Observations on the Estimation of Scavenging Activity of Phenolic Compounds Using Rapid 1,1-Diphenyl-2-picrylhydrazyl (DPPH$^{\bullet}$) Tests

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ABSTRACT: Rapid 1,1-diphenyl-2-picrylhydrazyl (DPPH$^{\bullet}$) tests are often applied to classify the scavenging activity of phenolic compounds (AH). Published analytical protocols differ in more than one experimental condition, and results for the relative order or magnitude of activity are often contradictory. In this work, parameters such as duration of test, [AH]/[DPPH$^{\bullet}$] molar ratio, and solvent effects were examined and discussed. The test duration and the value of the [AH]/[DPPH$^{\bullet}$] ratio did not influence the order of activity among tested antioxidants. Ethanol, commonly used as solvent in such tests, was compared with acetonitrile and *tert*-butyl alcohol. Solvent properties such as the ability to form hydrogen bonds with the AH seem to influence the level of the relative activity (%RSA). Higher %RSA values were observed in ethanol. The activity of the most polar compounds was affected the most, and in some cases (caffeic, dihydrocaffeic, and rosmarinic acids) the order of activity was changed owing to different kinetics. Standardization of the analytical protocol should include a 20-min reaction period and a molar ratio that permits attainment of a 60–80% RSA value for the most potent antioxidant. Solvent choice is critical for classifying activity. Safe classification can be based only on results from kinetic studies.

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KEY WORDS: DPPH$^{\bullet}$ test, phenolic antioxidants, radical scavenging activity, solvent effect.

The main mechanism of action of phenolic antioxidants (AH) is considered to be the scavenging of free radicals by hydrogen-atom donation, although other mechanisms may be involved (1). A methodology to estimate the scavenging of free radicals by the phenolic antioxidants that is rapid, inexpensive, easily applicable to a large number of samples (standard compounds or extracts), accurate, and robust is needed. Much effort has been devoted to the development of procedures based on reaction of AH with different radical species of biological significance such as $O_2^{\bullet -}$, OH$^{\bullet}$, NO$^{\bullet}$, or lipid peroxyl radicals LOO$^{\bullet}$ (2–4). Tests using the reduction of the 1,1-diphenyl-2-picrylhydrazyl radical (DPPH$^{\bullet}$) in the presence of phenolic compounds are also quite popular among food scientists (5–7). These tests are based on the decrease of the absorbance of the radical solution according to the reaction DPPH$^{\bullet}_{(purple)}$ + HA $\rightarrow$ DPPH-H$_{(yellowish)}$ + A$^{\bullet}$. This radical has been used for many decades to study the mechanism of hydrogen-atom donation to free radicals from certain substrates or the antioxidant activity of compounds carrying –SH, –OH, and –NH groups (8–10). DPPH$^{\bullet}$ does not dimerize, i.e., it remains in its monomeric form in solutions, exhibits a stable absorbance over a wide pH range, and resists oxidation. In addition, the reaction conditions are mild and, as reported, the results provide basic information on the reactivity of compounds with regard to their structure (5–7,10). All these characteristics explain the increasing popularity of applying rapid DPPH$^{\bullet}$ tests to foods either for screening or to highlight the mechanism of reaction with the AH. An overview of the literature reveals that the analytical protocols of these tests differ in more than one experimental condition. The latter may be the reason why the results concerning the relative order or the magnitude of the radical scavenging activity are often contradictory (11,12).

The aim of this work was to examine parameters of the rapid DPPH$^{\bullet}$ tests that may influence the scavenging activity of phenolic antioxidants. A wide range of phenolic compounds was used to support the observations and justify the proposals for the standardization of the analytical protocol.

MATERIALS AND METHODS

Standards, reagents, and solvents. Caffeic acid and 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) were from Riedel-de Haën (Seelze, Germany). Dihydrocaffeic acid, *o*-coumaric acid, *p*-coumaric acid, sinapic acid, chlorogenic acid, tyrosol, BHA, BHT, TBHQ, and DPPH$^{\bullet}$ were from Sigma Chemical Co. (St. Louis, MO). Ferulic acid and isoferulic acid were from Aldrich, Chemical Co. (Steinheim, Germany); *m*-coumaric acid was from Fluka Chemie (Buchs, Switzerland). Oleuropein and rosmarinic acid were from Röth (Karlsruhe, Germany). Hydroxytyrosol was a generous gift of Dr. G. Blekas. α-Tocopherol was from Merck (Darmstadt, Germany). Absolute ethanol and acetonitrile of HPLC grade were from Riedel-de Haën. *tert*-Butylalcochol was from Fluka Chemie.

Apparatus. A U-2000 Hitachi spectrophotometer (Tokyo, Japan) was used for the measurement of the reduction of DPPH$^{\bullet}$ absorbance at 516 nm.

Estimation of radical scavenging activity (%RSA) by the DPPH$^{\bullet}$ test. The %RSA activity of phenols was based on the method of Pekkarinen *et al.* (11). The decrease of the absorption at 516 nm of the DPPH$^{\bullet}$ solution after addition of the AH

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was measured in a glass cuvette (1 cm long). An aliquot (2960 μL) of 0.1 mM ethanolic DPPH• solution was mixed with 40 μL of a 1 mM AH solution so that the relative concentration of AH vs. the stable radical (mole AH/mole DPPH•) in the cuvette was 0.13. The absorption was monitored at the start and at 10 and 20 min. The results are expressed as %RSA = $[Abs_{516nm}(t = 0) - Abs_{516nm}(t = t') \times 100/Abs_{516nm}(t = 0)]$. Absorbance values were corrected for radical decay using blank solutions. The experimental procedure was also performed using AH solutions of 1.85 and 3.7 mM (mole AH/mole DPPH• = 0.25 and 0.5, respectively). Determination of the %RSA using the 1.85 mM solution of the AH was also carried out in acetonitrile or *tert*-butylalcohol. All tests were performed in triplicate at 25°C. Kinetic studies were undertaken in certain cases. The radical scavenging activity of the AH was determined using 0.1 mM DPPH• solution in ethanol or in acetonitrile. Reduction of DPPH• was followed by monitoring the decrease in absorbance at 516 nm until the reaction reached a plateau. Graphs were constructed showing the percentage of residual DPPH• vs. time. From these graphs the percentage of DPPH• remaining at the steady state was determined, and the values were transferred onto another graph showing the percentage of residual stable radical at the steady state as a function of the molar ratio of antioxidant to DPPH•. The latter was used to determine the efficient concentration (EC_{50}), that is, the amount of antioxidant necessary to decrease the initial [DPPH•] by 50%. The lower the EC_{50} is, the higher the antioxidant activity. Moreover, the reaction time needed to reach the steady state for EC_{50} (T_{EC50}) and antiradical efficiency, $AE = 1/EC_{50} \times T_{EC50}$ were also calculated (6).

Statistical analysis. Statistical comparisons of the mean values for each experiment were performed by one-way ANOVA, followed by the multiple Duncan test ($P \leq 0.05$ confidence level).

RESULTS AND DISCUSSION

In the present study a large number of phenolic compounds were examined, namely, hydroxycinnamic acid derivatives and secoiridoids as well as α-tocopherol and a group of synthetic antioxidants often used as a reference in autoxidation studies. The results of the effect of the test duration and of the relative concentration of the antioxidant on the ability of the compounds to scavenge the DPPH• are presented in Table 1. %RSA values are presented for 10-min, as proposed by Pekkarinen *et al.* (11), and 20-min reaction periods. According to these results, the order of DPPH• scavenging by the compounds was not affected by the reaction period. The %RSA values within each column indicated the different activity of each compound toward the stable radical. This difference in %RSA is due to the substituents in the aromatic ring that affect the reactivity of the compounds toward the sta-

TABLE 1
Effect of Reaction Period and Relative Concentration of the AH on the Ability of Phenolic Antioxidants to Scavenge the DPPH• Radical

	[AH]/[DPPH•] (mol/mol)					
	0.13		0.25		0.5	
	Reaction period (min)					
AH	10	20	10	20	10	20
	(%) RSA[a]					
Dihydrocaffeic acid	50.9 ± 0.3^{a,A}	63.2 ± 0.2^{a,B}	83.7 ± 1.2^{a,A}	93.9 ± 0.5^{a,B}	94.2 ± 0.1^{a,A}	94.6 ± 0.1^{a,A}
Rosmarinic acid	49.2 ± 0.8^{a,A}	56.9 ± 0.5^{b,B}	82.4 ± 0.1^{b,A}	88.4 ± 0.4^{a,B}	92.7 ± 0.4^{a,A}	93.4 ± 0.2^{a,B}
Caffeic acid	46.8 ± 0.2^{b,A}	47.7 ± 0.6^{c,A}	63.6 ± 0.8^{c,A}	76.6 ± 0.5^{b,B}	90.2 ± 0.5^{b,A}	92.7 ± 0.4^{a,A}
Chlorogenic acid	33.2 ± 1.1^{c,A}	34.2 ± 0.8^{d,A}	49.2 ± 0.6^{d,A}	52.0 ± 0.6^{c,A}	70.1 ± 1.5^{c,A}	79.2 ± 1.4^{b,B}
Sinapic acid	36.5 ± 0.7^{d,A}	37.5 ± 0.5^{e,A}	54.4 ± 2.1^{e,A}	56.1 ± 2.0^{d,A}	88.1 ± 2.0^{d,A}	88.4 ± 1.8^{c,A}
Ferulic acid	13.6 ± 0.9^{e,A}	17.2 ± 0.9^{f,A}	26.7 ± 2.1^{f,A}	30.9 ± 2.9^{e,A}	32.0 ± 0.9^{e,A}	37.3 ± 1.3^{d,B}
Isoferulic acid	3.2 ± 0.4^{f,A}	3.2 ± 0.4^{g,A}	3.2 ± 0.1^{g,A}	3.5 ± 0.1^{f,A}	4.3 ± 0.1^{f,A}	4.4 ± 0.1^{e,A}
o-Coumaric acid	2.9 ± 0.1^{f,A}	3.0 ± 0.1^{g,A}	3.6 ± 0.1^{g,A}	3.5 ± 0.3^{f,A}	4.3 ± 0.1^{f,A}	4.3 ± 0.3^{e,A}
m-Coumaric acid	2.0 ± 0.1^{f,A}	2.0 ± 0.1^{g,A}	2.5 ± 0.5^{g,A}	2.6 ± 0.6^{f,A}	1.5 ± 0.5^{g,A}	1.6 ± 0.6^{f,A}
p-Coumaric acid	2.4 ± 0.1^{f,A}	2.4 ± 0.1^{g,A}	3.6 ± 0.5^{g,A}	3.6 ± 0.4^{f,A}	3.3 ± 0.5^{f,A}	3.3 ± 0.4^{e,A}
trans-Cinnamic acid	0.5 ± 0.1^{g,A}	0.5 ± 0.1^{h,A}	0.5 ± 0.1^{h,A}	0.5 ± 0.2^{g,A}	0.5 ± 0.1^{h,A}	0.5 ± 0.1^{f,A}
Hydroxytyrosol	39.2 ± 2.6^{d,A}	39.4 ± 2.4^{e,A}	56.5 ± 1.2^{e,A}	57.0 ± 0.2^{d,A}	89.6 ± 0.2^{d,A}	89.6 ± 0.3^{c,A}
Oleuropein	25.0 ± 0.7^{h,A}	25.1 ± 0.7^{i,A}	41.3 ± 0.6^{i,A}	41.3 ± 0.2^{h,A}	81.9 ± 0.2^{i,A}	82.3 ± 0.3^{g,A}
Tyrosol	2.5 ± 0.5^{f,A}	2.5 ± 0.5^{f,A}	2.6 ± 0.2^{f,A}	2.7 ± 0.3^{f,A}	2.8 ± 0.3^{f,A}	2.8 ± 0.3^{f,A}
α-Tocopherol	41.4 ± 0.1^{d,A}	41.8 ± 0.4^{e,A}	52.8 ± 1.7^{e,A}	54.0 ± 1.9^{d,A}	72.5 ± 1.1^{c,A}	73.5 ± 1.1^{h,A}
Trolox	41.4 ± 0.1^{d,A}	41.5 ± 0.1^{e,A}	53.4 ± 1.2^{e,A}	53.4 ± 1.2^{d,A}	85.2 ± 1.1^{d,A}	86.0 ± 1.5^{c,A}
TBHQ	28.3 ± 0.7^{h,A}	31.7 ± 0.8^{j,B}	52.3 ± 0.7^{e,A}	58.7 ± 0.8^{d,B}	60.6 ± 1.1^{j,A}	69.6 ± 1.2^{h,B}
BHA	14.3 ± 0.5^{e,A}	21.5 ± 0.6^{k,B}	16.1 ± 0.3^{j,A}	22.3 ± 0.6^{i,B}	39.9 ± 0.8^{k,A}	47.6 ± 0.8^{i,B}
BHT	4.3 ± 0.4^{f,A}	5.1 ± 0.4^{f,A}	5.7 ± 0.5^{k,A}	8.0 ± 0.4^{j,A}	6.5 ± 0.4^{l,A}	9.9 ± 0.3^{j,A}

[a]Mean value of three measurements ± SD. Different lowercase letters as superscripts indicate significantly different values within each column. Different uppercase letters as superscripts indicate significantly different values within the same row at $P \leq 0.05$. AH, phenolic compound; DPPH•, 1,1-diphenyl-2-picrylhydrazyl; %RSA, percent relative activity; Trolox, 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid.

ble radical and, consequently, the kinetics of the reaction. As known, however, the reaction with certain compounds is completed within a few minutes, whereas slow kinetics have been reported for a great number of antioxidants owing to a more complex mechanism (5). Therefore, by performing a rapid test this piece of information cannot be revealed and results may not be directly used in structure–activity relationship studies. Kinetic studies using DPPH•, although time-consuming, may provide better insight into the real "potency" of each tested compound (5,7).

More critical was the relative concentration of the antioxidant ([AH]/[DPPH•]) ratio that affected the level of the %RSA. The %RSA value for most of the compounds increased concomitantly with the value of the [AH]/[DPPH•] ratio, most likely as a result of higher levels of AH molecules available to scavenge the same amount of the stable radical. This affected the level of the differences observed among the activities of the AH. At a high [AH]/[DPPH•] ratio, no or slight differences could be found among structurally similar AH. For example, dihydrocaffeic, caffeic, and rosmarinic acids had similar %RSA values, as did hydroxytyrosol and oleuropein. At the lowest [AH]/[DPPH•] ratio, these molecules gave rather low %RSA values (≤50%). Thus, optimization of the [AH]/[DPPH•] ratio is important with regard to the potency of the antioxidants under examination. This is expected when the maximal %RSA values range from 60 to 80%.

The environment of the reaction is expected to affect the hydrogen-atom transfer from AH to the free radical (13). However, this parameter seems not to be taken into consideration although in most applications ethanol or methanol is used as the solvent. The choice of solvent for preparing the radical solution could be important in structure–activity studies. In our study the scavenging of DPPH• was carried out in ethanol, and the results were compared with those in acetonitrile and in *tert*-butyl alcohol. These solvents have been used previously to study the kinetics of the phenolic hydrogen-atom transfer from α-tocopherol and phenol to DPPH• (13–15). The rate of this transfer was found to be affected by the ability of the solvent to form intermolecular hydrogen bonds with the AH (13–15). This ability is well described by the β_2^H constant (16). The β_2^H value is the same for ethanol and acetonitrile (0.44) and somewhat higher in *tert*-butyl alcohol (0.49). It is therefore implied that the activity of an AH would be similar in the first two solvents and lower in the third. The results of the effect of the reaction environment on the %RSA are presented in Table 2. Unexpectedly, the activity of the AH in acetonitrile was similar to that in *tert*-butyl alcohol. %RSA values were higher in ethanol and, in general, the relative order in the antioxidant activity seemed to be in line with the rules of structure–activity relationship. Compounds with more than one hydroxyl group (dihydrocaffeic acid, rosmarinic acid, caffeic acid) were the most active, followed by monophenols with methoxy (sinapic, ferulic) or alkyl substituents (e.g., BHA, BHT). Simple phenols without an aromatic ring substituent (coumaric acids, tyrosol) were almost inactive, in line with previous reports (17). In the presence of acetonitrile and *tert*-butyl alcohol, the %RSA values were significantly lower and differences among AH were evident. In some cases, the relative order of activity in the compounds changed.

TABLE 2
Solvent Effect on the Ability of Phenolic Antioxidants to Scavenge the DPPH• Radical[a]

AH	Solvent		
	Ethanol	Acetonitrile	*tert*-Butylalcohol
	(%) RSA[b]		
Dihydrocaffeic acid	93.9 ± 0.5^{a,A}	45.7 ± 0.4^{a,B}	46.3 ± 0.2^{a,B}
Rosmarinic acid	88.4 ± 0.4^{a,A}	71.2 ± 0.1^{b,B}	65.7 ± 0.2^{b,B}
Caffeic acid	76.6 ± 0.5^{b,A}	59.0 ± 0.2^{c,B}	46.9 ± 1.3^{a,B}
Chlorogenic acid	52.0 ± 0.6^{c,A}	37.5 ± 0.4^{d,B}	40.3 ± 1.5^{a,B}
Sinapic acid	56.1 ± 2.0^{d,A}	27.3 ± 1.8^{e,B}	23.7 ± 1.8^{c,B}
Ferulic acid	30.9 ± 2.9^{e,A}	8.7 ± 0.6^{f,B}	6.6 ± 0.1^{d,B}
Isoferulic acid	3.5 ± 0.1^{f,A}	1.8 ± 0.7^{g,B}	1.2 ± 0.1^{e,B}
o-Coumaric acid	3.5 ± 0.3^{f}	0	0
m-Coumaric acid	2.6 ± 0.6^{f}	0	0
p-Coumaric acid	3.6 ± 0.4^{f,A}	1.2 ± 0.5^{g,B}	0
trans-Cinnamic acid	1.5 ± 0.2^{g}	0	0
Hydroxytyrosol	57.0 ± 0.2^{d,A}	32.1 ± 0.8^{d,B}	31.2 ± 0.8^{f,B}
Oleuropein	41.3 ± 0.2^{h,A}	26.9 ± 0.2^{e,B}	24.6 ± 0.2^{c,B}
Tyrosol	2.7 ± 0.3^{f}	0	0
α-Tocopherol	54.0 ± 1.9^{d,A}	49.5 ± 0.6^{h,A}	51.8 ± 1.5^{g,A}
Trolox	53.4 ± 1.2^{d,A}	52.7 ± 1.5^{h,A}	60.5 ± 1.7^{h,B}
TBHQ	58.7 ± 0.8^{d,A}	45.8 ± 0.1^{a,B}	60.9 ± 1.7^{h,A}
BHA	22.3 ± 0.6^{i,A}	14.8 ± 0.7^{i,B}	19.6 ± 1.5^{i,A}
BHT	8.0 ± 0.4^{j,A}	2.5 ± 0.7^{g,B}	6.5 ± 0.7^{d,A}

[a]Reaction period 20 min, [AH]/[DPPH•] = 0.25.
[b]Mean value of three measurements ± SD. Different lowercase letters as superscripts indicate significantly different values within each column. Different uppercase letters as superscripts indicate significantly different values within the same row at $P \leq 0.05$. For abbreviations see Table 1.

In the case of cinnamic acid derivatives, the presence of a second catechol moiety did not infer higher reactivity as expected, since rosmarinic acid was found to be as active as dihydrocaffeic acid in ethanol. However, the effect of the number of the phenolic hydroxyl groups on the activity of the compounds was more obvious (rosmarinic acid was 1.2 and 1.6 times more active than caffeic and dihydrocaffeic acids, respectively) when acetonitrile or *tert*-butyl alcohol was used as a solvent. Caffeic acid remained more active than its esterified form with quinic acid (chlorogenic acid), although the relative difference depended heavily on the solvent. The behavior of dihydrocaffeic acid differed in the three solvent systems. In ethanol, it was the most active of all the derivatives, in *tert*-butyl alcohol its activity was equal to that of caffeic acid, whereas in acetonitrile it was less active than caffeic and rosmarinic acids. The relative order of reactivity was retained among methoxy-substituted phenols. Sinapic was more active than ferulic and isoferulic acids, but the relative differences varied from solvent to solvent. Monophenols with no substituents in the aromatic ring (coumaric acids) were inactive in all solvents.

The order of reactivity of secoiridoids was the same in all three solvents. Oleuropein, the esterified form of hydroxytyrosol with elenolic acid, was less active than hydroxytyrosol, whereas the monophenol, tyrosol, was inactive. Relative differences were of similar size in all solvents. The behavior of α-tocoph-

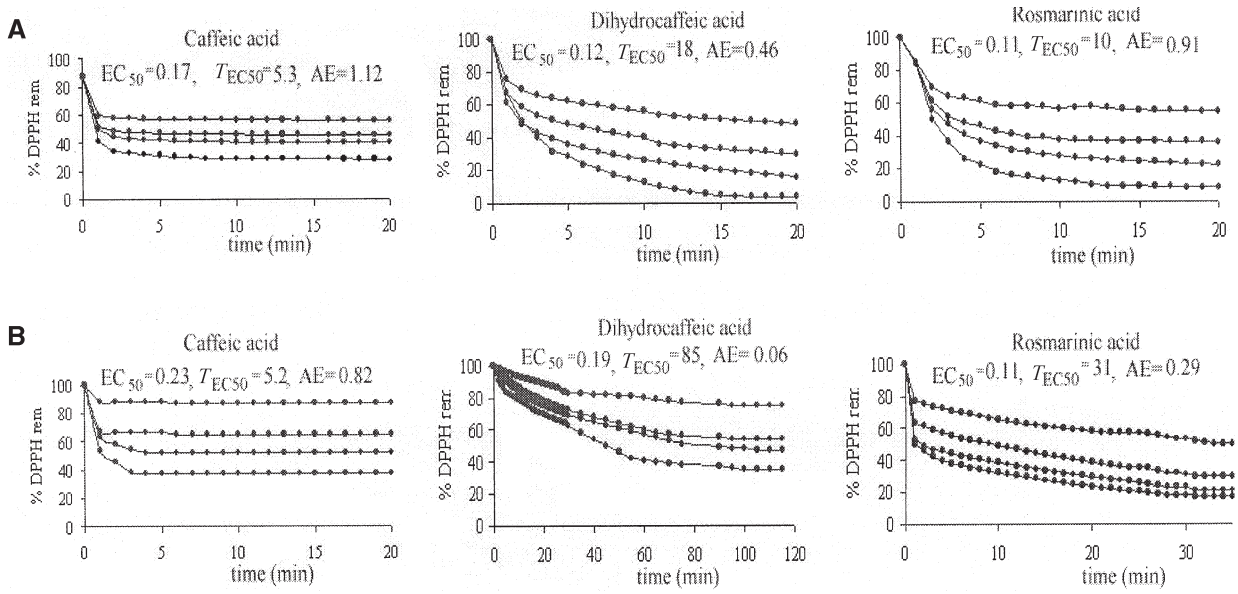

FIG. 1. Kinetic behavior of phenolic antioxidants at [AH]/[DPPH•] = 0.1, 0.15, 0.2, and 0.25 mol/mol. (A) in ethanol; (B) in acetonitrile. EC_{50}, efficient concentration, i.e., the amount of antioxidant necessary to decrease the initial [DPPH•] by 50%; T_{EC50}, reaction time needed to reach the steady state for EC_{50}; AE, antiradical efficiency = $1/EC_{50} \times T_{EC50}$; AH, phenolic compound; DPPH•, 1,1-diphenyl-2-picrylhydrazyl.

erol and synthetic AH was not so dependent on the reaction environment, although the %RSA values differed statistically in some cases. In general, the activity of the more polar compounds was seriously affected by the change of solvent, whereas that of the less polar and more sterically hindered molecules was affected less. The higher %RSA values in ethanol can be attributed to the enhancement of hydrogen-atom transfer as well as to secondary reactions that may take place (5). The lowest %RSA values were found in acetonitrile. Seemingly the role of solvent cannot be interpreted only on the basis of the β_2^H values, and more research is needed on this topic (18–20).

The activity of caffeic, dihydrocaffeic, and rosmarinic acids was also examined by carrying out kinetic studies in ethanol and in acetonitrile. Results are illustrated in Figure 1. Calculation of EC_{50} values for these compounds revealed that rosmarinic and dihydrocaffeic acids were equally active in ethanol; and both of them were more effective than caffeic acid (Fig. 1A). The results were in line with those of the rapid test. However, when the T_{EC50} was included in the calculation of AE value (Fig. 1A), caffeic acid was the most active of the three, whereas differences were revealed between rosmarinic and dihydrocaffeic acids. Slightly higher EC_{50} values were obtained in acetonitrile (Fig. 1B), but the order of activity was the same as in ethanol. When the AE values were taken into consideration, the order was reversed for caffeic acid and its saturated counterpart in this solvent (Fig. 1B). This was due to the slow kinetics observed for dihydrocaffeic acid (T_{EC50} 85 min), in agreement with findings indicating that in less polar solvents the time needed for the stoichiometric reaction was longer than that in polar media (19). Such observations could not be made using the rapid DPPH• test (Table 2). In summary, in rapid DPPH• tests the duration of the test, the [AH]/[DPPH•] molar ratio, and in particular the reaction environment may affect the level of the relative differences or the order of activity among compounds. Standardization of the analytical protocol should include a 20-min reaction period and a molar ratio efficient to reach a 60–80 %RSA for the most potent antioxidant. Solvent choice is critical for classifying antioxidant activity. Safe classification can be based only on results from kinetic studies.

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