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Reduction Kinetics of the Antiradical Probe 2,2-Diphenyl-1-picrylhydrazyl in Methanol and Acetonitrile by the Antiradical Activity of Protocatechuic Acid and Protocatechuic Acid Methyl Ester

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This work evaluates the reduction kinetics of the antiradical probe 2,2-diphenyl-1-picrylhydrazyl (DPPH$^\bullet$) in methanol and acetonitrile by the antiradical activity of protocatechuic acid (3,4-dihydroxybenzoic acid, **1**) and protocatechuic acid methyl ester (**2**). The reduction kinetics of DPPH$^\bullet$ in both solvents by the antiradical activity of the *p*-catechol group in **2** is regular, that is, coincide with the proposed standard kinetic model for the reduction kinetics of DPPH$^\bullet$ by the antiradical activity of an isolated *p*-catechol group. Therefore, the antiradical activity of **2** experimentally exhibits two rate−two stoichiometric constants in acetonitrile and three rate−three stoichiometric constants in methanol. In contrast, the reduction kinetics of DPPH$^\bullet$ in both solvents by the antiradical activity of the *p*-catechol group in **1** is perturbed, that is, deviate from the proposed standard kinetic model. The deviations arise from the presence of the reactive carboxylic acid function which, in methanol, induces an additional reversible side reaction and, in acetonitrile, turns an irreversible reaction reversible, thus modifying the otherwise regular reduction kinetics of DPPH$^\bullet$ by the antiradical activity of the *p*-catechol group in **1**. On the other hand, the approximated theoretical kinetic equation that applies for those *p*-catechol groups whose reduction kinetics is regular and that experimentally exhibit three rate−three stoichiometric constants has been derived and used for fitting.

KEYWORDS: Antiradical activity; reduction kinetics; *p*-catechol; protocatechuic acid; protocatechuic acid methyl ester; DPPH$^\bullet$

INTRODUCTION

Protocatechuic acid (3,4-dihydroxybenzoic acid) and its esters, as well as other families of naturally occurring compounds, such as ascorbic acid, flavonoids, chalcones, carotenoids, and anthocyanidins, are known to exhibit antiradical activity (*1*–*5*). To possess this activity is important, since there is much experimental evidence suggesting that most of these compounds are also bioactive against different free radical mediated diseases, such as cancer, cardiovascular diseases, diabetes, as well as premature body aging (*6, 7*). Hence, it is thought that the intake of antiradicals present in food could be an important health-protecting factor (*8*).

Rather than the full chemical structure of a given compound, its antiradical activity is due to a reduced number of "active antiradical groups" contained within this structure. Among these groups, the vinyl alcohol and the *p*-catechol (3,4-dihydroxybenzene) groups are recognized to exhibit intense antiradical activity (*9*). Moreover, the conjugation of active antiradical groups within a chemical structure results in a new extended antiradical group with enhanced activity (*10*).

The *p*-catechol, either isolated or conjugated with another antiradical group, is probably the most ubiquitous natural antiradical group, since it can be found in almost all families of naturally occurring compounds, such as flavonoids, chalcones, phenolic acids, and anthocyanins, exhibiting this activity. When using DPPH$^\bullet$ as the target probe, the antiradical activity of the *p*-catechol group also depends on the structural characteristics of the carrying molecule, while the corresponding reduction kinetics can be modified by the presence of reactive chemical groups lacking this activity (i.e., carboxylic acid function).

Saito et al. (*11*) studied the effect of alcoholic and nonalcoholic solvents on the antiradical activity of protocatechuic acid and its alkyl esters, using DPPH$^\bullet$ as the antiradical probe. The main results indicated that the scavenging activity (total stoichiometric constant, σ_t) of the alkyl esters was significantly greater in alcoholic solvents (i.e., methanol, $\sigma_t > 4$) than in nonalcoholic solvents (i.e., acetonitrile, $\sigma_t \approx 2$), whereas the scavenging activity of the acid, at the assayed reaction time (≈30 min), was almost the same in alcoholic as in nonalcoholic solvents ($\sigma_t \approx 2$). The enhanced scavenging activity of the alkyl

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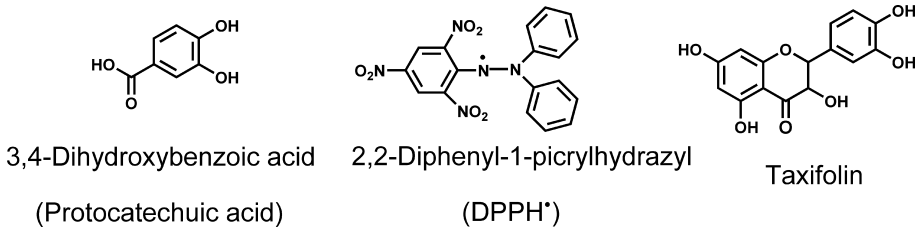

Figure 1. Chemical structures of the experimental and referenced compounds.

esters in alcoholic solvents, when compared to that in nonalcoholic solvents, was convincingly explained by Saito et al. (*12*) and is due to an adduct formation between an intermediate *o*-quinone and the alcoholic solvent. This reaction leads to regeneration of the *p*-catechol group and allows the reaction to go on. Concerning the decreased scavenging activity of protocatechuic acid in methanol when compared to those of its alkyl esters, Saito and Kawabata (*13*) studied the difference in scavenging activity between additional related phenolic acids and their esters. Results indicated that the scavenging activities of all the assayed acid/ester pairs were similar to that of the protocatechuic acid/protocatechuic acid methyl ester pair (pair **1/2**), that is, after a relatively short reaction time (≈30 min) the total stoichiometric constant of the acid slows down when compared to that of the corresponding ester, but as the reaction proceeds (at time >350 min), their values become almost identical. From these results, it was suggested that the relatively strong carboxylic acid function in the intermediate *o*-quinone dissociates to a carboxylate ion, thus lowering the susceptibility of the *o*-quinone to the nucleophilic attack by the alcoholic solvent.

Sendra et al. (*14*) studied the reduction kinetics of DPPH• in alcoholic and nonalcoholic solvents by the antiradical activity of the isolated *p*-catechol group in flavanone type structures. From the results, a standard kinetic model for the reaction of reduction of DPPH• by an isolated *p*-catechol group was proposed. Moreover, an approximated theoretical kinetic equation that applies for those *p*-catechol groups that follow the standard kinetic model, that is, with regular reduction kinetics, and that experimentally exhibit two rate−two stoichiometric constants was derived from the kinetic model. The use of this kinetic equation to fit the experimental data points from the assayed *p*-catechols allowed the determination of their corresponding rate and stoichiometric constants.

This work evaluates the reduction kinetics of DPPH• in methanol and acetonitrile by the antiradical activity of protocatechuic acid (**1**) and protocatechuic acid methyl ester (**2**). Results indicate that in both solvents the reduction kinetics of DPPH• by **1** and **2** is different. The reduction kinetics of DPPH• in both solvents by **2** is regular, that is, it coincides with the standard kinetic model. As a consequence, the antiradical activity of **2** experimentally exhibits two rate−two stoichiometric constants in acetonitrile and three rate−three stoichiometric constants in methanol, which can be determined by fitting using the corresponding approximated theoretical kinetic equation. In contrast, the reduction kinetics of DPPH• in both solvents by **1** is not regular, that is, it deviates from the standard kinetic model. As a consequence and since the corresponding theoretical kinetic equations are not known, the rate and stoichiometric constants cannot be determined by fitting. The deviations from the standard kinetic model arise from the presence of the reactive carboxylic acid function. This function induces an additional reversible side reaction in methanol and turns an irreversible reaction reversible in acetonitrile, thus modifying both the otherwise standard reduction kinetics of DPPH• by the *p*-catechol group in **1** and the time evolution of its antiradical activity.

MATERIALS AND METHODS

Reagents and Standards. Spectrophotometric grade methanol and acetonitrile as well as 3,4-dihydroxybenzoic acid (protocatechuic acid) were from Sigma (Sigma-Aldrich Co., St. Louis, MO). 3,4-Dihydroxybenzoic acid methyl ester (protocatechuic acid methyl ester) was from Chemos (Chemos GmbH, Regenstauf, Germany). 2,2-Diphenyl-1-picrylhydrazyl (DPPH•, 94.6% purity) was from Fluka (Fluka AG Chemische, Buchs, Switzerland). Anhydrous sodium sulfate was from Panreac (Panreac Química S.A., Barcelona, Spain).

Determination of the Antiradical Activity. The antiradical activity was determined according to the methodology described by Sendra et al. (*14*) as follows.

Sample Preparation. The solvent to be used (methanol or acetonitrile) was dried overnight over anhydrous sodium sulfate, and the working solutions of the antiradical and DPPH• were freshly prepared before analysis. A volume of the antiradical solution (between 5 and 15 μL) was added in situ, using a chromatographic syringe, into a thermostated (24 °C) and stirred (600 rpm) quartz spectrophotometric cuvette (3.5 mL of capacity and 1 cm path length) containing an appropriate volume of DPPH• to yield a final volume of 2 mL (the final concentration of DPPH• was around 100 μmol/L), and the spectrophotometric cuvette was immediately end-capped again. The analysis time commenced with the addition of the antiradical. As a general rule, those samples yielding an asymptotic value of the DPPH• concentration <10% or >90% of its initial concentration were discarded.

UV−Vis Analysis. Absorbance was measured using a model 8453 UV−vis spectrophotometer (Agilent Technologies GmbH, Karlsruhe, Germany) equipped with a diode array detector and a thermostated cell holder with magnetic stirring. Operating conditions were as follows: vis lamp, on; UV lamp, off; wavelength, 515 nm; slit width, 1 nm; and data acquisition rate, 2.1 s/data point in all cases excepting for protocatechuic acid in methanol for which it was 10 s/data point. Automatic acquisition of data was stopped after a reaction time of 40−360 min, depending on the speediness of the kinetics. All samples were analyzed in duplicate.

Prior to the experiments on antiradical activity, a calibration curve of absorbance versus concentration of DPPH• in both methanol and acetonitrile was obtained to determine the molar extinction coefficient (ε) of DPPH•. From the linear fitting of data, the values determined for ε were as follows: methanol, 1.18×10^4 L/(mol cm); acetonitrile, 1.15×10^4 L/(mol cm).

RESULTS AND DISCUSSION

Figure 1 shows the chemical structures of protocatechuic acid, DPPH• and taxifolin.

Antiradical Activity of Protocatechuic Acid Methyl Ester in Acetonitrile. Figure 2 shows the time evolution of the concentration of DPPH• in acetonitrile during its reduction by three different initial concentrations of **2**, as well as an inset showing the first minute of the reaction. Since the shape of the reduction curves was in all similar to that obtained from the reduction of DPPH• in acetonitrile by taxifolin (*14*), it was provisionally assumed that the reduction kinetics of DPPH• by

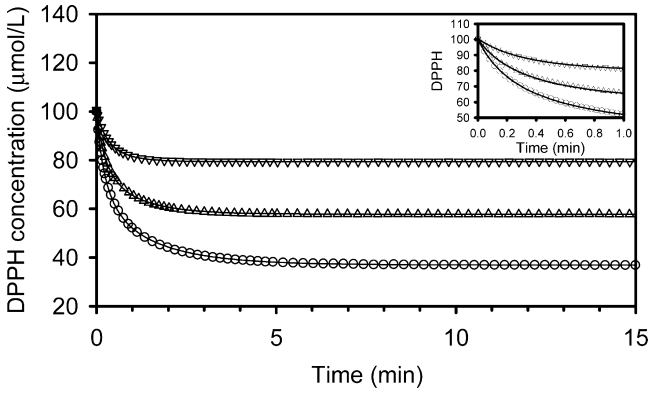

Figure 2. Time evolution of the concentration curves of DPPH• in acetonitrile during its reduction by three different initial concentrations of protocatechuic acid methyl ester. The inset corresponds to a zooming of the first minute of the reaction. See **Table 1** for the initial concentrations of DPPH• and protocatechuic acid methyl ester.

2 is regular, that is, coincides with the standard kinetic model (*14*). According to this kinetic model, which is schematized in **Figure 3** for a generic isolated and unperturbed *p*-catechol group, the full reaction of reduction can be viewed as composed by successive reaction steps. The first step of the reaction is a fast transfer of two consecutive hydrogen atoms [rate constants $k_1(1)$ and $k_1(2)$ and stoichiometric constants $\sigma_1(1) = 1$ and $\sigma_1(2) = 1$ for the substeps 1 and 2, respectively] from the *p*-catechol **a** to the DPPH•, with the subsequent transformations of **a** into the corresponding *o*-quinone **a*** and DPPH• into the corresponding hydrazine DPPH-H. Hence and irrespective of the solvent used, the global stoichiometric constant of this first step must be 2 [$\sigma_1 = \sigma_1(1) + \sigma_1(2) = 2$]. Concerning the rate constants, the possibility to experimentally differentiate between $k_1(1)$ and $k_1(2)$ depends on the solvent used: in nonalcoholic solvents such as acetonitrile, both rate constants can experimentally be differentiated; in alcoholic solvents such as methanol, on the contrary, both rate constants cannot experimentally be differentiated and only a global rate constant k_1 [$k_1 = k_1(1) + k_1(2)$] can be determined. In addition, the existence or not of additional reaction steps also depends on the solvent used: in nonalcoholic solvents, the reaction of reduction is already completed, that is, the reaction is single step (but composed of two distinguishable substeps) and there are no additional steps; in alcoholic solvents, on the contrary, the intermediate *o*-quinone **a*** quantitatively reacts with the alcohol (rate constant k_2 of formation of the adduct) and fully regenerates the *p*-catechol group **b** which, in its turn, rapidly reduces additional DPPH•, yielding the corresponding *o*-quinone **b*** (second step). When using methanol as solvent, the known experimental evidence suggests that all the reactions belonging to this second step are always fully completed and thus the value of the corresponding stoichiometric constant is 2 ($\sigma_2 = 2$). Similarly to the *o*-quinone **a***, the *o*-quinone **b*** can react with the solvent to regenerate the *p*-catechol group **c** which, in its turn, reduces additional DPPH•, yielding the corresponding *o*-quinone **c*** (third step). In this third step, however, either the reaction of adduct formation or the reaction of hydrogen transfer or both can be quantitative or not. If both reactions are quantitative, then the value of the corresponding stoichiometric constant is 2 ($\sigma_3 = 2$); if one or both reactions are not quantitative, then the value of the corresponding stoichiometric constant is a number, integer or not, within the range 0−2 ($2 > \sigma_3 > 0$). If the third step of the reaction is fully completed, then the *o*-quinone **c*** can react with the solvent, and so on, up to a maximum of four steps. Consequently, the standard kinetic model predicts that when using acetonitrile as solvent, the experimentally determined total stoichiometric constant for any isolated *p*-catechol group must be 2. In alcoholic solvents, in contrast, the experimentally determined total stoichiometric constant for an isolated *p*-catechol group must be a number, integer or not, within the range 4−8 ($8 \geq \sigma_t \geq 4$), depending on the extent of the reaction of reduction, that is, on both the alcohol used as solvent and the chemical structure of the antiradical.

From the standard kinetic model, two approximate theoretical kinetic equations were derived for those isolated *p*-catechol groups with regular reduction kinetics and whose reaction of reduction in methanol only extend over two steps (one step in acetonitrile, but composed of two substeps) and thus experimentally exhibit two rate−two stoichiometric constants (i.e., the *p*-catechol group in taxifolin). The coefficients of correlation from the fitting using both kinetic equations were almost identical, but due to practical reasons (*14*) the following was preferred:

$$y - y_s = \frac{y_1(y_o - y_1)}{y_1 - y_o(1 - e^{(k_1/\sigma_1)y_1 t})} + \frac{y_2(y_o - y_2)}{y_2 - y_o(1 - e^{(\rho_2/\sigma_2)y_2 t})} \quad (1)$$

with the constraint

$$y_2 = y_o + y_s - y_1$$

and the identities

$$y_1 = y_o - \sigma_1 a_o$$

$$y_2 = y_o - \sigma_2 a_o$$

$$y_s = y_o - (\sigma_1 + \sigma_2)a_o = y_o - \sigma_t a_o$$

where y is the time-dependent concentration of DPPH•, y_o is the initial concentration of DPPH•, a_o is the initial concentration of the antiradical, t is the reaction time, k_1 is the global rate constant corresponding to the first step of the reaction, y_1 is the asymptote that would be reached due solely to the antiradical activity of the first step of the reaction, ρ_2 is the pseudorate constant (which is directly correlated with the rate constant k_2 of formation of the first adduct) corresponding to the second step of the reaction, y_2 is the asymptote that would be reached due solely to the antiradical activity of the second step of the reaction, y_s is the experimental asymptote of the reaction, σ_1 and σ_2 are the stoichiometric constants of the first and second steps of the reaction, respectively, and σ_t ($=\sigma_1 + \sigma_2$) is the total stoichiometric constant of the reaction. It must be taken into account that in acetonitrile, where the full reaction of reduction is only the first step (composed of two substeps), the meaning of the adjustable parameters in eq 1 are (see **Figure 3**): $k_1 = k_1(1)$, $\rho_2 = k_1(2)$, $\sigma_1 = \sigma_1(1)$, and $\sigma_2 = \sigma_1(2)$.

Each set of experimental data points shown in **Figure 2** was fitted using eq 1. The fitting was excellent ($r^2 > 0.999$) for all sets of data and the adjusted values for the rate and stoichiometric constants are given in **Table 1**. These results confirm that the reduction kinetics of DPPH• in acetonitrile by the antiradical activity of **2** is regular since, according to the standard kinetic model, the adjusted values for the stoichiometric constants $\sigma_1(1) = 0.995 \pm 0.003$ and $\sigma_1(2) = 1.005 \pm 0.003$ were very close to 1 and the value of the total stoichiometric constant $\sigma_t = \sigma_1(1) + \sigma_1(2) = 2.000 \pm 0.006$ was very close to 2, independently of the assayed concentration of the antiradical. Concerning the adjusted values for the rate constants $k_1(1)$ and

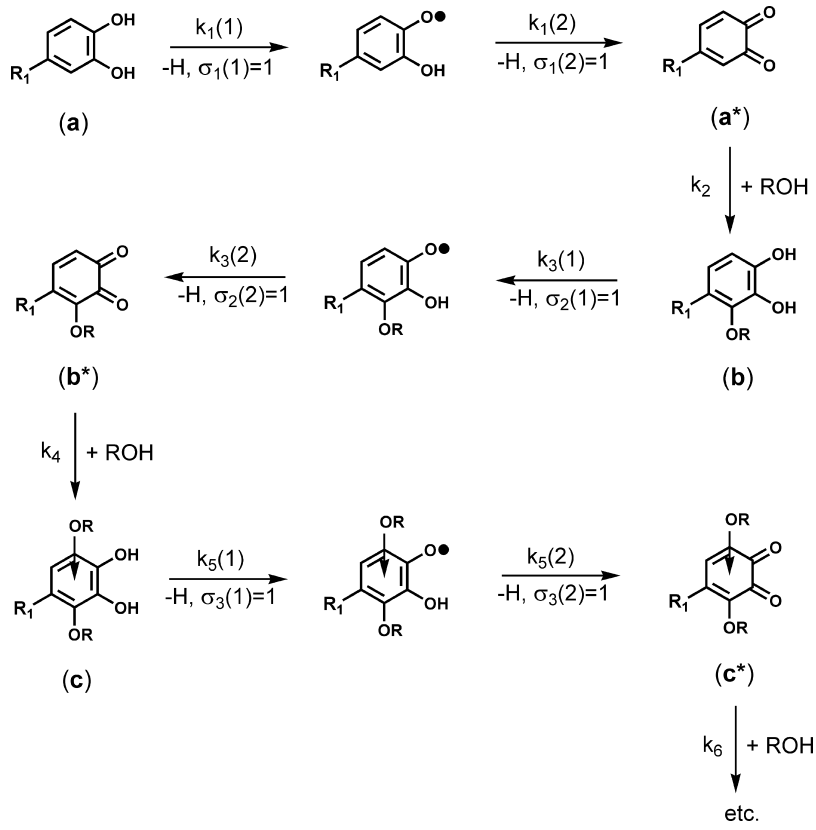

Figure 3. Standard kinetic model for the reduction kinetics of DPPH• by the antiradical activity of an isolated and unperturbed *p*-catechol group.

Table 1. Adjusted Values for the Rate and Stoichiometric Constants from the Reduction of DPPH• in Acetonitrile by Protocatechuic Acid Methyl Ester, Using Equation 1 for Fitting

initial concentration (μmol/L)			rate constants (L/mol min)		stoichiometric constants		global rate constant (L/mol min):	total stoichiometric constant:
	DPPH•	a_o	$k_1(1)$	$k_1(2)$	$\sigma_1(1)$	$\sigma_1(2)$	$k_1 = k_1(1) + k_1(2)$	$\sigma_1 = \sigma_1(1) + \sigma_1(2)$
○	100.108	31.695	51.4×10^3	8.7×10^3	0.991	1.001	60.1×10^3	1.993
△	100.099	21.130	49.0×10^3	11.5×10^3	0.997	1.007	60.5×10^3	2.003
▽	100.552	10.565	42.7×10^3	18.0×10^3	0.997	1.007	60.6×10^3	2.005

$k_1(2)$, they were dependent on the initial concentration of **2**. This result, which prevents the accurate determination of both rate constants, was not surprising at all since it is an inevitable consequence of using eq 1 for fitting. The theoretical but approximate eq 1 was derived under the assumption that the rate constant of a given step of the reaction is significantly greater than the rate constant from to the next step of the reaction. As a general rule, this assumption is true for alcoholic solvents, where $k_1(1)$ and $k_1(2)$ can only be determined together as the global cumulative rate constant k_1 [$=k_1(1) + k_1(2)$] and the pseudorate constant ρ_2, which corresponds to the second step of the reaction, is normally rather smaller than k_1, but the assumption cannot be true for acetonitrile, where the values of $k_1(1)$ and $k_1(2)$ are quite similar and, in addition, they can experimentally be distinguished. As a direct consequence, eq 1 is still a good kinetic equation for fitting but can only provide individually adjusted values for the rate constants that depend on the initial concentration of the antiradical. In any case, this is not a great trouble since, as can be seen in **Table 1**, the global cumulative rate constant of the reaction, that is, $k_1 = k_1(1) + k_1(2) = (60.4 \pm 0.28) \times 10^3$ L/(mol min), is a constant irrespective of the assayed initial concentration of the antiradical. Therefore, the antiradical activity in acetonitrile of any isolated *p*-catechol group with regular reduction kinetics can be perfectly characterized by giving its global cumulative rate [$k_1 = k_1(1) + k_1(2)$] and total stoichiometric ($\sigma_t = 2$) constants.

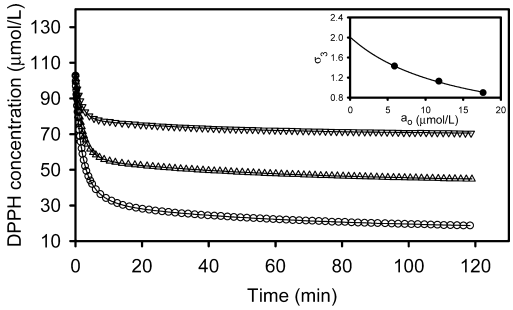

Figure 4. Time evolution of the concentration curves of DPPH• in methanol during its reduction by three different initial concentrations of protocatechuic acid methyl ester. The inset shows the dependence of the stoichiometric constant σ_3 versus the initial concentration of the antiradical. See **Table 2** for the initial concentrations of DPPH• and protocatechuic acid methyl ester.

Antiradical Activity of Protocatechuic Acid Methyl Ester in Methanol. Figure 4 shows the time evolution of the concentration of DPPH• in methanol during its reduction by three different initial concentrations of **2**. An approximate but very simple calculus, $\sigma_t = (y_o - y_n)/a_o$, where y_n is the DPPH• concentration of the last experimental data point from each set of data and y_o and a_o are the corresponding initial concentrations

Table 2. Adjusted Values for the Rate and Stoichiometric Constants from the Reduction of DPPH• in Methanol by Protocatechuic Acid Methyl Ester, Using Equation 2 for Fitting

	initial concentrations (μmol/L)		rate constants (L/mol min)			stoichiometric constants					
	DPPH•	a_o	k_1	ρ_2	ρ_3	σ_1	σ_2	σ_3	$k_1 + \rho_2$	$\sigma_1 + \sigma_2$	σ_t
○	102.824	17.66	17.2×10^3	5.9×10^3	157	1.991	2.001	0.897	23.1×10^3	3.993	4.890
△	103.261	11.78	14.7×10^3	7.5×10^3	195	2.001	1.991	1.124	22.3×10^3	3.993	5.116
▽	102.015	5.89	12.4×10^3	10.1×10^3	350	1.993	2.003	1.426	22.6×10^3	3.997	5.423

of DPPH• and **2**, reveals that the total stoichiometric constant takes a value within the range 4−6 ($6 > \sigma_t > 4$). Therefore and assuming that the reduction kinetics of DPPH• in methanol by **2** is also regular, the reaction of reduction extents over three steps and the antiradical activity of **2** should experimentally exhibit three rate−three stoichiometric constants (k_1, ρ_2, ρ_3, $\sigma_1 = \sigma_2 = 2$, and σ_3). The approximated theoretical kinetic equation that applies for these *p*-catechol groups has not been published yet, but taking into account the standard kinetic model as well as eq 1, which applies when the extent of the reaction is two steps (two rate−two stoichiometric constants), its derivation is immediate:

$$y - y_s = \frac{y_1(y_o - y_1)}{y_1 - y_o(1 - e^{(k_1/\sigma_1)y_1 t})} + \frac{y_2(y_o - y_2)}{y_2 - y_o(1 - e^{(\rho_2/\sigma_2)y_2 t})} + \frac{y_3(y_o - y_3)}{y_3 - y_o(1 - e^{(\rho_3/\sigma_3)y_3 t})} \quad (2)$$

with the constraint

$$y_3 = 2y_o + y_s - y_1 - y_2$$

and the identities

$$y_1 = y_o - \sigma_1 a_o$$

$$y_2 = y_o - \sigma_2 a_o$$

$$y_3 = y_o - \sigma_3 a_o$$

$$y_s = y_o - (\sigma_1 + \sigma_2 + \sigma_3)a_o = y_o - \sigma_t a_o$$

where the meanings of the new adjustable parameters, y_3, ρ_3, and σ_3, corresponding to the third step of the reaction, are evident.

Each set of experimental data points in **Figure 4** was fitted using eq 2. The fitting was excellent ($r^2 > 0.999$) for all sets of data, and the results are given in **Table 2**. These results confirm that the reduction kinetics of DPPH• in methanol by **2** is also regular, since the adjusted values for σ_1 (=1.995 ± 0.005) and σ_2 (=1.999 ± 0.006) were the theoretically expected values from the standard kinetic model. Concerning the rate constants k_1 and ρ_2, their adjusted values were rather similar, contrary to the adjusted values for k_1 and ρ_2 from the antiradical activity of the *p*-catechol in taxifolin that are very different (see later for a more extensive discussion). Therefore, they are dependent on the initial concentration of **2** and cannot accurately be determined. In any way and similarly as in acetonitrile, their addition gives a constant [$k_1 + \rho_2 = (22.7 \pm 0.42) \times 10^3$ L/(mol min)] that can be used to characterize the antiradical activity in these particular cases. The adjusted values of the pseudorate ρ_3 [157, 195, and 350 L/(mol min)] and stoichiometric σ_3 (0.897, 1.124, and 1.426) constants were dependent on the initial concentration of **2** (17.66, 11.78, and 5.89 μmol/L, respectively), indicating that the third step of the reaction is not fully completed. It is very significant, however, that, as the concentration of the antiradical decreases, the adjusted values for the corresponding ρ_3 and σ_3 increase. Consequently, it seems reasonable to conclude that the reaction between the regenerated *p*-catechol **c** and DPPH• is not completed because it is not irreversible but reversible. In fact, if this reaction is really reversible, then the experimentally determined value for σ_3 must tend toward 2 at infinite dilution of the antiradical. The inset in **Figure 4** shows the graphical representation of σ_3 versus the initial concentration (a_o) of **2**, as well as the fitting to a decreasing hyperbola ($r^2 > 0.998$). At $a_o = 0$, the adjusted value for σ_3 is 2.011.

As indicated previously, the antiradical activity of an isolated *p*-catechol group also depends on the structural characteristics of the carrying molecule. According to the standard kinetic model, the total antiradical activity in alcoholic solvents results from the addition of the partial antiradical activities from the different steps of the reaction. Except for the first step of the reaction, the antiradical activity of the following steps depend on the adduct formation between the corresponding intermediate *o*-quinone (**a***, **b***, etc.) and the alcoholic solvent. Consequently, any structural characteristic (or nonreactive functional group) that increases/decreases the susceptibility of the intermediate *o*-quinones toward the nucleophilic attack by the alcohol will increase/decrease the antiradical activity of the corresponding step. For instance, the *p*-catechol group in taxifolin is subjected

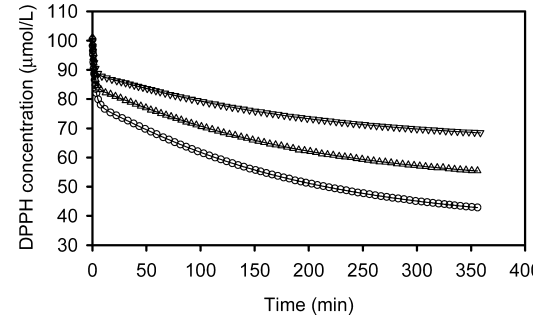

Figure 5. Time evolution of the concentration curves of DPPH• in methanol during its reduction by three different initial concentrations of protocatechuic acid. See **Table 3** for the initial concentrations of DPPH• and protocatechuic acid.

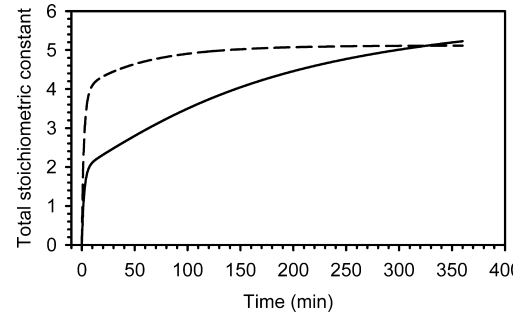

Figure 6. Time evolution of the total stoichiometric constant (σ_t) from protocatechuic acid methyl ester (– –, $y_o = 103.261$ μmol/L, $a_o = 11.780$ μmol/L) and protocatechuic acid (—, $y_o = 100.334$ μmol/L, $a_o = 11.027$ μmol/L) during the reduction of DPPH• in methanol.

Table 3. Adjusted Values for the Rate and Stoichiometric Constants from the Reduction of DPPH• in Methanol by Protocatechuic Acid, Using Equation 2 for Fitting

	initial concentrations (μmol/L)		rate constants (L/mol min)			stoichiometric constants			
	DPPH•	a_0	k_1	ρ_2	ρ_3	σ_1	σ_2	σ_3	σ_t
○	100.334	11.027	10.0×10^3	−218.0	397.0	1.966	−1.020	5.022	5.967
△	101.136	8.526	11.6×10^3	−204.8	375.1	2.013	−1.010	5.053	6.055
▽	100.028	5.765	11.7×10^3	−174.4	361.9	1.973	−0.988	5.133	6.117

to a rather intense steric hindrance. As a consequence, the first intermediate *o*-quinone **a*** is slowly attacked by the alcohol (the rate constant k_2 is rather slow), the value of the corresponding pseudorate constant ρ_2 [$=0.937 \times 10^3$ L/(mol min)] is smaller by far than that of k_1 [$=60.9 \times 10^3$ L/(mol min)] and hence both values can accurately be determined by fitting using eq 2. Even more, the next intermediate *o*-quinone **b*** is no longer able to react with the solvent to regenerate the *p*-catechol group ($k_4 = \rho_3 = \sigma_3 = 0$), and so the extent of the reaction is only two steps and the value of the total stoichiometric constant is 4 (*14*). In contrast, the *p*-catechol group in **2** is almost free of steric hindrance, the intermediate *o*-quinone **a*** is rapidly attacked by the alcohol (the rate constant k_2 is rather fast), and the value of the corresponding pseudorate constant ρ_2 is rather close to that of k_1 and so both rate constants cannot accurately be determined by fitting using eq 2. Even more, the next intermediate *o*-quinone **b*** is still able to react with the solvent to regenerate the *p*-catechol group **c** ($k_4 > 0$, $\rho_3 > 0$), and so the reaction extends over three steps ($\sigma_1 = \sigma_2 = 2$, $0 < \sigma_3 < 2$) and the total stoichiometric constant is >4.

Antiradical Activity of Protocatechuic Acid in Methanol. **Figure 5** shows the time evolution of the concentration of DPPH• in methanol during its reduction by three different initial concentrations of **1**. Comparison of **Figures 4** and **5** reveals at first glance that there is a remarkable difference between the shapes of the reduction curves from **1** and **2**. The DPPH• concentration curves in **Figure 4** monotonically decrease along the reaction time up to reach the asymptote, in accordance with the standard kinetic model. In contrast, the DPPH• concentration curves in **Figure 5** monotonically decrease during the first minutes of the reaction but suddenly there is a deceleration followed by a further acceleration of the reduction rate. This surprising behavior of the antiradical activity of **1** in methanol, which was already observed by Saito and Kawabata (*13*), can be better visualized by comparing the time evolution of the total stoichiometric constants from **1** and **2**, as shown in **Figure 6**. It seems evident that during the reduction of DPPH• in methanol by **1** and near the completion of the first step of the reaction ($t \approx 10$ min, $\sigma_t \approx 2$) something unexpected happens that slows down its scavenging activity when compared with that from **2**. Moreover and very interestingly, the decreased scavenging activity of **1** is only transitory since, after all, the total stoichiometric constant of **1** is even greater than that of **2** at long reaction times, that is, near the completion of the reaction. This result clearly indicates that the reduction kinetics of DPPH• by **1** is not regular due to the presence of the reactive carboxylic acid function, but it also suggests that this perturbed reduction kinetics is most probably a minor modification of the standard kinetic model.

In an attempt to elucidate why the antiradical activity of **1** and **2** behaves so differently in methanol and if this is a general trend for all acid/ester pairs in analogous phenolic acids, Saito and Kawabata (*13*) determined also the radical scavenging activity of sodium protocatechuate, 3,4-dihydroxybenzenesulfonic acid, 3,4-dihydroxyphenylphosphonic acid, and their esters. Results indicated that the sodium salt of **1** (the carboxylate ion from **1**) behaves as **1** and that all the assayed pairs acid/ester behave similarly to the pair **1/2**. Therefore it was concluded that the carboxylic acid function in the *o*-quinone acid **a***, that is a rather stronger acid than in the parent molecule **a**, dissociates to the carboxylate ion. Since the carboxylate ion is electron-donating, contrary to the carboxylic acid that is electron-withdrawing, it lowers the susceptibility of the *o*-quinone acid **a*** toward the nucleophilic attack by the alcohol (first adduct formation), thus delaying the scavenging activity due to the second step of the reaction. This mechanism, however, although very convincing, cannot explain by itself the observed time evolution of the antiradical activity from **1**. As indicated previously, the standard kinetic model envisages the full reaction of reduction as a concatenation of successive reaction steps. As a direct consequence, any rate constant within the whole reaction is limiting concerning the experimentally determined value for any of the following rate constants. Therefore, the standard kinetic model demands that, excepting when there exists an additional side reaction, the shape of the reduction curve of DPPH• must monotonically decrease along the reaction time to reach the asymptote (i. e., the reduction curves from **2** shown in **Figure 4**). Since the curves shown in **Figure 5** are clearly not monotonically decreasing, the above mechanism fails, because it does not modify the standard kinetic model at all, but only slows down the reaction of the first adduct formation.

Despite the fact that in this case eq 2 is not a "theoretical kinetic equation", in an attempt to gain additional insight about this kinetics and taking into account that $\sigma_t > 4$ for all the assayed concentrations of **1**, each set of experimental data points in **Figure 5** was fitted using eq 2. Since the unknown side reaction appears near the completion of the first step of the reaction, the side reaction in this fitting will behave as a pseudosecond reaction step (with the corresponding adjustable parameters ρ_2 and σ_2) and the values of the subsequent rate and stoichiometric constants (corresponding to the true second and third reaction steps) will be accumulated into the adjusted values for ρ_3 and σ_3, respectively. The fitting was very good ($r^2 > 0.999$) for all sets of data points, and the results are given in **Table 3**. As can be seen, the adjusted values for k_1 [$=(11.1 \pm 1) \times 10^3$ L/(mol min)] and σ_1 ($=1.984 \pm 0.025$), which correspond to the first step of the reaction, were independent of the initial concentration of **1** and within the range of expected values. Very interestingly, the adjusted value for ρ_2 [$=(-0.199 \pm 0.022) \times 10^3$ L/(mol min)] and σ_2 ($=-1.006 \pm 0.016$), which quantify the influence of the unknown side reaction, were negative, thus implying an increase of the color (or DPPH• concentration) of the reaction mixture. The adjusted values for ρ_3 [$=(0.378 \pm 0.018) \times 10^3$ L/(mol min)] and σ_3 ($=5.069 \pm 0.058$) were within the range of expected values, taking into account that they are cumulative values. Finally, the value of the total stoichiometric constant $\sigma_t = \sigma_1 + \sigma_2 + \sigma_3 = 6.047 \pm 0.075$, indicates that, after all and independently of the concentration of **1**, the extent of the reaction was in fact three completed steps.

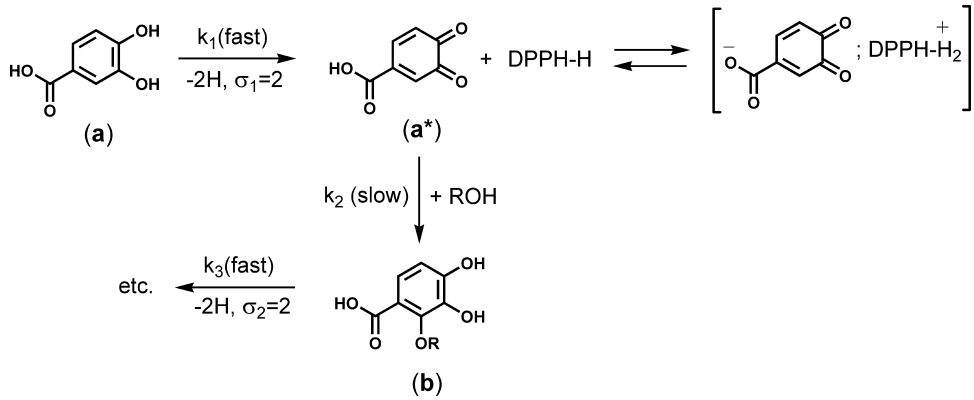

Figure 7. Proposed kinetic model for the reduction kinetics of DPPH• in methanol by the antiradical activity of protocatechuic acid.

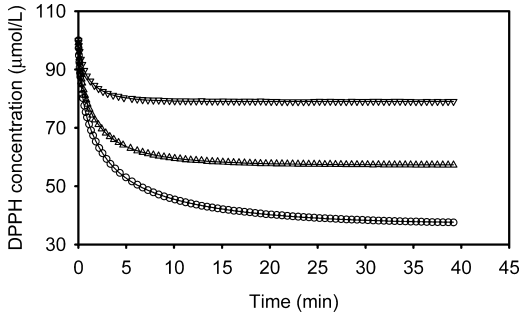

Figure 8. Time evolution of the concentration curves of DPPH• in acetonitrile during its reduction by three different initial concentrations of protocatechuic acid. See **Table 4** for the initial concentrations of DPPH• and protocatechuic acid.

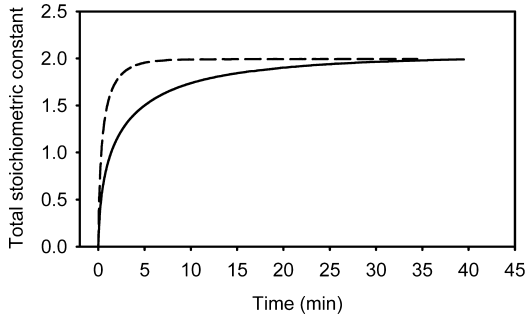

Figure 9. Time evolution of the total stoichiometric constant (σ_t) from protocatechuic acid methyl ester (– –, y_o = 100.108 μmol/L, a_o = 31.695 μmol/L) and protocatechuic acid (—, y_o = 99.960 μmol/L, a_o = 31.390 μmol/L) during the reduction of DPPH• in acetonitrile.

Taking into account these results and, over all, the very significant shape of the reduction curves, where the scavenging-activity of **1** is smaller than that of **2** at early reaction times but becomes almost identical at long reaction times, the modified standard reduction kinetics schematized in **Figure 7** is proposed. In agreement with the suggestion by Saito and Kawabata (*13*), the relatively strong carboxylic acid function in the *o*-quinone acid **a*** dissociates to a carboxylate ion, that is, reacts with DPPH-H (the already reduced DPPH• from the first step of the reaction) through a reversible reaction and forms a complex, most probably a salt. It is postulated that this complex also absorbs at 515 nm, thus leading, near to the completion of the first step of the reaction, to an increase of the color of the reaction mixture (that is, an apparent increase of the DPPH• concentration) or, what is equivalent, to an apparent decrease of the DPPH• reduction rate. As the reaction proceeds according to the standard kinetic model, the acidic *o*-quinone **a*** reacts with the alcohol and yields the acidic *p*-catechol **b**. However,

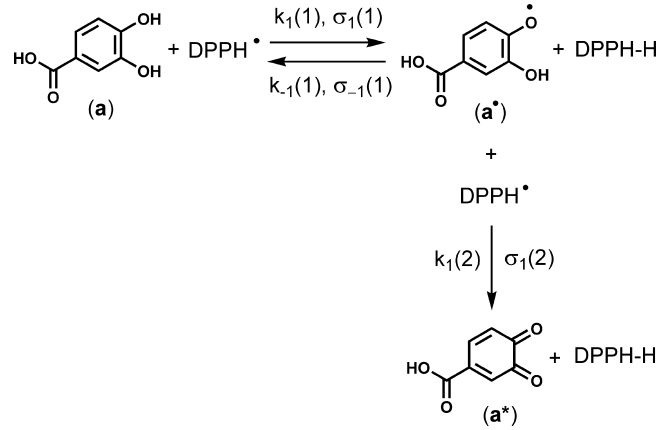

Figure 10. Proposed kinetic model for the reduction kinetics of DPPH• in acetonitrile by the antiradical activity of protocatechuic acid.

the carboxylic acid function in **b** is a weak acid (like in the parent molecule **a**) and thus unable to dissociate and forms the complex with DPPH-H. As a direct consequence, the reversible reaction is continuously forced backward, from approximately the ending of the first step of the reaction onward, and thus both the complex and its associated additional color slowly vanish as the reaction proceeds. This could explain the observed time evolution of the antiradical activity from **1** as well as the fact that its total stoichiometric constant is even greater than that from **2** at long reaction times.

Antiradical Activity of Protocatechuic Acid in Acetonitrile. Figure 8 shows the time evolution of the concentration of DPPH• in acetonitrile during its reduction by three different initial concentrations of **1**. Although at first glance it is not so evident than in methanol, the time evolution of these curves is quite different from those from **2** in the same solvent (**Figure 2**). This difference can be better visualized by comparing the time evolution of the total stoichiometric constants from **1** and **2** in acetonitrile, as it is shown in **Figure 9**. Similarly as in methanol, but now from approximately the beginning of the reaction, the increase of the total stoichiometric constant from **1** slows down when compared to that from **2**. In addition, the decreased scavenging activity of **1** is also transitory again since the asymptotic value for both stoichiometric constants is 2, that is, the theoretical value. As expected from the above, the fitting of the experimental data points using eq 1 was very poor for all sets of data, indicating that the reduction kinetics of DPPH• by **1** is not regular. Hence, something unexpected happens for **1**, near the beginning of the reaction and also modulated by the carboxylic acid function, that transitorily slows down its scavenging activity at early reaction times, but that vanishes as the reaction proceeds. In this case, however, the mechanism

Table 4. Adjusted Values for the Rate and Stoichiometric Constants from the Reduction of DPPH• in Acetonitrile by Protocatechuic Acid, Using Equation 2 for Fitting

	initial concentration (μmol/L)		rate constants (L/mol min)			stoichiometric constants			
	DPPH•	a_o	$k_1(1)$	$k_{-1}(1)$	$k_1(2)$	$\sigma_1(1)$	$\sigma_{-1}(1)$	$\sigma_1(2)$	σ_t
○	100.077	31.39	21.7×10^3	-1.05×10^3	6.14×10^3	0.493	−0.596	2.104	2.001
△	99.422	20.93	22.8×10^3	-1.68×10^3	8.31×10^3	0.535	−0.787	2.253	2.000
▽	100.110	10.46	22.6×10^3	-3.70×10^3	12.40×10^3	0.633	−0.979	2.345	2.000

must be different than that in methanol since (1) the slow down of the total stoichiometric constant from **1** starts near the beginning of the reaction, (2) the time evolution of the DPPH• reduction curves is right according to the standard kinetic model, that is, they monotonically decrease along the reaction time up to reach the asymptote, and (3) it is even more unlikely in acetonitrile than in methanol that the weak carboxylic acid function in **1** dissociates to a carboxylate ion. From all the above, the mechanism schematized in **Figure 10** is proposed, where the presence of the carboxylic acid function turns the irreversible reaction corresponding to the first substep of the reaction reversible. In other words, in this mechanism it is assumed that in the nonalcoholic solvent acetonitrile the presence of the electron-withdrawing carboxylic acid function in **1** (**a**) lowers so strongly the tendency of the *p*-catechol to transfer one hydrogen atom to the DPPH• that the reaction changes from irreversible to reversible or, what is equivalent, the tendency of the *p*-catechol in **1** (**a**) to reduce the DPPH• (yielding **a**• and DPPH-H) is comparable to the tendency of the radical **a**• to be reduced by the DPPH-H (yielding **a** and DPPH•). At the beginning of the reaction, the *p*-catechol in **1** (**a**) transfers one hydrogen atom to the DPPH•, yielding the radical **a**• and reduced DPPH-H, but since the reaction is reversible, the time evolution of the reduction rate of DPPH• is slower by far than if the reaction was irreversible (as from **2**). As the reaction proceeds, the radical **a**• transfers one additional hydrogen atom to the DPPH•, yielding the final *o*-quinone **a***, but since this reaction is irreversible, the reversible reaction is continuously forced forward up to completion of the reaction, that is, up to the reduction of the stoichiometric amount of DPPH•, which corresponds to a final stoichiometric constant value of 2.

Despite the fact that eq 2 is not a "theoretical kinetic equation" in this case, but taking into account that the reaction of reduction envisages "three rate−three stoichiometric constants", each set of experimental data points in **Figure 8** was fitted using eq 2. In this fitting, the values of the adjusted parameters k_1 and σ_1 would correspond to the forward reaction of the first substep, that is, $k_1(1)$ and $\sigma_1(1)$, respectively; the adjusted values for ρ_2 and σ_2 would correspond to the backward reaction of the first substep, that is, $k_{-1}(1)$ and $\sigma_{-1}(1)$, respectively; and the values of the adjusted parameters ρ_3 and σ_3 would correspond to the second substep of the reaction, that is, $k_1(2)$ and $\sigma_1(2)$, respectively. The fittings were very good ($r^2 > 0.999$) and the results are given in **Table 4**. As can be seen, the adjusted values of the rate and stoichiometric constants "corresponding" to the backward reaction, ρ_2 and σ_2, were negative for all curves, as expected, and the total stoichiometric constant of the reaction was 2 ($\sigma_t = 2.000 \pm 0.001$) independent of the initial concentration of the antiradical.

The results of this work indicate that, in both methanol and acetonitrile, the reduction kinetics of DPPH• by the antiradical activity of protocatechuic acid methyl ester follows the standard kinetic model, while the reduction kinetics of DPPH• by the antiradical activity of protocatechuic acid deviates from the standard kinetic model. The observed deviations arise from the presence of the reactive carboxylic acid function, which induces a new reversible side reaction in methanol and turns an irreversible reaction reversible in acetonitrile, leading to a transitory deceleration (apparent in the case of methanol) of the DPPH• reduction rate. In any case, the proposed mechanisms are only tentative and additional experimental work is needed to ascertain their validity. Finally, it seems that when using DPPH• as the antiradical probe, eq 2 behaves as an excellent "multipurpose" kinetic equation to fit antiradical activities. In fact, preliminary but very consistent data indicate that almost any antiradical activity coming from mixtures of antiradicals (i.e., juices, plant extracts) can be very well fitted using eq 2, but one must take into account that in these cases the adjusted values would correspond to average rate and stoichiometric constants.

GLOSSARY

a_o	initial concentration (μmol/L) of the antiradical
DPPH•	2,2-diphenyl-1-picrylhydrazyl
DPPH-H	2,2-diphenyl-1-picrylhydrazyne (reduced DPPH•)
k_1	in methanol, global rate constant [$=k_1(1) + k_1(2)$] [L/(mol min)] of the first step of the reaction; in acetonitrile, $= k_1(1)$
$k_1(1)$	in acetonitrile, rate constant [L/(mol min)] of the first substep of the single step reaction
$k_1(2)$	in acetonitrile, rate constant [L/(mol min)] of the second substep of the single step reaction
k_2	in methanol, rate constant [L/(mol min)] of the first adduct formation (second step)
k_4	in methanol, rate constant [L/(mol min)] of the second adduct formation (third step)
t	time (min); y, time dependent concentration (μmol/L) of DPPH•
y_o	initial concentration (μmol/L) of DPPH•
y_1	in methanol, DPPH• concentration asymptote (μmol/L) that would be reached due solely to the antiradical activity of the first step of the reaction; in acetonitrile, DPPH• concentration asymptote (μmol/L) that would be reached due solely to the antiradical activity of the first substep of the single step reaction
y_2	in methanol, DPPH• concentration asymptote (μmol/L) that would be reached due solely to the antiradical activity of the second step of the reaction; in acetonitrile, DPPH• concentration asymptote (μmol/L) that would be reached due solely to the antiradical activity of the second substep of the single step reaction
y_3	in methanol, DPPH• concentration asymptote (μmol/L) that would be reached due solely to the antiradical activity of the third step of the reaction
y_s	experimental DPPH• concentration asymptote (μmol/L)
ρ_2	in methanol, pseudorate constant [L/(mol min)] corresponding to the second step of the reaction, whose value is dependent on k_2; in acetonitrile, $=k_1(2)$

ρ_3 in methanol, pseudorate constant [L/(mol min)] corresponding to the third step of the reaction, whose value is dependent on k_4

σ_1 in methanol, global stoichiometric constant [$=\sigma_1(1) + \sigma_1(2) = 2$] of the first step of the reaction; in acetonitrile, $=\sigma_1(1)$

$\sigma_1(1)$ in acetonitrile, stoichiometric constant (=1) of the first substep of the single step reaction

$\sigma_1(2)$ in acetonitrile, stoichiometric constant (=1) of the second substep of the single step reaction

σ_2 in methanol, stoichiometric constant (=2) of the second step of the reaction; in acetonitrile, $= \sigma_1(2)$

σ_3 in methanol, stoichiometric constant [$2 \geq \sigma_3 \geq 0$] of the third step of the reaction

σ_t total stoichiometric constant ($=\sigma_1 + \sigma_2 + \sigma_3$) of the reaction (=2 in acetonitrile; $=4 + \sigma_3$ in methanol)

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