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Reducing Activity of Polyphenols with Stable Radicals of the TTM Series. Electron Transfer versus H-Abstraction Reactions in Flavan-3-ols

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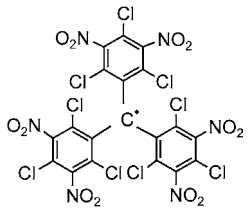
ABSTRACT



HNTTM

A new method to test the antioxidant activity of polyphenols by electron transfer reactions to a stable organic free radical, tris(2,4,6-trichloro-3,5-dinitrophenyl)methyl radical (HNTTM), is reported. Therefore, the activity of the natural flavanols, (–)-epicatechin, and two synthetic derivatives, 4β-(*S*-cysteinyl)epicatechin and 4β-(2-aminoethylthio)epicatechin, can be differentiated by their capacity to transfer hydrogen atoms to 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) and to transfer electrons to HNTTM.

Flavan-3-ols are natural polyphenols widely found in foods that show the ability to act as antioxidants.[1] Their free radical scavenging activity lies in the transfer of the phenolic H-atoms to oxygen free radicals such as hydroxyl and peroxyl radicals, chain-carrying species in autoxidation and initiators of many human degenerative diseases.[2] Recently, efforts have been made to obtain bio-based derivatives of flavan-3-ols with improved ability to transfer hydrogen atoms.[3] DPPH, a stable organic nitrogen-centered free radical with a high absorption in the visible part of the electronic spectrum, has been used to test this antioxidant activity by its ability to abstract hydrogens from polyphenols.[4] Recently, we have reported that the tris(2,4,6-trichloro-3,5-dinitrophenyl)methyl radical (**1**), a stable organic carbon-centered free radical of the TTM (tris(2,4,6-trichlorotriphenyl)methyl radical) series, is a good sensor to test the activity of polyphenols measuring their capacity to participate in electron transfer reactions.[5] Therefore, the activity of polyphenols may be

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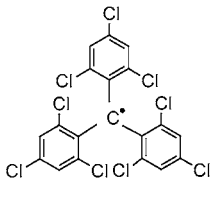
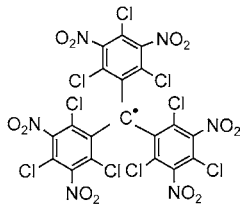
measured either by their ability to donate H-atoms or by their electron transfer properties, and, in general, both processes are correlated. Along with the antioxidant properties against destructive radicals, a disadvantage of many natural polyphenols such as catechins is that their low ionization potentials make them easily oxidized by atmospheric oxygen by electron transfer, generating $O_2^{\bullet -}$, a very active, electron-rich radical, and hydrogen peroxide under certain conditions.[6] This is why, nowadays, efforts are focused to prepare new polyphenols, more stable to oxygen but keeping the high tendency to transfer H-atoms.[7]

Radicals of the series of TTM are a kind of organic carbon-centered free radicals whose great persistence is mainly due to steric hindrance of six chlorine atoms around the trivalent carbon.[8] All these radicals are completely disassociated and very stable both in solid and in solution. Their inefficiency to abstract H-atoms from hydrogen-labile species is accounted for by steric hindrance, and therefore they are inoperative in these processes. However, they are very sensitive to electron transfer reactions; in the presence of electron donor species they are easily reduced to carbanions with stabilities comparable to their precursors, and their electrochemical behavior by cyclic voltammetry shows reversible reduction processes. It is worth noting the possibility of modulating the redox properties of these radicals by simply introducing different substituents into their aromatic structure.[5] Therefore, we have prepared radical **1**,[9] an oxidant ($E°$= 0.58 V vs NaCl-saturated calomel electrode), stronger than TTM and sensitive to the presence of flavan-3-ols.



TTM



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To get more insight into the electron transfer mechanism of the oxidation of natural polyphenols by radical **1**, the activity of this oxidant magnetic species was tested with two simple models, catechol (1,2-dihydroxybenzene) and resorcinol (1,3-dihydroxibenzene), as the presumably active moieties in the efficient antioxidant (−)-epicatechin, and the course of these reactions was monitored by electronic spectroscopy. The $\pi^*-\pi$ electronic transition characteristic of radical **1** and the broad and less energetic transition of anion $\mathbf{1}^-$ in chloroform−methanol (10%) appear at 384 and 497 nm, respectively (Figure 1).

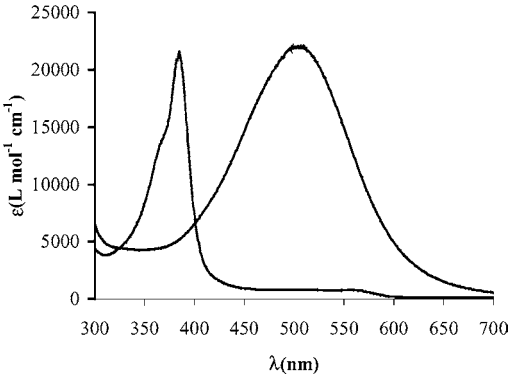

Figure 1. Absorption spectra of radical **1** in $CHCl_3$ ($\pi^*-\pi$ band at 387 nm) and anion $\mathbf{1}^-$ (band at 497 nm) from a solution of triphenylmethane **1H** and Bu_4NOH in THF.

When two equimolecular solutions of radical **1** and catechol are mixed together, the electronic spectrum of the mixture displays peaks of the radical **1** and the negatively charged species $\mathbf{1}^-$, which denotes the electron transfer from polyphenol to radical. Figure 2 shows the evolution of both absorptions with time. After 1 min of reaction, ∼35% of the radical was reduced, and ∼68% had reacted after 30 min. The decrease of the intensity of the peak at 497 nm corresponding to the carbanion during the reduction suggests that the electron transfer is followed by protonation of the anion in the course of the reaction, **1H** being the final product of the process. However, if experiments are carried out with resorcinol, as an alternative active moiety of (−)-epicatechin, the reaction practically does not take place. So, only ∼3% of the radical was reduced after 30 min of reaction and ∼15% after 4 h. This is in agreement with the results of other authors stressing the preponderant role of the catechol moiety.[10]

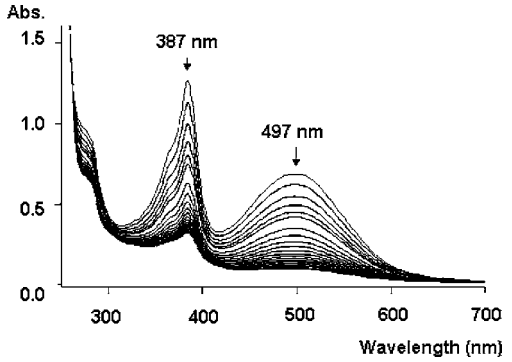

Figure 2. Evolution of the vis spectrum of a $\sim 10^{-4}$ M solution of radical **1** and catechol (1:1) in $CHCl_3$.

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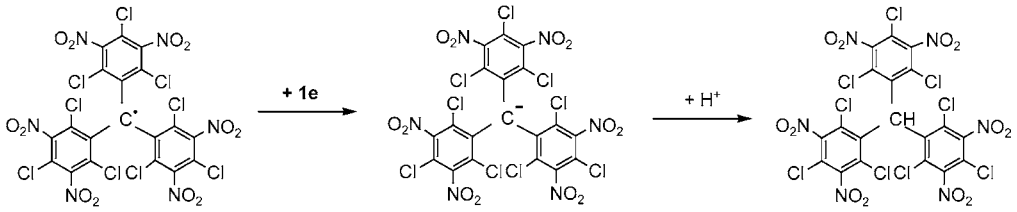
Scheme 1



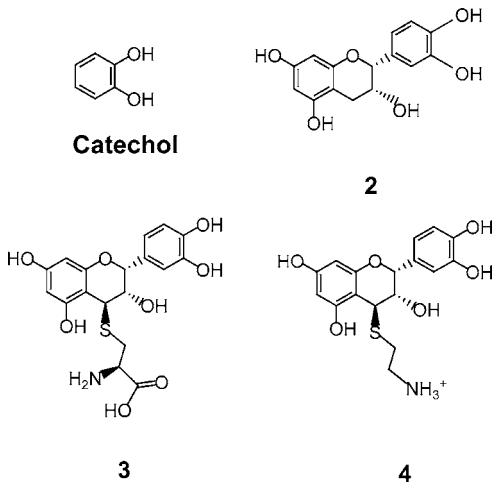
The two-step mechanism of reduction is consistent with the results found when radical **1** reacts with catechol (see Scheme 1).

The oxidant ability of radical **1** in the presence of polyphenolic antioxidants has also been evaluated by electron paramagnetic resonance (EPR),[11] measuring the decrease of the intensity of the radical signal from diluted solutions (60 μM) in chloroform−methanol (2:1) with variable concentrations of (−)-epicatechin (**2**) and two different synthetic derivatives, 4β-(*S*-cysteinyl)epicatechin[3c] (**3**) and 4β-(2-aminoethylthio)epicatechin[3b] (**4**). The results were expressed as the efficient dose ED_{50} standing for the necessary micromoles of polyphenol to react with half the amount of free radical divided by micromoles of initial radical **1**. The stoichiometric value is obtained by multiplying ED_{50} by two, and the inverse of this value represents the moles of **1** reduced by 1 mol of antioxidant or, in other words, the number of transferred electrons per molecule of polyphenol.[12] A comparison of the parameters of the free radical scavenging power of these polyphenols, measured by the DPPH method[3b,c] (number of transferred hydrogens per molecule of polyphenol), and the parameters of their reduction power, measured by the radical **1** method (number of transferred electrons per molecule of polyphenol), is summarized in Table 1. Parameters from catechol are also enclosed in Table 1 for comparative purposes.

Table 1. Free Radical Scavenging Power (Hydrogen Transfer) and Electron Transfer of Polyphenols

	DPPH method[a]		radical **1** method	
compound	stoichiometric value	H atoms per molecule	stoichiometric value	electrons per molecule
catechol	0.34	2.9	0.44	2.3
2	0.4	2.8	0.42	2.4
3	0.24	4.2	0.42	2.4
4	0.22	4.5	0.43	2.3

[a] Results reported in refs 3b,c.



Catechol, as a simple model, was able to transfer two electrons and reduce two molecules of radical **1** per molecule and is also suitable to transfer two hydrogens and reduce two DPPH molecules per molecule. In such a case, the stoichiometry of both processes is the same and the reactions proceed as follows (see Scheme 2).

The reactivity of (−)-epicatechin is quite similar to that of catechol (Table 1), corroborating that the more reactive moiety of the molecular structure in both electron transfer and hydrogen abstraction is the catechol ring. This is a first approximation of the reactivity of the polyphenols with **1**.

(9) Preparation of **1H**, as a precursor of radical **1**, and **1** are as follows: **Synthesis of Tris(2,4,6-trichloro-3,5-diphenyl)methane.** A mixture of tris(2,4,6-trichlrophenyl)methane (0.780 g), fuming nitric acid (40 mL), and sulfuric acid (30% SO_3) (10 mL) was stirred (80 °C) (72 h) and then poured into an excess of glass-water. The precipitate, separated by filtration and dried under reduced pressure, was tris(2,4,6-trichloro-3,5-diphenyl)methane (1.095 g; 94%). Selected data can be found in ref 5. **Synthesis of Tris(2,4,6-trichloro-3,5-dinitrophenyl)methyl Radical.** An aqueous solution of tetrabutylammonium hydroxide (1.5 M) (0.71 mL) was added to a solution of tris(2,4,6-trichloro-3,5-dinitrophenyl)methane (0.770 g) in acetone (40 mL) and stirred at room temperature (20 min). Solid chromium(VI) oxide (0.450 g) was added, and the mixture was stirred in argon and in the dark for a period of time (20 h). The mixture was poured into an excess of hydrochloric acid aqueous solution, and the precipitate was separated by filtration. The solid in chloroform was column chromatographed (silica gel), affording tris(2,4,6-trichlro-3,5-dinitrophenyl)methyl radical (0.541 g; 70%). Selected data can be found in ref 5.

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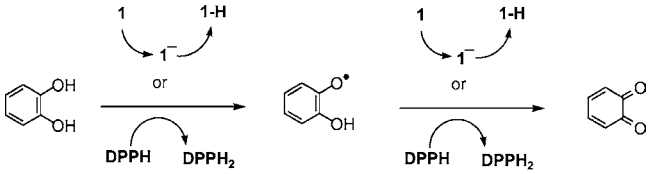
(11) EPR spectrum with spectral parameters of tris(2,4,6-trichloro-3,5-dinitrophenyl)methyl radical in CH_2Cl_2 is provided in ref 5.

(12) Analogous methodology has been used when the antioxidant activity of these polyphenols by H-abstraction was analyzed in the presence of DPPH (see ref 3c).

Scheme 2



The actual scavenging mechanism is clearly more complex, as evidenced by the stoichiometric values in Table 1 and the literature.[13,14] When (−)-epicatechin and derivatives such as **3** and **4** were examined, the results in Table 1 revealed remarkable differences among them. While the stoichiometries for both chemical processes with (−)-epicatechin and for the electron transfer process with **3** and **4** show similarity, the presence of mercapto substituents in position 4 of the heterocycle in **3** and **4** seems to affect the hydrogen abstraction process in both derivatives. These flavanols are capable of reducing at least one mole more of DPPH per mol of antioxidant relative to (−)-epicatechin. This increase in the number of labile hydrogens of the molecule may be ascribed to the presence of the long-chain substituent at C4.

However, to evaluate the effectiveness of the antioxidants, it is of more interest to provide the kinetics of their reactions with radical species. Thus, some preliminary results on kinetic studies of the reaction of radical **1** with the reported antioxidants have been carried out following the general kinetic model 1 reported by Dangles et al. to assess the ability of antioxidants to transfer H atoms to DPPH.[14] In our experiments, the electron transfer reaction to radical **1** from polyphenols in methanol was monitored at 25 °C by following the decrease of the absorbance (λ_{max} = 385 nm) in the electronic spectrum of **1** after the addition of variable concentrations of polyphenols. In contrast with what happens when DPPH is used, the two steps of the decay of the absorbance of DPPH, one fast and the other slow, in the course of the reaction with the antioxidant are hardly distinguishable in the case of using radical **1**. Values of the kinetic constants at three different concentrations of the reported polyphenols are shown in Table 2. An analysis of these results suggests that (−)-epicatechin is the more active electron-donating antioxidant, since the activity of the catechol and of the derivatives of (−)-epicatechin, **3** and **4**, are very similar. Consequently, although the stoichiometric factors of **3** and **4** are nearly twice the value of (−)-epicatechin, this antioxidant has shown to be more effective as electron donor to radical **1**. An estimation of the kinetics of **2** with DPPH as a stable radical in methanol at 25 °C was also performed. At the DPPH/**2** molar ratio of 12, the same as one of the molar ratios in Table 2, the observed rate constant is 3468 ± 311, much higher than the value reported for radical **1**. Detailed results of the rate constants of the other polyphenols with DPPH recorded under similar conditions are now in progress.

Table 2. Observed Rate Constants, k_1 (M^{-1} s^{-1}), for the Reaction of Radical **1** (60 μM) with Antioxidants at 25 °C in Methanol

antioxidant concentration (μM)	radical **1**/ antioxidant molar ratio	catechol	**2**	**3**	**4**
5	12.0	301 ± 61	815 ± 138	282 ± 27	214 ± 14
10	6.0	207 ± 21	669 ± 20	264 ± 23	260 ± 33
20	3.0	210 ± 3	659 ± 21	300 ± 16	300 ± 3

What has been said above is a first approximation of the reactivity of these polyphenols, because the overall mechanism of their antioxidant behavior is clearly complex, as evidenced by the stoichiometric values in Table 1, which in some cases do not fit to the required values. As reported before,[3c] the higher than theoretical number of hydrogens or electrons involved in these processes would be regarded as the result of the antioxidant activity of the oligomeric fractions derived from the first generated phenolic reactive radicals. It should also be underlined that the reported kinetic results are only indicative of the relative efficacy of the different antioxidants, as they refer to a stable carbon-centered radical instead of a very reactive oxygen-centered radical like those actually involved in autoxidation.

In summary, this manuscript reports a new method to distinguish between hydrogen- and electron-donating properties in polyphenols. Therefore, while polyphenols **3** and **4** preserve the same power as electron donors as (−)-epicatechin (**2**), they provide more hydrogens to be transferred to radical species. The ability of radicals of the TTM series and in particular of radical **1** to be reduced by electron transfer reactions to very stable negatively charged species detected by spectroscopy has made it possible to corroborate the reducing capacity of natural and synthetic polyphenols by electron transfer reactions. Kinetics and stoichiometric factors of the reactions of the reported polyphenols with this new stable radical **1** in different solvents and conditions are currently underway in our laboratories.

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