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Isolation of an in Vitro and ex Vivo Antiradical Melanoidin from Roasted Barley

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The antiradical properties of water-soluble components of both natural and roasted barley were determined in vitro, by means of DPPH• assay and the linoleic acid−β-carotene system, and ex vivo, in rat liver hepatocyte microsomes against lipid peroxidation induced by CCl_4. The results show the occurrence in natural barley of weak antioxidant components. These are able to react against low reactive peroxyl radicals, but offer little protection against stable DPPH radicals deriving from peroxidation in microsomal lipids. Conversely, roasted barley yielded strong antioxidant components that are able to efficiently scavenge free radicals in any system used. The results show that the barley grain roasting process induces the formation of soluble Maillard reaction products with powerful antiradical activity. From roasted barley solution (barley coffee) was isolated a brown high molecular mass melanoidinic component, resistant to acidic hydrolysis, that is responsible for most of the barley coffee antioxidant activity in the biosystem.

KEYWORDS: Antioxidants; melanoidins; natural and roasted barley; antiradical activity; lipid peroxidation

INTRODUCTION

Barley (*Hordeum vulgare* L.), a prized cereal in ancient Egypt and Babylon, was probably one of the first plants of its type to have been systematically cultivated. It was used for breadmaking, for decoction (to which Hippocrates and Galenic ascribed healthy properties), and for the soup with which Romans fed gladiators prior to combat. The Romans considered barley as having a high energy yield, and although in fact it contains less starch than most other cultivated used cereals, it does have a higher content of other carbohydrates (*1*) that release energy more immediately.

Today barley is considered to be a minor cereal and is largely used as fodder. However, it is still important for human nutrition; although it is almost completely supplanted by wheat in breadmaking, pearl barley is often used in the preparation of soup. In germinated form, it is the main ingredient in beer brewing. In recent years, due to its high content in β-glucans, tocols, and polyphenols, it has been used in the preparation of functional foods. Furthermore, the secular custom in China of drinking barley or malt coffee instead of traditional coffee as a tasty beverage has greatly spread in Western regions, because children and adults alike are highly susceptible to the negative effects of caffeine. Such beverages use roasted barley, or malt, the composition of which is different from that of natural products.

Natural barley grains contain a number of polyphenolic compounds, such as phenolic acids (benzoic and cinnamic acid derivatives) and flavonoids, proanthocyanidins, and tannins (*2*, *3*), all of which are known to possess antioxidant and antiradical properties. These compounds are partially destroyed during the roasting process, which, however, induces the formation of new compounds in roasted grains; of particular note in this respect are polymeric substances that are generally considered to be either caramels, when they do not contain nitrogen, or melanoidins, when they incorporate nitrogen atoms. Melanoidins consist of brown compounds that can be detected in biological material and, in particular, in heat-treated foods, where they are the main end products of the Maillard reaction, which in turn comprises a set of consecutive and parallel chemical reactions. The complex structure of melanoidins is largely unknown because polymerization is influenced both by the starting reactans and their concentrations and by the reaction conditions, such as pH, water activity, temperature, reaction time, and solvent used (*4*−*7*). Accordingly, most systems or foods (irrespective of whether they differ from, or resemble, each other) co-host a variety of polymeric products, each of which is characterized by differing structure, molecular mass (MM), and elemental composition. These polymeric compounds are classified as low MM or high MM melanoidins depending on their dialysis, as determined by means of 3500 or 12000 Da cutoff membranes. Generally, the MM of such polymeric

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compounds does not exceed 100000 Da (*8*), but Faist et al. (*9*) found MM of up to 200000 Da for the melanoidins formed in roasted barley malt. Some authors report that in a model system the C/N ratio in melanoidins is high in the initial reaction period and decreases as the reaction time increases (*10*). The C/N ratio also decreases at lower temperatures or pH values (*11*). Cammerer et al. (*12*) found that maltose forms melanoidins with a lower nitrogen content than do other disaccharides such as lactose and sucrose. With regard to foods, the nitrogen content in melanoidins isolated from barley was found to be high in low MM compounds (as much as 30%), whereas it was lower in the high MM polymers (~3% in compounds of 30000−60000 Da) (*9*, *13*).

Maillard reaction products (MRPs) largely influence food sensorial features and have a significant effect on color, taste, and aroma, which are important food attributes and key factors in consumer acceptance. Furthermore, for the past two decades, research on melanoidins has taken biological properties into account (*14*). Melanoidins were found to possess mutagenic (*15*) and antimutagenic activity (*16*, *17*). A number of workers found that melanoidins also possess strong antioxidant properties (*18*, *19*).

Substantial research has studied the antioxidant properties of coffee brew (*19*, *20*) and demonstrated that the roasting process induces strong antioxidant activity in coffee, both in chemical and, in particular, in biological systems (*18*).

Conversely, few reports deal with the antioxidant properties of water-soluble components in natural barley or, above all, in roasted barley, which is used in roasted barley coffee preparation. Duh used chemical systems to test the antioxidant properties of natural and roasted barley, for which Chinese methods use temperatures of up to 465 °C (*21*). He found that roasted barley possesses less antioxidant activity than natural barley. To our knowledge, the literature lacks investigations into the efficiency of barley coffee antioxidants in biological systems.

The aim of this work was to study the antiradical properties of barley coffee beverage as prepared in Western countries, both in vitro in chemical and ex vivo in biological systems.

Furthermore, we intended to isolate the compound(s) responsible for any activity we may have found and verify whether they are naturally occurring in barley grains or are produced during the roasting process as MRPs and wheter they can act as antioxidants in vivo, too.

MATERIALS AND METHODS

Chemicals. 3-*O*-Caffeoylquinic acid (3-*O*-CQA), borate buffer (pH 10), phosphate buffer (pH 7.4), phosphate buffer (pH 3.0), 2,2-dipehnyl-1-picrylhydrazyl radical (DPPH•), linoleic acid, β-carotene, Tween 20, ethylenediaminetetraacetic acid (EDTA), nicotinamide adenine dinucleotide phosphate (NADP), glucose-6-phosphate (G6P), and trichloroacetic acid (TCA) were purchased from Sigma-Aldrich S.r.l. Sodium chloride, sodium phosphate buffer, carbon tetrachloride (CCl_4), thiobarbituric acid (TBA), and methanol of spectrophotometric grade >99% were purchased from Carlo Erba.

Roasting Barley Conditions. Natural barley grains (var. Clarine, courtesy of Crastan-Pontedera) were roasted in a pilot roaster apparatus. They were heated to 70−80 °C in 3 min, to 200 °C in 30 min, and finally to 220 °C in 13 min; they were then cooled by water spray to 180−190 °C and finally to room temperature with air.

Barley Solution Preparation. One sample of either natural or roasted barley was ground in a laboratory scale mill and sieved through a no. 30 sieve. Barley solutions were prepared according to the common Italian procedure for brewed coffee. A 6-g sample of natural or roasted barley was boiled for 10 min in 100 mL of Millipore grade water. The solutions were then filtered on Millipore membrane of cellulose acetate/cellulose nitrate mixed esters (0.45 μm), and the pH value of each sample was measured immediately.

DPPH• Assay (Antiradical Activity, ARA%) (*22*, *23*). Antioxidant activity was determined using DPPH• as a free radical. A 100-μL aliquot of barley solutions (or of their diluted solutions) (sample) or a 100-μL aliquot of KH_2PO_4/NaOH buffer (pH 7.4) (control sample) was added to 3.9 mL of a 6×10^5 mol L^{-1} methanol/KH_2PO_4/NaOH buffer (50:50 v/v) DPPH• solution. The decrease in absorbance was determined at 515 nm when the reaction reached a steady state (after 20 min of reaction).

The percent scavenger activity (ARA%) against DPPH• was calculated in accordance with the following equation:

$$\text{ARA\%} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100 \quad (1)$$

The scavenger activity was also determined for a methanolic solution of Trolox C (Sigma), which was assayed at three different concentrations (100 μM ARA% = 47.25; 200 μM ARA% = 72.15; 250 μM ARA% = 73.00).

Linoleic Acid−β-Carotene Assay (Anti-Peroxyl Radical Activity, AA%). The antioxidant activities of the barley solutions, based on coupled oxidation of linoleic acid and β-carotene, were determined in accordance with a modified version (*18*, *23*) of the method of Taga et al. (*24*). Briefly, 5 mg of β-carotene (Merck) was dissolved in 50 mL of chloroform solution. A 3-mL aliquot of β-carotene chloroform solution was added to a conical flask along with 40 mg of linoleic acid (Merck) and 400 mg of Tween 20 (Merck). The chloroform was evaporated until dry under reduced pressure at low temperature (<30 °C). Millipore grade water (100 mL) was added to the dried mixture, and the mixture was shaken. Two aliquots (100 μL) of barley solutions were added to 5 mL of β-carotene emulsion in test tubes, and the mixture was thoroughly mixed (samples). In preliminary tests, the addition of barley solutions produced no significant change in the sample's pH. One of the sample's absorbance was immediately measured with the spectrophotometer set at 470 nm, and the other sample's absorbance was measured after 30 min of incubation in a water bath at 50 °C. Each sample was read against an emulsion prepared as described but without β-carotene (blank). To correct any influence that might be due to barley solution color in the calculation of the β-carotene degradation rate (dr), an aliquot of each barley solution was added to 5 mL of blank (blank sample). These mixtures were read spectrophotometrically, and the absorbance measured was subtracted from that of the corresponding sample. The dr of β-carotene was calculated by first-order kinetics

$$\ln(A_0/A_t)/t = \text{dr of sample} \quad (2)$$

where A_0 = absorbance of the sample − absorbance of blank sample at time 0 (absorbance was read immediately after the addition of barley solution), A_t = absorbance of the sample − absorbance of blank sample at time t, t = 30 min of incubation in a water bath at 50 °C.

$$\ln(a_0/a_t)/t = \text{dr of control sample} \quad (3)$$

where 100 μL of Millipore grade water was added to 5 mL of β-carotene emulsion and treated as the corresponding sample, a_0 = absorbance of the sample − absorbance of blank sample at time 0, a_t = absorbance of the sample − absorbance of blank sample at time t.

AA was expressed as the percent of inhibition relative to the control in accordance with the following equation:

$$\text{AA\%} = \frac{\text{dr}_{\text{control sample}} - \text{dr}_{\text{sample}}}{\text{dr}_{\text{control sample}}} \times 100$$

A Trolox C (Sigma) solution was also assayed for AA at three different concentrations (50 μM AA% = 68.67; 100 μM AA% = 84.50; 200 μM AA% = 84.62).

Biological Lipid Peroxidation Assay (Protective Activity, PA %). Liver microsomes were prepared from male Wistar rats weighing 200−250 g in accordance with a modified version (*18*, *25*) of the method of

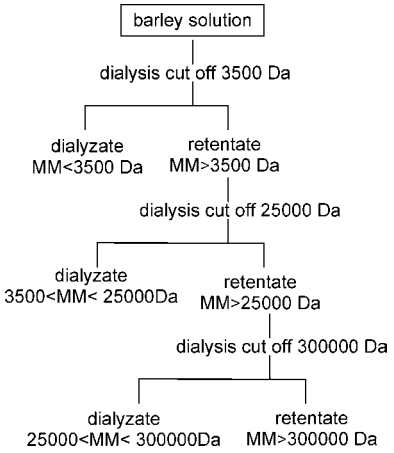

Figure 1. Sequential dialysis scheme.

Horie (*26*). The microsomal pellets obtained were suspended either in 0.1 M sodium phosphate buffer (pH 7.4) (control sample) or in barley solutions (sample) to make total respective volumes of 6 mL. An aliquot (0.1 mL) of the obtained suspension was immediately removed and used for determination of microsomal proteins (*27*).

The remaining preparation was added to NaCl (1 mL, 140 μM), EDTA (1 mL, 50 μM), and sodium phosphate buffer (1 mL, 0.1 M, pH 7.4) and then subdivided into two respective aliquots of 4 mL. All test tubes containing samples were stoppered, and N_2 was bubbled through the solutions at 37 °C for 15 min to obtain anaerobic conditions for the subsequent induction of lipid peroxidation. To one group of samples we then added NADP (0.5 mL, 250 μM) and CCl_4/EtOH (20 μL, 50% v/v). In contrast, we added an equivalent amount of buffer to the second group. Both samples were placed in a shaking water bath at 37 °C for 30 min, and equal volumes of 30% TCA at 0 °C and 0.75% TBA were subsequently added (*28*).

The reaction mixtures were heated in boiling water for 15 min, kept in ice for 5 min, and then centrifuged for 10 min at 3000 rpm to separate corpuscolate particles.

Absorbance of supernatants was read in a spectrophotometer (λ = 545 nm) using the second series of samples treated as above but without coenzymes to bring the spectrophotometer to zero. This was done to correct any interference deriving from the color of TBA reacting substances (TBA-RS) that naturally occur in barley solutions.

The protective activity was expressed as the percentage decrease in TBA-RS relative to the control in accordance with the equation

$$\mathrm{PA\%} = (a - b)/a \times 100$$

where *a* represents the TBA-RS in control sample and *b* the TBA-RS in sample.

The protective activity was also determined for an aqueous/methanolic solution of Trolox C (Sigma), which was assayed at four different concentrations (100 μM PA% = 50.61; 200 μM PA% = 58.34; 250 μM PA% = 62.34; 300 μM PA% = 62.93).

Dialysis. Dialysis was performed with Spectra/Por Biotech cellulose ester membrane and molecular weights cutoff at 3500, 25000, and 300000 Da (**Figure 1**). A 10-mL aliquot of the above-mentioned barley solutions was fractionated by dialysis in 1000 mL of Millipore grade water for 6 h at 4 °C. The dialysates and the retentates were brought to the initial volume of the barley solutions (10 mL) using Millipore grade water before to be tested for DPPH• assay, peroxyl radical assay, and protective activity assay. The 3-*O*-CQA used as a standard molecular weight marker was recovered at a percentage that was >95%.

Gel Filtration Chromatography (GFC). The GFC apparatus was a 655A-11 Merck−Hitachi liquid chromatograph with a wavelength UV monitor at 210 nm. The GFC separation of the roasted barley fraction with MM >300 kDa was performed with a Superformance Universal glass-cartridge system (300 × 10 mm) (Merck). The stationary phases were at first TSK gel Toyopearl HW-65F (exclusion limits 10−1000 kDa) (65GFC) and then TSK gel Toyopearl HW-75F (exclusion limits 1000−10000 kDa) (75GFC) (Tosoh Biosep GmbH). The mobile phase was Millipore grade water at a flow of 0.4 mL min^{-1}.

Table 1. Moisture of Natural and Roasted Barley, Dry Residue, and pH Values of Natural and Roasted Barley Solutions

sample	moisture (% ± SD)	dry residue (mg/mL ± SD)	pH (± SD)
natural barley	12.78 ± 0.049	11.48 ± 0.88	5.84 ± 0.24
roasted barley	8.33 ± 0.085	24.45 ± 0.98	4.54 ± 0.14

Capillary Electrophoresis (CE). The eluate corresponding to the 75GFC1 fraction was separately collected to give three subfractions (75GFC1-1, 75GFC1-2, 75GFC1-3). They were freeze-dried and each dissolved in 400 μL of Millipore grade water. All of the subfractions were then analyzed by CE (HP^{3D} CE system, Waldbronn, Germany, with built-in diode array detector). The length of the capillaries (fused silica capillary A, 25.5 cm L_{eff}, 34 cm L_{tot}; fused silica capillary B, L_{eff}, 58.5 cm L_{tot}), the pH of the background electrolyte (BGE) (borate buffer, 100 mM, pH 10; phosphate buffer, 100 mM, pH 7.4; phosphate buffer, 100 mM, pH 3.0), and injection times (50 mbar 15 s, 50 mbar 5 s) were changed to optimize the electrophoretic experiments. The analyses were carried out by applying a voltage of 10 kV, and the electrophoretic profiles and the areas were compared with the electrophoretic profiles obtained by contemporarily applying a pressure of 15 mbar, to be sure that the entire sample was completely eluted.

Hydrolysis. For hydrolytic investigations, 1 mL of MM > 300 kDa fraction (pH 4.70) obtained with TSK75 resin was treated with 1 mL of 2N HCl, and the mixture was heated for 2 h at 50 °C in a sealed tube. After rapid cooling, the pH of the mixture was adjusted to the initial MM > 300 kDa fraction pH. The mixture was then filtered before 75GFC analysis.

Statistical Analysis. The values represent the mean values of, respectively, at least 10 replications for the anti-DPPH• assay and the anti-peroxyl radical assay, and 5 independent experiments on triplicate samples for PA assay. Data were analyzed by analysis of variance (ANOVA) with the Statgraphic Plus (1998) statistical package. Means were separated with the LSD method at a confidence level of 99%.

RESULTS AND DISCUSSION

Table 1 shows the moisture and the water-soluble material contents of natural and roasted barley grains and the pH values of natural barley solution and of roasted barley solution (barley coffee), prepared as previously described. The natural barley grains contain a lot less soluble matter than do the roasted barley, indicating that the roasting process dramatically increases the water-soluble components in barley grain. The barley coffee pH is lower than the natural barley solution pH, indicating that the compounds generated during the roasting process have acidic properties.

The antioxidant properties of natural and roasted barley solutions were tested in chemical systems against (1) stable and colored radical DPPH and (2) low reactive peroxyl radicals in the linoleic acid−β-carotene system. In the former, antiradical activity was expressed as the percent decrease in the coloration of the sample solution relative to the control (ARA%); in the latter, the antioxidant activity was expressed as the percentage of inhibition of the β-carotene degradation rate (AA%). Thereafter, to improve simulation of in vivo conditions, antioxidant properties were also evaluated ex vivo against rat hepatocyte microsome lipid peroxidation measured as the percentage decrease in TBA-RS in the sample relative to the control. It was expressed as the percentage of protective activity (PA%).

With regard to ARA, the natural barley solution showed low anti-DPPH radical activity, whereas that of barley coffee was much higher, close to that of a 200 μM Trolox C solution used

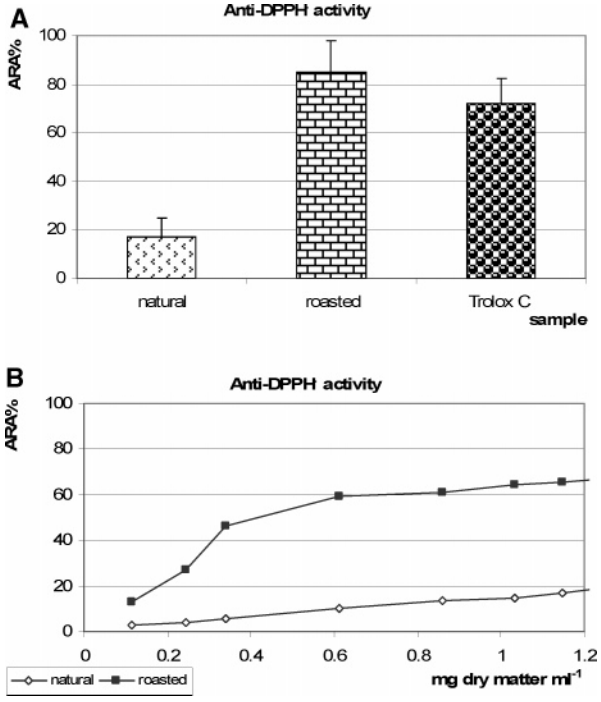

Figure 2. (**A**) Anti-DPPH activity (ARA%) of natural and roasted barley solution (6 g/100 mL) and of a Trolox C 200 μM standard solution. (**B**) Anti-DPPH• activity (ARA%) versus concentration of natural and roasted barley dry matter (SD values < 2.4%).

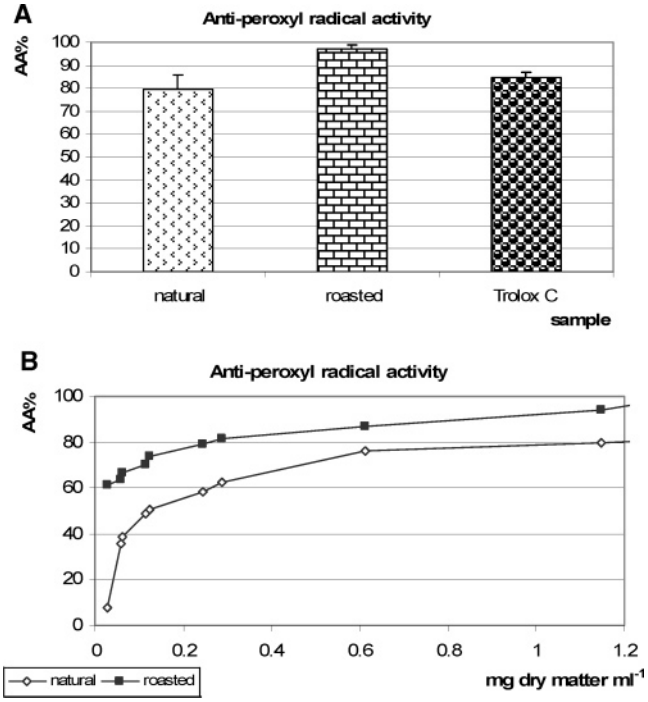

Figure 3. (**A**) Anti-peroxyl radical activity (AA%) of natural and roasted barley solution (6 g/100 mL) and of a Trolox C 100 μM standard solution. (**B**) Anti-peroxyl radical activity (AA%) versus concentration of natural and roasted barley dry matter (SD values < 3.7%).

as a standard (**Figure 2A**). To ensure that differences in activity given by the two solutions did not depend either on different concentrations of the same compound or on differences in the total soluble component amounts in the two solutions, we determined the dose−response relationship for both natural and roasted solutions using the same concentrations of dry matter for both of the samples (**Figure 2B**). The different ARA values and profile shown by the natural solution in comparison with roasted barley coffee in relation to the dry matter concentration indicate that the anti-DPPH radical activity is due to different compounds and not the same compound occurring in different amounts in the two solutions.

In the linoleic acid−β-carotene system, both solutions showed anti-peroxyl radical activity, with the barley coffee obtaining higher values (**Figure 3A**). Both of them are close to the AA values given by a 100 μM Trolox C solution. In this case, too, the dose−response relationship when dry matter was considered differed between natural and roasted barley (**Figure 3B**).

These results show the occurrence in natural barley of weak antioxidant components. The latter are able to react against low reactive peroxyl radicals, but they offer little protection against stable DPPH radicals when they are tested in chemical systems (ARA values are low for natural barley; AA values are close for natural and roasted barley solutions). Conversely, roasted barley yielded strong antioxidant components able to scavenge stable DPPH radicals efficiently.

With regard to the biological system, the PA values showed weak activity in natural barley, whereas the roasted barley beverage (PA = 100%) completely inhibited the formation of TBA-RS, indicating a strong activity in the protection of microsomial lipids from peroxidation, even when peroxidation is induced by CCl_4 (**Figure 4A**). In this biosystem, too, the difference in dose−response relationship between natural and roasted barley solutions showed that the roasted barley beverage

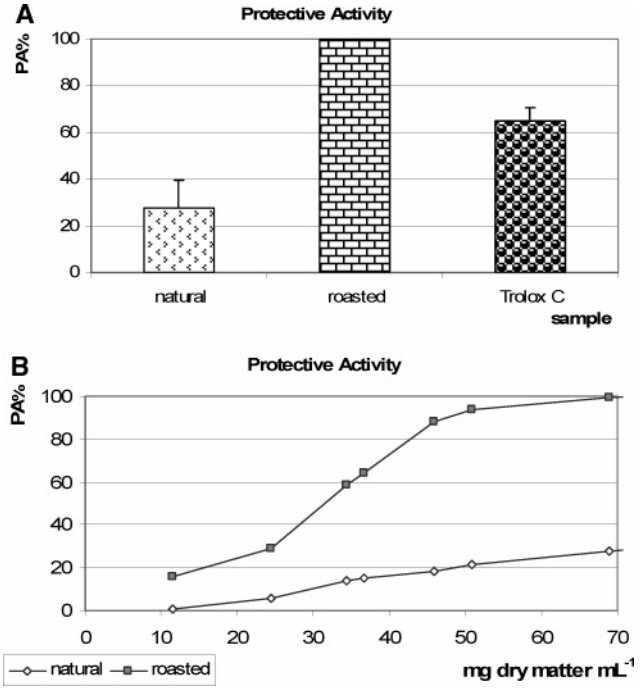

Figure 4. (**A**) Protective activity (PA%) of natural and roasted barley solution (6 g 100 mL^{-1}) and of a Trolox C 250 μM standard solution. (**B**) Protective activity (PA%) versus concentration of natural and roasted barley dry matter (SD values < 2.9%).

contained more active compounds having substantial activity even when their concentration was low in the system (**Figure 4B**).

We can thus conclude that all three methods used to test the antiradical activity of natural and roasted barley demonstrated that the roasting process induced the formation of soluble compounds with strong antiradical activity in barley grain.

Table 2. Anti-DPPH Radical Activity (ARA), Antiperoxyl Radical Activity (AA), and Protective Activity (PA) of Natural Barley and Its Dialysis Fractions (Cutoff = 3500 Da)

sample	ARA (% ± SD)	AA (% ± SD)	PA (% ± SD)
natural barley	16.87 ± 1.36	79.74 ± 4.64	27.46 ± 3.39
MW < 3500 Da	7.53 ± 0.32	75.89 ± 0.01	31.16 ± 3.58
MW > 3500 Da	8.83 ± 0.32	56.25 ± 0.71	0.36 ± 0.06

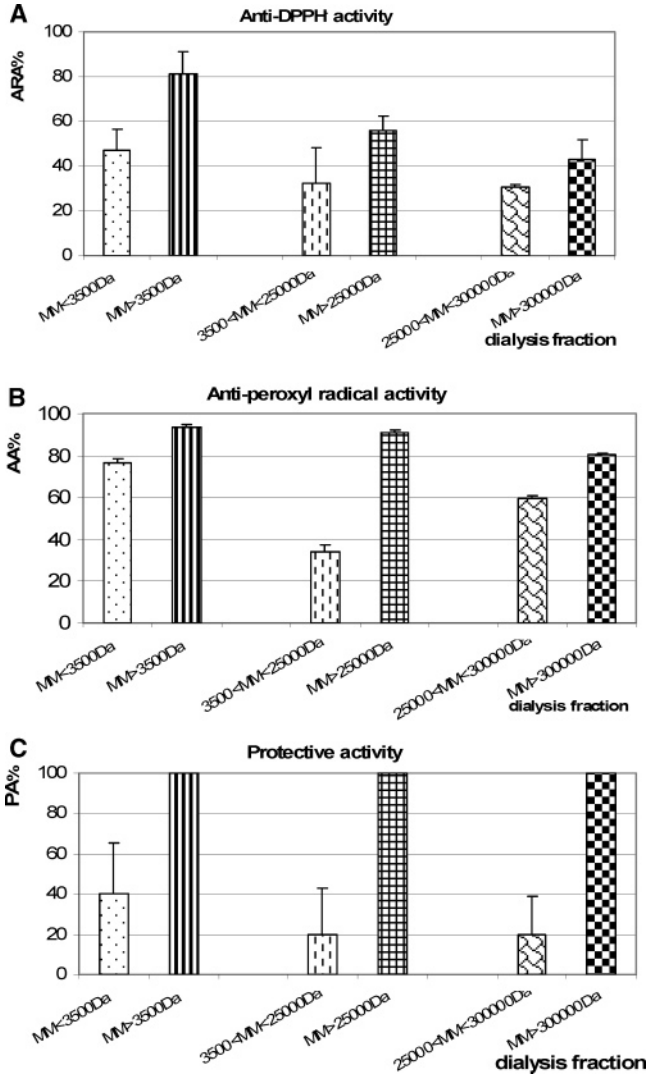

Figure 5. (**A**) Anti-DPPH• activity (ARA%) of roasted barley dialysis fractions. (**B**) Anti-peroxyl radical activity (AA%) of roasted barley dialysis fractions. (**C**) Protective activity (PA%) of roasted barley dialysis fractions.

To isolate the active components, and to have preliminary information on their MM, we dialyzed natural and roasted barley solutions with membranes of differing cutoffs, starting from 3500 Da. This procedure enabled the separation of the monomeric or oligomeric polyphenols from the higher MM components. Of these components, we note the high MM melanoidins that formed as MRPs in roasted barley. Each dialysate and retentate obtained, reported to the initial volume of the dialyzed barley solution, was submitted to the three cited assays.

With regard to natural barley (**Table 2**) in the DPPH• assay, both fractions obtained by dialysis (MM < 3500 Da and MM > 3500 Da) proved to have low activity. In the linoleic acid−β-carotene system, both dialysate and retentate showed good antioxidant activity; in the biological system, the MM > 3500 Da fraction is not protective, and the MM < 3500 Da fraction

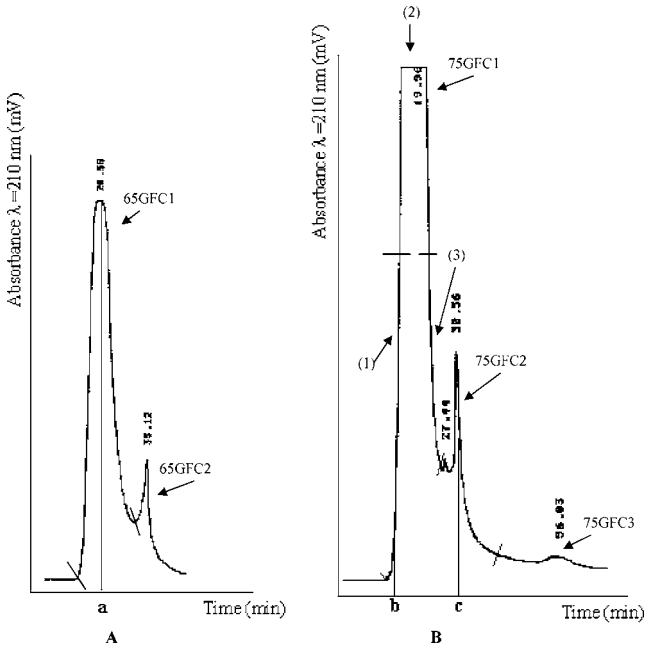

Figure 6. (**A**) GFC chromatogram of roasted barley MM fraction >300 kDa obtained with resin HW-65F (65GFC). (**B**) GFC chromatogram of roasted barley MM fraction >300 kDa obtained with resin HW-75F (75GFC). Procedures are described in the text: (**a**) Blue dextran MM 2000 kDa; (**b**) blue dextran; (**c**) dextran MM 500 kDa; (**1**) 75GFC1-1; (**2**) 75GFC1-2; (**3**) 75GFC1-3.

was weakly active, confirming the results previously obtained from natural barley solution.

With regard to roasted barley, the retentate fraction (MM > 3500 Da), containing an amount of dry matter corresponding to the 35.31% of roasted barley grain, showed significantly greater activity in all of the systems ($p < 0.01$) than did the dialysate fraction (MM < 3500 Da; dry matter = 10.35% of roasted barley grain). In particular, in the biological system, the components with nominal MM > 3500 Da completely protected microsomial lipids from peroxidation (**Figure 5**).

The retentate (MM > 3500 Da) was then submitted to sequential dialysis (**Figure 1**) with 25000 and 300000 Da membrane cutoff, and each dialysate and retentate obtained was again tested for its antioxidant activity in the three described assays. They all showed that most of the barley coffee antiradical activity is caused by the fraction with MM > 300000 Da (dry matter = 24.56% of roasted barley grain) (**Figure 5**). On this basis, we submitted the fraction containing the components with MM > 300000 Da to GFC; two different resins were used separately with respective exclusion limits of 10−1000 kDa (65GFC) and 100−10000 kDa (75GFC).

With the first resin, two chromatographic peaks were obtained, but only one (65GFC1) was active (PA = 100%). This active fraction presented the same retention time (RT) as blue dextran, which was used as a standard (V_0). This indicated that the 65GFC1 components have MM in excess of 1000 kDa (**Figure 6A**).

When the second resin was used, three fractions were obtained. In this case, too, only one active fraction (75GFC1) was found (PA = 100%). The RT of this fraction (the dry residue of this fraction consists of 12.40 mg mL^{-1} corresponding to 20.67% of roasted barley grain and 50.71% of barley coffee dry matter) was higher than that of blue dextran (RT = 19.08 min). We can therefore attribute an MM in the range of 1000−2000 KDa (**Figure 6B**) to the active component(s).

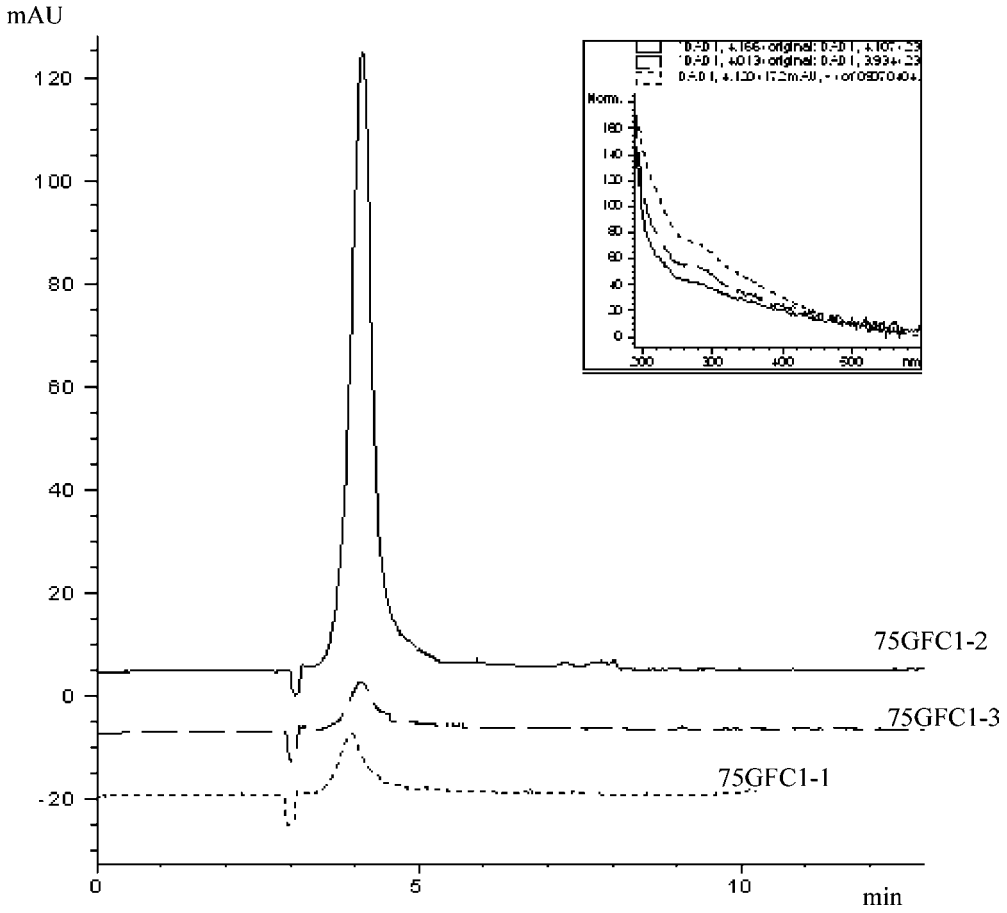

Figure 7. Comparison of the electrophoretic profiles obtained by injection of the three subfractions (75GFC1-1, 75GFC1-2, 75GFC1-3), from 75GFC1 in the capillary electrophoresis system. Conditions: capillary, fused silica capillary (L_{tot}, 34 cm; L_{eff}, 25.5 cm); BGE, borate buffer, 100 mM, pH 10; injection, 50 mbar, 5 s; voltage, 10 kV; λ, 200 nm. The effective mobility of the main peak is $-1.2 \times 10^{-4} \pm 0.09 \times 10^{4}$ cm^2/Vs.

Given this surprisingly high MM and the fact that some authors reported that polymerization of MRP could take place during the dialysis process, we dialyzed coffee barley for differing periods (0, 3, 6, 12, and 24 h) by submitting the respective retentates to GFC analysis. The peak corresponding to the lower MM components was strongly reduced after 6 h of dialysis, whereas the peaks corresponding to the high MM active components showed no change even after 24 h of dialysis. These results indicated that the high MM active components were actually present in roasted barley and did not derive by polymerization of lower MM polymers during dialysis.

Subsequently, to verify if the 75GFC1 fraction consisted of one or more compounds, the eluate corresponding to this peak was subdivided into three subfractions (75GFC1-1, 75GFC1-2, 75GFC1-3), as indicated in **Figure 6B**. These subfractions were analyzed by CE underthe previously described, differing analytical conditions. The resulting electrophoretic profiles were invariably the same for all three different 75GFC1 subfractions, indicating that the active 75GFC1 fraction consists of only one component (**Figure 7**). Therefore, we conclude that we have isolated the roasted barley MRP responsible for most of the barley coffee protective activity. Elementary analysis showed this component as consisting of C = 39.03%, H = 6.92%, and N = 1.12%. These data could indicate that polysaccharide chains bind the chromophore in this compound. Anyway, the nitrogen presence allowed us to define this component as a melanoidin. Our results agree with those of Cammerer et al. (*29*), who found intact carbohydrate structures within the melanoidin skeleton.

We then tested the stability of roasted barley antioxidant properties with regard to (i) gastric conditions and (ii) the spray-drying method used in soluble roasted barley preparation. In the first case the dialysis retentate with MM > 300 kDa was submitted to hydrolysis, as previously described. The obtained hydrolysis mixture was fractionated by means of GFC technique and TSK75 resin, and we again found three chromatographic peaks. Of these, only the first, which had the same RT as 75GFC1 fraction (RT = 19.96 min) and the same percent area as the first peak of nonhydrolyzed MM > 300 kDa fraction, proved to have the same ARA%, AA%, and PA% values as the 75GFC1 fraction. These results demonstrate that the hydrolysis conditions applied (2N HCl, 2 h, and 50 °C) do not affect the active component.

We also analyzed a commercial sample of instant soluble powder obtained from roasted barley. This type of product is now widely used, because it enables rapid and simple preparation of the beverage. The instant barley coffee was prepared with an amount of soluble powder that corresponded to the amount of soluble components present in barley coffee. In this case, too, in all three antiradical activity assays, the instant barley coffee showed about the same antiradical activity values as did the barley coffee (ARA% = 77.25 ± 4.24; AA% = 84.2 ± 3.56; PA% = 95.21 ± 6.89); it also presented the same chromatographic profile. These findings indicate that the isolated polymer and its antiradical properties were stable when they underwent the treatments applied to roasted barley in the preparation of instant soluble barley.

In conclusion, our results show (i) that natural barley contains water-soluble low MM compounds having weak antioxidant efficiency much lower than that of barley that is thermally treated at up to 220 °C and (ii) that in a variety of conditions, roasted barley contains water-soluble components which present powerful antioxidant properties. One of these components is able to completely inhibit lipid peroxidation in an ex vivo system consisting of rat hepatocyte microsomes, even when peroxidation is induced by CCl_4. This component has a brown color and a high MM, as shown by GFC analysis with a small percent content of non-amino acidic nitrogen. On the basis of these features, it can be considered as belonging to the group of melanoidins. Its unusually high MM and its low nitrogen content could be due to the relatively high polysaccharide and low protein content of barley grain and to specific reaction conditions applied during the roasting process, such as low water activity and lengthy roasting periods.

The persistence of the isolated melanoidin antioxidant properties, even after the melanoidin is treated in acidic medium in more severe conditions than those found in gastric medium, shows that it can act as an antioxidant in the gastrointestinal tract, reducing the oxidative stress in situ where organic and inorganic food contaminants and other compounds with oxidative and peroxidase activities commonly occur (*30*).

Considering that dietary antioxidant compounds are substantially reported in epidemiological studies as generally playing a protective role in chronic diseases, our findings seem to indicate that barley coffee and instant barley coffee consumption can be beneficial to human health.

ABBREVIATIONS USED

MRPs, Maillard reaction products; $DPPH^{\bullet}$, 2,2-diphenyl-1-picrylhydrazyl radical; CCl_4, carbon tetrachloride; EDTA, ethylenediaminetetraacetic acid; NADP, nicotinamide adenine dinucleotide phosphate; G6P, glucose-6-phosphate; TCA, trichloroacetic acid; TBA, thiobarbituric acid; TBA-RS, TBA reactive substances; $ROO^{\bullet}$, alkyl peroxyl radical; PA, protective activity; ARA, anti-DPPH radical activity; AA, anti-peroxyl radical activity; CE, capillary electrophoresis; GFC, gel filtration chromatography; ANOVA, analysis of variance; LSD, least significant difference; MM, molecular mass; BGE, background electrolyte; SD, standard deviation.

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