The Antioxidant Capacity of Complex Mixtures by Kinetic Analysis of Crocin Bleaching Inhibition

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ABSTRACT: The capability of a compound or of a mixture of compounds to quench peroxyl radicals was measured by analyzing the kinetics of the competition of a parallel reaction where peroxyl radicals bleach the carotenoid crocin. This kinetic approach, originally described for the analysis of antioxidants reacting with hydroxyl radicals in water, was modified by both decreasing the polarity of the solvent, thus allowing the analysis of lipophilic compounds, and by substituting a source of peroxyl radicals for the hydroxyl radical generating system. Single compounds as well as complex mixtures were analyzed by kinetic data processing. Overall antioxidant capacity, relative to that of α-tocopherol or of its soluble analog Trolox C, was calculated. As examples of the use of this test, the antioxidant capacities of a crude rosemary extract, Maillard reaction products, and virgin olive oils were measured.
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KEY WORDS: Antioxidant, crocin, diazo-compounds, Maillard reaction, olive oil, peroxyl radicals, rosemary.

The mechanism of polyunsaturated lipid oxidation has been extensively analyzed in the last thirty years (1–4) in relation to oxidative stability and development of off-flavors in foods (5–7), but also as an important mechanism of cellular function derangement in some diseases (8). Moreover, studies on the toxicity of lipid oxidation products (9) highlighted the relevance of food processing in lowering possible toxicity.

The natural antioxidant content of foods is considered a major health-protecting factor by supplying the human body with exogenous antioxidant protection (10–13). Furthermore, it is reasonable to assume that reduction of the content of oxidized toxic components in foods, brought about by antioxidants, must be relevant to human health as well.

The capability of measuring overall antioxidant capacity of a food, therefore, is a relevant task in food chemistry, to possibly expand information obtained from tests with single known antioxidants. An example of this concept is provided by virgin olive oil, where, although α-tocopherol is the most known antioxidant, the antioxidant capacity is dramatically increased by a complex series of polyphenolic compounds (14,15).

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The accelerated stability test is the usual procedure for evaluating food antioxidants. A lipid matrix is exposed to various oxidizing conditions, and the effect of the antioxidant is calculated from the induction period prior to the rapid oxidation phase (5). Oxidation is usually measured by oxygen consumption or by production of specific compounds. Although useful for practical purposes and widely adopted, these procedures have been questioned because of the variability of conditions to produce oxidation products or the precision of the analytical procedure applied (7,16).

This paper describes a simple procedure for analyzing the antioxidant capacity of complex matrices (oleoresins, extracts, oils, etc.), which is expressed relative to α-tocopherol or its soluble analog (Trolox C) on a weight basis.

The description of the method requires a brief definition of the peroxidative process and of the antioxidant mechanism to which the analysis has been addressed, highlighting the kinetics of reactions involved in the antioxidant effect. Polyunsaturated fatty acids (PUFA), after extraction of an allylic hydrogen atom by an initiator, react extremely fast with molecular oxygen (rate constant: $>2 \times 10^8$ M^{-1} s^{-1}) to produce a peroxyl radical (17). The initiation of peroxidation is accounted for by the production of free radicals by several mechanisms: unimolecular homolysis of weak bonds, radiolysis, photolysis or electron transfer from transition metal ions (18). The bond that most frequently undergoes homolytic or heterolytic decomposition through the above mechanisms is the O–O bond of a hydroperoxide (4). Thus, this mechanism of initiation of lipid peroxidation is usually referred to as "hydroperoxide-dependent" (19).

Peroxyl radicals react with other PUFA to yield hydroperoxides and a new carbon-centered radical, thus driving the peroxidation chain reaction (19). The reported rate constant for this propagation reaction is 36 M^{-1} s^{-1} for linoleic acid (20), and a fivefold increase is expected by increasing the number of double bonds to six (21). The length of the chain reaction is determined by availability of substrates (PUFA and oxygen) and by the ratio between propagation and termination reactions by radical-radical interaction (18).

Inhibition of initiation, a primary antioxidant effect, is brought about by physical or chemical procedures and prevents the formation of free radicals from preexisting lipid hydroperoxides (reduction of hydroperoxides, protection from

light, sequestration of metals, inhibition of lipoxygenases, etc.). On the other hand, free-radical scavengers or chain-breaking antioxidants are molecules that specifically quench peroxidation-driving radicals, thus shortening the chain reaction length. The theoretical targets of this antioxidant effect are carbon-centered radicals, alkoxyl radicals, and peroxyl radicals.

As a matter of fact, it is almost impossible for an antioxidant to compete with oxygen for the interaction with a pentadienyl radical because the reaction is extremely fast (17), although reversible (3). The same kinetic limitation applies to alkoxyl radicals, which react with PUFA with a rate constant that increases with the insaturation of the fatty acid chain from 8.8×10^6 to 2.05×10^7 M^{-1} s^{-1} (22). In other words, due to the reactivity of the radical involved, it is practically impossible for the antioxidant reaction to kinetically overwhelm the prooxidant reaction. Moreover, the actual involvement of the reaction between alkoxyl radicals of fatty acids and PUFA is questioned, owing to their rearrangement. In fact, alkoxy radicals may cyclize into the adjacent site of unsaturation to form, upon oxygen addition, an epoxide–peroxyl radical (23, 24), or undergo β-scission (25) to form, in subsequent reactions, aldehydes and peroxyl radicals (26). The propagation driving reaction between a peroxyl radical and PUFA, on the other hand, has a limited rate constant (20,21). Thus, a competitive reaction may take place in which an antioxidant, reacting with a much higher rate constant, can actually spare PUFA.

The first requirement for a free-radical scavenger to have an antioxidant effect is, therefore, the kinetic advantage of having a rate constant of reaction with a peroxyl radical that is much higher than that of the propagation reaction between a peroxyl radical and PUFA. The second requirement is that the radical of the antioxidant has to react with PUFA at a rate constant that is much lower than the one of peroxyl radical. This highlights the concept that the measurement of just the rate constant of the reaction between a compound and peroxyl radicals (often taken as an index of antioxidant capacity) could be misleading in terms of real antioxidant effects, when the radical produced is still reactive enough to substitute for peroxyl radicals in the propagation reaction. In the case of an efficient antioxidant, such as α-tocopherol, the rate constant of the antioxidant reaction is several orders of magnitude higher than the rate constants of the chain-transfer prooxidant reactions (27). The seleno compound Ebselen is an example of the opposite situation. This compound reacts with peroxyl radicals at a high rate constant, as measured by pulse radiolysis, but fails to show any significant antioxidant activity in a competition kinetics test, apparently because the interaction with a peroxyl radical produces a radical with the same reactivity (28).

The procedure for measuring antioxidant activity reported in this paper is based on kinetic constraints of the antioxidant effect: (i) the high rate constant of the reaction with peroxidation-driving peroxyl radicals, and (ii) the low rate constant of the propagation reaction (if any) for the radical of the antioxidant. By this procedure, the capability of a compound (or the mixture of compounds) to quench peroxyl radicals is measured in terms of α-tocopherol equivalents (by weight), by analyzing the kinetics of competition of a parallel reaction where peroxyl radicals bleach the carotenoid crocin. Crude rosemary extract, glucose and glycine undergoing a Maillard reaction, and virgin olive oils were analyzed by this procedure.

MATERIALS AND METHODS

2,2′-Azo-*bis*(2-amidinopropane) dihydrochloride (ABAP) and 2,2′-azo-*bis* (2,4-dimethylvaleronitrile) (AMVN) were purchased from Waco Chem. USA, Inc. (Richmond, VA); Trolox C, glucose, glycine, and dimethyl formamide (DMF) from Janssen Chimica (Geel, Belgium); DL α-tocopherol from ICN Biochemicals, Inc. (Costa Mesa, CA); potassium hydrogen phosphate from Carlo Erba (Milano, Italy); saffron from Sigma Chemical Co. (St. Louis, MO). All solvents [high-performance liquid chromatography (HPLC) grade] were from J.T. Baker (Milano Italy). Crocin was isolated from saffron by methanol extraction after repeated extraction with ethyl ether to eliminate possible interfering substances (29). The concentration of crocin in methanol was calculated from the absorbtion coefficient ($\varepsilon = 1.33 \times 10^5$ M^{-1} cm^{-1} at 443 nm). Rosemary Oleoresin Biopherol RE 3401 was a gift from Quest International Italia S.p.A. (Milano Italy). Olive oils were purchased from a local market.

Competition kinetic test. The kinetic test (29) was modified by introducing diazo-compounds to produce peroxyl radicals and by using solvents of different polarity to allow the analysis of either hydrophilic or lipophilic compounds.

Reactions were carried out at 40°C, and the bleaching rate of crocin, linear 1–1.5 min after the addition of the diazo-compound, was recorded for 10 min. Blanks without crocin were run to rule out spectral interferences between the molecule under analysis and crocin.

For hydrophilic compounds, ABAP was the radical generator. The hydrophilic reaction mixture contained 1 mL of 10% ethanol in water, 1 mL of 10 mM phosphate buffer pH 7, 12 μM crocin (from a 1.2 mM methanolic stock solution), and variable amounts of the sample, containing the antioxidant to be analyzed. Samples were added either in water or ethanol while maintaining the final concentration of the latter constant. The reaction was started by the addition of 5 mM ABAP (from a fresh 0.5 M solution in water) to the complete reaction mixture, which was preequilibrated at 40°C. This procedure was used for Trolox C (as reference compound) and for Maillard reaction products.

For lipophilic compounds, AMVN was used as the free-radical generator. Lipophilic reaction mixture I contained 12 μM crocin (from a 1.2 mM methanolic stock solution) and DMF to a final volume of 2.5 mL. Samples were added in DMF. The reaction was started by the addition of 2.5 mM AMVN from a freshly prepared 0.25 M solution in toluene. This procedure was used for α-tocopherol (as reference compound) and rosemary oleoresin.

Lipophilic reaction mixture II contained 12 μM crocin (from a 1.0 mM methanolic stock solution) in 2.5 mL of toluene/DMF, (1:4, vol/vol). Samples were added in toluene/DMF (1:1, vol/vol), and the final ratio 1:4 of toluene/DMF was kept constant by adding DMF. The reaction was started by the addition of 2 mM AMVN (from a freshly prepared 0.25 M solution in toluene). This procedure was used for α-tocopherol (as reference compound) and olive oils.

Carbon-centered radicals, generated by thermal decomposition of the diazo-compound (Reaction 1), add molecular oxygen-yielding peroxyl radicals (ROO•) in a diffusion-controlled reaction (Reaction 2) (30,31). These radicals bleach the carotenoid, crocin, thus allowing the measurement of the reaction rate by following the specific absorbance decrease at 443 nm (29). In the presence of an antioxidant, competing with crocin for the reaction with radicals, the bleaching rate (Reaction 3) slows down, providing that: (i) the antioxidant is able to react with peroxyl radicals (Reaction 4) and (ii) the rate of the interaction between the radical of the antioxidant and crocin (Reaction 5) is slower than the rate of Reaction 3:

$$\text{R–N=N–R} \xrightarrow{\text{heat}} 2\,\text{R}^{\bullet} + \text{N}_2 \qquad [1]$$

$$\text{R}^{\bullet} + \text{O}_2 \longrightarrow \text{ROO}^{\bullet} \qquad [2]$$

$$\text{ROO}^{\bullet} + \text{crocin} \longrightarrow \text{ROOH} + \text{crocin}^{\bullet}\ (\text{bleached}) \qquad [3]$$

$$\text{ROO}^{\bullet} + \text{antiOx} \longrightarrow \text{ROOH} + \text{antiOx}^{\bullet} \qquad [4]$$

$$\text{antiOx}^{\bullet} + \text{crocin} \longrightarrow \text{antiOx} + \text{Crocin}^{\bullet}\ (\text{bleached}) \qquad [5]$$

The crocin bleaching by a peroxyl radical ($-\Delta A_0$), corresponding to $V_o = K_c[C]$, decreases in the presence of an antioxidant that competes for the peroxyl radical and, according to competition kinetics (29,32), the new bleaching rate (V) corresponds to:

$$V = V_o \times \frac{K_c[C]}{K_c[C] + K_a[A]} \qquad [6]$$

where $V_o = K_1 \times [\text{ROO}^{\bullet}] \times [C]$; $V_a = K_2 \times [\text{ROO}^{\bullet}] \times [A]$; $K_c = K_1 \times [\text{ROO}^{\bullet}]$; $K_a = K_2 \times [\text{ROO}^{\bullet}]$; and V_o = rate of the reaction of crocin with ROO•; V_a = rate of the reaction of the antioxidant under study with ROO•; K_1 = rate constant for the reaction between ROO• and crocin; K_2 = rate constant for the reaction between ROO• and antioxidant; $[C]$ = concentration of crocin; and $[A]$ = concentration of antioxidant.

By transforming, the bleaching rate of crocin ($-\Delta A_o$) decreases in the presence of an antioxidant to a new value ($-\Delta A_a$) fitting the straight line equation:

$$\frac{-\Delta A_o}{-\Delta A_a} = \frac{V_o}{V} = \frac{K_c[C] + K_a[A]}{K_c[C]} = 1 + \frac{K_a}{K_c} \times \frac{[A]}{[C]} \qquad [7]$$

The slope K_a/K_c, calculated from the linear regression of the plot of $[A]/[C]$ vs. V_o/V, indicates the relative capacity of different molecules to interact with ROO•. When molecules, although reacting with peroxyl radicals, are transformed into radicals that are able to react with crocin, and thus, by analogy, to propagate peroxidation, this kinetic approach produces ratios K_a/K_c lower than the actual ratio between the absolute rate constants. Thus, this test averages the antioxidant capacity with a possible prooxidant effect of the sample.

Maillard reaction products. A procedure for preparing a mixture of Maillard reaction products (MRP) (33) was adopted by dissolving 13.3 g glucose and 6.7 g glycine in 80 mL water and heating the mixture at 90°C in closed vials. The antioxidant capacity was analyzed on samples taken at 0, 3, 6, and 15 h and diluted 1:10 with 0.1 M phosphate buffer of pH 7.

α-Tocopherol analysis. α-Tocopherol content in olive oils was measured by normal-phase HPLC with fluorimetric detection (excitation: 286 nm; emission: 330 nm). In brief, one gram of oil was dissolved in 10 mL hexane. Twenty-μL samples were injected into HPLC. A Hidar prepacked RT 250-4 column was used; the mobile phase was hexane/isopropanol (95.5:0.5); the flow rate was 1 mL/min. The determination was carried out with a standard curve of authentic α-tocopherol.

RESULTS AND DISCUSSION

Chain-breaking antioxidants are molecules that interact with free radicals produced during lipid peroxidation, thus inhibiting the free-radical chain propagation. Their efficiency depends on several factors, among which the kinetic constraints of the reaction involved are particularly relevant. The described kinetic procedure addresses the capacity of a compound or a complex mixture of compounds to react with peroxidation-driving peroxyl radicals, producing a hydroperoxide and the radical of the antioxidant, the reactivity of which affects the final result. Thus, this kinetic analysis provides information on the average between antioxidant and prooxidant effects. This extends information that is obtained just from the measurement of the rate constant of the interaction between antioxidant and peroxyl radical and could be particularly relevant in analyzing complex mixtures.

Competition kinetics has been used for the analysis of radical reactions that are induced by pulse radiolysis (32) and for the measurement of relative rate constants of antioxidants that react with hydroxyl and alkoxyl radicals (29). The latter test has been modified both by introducing diazo-compounds as a source of peroxyl radicals at a constant rate, a procedure that is widely adopted for analyzing antioxidants (31), and by using solvents of different polarity to expand the analysis to a large variety of compounds.

The analysis results of the antioxidant capacity of α-tocopherol in two lipophilic systems and of the water-soluble analog Trolox C in the hydrophilic system are reported in Figure 1. In these plots, V_o/V indicates the ratio of bleaching rates in the presence of different molar ratios between the antioxidant and crocin. These data indicate that experimental results fit the kinetic model (R values >0.98 and intercept close to 1). Thus, according to the kinetic equation, the slope of the regression fitting of experimental data indicates the antioxidant

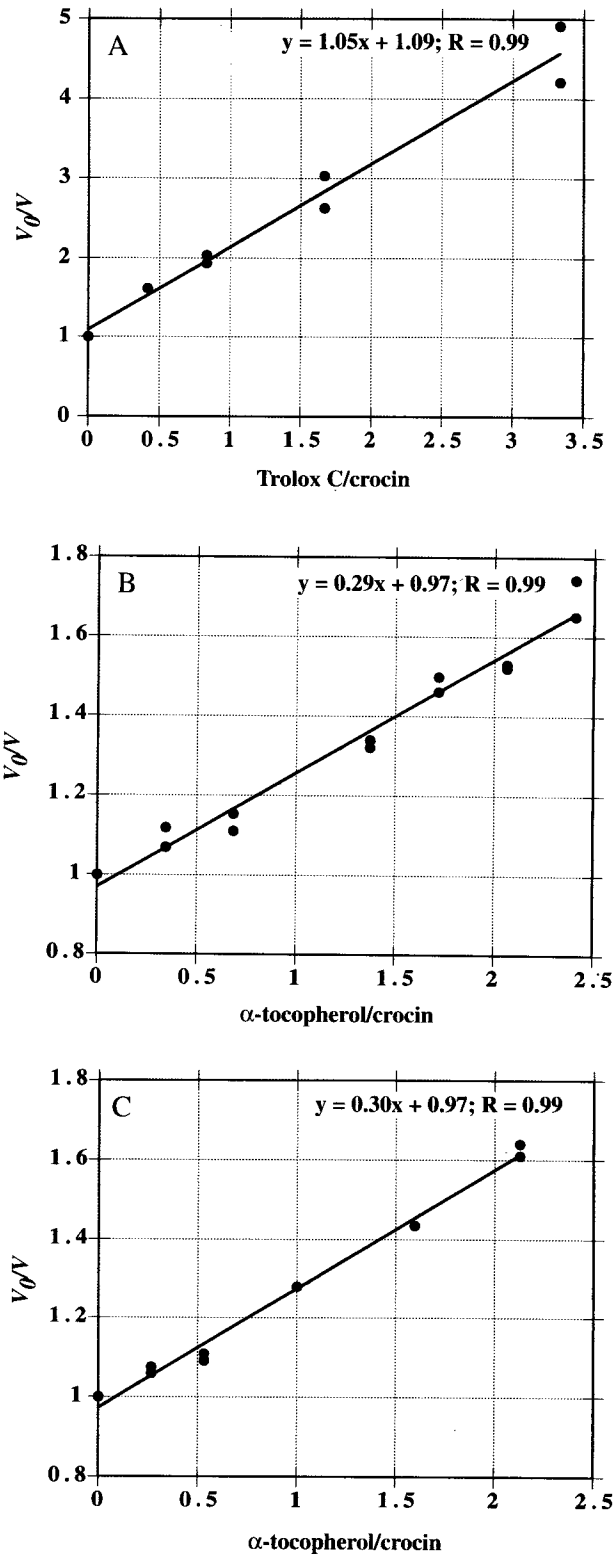

FIG. 1. Kinetic plot for the competition between crocin and Trolox C (Janssen Chimica, Geel, Belgium) or α-tocopherol for peroxyl radicals. A) antioxidant/Trolox C; hydrophilic reaction mixture; B) antioxidant/α-tocopherol; lipophilic reaction mixture I; C) antioxidant/α-tocopherol; lipophilic reaction mixture II. The slope of the regression fitting indicates, according to the kinetic equation, the antioxidant capacity of the antioxidant. In three repeated measurements the interassay variability was <15 %; V, the new bleaching rate; V_o, rate of reaction of crocin with ROO•.

capacity of the analyzed compound. In different solvents, the relative efficiency of the chromanol ring, in respect to crocin, slightly changes (compare the slopes in Fig. 1). This highlights the concept that different antioxidants must be compared under the same experimental conditions. Nevertheless, these observed differences of chromanol (or crocin) reactivity as a function of the solvent are minimal in comparison with the differences among different antioxidants (28,34,35).

Antioxidant activity of rosemary oleoresin and MRP. The described kinetic procedure, set up for the analysis of the antioxidant capacity of single molecules, was extended to complex mixtures (extracts, oleoresins) where neither the concentration of antioxidants present nor their actual number were known. In fact, the overall antioxidant capacity of a complex mixture corresponds to the sum of each antioxidant concentration times its rate constant for the interaction with a peroxyl radical. Moreover, compounds that are possibly present in the mixture, which produce new radicals, reactive with crocin by affecting crocin bleaching rate, decrease the antioxidant capacity of the mixture, as measured by the kinetic test. Thus, from the kinetics of crocin bleaching in the presence of a mixture of compounds, a value $K_a \times [A]$ can be calculated, where A is a theoretical antioxidant and K_a its rate constant for its interaction with peroxyl radicals. This value ($K_a \times [A]$) accounts for, by the average between antioxidant and prooxidant effects, all molecules present in the mixture that interact with radicals. By considering the whole amount of the sample as α-tocopherol, its "theoretical" concentration [pseudo α-tocopherol] is used to plot the results of crocin bleaching. The antioxidant capacity of the sample is calculated from the slope of the fitting of the kinetic equation:

$$\frac{V_o}{V} = 1 + \frac{K_{\text{pseudo }\alpha\text{-toc}}}{K_c} \times \frac{[\text{pseudo }\alpha\text{-tocopherol}]}{[C]} \qquad [8]$$

where $K_{\text{pseudo }\alpha\text{-toc}}$ is the rate constant for the interaction of pseudo α-tocopherol with peroxyl radicals. Finally, because

$$K_a \times [A] = K_{\text{pseudo }\alpha\text{-toc}} \times [\text{pseudo }\alpha\text{-tocopherol}] = K_{\alpha\text{-toc}} \times [\alpha\text{-tocopherol}] \qquad [9]$$

and because $K_{\alpha\text{-toc}}$ is known from independent measurements, the ratio [pseudo α-tocopherol]/[α-tocopherol] can be calculated. This ratio indicates the relative capacity, in terms of weight, of the sample vs. α-tocopherol to produce the same antioxidant effect.

This procedure was used to measure the overall antioxidant capacity of rosemary oleoresin (Fig. 2). In the plot, V_0/V indicates the ratio of bleaching rates in the absence and in the presence of different amounts of oleoresin expressed as pseudo α-tocopherol concentration. According to the kinetic model, the slope of the fitting of the regression indicates the relative antioxidant capacity of the oleoresin. Because the ratio between the rate constants of rosemary oleoresin, as pseudo α-tocopherol, and crocin was 6.03×10^{-2} and for real α-tocopherol it was 0.29 (Fig. 1B), the total antioxidant capacity of the oleoresin was $K_{\alpha\text{-toc}}/K_{\text{pseudo }\alpha\text{-toc}} = 0.29/0.0603$

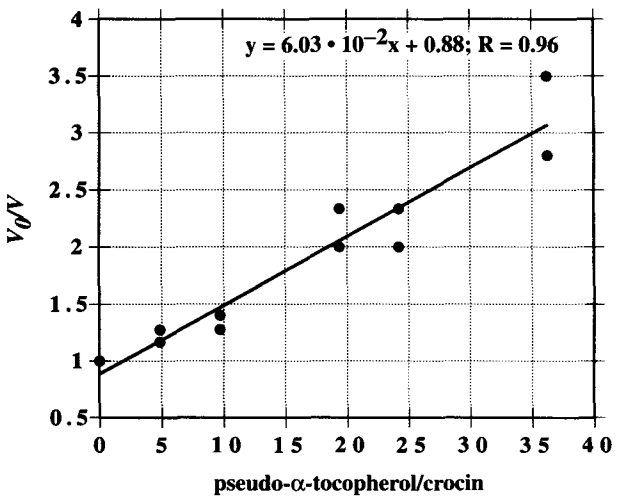

FIG. 2. Competition kinetic plot of rosemary oleoresin. The concentration of antioxidant is calculated by considering the whole sample as α-tocopherol [pseudo-α-tocopherol]. The slope of the regression fitting indicates the relative antioxidant capacity of the oleoresin. In three repeated measurements, the interassay variability was <20%. See Figure 1 for company source and abbreviations.

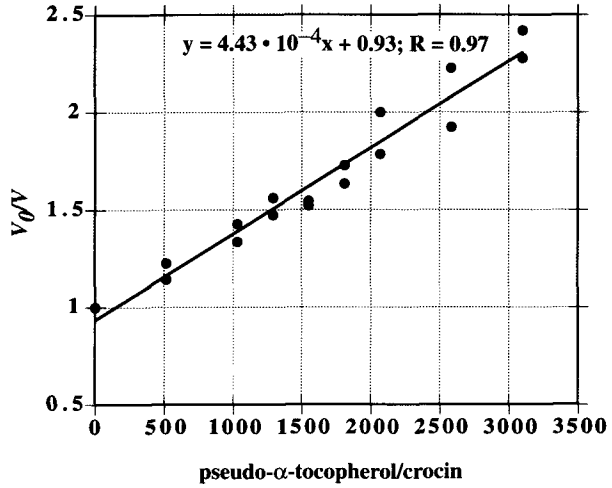

FIG. 3. Competition kinetic plot of an olive oil. The concentration of antioxidant is calculated by considering the whole sample as α-tocopherol [pseudo-α-tocopherol].The slope of the regression fitting indicates the relative antioxidant capacity of the oil (sample 3 in Table 2). See Figure 1 for company source and abbreviations.

= 4.81. According to the kinetic equation, this value corresponds to the ratio between pseudo-α-tocopherol and α-tocopherol concentration. Thus, because both concentrations have been calculated by using the molecular weight of α-tocopherol, the result is actually a ratio between weights and indicates that 4.81 g of the oleoresin are required to get the same antioxidant capacity as 1 g of α-tocopherol.

Trolox C as reference compound and the hydrophilic solvent have been used to analyze MRP that are known to play an antioxidant effect (36,37). The antioxidant capacity was measured on a mixture of glucose and glycine, heated to produce a mixture MRP and analyzed at 3, 6, and 15 h. In this test, the hydrophilic reaction mixture was used, and for the competition kinetics analysis, the molar concentration of the antioxidant was calculated by dividing the weight of the sum of glucose and glycine content by the molecular weight of Trolox C. Table 1 shows that the antioxidant capacity of the mixture progressively increases: after 3 h of heating, almost 1 kg of glucose–glycine contained the antioxidant effect of 1 g of Trolox, while just 50 g had the same effect after 15 h. This result was supported by controls, which showed that at room temperature, or when glucose or glycine were heated independently, no antioxidant effect was produced (not shown).

Antioxidant activity of olive oils. There is a growing interest in the antioxidant capacity of virgin olive oils, for both the oxidative stability of the oils, and their possible impact on human health. Several papers have reported the extraction, separation, and quantitative analysis of antioxidants from virgin olive oil (38,39); however, our simple test could provide a quantitative evaluation of the overall antioxidant capacity. Olive oils from seven Italian sources were analyzed with a hydrophobic reaction mixture and the hydrophobic diazo-compound AMVN (Fig. 3). The kinetic analysis was carried out by considering the whole oil as pseudo-α-tocopherol. Thus, the ratio between rate constants could be calculated and eventually the ratio that indicates how many grams of oil contain the antioxidant capacity of one gram of α-tocopherol. The total antioxidant capacities of seven Italian olive oils, calculated by this kinetic approach, are reported in Table 2.

TABLE 1
Kinetics of Crocin Bleaching Inhibition by Maillard Reaction Products (MRP)[a]

Sample time	Competition kinetics equation	R	MRP/Trolox C
MRP, 3 h	$y = 1.12 \times 10^{-3}x + 1.007$	0.99	937
MRP, 6 h	$y = 5.39 \times 10^{-3}x + 0.808$	0.99	195
MRP, 15 h	$y = 21.50 \times 10^{-3}x + 1.007$	0.99	49

[a]The hydrophilic reaction mixture was used. The ratio MRP/Trolox C (Janssen Chimica, Geel, Belgium) indicates the relative amount (by weight) of substrates of the Maillard reaction (glucose plus glycine) in which the antioxidant effect of Trolox C has been produced (by weight).

TABLE 2
Kinetics of Crocin Bleaching Inhibition by Virgin Olive Oils[a]

Oil	Competition kinetics equation	R	Oil/α-tocopherol
1	$y = 1.65 \times 10^{-4}x + 1.04$	0.98	1.82×10^{3}
2	$y = 1.85 \times 10^{-4}x + 1.15$	0.95	1.62×10^{3}
3	$y = 4.43 \times 10^{-4}x + 0.93$	0.97	0.68×10^{3}
4	$y = 1.67 \times 10^{-4}x + 1.12$	0.95	1.80×10^{3}
5	$y = 0.81 \times 10^{-4}x + 1.08$	0.97	3.70×10^{3}
6	$y = 2.13 \times 10^{-4}x + 1.08$	0.99	1.41×10^{3}
7	$y = 1.77 \times 10^{-4}x + 1.11$	0.93	1.69×10^{3}

[a]The lipophilic reaction mixture II was used. The ratio oil/α-tocopherol indicates the relative amount (by weight) of oil that exhibits the antioxidant effect of α-tocopherol .

TABLE 3
Percentage of the Overall Antioxidant Effect from α-Tocopherol in Virgin Olive Oils[a]

Oil	α-Tocopherol (mg/kg)	% of Overall antioxidant effect[a]
1	236	42.9
2	130	21.1
3	297	20.1
4	138	24.8
5	172	63.7
6	182	25.6
7	172	29.2

[a]Calculated from data reported in Table 2.

These data indicate that the antioxidant capacity corresponding to 1 g of α-tocopherol is contained in amounts of oil ranging from 680 to 3,700 g. For measuring antioxidant capacity of oils, relatively high amounts of substrate were used to obtain a detectable inhibition of crocin bleaching. This procedure introduced an imprecision in the analysis because triglycerides actually reduced the crocin bleaching rate, possibly by facilitating radical-radical interactions after thermal decomposition of the diazo-compound, which are in competition with oxygen addition (cage effect). However, this artifactual inhibitory effect, as measured on triolein and trilinolein, was minor. It increased the overall antioxidant capacity of different oils by no more than 10–15% (not shown) and was considered negligible.

The described kinetic procedure provides information on the amount of α-tocopherol that accounts for the overall antioxidant capacity of the oil. Thus, by measuring the actual α-tocopherol content, it is possible to define the percentage of the antioxidant effect of α-tocopherol itself. These results are reported in Table 3. Sample 3 showed both the highest content of α-tocopherol and the highest antioxidant capacity. Samples 5 and 7, although containing the same amount of α-tocopherol (172 mg/kg), dramatically differed in terms of total antioxidant capacity. This information highlights the effect of minor components of olive oil as possible antioxidants and could be relevant for assessing the oxidative stability and quality of the oil. For instance, an olive oil in which almost all antioxidant capacity is accounted for by α-tocopherol likely has been fully processed and may have had α-tocopherol added after processing.

REFERENCES

1. Porter, N.A., Autoxidation of Polyunsaturated Fatty Acids: Initiation, Propagation, and Product Distribution (basic chemistry), in *Membrane Lipid Oxidation*, Vol. I, edited by C. Vigo-Pelfrey, CRC Press, Boca Raton, 1990, pp. 33–62.
2. Chan, H.W.S., The Mechanism of Autoxidation, in *Autoxidation of Unsaturated Lipids*, Academic Press, London, 1987.
3. Simic, M., and M. Karel, *Autoxidation in Food and Biological Systems*, Plenum Press, New York, 1980.
4. Terao, J., Reactions of Lipid Hydroperoxides, in *Membrane Lipid Oxidation*, Vol. I, edited by C. Vigo-Pelfrey, CRC Press, Boca Raton, 1990, pp. 219–238.
5. Frankel, E.N., In Search for Better Methods to Evaluate Natural Antioxidants and Oxidative Stability in Food Lipids, *Trends in Food Science and Technology 4*:220–225 (1993).
6. Rossel, J.B., Measurement of Rancidity, in *Rancidity in Foods*, edited by J.C. Allen and R.J. Hamilton, Elsevier, Amsterdam, 1983, pp. 21–45.
7. Ragnarsson, J.O., and T.P. Labuza, Accelerated Shelf-Life Testing for Oxidative Rancidity in Foods—A Review, *Food Chem. 2*:291–298 (1977).
8. Halliwell, B., and M.C. Gutteridge, Role of Free Radicals and Catalytic Metal Ions in Human Disease: An Overview, *Meth. in Enzmol. 186*:1–85 (1990).
9. Addis, P.B., and G.J. Warner, The Potential Health Aspects of Lipid Oxidation Products in Food, in *Free Radicals and Food Additives*, edited by O.I. Aruoma and B. Halliwell, Taylor and Francis, London, 1991, pp. 77–119.
10. Ames, B.N., M.K. Shigenaga, and T.M. Hagen, Oxidants, Antioxidants, and the Degenerative Diseases of Aging, *Proc. Natl. Acad. Sci. 90*:7915–7922 (1993).
11. Diplock, A.T., Antioxidants Nutrients and Disease Prevention: An Overview, *Am. J. Clin. Nutr. 53*:189S–193S (1991).
12. Block, G., The Data Support a Role for Antioxidants in Reducing Cancer Risk, *Nutr. Rev. 50*:207–213 (1992).
13. Gey, K.F., P. Puska, P. Jordan, and U.K. Moser, Inverse Correlation Between Plasma Vitamin E and Mortality from Ischemic Heart Disease in Cross-Cultural Epidemiology, *Am. J. Clin. Nutr. 53*:326–334S (1991).
14. Fedeli, E., Lipids of Olives, *Prog. Chem. Fats Other Lipids 15*:57–74 (1977).
15. Papadopoulos, G., and D. Boskou, Antioxidant Effect of Natural Phenols on Olive Oil, *J. Am. Oil Chem. Soc. 68*:669–671 (1991).
16. Läubli, M.W., and P.A. Bruttel, Determination of the Oxidative Stability of Fats and Oils: Comparison Between the Active Oxygen Method (AOAC cd 12-57) and the Rancimat Method, *Ibid. 63*:792– 795 (1986).
17. Hasegawa, K., and L.K. Patterson, Pulse Radiolysis in Model Systems: Formation and Behaviour of Peroxy Radicals in Fatty Acids, *Photochem. Photobiol. 28*:817–823 (1978).
18. Pryor, W.A., The Role of Free Radical Reactions in Biological Systems, in *Free Radicals in Biology*, Vol. I, edited by W.A. Pryor, Academic Press, New York, 1976, pp. 1–49.
19. Ursini, F., M. Maiorino, and A. Sevanian, Membrane Hydroperoxides, in *Oxidative Stress: Oxidants and Antioxidants*, edited by H. Sies, Academic Press, London, 1991, pp. 319–336.
20. Barclay, L.R.C., K.A. Baskin, S.J. Locke, and M.R. Vinquist, Absolute Rate Constants for Lipid Peroxidation and Inhibition in Model Biomembranes, *Can. J. Chem. 67*:1366–1369 (1989).
21. Nagaoka, S.,Y. Okauchi, S. Urano, U. Nagashima, and K. Mukai, Kinatic and *ab initio* Study of the Prooxidant Effect of Vitamin E. Hydrogen Abstraction from Fatty Acid Esters and Egg Yolk Lecithin, *J. Am. Chem. Soc. 112*:8921–8924 (1990).
22. Small, R.D. Jr., J.C. Scaino, and L.K. Patterson, Radical Processes in Lipids. A Laser Photolysis Study of *t*-Butoxy Radical Reactivity Toward Fatty Acids, *Photochem. Photobiol. 29*:49–51 (1979).
23. Gardner, H.W., D. Weisleder, and R. Kleiman, Formation of *trans*-12,13-Epoxy-9-Hydroperoxy-*trans*-10-Octadecenoic Acid from 13-L-Hydroperoxy-*cis*-9, *trans*-11-Octadecadienoic Acid Catalyzed by Either a Soybean Extract or Cysteine-$FeCl_3$, *Lipids 13*:246–255 (1978).
24. Labeque, R., and L.J. Marnett, Reaction of Hematin with Allylic Fatty Acid Hydroperoxides: Identification of Products and Implication for Pathways of Hydroperoxide-Dependent Epoxydation of 7,8-Dihydroxy-7,8 dihydrobenzo [*a*] pyrene, *Biochemistry 27*:7060–7070 (1988).
25. Lillard, D.A., and E. Day, Degradation of Monocarbonyls from Autoxidizing Lipids, *J. Am. Oil Chem. Soc. 41*:549–554 (1964).

26. Esterbauer, H., H. Zollner, and R. Jörg Schaur, Aldehydes Formed by Lipid Peroxidation: Mechanisms of Formation, Occurrence, and Determination, in *Membrane Lipid Oxidation*, Vol. I, edited by C. Vigo-Pelfrey, CRC Press, Boca Raton, 1990, pp. 239–268.
27. Mukai, K., K. Sawada, Y. Kohno, and J. Terao, Kinetic Study of the Prooxidant Effect of Tocopherol. Hydrogen Abstraction from Lipid Hydroperoxides by Tocopheroxyls in Solution, *Lipids 28*:747–752 (1993).
28. Maiorino, M., A. Roveri, and F. Ursini, Antioxidant Effect of Ebselen (PZ51): Peroxidase Mimetic Activity on Phospholipid and Cholesterol Hydroperoxides vs. Free Radical Scavenger Activity, *Arch. Biochem. Biophys. 295*:404–409 (1992).
29. Bors, W., C. Michel, and M. Saran, Inhibition of Bleaching of the Carotenoid Crocin, A Rapid Test for Quantifying Antioxidant Activity, *Bioch. Biophys. Acta 796*:312–319 (1984).
30. Bors, W., M. Erben-Russ, and M. Saran, Fatty Acid Peroxyl Radicals: Their Generation and Reactivities, *Biolectrochem. Bioenergetics 18*:37–49 (1987).
31. Pryor, W.A., J.A. Cornicelli, L.J. Devall, B. Tait, B.K. Trivedi, D.T. Witiak, and M. Wu, A Rapid Screening Test to Determine the Antioxidant Potencies of Natural and Synthetic Antioxidants, *J. Org. Chem. 58*:3521–3532 (1993).
32. von Sonntag, C., and H.P. Schuchmann, Pulse Radiolysis, *Meth. in Enzymol. 233*:3–20 (1994).
33. Franzke, C., and H. Iwainsky, Zur Antioxydativen Wirksamkeit der Melanoidine, *Deut. Lebensm.- Rundschau. 50*:251–254 (1954).
34. van Amsterdam, F.T.M., A. Roveri, M. Maiorino, E. Ratti, and F. Ursini, Lacidipine: A Dihydropyridine Calcium Antagonist with Antioxidant Activity, *Free Radical Biol. Med. 12*:183–187 (1992).
35. Ursini, F., M. Maiorino, P. Morazzoni, A. Roveri, and G. Pifferi, A Novel Antioxidant Flavonoid (IdB 1031) Affecting Molecular Mechanisms of Cellular Activation, *Free Rad. Biol. Med. 16*:547–553 (1994).
36. Eichner, K., Antioxidative Effect of Maillard Reaction Intermediates, *Prog. Fd. Nutr. Sci. 5*:441–451 (1981).
37. Lingnert, H., and C.E. Eriksson, Antioxidative Effect of Maillard Reaction Products, *Prog. Fd. Nutr. Sci. 5*:453–466 (1981).
38. Nergiz, C., and K. Unal, Determination of Phenolic Acids in Virgin Olive Oil, *Food Chem. 39*:237–245 (1991).
39. Montedoro, G., M. Servili, M. Baldioli, and E. Miniati, Simple and Hydrolyzable Phenolic Compouns in Virgin Olive Oil 1—Their Extraction, Separation, and Quantitative and Semiquantitative Evaluation by HPLC, *J. Agric. Food. Chem. 40*:1571–1578 (1992).

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