Antioxidant Activity of Dietary Polyphenols As Determined by a Modified Ferric Reducing/Antioxidant Power Assay

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Most nonenzymatic antioxidant activity (scavenging of free radicals, inhibition of lipid peroxidation, etc.) is mediated by redox reactions. The antioxidant (AO) activity of polyphenols (PPs), as ferric-reducing power, was determined for the first time using a modified FRAP (ferric reducing/antioxidant power) assay. Reaction was followed for 30 min, and both Fe(II) standards and samples were dissolved in the same solvent to allow comparison. Selected representative PPs included flavonoids (quercetin, rutin, and catechin), resveratrol, tannic acid, and phenolic acids (gallic, caffeic, and ferulic). Carotenoids (β-carotene and zeaxanthine), ascorbic acid, Trolox, and BHA were included for comparison. Equivalent concentration 1 (EC_1), as the concentration of AO with a reducing effect equivalent to 1 mmol/L Fe(II), was used to compare AO efficiency. PPs had lower EC_1 values, and therefore higher reducing power, than ascorbic acid and Trolox. Tannic acid and quercetin had the highest AO capacity followed by gallic and caffeic acids. Resveratrol showed the lowest reducing effect. Carotenoids had no ferric reducing ability. Polyphenol's AO efficiency seemed to depend on the extent of hydroxylation and conjugation.

Keywords: *Antioxidant activity; dietary antioxidants; polyphenols; ferric reducing ability*

INTRODUCTION

Research in recent years has shown the implication of oxidative and free-radical-mediated reactions in degenerative processes related to aging (Ames et al., 1993; Harman, 1995) and diseases such as cancer, coronary heart disease, and neurodegenerative disorders such as Alzheimer's disease, etc. (Ames, 1983; Gey, 1990; Smith et al., 1996; Diaz et al., 1997). Antioxidant defenses in the organism against reactive oxygen species (prooxidants and free radicals) produced during normal cell aerobic respiration may be of endogenous (enzymatic and nonenzymatic) or dietary origin (vitamins, carotenoids, flavonoids, etc.) (Harman, 1995). When natural defenses are overwhelmed by an excessive generation of prooxidants, a situation of oxidative stress evolves; cellular and extracellular macromolecules (proteins, lipids, and nucleic acids) can suffer oxidative damage, causing tissue injury (Halliwell and Gutteridge, 1989; Halliwell and Aruoma, 1991; Halliwell and Chirico, 1993), and affecting immune function (Meydani et al., 1995; Hughes, 1999). Increased intakes of dietary antioxidants may help to maintain an adequate antioxidant status, defined as the balance between antioxidants and oxidants in living organisms (Halliwell et al., 1995). Epidemiological studies support this protective effect of dietary antioxidants: increased intakes of fruit and vegetables have been related to a reduction of the risk of cardiovascular disease and certain types of cancer (Gillman et al., 1995; Kohlmeier et al., 1995; Steinmetz and Potter, 1996; Hininger et al., 1997; Ness and Powles, 1997). Dietary antioxidants other than vitamins (i.e., polyphenolic compounds such as flavonoids) have been shown to be a major dietary factor responsible for such protective effects (Hertog, 1996; Hertog et al., 1994, 1995, 1997).

A number of methods have been developed to measure the efficiency of dietary antioxidants either as pure compounds or in food extracts, as well as to determine the antioxidant activity of plasma as an index of the antioxidant status in vivo. These methods focus on different mechanisms of the antioxidant defense system, i.e., scavenging of oxygen and hydroxyl radicals, reduction of lipid peroxyl radicals, inhibition of lipid peroxidation, or chelation of metal ions. Thus, some methods determine the ability of antioxidants to scavenge free radicals generated in the reaction medium such as the TEAC (Trolox equivalent antioxidant capacity; Miller et al., 1993), ORAC (oxygen radical absorbance capacity; Cao et al., 1995), or TRAP (total reducing ability of plasma; Ghiselli et al., 1995) assays. Other methods such as the DPPH$^•$ (2,2-diphenyl-1-picrylhydrazyl; Brand-Williams et al., 1995; Sanchez-Moreno et al., 1998) or DMPD$^•$ (Fogliano et al., 1999) measure the scavenging of stable radical species by antioxidants. The xanthine/xanthine oxidase assay determines the efficiency of antioxidants quenching singlet oxygen generated by the enzymatic system (Robak and Gryglewski, 1988). Other methods evaluate the inhibition of lipid peroxidation by antioxidants, quantifying products such as conjugated dienes (Esterbauer et al., 1989), lipid peroxides (Kikuzaki and Nakatani, 1993), or hydroperoxides (Nourooz-Zadeh et al., 1994), as well as products resulting from the decomposition of lipid peroxides such as malondialdehyde determined by the TBARS (thiobarbituric acid reactive substances; Plumb et al., 1996) method, just to mention some of the most commonly used methodologies.

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In most cases, irrespectively of the stage in the oxidative chain in which the antioxidant action is assessed, a common mechanism involving a redox reaction takes place. On this basis, Benzie and Strain (1996) developed a methodology to determine the reducing ability of plasma as a measure of its antioxidant power. This is a simple method of determining the reduction of a ferric−tripyridyltriazine complex to its ferrous, colored form in the presence of plasma antioxidants, i.e., the ferric reducing ability of plasma or FRAP assay. Initially designed to determine the antioxidant activity of plasma, it was also applied to other substrates such as tea and wine (Benzie and Strain, 1999; Benzie and Szeto, 1999) and renamed as the ferric reducing/antioxidant power assay (Benzie and Strain, 1999).

Most in vitro methods have shown that dietary polyphenols are important antioxidants. However, the reducing ability of these compounds, as a measure of their antioxidant capacity, has not been determined as yet. The present work was aimed at studying the ferric reducing efficiency of polyphenolic compounds representative of the most common structures occurring in plant foods. Other dietary antioxidants such as carotenoids and ascorbic acid as well as Trolox and BHA were also used for comparison. A modified FRAP method was used. To allow for a better comparison of results, a new parameter, the equivalent concentration 1 or EC_1 was defined.

MATERIALS AND METHODS

Standard and Samples. Flavonoids (quercetin, rutin, and catechin), the stilbene resveratrol, and phenolic acids (tannic acid, gallic acid, caffeic acid, and ferulic acid) were all from Sigma Chemical Co. (St. Louis, MO), as well as the carotenoids β-carotene and zeaxanthin. Ascorbic acid was purchased from Panreac Química S.A. (Barcelona, Spain). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble analogue of vitamin E, was from Aldrich Chemical Co. (Guillingham, Dorset, U.K.). BHA (3-*tert*-butyl-4-hydroxyanisole) was purchased from Merck Farma y Química S.A. (Madrid, Spain), and TPTZ (2,4,6-tripyridy-*s*-triazine) from Fluka Chemicals (Madrid, Spain). All other reagents were of analytical grade.

Aqueous and methanolic solutions were prepared daily, as were the reagents for the FRAP assay. Ascorbic acid was prepared and used within the hour.

FRAP Assay. The antioxidant capacity of each standard (aqueous or methanolic solutions) was estimated according to the procedure described by Benzie and Strain (1996) with some modifications. Briefly, 900 μL of FRAP reagent, prepared freshly and warmed at 37 °C, was mixed with 90 μL of distilled water and 30 μL of test sample, water, or methanol as appropriate for the reagent blank. The final dilution of the test sample in the reaction mixture was 1/34. The FRAP reagent contained 2.5 mL of a 10 mmol/L TPTZ solution in 40 mmol/L HCl plus 2.5 mL of 20 mmol/L $FeCl_3 \cdot 6H_2O$ and 25 mL of 0.3 mol/L acetate buffer, pH 3.6 (Benzie and Strain, 1996).

Readings at the absorption maximum (595 nm) were taken every 15 s using a Beckman DU-640 spectrophotometer (Beckman Instruments Inc., Fullerton, CA) equipped with a thermostatized auto-cell-holder. Temperature was maintained at 37 °C and the reaction monitored for up to 30 min.

Aqueous and methanolic solutions of known Fe(II) concentrations in the range of 100−2000 μmol/L ($FeSO_4 \cdot 7H_2O$) were used for calibration.

The parameter *Equivalent Concentration 1* or EC_1 was defined as the concentration of antioxidant having a ferric−TPTZ reducing ability equivalent to that of 1 mmol/L $FeSO_4 \cdot 7H_2O$. EC_1 was calculated as the concentration of antioxidant giving an absorbance increase in the FRAP assay equivalent to the theoretical absorbance value of a 1 mmol/L concentration of Fe(II) solution determined using the corresponding regression equation.

Table 1. Comparison of the FRAP Values at 4 min (μmol equiv of Fe(II)/L) of Aqueous and Methanolic Solutions of Polyphenols (250 μmol/L) Calculated with the Respective Calibration Curves of $FeSO_4$ in Water or Methanol (Mean Values ± STD; $n = 3$)[a]

	aqueous solutions[b]	methanolic solutions[c]
gallic acid	$1281 \pm 27^{a,\alpha}$	$1374 \pm 22^{a,\beta}$
tannic acid	$1283 \pm 77^{a,\alpha}$	$1625 \pm 36^{b,\beta}$
catechin	$579 \pm 13^{b,\alpha}$	$642 \pm 29^{c,\beta}$

[a] Values within columns followed by different Latin superscript letters are statistically different ($P < 0.05$). Values within rows followed by different Greek superscript letters are statistically different ($P < 0.05$). [b] Polyphenols and Fe(II) in water. [c] Polyphenols and Fe(II) in methanol.

Statistical Analysis. Samples were analyzed in triplicate. Simple regression analysis was performed to calculate the dose−response relationship of the standard Fe(II) solutions used for calibration as well as of the test samples. One-way analysis of variance (ANOVA) followed by Duncan's multiple comparison test was performed to assess the influence of solvent media in the FRAP assay (significance level $P < 0.05$). A Statgraphics Plus program version 2.1 (Statistical Graphics Corp., Rockville, MD) was used.

RESULTS AND DISCUSSION

The FRAP assay was developed to determine the ferric reducing ability of biological fluids and aqueous solutions of pure compounds. However, due to the characteristics of the standards used in the present study, some of them had to be dissolved in an alcoholic solvent (i.e., methanol). Although the final dilution of the sample in the reaction mixture was high (1/34), we wanted to check for any possible influence of the solvent on the reaction. Therefore, aqueous and methanolic solutions of the $FeSO_4 \cdot 7H_2O$ standard used for calibration were prepared and analyzed with the FRAP reagent. Ferrous sulfate dissolved well in both solvents, and there was no precipitation problem. Equations defining the regression curves of the aqueous and alcoholic Fe(II) solutions showed different values for the intercepts (-20.51×10^3 vs -1.90×10^3) and slopes (0.65×10^3 vs 0.43×10^3) for the aqueous and methanolic solutions, respectively. This points out the importance of the dissolvent influencing the redox potential. Differences in the FRAP values of the samples were also shown (Table 1) when polyphenols were dissolved in distilled water or methanol and the FRAP values calculated using the respective Fe(II) calibration curves (aqueous or methanolic Fe(II) solutions). Therefore, care should be taken when nonaqueous samples are analyzed and calculations of FRAP values are compared with those of the corresponding Fe(II) solutions.

To further illustrate this point, Table 2 shows the FRAP values of the studied antioxidants (polyphenols, ascorbic acid, and Trolox) in methanol calculated using the calibration curves obtained from aqueous and methanolic solutions of the Fe(II) standard. FRAP values were consistently higher when referred to the corresponding Fe(II) methanolic standard. On the contrary, an underestimation of the ferric reducing ability of samples in alcoholic media occurred when compared to an Fe(II) aqueous standard curve. To compare the ferric reducing ability of the antioxidants used in the present study, standards were dissolved in methanol

Table 2. Comparison of FRAP Values at 4 min (μmol equiv of Fe(II)/L) of Methanolic Solutions of Standards (250 μmol/L) Calculated Using Fe(II) Calibration Curves in Methanol and Distilled Water (Mean Values ± STD; *n* = 3)[a]

	Fe(II) in methanol	Fe(II) in distilled water
quercetin	$1926 \pm 43^{a,\alpha}$	$1338 \pm 29^{a,\beta}$
tannic acid	$1625 \pm 36^{b,\alpha}$	$1115 \pm 24^{b,\beta}$
caffeic acid	$1430 \pm 62^{c,\alpha}$	$985 \pm 41^{c,\beta}$
gallic acid	$1374 \pm 22^{c,\alpha}$	$948 \pm 41^{c,\beta}$
Trolox	$709 \pm 67^{d,\alpha}$	$506 \pm 45^{d,\beta}$
ascorbic acid	$647 \pm 53^{d,\alpha}$	$465 \pm 35^{d,\beta}$
catechin	$642 \pm 29^{d,\alpha}$	$461 \pm 19^{d,\beta}$
resveratrol	$343 \pm 10^{e,\alpha}$	$262 \pm 6^{e,\beta}$

[a] Values within columns followed by different Latin superscript letters are statistically different ($P < 0.05$). Values within rows followed by different Greek superscript letters are statistically different ($P < 0.05$).

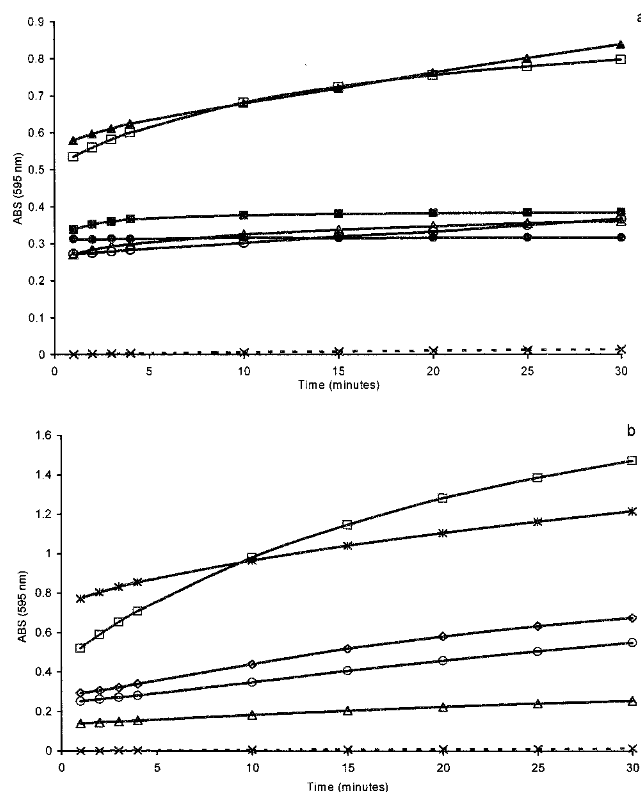

Figure 1. FRAP reaction kinetics of reagent blanks (×) and 250 μmol/L methanolic solutions of antioxidants: (a) ▲, caffeic acid; □, gallic acid; ■, BHA; ●, Trolox; △, ferulic acid; ○, ascorbic acid; (b) *, quercetin;, □, tannic acid; ◇, rutin; ○, catechin; △, resveratrol.

and FRAP values calculated using an Fe(II) calibration curve also in methanol.

Conditions for the determination of the ferric reducing ability of antioxidants in the original method established a 4 min interval as suitable for such measurements, since the absorbance of the reduced ferrous−TPTZ complex was stable at this time (Benzie and Strain, 1996). When these conditions were used in the present study, we observed that the reaction had not finished after 4 min and reduction of the ferric−TPTZ complex continued. Except for Trolox and BHA (Figure 1a), as well as uric acid (results not shown) in which the absorbance did not increase after 4 min, agreeing with data reported by Benzie and Strain (1996), all the other assayed compounds showed a steady absorbance increase with time. We prolonged the reaction for several hours and observed a continuos increment of absorbance at 595 nm at different time intervals. However, after the initial 30 min, the order of antioxidant efficiency of the studied samples was maintained. Therefore, we selected this time for absorbance readings, yet also keeping the 4 min absorbance recording established in the original method for comparison.

Figure 1 shows the reaction kinetics of the studied samples. As can be seen, some antioxidants even doubled their initial absorbance after 30 min of reaction, as was the case with tannic acid (Figure 1b). Nevertheless, these increments in the absorbance readings were not caused by alterations of the reaction mixture with time, since blank samples showed no significant modification of their initial absorbance.

Following these analytical conditions (i.e., 30 min reaction time and samples in methanolic solutions), the ferric reducing ability of different concentrations of antioxidants was determined. However, because carotenoids were insoluble in methanol, they had to be dissolved in tetrahydrofuran (THF). This nonpolar reagent showed no interference in the FRAP assay as verified when polyphenols were added to the reaction mixture in the presence of THF. Nevertheless, none of the assayed carotenoids (β-carotene and zeaxanthin) showed a positive reaction in the FRAP assay, the absorbance values remaining unchanged and similar to the blank reading during the 30 min interval. Therefore, it was not possible to determine the reducing ability of carotenoids as an index of their antioxidant capacity using the present methodology. This implies that carotenoids do not contribute to the ferric reducing ability of plasma.

Carotenoids have shown contradictory results in different methods to assess their inhibitory effect on low-density lipoprotein (LDL) oxidation. Some authors reported no protection of β-carotene to copper-mediated LDL oxidation (Princen et al., 1992; Reaven et al., 1993) or even a prooxidant effect (Gaziano et al., 1995). On the contrary, other in vitro studies suggest a protective effect of β-carotene (Jialal et al., 1991; Packer, 1993). However, this protective action of carotenoids differed from that of vitamin E in that it was not based on redox reactions (Packer, 1993), which agrees with the nonreducing ability of carotenoids in the FRAP assay reported here.

Table 3 shows the regression equations for all the antioxidants tested (polyphenols, ascorbic acid, Trolox, and BHA), including $FeSO_4$, as well as the correlation coefficients and concentration intervals assayed at both 4 and 30 min of reaction. In all cases, a linear dose−response relationship was observed over a wide range of concentrations. The $FeSO_4$ and Trolox curves showed similar regression equations at 4 and 30 min, with no changes in their slopes as expected for these samples, showing no alterations of their ferric reducing ability with time (Figure 1a). All the other samples had different equations at 4 and 30 min, with higher slope values at 30 min denoting higher ferric reducing ability at this time as compared with the values at 4 min. Slope values allow for a comparison of the antioxidant efficiencies of the standards. However, a more straightforward way to compare the antioxidant efficiencies should be envisaged to facilitate such comparisons. Thus, we propose the equivalent concentration 1 or EC_1 as the concentration of antioxidant having a ferric−TPTZ reducing ability equivalent to that of a 1 mmol/L

Table 3. Assayed Concentration Intervals (μmol/L) and Regression Equations with correlation coefficients (r) of $FeSO_4$ and Different Antioxidants at 4 and 30 min of Reaction with the FRAP Reagent[a]

		4 min			30 min		
standard	lineal interval (μmol/L)	intercept (x10^3)	slope (x10^3)	r	intercept (x10^3)	slope (x10^3)	r
$FeSO_4$	100−2000	1.90	0.43	0.9977	4.29	0.43	0.9978
quercetin	25−500	−26.29	3.69	0.9969	−36.02	5.19	0.9981
tannic acid	25−500	52.95	2.40	0.9932	143.81	4.59	0.9898
gallic acid	50−500	−38.02	2.64	0.9980	−36.22	3.35	0.9986
caffeic acid	25−750	53.88	1.96	0.9950	60.31	2.74	0.9953
BHA	50−750	−7.05	1.49	0.9955	−10.55	1.59	0.9951
rutin	50−1000	−0.36	1.38	0.9973	45.19	2.48	0.9954
trolox	50−1000	−25.42	1.44	0.9958	−22.79	1.44	0.9957
catechin	50−750	−28.17	1.34	0.9987	8.65	2.25	0.9991
ferulic acid	50−1000	15.04	1.08	0.9933	19.51	1.32	0.9928
ascorbic acid	50−1000	0.45	1.11	0.9989	15.34	1.37	0.9991
resveratrol	100−1000	9.31	0.58	0.9971	51.35	0.78	0.9950

[a] Equations were calculated with six different concentrations assayed in triplicate ($P < 0.01$). All equations followed a lineal regression model.

Table 4. EC_1 Values and Confidence Limits of Antioxidants at 4 and 30 min of Reaction with the FRAP Assay[a]

	4 min		30 min	
standard	EC_1 (μmol/L)	confidence limits (95%)	EC_1 (μmol/L)	confidence limits (95%)
quercetin	126	119−133	92	86−98
tannic acid	160	150−170	66	52−80
gallic acid	180	174−186	142	137−147
caffeic acid	196	183−209	139	125−154
BHA	299	284−313	283	268−298
rutin	316	303−330	162	140−183
trolox	322	305−339	322	305−340
catechin	348	340−356	192	186−198
ferulic acid	390	368−411	319	296−343
ascorbic acid	392	384−400	310	302−318
resveratrol	729	714−744	497	481−513

[a] EC_1 = concentration of antioxidant having a ferric reducing ability equivalent to that of 1 mmol/L $FeSO_4 \cdot 7H_2O$. Data are the mean values of three different determinations.

concentration of $FeSO_4 \cdot 7H_2O$. The higher the EC_1 value, expressed as μmol/L, the lower the antioxidant activity.

Table 4 shows the EC_1 values and confidence limits at 4 and 30 min of the studied antioxidants. EC_1 values, and therefore ferric reducing ability, were lower at longer reaction times as expected from the kinetic behavior of the samples. Some polyphenols such as tannic acid, rutin, and catechin almost doubled their antioxidant capacity at 30 min. This resulted in a different classification in terms of antioxidant efficiency of the studied compounds at short versus long reaction times. In both cases, quercetin and tannic acid showed the lowest EC_1 values, followed by gallic and caffeic acids, although the order changed at 4 and 30 min. Trolox had an intermediate antioxidant activity at 4 min, but was surpassed by most compounds after 30 min. Ascorbic acid had a ferric reducing ability lower than those of most polyphenols. Resveratrol, a stilbene present in grapes and wine, showed the lowest reducing ability of all the studied antioxidants, which agrees with results reported by Frankel et al. (1993). These authors showed that resveratrol was less effective than quercetin or epicatechin toward reduction of hexanal generation from oxidized LDL.

In general, the classification of antioxidants according to their ferric reducing ability agreed with results reported by other authors using different methods to estimate antioxidant power. Thus, Cuvelier et al. (1992) found that the ability of phenolic compounds to inhibit the autoxidation of methyl linoleate decreased in the order caffeic acid > gallic acid > BHA > ferulic acid. Wang and Goodman (1999) reported a decreased effect of polyphenols inhibiting peroxidation of LDL in the order quercetin > caffeic acid > rutin > gallic acid > ferulic acid. Brand-Williams et al. (1995) found that gallic acid was the most effective polyphenol scavenging the $DPPH^{\bullet}$ radical, followed by caffeic acid, BHA, ferulic acid, and vitamin C. In a recent work using the same method to estimate the antiradical power, we found the following order of antioxidant efficiency: gallic acid > tannic acid > ascorbic acid > quercetin > BHA > rutin > ferulic acid > DL-α-tocopherol > resveratrol (Sanchez-Moreno et al., 1998). Finally, Rice-Evans et al. (1995) using the TEAC method observed a decreased efficiency of polyphenols toward the scavenging of the $ABTS^{\bullet+}$ radical in the order quercetin > rutin > catechin > ascorbic acid > DL-α-tocopherol. The fact that all these methods showed results in terms of antioxidant efficiency of polyphenols similar to the ones obtained using the FRAP assay suggests that the reducing ability of polyphenols seems to be an important factor dictating the antioxidant and free-radical-scavenging capacity of these compounds.

Each one of the methods mentioned above measures the effect of antioxidants in different steps of the oxidative chain (i.e., inhibition of lipid peroxidation and scavenging of free radicals), and thus the mechanism of antioxidative action is different. However, it has been seen that the ability of monomeric phenolics as antioxidants depends on the degree of hydroxylation and extent of conjugation (Hodnick et al., 1988). Specifically for flavonoids, it has been suggested that the radical scavenging ability increases when the following conditions are met: (i) the presence of a 3′,4′-dihydroxy structure in the B ring; (ii) the presence of a 2,3-double bond in conjunction with the 4-oxo group in the heterocycle, allowing for conjugation between the A and B rings; (iii) the presence of 3- and 5-hydroxyl groups in the A ring together with a 4-oxo function in the A and C rings (Bors et al., 1990; Rice-Evans et al., 1996). Our results with flavonoids, as those of other authors mentioned above, agree with these criteria: quercetin, meeting all three conditions (Figure 2), was more potent than rutin (meeting criteria i and ii) and catechin (meeting i and iii). In flavonoids, the redox potential seems to be controlled by the catechol-type structure in the B ring (with an *o*-dihydroxy substitution pattern), and the presence of a 2,3-double bond in the C ring

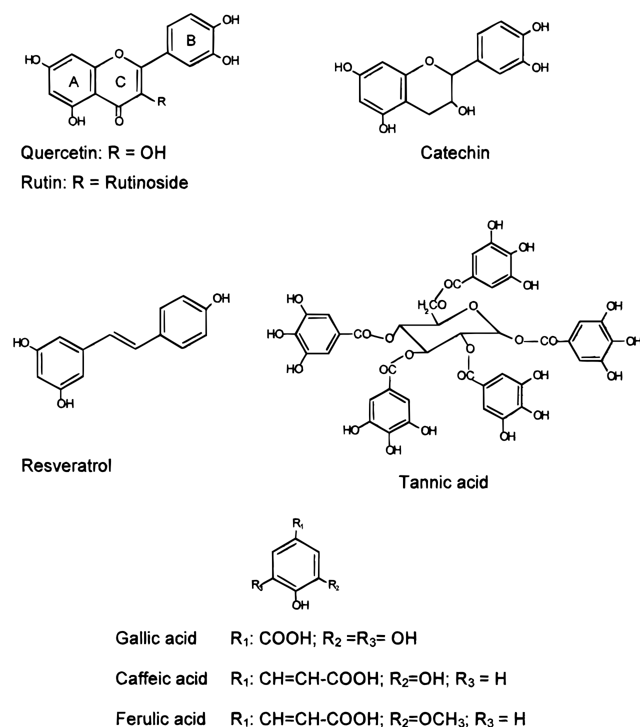

Figure 2. Chemical structures of the assayed polyphenols.

(Rice-Evans et al., 1996). This agrees with the higher reducing ability of rutin, with the 3-hydroxy group blocked by glycosilation, as compared with catechin, with no 2,3-double bond. As to the phenolic acids, the number and position of hydroxyl groups rule their antioxidant activity (Rice-Evans et al., 1996). The hydrolyzable tannin tannic acid, with the highest degree of hydroxylation, showed the highest reducing ability at the end of the assay. Gallic and caffeic acids had similar ferric reducing power, much higher than that of ferulic acid, the methoxyl group greatly reducing the antioxidant power of this hydroxycinnamic acid as also shown with other methods (Brand-Williams et al., 1995; Wang and Goodman, 1999). The stilbene resveratrol, although having three hydroxyl groups and conjugation between both aromatic rings (Figure 2), has a structure different from the diphenylpropane skeleton of flavonoids, and no *o*-dihydroxy phenolic structure in the B ring. This may account for the low reducing power of this compound, the polyphenol with the lowest antioxidant capacity, which was in agreement with previous results using the $DPPH^{\bullet}$ method (Sanchez-Moreno et al., 1998).

The antioxidant efficiency determined by the present FRAP assay depends on the redox potentials of the compounds under study, characterized by the complexity of their molecules. From our results, it is apparent that the reducing ability of polyphenols, as determined by the FRAP assay, seems to depend on the degree of hydroxylation and extent of conjugation of the phenolic compounds.

Concerning the implication of the time-dependent increase in the ferric reducing ability of polyphenols, some redox reactions in which antioxidants intervene, such as the scavenging of the extremely reactive hydroxyl radical ($OH^{\bullet}$), are very rapid. In this case, antioxidants would also act rapidly, the most efficient likely being those compounds that showed higher Fe(III) reduction at the beginning of the reaction (Figure 1) or after 4 min. However, the fact that the studied antioxidants retain and even increase their reducing ability with time might imply an ability to maintain their antioxidant activity for longer times, helping to maintain an adequate antioxidant status in vivo. When these polyphenols are present in foods naturally or as food ingredients, an increased reducing ability with time might signify a longer protecting effect of polyphenols against oxidative damage of the food material.

As to the biological significance of the results obtained with the studied antioxidants, this is difficult to predict. None of the methodologies described in the literature to determine the antioxidant status in plasma perform such determinations in physiological conditions. This was also the case for the FRAP assay, which is performed at very low pH (3.6), different from the pH found in biological fluids. However, this method has the advantage of determining the antioxidant activity directly in whole plasma, it is not dependent on enzymatic or nonenzymatic methods to generate free radicals prior to the evaluation of the anti-radical efficiency of plasma, no isolation of plasma fractions such as LDL is required, etc. Therefore, methodologically the FRAP assay has advantages over other methodologies commonly used, and the results obtained are comparable, as shown in the present work with pure compounds. On the other hand, the redox potentials of polyphenolic compounds are more negative at higher pH (Simic and Jovanovic, 1994; Hagerman et al., 1998). The lower the redox potential, the higher the antioxidant efficiency against free radicals such as the peroxyl or hydroxyl radicals, which have more positive redox potentials (about 1000 and 2310 mV at pH 7, respectively) (Simic and Jovanovic, 1994). Therefore, it should be expected that these compounds keep their antioxidant activity at physiological pH. Although the relative efficiencies in those conditions are not predictable, the results reported here might be representative of biological activity.

CONCLUSIONS

The FRAP assay is a simple and reproducible method which can be applied not only to the study of the antioxidant activity of plasma or antioxidants in food extracts and beverages, but also to the study of the antioxidant efficiency of pure dietary antioxidants with results comparable to those obtained with other more complex methodologies, as shown here. Care should be taken and homogeneity pursued in the use of solvents used to prepare samples and the Fe(II) calibration standard to avoid possible errors. Reaction times longer than 4 min showed that antioxidants followed different kinetic behavior with time-dependent increases in their ferric reducing ability.

The degree of hydroxylation and extent of conjugation seemed to be the criteria ruling the reducing power of dietary polyphenols. From the results obtained here and from results reported by other authors, it was apparent that polyphenols have higher in vitro antioxidant capacity than ascorbic acid and DL-α-tocopherol. This, along with the fact that these compounds are present in significant amounts in most fruits and vegetables, emphasizes the significance of polyphenols as dietary antioxidants, and stresses the importance of deepening the understanding of their bioavailability and effects in vivo.

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