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Fast and Simple Method for the Simultaneous Evaluation of the Capacity and Efficiency of Food Antioxidants in Trapping Peroxyl Radicals in an Intestinal Model System

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A simple oxygraphic method, for which the theoretical and experimental bases have been recently revised, has been successfully applied to evaluate the peroxyl radical chain-breaking characteristics of some typical food antioxidants in micelle systems, among which is a system that reproduces conditions present in the upper part of the digestive tract, where the absorption and digestion of lipids occur. This method permits one to obtain from a single experimental run the peroxyl radical trapping capacity (PRTC, that is, the number of moles of peroxyl radicals trapped by a given amount of food), the peroxyl radical trapping efficiency (PRTE, that is, the reciprocal of the amount of food that reduces to half the steady-state concentration of peroxyl radicals), and the half-life of the antioxidant ($t_{1/2}$) when only a small fraction of peroxyl radicals reacts with the antioxidants present in foods. Examples of application of the method to various types of foodstuffs have been reported, assessing the general validity of the method in the simple and fast evaluation of the above-reported fundamental antioxidant characteristics of foods.

KEYWORDS: Food antioxidants; lipid peroxidation; radical scavenging; peroxyl radical trapping capacity; peroxyl radical trapping efficiency

INTRODUCTION

The presence of antioxidants in food is of special interest because recent epidemiological studies have described many beneficial health effects of these compounds for protection against cancer, cardiovascular disease, and aging (*1–4*). As the attention given to antioxidants is rising, methods to evaluate the various aspects of their action and to compare results between laboratories are needed (*5*).

Most methods to evaluate the antioxidant activity rely on the titration of the antioxidant with various types of reagents such as DPPH, molybdotungstate (Folin−Ciocalteu), ABTS, and highly reactive radicals such as peroxyl radicals, Fe(III), and Cu(II) (*6–11*). These titrations usually give different results according to the type of reaction (H or electron abstraction) (*12, 13*), the reactivity of the titrant, the titration conditions, and the end point (kinetic or equilibrium). A small number of antioxidant activity methods evaluate the efficiency, that is, the reactivity of the antioxidant in trapping reactive species (*14, 15*). Furthermore, only a few of the developed methods offer the possibility to evaluate simultaneously both efficiency and capacity (*16–19*); however, they suffer from drawbacks that make it very difficult and sometimes impossible to obtain correct results (*20*). Finally, it must be considered that the chemistry and experimental conditions of the majority of the proposed methods to test the antioxidant activity are very far from those occurring in the systems where the antioxidants play their role, that is, in human beings, fruits, processed foods, etc.

Recently we have revised and perfected a simple oxygraphic method that overcomes most of these problems and permits us to evaluate correctly and simultaneously both the efficiency and capacity of antioxidants in the inhibition of the propagation of peroxyl radical chain (*20*). The latter process, due to the presence of lipid peroxides and heme derivatives (*21, 22*), occurs easily in the small intestine, where the oxygen concentration is rather high (*23–25*). In fact, in humans molecular oxygen present in swallowed food, after ingestion, equilibrates with gastrointestinal mucosal membranes, where the oxygen tension, according to reflectance spectroscopy measurements, is about 40% of the

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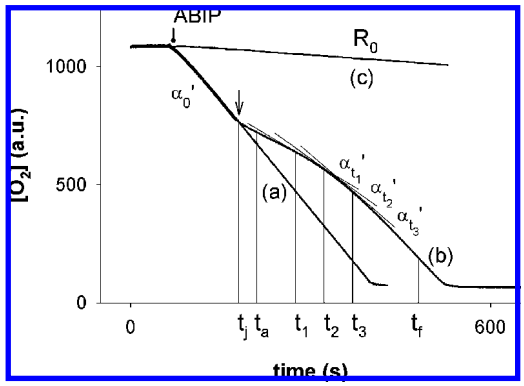

Figure 1. Representative oxygraphic records of linoleic acid peroxidation in a micelle system in the presence and in the absence of an antioxidant. The peroxidation of 2 mM LH was carried out at 37 °C by decomposition of 4 mM ABIP in a micelle system containing 5 mM DCA and 0.5 mM LG, in the absence of antioxidant (curve a) and after injection (arrow) of 0.3 μM chlorogenic acid as a water-soluble antioxidant (curve b). The slope α_t' at various times t was measured in the range t_a-t_f. Curve c is the oxygraphic trace obtained during the decomposition of 4 mM ABIP in the DCA-LG micelle system in the absence of LH. The solutions were initially equilibrated with air.

oxygen tension of room air in the case of the duodenum (*26*). Furthermore, in this body region, according to Carey et al. (*27, 28*), during duodenal fat digestion the pH ranges from 6.1 to 8.1, whereas the mixed intestinal lipids are principally partially ionized fatty acids.

We propose to extend the revised oxygraphic method to evaluate the antiradical performance of foods, characterized by antioxidant properties, in micelle systems and in particular in a micelle system reproducing conditions as close as possible to those occurring in the upper part of the intestine (*27, 28*), that is, in a body region where bioavailability problems are absent and where lipid peroxidation may easily occur (*29*).

Basic Elements for Theoretical Treatment of Experimental Data. With reference to the general scheme of free radical peroxidation of fatty acids, such as linoleic acid (LH) in the presence of an antioxidant (IH), three groups of reactions must be considered in the presence of a source generating peroxyl radicals (such as azo-compounds) with a constant rate R_i. The three groups of reactions, which we have discussed in detail in a previous paper (*20*), are (i) the propagation reaction

$$LOO^\bullet + LH \rightarrow LOOH + L^\bullet \quad (1)$$

$$L^\bullet + O_2 \rightarrow LOO^\bullet \quad (2)$$

which leads to O_2 disappearance; (ii) the termination process by the mutual reaction of $LOO^\bullet$ radicals

$$LOO^\bullet + LOO^\bullet \rightarrow \text{products} \quad k_{LL} \quad (3)$$

and (iii) the breaking of the chain process of LH peroxidation, which occurs according to the following reactions

$$LOO^\bullet + IH \rightarrow LOOH + I^\bullet \quad k_i \quad (4)$$

$$(n-1)LOO^\bullet + I^\bullet \rightarrow \text{products} \quad k'_i \quad (5)$$

where, in the case of a water-soluble and H-donor antioxidant, reaction 4 is first-order with respect to the antioxidant concentration and k'_i refers to all possible reactions of the antioxidant molecule with $(n-1)$ peroxyl radicals occurring after the abstraction of the first H-atom, k_i' being $\gg k_i$ and n the total number of peroxyl radicals scavenged by a molecule of the antioxidant.

According to this simple scheme and the oxygraphic traces reported in **Figure 1**, we can write

$$\alpha_0 = \alpha_0' - R_0 = -\left(\frac{d[O_2]}{dt}\right)_{IH=0} = k_p[LOO^\bullet]_{IH=0}[LH] = \text{constant} \quad (6)$$

$$\alpha_t = \alpha_t' - R_0 = -\left(\frac{d[O_2]}{dt}\right)_{IH\neq 0} = k_p[LOO^\bullet]_{IH\neq 0}[LH] \quad (7)$$

where α_0' and α_t' are the experimental rates of oxygen consumption in the absence and in the presence of IH, respectively, and R_0 is the rate of O_2 consumption due to the cage escape of the geminate pair of initiator radicals generated by the decomposition of the azo-compound used as a source of free radicals, R_0 being $> R_i$ (*30*).

Therefore, the ratio between the rate of oxygen consumption due to LH peroxidation process in the absence (α_o) and in the in the presence (α_t) of IH at variable times (t, t_j, t_a, t_f, ..., see **Figure 1**) is

$$x_t = \frac{\alpha_t}{\alpha_0} = \frac{[LOO^\bullet]_{IH\neq 0}}{[LOO^\bullet]_{IH=0}} \quad (8)$$

Introducing the $LOO^\bullet$ concentration obtained on the basis of eqs 1–5, applying the steady-state approximation to $LOO^\bullet$, and taking into account that only the fraction η of $LOO^\bullet$ radicals reacts with IH

η = rate of disappearance of $LOO^\bullet$ by reaction with IH/ rate of disappearance of $LOO^\bullet$ by reaction with IH and with itself

$$\eta = \frac{n\,k_i[LOO^\bullet]_{IH\neq 0}[IH]}{n\,k_i[LOO^\bullet]_{IH\neq 0}[IH] + 2k_{LL}[LOO^\bullet]^2_{IH\neq 0}} \quad (9)$$

we found a function $F(x_t)$ that depends linearly on time (*20*), that is

$$F(x_t) = C\,(t - t_a) \quad (10)$$

where

$$F(x_t) = \left(\frac{1}{x_a} - \frac{1}{x_t}\right) + \ln\frac{(1-x_a)}{(1+x_a)} + \ln\frac{(1+x_t)}{(1-x_t)} \quad (11)$$

and

$$C = \sqrt{\frac{R_i}{2k_{LL}}}\,k_i \quad (12)$$

is the slope of the plot $F(x_t)$ versus t. Therefore, by the measurement of the experimental slope of the oxygraphic trace at various times t, eq 10 permits us to calculate the value of C and consequently the ratio $k_i/\sqrt{2k_{LL}}$.

According to eq 12, C is a parameter independent of the concentration of IH, and because the term $\sqrt{R_i/2k_{LL}}$ of eq 12 is the steady-state concentration of $LOO^\bullet$ radicals in the absence of IH, C could be considered as the pseudo-first-order kinetic rate constant of the IH decay in the presence of a source

generating peroxyl radicals with a constant rate R_i, when the rate of reaction 4 is negligible with respect to that of reaction 3. Therefore, we can write

$$t_{1/2} = \frac{\ln 2}{C} \tag{13}$$

that is, the half-life of the antioxidant under the conditions above stated. Taking into account the fraction η of $LOO^{\bullet}$ radicals reacting with IH, the value n is given by (*20*)

$$n = \left(x_j^{-1} - x_j\right)\frac{R_i}{z_j C} \tag{14}$$

where x_j and z_j are the values of the ratio x and of the concentration z of the antioxidant IH at the injection time t_j, respectively.

Because the injection of the antioxidant may generate perturbation in the system (different concentrations of O_2 between the injected solution and the reaction system, distribution of IH in micelles, etc.), we prefer to calculate x_j by extrapolating the oxygraphic trace at time t_j from a time interval where perturbations are absent.

The extrapolation is performed by substituting x_j and t_j for x_t and t in eqs 10 and 11 and rewriting these equations in the form

$$C(t_a - t_j) + \frac{1}{x_a} + \ln\frac{(1-x_a)}{(1+x_a)} = \frac{1}{x_j} + \ln\frac{(1-x_j)}{(1+x_j)} \tag{15}$$

The value B_j of the left-hand side of the following equation

$$B_j = C(t_a - t_j) + \frac{1}{x_a} + \ln\frac{(1-x_a)}{(1+x_a)} \tag{16}$$

can be easily obtained on the basis of the experimental value x_a and of the calculated value of C and is used to obtain x_j according to the equation

$$\left(\frac{1}{x_j}\right) + \ln\frac{(1-x_j)}{(1+x_j)} = B_j \tag{17}$$

Because this equation is transcendental, in **Table 1** we provide the values of x_j and the corresponding values B_j, obtained by numerical approximation.

The calculated values of x_j and C can be used to obtain the n and IC_{50} values according to eq 14 and the following equation, respectively

$$IC_{50} = 1.5\,\frac{R_i}{n\,C} \tag{18}$$

Calculation Procedure. *Calculation of the C Value.* From the experimental oxygraphic trace the rates of oxygen depletion α_t' and the corresponding values of x_t were calculated at various times t in the range $t_a - t_f$ by a graphic procedure (**Figure 1**) or by using software developed in our laboratory. As we reported in a previous paper (*20*), in which we developed the procedure leading to eqs 6–18, we found that the best and most reliable values of C, n, and IC_{50}^{-1} were obtained by choosing t_a as close as possible to t_j, avoiding the region where perturbation of the oxygraphic trace is present, and by setting the range $t_f - t_a$ as large as possible, avoiding t_f values below 10% residual O_2 concentration (small deviations from the expected behavior may occur in this final part of the kinetic experiment).

The values of C, n, and IC_{50}^{-1} can be obtained (i) in a few seconds by processing the x_t data, according to eqs 10–18, using the software we have developed or (ii) by inserting the x_t data (which can be easily calculated by a graphic procedure) in these equations and solving them numerically.

Table 1. Calculated B_j Values[a]

x_j	B_j	x_j	B_j	x_j	B_j	x_j	B_j
0.01	99.98	0.26	3.31	0.51	0.84	0.76	−0.68
0.02	49.96	0.27	3.15	0.52	0.77	0.77	−0.74
0.03	33.27	0.28	3.00	0.53	0.71	0.78	−0.81
0.04	24.92	0.29	2.85	0.54	0.64	0.79	−0.88
0.05	19.90	0.30	2.71	0.55	0.58	0.80	−0.95
0.06	16.55	0.31	2.58	0.56	0.52	0.81	−1.02
0.07	14.15	0.32	2.46	0.57	0.46	0.82	−1.09
0.08	12.34	0.33	2.34	0.58	0.40	0.83	−1.17
0.09	10.93	0.34	2.23	0.59	0.34	0.84	−1.25
0.10	9.80	0.35	2.13	0.60	0.28	0.85	−1.34
0.11	8.87	0.36	2.02	0.61	0.22	0.86	−1.42
0.12	8.09	0.37	1.93	0.62	0.16	0.87	−1.52
0.13	7.43	0.38	1.83	0.63	0.10	0.88	−1.62
0.14	6.86	0.39	1.74	0.64	0.05	0.89	−1.72
0.15	6.36	0.40	1.65	0.65	−0.01	0.90	−1.83
0.16	5.93	0.41	1.57	0.66	−0.07	0.91	−1.96
0.17	5.54	0.42	1.49	0.67	−0.13	0.92	−2.09
0.18	5.19	0.43	1.41	0.68	−0.19	0.93	−2.24
0.19	4.88	0.44	1.33	0.69	−0.25	0.94	−2.41
0.20	4.59	0.45	1.25	0.70	−0.31	0.95	−2.61
0.21	4.34	0.46	1.18	0.71	−0.37	0.96	−2.85
0.22	4.10	0.47	1.11	0.72	−0.43	0.97	−3.15
0.23	3.88	0.48	1.04	0.73	−0.49	0.98	−3.57
0.24	3.68	0.49	0.97	0.74	−0.55	0.99	−4.28
0.25	3.49	0.50	0.90	0.75	−0.61		

[a] B_j values are calculated according to the equation $B_j = 1/x_j + \ln(1 - x_j)/(1 + x_j)$ for (x_j) ranging from 0.01 to 0.99.

The R_o value was obtained by measuring the rate of oxygen consumption in the presence of the azo-compound and in the absence of LH (**Figure 1**, curve c), whereas the R_i value was obtained from experiments carried out with Trolox as inhibitor by using eq 14, where $n = 2$ was set (*31*).

Parameters Characterizing the Peroxyl Radical Scavenging Properties of Food Antioxidants. To evaluate the antioxidant properties of foodstuff, we found it useful to consider the PRTC and PRTE values instead of n and IC_{50}^{-1}, respectively, PRTC being the peroxyl radical trapping capacity, that is, the number n of moles of peroxyl radicals trapped by a given amount of food (usually micromoles of peroxyl radicals per gram of food), and PRTE being the peroxyl radical trapping efficiency, that is, the reciprocal of the concentration of food which halves the steady-state concentration of peroxyl radicals (usually grams of food per liter).

MATERIALS AND METHODS

Reagents. The chemicals were of analytical reagent grade and were used without further purification. 2,2′-Azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride (ABIP) was a kind gift of Wako Chemicals (Germany). Rac1-lauroylglycerol (LG), (±)-α-tocopherol, gallic acid, and protocatechuic acid were obtained from Sigma-Aldrich (Milano, Italy). Linoleic acid, deoxycholic acid sodium salt monohydrate (DCA), (+)-catechin, caffeic acid, ferulic acid, chlorogenic acid, ellagic acid, quercetin, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), and ascorbic acid were purchased from Fluka (Buchs, Switzerland) and were of the highest available quality. Cichoric acid was obtained from PhytoLab GmbH & Co. KG (Hamburg, Germany). Anthocyanins (HPLC grade) were obtained from Extrasynthese (Genay, France). All of the antioxidant standard solutions were prepared in absolute ethanol. Deionized water, quartz bidistilled, was used to prepare all of the aqueous solutions. When necessary, these solutions were purified by a Chelex-100 column (Bio-Rad, Richmond, CA) to minimize the concentration of heavy metal ions. Vegetables, fruits, and wines were purchased from a local market.

Table 2. Peroxyl Radical Chain-Breaking Activity of Some Antioxidants Present in Food, Measured under the Conditions Occurring in the Upper Part of the Small Intestine[a]

	C (min^{-1})	R^2	$t_{1/2}$ (min)	IC_{50}^{-1} (μM^{-1})	n	PRTE (L/mg)	PRTC (μmol/mg)	C/C_{tx} ($k_i/k_{i,Trolox}$)
Trolox	0.87 ± 0.02	0.992	0.80 ± 0.02	1.16 ± 0.03	2.00 ± 0.01	4.64	7.99	1.00
ascorbic acid	4.11 ± 0.56	0.977	0.17 ± 0.03	5.62 ± 0.59	2.09 ± 0.10	31.92	11.87	4.72
caffeic acid	0.83 ± 0.11	0.982	0.83 ± 0.16	5.93 ± 0.66	10.60 ± 0.08	32.93	58.84	0.96
chlorogenic acid	0.95 ± 0.08	0.987	0.73 ± 0.09	7.12 ± 0.47	11.00 ± 1.12	20.10	31.05	1.09
cichoric acid	1.04 ± 0.29	0.984	0.67 ± 0.27	14.43 ± 3.71	21.80 ± 0.31	30.43	45.96	1.19
ferulic acid	0.18 ± 0.02	0.983	3.87 ± 0.64	0.70 ± 0.04	5.82 ± 0.47	3.60	29.97	0.21
ellagic acid	4.00 ± 0.76	0.975	0.17 ± 0.05	13.02 ± 1.91	4.40 ± 0.42	43.10	14.56	4.60
gallic acid	0.66 ± 0.09	0.967	1.04 ± 0.21	1.24 ± 0.14	2.50 ± 0.36	7.30	14.70	0.76
protocatechuic acid	0.29 ± 0.06	0.987	2.39 ± 0.67	0.97 ± 0.12	5.15 ± 0.67	6.38	33.86	0.33
catechin	0.92 ± 0.28	0.981	0.76 ± 0.33	6.28 ± 0.37	10.67 ± 2.45	21.64	36.76	1.05
quercetin	3.42 ± 0.42	0.975	0.20 ± 0.04	2.54 ± 0.24	1.14 ± 0.15	8.41	3.77	3.93
cyanidin 3-glucoside[b]	5.12 ± 0.28	0.968	0.14 ± 0.01	19.07 ± 1.36	5.60 ± 0.23	42.46	12.47	5.89
malvidin 3- glucoside[b]	2.64 ± 0.04	0.986	0.26 ± 0.01	7.03 ± 0.46	4.01 ± 0.30	14.25	8.13	3.03
delphinidin 3-glucoside[b]	4.98 ± 0.28	0.982	0.14 ± 0.01	7.80 ± 0.46	2.35 ± 0.16	16.76	5.05	5.73
delphinidin[b]	1.57 ± 0.35	0.989	0.44 ± 0.14	2.37 ± 0.26	2.30 ± 0.31	7.83	7.58	1.81

[a] The experiments were carried out in phosphate buffer, pH 7.4, containing 5 mM DCA, 0.5 mM LG, 2 mM LH, and 4 mM ABIP, at 37 °C. Data were normalized to R_i = 1.0 μM/min. Mean values and standard deviations were obtained from experimental runs carried out at four different antioxidant concentrations. [b] Before injection, the anthocyanin solution in absolute ethanol was diluted in ethanol/water (85:15) containing 0.1 M HCl.

Extract Preparation. About 50 g of the edible parts of fresh vegetables or fruits (only leaves in the case of chicory, both pulp and skin in the case of apple, and the whole fruit in the case of blueberry) were randomly sampled from raw material and homogenized in 200 mL of ethanol/water solution (85:15 v/v) containing 0.1 M HCl. The homogenized samples were centrifuged, and the clear solutions were stored at −80 °C until activity measurements.

Measurement of the Peroxyl Radical Scavenging Activity. The rate of peroxidation was measured from the rate of O_2 disappearance by a Metrohm 663 VA stand equipped with a Yellow Springs oxygen electrode, inserted into a thermostated oxygraphic cell (volume = 3 mL). The current was recorded both on a $Y-t$ chart recorder (Lynseis, Italy) and by a personal computer equipped with a data acquisition board (DAQ PCI-6221, M series, National Instruments, Austin, TX). The working electrode was poised at −800 mV versus Ag/AgCl. The experimental oxygraphic traces were automatically processed by means of a computational procedure to obtain oxygen consumption rates and C, n, and IC_{50}^{-1} values or PRTC and PRTE values.

ABIP was used as a constant source of peroxyl radicals. In fact, this azo-compound, under our experimental conditions, slowly decomposes according to a first-order kinetic rate law, the kinetic rate constant being (2.44 ± 0.13) × 10^{-4} min^{-1} at 37 °C (*32*).

The reaction mixture was prepared by drying 350 μL of 10 mM LG in dichloromethane and by dissolving the obtained film in 7 mL of 5 mM DCA in 20 mM phosphate buffer, pH 7.4. Linoleic acid (2 mM final concentration) was then added, and the resulting test solution was equilibrated with atmospheric oxygen by continuous stirring in the oxygraphic cell, thermostated at 37 ± 0.1 °C. After thermal equilibrium was achieved, 0.5 M ABIP was added (4 mM final concentration) and the rate of oxygen consumption due to the uninhibited autoxidation of linoleic acid (α_0') was recorded for some minutes. At the end of this period the antioxidant or the extract was added to the test solution and the rate of the inhibited reaction (α_i') was recorded. The rates of oxygen consumption were calculated from the slope of the oxygraphic record, on the basis of the initial oxygen concentration.

RESULTS AND DISCUSSION

Peroxyl Radical Scavenging Capacity and Efficiency of Some Antioxidants Present in Foods. A representative variety of water-soluble antioxidants present in foods, characterized by good antioxidant properties, was tested in a micelle system reproducing the conditions occurring in the upper small intestine in humans (see **Table 2**). For all of these compounds we found that eqs 10 and 11 fit the experimental data, the R^2 values being usually ≥0.98 (see **Table 2**, column 3). For these antioxidants the number n of peroxyl radicals scavenged by one molecule of IH and by its reaction products and the reciprocal of the concentration of IH that halves the steady-state concentration of $LOO^{\bullet}$ radicals (IC_{50}^{-1}) were calculated and are reported in **Table 2** together with the $t_{1/2}$ values. For the sake of comparison, in this table, the corresponding PRTC and PRTE values are reported in addition to the n and IC_{50}^{-1} values.

In general, we found that, if the inhibition process is first order with respect to the antioxidant concentration as in the case of most water-soluble antioxidants, eqs 10 and 11 fit the experimental data independently of the micelle system used to carry out the peroxidation reaction (DCA−LG−LH, SDS−LH, or CHAPS−LH) and of the phase where the peroxyl radicals are generated (aqueous or lipid phase) (*20*). However, in some cases (anthocyanins, gallic acid, etc.) the fitting of the experimental data to eq 10 was less satisfactory ($R^2 < 0.98$). We attributed this behavior to other processes involving the antioxidant molecule, occurring along with the inhibition of the peroxidation reaction. In particular, the injection of the alcoholic solution of anthocyanins is followed by the transformation of these molecules in their pseudobase form with different antioxidant characteristics (*33*), leading to a poor fitting, whereas the injection of stabilized forms of these molecules permitted us to obtain good fitting ($R^2 > 0.98$), demonstrating the fine sensitivity of the method we have set up.

Because, according to eqs 12 and 18, the C and IC_{50} values increase linearly with the square root of R_i, in **Table 2** the values of C and IC_{50}^{-1} normalized to R_i = 1 μM/ min are reported. Finally, in the last column of **Table 2** we report the C values normalized to Trolox, taken as reference compound, (C/C_{Trolox}). According to eq 12, C/C_{Trolox} gives the ratio $k_i/k_{i,Trolox}$ for the various antioxidants. Some of the C/C_{Trolox} ratios of **Table 2** appear to be quite different from those reported by other authors (*34*) because the reactivity of the antioxidants depends on many experimental variables such as the temperature and composition of micelle system. In fact, it appears that the main events determining the rate and inhibition of radical-promoted lipid peroxidation occur at the interface between the lipid and water phase of the micelle system (*35*), and therefore the rate of the inhibition process will depend on the fluidity and dynamics of the micelles and on the presence of electric charge on the micelle surface. A very modest influence should have the phase from where the chain of peroxidation process starts. In fact, we found that the use of water-soluble or lipid-soluble azo-compounds does not change significantly the values of n and IC_{50}^{-1} in the case of water-soluble antioxidants (*20, 36*).

Table 3. Peroxyl Radical Chain-Breaking Activity of Trolox at Various Oxygen Partial Pressures[a]

P_{O_2} (Torr)	n	IC_{50}^{-1} (μM^{-1})
760	2.01	1.09
160	1.99	1.09
30	2.16	1.08

[a] The experiments were carried out with 2 μM Trolox, equilibrating the solution with various oxygen partial pressures, before the ABIP injection. For other experimental conditions, see **Table 2**.

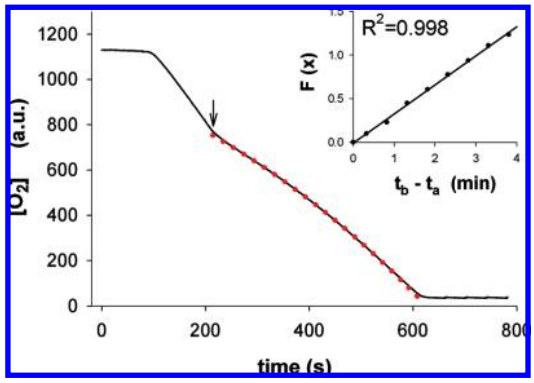

Figure 2. Inhibition of LH peroxidation by red wine. An amount of 0.6 μL of Cabernet Franc red wine was added (arrow) in the oxygraphic cell (0.2 g/L final concentration). The continuous line is the experimental trace of oxygen consumption due to LH peroxidation, whereas the dotted line is the calculated trace on the basis of eqs 10 and 11. (Inset) Plot of $F(x_t)$ versus time. The peroxidation of LH was carried out in a solution containing 5 mM DCA, 0.5 mM LG, 2 mM LH, and 4 mM ABIP at 37 °C, pH 7.4.

Because in the duodenum the pH may range from 6.1 and 8.1 and the O_2 partial pressure may decrease with respect to air saturation along the gastrointestinal tract, some inhibition experiments (Trolox) were carried out at pH 7.0 and at various oxygen partial pressures (see also **Table 3**). No significant change of the results with respect to those found at pH 7.4 and under air equilibration conditions were found. This behavior in the case of oxygen is expected because the peroxidation chain reaction of fatty acids appears to be of zero order with respect to the O_2 concentration, as it clearly appears from the linear disappearance of the oxygen in the absence of inhibition (see **Figure 1**, curve a).

With regard to the possibility of the reaction of peroxyl radicals with fundamental components of foods such as carbohydrates and amino acids, we found that, under our experimental conditions, whereas phytochemicals generally inhibit the peroxidation of LH at concentrations of ≤ 10 μM, carbohydrates and amino acids at concentrations of $\gg 10^{-4}$ M do not significantly influence the peroxidation rate.

Peroxyl Radical Scavenging Activity of Some Typical Foodstuffs Characterized by the Presence of a Significant Amount of Antioxidants. Because the micelle system and the experimental conditions influence the PRTC and PRTE values (*20*), to evaluate the antioxidant characteristics of food it appears very important to simulate conditions as close as possible to those occurring in the system where the antioxidant plays a role. Therefore, we tested some typical foodstuffs containing a relatively large amount of antioxidants in conditions close to those occurring in the upper part of the small intestine in humans, where lipid peroxidation may easily occur (*27, 28*).

Accordingly, inhibition experiments of lipid peroxidation were performed by adding small and variable amounts of some foods rich in antioxidants, or their extracts, in the oxygraphic cell containing the test solution. As representatives of these foodstuffs we considered red wine, apple, chicory, and blueberry, which are characterized by the presence of various classes of water-soluble polyphenols with antioxidant properties (*32, 37–45*). Equations 10 and 11 fit the experimental data of all the tested foods (see the examples reported in **Figures 2–4**, where the oxygraphic traces of LH peroxidation in the presence of red wine, apple juice, and chicory extract are shown). In particular, eq 10 fits very well the experimental data: usually regression coefficients $R^2 > 0.99$ were calculated (see also **Figures 2–4** insets). It is interesting to note the better fitting of eq 10 to the experimental data of foods than to those of pure compounds. This behavior can be due to the presence of the stabilized form of antioxidants in the tested food.

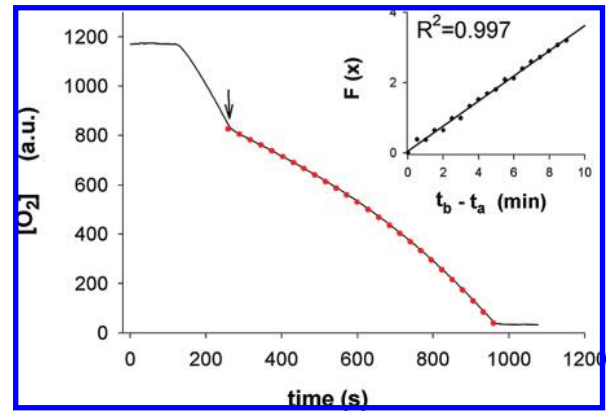

Figure 3. Inhibition of LH peroxidation by apple extract. Renetta apple extract was added (arrow) in the oxygraphic cell at final concentration of 0.17 g of fresh weight/L. The continuous line is the experimental trace of oxygen consumption due to LH peroxidation, whereas the dotted line is the calculated trace on the basis of eqs 10 and 11. (Inset) Plot of $F(x_t)$ versus time. For experimental conditions see **Figure 2**.

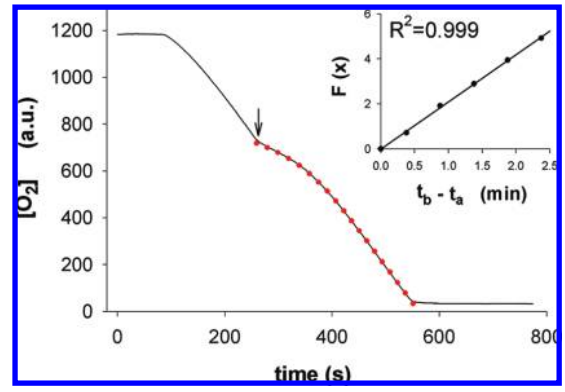

Figure 4. Inhibition of LH peroxidation by red chicory extract. Verona red chicory extract was added (arrow) in the oxygraphic cell at final concentration 0.03 g of fresh weight/L. The continuous line is the experimental trace of oxygen consumption due to LH peroxidation, whereas the dotted line is the calculated trace on the basis of eqs 10 and 11. (Inset) Plot of $F(x_t)$ versus time. For experimental conditions see **Figure 2**.

On the basis of the fitting of the oxygraphic trace according to the procedure we are proposing, it was possible to calculate for each food the PRTC, PRTE, and $t_{1/2}$. Such values were found to be independent of the concentration of the food injected in the oxygraphic cell, as expected. In particular, very good fits were obtained when the amount of food injected in the oxygraphic cell gave x_j values in the range of 0.2–0.4.

It is very interesting to observe that from the kinetic point of view, although in every food we have tested a variety of

Table 4. Peroxyl Radical Chain-Breaking Activity of Some Typical Foodstuffs Measured under the Conditions Occurring in the Upper Part of the Small Intestine[a]

food		C (min^{-1})	R^2	$t_{1/2}$ (min)	PRTE (L/g)	PRTC (μmol/g)	ePRTE (mg_{Trolox}/g)	ePRTC (mg_{Trolox}/g)
red wine	Cabernet Franc	0.335 ± 0.022	0.994	2.07 ± 0.20	9.5 ± 0.7	42.5 ± 5.0	2.0	5.3
red wine	Pinot Noir	0.243 ± 0.021	0.994	2.85 ± 0.35	11.2. ± 1.2	70.6 ± 9.5	2.4	8.8
red wine	Syrah	0.258 ± 0.035	0.990	2.68 ± 0.52	14.1 ± 1.7	82.6 ± 3.3	3.0	10.3
apple	Renetta	0.338 ± 0.021	0.995	2.05 ± 0.18	20.9 ± 2.1	93.1 ± 3.8	4.5	11.7
apple	Stark Delicious	0.349 ± 0.031	0.996	1.98 ± 0.25	8.2 ± 0.7	35.4 ± 0.7	1.8	4.4
apple	Fuji	0.267 ± 0.017	0.993	2.60 ± 0.24	3.8 ± 0.2	21.2 ± 0.6	0.8	2.7
red chicory	Verona	1.855 ± 0.102	0.997	0.37 ± 0.03	89.0 ± 5.9	72.2 ± 2.7	19.2	9.0
red chicory	Treviso	1.345 ± 0.033	0.987	0.52 ± 0.02	23.1 ± 0.2	25.7 ± 0.8	5.0	3.2
white chicory	Chioggia	1.460 ± 0.095	0.994	0.47 ± 0.04	20.1 ± 1.8	20.8 ± 0.6	4.3	2.6
blueberry	*Vaccinium myrtillus*	2.516 ± 0.161	0.982	0.28 ± 0.03	80.9 ± 4.9	48.5 ± 5.5	17.4	6.1
blueberry	American	1.709 ± 0.097	0.981	0.41 ± 0.03	83.4 ± 1.3	73.4 ± 3.3	18.0	9.2

[a] The experiments were carried out in 5 mM DCA, 0.5 mM LG, 2 mM LH, and 4 mM ABIP. Data were normalized to $R_i = 1.0$ μM/min. Mean values and standard deviations were obtained from experimental runs carried out at four different antioxidant concentrations.

antioxidants was present, these foods behave as they should contain a single antioxidant, and therefore each food can be characterized by its peculiar PRTC, PRTE, and $t_{1/2}$ values. These values, for a given foodstuff, may range over a large interval, as shown in **Table 4**, and therefore they are very useful to characterize the peroxyl radical trapping activity of foods. According to the data reported in **Table 4**, no relationship was found between the PRTC and PRTE values of the various foodstuffs we have considered ($R^2 < 0.4$), as expected.

From the results we have reported in **Figures 2–4** and in **Table 4** it appears that the procedure we are proposing, on the basis of the analysis of a simple oxygraphic curve, permits us to characterize the radical chain breaking activity of various foods by three parameters, those being PRTC, PRTE, and $t_{1/2}$. The relatively low standard deviation characterizing these parameters may be slightly improved using the three-digit table reported previously (supplementary data of ref *20*) instead of **Table 1** (two digits).

Finally, the C, PRTE, and PRTC of food can be normalized in various ways. For example, normalization can be carried out as follows:

(a) The experimental C and PRTE can be normalized to a reference value of R_i (for instance, 1 μM $LOO^{\bullet}$/min).

(b) The PRTC (micromoles of $LOO^{\bullet}$ trapped by 1 g of given food) can be divided by the PRTC of a reference compound (for example, Trolox, catechin) obtained under the same experimental conditions and expressed in this case as micromoles of $LOO^{\bullet}$ trapped by 1 mg of antioxidant. In this way, we can assign to each food a value of equivalent capacity (ePRTC) in terms of milligrams of reference compound present in 1 g of fresh food.

(c) A similar procedure can be applied to the PRTE, assigning in this way to each food a value of equivalent efficiency (ePRTE) in terms of milligrams of reference compound present in 1 g of fresh food.

These normalization procedures were applied to the data of **Table 4**, in particular, in the last two columns, where the normalized values of PRTC and PRTE with respect to Trolox are reported ($ePRTC_{Trolox}$ and $ePRTE_{Trolox}$).

In conclusion, from the results we have presented it appears that each food behaves as a well-defined compound in the inhibition of radical peroxidation of LH. Therefore, from the analysis of the oxygraphic trace, obtained in an experiment lasting a few minutes, the three different parameters characterizing the chain-breaking activity of a food can be easily obtained with good precision. This will facilitate the intercomparison of foods or the study of inhibition of lipid peroxidation under various experimental conditions. These conditions can be those occurring in systems where the antioxidant activity of various foods must be evaluated or in various body regions, for example, in the upper part of the small intestine that we have considered in this paper. Finally, it must be observed that the assay procedure can be readily automated.

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