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Assessment of Pro-oxidant Activity of Foods by Kinetic Analysis of Crocin Bleaching

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The pro-oxidant activity of potent oxidants and foods was determined using the kinetic analysis of crocin bleaching. In its reduced form, crocin has an absorption band at 443 nm, which disappears upon oxidation by a generic radical species. Hydroxyl radicals generated by hydrogen peroxide, peroxyl radicals from ABAP, and the stable free radical DPPH• were allowed to react with crocin in an aqueous solution at 40 °C. Pro-oxidant activity was taken as the ratio between the decrease in crocin absorbance at 5 min and the relevant oxidant concentration. The test proposed was used to evaluate the pro-oxidant activity of widely consumed foods such as pasteurized skim milk and bread. They both exerted significant pro-oxidant activities, which were attributed to the early nonenzymatic browning products formed upon heat treatment.

KEYWORDS: Pro-oxidant activity; crocin; redox potential; milk; bread; Maillard reaction

INTRODUCTION

Nowadays, molecular and cellular damage due to free radicals is widely believed to be a major cause of chronic degenerative diseases including heart disease and cancer (*1*−*3*). Radicals can actually damage biomolecules and create toxic products by altering gene expression and disrupting the physiological repair mechanisms (*4*−*7*). The hypothesis that diet can affect in vivo oxidative damage has a strong theoretical and epidemiological basis (*8*−*15*). The initial excited enthusiasm arising from the awareness that foods contain a number of antioxidants with antiradical properties has been followed by a growing concern due to the continuous identification of radicals in widely consumed and before unsuspected foods (*16*−*23*). In fact, pro-oxidant molecule formation has been observed in model systems and foods during the early phases of Maillard browning but also as a consequence of the reaction between antioxidants and peroxyl radicals generated by lipid oxidation (*24*−*33*). In addition, most antioxidants present in foods (e.g., ascorbic acid, α-tocopherols, flavonoids, and catechins) are capable of exerting pro-oxidant actions depending on the reaction conditions (*34*).

Despite abundant methods widely used to assess antioxidant activity, it is surprising that only a few methodologies are available to detect the pro-oxidant activity of foods. Kinetic measurements have been carried out by detecting the rate of lipid oxidation in the presence of the sample or by biological tests evaluating its genotoxicity (*35*−*37*). In addition, studies of electron resonance spectroscopy (EPR) have been used to directly identify the presence of radicals in model systems and foods (*24*, *29*, *30*, *38*).

The present research was addressed to evaluate the possibility of assessing the overall pro-oxidant activity of foods by a simple and rapid spectrophotometric method. In particular, the sample was allowed to react with the carotenoid crocin in an aqueous solution. In its reduced form, crocin absorbs at 443 nm, but upon oxidation by a generic radical species (R•), its absorbance disappears:

$$\text{crocin} - \text{H} + \text{R}^{\bullet} \rightarrow \text{crocin}^{\bullet}\ (\text{bleached}) + \text{RH} \qquad (1)$$

The direction of this elementary reaction is straightforward and predictable on the basis of its thermodynamics. Due to its relatively low redox potential, crocin can be easily oxidized by potent as well as weak oxidants, such as those contained in foods, leading to a possible kinetic evaluation of their pro-oxidant activity.

After the procedure had been validated by testing the pro-oxidant activity of potent oxidants, the test proposed was used to evaluate the pro-oxidant activity of widely consumed foods such as milk and bread.

MATERIALS AND METHODS

Sample Preparation. A 30% (w/w) hydrogen peroxide aqueous solution was purchased from Sigma (Sigma Aldrich Chemicals, Milan, Italy). A 9.95×10^{-2} M aqueous solution of 2,2′-azobis(2-amidinopropane) dihydrochloride (ABAP) (Wako Chemicals GmbH, Neuss, Germany) and a 6.1×10^{-5} M 2,2-diphenyl-1-picrylhydrazyl (DPPH•) (Sigma Aldrich Chemicals, Milan, Italy) methanol solution were prepared. Pasteurized skim milk and fresh bread (standard recipe) were purchased from a local market. Before analysis, bread was ground in a mill (Moulinex, model 505, Paris, France) and submitted to solid−liquid extraction using 0.1 M phosphate buffer, pH 7.0 (Sigma Chemical Co., St. Louis, MO) (1:10 w/w) at 25 °C for 12 h. The extract was then filtered through Whatman No. 4 filter paper.

Total Solid Content. Total solid content determinations of milk and bread extract were carried out according to AOAC methods (*39*).

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pH. The pH was measured at 25 °C using a pH meter (Hanna Intruments, model 8417, Singapore).

Redox Potential. Measurements were made with a platinum indicating electrode and an Ag/AgCl, Cl^-_{sat} reference electrode connected with a voltmeter (Crison, model 2002, Alella, Spain). Calibration was performed against a redox standard solution (E = 468 mV at 25 °C, Reagecon, Shannon, Clare, Ireland). Electrodes were inserted into a 50 mL three-neck flask containing a volume of 20 mL of sample. Prior to analysis, oxygen was removed from the system by continuous nitrogen flushing for 10 min. Data were recorded for at least 5 min, until a stable redox potential was reached. A stable redox potential was arbitrarily defined as a change of <1 mV in a 5 min period.

Pro-oxidant Activity. The pro-oxidant activities were determined using crocin as a radical quencher. Crocin was isolated from saffron (Sigma Chemical Co, St. Louis, MO) by methanol extraction after repeated washings with ethyl ether. The crocin solution was diluted with 0.1 M phosphate buffer, pH 7.0 (Sigma Chemical Co.) in order to obtain a 1.35×10^{-5} M crocin solution (the absorption coefficient of crocin at 443 nm is 1.33×10^5 mol^{-1} cm^{-1}). The bleaching rate of crocin at 443 nm in the presence of the sample was monitored at 40 °C by a Uvikon 860 (Kontron Instruments, Milan, Italy) spectrophotometer. The reaction was started by the addition of increasing amounts of sample (0−100 μL) to 2 mL of crocin aqueous solution in a 3 mL capacity cuvette (1 cm length). The decrease in absorbance was determined every 30 s for 10 min. These conditions were chosen because they had already been validated to assess the antioxidant capacity of foods by determining the inhibition of crocin bleaching (*40*−*43*). In the case of crocin bleaching by peroxyl radicals generated from ABAP, the decrease in absorbance was recorded after 2 min of incubation. In fact, whereas hydrogen peroxide and DPPH• immediately reacted with crocin, the reaction with ABAP presented a lag phase of ~2 min. This short period corresponds to the time required to activate ABAP in order to generate peroxyl radicals (*40*). The pro-oxidant activity was expressed as the ratio between the decrease in crocin absorbance at 443 nm after 5 min of reaction and the relevant sample concentration (ΔOD_{5min} mg_{dm}^{-1}). In the case of milk and bread extract, all of their dry matter was assumed to possess pro-oxidant activity. The stoichiometric value of DPPH• was defined as the number of crocin moles oxidized by 1 mol of DPPH•. The number of crocin moles consumed was calculated from the difference between initial crocin concentration and the concentration when the reaction had gone to completion.

Statistical Analysis. The results reported in this work are the average of at least three measurements, and the coefficients of variation, expressed as the percentage ratio between the standard deviation and the mean value, were found to be <3% for total solid content, <4% for redox potential, and <8% for pro-oxidant activity. Linear regression analysis was performed using Statistica for Windows software (version 5.1, Statsoft, Inc., Tulsa, OK, 1997).

RESULTS AND DISCUSSION

Some potent oxidants, such as the hydroxyl radicals generated by hydrogen peroxide (H_2O_2), as well as the peroxyl radicals from ABAP and the stable free radical DPPH•, were chosen to evaluate the possibility of assessing their pro-oxidant activity by allowing them to react with crocin. In addition, pasteurized skim milk and bread, in which intense radicals have been recently observed by EPR spectroscopy (*30*), were considered to determine if such a methodology could be used to measure the eventual pro-oxidant activity of foods.

Table 1 shows the redox potentials of crocin and of the selected oxidants and foods. Due to its relatively low redox potential, crocin is expected to be easily oxidized by strong as well as weak oxidants (i.e., food oxidants), allowing the kinetic assessment of their pro-oxidant activity.

The kinetic effectiveness of increasing amounts of hydrogen peroxide and ABAP in promoting crocin bleaching is shown in **Figures 1** and **2**. It can be noted that both oxidants caused the quick oxidation of crocin. Crocin bleaching in the presence of these oxidants followed the zero-order kinetic model for all concentrations tested ($R > 0.97$, $P < 10^{-3}$). The zero-order kinetic constants (k) relevant to crocin bleaching in the presence of increasing amounts of the tested oxidants are shown in **Table 2**. These results are consistent with a previous observation by Buettner (*44*) indicating that, because of their high reactivity, radicals often undergo elementary reactions, which exert a second-order kinetic at most. Crocin bleaching was found to be proportional to the sample concentration added in the medium ($R > 0.98$, $P > 10^{-4}$), and the slopes obtained by linear regression analysis of the zero-order kinetic rates of crocin bleaching (k) as a function of the oxidant concentration were found to be 0.011 and 30.01 ΔOD min^{-1} mg_{dm}^{-1} for hydrogen peroxide and ABAP, respectively.

Figure 3 shows the changes in crocin absorbance as a function of reaction time in the presence of increasing amounts

Table 1. Redox Potential[a] and pH at 25 °C of Hydrogen Peroxide, ABAP, DPPH•, Milk, Bread Extract, and Crocin

	redox potential (Ag/AgCl) (mV ± SD)	pH
H_2O_2	320 ± 2	7
DPPH•	229 ± 3	6.9
ABAP	216 ± 2	7.5
milk	273 ± 6	6.8
bread extract	150 ± 3	7
crocin	120 ± 5	7

[a] Mean values of three determinations and standard deviation.

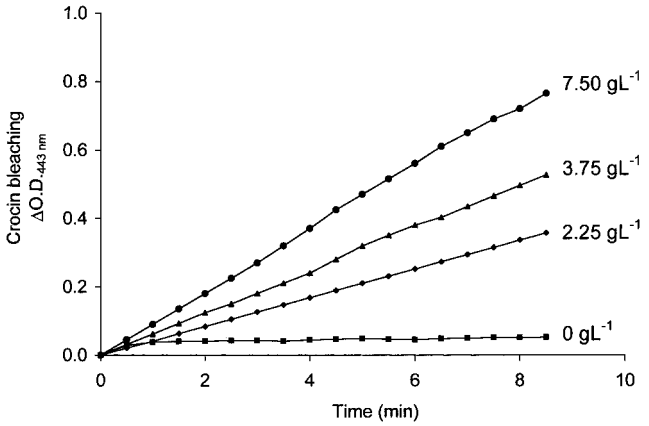

Figure 1. Crocin bleaching as a function of reaction time in the presence of increasing amounts of hydrogen peroxide.

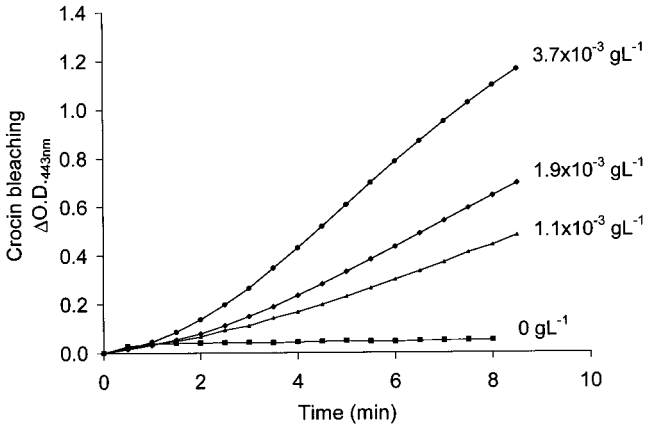

Figure 2. Crocin bleaching as a function of reaction time in the presence of increasing amounts of ABAP.

Table 2. Zero-Order Kinetic Constants (k) of Crocin Bleaching in the Presence of Increasing Concentration of Hydrogen Peroxide and ABAP[a]

oxidant	concentration M	concentration g L^{-1}	k (ΔOD $min^{-1} \pm$ SD)
H_2O_2	0	0	0.004 ± 0.002
	0.07	2.25	0.042 ± 0.002
	0.11	3.75	0.062 ± 0.003
	0.22	7.50	0.090 ± 0.002
ABAP	0	0	0.004 ± 0.002
	4.1×10^{-6}	1.12×10^{-3}	0.069 ± 0.004
	6.9×10^{-6}	1.87×10^{-3}	0.105 ± 0.005
	13.8×10^{-6}	3.74×10^{-3}	0.157 ± 0.004

[a] Number of samples, $n = 20$, $R > 0.97$, $P < 10^{-3}$; mean values of three determinations and standard deviation.

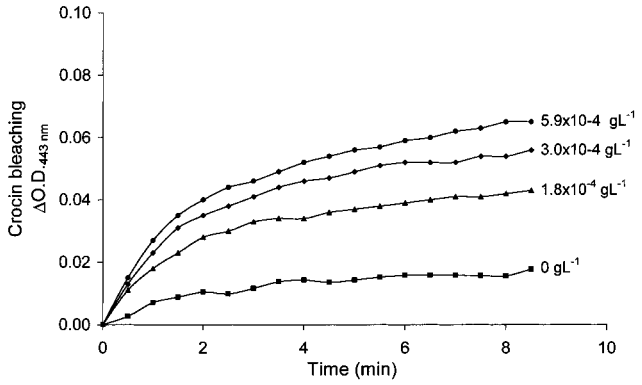

Figure 3. Crocin bleaching as a function of reaction time in the presence of increasing amounts of DPPH•.

of DPPH•. It is noteworthy that the evolution of the reaction kinetic of DPPH• was clearly different from those observed when hydrogen peroxide and ABAP were tested, because the former reacted with crocin following a hyperbolic curve. Such a behavior is in agreement with previous data showing that DPPH• reacts with most antioxidants undergoing nonelementary reactions and thus leading to typical hyperbolic curves (*45*, *46*). In addition, the stoichiometric value of DPPH•, which approximately corresponds to the number of crocin moles oxidized by 1 mol of DPPH•, was found to be <1, indicating that the mechanism of reaction can be very complicated and hardly predictable. In these conditions, crocin consumption by the sample accounts for the overall oxidative reactions occurring between crocin and the complex mixture of pro-oxidants, whether they are originally present in the sample or generated as a consequence of the reactions with crocin itself. In fact, a remarkable aspect of the electron transfer reaction is that not only can oxidants generate oxidants but oxidants can also beget reductants and vice versa (*33*). DPPH• can thus react with crocin, generating less oxidizing molecules that retain pro-oxidant activity and are able to further react with crocin. It is likely that crocin bleaching by DPPH•, and probably also by complex mixtures of pro-oxidants such as those contained in foods, follows multistep, generally parallel or consecutive, reactions, which are described by higher reaction orders. To obtain a feasible kinetic indicator of pro-oxidant activity, the consumption of crocin by increasing concentrations of DPPH• was fitted according to different kinetic models up to the fourth reaction order. In all cases, the kinetic analyses were found to be unsatisfactory as correlation coefficients were <0.80 ($P > 10^{-3}$). It was therefore decided to analyze data at a set reaction time. For this reason, the decrease in crocin absorbance after 5 min of reaction was arbitrarily chosen as an index of crocin

Table 3. Pro-oxidant Activity of Hydrogen Peroxide, DPPH•, and ABAP[a]

oxidant	pro-oxidant activity (ΔOD_{5min} $mg_{dm}^{-1} \pm$ SD)
H_2O_2	0.030 ± 0.002
DPPH•	35.167 ± 1.979
ABAP	96.138 ± 0.644

[a] Mean values of six determinations and standard deviation. See Materials and Methods for explanation of pro-oxidant activity expression.

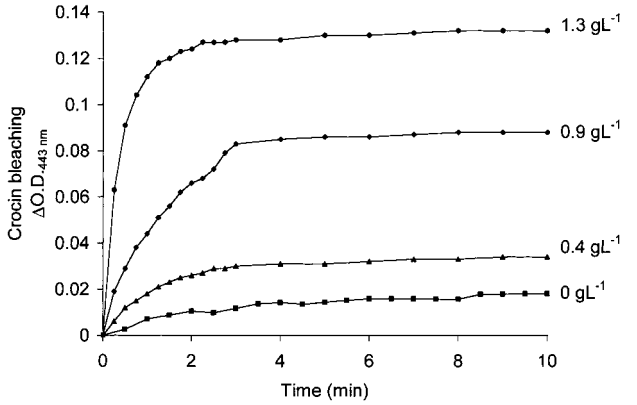

Figure 4. Crocin bleaching as a function of reaction time in the presence of increasing amounts of pasteurized skim milk.

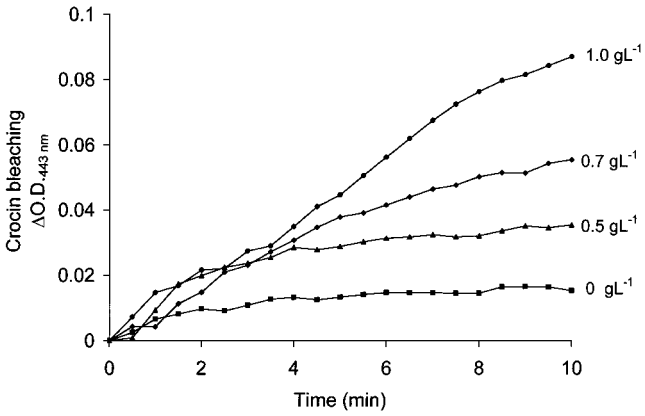

Figure 5. Crocin bleaching as a function of reaction time in the presence of increasing amounts of bread extract.

bleaching. Because this index was found to be proportional to the oxidant concentration ($R > 0.99$, $P < 10^{-3}$), pro-oxidant activity was expressed as the ratio between the decrease in crocin absorbance after 5 min and the relevant sample concentration (ΔOD_{5min} mg_{dm}^{-1}). To compare the pro-oxidant activity of the molecules tested, similar evaluations were also performed for hydrogen peroxide and ABAP (**Table 3**). It can be noted that conceptually different and complementary information can be obtained by comparing redox potential and pro-oxidant activity data (**Tables 1** and **3**). In fact, low pro-oxidant activity values were associated with high values of redox potential. For example, hydrogen peroxide presents the highest redox potential despite having the lowest pro-oxidant activity, indicating that oxidants can slowly react even with molecules having a considerably lower redox potential.

This approach was adopted to evaluate the pro-oxidant activity of foods that were indicated to contain radical compounds. In particular, commercial pasteurized milk and bread were chosen because they are highly suspected to develop protein-bound radical cations upon thermal treatment (*30*). Data shown in **Figures 4** and **5** clearly indicate that milk and bread

Table 4. Pro-oxidant Activity of Milk and Bread[a]

sample	pro-oxidant activity ($\Delta OD_{5min}\ mg_{dm}^{-1} \pm SD$)
milk	0.044 ± 0.008
bread	0.011 ± 0.001[b]

[a] Mean values of six determinations and standard deviation. See Materials and Methods for explanation of pro-oxidant activity expression. [b] Referred to water soluble compounds.

exert a significant pro-oxidant activity following nonlinear kinetic models. The pro-oxidant molecules contained in these foods are reasonably represented by a number of compounds having different natures and hence different reactivities toward crocin. In addition, these products may contain naturally occurring antioxidants as well as those formed as a consequence of heat treatments, such as nonenzymatic browning reaction products (*26*, *42*, *43*, *47*−*49*). These antioxidants can act as reductants, depending on their thermodynamic and kinetic properties, making the reaction kinetics even more complex. The concomitant development of these different pools of reactions can explain a kinetic behavior that was not satisfactorily described even by high-reaction-order models. In fact, as in the case of DPPH$^{\bullet}$ (**Table 3**), the correlation coefficients and probability values obtained from increasing reaction-order regression of the crocin bleaching curves in the presence of milk and bread were lower than 0.80 and 10^{-2}, respectively. In light of these findings, the pro-oxidant activity of the foods was taken as the ratio between the decrease in crocin absorbance after 5 min of reaction and the sample concentration (**Table 4**). In particular, milk pro-oxidant activity was expressed in relation to its dry matter, whereas, in the case of bread, pro-oxidant activity was referred to the water soluble compounds. As observed for the strong oxidants (**Table 3**), also for the foods considered, this index was found to be proportional to the sample concentration ($R > 0.98$, $P < 10^{-3}$). It can be noted that milk showed a pro-oxidant activity value significantly higher than that measured for the bread extract. These results could find a possible explanation considering the different intensities of the heat treatments applied in their production. In fact, whereas milk pasteurization is carried out with minimized development of nonenzymatic browning reactions, bread is cooked so that browning is achieved. It is known that, depending on the extent of the reaction, pro-oxidant or antioxidant molecules are expected to be formed (*27*). In particular, highly reactive radicals are formed in the early phases of the Maillard reaction, just prior to the Amadori rearrangement, whereas strong antiradical properties are attributable to the high molecular weight brown compounds formed in the advanced phases of the reaction. The latter have been shown to be abundant in bakery products but are reasonably present to a minimum extent in pasteurized milk (*37*, *41*, *50*−*51*). Because the pro-oxidant activity, as measured by the crocin bleaching test, is the result of the balance between the activity of pro-oxidant and antioxidant species, a lower pro-oxidant activity can be due to either less active oxidants or more efficient antioxidants.

CONCLUSIONS

The described spectrophotometric assay seems to be well-suited for determining the overall pro-oxidant activity of radicals as well as of complex mixtures of oxidants such as those contained in foods. In fact, information about the pro-oxidant capability of foods can be achieved by considering not only the thermodynamic properties of the reactants (i.e., their redox potential) but also the rate of the reactions involved. The reducing potential can predict if a compound, or a mixture of compounds, is thermodynamically capable of stealing an electron from any other species of known redox potential (*44*). However, a thermodynamically possible reaction may not be kinetically feasible because its rate constant may be too low to be significant. Although redox potential allows one to estimate the reducing properties of the sample, kinetic measurements evaluate its reaction rate toward a specific redox couple, taken as a reference. Also, kinetic methods present the additional advantage of accounting not only for the rate of the direct redox reaction between sample and reference standard but also for the eventual interactions among reactants, reaction intermediates, and products. In other words, a kinetic measure evaluates the rate of the reaction pool that is generated by the sample, allowing its overall activity to be assessed.

The method presented in this paper delivers rapid information about the kinetic capability of the sample to react in an aqueous solution with the reference redox couple crocin−H/crocin$^{\bullet}$. Compared with the existing kinetic measurements based on the detection of the rate of lipid oxidation or genotoxicity, the procedure described here appears to be simple, time-saving, and economic (*34*−*37*). In addition, the analyses do not involve the use of organic solvents or radical species in both reactant and sample preparation.

It must be noted that, like all kinetic measurement of oxidative capability, the assessment of pro-oxidant activity by crocin bleaching produces no direct evidence of the presence of specific radicals. The latter can be detected using chemical methods or identified in dynamic processes by electron resonance spectroscopy (*24*, *29*−*31*, *38*). Although allowing a deep insight in the radical-assisted mechanisms of reaction, these methodologies provide no information about the kinetic reactivity of the sample.

In conclusion, the possibility of dealing with a simple kinetic analysis of crocin bleaching can represent a useful tool to rapidly identify eventual pro-oxidant activity in foods as well as to evaluate whether antioxidant changes detected during food processing are attributable to the consumption of antioxidant species or to the formation of pro-oxidant molecules. However, it is evident that proper conclusions about the pro-oxidant capability of foods should arise by concomitant use of various methodologies, each providing different information about (i) sample reducing properties, (ii) kinetic activities in both chemical and biological systems, and (iii) the eventual presence of specific radicals.

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