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ARTICLE

New Tool To Evaluate a Comprehensive Antioxidant Activity in Food Extracts: Bleaching of 4-Nitroso-*N*,*N*-dimethylaniline Catalyzed by Soybean Lipoxygenase-1

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In this study the 4-nitroso-*N*,*N*-dimethylaniline (RNO) bleaching associated with linoleic acid hydroperoxidation catalyzed by the soybean lipoxygenase (LOX)-1 isoenzyme (LOX/RNO reaction) was used to determine the antioxidant activity (AA) of hydrophilic and lipophilic pure antioxidant compounds and of mixtures of antioxidants extracted from durum wheat whole flour (DWWF). By means of a simple and rapid experimental protocol (about 3 min/assay), the LOX/RNO reaction may simultaneously detect many antioxidant functions (scavenging of some physiological radical species, iron ion reducing and chelating activities, inhibition of the pro-oxidant apoenzyme), thus providing a comprehensive AA evaluation. Consistently, the LOX/RNO assay was very sensitive to hydrophilic, lipophilic, and phenolic antioxidant extracts from DWWF, providing AA values at least 35 and 30 times higher than those by TEAC and ORAC methods, respectively. Moreover, the new method was able to highlight synergism (among extracts) 3 times more than the ORAC method, whereas TEAC did not measure synergism under our experimental conditions.

KEYWORDS: Antioxidant activity; antioxidant synergism; durum wheat whole grains; 4-nitroso-*N*,*N*-dimethylaniline (RNO) bleaching; soybean lipoxygenase

INTRODUCTION

In the past two decades food antioxidants have become a topic of increasing interest, and nowadays the assessment of food antioxidant activity (AA) still represents a very popular objective of food science research. Several methods to measure AA of food extracts and biological samples have been developed; these assays differ from each other in terms of reaction mechanisms, oxidant and target/probe species, reaction conditions, spectroscopic or chromatographic methodology used for monitoring the reaction progress, and quantification methods (*1*); therefore, the results from different assays are extremely difficult to compare. Some assays involve single electron transfer (SET) redox reactions and mainly measure the capacity of an antioxidant to reduce an oxidant (also representing the probe for monitoring the reaction), which changes color when reduced (*2*). These assays are considered to be very easy, rapid, and accurate methods with regard to measuring the antioxidant capacity of food samples (*2*), but the SET redox reactions are known to play a minor role in vivo. Other methods are based on hydrogen atom transfer (HAT) reactions; the majority of these assays apply a competitive reaction scheme, in which an antioxidant and an oxidizable substrate (or a molecular probe) compete for thermally generated peroxyl radicals through the decomposition of an azo radical initiator (*2*). These methods have found even broader application for measuring AA of food extracts and biological samples. Unfortunately, the above-mentioned assays do not measure an actual total AA, because they highlight only one or a few possible mechanisms of antioxidant protection against oxidative damage. The majority of the commonly used AA assays, in fact, are able to measure only the capacity of antioxidant compounds to break the radical propagation chain by hydrogen atom or electron donation or of reducing oxidant species, failing to detect also the antioxidant capacity of chelating or reducing metal ions involved in the radical generation and of inhibiting pro-oxidant enzymes (*3*). Moreover, according to the majority of the published assays, measurements are carried out under experimental conditions (radical and oxygen concentration, reaction time, pH) not resembling physiological ones; last but not least, the antioxidant capacity toward nonphysiological radical species is often evaluated.

To overcome these problems, we developed a new assay for AA assessment in food extracts, based on the bleaching of 4-nitroso-*N*,*N*-dimethylaniline [abbreviated RNO (*4*), because it belongs to the family of *C*-nitroso compounds (RN═O, R = alkyl or aryl group)] catalyzed by soybean lipoxygenase. Lipoxygenases (linoleate:oxygen oxidoreductase, EC 1.13.11.12) (LOXes) constitute a large gene family of non-heme-iron-containing fatty acid

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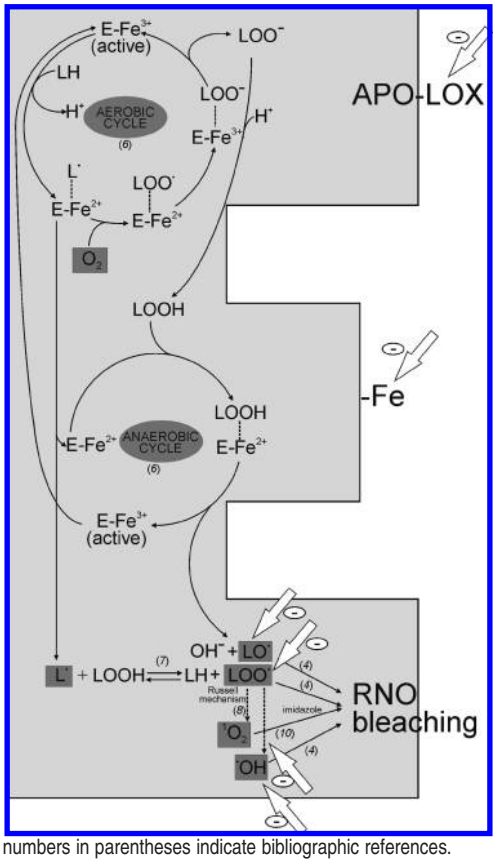
dioxygenases, which occur in both the plant and animal kingdoms. LOXes catalyze the regio- and stereospecific insertion of molecular oxygen into polyunsaturated fatty acids containing 1,4-*cis*,*cis*-pentadiene structures to produce the corresponding hydroperoxy derivatives. Some relevant information about plant LOXes is provided in ref *5*. In **Scheme 1** the aerobic cycle of linoleate (LH) dioxygenation to 13-hydroperoxylinoleate (LOOH) catalyzed by the soybean LOX-1 isoenzyme is shown. The apoenzyme (APO-LOX) is schematized as E-shaped. The key role of the non-heme iron atom (Fe), cycling from the oxidized form (3+) of the active enzyme to the reduced one (2+), is also well highlighted (*6*). Under conditions of limited oxygen, reached with the progress of the reaction, LOX-1 is also involved in several secondary anaerobic reactions that generate, in the presence of LH and LOOH preformed in the course of the aerobic cycle, some radical species, which also occur in vivo (and so referred to as "physiological"): fatty acid radicals, including the linoleate alkylic (L$^{\bullet}$), alkoxyl (LO$^{\bullet}$), and peroxyl (LOO$^{\bullet}$) radicals, and oxygen radical species, such as the hydroxyl radical ($^{\bullet}$OH) and the singlet oxygen ($^1O_2$) (*6*−*8*). It is known that these radicals may cause the oxidation of plant pigments (ref *9* and references cited therein) and the oxodiene generation (*4*, *7*). Moreover, for some of these radicals [LO$^{\bullet}$, LOO$^{\bullet}$, and $^{\bullet}$OH, as well as $^1O_2$, but only in the presence of imidazole (*10*)] the capability to induce the bleaching of RNO in a biochemical pathway coupled with oxodiene formation has been demonstrated (*4*). As shown in **Scheme 1**, the soybean LOX-1-dependent RNO bleaching may be delayed, inhibited, or even prevented by antioxidant compounds by different mechanisms (white arrows): scavengers of one or more free radical species; chelating or reducing agents of the iron ion essential for the catalysis; inhibitors of the apoenzyme (antiperoxidative action). Therefore, the peculiarity of the soybean LOX-1-mediated RNO bleaching (LOX/RNO reaction) is to simultaneously detect, under conditions of low oxygen supply, the scavenging capacity toward "physiological" and biologically relevant radical species together with other important antioxidant functions. Thus, a method based on the LOX/RNO reaction (LOX/RNO method) is expected to provide more integrated and comprehensive information about the food antioxidant capacity.

The objective of this study was to define the new LOX/RNO method and to evaluate the performances of this method with respect to the AA determination of both pure antioxidant molecules and complex mixtures of phytochemicals extracted from durum wheat (*Triticum durum* Desf.) grains, which is known as a relevant source of antioxidants. To evaluate synergistic interaction among different antioxidant extracts from durum wheat whole flour, the LOX/RNO method was also compared with the well-known Trolox equivalent antioxidant capacity (TEAC) (*11*) and the oxygen radical absorbance capacity (ORAC) assays (*12*), which measure the capability of antioxidants to reduce the radical cation ABTS$^{\bullet+}$ and the chain-breaking antioxidant capacity against peroxyl radicals, respectively.

MATERIALS AND METHODS

2.1. Chemicals. All reagents at the highest commercially available purity (usually ranging from 97 to 99%) were purchased from Sigma Chemical Co. (St. Louis, MO). Gallic acid, ferulic acid, glutathione, ascorbic acid, inulin, and RNO were dissolved in 80 mM sodium borate buffer, pH 9.00, and resveratrol, catechin, and α-tocopherol in absolute ethanol. Apigenin was dissolved in 1 M KOH solution; 3′,6′-dihydroxyspiro[isobenzofuran-1[3*H*],9′[9*H*]-xanthen]-3-one (fluorescein) and 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) were dissolved in 75 mM sodium phosphate buffer, pH 7.40; and

Scheme 1. Schematic Representation of the Aerobic and Anaerobic Cycles Catalyzed by Soybean Lipoxygenase (LOX)-1 Isoenzyme Explaining the Reaction of the 4-Nitroso-*N*,*N*-dimethylaniline (RNO) Bleaching[a]



[a] The numbers in parentheses indicate bibliographic references.

(±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) was dissolved in the medium required by the assay used to determine AA. An ammonium sulfate suspension of soybean LOX type V (LOX-1 isoenzyme) was used, the activity of which ranged from 0.07 to 0.1 enzymatic units (EU) μg^{-1} of protein in different stocks as determined by means of the LOX assay described in ref *13*; a 1:10−1:15 dilution of this suspension with 80 mM sodium borate buffer, pH 9.00, was generally used.

2.2. Plant Material. Plant material used in this study was represented by whole grains of durum wheat (*T. durum* Desf. cv. Ofanto). Whole grain samples were stored at 4 °C, and before use, they were milled by means of a Cyclotec 1093 sample mill (1 mm sieve).

2.3. Preparation of Aqueous Solutions of Linoleate and *β*-Carotene. The linoleate solution was prepared according to the method given in ref *13*, by dissolving 45 mg of sodium linoleate and 150 μL of Tween 20 in 5 mL of 80 mM sodium borate buffer, pH 9.00. The exact linoleate concentration was determined by means of the LOX assay (*13*). The β-carotene solution was prepared according to the method given in ref *13* (and references cited therein), by dissolving 25 mg of β-carotene and 900 μL of Tween 80 in 25 mL of chloroform; 1 mL of this solution was dried under vacuum at 25 °C for 15 min, and the residue was dissolved in 10 mL of 80 mM sodium borate buffer, pH 9.00. The β-carotene concentration was spectrophotometrically determined by means of a Perkin-Elmer λ 45 UV−vis spectrometer (Perkin-Elmer, Wellesley, MA) as described in ref *13* (and references cited therein).

2.4. Enzymatic Assays. *2.4.1. Oxygen Uptake and Oxodiene Formation Catalyzed by Soybean LOX-1.* A parallel investigation of RNO bleaching (section 2.4.2) and oxygen uptake due to linoleate hydroperoxidation reaction by soybean LOX-1 was performed in the experiment reported in **Figure 1A**. To do this, oxygen uptake was monitored as described in ref *4*, by means of a Gilson Oxygraph model 5/6-servo Channel pH 5, equipped with a Clark-type electrode (5331 YSI, Yellow Springs, OH). The reaction medium (2 mL) consisted of 80 mM

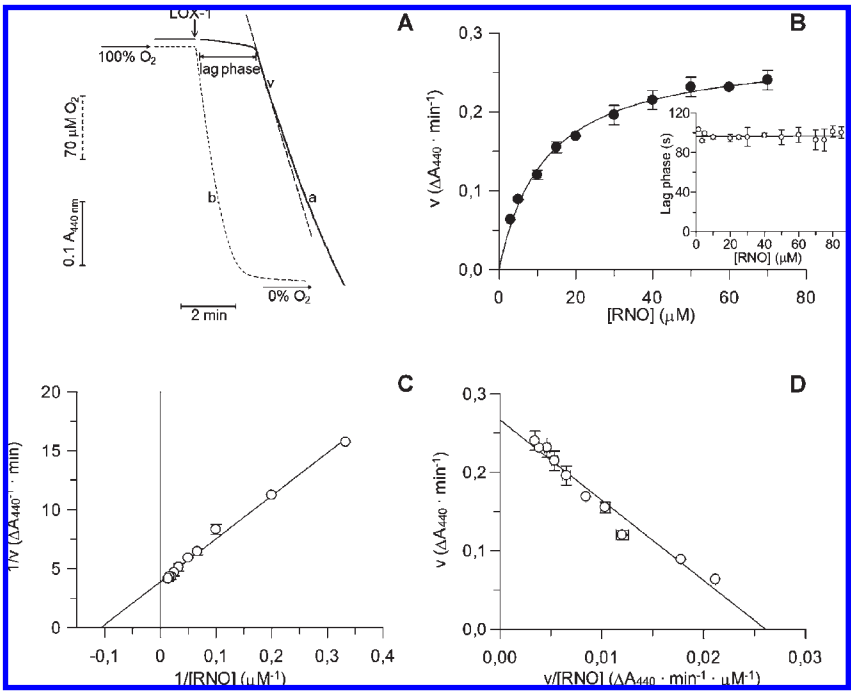


Figure 1. Kinetics of the RNO bleaching catalyzed by soybean LOX-1. (**A**) The LOX-1-dependent RNO bleaching (curve a) was spectrophotometrically monitored, as described under Materials and Methods (section 2.4.2). Simultaneous oxygen uptake during soybean LOX-1 reaction (dotted line, curve b) was oxygraphycally monitored, as described under Materials and Methods (section 2.4.1). Both reactions were started by adding 0.3 EU of soybean LOX-1 (Sigma type V). The rate of the RNO bleaching (v) and the lag phase are indicated. (**B**−**D**) Measurements were carried out as described in **A**, in the presence of different RNO concentrations. The rates of RNO bleaching, expressed as ΔA_{440} min^{-1}, are reported as Michaelis−Menten (**B**), Lineweaver−Burk (**C**), and Eadie−Hofstee (**D**) plots. Data are expressed as mean value ± standard deviation (n = 3). In the inset of **B** the dependence of the lag phase on RNO concentration is reported.

sodium borate buffer, pH 9.00, 1 mM sodium linoleate, per μmol of linoleate; the reaction was started by adding 0.3 EU of soybean LOX-1.

In the experiment reported in **Figure 2** (inset), oxodiene formation in the course of linoleate hydroperoxidation by soybean LOX-1 was spectrophotometrically monitored at 25 °C according to the procedure described in ref *7*, modified as in ref *4*, by measuring the absorbance increase at 285 nm. The reaction mixture (2 mL) contained 80 mM sodium borate buffer, pH 9.00, 1 mM sodium linoleate, and 1.5 μL of Tween 20 per μmol of linoleate; the reaction was started by adding 0.3 EU of soybean LOX-1. The rate of oxodiene generation, expressed as ΔA_{285} min^{-1}, was calculated as the tangent of the experimental curve at which the highest variation of absorbance per min was reached.

2.4.2. RNO Bleaching Catalyzed by Soybean LOX-1. RNO bleaching in the course of linoleate hydroperoxidation by soybean LOX-1 (LOX/RNO reaction) was spectrophotometrically monitored, as described in ref *4*, by measuring the RNO absorbance decrease at 440 nm and 25 °C. The reaction mixture (2 mL) contained 80 mM sodium borate buffer, pH 9.00, 1 mM sodium linoleate, 1.5 μL of Tween 20 per μmol of linoleate, and different RNO concentrations (usually 15 μM when not differently specified); the reaction was started by adding 0.3 EU of soybean LOX-1. The rate of the LOX/RNO reaction, expressed as ΔA_{440} min^{-1}, was calculated as the highest slope to the experimental curve. The lag phase (i.e., the time occurring between enzyme addition to the test sample and the start of the reaction) and the $tA_{75\%\text{ or }50\%}$ (i.e., the time occurring to reach a RNO absorbance equal to 0.75 or 0.5 times the initial value, respectively) were expressed in seconds. The $tA_{75\%\text{ or }50\%}$ is a function of both the rate and the lag phase of the LOX/RNO reaction, so it may give an integrated and simultaneous evaluation of the changes of these two parameters. Both the rate and the lag phase of the LOX/RNO reaction are affected by ethanol, but no clear dose−effect response has been observed. Therefore, the control reaction should be carefully evaluated at the exact ethanol concentration used in the assay every time this solvent is used to dissolve antioxidant compounds.

The LOX/RNO reaction was studied in the absence and presence of pure antioxidant compounds. With regard to glutathione, which chemically consumes RNO, the effect on secondary LOX reactions was evaluated by studying the oxodiene generation (see section 2.4.1 and Results and Discussion). With respect to apigenin, the absorbance spectrum of which partially overlaps the one of RNO and also shows a maximum at 310 nm, the effect on the LOX/RNO reaction, which can be studied neither at 440 nm nor as oxodiene formation, was evaluated by monitoring the RNO absorbance changes at 475 nm; at this wavelength apigenin absorbance approaches zero, whereas that of RNO is still sufficiently high. With regard to β-carotene, which is subjected to absorbance bleaching by LOX and the absorbance spectrum of which largely overlaps the one of RNO, the LOX/RNO reaction was studied by measuring the RNO absorbance changes as absorbance difference $\Delta A_{394-512}$ by means of a Jasco V-560 properly modified in a double-wavelength spectrophotometer.

2.5. Extraction of Hydrophilic, Lipophilic, and Phenolic Compounds from Daily Milled Whole Flour. *2.5.1. Hydrophilic Extracts.* The whole flour was suspended in deionized water at a (w/v) ratio equal to 1 g/3 mL. The suspension was placed in an ice−water bath for 1 h, stirred at 15 min intervals, and then centrifuged twice at 18700*g* for 20 min at 4 °C. The final supernatant represents the hydrophilic extract.

2.5.1.1. Determination of Protein and Flavonoid Content in Hydrophilic Extracts. Determinations were performed according to the procedures described in refs *14* and *15*, respectively.

2.5.1.2. Determination of Endogenous LOX Activity in Hydrophilic Extracts. Because the hydrophilic extracts may contain LOX, which may affect the LOX/RNO assay, a preliminary investigation is necessary to ascertain the occurrence of an endogenous LOX activity. It was performed by measuring linoleate hydroperoxidation activity of extract as described in ref *13*.

2.5.2. Lipophilic Extracts. Lipophilic compounds were extracted according to the procedure described in ref *16* with minor modifications. Briefly, whole wheat flour (2 g) was saponified with 60% (w/v) KOH under nitrogen at 70−80 °C for 45 min, and then the suspension was extracted twice with 15 mL of *n*-hexane/ethyl acetate 9:1 (v/v). The organic phases were collected, combined, and partitioned into

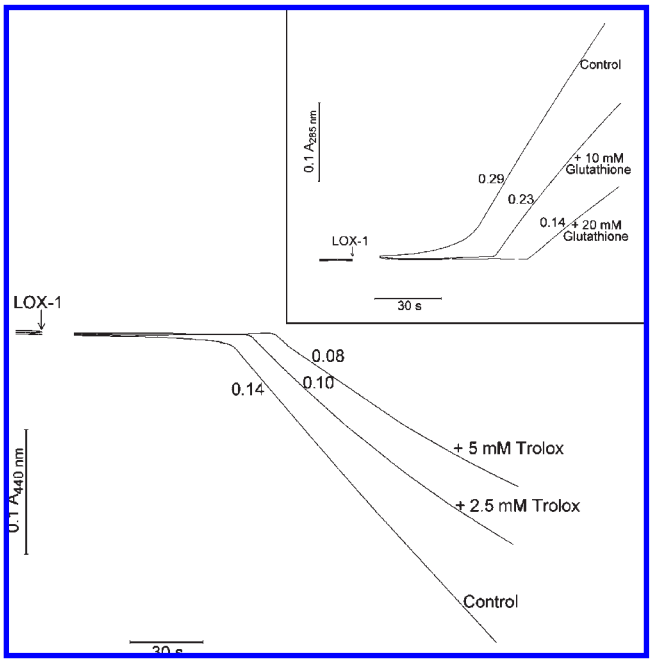


Figure 2. Inhibition of the LOX/RNO reaction by Trolox and of the LOX-dependent oxodiene formation by glutathione (inset). The LOX/RNO reaction was carried out as described in **Figure 1A**, in both the absence (control) and presence of either 2.5 or 5 mM Trolox. (Inset) The LOX-dependent oxodiene formation was spectrophotometrically monitored, as reported under Materials and Methods (section 2.4.1). Measurements were carried out both in the absence (control) and in the presence of either 10 or 20 mM glutathione. All of the reactions were started by adding 0.3 EU of soybean LOX-1. The numbers alongside the curves represent the reaction rates expressed as $\Delta A_{440\ or\ 285}\ min^{-1}$.

two equal volumes that were separately evaporated to dryness under vacuum at 40 °C using a Buchi evaporator. For the AA assessment by the LOX/RNO method, the first dry residue was reconstituted in 10 mL of 80 mM sodium borate buffer, pH 9.00, containing 2 mM sodium linoleate and 1.5 μL of Tween 20 per μmol of linoleate; for AA measurement by the TEAC and ORAC methods, the second residue was reconstituted in 1 mL of ethanol.

2.5.2.1. Determination of Total Carotenoid, Tocotrienol, and Tocopherol Content in Lipophilic Extracts. Determinations were performed by means of HPLC analysis, as reported in ref *16*.

2.5.3. Phenolic Extracts. Insoluble-bound phenolic compounds were extracted from whole wheat flour samples according to the procedure described in ref *17*, with some modifications. Whole wheat flour (1 g) samples were extracted twice with 10 mL of 80% (v/v) ethanol for 10 min at room temperature and centrifuged at 5000*g* for 10 min at 20 °C. The residue from ethanol extraction was digested with 20 mL of 2 M NaOH at room temperature for 1 h under nitrogen. The resultant hydrolysate was acidified to pH 5−6 with acetic acid and centrifuged at 5000*g* for 15 min at 20 °C, and the supernatant was retained; the residue was washed twice with 10 mL of water and centrifuged at 5000*g* for 10 min at 20 °C. The combined supernatants were concentrated under vacuum at 40 °C to 15 mL, adjusted to pH 2−3 with 6 M HCl, and extracted twice with *n*-hexane to remove interfering lipids. The organic phases were discarded, whereas the water phases were collected and combined and then submitted to three extractions with ethyl acetate to obtain phenolic compounds. The ethyl acetate fractions were combined and evaporated to dryness under vacuum at 40 °C, and the dry residue was reconstituted in 2 mL of deionized water, obtaining the extract referred to as insoluble-bound phenolic extract.

2.6. Determination of Total Phenolics and Phenolic Acid Content of Hydrophilic and Phenolic Extracts. Total phenolics of both hydrophilic (section 2.5.1) and insoluble-bound phenolic extracts (section 2.5.3) were determined using the colorimetric Folin−Ciocalteu method described in ref *18* and quantified by means of a proper calibration on a ferulic acid basis.

Phenolic acid content was determined by HPLC analysis according the procedure described in ref *19*. Briefly, 20 μL of the extract was analyzed by HPLC (Shimadzu LC10, Japan) with a diode array detector (SPD-M10A Shimadzu, Japan) and a Prodigy column (5 μm ODS3 100A, 250 × 4.60 mm; Phenomenex, Torrance, CA) at a flow rate of 1 mL min^{-1}. The mobile phase was a mixture of water/formic acid (95:5, v/v) and methanol. Peak identification in the HPLC chromatogram of the extracts was obtained by comparing the retention times of eluted compounds with those of pure standards at 325 nm.

2.7. AA Determination of Hydrophilic, Phenolic, and Lipophilic Extracts from Durum Wheat Whole Flour by Means of the LOX/RNO, TEAC, and ORAC Methods. *2.7.1. LOX/RNO Method.* The LOX/RNO reaction was carried out as described in section 2.4.2, in both the absence (control) and presence of sample (extract or standard antioxidant). Measurements were carried out in duplicate at each separate (at least three) amount of extract or concentration of standard antioxidant. The inhibition of the LOX/RNO reaction was determined by calculating the decrease of the rate of the RNO bleaching (%) measured in the presence of extract (or standard antioxidant) (v_a) with respect to the control (v_c), according to the following equation: inhibition (% of the control) = $[1 - (v_a/v_c)] \times 100$. AA was calculated by means of a dose−response curve prepared with Trolox by plotting the decrease of the rate of RNO bleaching (%) as a function of the standard antioxidant concentration and expressed as micromoles of Trolox equivalents per gram of dry whole flour. With regard to the lipophilic extract, because it already contained sodium linoleate (see section 2.5.2), every time this extract was tested, considering the volume of the assayed extract, a further appropriate linoleate concentration was added to the assay reaction mixture to obtain a 1 mM linoleate final concentration.

Determination of AA was carried out at 25 °C (see section 2.4.2). Although no significant changes in assay accuracy are presumed to occur at 37 °C or other temperatures, the sensitivity to different antioxidant compounds may vary as a result of temperature changes; in fact, temperature may differently affect the binding of each single antioxidant with the ternary complex enzyme−radical−RNO. Therefore, temperature should be carefully maintained in the course of the assay.

2.7.2. TEAC (Trolox Equivalent Antioxidant Capacity) Method. The TEAC assay described in ref *11* was applied with slight modifications. The colored radical monocation $ABTS^{\bullet+}$ was generated by ABTS [2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)] oxidation with potassium persulfate. The $ABTS^{\bullet+}$ solution was diluted with either 5 mM sodium phosphate buffer, pH 7.40 (for AA determination of both hydrophilic and phenolic extracts), or ethanol (for AA determination of lipophilic extract), to obtain an absorbance at 734 nm of 0.70 ± 0.20. The assay mixture contained 1.0 mL of the $ABTS^{\bullet+}$ diluted solution, the extract (or standard antioxidant), and an appropriate volume of sodium phosphate buffer, pH 7.40 (or ethanol), to obtain a final volume of the assay mixture equal to 1.1 mL. The absorbance at 25 °C and 734 nm (A_{734}) was read exactly 5 min after extract (or standard antioxidant) addition. Measurements were carried out in triplicate at each separate (at least three) amount of extract or concentration of standard antioxidant: in particular, amounts of hydrophilic, phenolic, and lipophilic extracts ranging from 0.3 to 1.0 mg, from 0.5 to 1.0 mg, and from 15 to 40 mg of dry whole flour, respectively, were tested; with regard to standard antioxidant, Trolox was added to obtain a final concentration ranging from 5 to 15 μM. The decrease of A_{734} (%) measured 5 min after the extract (or standard antioxidant) addition with respect to A_{734} of the radical cation solution (blank) was used to quantify AA by means of a proper concentration−response curve prepared with Trolox by plotting the decrease of A_{734} (%) as a function of the standard antioxidant concentration.

2.7.3. ORAC (Oxygen Radical Absorbance Capacity) Method. The ORAC protocol described in ref *12* was applied with some modifications. The assay mixture (2 mL), preincubated at 37 °C for 15 min, contained 75 mM sodium phosphate buffer, pH 7.40, and 6.3 nM fluorescein; the reaction was started by adding 38 mM AAPH. Because lipophilic antioxidants extracted from wheat flour were reconstituted in ethanol (see section 2.5.2), in this case a constant volume of ethanol was maintained in the assay mixture. Fluorescence intensity decay due to

fluorescein oxidation by peroxyl radicals generated by AAPH thermal decomposition was continuously monitored by using time drive application (collecting fluorescence data every 0.1 s) at 37 °C at excitation and emission wavelengths of 485 and 515 nm, respectively, by means of a Perkin-Elmer LS 55 spectrofluorometer. Measurements were carried out in the absence (blank) and presence of sample (extract or standard antioxidant). Sample fluorescence decay curves were first normalized to the blank curve by multiplying original data by the factor initial $\text{fluorescence}_{\text{blank}}$/initial $\text{fluorescence}_{\text{sample}}$. Then, the areas under the curves (AUC) corresponding to the sample ($\text{AUC}_{\text{sample}}$) and to the blank ($\text{AUC}_{\text{blank}}$) were calculated, and the AUC_{net} was obtained by subtracting the $\text{AUC}_{\text{blank}}$ from the $\text{AUC}_{\text{sample}}$. AA was determined using a standard curve obtained by plotting the AUC_{net} as a function of Trolox concentration.

2.8. Evaluation by Means of the LOX/RNO, TEAC, and ORAC Methods of Synergistic Interaction among Different Antioxidant Extracts Obtained from Durum Wheat Whole Flour. In **Figure 6**, hydrophilic, lipophilic, and phenolic extracts (section 2.5) were prepared from the same wheat flour sample and in the same experiment. AA of each extract was quantified by means of the LOX/RNO, TEAC, and ORAC methods and expressed as micromoles of Trolox equivalents. An amount was determined for each extract, able to induce a small but significant inhibition of the LOX/RNO or TEAC or ORAC assays. The mathematical sum of AA values of the three extract amounts was calculated (AA_{sum}). Then, the three extract amounts were mixed and AA was determined again (AA_{mix}). Synergistic action was evaluated as percentage increase of AA_{mix} value with respect to AA_{sum} value according to the following equation: $\text{synergistic increase}\,(\%) = [(\text{AA}_{\text{mix}}/\text{AA}_{\text{sum}}) - 1] \times 100$.

2.8.1. LOX/RNO Method. AA of the hydrophilic, phenolic, and lipophilic extracts was determined as previously described (section 2.7.1). With regard to the mix, because it contained the lipophilic extract obtained from 70 mg of dry whole flour together with the hydrophilic and phenolic extracts from only 6 and 9 mg of dry whole flour, respectively, AA_{mix} determination was carried out as described for the lipophilic extract.

2.8.2. TEAC Method. AA of each extract and AA_{mix} were determined as previously described for hydrophilic and phenolic extracts (section 2.7.2). The mix used to evaluate the antioxidant synergism contained hydrophilic, phenolic, and lipophilic extracts obtained from 0.3, 0.5, and 15 mg of dry whole flour, respectively.

2.8.3. ORAC Method. All of the determinations were performed as previously described for lipophilic extracts (section 2.7.3). The mix used to evaluate the antioxidant synergism contained hydrophilic, phenolic, and lipophilic extracts obtained from 0.7, 2.2, and 4.3 mg of dry whole flour, respectively.

2.9. Statistical Analysis. Interpolation of the data reported in the figures and the kinetic parameter calculations were obtained by means of GRAFIT 5.0 (ERITHACUS) software. Statistical analysis was obtained by means of MSTAT-C statistical package (version 2.1, 1991; Crop and Soil Sciences Department, Michigan State University, East Lansing, MI). All data are reported as mean value ± standard deviation ($n = 3$, when not differently specified), except data in the antioxidant/Trolox column of **Table 1**, which are reported as mean value ± standard error.

RESULTS AND DISCUSSION

In this study a new LOX/RNO method for in vitro AA measurement is developed, based on the RNO bleaching associated with linoleic acid hydroperoxidation catalyzed by the soybean LOX-1 isoenzyme. In a previous study (*4*) the UV−vis absorbance spectra recorded in the course of the LOX/RNO reaction at pH 9.00 (soybean LOX-1 optimum) were already reported; it should be noted that the most important bands were unchanged when the absorbance spectra were recorded in the investigated physiological pH range of 7.00−7.40 (data not shown). The soybean LOX-1-dependent RNO bleaching (LOX/RNO reaction) can be easily photometrically monitored by continuously measuring the RNO absorbance decrease at 440 nm. In the experiment of **Figure 1A** the LOX-1-mediated RNO bleaching (curve a) and oxygen uptake (dotted curve, curve b) were simultaneously monitored. It should be noted that LOX-1 addition to the reaction mixture, containing excess linoleate (1 mM) and limited oxygen (256 μM), caused a rapid oxygen consumption due to linoleate dioxygenation, whereas no simultaneous RNO absorbance change was observed. The RNO bleaching, indicated by a rapid RNO absorbance decrease as a function of time, started about 1.5−2 min after enzyme addition, that is, only when a very low oxygen concentration approaching anaerobiosis was reached in the test sample [ranging between 20 and 50 μM in different experiments (*4*)]. The lag phase, that is, the time occurring between enzyme addition to the assay mixture and the start of the RNO bleaching, represents the time necessary to consume oxygen during the primary aerobic LOX-1 reaction of linoleate peroxidation (*4*), whereas the RNO bleaching is essentially an anaerobic phase, which has been demonstrated to depend on some "physiological" radical species generated in the course of secondary anaerobic reactions mediated by LOX-1 (*4*) (see also **Scheme 1**). The lag phase was expressed in seconds, whereas the rate of RNO bleaching was calculated as the highest slope of the experimental curve and expressed as $\Delta A_{440}\ \text{min}^{-1}$. In ref *4* the enzymatic nature of the LOX/RNO reaction has been demonstrated on the basis of the dependence of the reaction rate on LOX-1 amount, linoleate concentration, pH, and temperature and of the sensitivity to the powerful LOX inhibitor *n*-propylgallate (*n*-PG). Here, the dependence of the reaction rate and of the lag phase on RNO concentration was investigated. The rate of the LOX/RNO reaction was found to exhibit hyperbolic dependence on RNO concentration as predicted by the Michaelis−Menten equation (**Figure 1B**). Saturation kinetics were confirmed by plotting the data according to Lineweaver−Burk (**C**), Eadie−Hofstee (**D**), Eadie−Scatchard, and Hanes (data not shown) plots. In this experiment, in which 0.3 EU of soybean LOX-1 was used, the V_{max} and K_{m} mean values from all of these plots were equal to $0.270 \pm 0.096\ \Delta A_{440}\ \text{min}^{-1}$ and 11.91 ± 1.12 μM, respectively. In different experiments carried out by using the same LOX amount from different enzyme stocks, the V_{max} and K_{m} values ranged between 0.26 and 0.30 $\Delta A_{440}\ \text{min}^{-1}$ and between 10 and 15 μM, respectively. As expected, the time of the lag phase showed no dependence on RNO concentration (inset of **Figure 1B**). These results substantiate the enzymatic nature of the LOX/RNO reaction, with a lag phase dependent on the aerobic linoleate hydroperoxidation by LOX-1 and a RNO bleaching phase due to the LOX-1-mediated generation of radicals under anaerobiosis. The occurrence of a hyperbolic dependence of the reaction rate on RNO concentration indicates that the reaction cannot occur in the bulk phase of the reaction mixture, but at the LOX enzyme, in particular, at the level of a definite number of RNO binding sites, generating a ternary complex enzyme−radical−RNO. Therefore, in this case, the effect of an antioxidant may be considered more physiological, as it refers to a reaction taking place in the body of a biological macromolecule.

Interestingly, a new AA assay involving the β-carotene bleaching in soybean LOX-induced linoleic acid co-oxidation has been recently proposed (*3*). Unfortunately, in this case, no study of the kinetics of the reaction was reported. Moreover, β-carotene is an inhibitor of LOXes (*20*); therefore, at the same time it is the probe to monitor the reaction and an inhibitor of the reaction itself, thus making it very difficult to set the actual role of the enzyme in AA determination. It should be also noted that, with respect to this new AA assay, the LOX/RNO reaction shows some advantages: it utilizes the RNO, a highly water-soluble compound; moreover, the RNO bleaching looks at a series of biologically relevant oxidant species generated by soybean LOX-1 under low oxygen concentration, whereas the β-carotene oxidation was reported to

Table 1. Effect of Different Compounds on the LOX/RNO Reaction*

compound	nature of inhibition	K_i or IC_{50}	increase of the lag phase (% of the control)	antioxidant/Trolox[e]
inulin	no inhibition		no increase	
α-tocopherol	competitive	1.1 ± 0.1 mM[a,b]	11 ± 2[a,b]	3.43 ± 0.25[c,d]
Trolox	noncompetitive	7.0 ± 1.1 mM	33 ± 8	1.00
resveratrol	uncompetitive	1.7 ± 0.2 mM[e]	no increase[f]	4.57 ± 0.33
ferulic acid	non competitive	10.7 ± 2.1 mM	30 ± 7	0.99 ± 0.11
gallic acid	competitive	6.7 ± 1.3 mM	22 ± 3	0.61 ± 0.05
apigenin[g]	uncompetitive	3.4 ± 0.5 mM	45 ± 8	1.49 ± 0.09
catechin	competitive	13.6 ± 1.8 mM[h]	24 ± 6[i]	0.42 ± 0.03
L-ascorbic acid	non competitive	14.0 ± 1.9 mM	34 ± 7	0.83 ± 0.09
β-carotene	nd	7.8 ± 0.9 μM[j]	38 ± 6	nd
glutathione	nd	19.7 ± 3.2 mM[j]	68 ± 11	nd

* The LOX/RNO reactions were carried out as described in **Figure 1A**, in the absence and presence of the listed compounds. Studies about the nature of inhibition and calculations of K_i were carried out as in **Figure 3** at different RNO and antioxidant concentrations. With regard to β-carotene, the RNO (15 μM) bleaching was monitored by means of a double-wavelength spectrophotometer, as described under Materials and Methods. The effect of glutathione on secondary LOX reactions was studied by monitoring the oxodiene generation (see text) at 285 nm, as shown in the inset of **Figure 2**. The IC_{50} values of β-carotene and glutathione represent the antioxidant concentration able to make half the rates of the LOX/RNO reaction or that of LOX-mediated oxodiene generation, respectively. The effect of the different compounds on the lag phase at the K_i or IC_{50} concentration is also reported, as is the antioxidant activity compared with that of Trolox (antioxidant/Trolox). [a] Mean value ± standard deviation ($n = 4$). [b] Data obtained from measurements carried out in the presence of 24 μL of ethanol. [c] Data were obtained according to ref *11*, by calculating the ratio between the gradient of the plot reporting the decrease of the rate of RNO bleaching (%) as a function of the antioxidant concentration and the gradient of the same plot relative to Trolox (**Figure 3E**). [d] In the antioxidant/Trolox column values are reported as mean value ± standard error. [e] Data obtained from measurements carried out in the presence of 20 μL of ethanol. [f] Data obtained from measurements carried out in the presence of 90 μL of ethanol [g] The LOX/RNO reaction was monitored at 475 nm, as described under Materials and Methods. [h] Data obtained from measurements carried out in the presence of 40 μL of ethanol. [i] Data obtained from measurements carried out in the presence of 80 μL of ethanol. [j] IC_{50} values. nd = not determined.

occur under aerobic conditions essentially due to peroxyl radicals produced during linoleate oxidation (*21*).

Inhibition of the LOX/RNO Reaction by Pure Antioxidant Compounds. The possible inhibition of the LOX/RNO reaction by antioxidants was evaluated. First, the sensitivity of the LOX/RNO reaction to the standard antioxidant, Trolox, the α-tocopherol analogue with enhanced water solubility, was tested. In **Figure 2** the experimental curves of the LOX/RNO reactions carried out in both the absence (control) and presence of two different concentrations (2.5 and 5 mM) of Trolox are reported. It should be outlined that generally a RNO concentration (15 μM) close to K_m was used to avoid a saturating RNO concentration that might hide inhibitors acting by a competitive mechanism. Trolox was found to inhibit the LOX/RNO reaction by causing both some increase of the lag phase with respect to the control and a much more evident decrease of the reaction rate: 2.5 and 5 mM Trolox induced about 30 and 40% decreases of the RNO bleaching rate and about 15 and 30% increases of the lag phase, respectively. Trolox-dependent inhibition of the rate of the RNO bleaching may indicate antiradical activity, whereas the increase of the lag phase suggests the capability to inhibit LOX-1 hydroperoxidative activity (*22*).

In light of the enzymatic nature of the LOX/RNO reaction, the Trolox-dependent inhibition was kinetically characterized (**Figure 3**) by measuring the rate of the LOX/RNO reaction at different RNO and Trolox concentrations. Rates of RNO bleaching are reported as Michaelis−Menten (**A**), Lineweaver−Burk (**B**), Eadie−Hofstee (**C**), and Dixon (**D**) plots. The data of **Figure 3** clearly show that Trolox inhibits the rate of the LOX/RNO reaction in a noncompetitive manner, with an inhibition constant (K_i) value equal to 7.0 ± 1.1 mM. In these experiments, the lag phase was not studied, being independent of RNO concentration, as shown in the inset of **Figure 1B**; this makes this parameter not useful for kinetic studies of the LOX/RNO reaction. In (**E**) the Trolox-dependent inhibition, expressed as decrease with respect to the control of the rate of the RNO bleaching (%) measured at 15 μM RNO, is reported as a function of the standard antioxidant concentration: a linear dependence of the inhibition from 15 to 55% on Trolox concentration ranging between 1 and 7.5 mM was obtained, described by the equation $y(\text{inhibition}) = 5.838x(c_{\text{Trolox}}) + 11.374$ ($r = 0.996$ ***), where the inhibition is expressed in percent and the concentration of Trolox in millimolar. As expected, extrapolation to the *Y*-axis of the calibration curve cannot coincide with zero, in light of the kinetics of the Trolox-dependent inhibition of the LOX/RNO reaction.

A Trolox-based calibration curve of inhibition was also verified by using two other parameters, such as the lag phase and the $tA_{75\%}$ (see Materials and Methods), but lower [$r = 0.544$ * ($n = 32$) and $r = 0.660$ ** ($n = 32$), respectively] statistically significant correlations with Trolox concentration were found. Many other compounds, including inulin (a polymer unable to exhibit AA), used as a negative control, and the different well-known pure antioxidant compounds, both hydrophilic and lipophilic, listed in **Table 1**, were investigated with respect to the capability to affect the LOX/RNO reaction. The studies of kinetics regarding these antioxidants were performed as reported in **Figure 3**. All of the tested compounds, except inulin, were found to affect the LOX/RNO assay by inducing, with different mechanisms and effectiveness, an inhibition of the reaction rate. In **Table 1**, the nature of the inhibition and K_i (or IC_{50}) value for each antioxidant are reported. In the case of β-carotene, which is subjected to absorbance bleaching by LOX and the absorbance spectrum of which largely overlaps the one of RNO, the IC_{50} was calculated by studying the RNO bleaching reaction by means of a double-wavelength spectrophotometer as reported under Materials and Methods.

It should be noted that some antioxidants may interfere with RNO in an undesired manner. For example, glutathione chemically consumes RNO. This problem may be bypassed, because it is known that the RNO bleaching by soybean LOX-1 is coupled to oxodiene formation: these reactions, in fact, occur synchronously under conditions of limited oxygen and show similar dependence on LOX amount, pH, temperature, linoleate concentration, and *n*-PG sensitivity (*4*). Therefore, the oxodiene generation may be advantageously followed as absorbance increase at 285 nm in the experimental conditions in which the RNO bleaching reaction cannot be monitored. In the inset of **Figure 2**, it is shown how glutathione may affect secondary anaerobic LOX-1 reactions by monitoring the oxodiene generation. Glutathione at 10 and 20 mM induced about 20 and 50% decreases of the oxodiene generation rate and about 25 and 70% increases of the lag phase, respectively. The IC_{50} of glutathione was evaluated by following the rate of oxodiene formation.

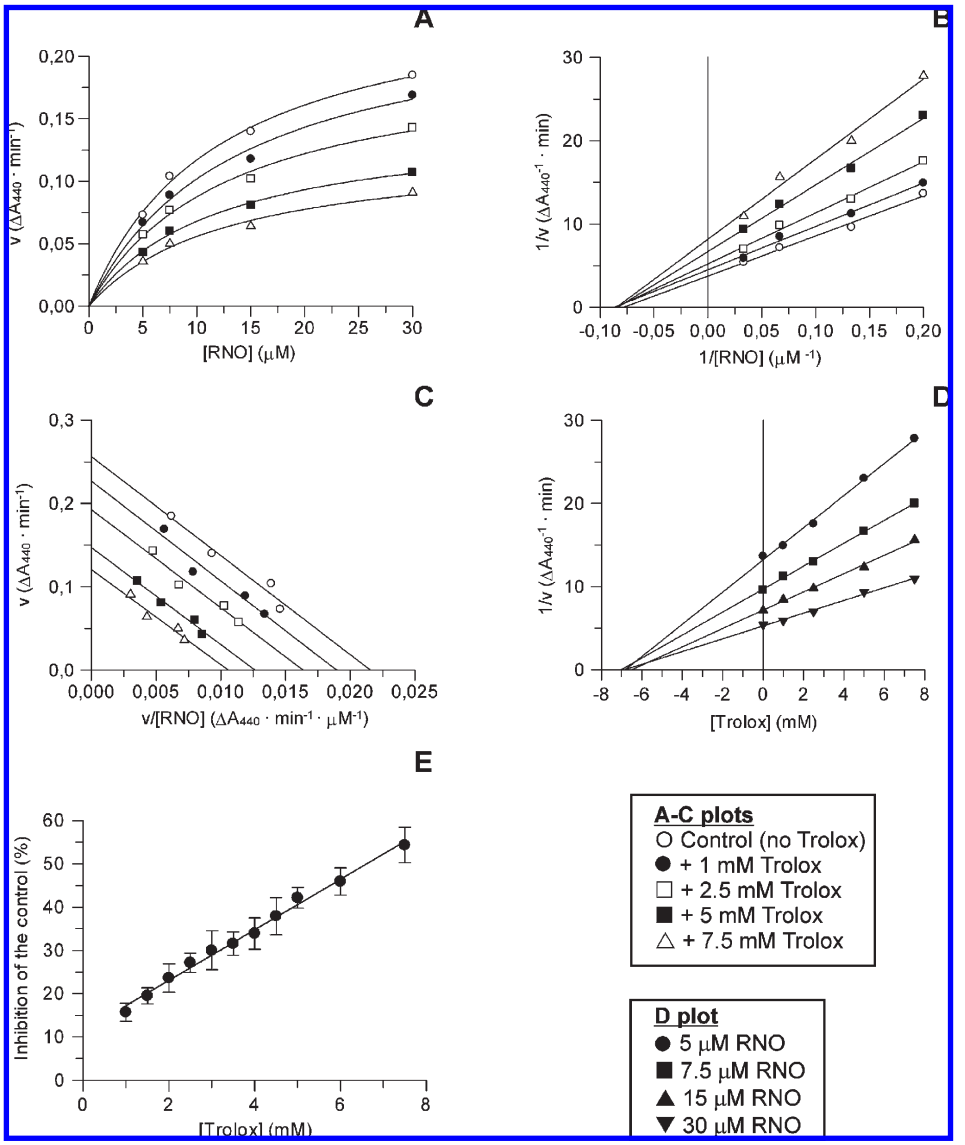


Figure 3. Kinetics of the Trolox-inhibited LOX/RNO reaction. The LOX/RNO reaction was carried out as described in **Figure 1A**, in the presence of different RNO and Trolox concentrations. The rates of RNO bleaching, expressed as ΔA_{440} min^{-1}, are reported as Michaelis−Menten (**A**), Lineweaver−Burk (**B**), Eadie−Hofstee (**C**), and Dixon (**D**) plots. (**E**) Calibration curve: the Trolox-dependent inhibition, expressed as decrease with respect to the control of the rate of RNO bleaching (%) measured at 15 μM RNO in the presence of Trolox, is reported as a function of the standard antioxidant concentration; data are expressed as mean value ± standard deviation (n = 3).

Interestingly, K_i and IC_{50} values in a millimolar range from **Table 1** indicate a general low sensitivity of LOX to single pure antioxidants with respect to other AA assays, with the notable exception of β-carotene. In **Table 1** the effect of each compound on the lag phase at K_i (or IC_{50}) concentration is also reported; the observation that some compounds are able to inhibit the primary peroxidative reaction of the enzyme is in agreement with inhibition of LOXes from different sources by antioxidants (*13*, *20*, *22*−*25*).

Similarly to Trolox (**Figure 3E**), also the other investigated antioxidants showed a linear plot describing the dependence of the inhibition of the rate of RNO bleaching (%) on antioxidant concentration. Therefore, according to ref *11*, the antioxidant activity of each compound was compared with that of Trolox (**Table 1**, column antioxidant/Trolox) by calculating the ratio between the gradient of the plot relative to the antioxidant and the gradient of the plot relative to Trolox. Resveratrol, α-tocopherol, and apigenin displayed higher activity than Trolox, ferulic and L-ascorbic acids similar activity, and gallic acid and catechin less activity.

From a technical point of view, it should be underscored that, unlike the majority of the commonly used AA assays, the LOX/RNO reaction allows testing of both water-soluble and fat-soluble pure antioxidant compounds in the same assay mixture. This is possible in light of the presence of Tween 20 in the reaction mixture, which under our experimental conditions is able to solubilize both linoleate and lipophilic antioxidants.

Setting a LOX/RNO Reaction-Based Method To Measure AA of Food Extracts. The sensitivity of the LOX/RNO reaction to many different classes of antioxidant compounds prompted us to investigate the possibility of using this reaction as a new tool to assess AA of food extracts. To set up the new LOX/RNO method, first, a suitable experimental protocol was checked. For this purpose, the inhibition exerted by 3 mM Trolox on the LOX/RNO reaction was measured in five different experimental conditions, in which two enzyme amounts (0.15 and 0.3 EU), RNO concentrations (15 μM, a concentration value close to K_m, and 70 μM, a saturating concentration, see **Figure 1B**), and pH values (9.00 and 7.00, representing the soybean LOX-1 pH

Table 2. Inhibition of the LOX/RNO Reaction by Trolox in Different Experimental Conditions[a]

	A 0.3 EU LOX-1, 15 μM RNO, pH 9.00	B 0.15 EU LOX-1, 15 μM RNO, pH 9.00	C 0.3 EU LOX-1, 15 μM RNO, pH 7.00	D 0.3 EU LOX-1, 70 μM RNO, pH 9.00	E 0.15 EU LOX-1, 15 μM RNO, pH 7.00	P^b (%) B/A	C/A	D/A	E/A
control, v (ΔA_{440} min^{-1})	0.145 ± 0.002	0.079 ± 0.001	0.066 ± 0.001	0.247 ± 0.016	0.035 ± 0.005	54 ***	46 ***	170 **	24 **
+ 3 mM Trolox, v (ΔA_{440} min^{-1})	0.101 ± 0.003	0.052 ± 0.002	0.033 ± 0.002	0.176 ± 0.001	0.014 ± 0.001	51 **	33 ***	174 ***	14 ***
inhibition (%)	30 ± 2.33	34 ± 2.82	50 ± 3.11	29 ± 0.14	60 ± 2.12	113 ns	167 *	97 ns	200 **
time of measure (s)	173 ± 4	357 ± 12	417 ± 1	484 ± 10	677 ± 57	206 **	241 ***	280 ***	391 ***

[a] The LOX/RNO reactions were carried out as described in **Figure 1A**, in the absence (control) and in the presence of 3 mM Trolox. Measurements were carried out using two different enzyme amounts, RNO concentrations, and pH values (see text). For each experimental condition (i) the rates of RNO bleaching (v), expressed as ΔA_{440} min^{-1}, measured in the absence and presence of Trolox, (ii) the Trolox-dependent inhibition, expressed percentage decrease of v with respect to the control, and (iii) the time of measure, i.e. the $tA_{50\%}$ relative to the control reaction (see Materials and Methods), are shown. Data are reported as mean value ± standard deviation (n = 3). A percentage and statistical comparison according to Student's t test among the different conditions is also shown. [b] P represents the probability level: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ns, not significant.

optimum and the physiological pH values, respectively) were combined (**Table 2**). In each experimental condition the rates of RNO bleaching in the absence and presence of Trolox, the Trolox-dependent inhibition (%), and the time necessary to make half the absorbance of the control reaction were evaluated. In the experimental conditions reported in **B**, **C**, and **E**, statistically significant decreases in reaction rates measured both in the absence and in the presence of Trolox, as well as a resulting increase of the time of measure, were obtained with respect to that measured in the conditions described in **A**, whereas in **D** a highly statistically significant increase in both the reaction rates and time of measure was observed. The **A** condition combines an inhibition (%) sufficiently high to be easily detected with a fast time of measure, which is necessary for analyzing numerous samples in a short time, so this was usually used to test AA of samples. It should be noted that, according to this condition, the pH of the reaction mixture was 9.00, that is, the optimum for LOX-1 activity. The adoption of a physiological pH 7.00 (**C** condition) is possible, and at this pH the LOX/RNO assay is even more sensitive to Trolox inhibition, although a slowing (2−2.5-fold) of time of measure is observed. Moreover, by adopting the **C** condition, the pH value should be more carefully controlled after sample (food extract) addition to the reaction mixture: in fact, also small pH changes may induce undesired changes in the RNO bleaching activity of LOX-1 (see the dependence activity vs pH in ref *4*).

To evaluate the capability of the LOX/RNO method to assess AA of complex mixtures of antioxidants present in food, it was tested on extracts obtained from durum wheat grains. Durum wheat, a cereal widely cultivated in the Mediterranean area, was chosen for the high and unique content of grains in both fat-soluble and water-soluble phytochemicals (*26*). Hydrophilic, lipophilic, and phenolic antioxidant compounds were extracted from durum wheat whole flour and then tested with respect to the ability to affect the LOX/RNO reaction. It was preliminarily verified that, under the adopted experimental conditions, the wheat grain extracts did not significantly affect the pH of the assay medium and the RNO spectral properties and that the hydrophilic ones did not show significant endogenous LOX activity. On the other hand, it should be noted that endogenous LOX activity was found in hydrophilic extracts obtained from legume grains (data not shown). In these cases, a thermal treatment able to inactivate the enzyme, without inducing important loss of AA, should be performed; for example, hydrophilic extract from chickpea grain may be treated at 90 °C for 3 min to completely abolish endogenous LOX activity, with about 20−25% loss of AA as evaluated by means of the TEAC assay (*11*).

As shown in **Figure 4A**, hydrophilic compounds extracted from durum wheat grains, besides inducing some increase of the lag phase, were found to inhibit the rate of the LOX/RNO reaction: in particular, hydrophilic extract from 70 mg of dry whole flour induced an about 40% decrease of rate of the RNO bleaching. A linear dependence in the 27−84 mg of dry whole flour range was observed between inhibition of the reaction rate and amount of extract (**Figure 4B**). The inhibition of **Figure 4A** corresponds, under our experimental conditions, to 125 ± 8 μmol of Trolox equiv/g of whole flour, as determined by the calibration curve of **Figure 3E**. This represents a highly active sample of durum wheat whole flour. Interestingly, the same sample showed AA values equal to 3.5 ± 0.3 and 3.9 ± 0.5 μmol of Trolox equiv/g of dry whole flour when assayed by means of the TEAC (*11*) and ORAC (*12*) methods, respectively. Similarly, lipophilic and phenolic extracts from the same grain sample were also found to linearly inhibit the rate of the LOX/RNO reaction in a dose-dependent manner (data not shown). As for AA values, the LOX/RNO, TEAC, and ORAC methods measured about 65 ± 3, 0.35 ± 0.02, and 0.95 ± 0.08 μmol of Trolox equiv/g of dry whole flour, respectively, in the case of lipophilic extract and 1240 ± 70, 6.2 ± 0.5, and 10.9 ± 0.9 μmol of Trolox equiv/g of dry whole flour, respectively, in the case of the phenolic one. Therefore, depending on the kind of extract, the LOX/RNO method showed AA values about 35−210 and 30−115 times higher than those obtained with TEAC and ORAC methods, respectively; this is in line with the fact that it simultaneously highlights different antioxidant functions (**Scheme 1**). In particular, the LOX/RNO reaction appears to be a suitable tool to measure high the AA of phenolic compounds.

Ability of the LOX/RNO Method To Highlight the Synergistic Action of Antioxidants in Durum Wheat Whole Grain Extracts. The LOX/RNO method is the first AA assay not much sensitive to single pure antioxidants, but, on the contrary, highly sensitive toward food extracts. This result strongly suggests the suitability of the new method to highlight the synergism among antioxidants when they are present as a mix in food extracts. To test this hypothesis, two different kinds of experiments (**Figures 5** and **6**) were planned using the three different extracts from durum wheat whole flour. As for the antioxidant content of the different extracts, the most abundant phenolic compound, in the insoluble-bound phenolic extract was ferulic acid (90%), followed by sinapic (5%), vanillic (3%), and protocatechuic (2%) acids, as determined by HPLC analysis (*19*). Moreover, Folin−Ciocalteu reagent (*18*) measured about 2.51 ± 0.09 μmol of ferulic acid equiv/g of dry whole flour. The hydrophilic extract was characterized by a high protein content (22 ± 1 mg of BSA/g of dry whole flour) as determined according to ref *14*; Folin−Ciocalteu reagent measured a value equal to 1.28 ± 0.03 μmol of ferulic equiv/g of dry whole flour (corrected for the protein content), with a phenolic acid profile similar to that obtained for the

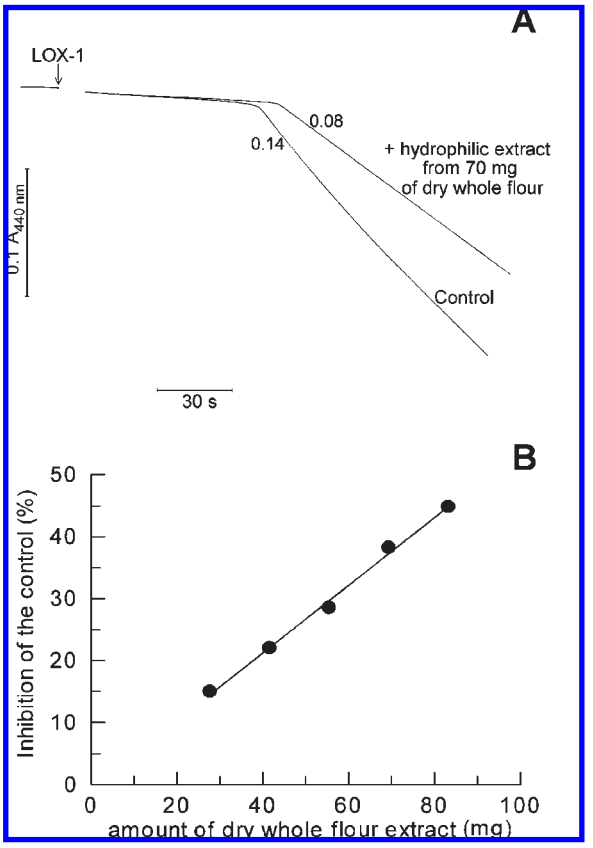


Figure 4. Inhibition of the LOX/RNO reaction by a highly active hydrophilic extract from durum wheat whole flour. (**A**) The LOX/RNO reaction was carried out as described in **Figure 1A**, both in the absence (control) and in the presence of the hydrophilic extract obtained from 70 mg of whole wheat flour. The numbers alongside the curves represent the rates of the RNO bleaching, expressed as ΔA_{440} min^{-1}. (**B**) The inhibition, expressed as decrease with respect to the control of the rate of RNO bleaching (%), is reported as a function of amount of dry whole wheat flour extract.

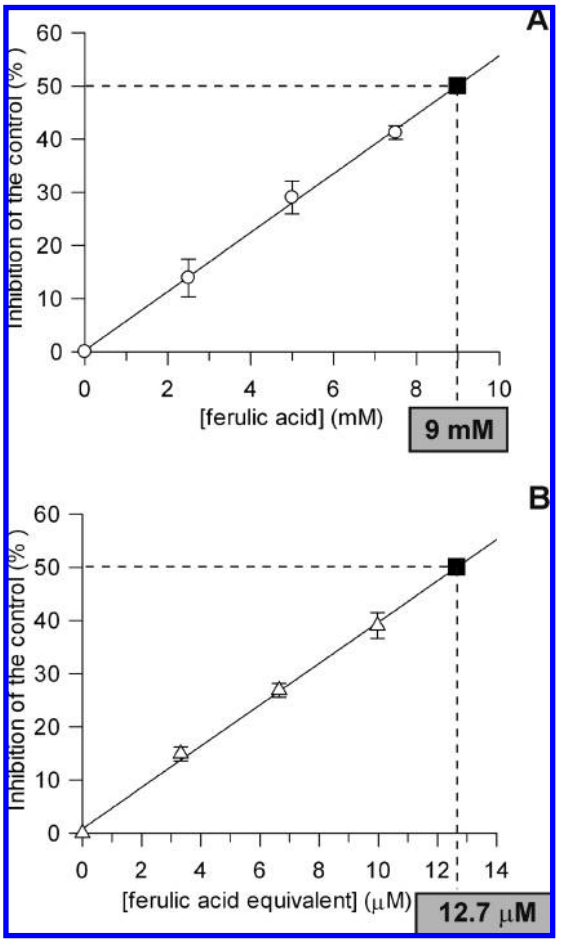


Figure 5. Possible synergism among phenolic compounds from durum wheat whole flour as evaluated by the LOX/RNO reaction. Effectiveness of inhibition of the LOX/RNO reaction induced by either pure ferulic acid (**A**) or a phenolic extract from durum wheat whole flour (**B**) was assessed on an IC_{50} basis. The phenolic extract was a mixture of insoluble-bound phenolic compounds extracted from whole wheat flour as reported under Materials and Methods (section 2.5.3). Measurements were carried out as reported in **Figure 4A**, both in the absence and in the presence of either different concentrations of pure ferulic acid (**A**) or different amounts of phenolic extract (**B**). The inhibitions are expressed as decrease with respect to the control of the rate of the RNO bleaching (%) and are reported as a function of either pure ferulic acid concentration (**A**) or ferulic acid equivalent concentration, obtained by assuming the total phenolic content of the extract as ferulic acid (**B**). Data are expressed as mean value ± standard deviation (n = 3). The two different IC_{50} values are pointed out.

phenolic extract; moreover, flavonoids were present at a concentration of about 0.23 ± 0.01 μmol of catechin equiv/g of dry whole flour, as measured according to ref *15*. The lipophilic extract essentially contained 0.37 ± 0.01 mg of carotenoids/g of dry whole flour, 1.31 ± 0.04 mg of tocopherols/g of dry whole flour, and 3.83 ± 0.06 mg of tocotrienols/g of dry whole flour, as evaluated by means of HPLC analysis (*16*).

In **Figure 5** the possible interaction among antioxidant compounds in the same type of extract was evaluated. For this purpose, the insoluble-bound phenolic extract from durum wheat grains was used, in light of its highly homogeneous composition. To compare the inhibition induced by the phenolic mixture with that exerted by pure ferulic acid [(the most abundant phenolic acid in wheat grains (see also ref *26*)], the LOX/RNO reaction was studied either in the presence of different amounts of the phenolic extract from durum wheat grains or at increasing pure ferulic acid concentrations. The inhibitions by either pure ferulic acid (**A**) or the phenolic extract expressed in terms of ferulic acid equivalents (see Materials and Methods) (**B**) are reported. Interestingly, under our experimental condition, a 50% inhibition (IC_{50}) of the LOX/RNO reaction is induced by either 9 mM pure ferulic acid or 12.7 μM ferulic acid equiv from insoluble-bound phenolic extracts, that is, a concentration about 700 times lower than the one of pure compound. Therefore, the effect on the LOX/RNO reaction of the complex phenolic mixture is much higher than that of a single pure phenolic compound; this result is in line with the idea that the LOX/RNO method should be able to detect the synergism among different phenolic compounds in the extract; further detailed studies aimed to fully verify this hypothesis should be performed.

In a second kind of experiment (**Figure 6**), the capability of the LOX/RNO method to highlight synergistic AA was studied in some detail by using complex mixtures of the hydrophilic, phenolic, and lipohilic antioxidant extracts from durum wheat grains. The results were compared with that obtained using the TEAC and ORAC methods. As for the LOX/RNO method (**A**), amounts of hydrophilic, phenolic, and lipophilic extracts showing AA values of 2.08, 0.98, and 0.83 μmol of Trolox equiv, respectively, were mixed; interestingly, an AA value equal to 8.1 μmol of Trolox equiv was obtained for the resultant mixture (AA_{mix}), about 2 times higher than the mathematical sum of the

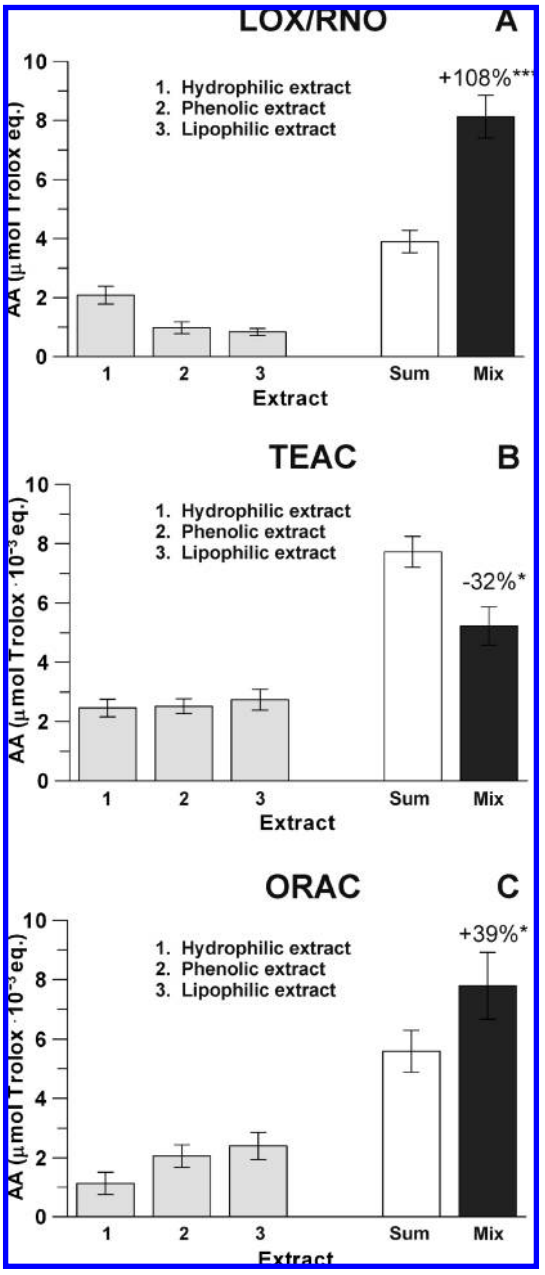


Figure 6. Synergism among hydrophilic, phenolic, and lipophilic antioxidant extracts from durum wheat whole flour as evaluated by means of the LOX/RNO method and comparison with the TEAC and ORAC methods. Hydrophilic, insoluble-bound phenolic, and lipophilic extracts (see Materials and Methods, section 2.5) were obtained in the same experiment from the same whole wheat flour. AA of each extract was quantified by means of the LOX/RNO, TEAC, and ORAC methods. For each extract, an amount able to induce a small but significant inhibition of the LOX/RNO, TEAC, or ORAC reaction was determined; AA of each amount was calculated and expressed as μmol of Trolox equiv. The mathematical sum of the three AA values was calculated (AA_{sum}). Then, the same three extract amounts were mixed and AA was determined again (AA_{mix}). In each plot, the AA values of the three extracts as well as the AA_{sum} and AA_{mix} values are shown. Data are reported as mean value $\pm$ standard deviation (n = 3). In this experiment, synergism, expressed as percentage increase of the AA_{mix} value with respect to the AA_{sum} (see Materials and Methods), was +108% for the LOX/RNO method and +39% for the ORAC method. Antagonism (−32%) was instead observed in the case of the TEAC method. The probability level (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; n.s. = not significant) is also reported.

AA values of the three extract amounts equal to 3.9 μmol of Trolox equiv (AA_{sum}). Therefore, under these experimental conditions, the LOX/RNO method was found to be capable of measuring an AA increase due to the synergistic interaction of different antioxidants: in this experiment, a highly statistically significant synergistic increase equal to 108% was obtained. On the contrary, in the same experiment, the TEAC assay shows a 32% decrease of AA_{mix} value with respect to AA_{sum}, thus indicating that, under these experimental conditions, the TEAC method is not useful to measure synergistic effect of antioxidant mixtures (**B**). On the contrary, the ORAC assay highlights a synergistic increase of AA of only about 39% (**C**). In a different version of the experiment of **Figure 6A**, small amounts of phenolic and lipophilic extracts having per se not measurable AA (inactive) were mixed to an active hydrophilic extract; this caused an about 85% increase of AA_{mix} with respect to AA_{sum}. An about 60% increase was also measured when small amounts (inactive) of hydrophilic and phenolic extracts were added to an active lipophilic extract. On the whole, the LOX/RNO method is able to detect synergistic-dependent AA increases, much higher than that obtained, under the same experimental conditions, with other commonly used AA assays. Obviously, the magnitude of this synergistic effect will depend on the kind of molecules extracted, on their concentrations, and on their relationship in the tested mixture. To the best of our knowledge, only a few data are available reporting so unambiguous an assessment of synergistic activity by an AA assay in food extracts: as reported in ref *27*, the ORAC values of methanol prune (*Prunus domestica* L.) extracts were from 1.5 to 2.9 times higher than the mathematical sum of AA of single constituents; the TEAC assay revealed 16−23% of synergistic activity in different mixtures of 12 phenolic compounds in typical concentrations found in red wines (*28*); AA of pomegranate (*Punica granatum* L.) fruit extracts evaluated by means of the TEAC assay was superior compared to those of single purified polyphenols (*29*). Our data have the added dimension that three different methods were reported to show different abilities to highlight synergistic effect under the same experimental conditions.

In conclusion, the LOX/RNO method shows an experimental protocol sufficiently simple, rapid, and inexpensive. Moreover, AA evaluation by means of the LOX/RNO method shows several advantages with respect to other widely used AA assays. It may provide an assessment of AA more reliable from a physiological point of view. In fact, (i) the LOX/RNO reaction produces more than one oxidant species having relevant physiological significance, (ii) the oxidant−antioxidant competition is evaluated under condition of low oxygen concentration, and (iii) it occurs at the surface of a biological macromolecule (the LOX enzyme) rather than in the bulk phase of the reaction mixture. All of these conditions approach the cellular ones. Moreover, the LOX/RNO method is able to assess the antioxidant ability of either single pure antioxidant compounds or food extracts, both fat-soluble and water-soluble, by using exactly the same medium of reaction. Therefore, the main requirements/criteria for standardization of AA determination (*30*) may be considered sufficiently satisfied by the LOX/RNO method. On the other hand, the new method shows some limitations when (i) food extracts display high endogenous LOX activity (for example, legume grains), (ii) antioxidant compounds are dissolved or extracted in ethanol, and (iii) antioxidant compounds chemically interact with RNO or have absorbance spectra overlapping that of RNO; another limitation is that (iv) a nonphysiological pH 9 is adopted. Anyway, to better fit cellular condition, a pH 7 condition may be used in the assay, although this may approximately double the time of measurement. In light of the unique capability to simultaneously

detect many antioxidant functions, the LOX/RNO method is able to highlight high AA values in durum wheat grains, as well as synergistic interactions among antioxidants from different extracts, and so provide a more comprehensive and integrated determination of AA. This is essential for a total AA assessment, which may provide a more realistic quantification of food antioxidant effectiveness in preventing diseases.

ABBREVIATIONS USED

AA, antioxidant activity; AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); AUC, area under curve; LOX, lipoxygenase; fluorescein, 3′,6′-dihydroxyspiro[isobenzofuran-1[3*H*],9′[9*H*]-xanthen]-3-one; EU, enzymatic units (μmol of substrate transformed min^{-1}); RNO, 4-nitroso-*N*,*N*-dimethylaniline; Trolox, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid.

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LITERATURE CITED

(1) Magalhãesa, L. M.; Segundo, M. A.; Reisa, S.; Lima, J. L. F. C. Methodological aspects about in vitro evaluation of antioxidant properties. *Anal. Chim. Acta* **2008**, *613*, 1–19.

(2) Huang, D.; Ou, B.; Prior, R. L. The chemistry behind antioxidant capacity assays. *J. Agric. Food Chem.* **2005**, *53*, 1841–1856.

(3) Chaillou, L. L.; Nazareno, M. A. New method to determine antioxidant activity of polyphenols. *J. Agric. Food Chem.* **2006**, *54*, 8397–8402.

(4) Pastore, D.; Trono, D.; Padalino, L.; Di Fonzo, N.; Passarella, S. *p*-Nitrosodimethylaniline (RNO) bleaching by soybean lipoxygenase-1. Biochemical characterization and coupling with oxodiene formation. *Plant Physiol. Biochem.* **2000**, *38*, 845–852.

(5) Baysal, T.; Demirdöven, A. Lipoxygenase in fruits and vegetables: a review. *Enzyme Microb. Technol.* **2007**, *40*, 491–496.

(6) Sanz, L. C.; Pérez, A. G.; Olías, J. M. La lipoxigenasa en el reino vegetal. I. Propiedades. *Grasas Aceites* **1992**, *43*, 231–239.

(7) Garssen, G. J.; Vliegenthart, J. F. G.; Boldingh, J. An anaerobic reaction between lipoxygenase, linoleic acid and its hydroperoxides. *Biochem. J.* **1971**, *122*, 327–332.

(8) Kanofsky, J. R.; Axelrod, B. Singlet oxygen production by soybean lipoxygenase isozymes. *J. Biol. Chem.* **1986**, *261*, 1099–1104.

(9) Siedow, J. N. Plant lipoxygenase: structure and function. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1991**, *42*, 145–188.

(10) Kraljić, I.; El Mohsni, S. A new method for the detection of singlet oxygen in aqueous solutions. *Photochem. Photobiol.* **1978**, *28*, 577–581.

(11) Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biol. Med.* **1999**, *26*, 1231–1237.

(12) Ou, B.; Hampsch-Woodill, M.; Prior, R. L. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *J. Agric. Food Chem.* **2001**, *49*, 4619–4626.

(13) Pastore, D.; Trono, D.; Padalino, L.; Simone, S.; Valenti, D.; Di Fonzo, N.; Passarella, S. Inhibition by α-tocopherol and L-ascorbate of linoleate hydroperoxidation and β-carotene bleaching activities in durum wheat semolina. *J. Cereal Sci.* **2000**, *31*, 41–54.

(14) Waddel, W.; Hill, C. A simple ultraviolet spectrophotometric method for the determination of protein. *J. Lab. Clin. Med.* **1956**, *48*, 311–314.

(15) Dewanto, V.; Wu, X.; Adom, K. K.; Liu, R. H. Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. *J. Agric. Food Chem.* **2002**, *50*, 3010–3014.

(16) Panfili, G.; Fratianni, A.; Irano, M. Normal phase high-performance liquid chromatography method for the determination of tocopherols and tocotrienols in cereal foods. *J. Agric. Food Chem.* **2003**, *51*, 3940–3944.

(17) Sosulski, F.; Krygier, K.; Hogge, L. Free, esterified, and insoluble-bound phenolic acids. 3. Composition of phenolic acids in cereal and potato flours. *J. Agric. Food Chem.* **1982**, *30*, 337–340.

(18) Singleton, V. L.; Orthofer, R.; Lamuela-Raventos, R. M. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods Enzymol.* **1999**, *299*, 152–178.

(19) Miglio, C.; Chiavaro, E.; Visconti, A.; Fogliano, V.; Pellegrini, N. Effects of different cooking methods on nutritional and physicochemical characteristics of selected vegetables. *J. Agric. Food Chem.* **2008**, *56*, 139–147.

(20) Lomnitski, L.; Bar-Natan, R.; Sklan, D.; Grossman, S. The interaction between β-carotene and lipoxygenase in plant and animal systems. *Biochim. Biophys. Acta* **1993**, *1167*, 331–338.

(21) Barimalaa, I. S.; Gordon, M. H. Cooxidation of β-carotene by soybean lipoxygenase. *J. Agric. Food Chem.* **1988**, *36*, 685–687.

(22) Maccarrone, M.; Veldink, G. A.; Vliegenthart, J. F. G.; Finazzi Agrò, A. Inhibition of soybean lipoxygenase-1 by chain-breaking antioxidants. *Lipids* **1995**, *30*, 51–54.

(23) Grossman, S.; Waksman, E. G. New aspects of the inhibition of soybean lipoxygenase by α-tocopherol. Evidence for the existence of a specific complex. *Int. J. Biochem.* **1984**, *16*, 281–289.

(24) Trono, D.; Pastore, D.; Di Fonzo, N. Carotenoid dependent inhibition of durum wheat lipoxygenase. *J. Cereal Sci.* **1999**, *29*, 99–102.

(25) Nijveldt, R. J.; van Nood, E.; van Hoorn, D. E.; Boelens, P. G.; van Norren, K.; van Leeuwen, P. A. Flavonoids: a review of probable mechanisms of action and potential applications. *Am. J. Clin. Nutr.* **2001**, *74*, 418–425.

(26) Adom, K. K.; Liu, R. H. Antioxidant activity of grains. *J. Agric. Food Chem.* **2002**, *50*, 6182–6187.

(27) Kayano, S.; Kikuzaki, H.; Fukutsuka, N.; Mitani, T.; Nakatani, N. Antioxidant activity of prune (*Prunus domestica* L.) constituents and a new synergist. *J. Agric. Food Chem.* **2002**, *50*, 3708–3712.

(28) de Beer, D.; Joubert, E.; Marais, J.; Manley, M. Unravelling the total antioxidant capacity of pinotage wines: contribution of phenolic compounds. *J. Agric. Food Chem.* **2006**, *54*, 2897–2905.

(29) Seeram, N. P.; Adams, L. S.; Henning, S. M.; Niu, Y.; Zhang, Y.; Nair, M. G.; Heber, D. In vitro antiproliferative, apoptotic and antioxidant activities of punicalagin, ellagic acid and a total pomegranate tannin extract are enhanced in combination with other polyphenols as found in pomegranate juice. *J. Nutr. Biochem.* **2005**, *16*, 360–367.

(30) Prior, R. L.; Wu, X.; Schaich, K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J. Agric. Food Chem.* **2005**, *53*, 4290–4302.

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