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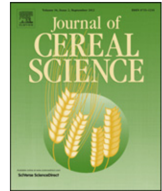

# Dissection of antioxidant activity of durum wheat (*Triticum durum* Desf.) grains as evaluated by the new LOX/RNO method

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ABSTRACT

Antioxidant activity (AA) of durum wheat (*Triticum durum* Desf.) grains was studied using the innovative LOX/RNO method, able to simultaneously detect different antioxidant mechanisms, and the TEAC assay, one of the most widely used assays. Insoluble-bound and free-soluble phenols, hydrophilic and lipophilic compounds were extracted from eight different whole flour samples; extracts were analyzed for AA and their content in several antioxidants. The LOX/RNO method measured very high AA values, with the highest ones [850–1500 μmol Trolox eq./g whole flour (dry weight)] for insoluble-bound phenolic extracts, highly correlated to total phenolic ($r = 0.761$, $P < 0.001$) and ferulic acid ($r = 0.816$, $P < 0.001$) contents. Hydrophilic and lipophilic extracts showed lower AA [70-140 and 40–60 μmol Trolox eq./g (dry weight), respectively], highly correlated to flavonoid ($r = 0.583$, $P < 0.01$) and protein ($r = 0.602$, $P < 0.01$), as well as β-tocotrienol ($r = 0.684$, $P < 0.05$) contents, respectively. Interestingly, the LOX/RNO method suggests that insoluble-bound phenolic compounds may exert very strong synergistic interactions within the extract. Contrarily, the TEAC assay did not correlate to any antioxidant content, resulted unable to highlight differences among samples, measured much lower AA values and did not suggest synergism. The use of the LOX/RNO method is useful to unearth new properties of phytochemicals from durum wheat grains, potentially giving health benefits.

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## 1. Introduction

Wheat is one of the major cereal crops in the temperate zones and represents an important component of the human diet because of the universal use of its grains for production of flour and semolina, the basic ingredients of bread and other bakery products and pasta (Adom et al., 2003 and refs. therein). Besides starch, proteins, dietary fiber and minerals, whole wheat grains are known for their unique healthy value due to their high and peculiar content in non-nutrient biologically active compounds, known as phytochemicals. They include a wide variety of both water- and fat-soluble compounds: phenolic acids (belonging to the benzoic and cinnamic acid families), flavonoids, anthocyanidins, lignans, carotenoids, tocotrienols, tocopherols, phytosterols (Liu, 2007). Antioxidant properties of whole grain phytochemicals have been suggested to be strongly related to health-beneficial properties of high whole grain consumption, such as reduced total mortality and reduced incidence of degenerative diseases (Liu, 2007 and refs. therein). Therefore, current interest in health benefits of whole

*Abbreviations:* AA, antioxidant activity; AAPH, 2, 2′-azobis(2-amidinopropane); ABTS, 2, 2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid); AUC, area under curve; BSA, bovine serum albumin; DMPD, 4-amino-*N*, *N*-dimethylaniline; DPPH, 2, 2-diphenyl-1-picrylhydrazyl; d.w., dry weight; EU, Enzymatic Units (μmol of substrate transformed/min); fluorescein, 3′, 6′-dihydroxyspiro[isobenzofuran-1 [3*H*], 9′[9*H*]-xanthen]-3-one; LOX, lipoxygenase (linoleate: oxygen oxidoreductase, EC 1.13.11.12); ORAC, Oxygen Radical Absorbance Capacity; RNO, 4-nitroso-*N*, *N*-dimethylaniline; TEAC, Trolox Equivalent Antioxidant Capacity; TOSC, Total Oxyradical Scavenging Capacity; Trolox, (±)-6-hydroxy-2, 5, 7, 8-tetramethylchromane-2-carboxylic acid.

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grain dietary intake has promoted many studies aimed at evaluating antioxidant composition of wheat grains with particular attention given to the effect of genotype, growing environment and milling process (Adom et al., 2003; Fratianni et al., 2005; Heimler et al., 2010; Liyana-Pathirana and Shahidi, 2007a, 2007b and refs. therein; Moore et al., 2006 and refs. therein; Mpofu et al., 2006; Panfili et al., 2003, 2004; Yu et al., 2002).

Generally, a central point in these kind of studies is the difficulty to relate analytical determinations of individual dietary antioxidants to the true whole antioxidant activity (AA) in food, since the complex mixture of phytochemicals acts through a combination of additive and/or synergistic effects (Liu, 2007 and refs. therein). Therefore, the direct determination of AA of food samples has gained an increasing interest as a tool in exploring the putative role of antioxidant-rich products in enhancing human health (Serafini et al., 2002 and refs. therein). Moreover, the more the AA assessment is able to highlight synergistic interactions of dietary antioxidants, the more biologically relevant information about the health promoting potential of foods may be provided.

In this regard, we have recently developed an innovative and advanced assay for *in vitro* AA assessment of food extracts, the lipoxygenase/4-nitroso-*N,N*-dimethylaniline (LOX/RNO) method (Pastore et al., 2009). It uses the RNO bleaching reaction due to some radical species, namely alkoxyl, peroxyl and hydroxyl radicals, as well as, in the presence of imidazole, singlet oxygen. These reactive species are generated by anaerobic reactions catalyzed by soybean LOX-1 isoenzyme, occurring when the main reaction of polyunsaturated fatty acid hydroperoxidation has consumed oxygen (Pastore et al., 2000a). With respect to the majority of AA assays, the peculiarity of the LOX/RNO method is to simultaneously detect the food scavenging capacity towards some radical species having relevant cellular significance, by means of a simple and rapid experimental protocol, as well as under experimental conditions resembling the physiological ones. Moreover, the new method may also highlight other important mechanisms of dietary antioxidant protection, including activity of both metal chelation and reduction (because an oxidized iron ion is essential for LOX catalysis), as well as direct inhibition of LOX apo-enzyme accounting for an antiperoxidative activity. In the light of this, the LOX/RNO method may provide a more integrated and comprehensive information about AA of food extracts. Above all, it has been proposed that the new method may highlight, better than other well-known AA assays, the synergistic effects of complex mixtures containing different antioxidant compounds (Pastore et al., 2009).

In this study, the new LOX/RNO method has been used to focus AA of durum wheat (*Triticum durum* Desf.) grains, since, to date, only few data concerning this species are available (Adom et al., 2003; Heimler et al., 2010; Liyana-Pathirana and Shahidi, 2007a, 2007b). In these studies, AA of grains from different varieties and years of production (Adom et al., 2003; Heimler et al., 2010) and of different milling fractions (Liyana-Pathirana and Shahidi, 2007a, 2007b) was evaluated, by using assays able to measure each time one possible mechanism of antioxidant protection against oxidative damage: mainly a scavenging activity or a reducing capacity towards single radical species or a metal chelating activity. By using these assays it may be more difficult to highlight synergistic interactions among phytochemicals. Moreover, some of these assays used radical species and experimental conditions that are strongly non-physiological; above all, these assays often measured AA values not clearly related to any antioxidant compound (Heimler et al., 2010). The availability of the LOX/RNO method, able to give useful information from a physiological point of view, may unearth new properties of phytochemicals from durum wheat grains, potentially related to health benefits.

In this study, the performances of the LOX/RNO method were compared with that of the widely used Trolox Equivalent Antioxidant Capacity (TEAC) assay (Re et al., 1999), that measures the capacity of antioxidants to induce the bleaching of the non-physiological 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation. This assay is generally classified as based on direct reduction via single electron transfer (SET) reaction, however, neutralization of $ABTS^{\bullet+}$ radical cation by radical quenching via hydrogen atom transfer (HAT) reaction has been also reported (Prior et al., 2005 and refs. therein). Hydrophilic, insoluble-bound and free-soluble phenolic, as well as lipophilic extracts, were prepared from eight different whole wheat flour samples and then analyzed for AA and for their content in several antioxidant compounds. An investigation of possible synergistic interactions among insoluble-bound phenols, the most active antioxidant compounds in cereal whole grains (Adom and Liu, 2002; Liu, 2007 and refs. therein), was also carried out by means of the LOX/RNO method. As a comparison, different methods were used: the TEAC assay, as well as the Oxygen Radical Absorbance Capacity (ORAC) protocol (Ou et al., 2001) and the 4-amino-*N,N*-dimethylaniline (DMPD)-based method (Fogliano et al., 1999), measuring the chain-breaking antioxidant capacity against peroxyl radicals and the capability of antioxidants to induce the bleaching of the $DMPD^{\bullet+}$ radical cation, respectively.

## 2. Material and methods

### 2.1. Chemicals

All reagents at the highest commercially available purity were purchased from SIGMA Chemical Co. (St. Louis, MO, USA). RNO was dissolved in 80 mM sodium borate buffer pH 9.00; catechin, gallic acid, bovine serum albumin (BSA), ABTS, potassium persulfate, DMPD and ferric chloride were dissolved in deionized water; 3′,6′-dihydroxyspiro[isobenzofuran-1[3*H*], 9′[9*H*]-xanthen]-3-one (fluorescein) and 2,2′-azobis(2-amidinopropane) (AAPH) in 75 mM sodium phosphate buffer pH 7.40. Ferulic acid and Trolox [(±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid] were dissolved in different media depending on the AA assay used. An ammonium sulfate suspension of soybean LOX type V (LOX-1 isoenzyme) was used, properly diluted with 80 mM sodium borate buffer pH 9.00.

### 2.2. Plant material

Plant material consisted of eight different durum wheat (*T. durum* Desf.) whole grain samples, kindly provided by Prof. Z. Flagella (University of Foggia), deriving from an agronomic trial, in which two cultivars (Simeto = S and Ofanto = O) were subjected to two levels of sulfur fertilization (0 and 33 kg/ha) and two levels of watering [irrigated = I (two irrigation treatments of 720 $m^3$/ha) and non-irrigated = NI]. Samples were indicated by Arabic numbers as follows: 1 = S0I; 2 = S33I; 3 = S0NI; 4 = S33NI; 5 = O0I; 6 = O33I; 7 = O0NI; 8 = O33NI.

Whole grain samples were stored as vacuum-packaged at 4 °C for no longer than 3 months; before use whole grains were daily milled by means of a Cyclotec 1093 Sample Mill (1 mm sieve).

### 2.3. Preparation of aqueous solutions of linoleate

The sodium linoleate solution was prepared as described in Pastore et al. (2009) and the exact linoleate concentration was determined by means of the LOX assay (Pastore et al., 2000a, 2000b and refs. therein), by using a Perkin–Elmer λ45 UV–Vis Spectrophotometer (Perkin–Elmer, Wellesley, MA).

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