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Evaluation of Antioxidant Activity of Some Natural Polyphenolic Compounds Using the Briggs−Rauscher Reaction Method

RINALDO CERVELLATI,*,† CECILIA RENZULLI,‡ MARIA CLELIA GUERRA,‡ AND ESTER SPERONI‡

Dipartimento di Chimica "G. Ciamician", Università di Bologna, Via Selmi 2, I-40126 Bologna, Italy, and Dipartimento di Farmacologia, Università di Bologna, Via Irnerio 48, I-40126 Bologna, Italy

A new method based on the inhibitory effects of antioxidants on the oscillations of the hydrogen peroxide, acidic iodate, malonic acid, and Mn(II)-catalyzed system (known as the Briggs−Rauscher reaction), was used for the evaluation of antioxidative capacity. With this method, which works near the pH of the fluids in the stomach (pH ≈2), a group of natural compounds present in fruits and vegetables or in medicinal plants assumed to have antioxidant capacity, was tested successfully. The aim of the present study is to evaluate the antioxidative properties of some active principles contained in vegetables and aromatic plants, namely, cynarin (from *Cynara scolymus*), rosmarinic acid (from *Rosmarinus officinalis*), echinacoside (from *Echinacea* species), puerarin (from *Pueraria lobata*), and oleuropein (from *Olea europea*). Also studied with the Briggs−Rauscher reaction method was the antioxidant activity of cyanidin 3-*O*-β-glucopyranoside (from *Citrus aurantium*) in order to compare the results with those obtained by other methods. The conclusions on the dependency of the antioxidative activity on the pH of the testing system are given.

KEYWORDS: Antioxidants; phenolics; fruit; aromatic plants; health benefits; Briggs−Rauscher reaction

INTRODUCTION

Free radicals (FR) and reactive oxygen species (ROS) including lipid peroxides have been suggested as potentially being important causative agents of aging and several human diseases such as cancer, inflammatory and degenerative diseases, emphysema, central nervous system injury, autoimmune disease, anemia, ischemia, and reperfusion injury (*1*−*4*). They can also be responsible for the toxicity of environmental xenobiotics, some anti-neoplastic agents, and radiations (*5*). The use of antioxidants for the prevention of damage caused by free radicals thereby assumes great importance for health and traditional medicine (*6*). In the past few years, there has been considerable interest in natural products endowed with antioxidant properties. A lot of traditionally used medicinal plants have been proposed for their interesting antioxidant activities and recommended for people's normal diets, as Rice-Evans et al. noted (*7*). In fact, it is widely accepted that diets rich in fruits and vegetables are protective against several human diseases. The protective effects have been attributed to the antioxidant nutrients present such as vitamins C and E but also to the minor polyphenolic components of plants. These components contain a number of phenolic hydroxyl groups attached to ring structures responsible for antioxidant activity (*8*) and often occur in the glycosidic form. For example, the antioxidant properties of cyanidin [2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxyflavonol] contained in cherries, raspberries, strawberries, and grapes as 3-glucoside have been studied in vitro both with the original Trolox equivalent antioxidant capacity (TEAC) assay (*7, 9*) and with the improved one (*10*). Among the several testing methods available to determine the relative antioxidant activity of pure compounds, plant extracts, drinks, etc., the TEAC assay was recently found to be the best under instrumental requirements and time aspects (*11*). A commonly used test, based on TEAC, is the determination of the total antioxidant status (TAS) using a test kit purchased from Randox Laboratories. The TEAC assay and many other methods work at the pH of blood (pH 7.4), but there are also methods working at higher pH values (*12*).

All of the methods are based on the formation of free radicals in a reaction mixture and the effects of added antioxidants on some properties of the radicals (or of the mixture): absorbance at a given wavelength, quenching of chemiluminescence, electric potential of the mixture, etc. In the presence of antioxidants these properties change depending on the amount of antioxidant added with respect to those of a reference mixture.

A new method for the measurement of antioxidative capacity reported by Cervellati et al. (*13*) is based on the inhibitory effects of antioxidants on the oscillations of the hydrogen peroxide, acidic iodate, malonic acid, and Mn(II)-catalyzed system, known as the Briggs−Rauscher (BR) reaction (*14*). This method works near the pH of the fluids in the stomach (pH ≈2) and was tested successfully on a number of white wines (*15*). Because fruits, vegetables, some drinks (teas, fruit juices, etc.), healthy foods,

* Corresponding author (telephone +390512099467; fax +390512099456; e-mail rcerv@ciam.unibo.it).
† Dipartimento di Chimica "G. Ciamician".
‡ Dipartimento di Farmacologia.

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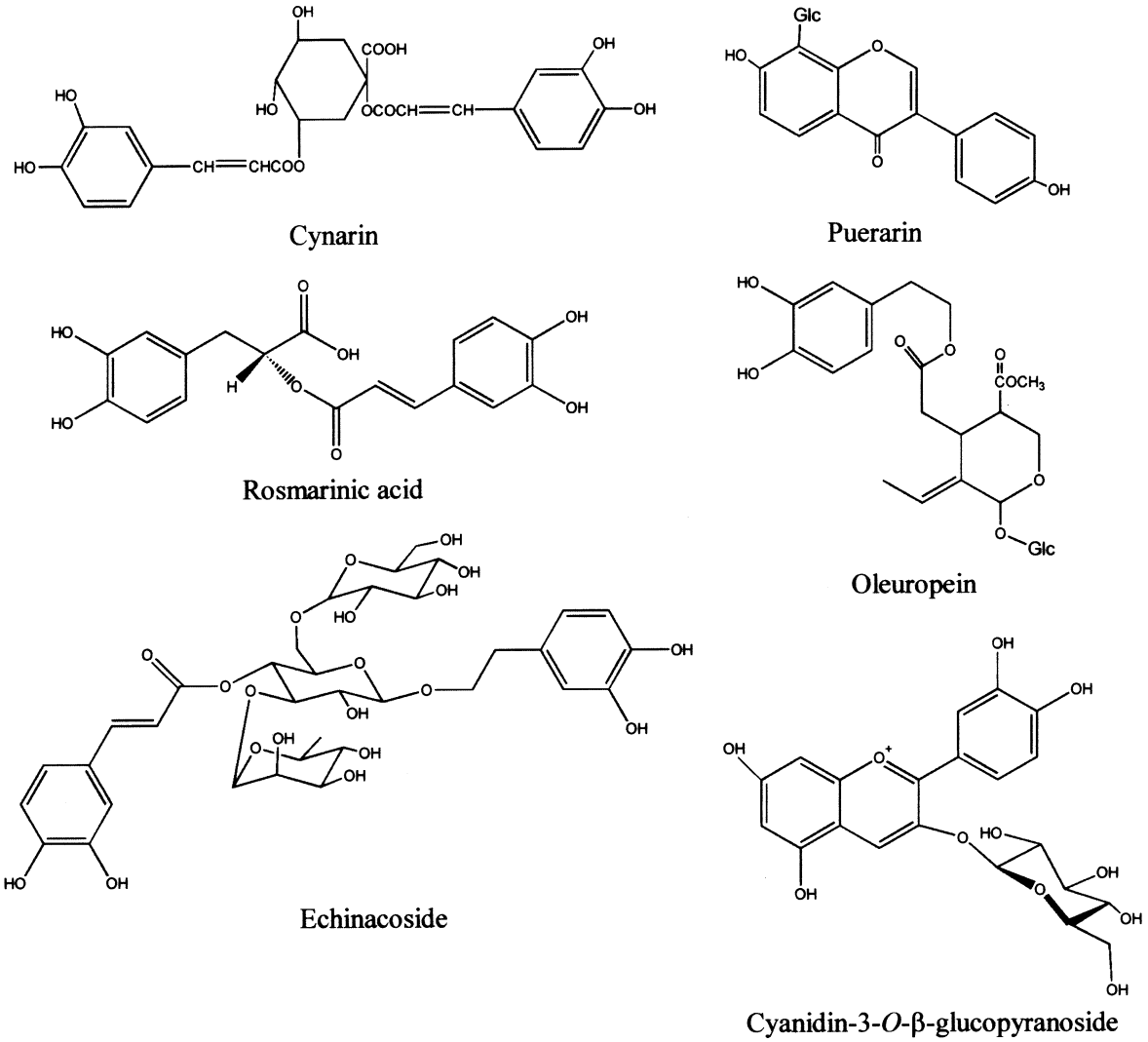

Figure 1. Structural formulas of the antioxidants studied.

and vitamins are usually assumed per os, it is conceivable that they exhibit their first antioxidant capacity against free radicals produced in the stomach, thus preventing medical problems of this organ. Therefore, the BR reaction method can give useful information on the antioxidant activity at acidic pH values.

The aim of the present study is to evaluate the antioxidative properties of some active components contained in vegetables and aromatic plants, namely, cynarin (from *Cynara scolymus*), rosmarinic acid (from *Rosmarinus officinalis*), echinacoside (from *Echinacea* species), puerarin (from *Pueraria lobata*), and oleuropein (from *Olea europea*). We also studied with the BR reaction method the antioxidant activity of cyanidin 3-*O*-*β*-glucopyranoside (from *Citrus aurantium*) in order to compare the results with those obtained with the TEAC method. The antioxidant properties of rosmarinic acid were also studied at pH 7.4 but with a different assay: the ORAC method (*16*). The present investigation provides conclusions on the dependency of the antioxidative activity on the pH of the testing system.

The structures of the six compounds studied are reported in **Figure 1**. The principles of the BR reaction method are briefly sketched in the following section.

PRINCIPLES OF THE BR OSCILLATING REACTION METHOD

Oscillating chemical reactions are always complex and involve a large number of chemical species, which can be categorized as reactants, products, intermediates, or catalysts (*17*). During a usual chemical reaction, the concentrations of reactants monotonically decrease, the concentrations of products monotonically increase, and the concentrations of intermediates approach low, relatively constant steady-state values, where their rate of formation is essentially equal to their rate of disappearance. During an oscillating chemical reaction the concentrations of reactants still must monotonically decrease and the concentrations of products still must monotonically increase, but the concentrations of intermediates or catalysts species show periodic behavior (i.e., they oscillate) as the conversion of reactants to products approaches equilibrium. This scheme (*17*) is shown below:

$$\underset{\{\text{reactants disappear}\}}{R_1 + R_2 + ... + C^{n+}} \rightleftarrows \underset{\substack{\{\text{intermediates either reach}\\ \text{a steady state or oscillate}\}}}{X, Y, ... C^{n+}/C^{(n+1)} +} \rightleftarrows \underset{\{\text{products appear}\}}{P_1 + P_2 + ... + C^{n+}}$$

Thermodynamic and kinetic conditions under which a chemical system can show oscillatory behavior have been discussed elsewhere (*17*, *18*).

The BR oscillating system was discovered in 1973 (*14*) and consists of the iodination and oxidation of an organic substrate (in general, malonic acid or its derivatives) by acidic iodate in

the presence of hydrogen peroxide with Mn^{2+} ion as catalyst. This system shows, under appropriate conditions, a periodic variation of the concentrations of intermediates and catalyst (this latter oscillates between the two oxidation states 2+ and 3+). After 1973, much work has been done on this system to elucidate its reaction mechanism as well (*19*−*23*).

The main intermediates for which concentrations oscillate in the BR reaction are iodine; the iodide ion; the oxyiodine species HOI, HOIO, and $IO_2^{\bullet}$; and the hydroperoxyl radical $HOO^{\bullet}$. The involvement and important role played by $HOO^{\bullet}$ in the onset of oscillations has been recognized in previous works (*24*, *25*).

Oscillatory behavior in the BR system can be easily followed potentiometrically using a bright platinum electrode coupled with a suitable reference electrode. In fact, the electric potential of the reactant mixture oscillates because of the periodic variation of the quantity log{[Mn(III)]/[Mn(II)]}.

When an antioxidant scavenger of free radicals is added to an active oscillating BR mixture, there is an immediate quenching of oscillations, an inhibition time that linearly depends on the concentration of the antioxidant added, and subsequent regeneration of oscillations (**Figure 2a**,**b**). Linearity was found in a wide concentration range of antioxidant added (*13*).

The cessation of oscillations is due to the scavenging action of the antioxidant added against the hydroperoxyl radicals forming in the BR reaction. Eventually, when the concentration of antioxidant decreases to the point at which $HOO^{\bullet}$ radicals can multiply, the oscillations resume (*13*).

Relative antioxidative activities of antioxidants with respect to a substance chosen as standard can then be determined on the basis of inhibition times. The inhibition time is defined as the time elapsed between the end of the addition of the antioxidant and the first regenerated oscillation.

The mechanism of the BR reaction is quite complex: very recently Furrow et al. (*25*) proposed a mechanism that takes into account the experimental evidence of the important role played by $HOO^{\bullet}$ radicals in the oscillatory behavior of the BR reaction (*24*). With this mechanism, Furrow et al. (*25*) obtained not only a better agreement between experimental results and simulations for malonic acid and its derivatives but were able to simulate oscillations in BR systems with substrates that are iodinated in a different way from malonyl derivatives. Moreover, the mechanism is able to reproduce inhibition times very well with the addition of two inhibitory steps (*13*).

EXPERIMENTAL PROCEDURES

Materials and Apparatus. Malonic acid (MA, Merck; reagent grade, >99%), manganese(II) sulfate monohydrate (Merck; reagent grade, >99%), and $NaIO_3$ (Merck; reagent grade, >99.5%) were used without further purification. $HClO_4$, H_2O_2, and other chemicals were of analytical grade. All stock solutions were prepared from doubly distilled, deionized H_2O. Perchloric acid was analized by titration versus standard 0.1 M NaOH (Merck). H_2O_2 was standardized daily by manganometric analysis. Antioxidants used were cyanidin 3-*O*-*β*-glucopyranoside (Apin Chemicals Ltd., ≥99%, MW = 485.5 g mol^{-1}), cynarin (Apin Chemicals Ltd., ≥99%, MW = 516.46 g mol^{-1}), rosmarinic acid (Apin Chemicals Ltd., ≥95%, MW = 360.23 g mol^{-1}), echinacoside (Apin Chemicals Ltd., ≥ 95%, MW = 786.64 g mol^{-1}), puerarin (Sigma, ∼80%, MW = 416.4 g mol^{-1}), and oleuropein (≥95%, MW = 540.53 g mol^{-1}, donated by Prof. A. Minghetti, University of Bologna, Bologna, Italy). In the concentration range used in this work all of these antioxidant are water soluble.

Oscillations in the BR mixtures were followed potentiometrically by recording the potential of the mixture using a coupleed bright platinum electrode (Hamilton, model P/N 238 945)−reference electrode (double-junction Ag/AgCl, Ingold, model 373-90-WTE-ISE-S7). Electrodes were connected to a pH multimeter (WTW, model pH 540 GLP) controlled by an IBM-compatible PC. The accuracy of the multimeter was ±1 mV. The suitable data acquisition Multi Achat II (WTW) was used. The multimeter was equipped with a temperature sensor with an accuracy of ±0.1 °C.

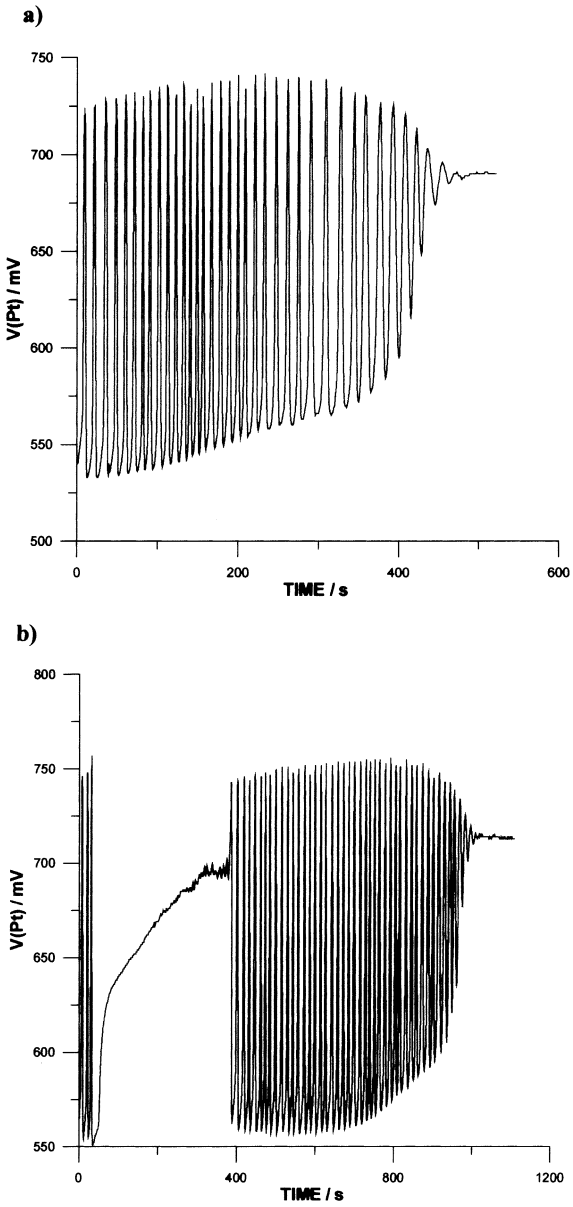

Figure 2. (a) Recording of the oscillations of the bright Pt electrode potential in a BR reference mixture. Initial composition: $[H_2O_2] = 1.20$ M, $[IO_3^-] = 0.0667$ M, $[HClO_4] = 0.0266$ M, [malonic acid] = 0.05 M, $[Mn^{2+}] = 0.00667$ M. 1.0 mL of doubly distilled water was added after the third oscillation. (b) Recording of the oscillations of the bright Pt electrode potential when 1.0 mL of a solution of rosmarinic acid (0.01 mg/mL) was added to a BR mixture of the same initial composition after the third oscillation. Temperature = 25.0 ± 0.1 °C.

All solutions and reaction mixtures were maintained at constant temperature by means of a suitable thermostating system (accuracy of ±0.1 °C).

Procedure. BR mixtures were prepared by mixing the appropriate amounts of stock solutions of reagents using pipets or burets in a 100 mL beaker to a total volume of 30 mL. The order of addition was malonic acid, $MnSO_4$, $HClO_4$, $NaIO_3$, and H_2O_2. Oscillations start after the addition of H_2O_2. The reaction mixtures were continually well stirred using a magnetic stirrer. After the third oscillation, 1.0 mL of a suitably diluted solution of an antioxidant was added using a micropipet. The inhibition time was then measured from the recordings (see **Figure**

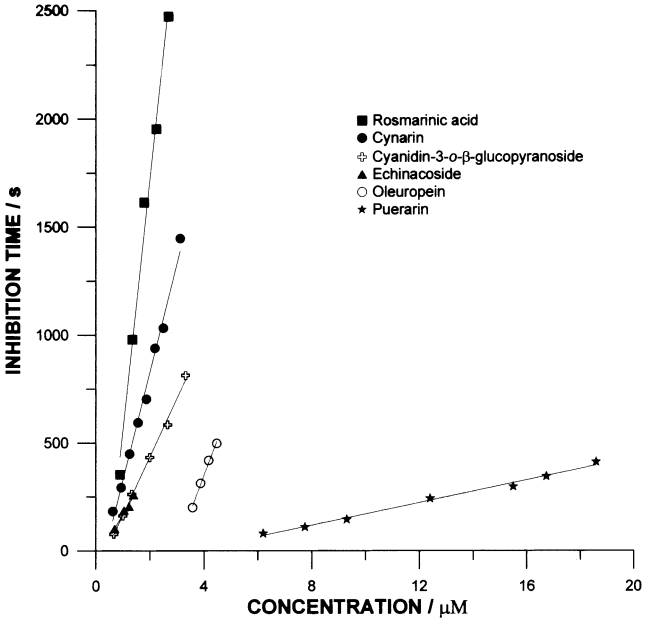

Figure 3. Straight lines of t_{inhib} versus concentration for the antioxidants studied.

2b) or the data worksheet. Before each series of measurements, we recorded the oscillations of a reference BR mixture to which 1.0 mL of doubly distilled water was added after the third oscillation. The initial composition of the BR mixture is [MA] = 0.05 M, $[Mn^{2+}] = 0.00667$ M, $[HClO_4] = 0.0226$ M, $[IO_3^-] = 0.0667$ M, and $[H_2O_2] = 1.20$ M.

RESULTS AND DISCUSSION

For each antioxidant, we studied the dependence of the inhibition time on the concentration. The graphs t_{inhib} versus concentration are reported in **Figure 3**.

As can be observed the experimental data are well fitted by straight lines. Even if the explored concentration range for echinacoside is narrow, comparing the behavior of echinacoside's straight line with that of cyanidin 3-*O*-*β*-glucopyranoside, it is conceivable that the activity of echinacoside would linearly increase until a concentration of at least 3.5 μM.

Below a certain concentration of antioxidants added (different for each antioxidant) the behavior deviates from linearity. In fact, at low concentration of antioxidant added, the inhibition time becomes too low to be measured (*24*). There is a threshold under which inhibition time cannot be detected. We believe that, under these lower limits, the straight lines curve toward 0. At high concentration of antioxidant the amplitude of the resumed oscillations becomes too low, until up to a given concentration (different for each antioxidant) oscillations do not restart. This means that the reaction reaches its end not being able to produce radicals anymore.

As shown in **Figure 3**, the slopes of the straight lines are different, so the calculation of the relative antioxidant activity will depend on the substance chosen as standard and the concentration of the sample [this limit is common also to the original TEAC method (*13*)]. The parameters of the straight lines together with the R^2 values are reported in **Table 1**.

It was found (*13*) that a way to calculate the relative antioxidant activity is to compare the concentrations of a sample and a chosen standard that give the same inhibition time, called the *relative activity with respect to concentrations* (rac), that is, the ratio

$$\text{rac} = [\text{std}]/[\text{smp}]$$

Table 1. Parameters of the Straight-Line Equations ($t_{inhib} = m[\text{Antioxidant}] + q$) and R^2 Values

substance	m (μM^{-1} s)	q (s)	R^2
rosmarinic acid	1164	−610.1	0.989
cynarin	501.1	−175.2	0.991
cyanidin 3-*O*-*β*-glucopyranoside	319.0	−105.6	0.996
echinacoside	213.7	−45.50	0.976
puerarin[a]	26.21	−1179	0.992
oleuropein	334.2	−988.5	0.994

[a] Eighty percent purity of puerarin was taken into account in the calculations.

Table 2. rac Values for the Antioxidants Studied

antioxidant	inhibition time (s)	concn (μM)	rac[a]	$(\text{rac})_m$[a]
echinacoside	350	1.851	1.78 ± 0.05	
cyanidin 3-*O*-*β*-glucopyranoside	350	1.685	1.95 ± 0.06	
cynarin	350	1.048	3.14 ± 0.08	
rosmarinic acid	350	0.825	3.99 ± 0.08	
oleuropein	310	3.885	0.82 ± 0.02	
	350	4.005	0.82 ± 0.02	0.82 ± 0.01
	420	4.214	0.82 ± 0.02	
	490	4.424	0.81 ± 0.02	
puerarin[b]	310	15.30	0.21 ± 0.01	
	350	16.83	0.20 ± 0.01	0.192 ± 0.005
	400	18.74	0.18 ± 0.01	
	420	19.50	0.17 ± 0.01	

[a] Quoted errors calculated according to the procedure suggested by Harris (*26*). [b] Eighty percent purity of puerarin was taken into account in the calculations.

where [smp] is the concentration of the sample added to the mixture giving a certain inhibition time and [std] is the concentration of the standard that should give the same inhibition time. The latter concentration is obtained from the straight-line equation of the substance chosen as standard. The inhibition time must be specified together with the rac values. The inhibition time for an rac calculation must be in the linear concentration range of the standard and of all of the examined substances.

In implementing the assay method based on the BR reaction, Cervellati et al. (*13*) used 10 diphenols and resorcinol was chosen as standard. The straight line for resorcinol is

$$t_{inhib}\,(\text{s}) = 465.1\,(\mu M^{-1}\,\text{s}) \times \text{conc}\,(\mu M) - 1181\,(\text{s})$$
$$R^2 = 0.994$$

in the concentration range 3−14 μM.

When possible, it is convenient to calculate a mean value of rac in the linear concentration range of the sample and the standard. This mean value, $(\text{rac})_m$, is more significant than the rac value calculated at only one inhibition time.

The obtained rac values are reported in **Table 2**. Then, the order of relative activity for the antioxidants studied at an inhibition time of 350 s is

rosmarinic acid (3.99 ± 0.08) > cynarin (3.14 ± 0.08) > cyanidin 3-*O*-*β*-glucopyranoside (1.95 ± 0.06) > echinacoside (1.78 ± 0.05) > resorcinol (std = 1.0) > oleuropein (0.82 ± 0.02) > puerarin (0.20 ± 0.01)

As can be observed, in acidic medium, rosmarinic acid has the highest antioxidant capacity, whereas puerarin has the lowest.

It is almost impossible to compare these values with those obtained using other analytical methods. Recently Schlesier et al. published the assessment of the antioxidant activity by using six different in vitro methods (*27*). The six usual tests for measuring antioxidant capacity (TEAC I−III, TRAP, DPPH, DMPD, PCL, and FRAP) were evaluated by comparing the results of four antioxidants and applying the tests to several beverages. The six assays differed with regard to pH (3.3−10.5) and in the nature and type of production of radicals. Some assays are suitable for hydrophilic antioxidants, others for hydrophobic substances when the solvent of the system is changed. The results showed that these six methods were not comparable because the ranking order of the antioxidant activity of the examined antioxidants differed from assay to assay. The authors concluded that this fact is probably caused by different pH values. Also, the ranking order of the examined beverages differed from assay to assay. These findings led to the conclusion that it is possible to use a ranking order of each assay only when antioxidant activities are compared. However, Schlesier et al. claimed that the results from each assay can give an idea of the protective efficacy of secondary plant components (*27*).

Galvano and co-workers (*28*) studied in detail the activity and mechanism of the antioxidant properties of cyanidin 3-*O*-*β*-glucopyranoside, the main anthocyanin present in the juice of pigmented oranges. The antioxidant activity of cyanidin 3-*O*-*β*-glucopyranoside was measured by studying the inhibitory effects on copper-induced LDL oxidation. The results together with the evaluation of the direct interaction with reactive oxygen species, such as $O_2^{\bullet -}$ and $HO^{\bullet}$, led the authors to the conclusion that cyanidin 3-*O*-*β*-glucopyranoside is a highly efficient oxygen free radical scavenger (*28*). Our findings strongly support this conclusion.

Galvano and co-workers (*28*) also found that cyanidin 3-*O*-*β*-glucopyranoside is much more active than vitamin C. Indeed, it was reported that studies in in vitro systems showed that the antioxidant activities of flavonoids and their glycosides are higher than those of vitamins C and E and many carotenoids on a molar basis (*29*). In fact, these substances (with the exception of vitamin E) do not contain phenolic group(s) in their molecules.

The mechanism for the reaction between a generic antioxidant, Ar−OH, and $HOO^{\bullet}$ radicals in the aqueous BR system is probably the following: the first step consists of an electron transfer from the antioxidant to the hydroperoxyl radical to form the radical cation of the antioxidant and a hydroperoxyl anion:

$$Ar{-}OH + HOO^{\bullet} \rightarrow Ar{-}OH^{\bullet +} + HOO^{-}$$

This reaction is followed by a rapid step:

$$Ar{-}OH^{\bullet +} \rightarrow Ar{-}O^{\bullet} + H^{+}$$

The proton reacts immediately with the hydroperoxyl anion to form hydrogen peroxide:

$$H^{+} + HOO^{-} \rightarrow H_2O_2$$

The antioxidant radical reacts with oxygen or other intermediates of the reaction to form stable derivatives. From this mechanism it is evident that the antioxidant activity in aqueous medium is influenced by the pH.

As stated above, the BR method works for water-soluble antioxidants in acidic medium at a pH value (≈2) that is similar to that of the fluids in the human stomach. It is known that a vegetarian diet can reduce the risk of stomach cancers (*30*), and it is therefore interesting to determine the activity of antioxidants at low pH values, which has been difficult prior to the advent of this new method.

For the study of antioxidative activity some factors of the method have to be considered such as the practicality, instrumental requirements, time, expertise, and cost necessary for the analysis. Concerning these factors, the BR method used here has many advantages: the analysis is inexpensive and rapid, and reagents and apparatus are in common use in all chemical laboratories.

ABBREVIATIONS USED

TEAC, Trolox equivalent antioxidant capacity; ORAC, oxygen radical absorbance capacity; TRAP, total radical-trapping antioxidant parameter; DPPH, 2,2-diphenyl-1-picrylhydrazyl; DMPD, *N*,*N*-dimethyl-*p*-phenylenediamine; PCL, photochemiluminescence; FRAP, ferric reducing ability of plasma; LDL, low-density lipoproteins.

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