameters obtained in competitive experiments are strongly influenced of stoichiometric factors and also by the reactivity of the reference compound employed.[24,25]

In summary, we propose the use of persistent substituted nitroxide radicals in micellar systems as a new and simple tool to evaluate antioxidant efficiency; this approach takes into account both the hydrophobicity of the antioxidant and also the high selectivity of the nitroxide radicals toward very reactive phenols such as flavonoids. Further, the use of different chromophore units tethered to nitroxide moieties, of different hydrophobicity,[26–29] can be employed to control the partition constant, and also the location of the nitroxide prefluorescent probe within the micelles.

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**Supporting Information Available:** Synthetic procedures and full spectroscopy data for all new compounds, an example of kinetic determination using fluorescence spectroscopy, and procedures for the determination of partition constants. This material is available free of charge via the Internet at http://pubs.acs.org.

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