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ARTICLE

Quantification of the Antioxidant Capacity of Different Molecules and Their Kinetic Antioxidant Efficiencies

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A kinetic method has been developed to determine the antioxidant capacity of a variety of molecules. In this method, named the enzymatic kinetic method, the free radical of ABTS is generated continuously in the reaction medium by a peroxidase/ABTS/H_2O_2 system. The presence of an antioxidant in the solution provokes a lag period in the accumulation of the free radical in the medium, and by studying this lag period it is possible to calculate the antioxidant capacity of the molecule in question. This antioxidant capacity, named the primary antioxidant capacity, will be quantified by *n*, the number of electrons donated per molecule of antioxidant, the effective concentration, EC50, and the antioxidant or antiradical power (ARP) (ARP = 1/EC50 = 2*n*). If the products arising from the reaction between the antioxidant and the free radical evolve by consuming more radical, a secondary antioxidant capacity is generated. To calculate this, a nonenzymatic test is proposed.

KEYWORDS: Enzymatic kinetic method; antioxidant capacity; antiradical power

INTRODUCTION

Numerous antioxidants, including phenols, flavonoids, anthocyanins, tannins, vitamins, etc., are contained in fruits and vegetables, although their proportions vary from one food to another. Their composition can also be profoundly altered by processing, storage, or cooking practices. Polyphenols, which are widely distributed in plants, are known to act as antioxidants (*1*).

Several methods to determine free radical scavenging have been reported (*2*), and a comparative evaluation of various total antioxidant capability assays applied to phenolic compounds using the CUPRAC assay is the subject of a more recent review (*3*). Among the methods used to determine antioxidant power that have proved to be most successful are those that use two stable free radicals as indicators of the antioxidant activity: α,α′-diphenyl-β-picrylhydrazyl radical (DPPH$^{\bullet}$) (*4*) and 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) radical cation (ABTS$^{\bullet+}$) (*5*). ABTS$^{\bullet+}$ has been used in the determination of antioxidant activity of single compounds (*6*) and in various mixtures (e.g., body fluids (*7*) and foods (*8*)). Subsequently, improvements were made in the ABTS$^{\bullet+}$ decoloration assay (*9*). From electrochemical measurements, the redox potential of several antioxidants has been determined with the aim of correlating this with antioxidant capacity, for example, that determined for DPPH radical scavenging activity (*10*). No clear correlation was found between the oxidation potentials of flavonoids and their DPPH scavenging activities (*10*). Moreover, the number of electrons (*n*) involved in the redox reaction of polyphenols determined by means of continuous flow-column electrolysis has been related with the antioxidant activities of polyphenols (*11*−*13*). In such cases, the high number obtained for *n* has been related with the existence of dimerization and polymerization reactions of the reaction products. The correlation study of *n* values at a slow flow-column electrolysis rate with the DPPH radical scavenging activity has served to support the involvement of slow chemical reactions that follow the initial oxidation of polyphenols (*13*).

Kinetic studies have been published on antioxidant activity using the DPPH$^{\bullet}$ free radical methods (*4*, *14*, *15*). These studies point to the biphasic behavior of many antioxidants, leading to their classification into three groups (rapid, intermediate, and slow) depending on the time they take to reach the steady state (the free radical does not vary with time) (*4*, *14*). On the basis of these studies, three parameters have been proposed to characterize an antioxidant: effective concentration (EC50), which is defined by the ratio of the antioxidant concentration necessary for decreasing the initial DPPH$^{\bullet}$ concentration by 50% to the initial DPPH$^{\bullet}$ concentration; T_{EC50}, the time needed to reach the steady state at the concentration corresponding to EC50; and antiradical efficiency (AE), defined as AE = 1/(EC50 × T_{EC50}), which takes in both effects (concentration and time) (*14*, *15*).

Several investigators have examined the kinetics of the reaction of ABTS$^{\bullet+}$ with antioxidants (*16*). Most plant polyphenols studied have demonstrated a biphasic kinetic pattern, involving fast and slow steps (*16*). Evidence suggests that the ABTS$^{\bullet+}$ initially extracts an electron or hydrogen atom from polyphenols,

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No.	Compound	Substituent	Structures
	Benzenes		
1	Catechol	1,2-OH	
2	*p*-Hydroquinone	1,4-OH	
3	Pyrogallol	1,2,3-OH	
4	Phenol	1-OH	
5	Guaiacol	3-OH,4-OCH_3	
6	Eugenol	1-CH_2-CH_2-CH_2,3-OCH_3,4-OH	
	Benzoic acids		
7	*p*-Hydroxybenzoic acid	4-OH	
8	Vanillin	3-OCH_3,4-OH	
9	Syringic acid	3,5-OCH_3,4-OH	
10	Protocatechuic acid	3,4-OH	
11	Gallic acid	3,4,5-OH	
	Cinnamic acids		
12	*p*-Coumaric acid	4-OH, R = H	
13	Ferulic acid	3-OCH_3,4-OH, R = H	
14	Sinapinic acid	3,5-OCH_3,4-OH, R = H	
15	Caffeic acid	3,4-OH, R = H	
16	Chlorogenic acid	3,4-OH, R = quinic acid	
	Flavans		
17	(+)-Catechin	3,5,7,3′,4′-OH	
18	(-)-Epicatechin	3,5,7,3′,4′-OH	
	Flavonols		
19	Quercetin	3,5,7,3′,4′-OH	
20	Rutin	5,7,3′,4′-OH, 3′-rutinoside	
	Tannin		
21	Ellagic acid		
	Others		
22	Trolox		
23	3-*tert*-butyl-4-hydroxyanisole		
	Non-polyphenols		
24	L-cysteine		
25	L-ascorbic acid		
26	D-ascorbic acid		

Figure 1. Structures of antioxidants.

resulting in the formation of a semiquinone (*16*). Evidence has been put forward to suggest that the semiquinone may dimerize upon reaction with $ABTS^{\bullet+}$ and that the resulting dimers may retain antioxidant activity. It has been demonstrated recently that dimerization may contribute to the biphasic pattern observed with some phenolics (*16*). Also, recent data demonstrate that some polyphenols react with $ABTS^{\bullet+}$ to form covalent adducts (*16*). In some cases, these adducts have been seen to retain antioxidant activity and may contribute to the complex kinetic behavior that has been observed.

The aim of the present study was to develop an enzymatic kinetic method that will permit the characterization of the antioxidant capacity of a compound. In cases when a secondary antioxidant activity exists, a nonenzymatic kinetic test is proposed, consisting of a kinetic analysis of how the concentration of the excess of free radical varies when it reacts with the initial product generated by the reaction of the antioxidant with the free radical.

MATERIALS AND METHODS

Enzyme Source. Horseradish peroxidase (POD; 251 U/mg) type VI, Rz ($A_{430\ nm}/A_{275\ nm}$) = 3.1, was obtained from Sigma Chemical (St. Louis, MO). The enzyme concentration was determined by measuring the absorbance at 403 nm using $\varepsilon_{403} = 100000/(M \times cm)$ (*17*).

Reagents. The compounds (**Figure 1**) *p*-hydroquinone (1,4-dihydroxybenzene), pyrogallol (1,2,3-trihydroxybenzene), *p*-hydroxybenzoic acid, protocatechuic acid (3,4-dihydroxybenzoic acid), *p*-coumaric acid (*trans*-4-hydroxycinnamic acid), ferulic acid (*trans*-4-hydroxy-3-methoxycinnamic acid), sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid), quercetin (3,3′,4′,5,6-pentahydroxyflavone), rutin (quercetin-3-rutinoside), L-cysteine [(*R*)-2-amino-3-mercaptopropionic acid], L-ascorbic acid

(L-threoascorbic acid), D-ascorbic acid (D-threoascorbic acid), 3-*tert*-butyl-4-hydroxyanisole, phenol (hydroxybenzene), guaiacol (2-methoxyphenol), eugenol [2-methoxy-4-(2-propenyl)phenol], Trolox (6-hydroxy-2,5,7,8-tetramethylchrome-2-carboxylic acid), (+)-catechin (*trans*-3,3′,4′,5,7-pentahydroxyflavane), and ABTS [2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)] were obtained from Sigma Chemical. Catechol (1,2-benzenediol), syringic acid (3,5-dimethoxy-4-hydroxybenzoic acid), chlorogenic acid [1,3,4,5-tetrahydroxycyclohexanecarboxylic acid 3-(3,4-dihydroxycinnamate)], (−)-epicatechin [(−)-*cis*-3,3′,4′,5,7-pentahydroxyflavane], gallic acid (3,4,5-trihydroxybenzoic acid), and ellagic acid (4,4′,5,5′,6,6′-hexahydroxydiphenic acid 2,6,2′,6′-dilactone) were obtained from Fluka, and caffeic acid (3,4-dihydroxycinnamic acid) was obtained from Aldrich. Stock solutions of the reducing substrates were prepared in 0.15 mM phosphoric acid to prevent autoxidation. The buffer used was 30 mM sodium phosphate buffer (pH 6.0−7.5).

Spectrophotometric Assays. Absorption spectra were recorded on a Perkin-Elmer Lambda 35 UV−vis spectrophotometer, at a scanning speed of 60 nm/s controlled by UV-Winlab software. The temperature was maintained at 25 °C using a Haake D16 circulating water bath with a heater/cooler and checked using a Cole-Parmer digital thermometer with a precision of 0.1 °C. Kinetic assays were also carried out with the above instruments following the appearance (kinetic enzymatic method) (*8*) or disappearance (nonenzymatic kinetic test) of $ABTS^{\bullet+}$ at $\lambda = 734$ nm [$\varepsilon = 15000/(M \times cm)$] (*18*). In the enzymatic kinetic method, the cuvette (final volume of 1 mL) contained 30 mM sodium phosphate buffer (pH 7.0), H_2O_2, and ABTS at a concentration at which POD is saturated by them (0.1 and 5 mM, respectively) (*17*), and the antioxidant was varied in the range specified in each figure. The reaction was started by adding enzyme (POD). In the nonenzymatic kinetic test, the cuvette (final volume of 1 mL) contained 30 mM sodium phosphate buffer (pH indicated in each figure) and ABTS at a concentration whereby POD is saturated (5 mM) (*17*). H_2O_2 was the limiting reagent and was totally consumed in each experiment after the addition of enzyme, generating the desired free radical. Once this free radical is generated, the reaction with the antioxidant (A) begins after the latter is added. Below, we describe in detail each of these methods.

Enzymatic Kinetic Method. The following six-step procedure is recommended to carry out this method.

Step 1. Select the experimental conditions. The initial concentration of ABTS, $[ABTS]_0$, should be high so that (i) there is a negligible variation of [ABTS] when ABTS evolves to $ABTS^{\bullet+}$ during the experiment (taking into consideration the value of K_m^{ABTS} (0.64 mM), a 5 mM concentration is suggested (*17*)) and (ii) the molecules of antioxidant (which could be substrates of the enzyme) should not have access to the enzyme (taking into account the low value of $K_m^{H_2O_2}$ (2.5 μM) (*17*), an initial concentration of H_2O_2, $[H_2O_2]_0$, of 0.1 mM should be chosen so that the enzyme is saturated by both substrates (ABTS and H_2O_2)). Note that the use of myoglobin has been reported (*19*); however, the ratio of the initial concentrations of ABTS and H_2O_2 was low, which led to deviations in the experimental measurements. The initial concentration of enzyme, $[E]_0$, should be such that, in the absence of antioxidant, it generates a quantity of radical acceptable with regard to its absorbance and which, in the presence of low concentrations of the same, gives rise to a measurable lag period outside the dead time of the measurement.

Step 2. Vary the initial concentration of antioxidant, A_0, so that it gives rise to short lag periods but well-defined with respect to the dead time of the measurement.

Step 3. Control the return to the steady state by referring to the parallelism of the straight lines. This is a fundamental test when using the enzymatic kinetic method.

Step 4. Analyze the lineal portion of the progress curves to determine V_0 (confirming the test) and τ, the lag period.

Step 5. Fit $V_0\tau$ versus $[A]_0$ by linear regression to determine the number of electrons (n, stoichiometric factor) (*13*, *15*, *20*).

Step 6. Calculate the effective concentration, EC50 [EC50 = 1/(2n)], and antioxidant capacity, ARP (ARP = 1/EC50), as shown in **Table 1**.

Nonenzymatic Kinetic Test. Step 1. Generate the free radical. First, radical $ABTS^{\bullet+}$ must be generated, for which several methods have been suggested: the chemical oxidation of ABTS with potassium persulfate solution (*12*, *13*) or enzymatically with POD/H_2O_2 (*17*). In this study, $ABTS^{\bullet+}$ is generated in the same reaction medium with POD/ABTS/H_2O_2, limiting H_2O_2, and a great excess of ABTS; the radical thus generated can be followed spectrophotometrically at $\lambda = 734$ nm [$\varepsilon = 15000/(M \times cm)$] or at $\lambda = 414$ nm [$\varepsilon = 30000/(M \times cm)$], the wavelength depending on any interferences that may exist.

Table 1. Characterization of Compounds with Monophasic Antioxidant Capacity

	primary antioxidant capacity		
substrate	n (electrons)	EC_p50	ARP_p
pyrogallol	5.81 ± 0.51	0.08 ± 0.01	12.51 ± 1.13
gallic acid	4.74 ± 0.42	0.11 ± 0.01	9.09 ± 1.01
catechol	2.91 ± 0.11	0.17 ± 0.01	5.88 ± 0.38
sinapinic acid	2.82 ± 0.11	0.18 ± 0.02	5.56 ± 0.21
BHA	2.82 ± 0.14	0.18 ± 0.02	5.56 ± 0.35
protocatechuic acid	2.26 ± 0.13	0.22 ± 0.02	4.55 ± 0.36
syringic acid	2.18 ± 0.12	0.23 ± 0.02	4.35 ± 0.21
L-ascorbic acid	2.01 ± 0.12	0.25 ± 0.03	4.00 ± 0.21
D-ascorbic acid	1.99 ± 0.16	0.25 ± 0.03	4.00 ± 0.25
Trolox	1.98 ± 0.12	0.25 ± 0.03	4.00 ± 0.24
ferulic acid	1.35 ± 0.09	0.37 ± 0.04	2.70 ± 0.10
p-hydroquinone	2.30 ± 0.11	0.38 ± 0.04	2.63 ± 0.19
vanillin	1.18 ± 0.06	0.42 ± 0.04	2.38 ± 0.21
L-cysteine	1.01 ± 0.05	0.49 ± 0.05	2.04 ± 0.12
phenol	0.95 ± 0.06	0.52 ± 0.05	1.92 ± 0.09
p-coumaric acid	0.66 ± 0.03	0.75 ± 0.08	1.33 ± 0.02
p-hydroxybenzoic acid	0.35 ± 0.02	1.42 ± 0.15	0.71 ± 0.01

Step 2. Choose the antioxidant concentration. The initial concentration of antioxidant should be such that a quantity of product (B) is generated, the evolution of which consumes a substantial quantity of free radical. Furthermore, the following must be fulfilled $n[A]_0 \ll [ABTS^{\bullet+}]_0$, so that free radical remains in excess. With regard to different antioxidants, the concentration of excess $ABTS^{\bullet+}$ must be the same.

Step 3. Select the initial concentration of free radical. The concentration of free radical ($[ABTS^{\bullet+}]_0$) generated must be greater than $n[A]_0$. For different antioxidants to be compared by the kinetic test, $[ABTS^{\bullet+}]_0 - n[A]_0 = [ABTS^{\bullet+}]$ should be equal in all cases; therefore, depending on the value of n, because $[A]_0$ is constant, $[ABTS^{\bullet+}]_0$ must be varied for each antioxidant studied.

Step 4. Generate the desired $[ABTS^{\bullet+}]_0$ value in the medium. Free radical is generated with the POD/H_2O_2/ABTS system. The H_2O_2 must be in defect with respect to ABTS and the stoichiometry should be $1H_2O_2$: $2ABTS^{\bullet+}$.

Step 5. React the free radical with the antioxidant. First, the desired concentration of $[ABTS^{\bullet+}]_0$ is generated, and then $[A]_0$ is added. The disappearance of free radical is measured spectrophotometrically.

Step 6. Analyze the progress curve by nonlinear fitting based on the initial fall (this corresponds to the primary antioxidant power) according to eq **4**. In this way, the parameters n_{app} and k_2^{app} are determined in the work conditions.

Step 7. Study the different antioxidants. Repeat steps 5 and 6 for each compound in the fixed experimental conditions, which are the same $[A]_0$ value and the same free radical in excess, that is, the same value of the difference $[ABTS^{\bullet+}]_0 - n[A]_0$.

RESULTS AND DISCUSSION

ABTS is a substrate of POD which, on oxidation, generates a quite stable radical cation, $ABTS^{\bullet+}$, a characteristic that has led to its use in kinetic studies of POD (*17*). In addition, we use ABTS to determine the total antioxidant status of foods, either generating it in situ with the POD/ABTS/H_2O_2 system and determining the lag period resulting from the presence of an antioxidant such as ascorbic acid (*8*) or applying a final point method, starting with a predetermined quantity of radical $ABTS^{\bullet+}$ (*18*). In both cases, at the working pH and with the compounds studied, the reaction was immediate and there was no subsequent evolution. However, two facts drew our attention: on the one hand, the appearance of a series of studies on the antioxidant capacity of polyphenols, together with the demonstration of the existence of slow chemical

Scheme 1

$$n\,ABTS^{\bullet+} + A \xrightarrow{k_1} n\,ABTS + B$$

reactions coupled to the process of initial oxidation (*15*, *20*−*22*), and, on the other, the consideration that many of these compounds are substrates of tyrosinase (catechins of green tea (*21*) or chlorogenic acid (*22*))). Taking these aspects into account, we consider that it is important to approach the characterization of the antioxidant capacity of polyphenols and related compounds using the POD/ABTS/H_2O_2 system.

Enzymatic Kinetic Method. The method is based on the accumulation of the radical ($ABTS^{\bullet+}$) generated by the POD/ABTS/H_2O_2 system according to

$$2ABTS + H_2O_2 \xrightarrow[V_0]{POD} 2H_2O + 2ABTS^{\bullet+} \qquad (1)$$

In these conditions, the system rapidly reaches the steady state with a velocity of V_0. If there is an antioxidant (A) in the medium, the free radical $ABTS^{\bullet+}$ could react with it according to **Scheme 1**. If the k_1 is high and the antioxidant concentration low, the velocity of accumulation of $ABTS^{\bullet+}$ returns to the steady state after a lag period to reach the same velocity V_0.

From eq **1** and **Scheme 1**, the balance of matter is

$$[ABTS^{\bullet+}] = V_0 t - n[A]_0 \qquad (2)$$

Figure 2A shows a series of assays of the activity of the POD/ABTS/H_2O_2 system in the presence of the antioxidant, Trolox. Recordings b−g of **Figure 2A** correspond to assays with increasing quantities of Trolox. Data analysis, according to eq **2**, gives the values of V_0 and τ, whereas the inset of **Figure 2A** confirms the linearity. From the slope the value of n, the stoichiometric factor, can be calculated. In this case, no chemical reactions coupled to the reaction of A with $ABTS^{\bullet+}$ exist, and so the parameters EC50 and the antioxidant capacity, ARP, can be calculated from the value of n (**Table 1**). The degree of parallelism between the straight lines is a test of the validity of the assay. **Figure 2B** shows the same experiments for L-ascorbic acid. **Table 1** shows a series of molecules characterized by this enzymatic method.

The following precautions should be taken in the application of the enzymatic kinetic method:

(a) Because the parallelism of the straight lines is the only way of validating the method, the velocity of the steady state must be the same, for which the substrates must vary as little as possible. In the case of ABTS, this is ensured because the radical $ABTS^{\bullet+}$ is reduced to ABTS. The other substrate, H_2O_2, can vary, but the POD/H_2O_2/ABTS system has the advantage that $K_m^{H_2O_2}$ is very small (*17*) and so the enzyme works at concentrations of H_2O_2 of 100 μM in zero-order kinetics. In this way, at low enzyme and antioxidant concentrations, curves like those shown in **Figure 2** are obtained.

(b) The antioxidant or its products are very powerful inhibitors of the enzyme, that is, they inhibit POD at very low concentrations; consequently, the parallelism shown in **Figure 2A** is not attained and the method cannot be applied.

(c) Due to possible competition of the antioxidant as substrate with the substrates of the enzyme, ABTS and H_2O_2, this situation is difficult because the enzyme is saturated by ABTS and H_2O_2. Moreover, using data from refs *17* and *23*, we can compare the pseudo-first-order rate constant of the attack of compound II on the ABTS and phenol. Bearing in mind that $k_5 = 7.82 \times 10^4$ 1/(M × s) and $k_5^* = 2.5 \times 10^5$ 1/(M × s) and because $[ABTS]_0 = 5 \times 10^{-3}$ M and $[phenol]_0 = 5 \times 10^{-6}$ M, the pseudo-first-order rate constant will be $k_5 \times [ABTS]_0$ and $k_5^* \times [phenol]_0$ for ABTS and phenol, respectively. The relation of the pseudo-first-order rate constants will be 200, so that the antioxidant probably does not interfere in these assays.

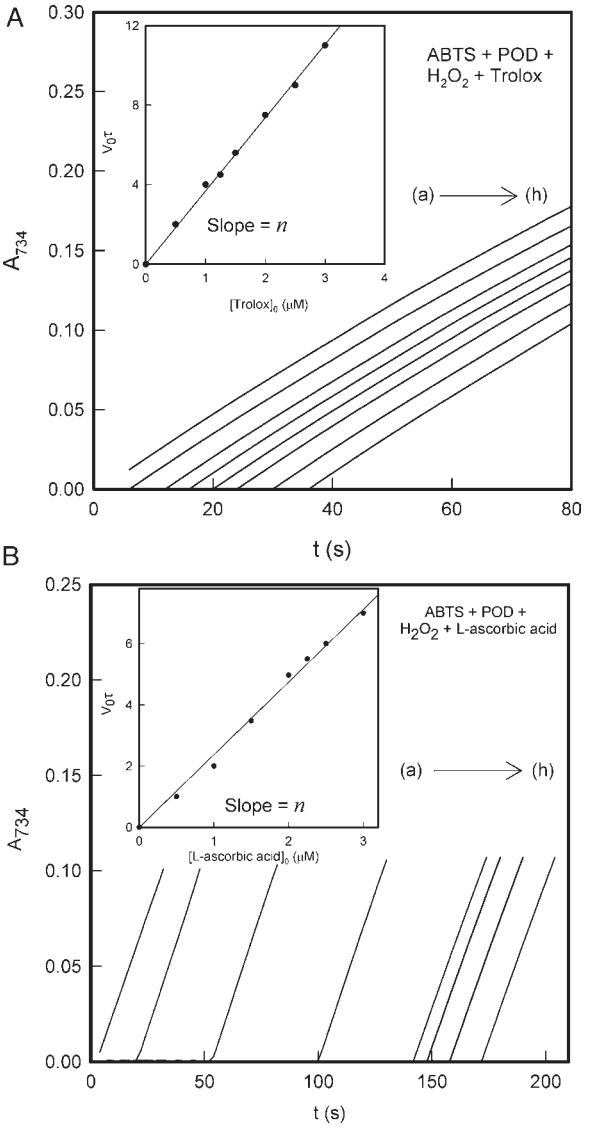


Figure 2. (**A**) Characterization of compounds with monophasic antioxidant activity. Time course of $ABTS^{\bullet+}$ accumulation in the presence of POD (0.25 nM), ABTS (5 mM). and H_2O_2 (0.1 mM) in sodium phosphate buffer 30 mM (pH 6.5), at 25 °C. The reaction was followed by measuring ΔA_{734}. Recording a, control; recordings b−h, time courses of the same reaction as in (a) but with 0.5, 1.0, 1.25, 1.5, 2.0, 2.5, and 3 μM Trolox added, respectively. (Inset) Representation of $V_0\tau$ versus $[Trolox]_0$. (**B**) Characterization of compounds with monophasic antioxidant activity. Time course of $ABTS^{\bullet+}$ accumulation in the presence of POD (0.25 nM), ABTS (5 mM), and H_2O_2 (0.1 mM) in sodium phosphate buffer 30 mM (pH 6.5), at 25 °C. The reaction was followed by measuring ΔA_{734}. Recording a, control; recordings b−h, time courses of the same reaction as in (a) but with 0.5, 1.0, 1.5, 2.0, 2.25, 2.5, and 3 μM L-ascorbic acid added, respectively. (Inset) Representation of $V_0\tau$ versus $[L\text{-ascorbic acid}]_0$.

(d) Deviations in the parallelism increase as the concentration of antioxidant in the medium increases. This is clear from **Figure 3A**, when quercetin acts as antioxidant, and indicates the secondary antioxidant capacity. Note that in this case the experiments at very low antioxidant concentrations fulfill the parallelism and, from the data analysis, a first estimation of the value of n, the stoichiometric factor, can be obtained **Figure 3A**, inset.

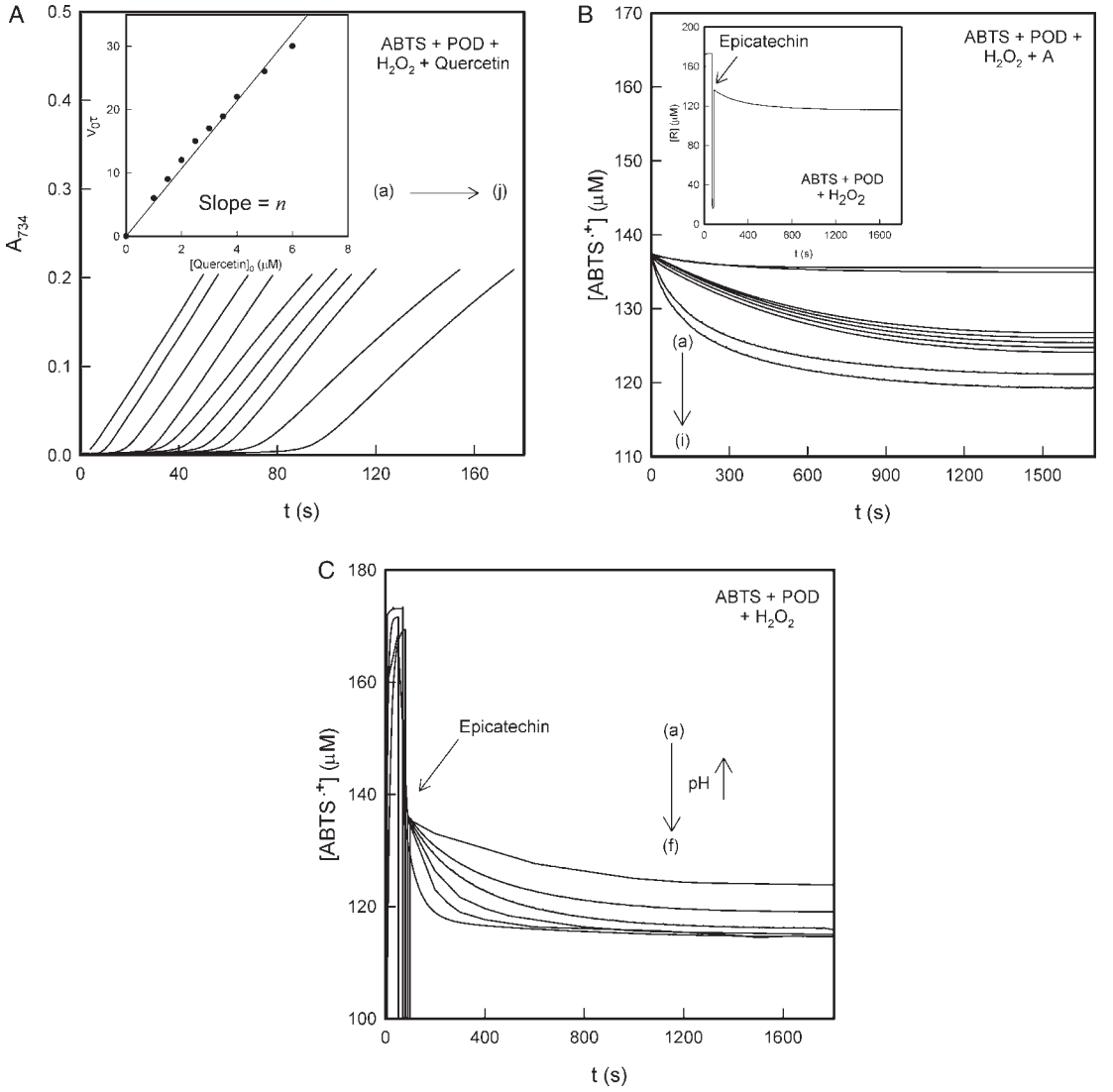
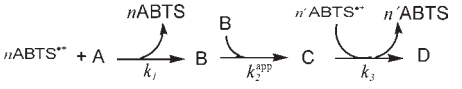
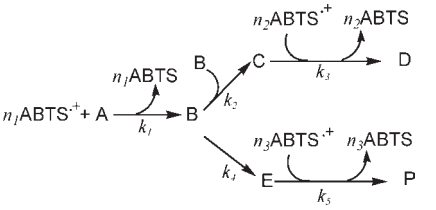


Figure 3. (**A**) Characterization of compounds with biphasic antioxidant activity: time course of $ABTS^{\bullet+}$ accumulation in the presence of POD (0.25 nM), ABTS (5 mM) and H_2O_2 (0.1 mM) in sodium phosphate buffer 30 mM (pH 6.5), at 25 °C. The reaction was followed by measuring ΔA_{734}. Recording a, control; recordings b−j, time courses of the same reaction as in (a) but with 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0 μM quercetin added, respectively. (Inset) Representation of $V_0\tau$ versus $[quercetin]_0$. (**B**) Characterization of compounds with biphasic antioxidant activity: nonenzymatic kinetic test to calculate n_{app} and k_2^{app}. The buffer used was 30 mM sodium phosphate buffer (pH 7.0), at 25 °C. The radical $ABTS^{\bullet+}$ was generated with the POD/ABTS/H_2O_2 system. The concentration of POD was 0.3 nM, $[ABTS]_0$ = 5 mM, and the $[H_2O_2]_0$ values (μM) were (a) rutin, 71; (b) eugenol, 64; (c) chlorogenic acid, 67; (d) quercetin, 73; (e) guaiacol, 65; (f) ellagic acid, 75; (g) caffeic acid, 63; (h) catechin, 68; and (i) epicatechin, 70. At t = 0, a 5 μM concentration of antioxidant was added and the changes in absorbance at 734 nm were recorded from the initial fall. (Inset) Recording of the change in absorbance at 734 nm from the beginning of the reaction in the case of epicatechin. (**C**) Characterization of compounds with biphasic antioxidant activity: study of the pH effect on epicatechin antioxidant capability. The buffers used were 30 mM sodium phosphate buffer pH (a) 6.25, (b) 6.5, (c) 6.75, (d) 7.0, (e) 7.25, and (f) 7.5. The concentrations (μM) of epicatechin and $ABTS^{\bullet+}$ were 5 and 150, respectively. The radical $ABTS^{\bullet+}$ was generated with the POD/ABTS/H_2O_2 system. The concentrations of POD and H_2O_2 were 0.3 nM and 75 μM, respectively. At t = 0, the antioxidant epicatechin was added at 5 μM. The reaction was recorded at 734 nm from the initial fall.

Scheme 2



Scheme 3



(e) The fact that the behavior of an antioxidant fits the enzymatic kinetic method does not mean that no chemical reactions exist subsequent to the formation of antioxidant radical, but that, if they do exist, they must be very rapid (see **Schemes 2** and **3**), the system returning in little time to the steady state (**Figure 2**).

The compounds described in **Table 1** fit the enzymatic kinetic method very well and should therefore reflect their antioxidant capacity because of their chemical structure. Such is the case with many of them, that is, the n obtained experimentally corresponds to what might be expected from their structure; for example, in the case of *p*-hydroquinone, protocatechuic acid, L-ascorbic acid,

D-ascorbic acid, L-cysteine, or phenol. Other compounds, such as *p*-hydroxybenzoic acid or *p*-coumaric acid, have much lower values also due to their chemical structures, in which withdrawing groups in para position diminish the capacity to be oxidized to the corresponding phenoxyl radicals. Finally, there are compounds that show high *n* values and therefore high ARP values (**Table 1**). For example, it has been proposed that the main oxidation mechanism for catechol involves donation of a single electron to the radical cation, resulting in the formation of a semiquinone, which can donate a further electron to form the quinone. In turn, this can suffer nucleophilic attack by the OH group, followed by a new oxidation before being polymerized (*24*); pyrogallol shows good antioxidant capacity but has also been used to form superoxide anions, meaning that it also acts as a pro-oxidant (*25*); gallic acid (as seen by NMR), when it reacts with DPPH radical, rapidly forms dimers from galloquinones, which explains its higher *n* value of 3 (*25*); in the case of ferulic, syringic, and sinapinic acids, which have been described as having a higher antioxidant capacity than *p*-coumaric acid, the presence of methoxyl groups in the ortho position means that the OH groups are more easily oxidizable and that they also stabilize the phenoxyl radical, which may react with another molecule of $ABTS^{\bullet+}$, explaining why the *n* value would be greater than 1 (*24*); BHA contains a methoxy group in the para position with respect to the OH group and a *tert*-butyl group on the ortho position; this renders it easily oxidizable, perhaps acting through a mechanism similar to that described for ferulic acid, and as may occur in Trolox due to the effect of the methyl groups. The interpretation of these results (compounds with a higher than expected value of *n*) should consider the possibility of rapid coupled chemical reactions (i.e., **Schemes 2** and **3**), in which free radical is consumed after an initial reaction of the compound with the free radical. This suggests that the chemical structure of a compound provides only an approximate idea of its antioxidant capacity toward a given free radical, as is the case with the oxidation/reduction potentials that, as has been seen, do not exactly correlate with the antioxidant capacity (*11*).

When an antioxidant is studied by the enzymatic kinetic method and deviations are observed such as those in **Figure 3A**, the compound has secondary antioxidant capacity and a nonenzymatic kinetic test is proposed, the basis of which consists of making the radical $ABTS^{\bullet+}$ be in excess with regard to the concentration of the antioxidant used and of following the decrease in absorbance of the free radical with time. This decrease in absorbance will have two phases: first, a very rapid one corresponding to the antioxidant reacting with the free radical (primary antioxidant capacity) and reflecting the *n* value, followed by coupled chemical reactions of the type indicated in **Schemes 2** and **3** (second order) as the radical continues reacting with the products until the reaction is exhausted.

The literature describes slow dimerization reactions during the reaction of the products of the antioxidant with the free radical (*11*−*13*). In our experiments with compounds showing a secondary antioxidant capacity, we demonstrated the second-order dependence of the disappearance of free radical mainly. However, the kinetics is complex, and only if the simplification of **Scheme 2** is accepted can an analytical expression be deduced to fit the experimental data. Although offering only apparent parameters and constants, this fit can be used for comparative purposes.

If the reactions of radical $ABTS^{\bullet+}$ with A and C are rapid, so that $k_2^{app} \ll k_1, k_3$, **Scheme 2** is kinetically equivalent to

$$2B \xrightarrow{k_2^{app}} D \qquad (3)$$

It can be demonstrated that the expression of the variation of free radical with time corresponds to the expression

$$[ABTS^{\bullet+}] = [ABTS^{\bullet+}]_\infty + \frac{ab}{b+t} \qquad (4)$$

with $a = n_{app}[A]_0$ and $b = 1/(k_2^{app}[A]_0)$.

A fit of the experimental $[ABTS^{\bullet+}]$ values to eq **4** gives $[ABTS^{\bullet+}]_\infty$, *a*, and *b*, which enables n_{app} and k_2^{app} (second-order rate constant) to be calculated. The velocity of the process is governed by k_2^{app}, but, in this case, being a second-order reaction (limiting), the $t_{1/2}^{app}$ can be defined, after reaction of the radical with the antioxidant, as the time necessary for the free radical (in excess) to fall to 50% of its total fall, at a fixed initial antioxidant concentration. The parameter $t_{1/2}^{app}$ depends on the concentration of antioxidant, according to $t_{1/2}^{app} = 1/(k_2^{app}[A]_0)$. Therefore, to characterize an antioxidant of this nature, the experiments to calculate the time factor should not be carried out with the particular EC_T50 value of each molecule as suggested in refs *15* and *16*, but at the same concentration of A, so that $t_{1/2}^{app} = 1/(k_2^{app} \times [A]_0)$ is a comparative parameter.

From the values obtained for n_{app} and k_2^{app}, the apparent kinetic antiradical efficiency (KAE_{app}) can be defined as $KAE_{app} = 1/(EC_T50_{app} \times t_{1/2}^{app})$, which includes both effects (concentration and time). Note that to compare the antioxidant capacity of different compounds in their reaction with the radical $ABTS^{\bullet+}$, the most interesting parameters for the primary antioxidant capacity and for the total antioxidant capacity are ARP_p and KAE_{app}, respectively.

The expression for the new parameter proposed in this study, the apparent kinetic antiradical efficiency (KAE_{app}), takes the form

$$KAE_{app} = \frac{1}{EC_T50_{app} \times t_{1/2}^{app}} = 2(n + n_{app})k_2^{app}[A]_0 \qquad (5)$$

This is equivalent to the number of electrons transferred per second by a 1 M antioxidant solution.

If the kinetic study points to second-order kinetic regardless of the concentration of free radical, analysis of the data provides the absolute values of n_{app} and k_2^{app}, which permits the real secondary antioxidant power to be characterized. In our case, in all of the compounds shown in **Table 2** there is a kinetic dependence on the concentration of free radical and, therefore, only the nonenzymatic kinetic test can be used to compare the different molecules, for which we propose the experimental design described under Material and Methods.

The enzymatic kinetic method was applied to the compounds shown in **Tables 1** and **2**. Those in **Table 2** deviate from linearity, as shown in **Figure 3A** for quercetin, meaning that they present a secondary antioxidant capacity. The nonenzymatic kinetic test was applied to this group of compounds, using the experimental conditions specified in **Figure 3B**. The same figure shows the spectrophotometric recordings for the disappearance of free radical with time. Fitting, by nonlinear regression, of these curves to eq **4** gives the n_{app} and k_2^{app} (see **Table 2**). From the values obtained for n_{app} with respect to the secondary antioxidant, the parameters EC_s50_{app} and ARP_s^{app}, corresponding to the secondary effective concentration and secondary antiradical power, can be calculated by comparison with the primary antioxidant capacity and, by extension, the total antioxidant capability in these assay conditions can be characterized (see **Table 2**).

The data shown in **Table 2** for the primary and secondary antioxidant capacities are better reflected in the new parameter we propose in this study, the apparent antiradical kinetic efficiency (KAE_{app}), which includes the information referring to the

Table 2. Characterization of Compounds with Biphasic Antioxidant Capacity

	primary antioxidant capacity			secondary antioxidant capacity			total antioxidant capacity				
substrate	n (electrons)	EC_p50	ARP_p	n_{app} (electrons)	EC_s50_{app}	ARP_s^{app}	$n + n_{app}$ (electrons)	EC_T50_{app}	ARP_T^{app}	k_2^{app} (M^{-1} s^{-1})	$KAE_{app} \times 10^{-4}$ (electrons/s)[a]
epicatechin	5.2 ± 0.5	0.09 ± 0.01	11.1 ± 0.4	7.2 ± 0.8	0.07 ± 0.01	14.4 ± 0.7	12.3 ± 1.3	0.04 ± 0.01	24.6 ± 1.3	2089 ± 27	5.1 ± 0.4
catechin	4.7 ± 0.4	0.11 ± 0.01	9.1 ± 0.1	6.9 ± 0.8	0.07 ± 0.01	14.3 ± 0.8	11.6 ± 1.2	0.04 ± 0.01	25.0 ± 1.2	2025 ± 25	4.7 ± 0.5
quercetin	6.4 ± 0.5	0.08 ± 0.01	12.5 ± 0.9	5.6 ± 0.6	0.09 ± 0.01	11.1 ± 0.7	12.0 ± 1.1	0.04 ± 0.01	25.0 ± 1.5	1907 ± 21	4.6 ± 0.8
ellagic acid	7.6 ± 0.7	0.07 ± 0.01	14.3 ± 0.6	6.2 ± 0.7	0.08 ± 0.01	12.5 ± 0.7	13.8 ± 1.4	0.03 ± 0.01	33.3 ± 1.9	1348 ± 15	3.7 ± 0.5
guaiacol	3.1 ± 0.3	0.16 ± 0.01	6.3 ± 0.4	5.9 ± 0.6	0.08 ± 0.01	12.5 ± 0.6	9.0 ± 0.9	0.05 ± 0.01	20.0 ± 1.1	1997 ± 20	3.6 ± 0.4
chlorogenic acid	4.1 ± 0.3	0.12 ± 0.01	8.3 ± 0.4	5.3 ± 0.6	0.09 ± 0.01	11.1 ± 0.6	9.4 ± 0.9	0.05 ± 0.01	20.0 ± 1.2	1857 ± 29	3.5 ± 0.6
caffeic acid	2.5 ± 0.2	0.19 ± 0.02	5.3 ± 0.2	7.0 ± 0.7	0.07 ± 0.01	14.3 ± 0.8	9.5 ± 0.9	0.05 ± 0.01	20.0 ± 1.0	1359 ± 14	2.6 ± 0.3
rutin	5.6 ± 0.5	0.09 ± 0.01	11.1 ± 0.7	1.4 ± 0.2	0.35 ± 0.03	2.8 ± 0.1	7.0 ± 0.7	0.07 ± 0.01	14.3 ± 0.9	1150 ± 17	1.7 ± 0.3
eugenol	2.8 ± 0.2	0.17 ± 0.01	5.9 ± 0.1	1.5 ± 0.2	0.33 ± 0.03	3.03 ± 0.1	4.3 ± 0.4	0.11 ± 0.01	9.1 ± 0.6	1100 ± 19	0.9 ± 0.2

[a] For a 1 M antioxidant solution.

Table 3. Effect of pH on the Biphasic Antioxidant Capacity for Epicatechin

	primary antioxidant capacity			secondary antioxidant capacity			total antioxidant capacity				
pH	n (electrons)	EC_p50	ARP_p	n_{app} (electrons)	EC_s50_{app}	ARP_s^{app}	$n + n_{app}$ (electrons)	EC_T50_{app}	ARP_T^{app}	k_2^{app} (M^{-1} s^{-1})	$KAE_{app} \times 10^{-4}$ (electrons/s)[a]
6.25	5.2 ± 0.5	0.09 ± 0.01	11.1 ± 0.4	6.899 ± 0.324	0.07 ± 0.01	13.80 ± 0.97	12.10 ± 1.19	0.04 ± 0.01	24.20 ± 1.98	229 ± 11	5.52 ± 0.65
6.50	5.2 ± 0.5	0.09 ± 0.01	11.1 ± 0.4	7.082 ± 0.393	0.07 ± 0.01	14.16 ± 0.89	12.28 ± 1.02	0.04 ± 0.01	24.56 ± 1.58	709 ± 12	17.41 ± 1.84
6.75	5.2 ± 0.5	0.09 ± 0.01	11.1 ± 0.4	7.105 ± 0.593	0.08 ± 0.01	14.21 ± 0.89	12.30 ± 0.99	0.04 ± 0.01	24.60 ± 1.38	1910 ± 61	46.71 ± 4.10
7.00	5.1 ± 0.5	0.09 ± 0.01	11.1 ± 0.4	7.223 ± 0.810	0.07 ± 0.01	14.41 ± 0.71	12.31 ± 1.30	0.04 ± 0.01	24.62 ± 1.33	2089 ± 27	51.00 ± 4.03
7.25	5.2 ± 0.5	0.09 ± 0.01	11.1 ± 0.4	7.190 ± 0.726	0.07 ± 0.01	14.28 ± 1.12	12.39 ± 1.26	0.04 ± 0.01	24.68 ± 1.87	2250 ± 14	55.72 ± 4.58
7.50	5.2 ± 0.5	0.09 ± 0.01	11.1 ± 0.4	7.651 ± 0.798	0.06 ± 0.01	16.67 ± 0.87	12.85 ± 1.98	0.04 ± 0.01	25.70 ± 1.99	2502 ± 17	64.32 ± 6.99

[a] For a 1 M antioxidant solution.

effective concentration and the time the process takes, but through $t_{1/2}^{app}$ (see **Table 2**).

In the case of caffeic and chlorogenic acids, dimerizations (*12*, *13*) have been proposed, whereas dimerizations and reducing nucleophilic attacks have been proposed in the case of catechin and epicatechin (*26*), although in the latter cases the primary antioxidant capacity corresponds to their chemical structure. Furthermore, because the k_2^{app} is high, the KAE_{app} value is the highest of all those studied in this work, meaning that epicatechin is the strongest antioxidant. In the case of quercetin, several types of reactions have been described (*27*); for example, depending on the type of oxidant, quercetin has been demonstrated to originate different products, including heterodimers, which would explain the high number of electrons (*27*). For the polyphenol rutin, a slightly lower result than for quercetin is to be expected, as can be seen from the value of n described in **Table 2**, although the subsequent polymerization is presumably less favored because of the glycoside chain (*26*). The case of guaiacol and eugenol is of note because of the high n value, because they are monophenols, but both give rise to coupled reactions that increase their antioxidant capacity. With regard to guaiacol, tetraguaiacol is formed during its oxidation. Finally, the high antioxidant capacity shown by ellagic acid can be attributed to rapid dimerization reactions (*28*).

Considering the data of **Table 2** and in light of this new parameter, it can be appreciated how ellagic acid, which shows higher electron consumption, passes to fourth place, because its k_2^{app} is the lowest of all. However, although the number of electrons consumed is lower, catechin passes to second place because of its high k_2^{app}.

For the test to be comparable for different antioxidants, the initial concentration of free radical should be varied so that the difference between this initial value and that consumed in the primary antioxidant capacity by the reaction with the antioxidant remains constant. From this kinetic study, the apparent second-order rate constant k_2^{app} can be calculated along with the number of electrons consumed in this secondary antioxidant capacity n_{app} in the study conditions used. This additional information permits us to define a new parameter, the apparent kinetic antiradical efficiency (KAE_{app}), which provides information on the effective concentration (EC_T50_{app}) and $t_{1/2}^{app}$. Once the radical has reacted with the antioxidant, this $t_{1/2}^{app}$ may be defined as the time necessary for the free radical in excess to fall to 50% of its total decline, at a fixed initial antioxidant concentration. This new parameter serves to compare different antioxidants if the kinetic conditions described above are fulfilled.

Study of the pH Effect on the Antioxidant Capacity. The literature describes how the antioxidant capacity of different molecules varies with the pH (*11*). In this work, we have studied the effect of pH on the antioxidant capacity of epicatechin. The experimental recordings of the disappearance of free radical are shown in **Figure 3C**. Note that the primary antioxidant capacity does not vary (see **Table 3**) and that at pH < 5 the chemical evolution of the coupled reactions is very slow (data not shown). However, the secondary antioxidant capacity increases as the pH (pH values of 6.25−7.5) of the medium increases due to the increasingly strong coupled chemical reactions, which lead to an increasing k_2^{app} (the apparent second-order rate constant that governs the process). This means that the KAE_{app} increases with pH (**Table 3**). For comparison purposes, the antioxidant capacities of the different compounds should be studied at the same pH value.

On the other hand, the formal redox potential of $ABTS^{\bullet+}$/ABTS is 0.490 V (*29*), whereas for $DPPH^{\bullet}$/DPPH it is 0.340 V (*30*), when both redox pairs are studied in a glassy carbon electrode and are expressed versus Ag/AgCl reference electrode (pH 7.0), meaning that the former system is applicable to a much greater number of antioxidants. **Figure 4** shows the greater power of $ABTS^{\bullet+}$ because the number of electrons transferred is slightly higher with the series of molecules studied.

In conclusion, this study proposes a kinetic method for characterizing different antioxidants and a nonenzymatic kinetic

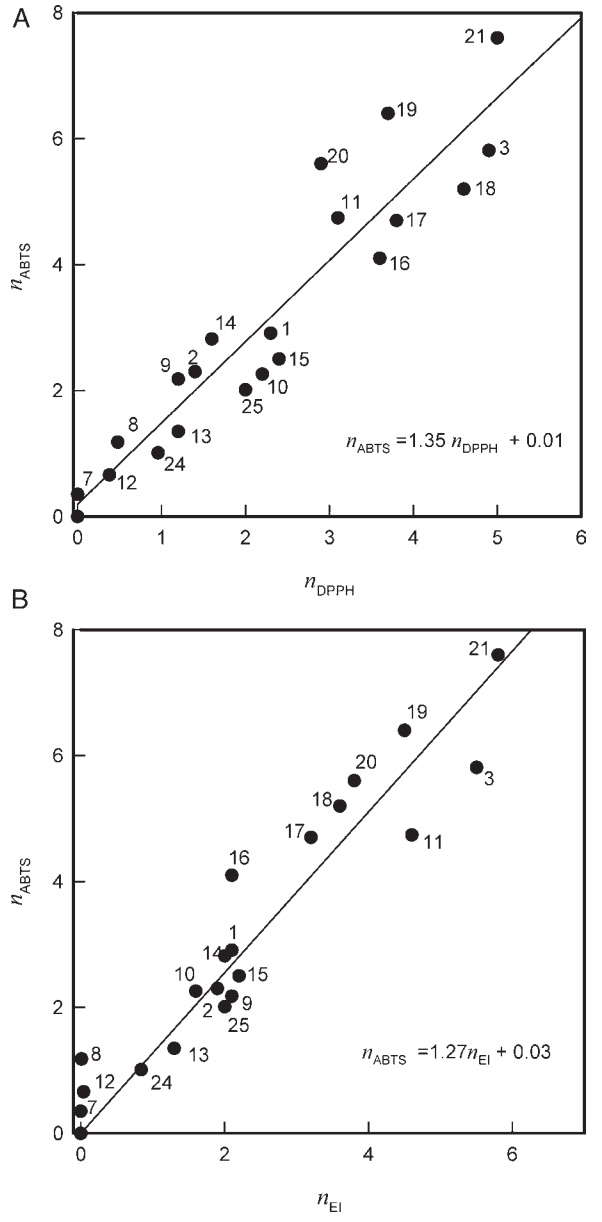


Figure 4. (**A**) Correlation of the number of electrons obtained by the ABTS method (n_{ABTS}) and by the DPPH method (n_{DPPH}), using data from ref *13*. (**B**) Correlation of the number of electrons yielded by different antioxidants obtained by the ABTS method (n_{ABTS}) with the number determined by flow-column electrolysis (n_{EI}) at a slower flow rate (0.05 mL min^{-1}), using data from ref *22*. The molecules were (1) catechol, (2) *p*-hydroxyquinone, (3) pyrogallol, (7) *p*-hydroxybencoic acid, (8) vanillin, (9) syringic acid, (10) protocatechuic acid, (11) gallic acid, (12) *p*-coumaric acid, (13) ferulic acid, (14) sinapinic acid, (15) caffeic acid, (16) chlorogenic acid, (17) catechin, (18) epicatechin, (19) quercetin, (20) rutin, (21) ellagic acid, (24) L-cysteine, and (25) L-ascorbic acid.

test. The enzymatic method uses POD, ABTS, and H_2O_2 to generate the radical ABTS$^{\bullet+}$ in situ. The kinetic test uses the ABTS$^{\bullet+}$ previously generated with POD. The combination of both can be used to characterize and compare the action of a series of antioxidants. We introduce the concept of primary antioxidant power (at short times) and secondary antioxidant power (at longer times) through the values of the number of electrons n and n_{app} for each process and the values EC_p50 and ARP_p for the first process and of EC_s50_{app} and ARP_s^{app} for the second process. From these values, the total values of the process, EC_T50_{app} and ARP_T^{app}, can be obtained. An experimental design is presented, which enables the value of the apparent second-order rate constant of the limiting step (k_2^{app}) to be obtained. Finally, all of the information obtained gives a new parameter, which we denominate the apparent kinetic antioxidant efficiency (KAE_{app}), which is the inverse of the product of the EC_T50_{app} and $t_{1/2}^{app}$ of the reaction and related directly with the total efficient concentration and time that the process takes. The parameter KAE_{app} is useful for comparing antioxidants.

ABBREVIATIONS USED

Species and Concentrations: ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); ABTS$^{\bullet+}$, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation; Phe-OH, phenolic compound; [ABTS$^{\bullet+}$], instantaneous concentration of ABTS$^{\bullet+}$; [ABTS$^{\bullet+}$]$_0$, initial concentration of ABTS$^{\bullet+}$; [ABTS$^{\bullet+}$]$_\infty$, remaining concentration of ABTS$^{\bullet+}$ at $t \rightarrow \infty$; A, antioxidant; [A]$_0$, initial concentration of A; B, product of the reaction of ABTS$^{\bullet+}$ with A; [B], instantaneous concentration of B; C, product of the reaction of B + B (second order); [C], instantaneous concentration of C; D, product of the reaction of C with ABTS$^{\bullet+}$; [D], instantaneous concentration of D; E, product of the evolution of B (first order); [E], instantaneous concentration of E; P, product of the reaction of E with ABTS$^{\bullet+}$; [P], instantaneous concentration of P.

Parameters and Kinetic Constants: n, number of electrons yielded by the antioxidant, which coincides with the number of radical molecules consumed by electron-donating action of one molecule of A; n', number of electrons yielded by the antioxidant, which coincides with the number of radical molecules consumed by electron-donating action by one molecule of C in the assay conditions; n_{app}, number of electrons yielded by the antioxidant, which coincides with the number of radical molecules consumed by electron-donating action during the evolution of one molecule of the product of the reaction of ABTS$^{\bullet+}$ with A or its evolution in the assay conditions used; τ, lag period; EC50, effective concentration, EC50 = 1/(2n), and is defined as the ratio between the concentration of antioxidant that decreases the concentration of radical by 50%; ARP, antioxidant or antiradical power, ARP = 2n; EC_p50, primary effective concentration; ARP_p, primary antiradical power; EC_s50_{app}, apparent secondary effective concentration; ARP_s^{app}, apparent secondary antiradical power; EC_T50_{app}, apparent total effective concentration; ARP_T^{app}, apparent total antiradical power; $n + n_{app}$, total number of electrons yielded by the antioxidant, which coincides with the number of radical molecules consumed by electron-donating action of one molecule of A, during its evolution in the kinetic conditions described in the assay conditions used; k_2^{app}, apparent rate constant of the limiting step of the coupled chemical reactions; $t_{1/2}^{app}$, half-life of the reaction, $t_{1/2}^{app} = 1/(k_2^{app}[A]_0)$; k_d^{app}, apparent decomposition constant of the radical ABTS$^{\bullet+}$; KAE_{app}, apparent kinetic antiradical efficiency, $KAE_{app} = 1/(EC_T50_{app}\ t_{1/2}^{app})$; T_{EC50}, time needed to reach steady state at the concentration corresponding to EC50; AE, antiradical efficiency, AE = 1/(EC50 × T_{EC50}).

Supporting Information Available: Kinetic analysis of the enzymatic kinetic method and description of the nonenzymatic kinetic test. This material is available free of charge via the Internet at http://pubs.acs.org.

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