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A New Method to Study Antioxidant Capability: Hydrogen Transfer from Phenols to a Prefluorescent Nitroxide

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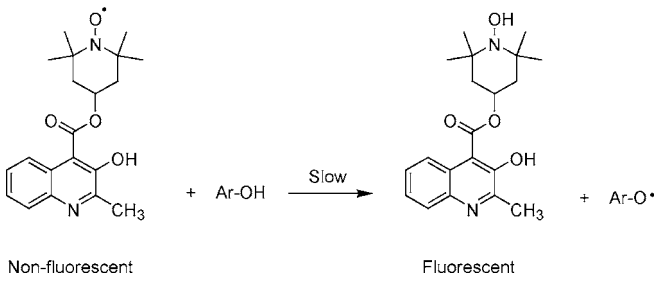
ABSTRACT



Rate constants for hydrogen abstraction from phenols by a prefluorescent-TEMPO probe are reported. The nitroxide is employed as a potential model of peroxyl radicals. The probe works by nitroxide suppression of the fluorescence of the chromophore. The fluorescence is restored when the nitroxide abstracts a hydrogen atom to produce the diamagnetic hydroxylamine. The phenols studied in this project exhibited rate constants between 0.003 and 0.2 M^{-1} s^{-1}. A deuterium isotope effect of 10 for TROLOX confirms that the mechanism is dominated by hydrogen transfer.

Phenols have been widely used as scavengers for free radicals involved in oxidative stress in living organisms. Their chain-breaking antioxidant action is based on the trapping of radicals involved in the oxidative chain by efficient hydrogen transfer.[1−3] Rate constants for hydrogen abstraction from phenols by peroxyl-, alkoxyl-, nitrogen-, and carbon-centered radicals have been reported.[4−7] However, due to the high reactivity of some of these radicals, they present low selectivity toward compounds with similar hydrogen-donating properties. This is particularly true in the evaluation of hydrogen-transfer reactions of highly reactive polyphenols such as flavonoids.

Different methodologies have been developed to evaluate antioxidant capability of individual compounds or in complex mixtures rich in polyphenols, such as wines and infusions.[8] Those methodologies are based on (i) indirect measurement of oxygen consumption,[9,10] or competitive reactions with a highly reactive reference compound, (e.g., c-phycocyanin) employing peroxyl radicals,[11] or (ii) direct monitoring of the consumption of a persistent free radical such as DPPH or ABTS.[12,13] The information obtained has allowed the de-

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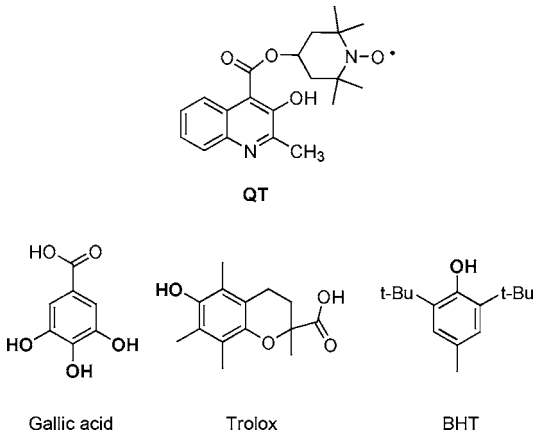
scription of the quality (reactivity) and the quantity (stoichiometry) for the added antioxidant reacting with the radicals used. However, those studies do not provide information about the type of radical trapped and the antioxidant mechanism involved. This is particularly true in studies employing DPPH and ABTS, where reactions of hydrogen abstraction and electron transfer have been proposed.[12−14]

Fast reaction of nitroxide radicals with ubiquinol-9, phenolic, and thiol antioxidants have been reported.[15,16]

Recently, Aliaga et al.[17] using ESR spectroscopy and product studies have shown a very slow reaction between TROLOX and tempol in aqueous solvents and suggested that traces of metal ions could catalyze the reaction. In the absence of metal ions, the kinetics of this process are believed to reflect hydrogen transfer.

In this paper, we evaluate the ability of 4-(3-hydroxy-2-methyl-4-quinolinoyloxy)-2,2,6,6-tetramethylpiperidine-1-oxyl free radical (QT), a prefluorescent TEMPO probe, to monitor hydrogen transfer from reactive phenols (Scheme 1)[18] The mechanism by which this prefluorescent nitroxide probe[19] works is based on the suppression of the intramolecular quenching of the fluorescent chromophore by the nitroxide group. The fluorescence is restored when the nitroxide moiety is trapped by a reducing agent, such as a hydrogen donor, to produce the diamagnetic hydroxylamine (QTH).[19] This methodology has been successfully used to detect and quantify directly primary radical concentrations generated by pulsed laser photolysis in homogeneous solvents.[20] These studies have allowed the quantification of very low concentrations of radicals that usually are not detected by conventional spectroscopic techniques or difficult by ESR.

Scheme 1. Phenols and Polyphenols Studied and Probe Structure



We propose here the use of QT as a probe mimicking peroxyl radical reactivity[21] in order to evaluate the antioxidant activity of phenolic compounds and to obtain kinetic parameters involved in the hydrogen-transfer process. Nitroxide compounds are orders of magnitude less reactive than peroxyl radicals;[4] this characteristic is perceived as an advantage since it allows evaluation of the selectivity for highly reactive phenols. A similar methodology has previously proposed to quantify vitamin C in biological fluids,[22] where an electron-transfer mechanism was proposed. In our systems (vide infra), the mechanism is believed to be hydrogen transfer; further, the enhancement of fluorescence for QT is much larger than for the dansyl probe reported. [22]

Most reactions were carried out under an inert atmosphere in methanol, water, and phosphate buffer (50 mM, pH 7.0). The aqueous solvents were pretreated with Chelex100 in the presence of 0.1 mM diethylenetriaminepentaacetic acid (DTPA) in order to minimize the concentration of free metal ions. The rate constants observed (k_{obs}) were obtained by monitoring the growth of the fluorescence according to eq 1, where I^{∞}, I^0, and I^t represent the fluorescence intensities in the plateau region, initially and at time "t", respectively.

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

Kinetic analysis, under pseudo-first-order reaction conditions, leads to the rate constant for hydrogen abstraction directly from the slope of Figure 1B (eq 2). The rate expression derived under these experimental conditions is shown in eq 3

$$\text{R-NO}^{\bullet} + \text{PhOH} \rightarrow \text{R-NOH} + \text{PhO}^{\bullet} \qquad (2)$$

$$\frac{x_e}{(2[A_0] - x_e)}\ln\left(\frac{x_e}{x_e - x}\right) = k_1 t \qquad (3)$$

where A_0 represents the initial nitroxide and x_e is the concentration of the corresponding diamagnetic *N*-hydroxylamine in the equilibrium, since eq 3 allows for possible reversibility in reaction 2.

In fact, similar considerations allow the evaluation of the equilibrium constant from the fluorescence plateau intensity reached at long times at different concentrations; a value of 8×10^{-3} is obtained for TROLOX. Despite the apparently small equilibrium constant, the equilibrium should be largely

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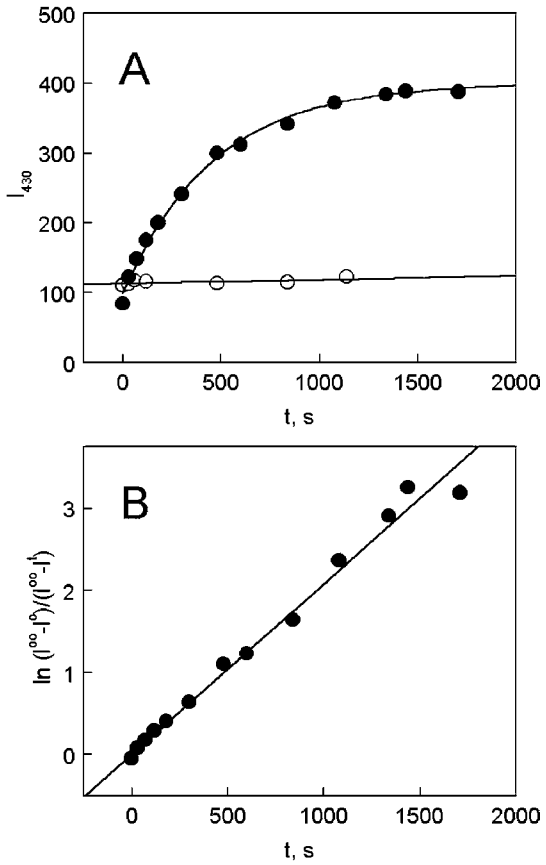

Figure 1. (A) QT/QTH Fluorescence profile evaluated at 430 nm after TROLOX addition (●) and in the absence of TROLOX (○) with time (λ_{exc}360 nm). (B) Fluorescence kinetic adjusted. Experimental conditions: 10 μM QT, 10 mM TROLOX, 50 mM phosphate buffer, pH 7.0.

on the product side, since the concentration of phenolic compound is typically 3 orders of magnitude larger than that of QT. We anticipate that phenoxyl radicals, while long-lived, will ultimately yield nonradical products[23] by either self-reaction or cross-reactions with QT. It is worth noting that the calculation of the equilibrium constant for BHT could be affected due to the fact that sterically hindered phenols are efficient light quenchers.[24] However, a rate constant evaluated at a large concentration is a reliable value, since the methodology is based on relative fluorescence intensities (eq 1).

The data for the rate constants for the different phenolic compounds are collected in Table 1. Note that the slow reaction between the probe and the phenolic compound makes it possible to evaluate substrates with high reactivity using conventional spectroscopic instrumentation, in contrast with the case of ABTS[25,26] where the most reactive substrates

Table 1. Experimental Rate Constants of Hydrogen Transfer from Phenolic Compounds to QT Probe

substrate	solvent	k, M^{-1} s^{-1}
TROLOX	buffer pH 7	0.2
	methanol	0.19
	methanol-*O*-*d*	0.018
gallic acid	buffer pH 7	8.8×10^{-3}
	H_2O metal free	5.5×10^{-3}
	methanol	4.6×10^{-3}
	methanol-*O*-*d*	1.3×10^{-3}
	D_2O	1.4×10^{-3}
BHT	methanol	3.3×10^{-3}
	methanol-*O*-*d*	1.3×10^{-3}

could not be evaluated kinetically. Further, we assume that reactions occur predominantly at the most reactive hydrogen atom from every structure.

In principle, at least three mechanisms can be proposed to rationalize the reaction of QT with phenolic hydrogen donors, i.e., (a) hydrogen abstraction, (b) electron transfer followed by proton transfer, and (c) substrate ionization followed by electron transfer from the phenoxide to QT, as recently demonstrated by Litwinienko and Ingold for reactions of DPPH in hydroxylic solvents.[27] The nitro substitution in DPPH clearly favors this type of behavior. To address this question, we examined the reactivity of TROLOX in methanol and methanol-*O*-*d*, since in the latter solvent the phenolic position in TROLOX should be fully deuterated. The results are shown in Figure 2. From these data we obtain

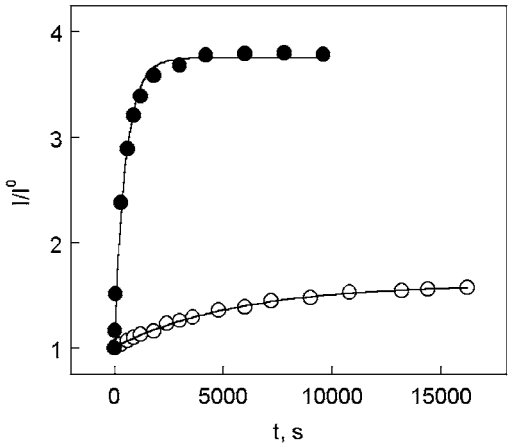

Figure 2. Relative fluorescence increase observed in methanol (●) and methyl alcohol-*d* (○). Experimental conditions: 10 μM QT, 10 mM TROLOX (λ_{exc} 360 nm, λ_{em} 400 nm).

a deuterium isotope effect of 10.5, whereas a factor of ~4 is observed for gallic acid. While large, values of this magnitude for reactions of phenols have been known for over 40 years.[28]

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Interestingly, the rate constants for TROLOX are essentially the same in water and in methanol; product stabilization in the former probably compensates for reactivity reductions due to stronger hydrogen bonding. However, the rate constant observed for gallic acid (a polyphenol) in buffer is larger than in methanol. Further, the fact that the less reactive substrate (gallic acid) shows a smaller isotope effect suggests that some degree of charge transfer may be involved in this example, probably mediated by the deprotonation of the acidic group. Additional studies of polyphenols could clarify this point.

The use of DPPH as a probe for radical reactions with phenols has close to a half-century history,[29] and in a recent publication Ingold reports that publications on DPPH and antioxidants approach 150 per year.[27] Despite this, concerns still remain, particularly in relation to the value of kinetic parameters determined with DPPH in hydrogen-bonding solvents. This is in part related to the strongly electron-deficient radical center in DPPH. Nitroxide radicals, while electrophilic (most oxygen centered radicals are), are not as electron deficient as DPPH, and in this sense are better model systems.

In summary, we report here a convenient approach for the determination of the hydrogen-donor ability of phenols which is very sensitive and appears less prone to suffer from the problems of highly electron-deficient probes such as DPPH. Prefluorescent probes offer a combination of high sensitivity, reaction selectivity, and in many cases simple synthetic routes; further, the concept can be easily exploited while optimizing required properties such as hydrophobicity/hydrophilicity, excitation wavelength, etc.

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