SHORT COMMUNICATION

Evaluation of synergistic interactions of antioxidants from plant foods by a new method using soybean lipoxygenase

MAURA N. LAUS – MARIO SOCCIO – DONATO PASTORE

Summary
A proper evaluation of synergism among antioxidants remains so far rather difficult to obtain. We recently reported that a new method for antioxidant activity measurement, based on the secondary reaction of soybean lipoxygenase (LOX)-1 isoenzyme with 4-nitroso-*N,N*-dimethylaniline (RNO), so-called LOX/RNO method, may assess very well the synergism among antioxidants from durum wheat whole semolina. To evaluate whether this behaviour is generalizable to different food matrices, herein, antioxidants from other very different sources were analysed. For this purpose, antioxidant activity of food-grade preparations enriched in catechins, quercetin, resveratrol, tyrosol/hydroxytyrosol and lycopene was evaluated by the LOX/RNO method in comparison with the Trolox equivalent antioxidant capacity (TEAC) assay. The antioxidant activity values obtained by LOX/RNO method were 2–90-times higher than those obtained by the TEAC protocol, depending on the tested antioxidant. Synergism was evaluated by comparing antioxidant activity of the mixture of compounds (AA_{mix}) with the sum of antioxidant activity values of individual compounds (AA_{sum}). The LOX/RNO method revealed a strong synergism, being AA_{mix} 5.69 ± 0.31 times higher than AA_{sum} (statistically significant, $p < 0.001$), while the TEAC method showed a synergistic increase of only 0.31 ± 0.04 (statistically significant, $p < 0.01$). These findings suggest that the LOX/RNO method is able to assess very well the synergism in various food samples.

Keywords
plant foods; antioxidants; synergism; LOX/RNO method; Trolox equivalent antioxidant capacity

It is widely accepted that regular consumption of fruits, vegetables and whole grains, containing high levels of a wide variety of potential chemopreventive substances (phytochemicals), including polyphenols, vitamins and carotenoids, is strongly associated with reduced risk of developing chronic diseases [1]. It has been also established that consistent protective health benefits of fruits, vegetables and grains, that are exhibited by whole foods and not by purified phytochemicals administered as dietary supplements alone, are mostly dependent on the additive and synergistic effects of the contained antioxidant compounds [2–4]. In the light of this, in order to give a more biologically relevant information about the health-promoting potential of foods, a direct assessment of antioxidant activity of food samples is requested, able to highlight possible synergistic interaction of dietary antioxidants.

Several methods to measure antioxidant activity of food extracts and biological samples were developed in the last 20 years. Unfortunately, the majority of published assays do not measure an actual antioxidant activity of food samples, since they consider only a few possible mechanisms of dietary antioxidant protection, mainly the food scavenging capacity towards radical species (often non-physiological), evaluated under experimental conditions not resembling the physiological ones. Recently, we have developed a new assay for assessment of the antioxidant activity of food extracts [5]. The method is based on bleaching of 4-nitroso-*N,N*-dimethylaniline (RNO) due to radical species generated under anaerobic reactions associated to linoleic acid (LH) hydroperoxidation catalysed by soybean lipoxygenase (LOX)-1 isoform [6]. The peculiarity of the LOX/RNO method is to detect the scavenging capacity towards physiological and

Maura N. Laus, Mario Soccio, Donato Pastore, Department of Agricultural, Food and Environmental Sciences, University of Foggia, Via Napoli 25, 71122 Foggia, Italy.

Correspondence author:
Donato Pastore, tel.: +39(0)881-589427; fax: +39(0)881-587108; e-mail: d.pastore@unifg.it

biologically relevant radical species produced in the course of secondary LOX reactions, such as alkoxyl ($LO^{\bullet}$), peroxyl ($LOO^{\bullet}$), hydroxyl ($^{\bullet}OH$) and perhaps alkylic ($L^{\bullet}$) radicals, as well as singlet oxygen ($^1O_2$), but this last only in the presence of imidazole [5–6]. Moreover, other important antioxidant functions may be simultaneously detected, including chelating or reducing activities of iron ions essential for the catalysis and generation of radical species, as well as any possible inhibition of the apoenzyme resulting in an antiperoxidative effect [5]. In the light of this, the LOX/RNO method may be expected to provide a more integrated and comprehensive information about the food antioxidant capacity and to better highlight the additive and/or synergistic effects of complex antioxidant mixtures. Consistently, the LOX/RNO method has been found to reveal very well synergism among antioxidant compounds obtained from whole semolina of durum wheat, which is a plant species of great commercial interest, well adapted in Mediterranean area and able to counteract oxidative stress at cellular and subcellular level [7–8]. In particular, synergism has been observed among different classes of antioxidants extracted by using solvents of different polarities [5], as well as among phenolic compounds within the same type of extract [9]. Anyway, since results of antioxidant activity measurement may vary markedly depending on food matrix composition [10], the question arises whether the ability of the LOX/RNO method to detect synergism may be somehow related to the kind of food under study, so being confined to the analysis of whole semolina from wheat grains, or it may be considered irrespective of the investigated sample.

In order to shed light on this point, the aim of this study was to evaluate the performance of the LOX/RNO method with respect to the capability to detect antioxidant synergism using food-grade polyphenols obtained from different sources, including flavonoid compounds such as catechins and quercetin, as well as the non-flavonoids resveratrol and tyrosol/hydroxytyrosol mixture (contained in the olive fruit extract OLIPLUS®, Nutraceutica, Monterenzio, Italy); as a lipophilic compound, lycopene was also examined. Results were compared with those obtained with the widely used Trolox equivalent antioxidant capacity (TEAC) assay [11], based on the scavenging capacity against the radical cation 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonate ($ABTS^{\bullet+}$), that showed no synergism among antioxidants from whole semolina [5, 9].

MATERIALS AND METHODS

Chemicals and food-grade antioxidants

Chemicals were purchased from Sigma Chemical (St. Louis, Missouri, USA). As for food-grade antioxidants, the following preparations were used: catechins (50%, comprising 15% epicatechin gallate and 30% epigallocatechin gallate) extracted from *Camelia sinensis* Kuntze (green tea); quercetin (98%) from *Sophora japonica* L.; resveratrol (98%) from *Polygonum cuspidatum* Siebold and Zucc.; a mixture of polyphenols (45%) from an *Olea europaea* L. (olive) fruit extract (OLIPLUS®, containing 0.3% tyrosol, 2% hydroxytyrosol and 6% oleuropeine); lycopene (15%) from *Solanum lycopersicum* L. (tomato). They were obtained from Nutraceutica (Monterenzio, Bologna, Italy). Working stock solutions of all antioxidants were prepared, containing 3% (v/v) ethanol and 50 $mmol \cdot l^{-1}$ NaOH.

Determination of antioxidant activity

LOX/RNO method

The LOX/RNO reaction was spectrophotometrically monitored, as described by Pastore et al. [5], by measuring the RNO absorbance decrease at 440 nm and 25 °C. Antioxidant activity was quantified by calculating the decrease percentage of the rate of RNO bleaching measured in the presence of a single food-grade antioxidant or antioxidant mixture with respect to the rate of the control reaction.

TEAC method

The TEAC assay described by Re et al. [11], modified by Pastore et al. [5], was applied. The coloured radical cation $ABTS^{\bullet+}$ was generated by oxidation of ABTS aqueous solution with potassium persulfate. Antioxidant activity was quantified by calculating the decrease percentage of absorbance at 734 nm (A_{734}) measured after 5 min incubation of a single food-grade antioxidant or antioxidant mixture with the $ABTS^{\bullet+}$ solution (diluted in sodium phosphate buffer 5 $mmol \cdot l^{-1}$, pH 7.4) with respect to A_{734} of the uninhibited radical cation solution (blank).

Since antioxidants were reconstituted in stock solutions containing ethanol and NaOH, antioxidant activity measurements of both individual antioxidants and antioxidant mixture were carried out in the presence of constant ethanol and NaOH concentrations (0.2% and 2.5 $mmol \cdot l^{-1}$ and 0.1% and 1.4 $mmol \cdot l^{-1}$ for the LOX/RNO and TEAC assays, respectively). For both methods, antioxidant activity was calculated by means of a proper calibration curve obtained using 6-hydroxy-2,5,7,8-

tetramethylchromane-2-carboxylic acid (Trolox) as a standard antioxidant and expressed as micromoles of Trolox per milligram of antioxidant compound.

Statistical analysis

Data are reported as mean value ± standard deviation ($n = 4$ independent experiments). Statistical analysis was performed by using Statistica (data analysis software system), version 7 (StatSoft, Tulsa, Oklahoma, USA). Data distribution was evaluated by using the Shapiro-Wilk test. Homogenity of variances was verified by the Bartlett's test. Data of Fig. 1 were submitted to a "two-factor" analysis of variance (ANOVA) model and the mean separation was tested by the Tukey's test at a level of significance $p = 0.001$. Data of Fig. 2 were submitted to the Student's t-test at levels of significance $p = 0.01$ and $p = 0.001$.

RESULTS AND DISCUSSION

Antioxidant activity of food-grade antioxidants was evaluated by the LOX/RNO reaction and, for comparison, with the TEAC assay (Fig. 1). Antioxidant activity measurement results of the LOX/RNO method were much higher than those of the TEAC method, about 2–90 times higher, depending on the tested antioxidant. Moreover, the two assays gave different activity ranks. This finding is in line with previous results [5, 9, 12] and, as stated in the Introduction, may be dependent on the ability of the LOX/RNO method to simultaneously detect different modes by which an antioxidant may exert its activity in the sample. Differently, the TEAC method only assesses the capability of antioxidants to scavenge the $ABTS^{\bullet+}$ radical. Interestingly, the LOX/RNO method was able to measure very well antioxidant activity of lycopene, similar to other lipophilic compounds, while the TEAC and the oxygen radical absorbance capacity (ORAC) methods could not [9, 12]. This is probably dependent on both the chemical basis of the method and on the polarity of the reaction media.

In another experiment, antioxidant activity of resveratrol, OLIPLUS®, quercetin, catechin and lycopene was assayed at concentrations 15, 7, 6.25, 3.12 and 0.27 $\mu g \cdot ml^{-1}$, respectively, that are concentrations from 4- to 70-fold lower than those able to induce toxic effects in human cells (astrocytes) [13]. Lycopene was assayed at the lowest concentration equal to 0.27 $\mu g \cdot ml^{-1}$, i.e. at the human plasma concentration [14]. Antioxidant activity was measured by the LOX/RNO and TEAC assays, both showing in this case a rank almost depending on compound concentration (Fig. 2). In order to evaluate possible synergism, the sum of antioxidant activity values of single compounds was calculated (AA_{sum}) and compared with antioxidant activity measured after mixing all compounds at the reported concentrations (AA_{mix}). Interestingly, as for the LOX/RNO method, AA_{mix} was 5.69 ± 0.31-fold higher than AA_{sum} (statistically significant, $p < 0.001$), thus indicating a very strong synergism among different antioxidant compounds from very different sources. Under the same experimental conditions, the TEAC method also revealed a statistically significant ($p < 0.01$) synergistic increase of AA_{mix} with respect to AA_{sum}, but it was only 0.31 ± 0.04-fold higher. It should be also considered that, in these experimental conditions, an underestimation of results

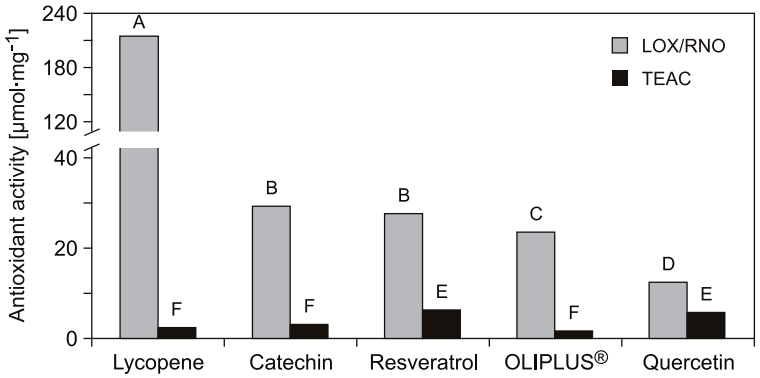

Fig. 1. Antioxidant activity of some food-grade antioxidants, as evaluated by means of the LOX/RNO and TEAC methods.

Data are reported as mean value ($n = 4$). Different capital letters indicate significant differences at $p = 0.001$ level, according to Tukey's test. Antioxidant activity is expressed as micromoles of Trolox per milligram.

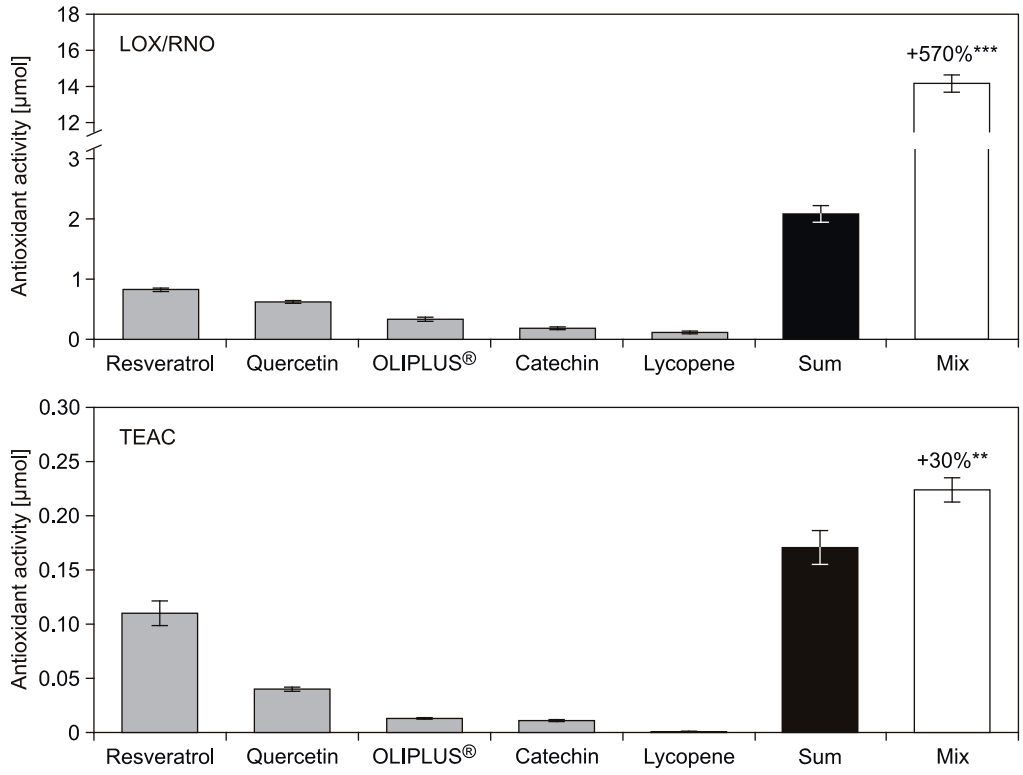

Fig. 2. Synergism among antioxidant compounds as evaluated by means of the LOX/RNO and TEAC methods.

Data are reported as mean value ± standard deviation ($n = 4$). ** – $p < 0.01$, *** – $p < 0.001$, where p represents the probability level relative to the comparison between antioxidant activity measured after mixing all compounds (AA_{mix}) and the sum of antioxidant activity values of single compounds (AA_{sum}) according to the Student's t-test. Antioxidant activity is expressed as micromoles of Trolox.

obtained by the TEAC method was possible. Certainly, TEAC values of many pure hydrophilic and lipophilic antioxidant compounds as well as of many phenolic compounds and of food and biological samples, have been reported [15]. Anyway, if the tested sample contains compounds with redox potential unfavourable to the direct reduction of the $ABTS^{\bullet+}$ radical (via electron transfer), its activity will not be detected by the TEAC protocol. This feature may limit quantitative antioxidant activity evaluation using TEAC method and it may explain its different behaviour than the LOX/RNO method at evaluating the antioxidant synergism.

The LOX/RNO method, probably thanks to its capability to simultaneously detect several antioxidant functions, indicated much higher synergistic interactions among antioxidants than other assays. For example, the ORAC method showed some ability to highlight synergism. However, under different experimental conditions, the obtained values were lower than those obtained by the LOX/RNO method, ORAC being less effective than LOX/RNO from 2.5 [5] to 150 times [9]. The TEAC method showed no synergism previously [9], while, under the same experimental conditions, the method based on the use of *N,N*-dimethyl-*p*-phenylenediamine (DMPD) showed antagonism [9]. In the whole, the new LOX/RNO method produced in various samples results suggesting for strong synergism. Future investigation aimed at clarification of the dependence of the magnitude of these synergistic/antagonistic effects on the types of mixed compounds, on their concentration in the tested mixture and on their interactions in relationship to their solubility is worthwhile.

Acknowledgments

Dr. Rocco Rossano of the University of Basilicata (Potenza, Italy) is gratefully acknowledged for provision of some antioxidant compounds and for the interesting contribution in experimental planning.

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Received 30 March 2013; revised 13 June 2013; accepted 17 July 2013; published online 28 November 2013.