JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

A New Procedure To Measure the Antioxidant Activity of Insoluble Food Components

ARDA SERPEN,[†] EDOARDO CAPUANO,[‡] VINCENZO FOGLIANO,[‡] AND VURAL GÖKMEN*,[†]

Department of Food Engineering, Hacettepe University, 06800 Beytepe, Ankara, Turkey and Dipartimento di Scienza degli Alimenti, Università di Napoli "Federico II", Parco Gussone 80055 Portici, Napoli

The measurement of antioxidant activity was limited to soluble components to date. Functional groups, which are bound to insoluble matters, may exert antioxidant activity by a surface reaction phenomenon. This hypothesis was tested on the insoluble matters of foods, food ingredients, and Maillard reaction products (MRPs). Insoluble matters were prepared by consecutive washes with water and methanol followed by a lyophilization of the insoluble residue. The measurement was performed by a new procedure using 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazil (DPPH) colored radicals. These insoluble matters always showed antioxidant activity. Alkali hydrolysis reduced up to 90% the antioxidant activity of cereal-based insoluble matters, thus confirming that fiber-bound compounds have a major role in their antioxidant activity. The antioxidant activity of the insoluble MRPs was not significantly affected by processing conditions, but severe treatments increased the ratio between insoluble and soluble matters. The contribution of insoluble matter to total antioxidant activity was limited for fruits and vegetables, but it was relevant for cereal-based foods and increased over 50% for dietary-fiber-rich ingredients.

KEYWORDS: antioxidant activity; insoluble food components; ABTS; DPPH; melanoproteins; melanoidins

INTRODUCTION

The assessment of food antioxidant activity has become a very popular objective of food science research, as it conjugated the issues related to shelf life and sensorial quality to those of health and nutrition. The antioxidant activity of hundreds of food items has been measured, creating databases that can be used to calculate the intake of antioxidant equivalents for an overall diet or for a specific meal (*1*–*3*). A relationship between the antioxidant equivalents intake and some chronic diseases has also been proposed (*4*, *5*).

Several methods to measure the antioxidant activity have been published in the past 20 years, and the discussion about the real meaning and the usefulness of this kind of measurement is still open (*6*–*10*). In addition to the traditional methods based on fat oxidation monitoring (*11*), conjugate diene formation (*12*), and measure of thiobarbituric reactive substances (*13*), the use of colored radical compounds was largely adopted as a result of low cost and good reproducibility of the developed procedures (*14*–*17*).

To date, the measurement of antioxidant activity has been limited to soluble material, and consequently the extraction procedure was considered a critical step in all these works. Many attempts using solvent mixtures or physical treatments have been made to increase the solubility of food components in order to assess their antioxidant activity (*18*–*21*). Despite these efforts, it is clear that many food items have insoluble components that cannot be solubilized without altering their molecular nature by chemical or enzymatic treatments (*22*–*25*). However, the insoluble components are not necessarily chemically inert. Functional groups, which can be bound to the insoluble fractions of various food components, may exert an antioxidant activity by quenching free radicals that are present in the solvent matrix. The possibility that reactions occur at the interface between two heterogeneous phases is at the basis of many chemical applications where surface reaction phenomena have a well-recognized and fundamental role (*26*, *27*).

In this work, the possibility that antioxidant functional groups, bound to the insoluble components of different food items, are able to exert an antioxidant action was examined using a new procedure based on two well-known colored radicals: 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (*15*) and 2,2-diphenyl-1-picrylhydrazil (DPPH) (*14*). Some pertinent items such as cereals, fruit or vegetable dietary fibers, as well as insoluble final products of the Maillard reaction (melanoidins or melanoproteins) were selected for this purpose. Results indicated that many food insoluble components have a signifi-

* To whom correspondence should be addressed. E-mail: vgokmen@hacettepe.edu.tr.
[†] Hacettepe University.
[‡] Università di Napoli "Federico II".

10.1021/jf071291z CCC: $37.00
Published on Web 08/21/2007

cant antioxidant activity, which in some cases can also have a relevant nutritional meaning.

MATERIALS AND METHODS

Chemicals and Reagents. All chemicals and solvents were of HPLC or analytical grade. D-(+)-Glucose (min 99.5%), D-(+)-fructose (min 99%), and L-glycine (min 99%) were obtained from Sigma (St. Louis, MO). L-Lysine monohydrochloride, cellulose (powder from spruce), and casein hydrolysate were purchased from Fluka Chemie AG (Buchs, Switzerland). Whey protein (lactoserum protein concentrate) was from NZMP (Germany). Soybean proteins were obtained from Chimpex (Caivano, Italy).

Methanol was purchased from Merck (Darmstad, Germany), and ethanol, acetone, and chloroform were from Carlo Erba (Italy). ABTS was from Fluka (St. Louis, MO), and DPPH, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and potassium persulfate (dipotassium peroxdisulfate) were obtained from Sigma-Aldrich (Steinheim, Germany). Ultrapure water was used throughout the experiments (MilliQ system, Millipore, Bedford, MA).

Foods and Food Ingredients. Durum wheat fiber was obtained from Barilla G. & R. F.lli S.p.A (Parma Italy); barley fiber was obtained by Ringnes AS (Oslo, Norway), coffee silverskin was a gift from Illycaffè (Trieste, Italy), and cocoa husks were from Trucillo SpA (Salerno, Italy). Lemon fiber was purchased by Prodotti Gianni (Milan, Italy) in powder form. Other food samples were obtained from local markets and processed as follows. Bread crust was obtained from a 1-kg bread obtained by sourdough fermentation and baking at 220 °C for 90 min. Bread crust was separated from the crumb with a kitchen knife, freeze-dried, and ground in a mill.

Biscuits (6.5% protein, 4% dietary fiber), snacks, and breakfast cereals (8.0 % protein, 5.1% dietary fiber) were finely ground in a Waring blender and passed through a sieve. Lettuce and tomato were homogenized with water in an Ultra Turrax T25 blender at 8000 *g* for 3 min. Orange juice (0.7% protein, 0.3% dietary fiber) and wholemeal oat flour were used as is. Gluten was prepared by extensive washing under tap water of dough obtained from *Triticum aestivum* flour containing 9.5% proteins. The gluten was freeze-dried, and the solid residue was finely ground in a Waring blender and passed through a sieve. All samples were stored at 4 °C prior to analysis.

Preparation of Soluble and Insoluble Fractions of MRPs. Model systems composed of reducing sugars and amino acids (melanoidins) or proteins (melanoproteins) were used to obtain soluble and insoluble MRPs. For melanoidins, 50 μmol of glucose or fructose was combined with 50 μmol of glycine or lysine to a final volume of 50 μL in a test tube (Pyrex). For melanoproteins, 50 μmol of glucose was combined with 10 mg of protein (casein, gluten, soybean, or whey protein) in a test tube (Pyrex). The test tubes containing the reactants were placed in an oil bath. The reactions were performed at 110 and 130 °C for up to 60 min. After the predefined heating periods of 5, 30, and 60 min, the tubes were immediately placed on cold water.

The pyrolysates were suspended in 1.0 mL of water, and the aqueous extract was obtained by vortexing for 1 min. The mixture was transferred into an eppendorf and centrifuged at 9200 *g* for 5 min. The clear supernatant was separated and lyophilized to obtain the water-soluble fraction of MRPs in the solid state. The washing procedure was ended after 10 washing cycles with 50 mL of water and 3 washing cycles with 50 mL of methanol. Each washing step was followed by a centrifugation at 1500 *g* for 10 min. Remaining precipitate was suspended in 1 L of water and allowed to settle for 12–15 h. After centrifugation, the final precipitate was lyophilized to obtain the insoluble fraction of MRPs in the solid state. Both soluble and insoluble fractions of MRPs were kept at 4 °C prior to antioxidant activity measurement.

Preparation of Insoluble Fractions of Foods and Food Ingredients. One gram of each powdered or homogenized sample was consecutively washed with water, methanol, and water. The washing procedure was ended after 5 washing cycles with 50 mL of water, 3 washing cycles with 50 mL of methanol, and 3 washing cycles with 50 mL of water. Each washing step was followed by a centrifugation at 1500 *g* for 10 min. For tomatoes, methanol was replaced with acetone during washing. After final washing and centrifugation, the residual white precipitate was lyophilized to obtain the insoluble fraction of dietary fibers and food samples, which was kept at 4 °C prior to antioxidant activity measurement.

Durum wheat bran and barley fiber were also treated with alkali under conditions that are known to hydrolyze the ester bonds with associated phenolic compounds (*22*). Because cereal fibers are known to have phenolic compounds with high antioxidant capacity that may be free or bound to an insoluble polysaccharide component (*23*), they were used as the food material to test our hypothesis. One gram of powder was mixed with 75 mL of 2 N NaOH in a glass flask. The mixture was gently shaken for 90 min. Then alkali-treated samples were washed using the procedure described above.

Measurement of Antioxidant Activity. *ABTS Method.* Antioxidant activities of the insoluble fractions obtained from the MRPs, dietary fibers, and foods were measured using the ABTS method (*15*) with some modifications. Lyophilized fractions were powdered and sieved prior to measurement. A 10 mg portion of the powdered sample was transferred to an eppendorf, and the reaction was started by adding 1.7 mL of ABTS reagent. The mixture was vortexed for 2 min to facilitate the surface reaction between the insoluble matter and the ABTS reagent. Following centrifugation at 9200 *g* for 2 min, the absorbance of the optically clear supernatant was measured at 734 nm using a Shimadzu model 2100 variable wavelength UV–vis spectrophotometer. All measurements were performed exactly 6 min after mixing the insoluble matter with the ABTS reagent.

DPPH Method. Antioxidant activities were also measured using the DPPH method (*14*) with some modifications. Lyophilized fractions were powdered and sieved prior to measurement. A 10 mg portion of the powdered sample was transferred to an eppendorf, and the reaction was started by adding 1.7 mL of DPPH reagent. The mixture was vortexed for 3 min at 0, 15, and 25 min to facilitate the surface reaction between the insoluble matter and the DPPH reagent. Following centrifugation at 9200 *g* for 2 min, the absorbance of the optically clear supernatant was measured at 517 nm using a Shimadzu model 2100 variable wavelength UV–vis spectrophotometer. All measurements were performed at exactly 30 min after mixing the insoluble matter with the DPPH reagent.

The antioxidant activity was expressed as millimole of Trolox equivalent antioxidant capacity (TEAC) per kilogram sample by means of a dose–response curve for Trolox. The solid-state samples were diluted prior to measurement if measured absorbance values were outside the linear response range of the radical discoloration solution. For insoluble fractions, dilution was performed with cellulose powder, which was found to be inert toward both ABTS and DPPH reagents. Dilution with cellulose allowed weighing 10 mg per sample, thus ensuring good reproducibility also for high antioxidant materials. For soluble fractions, dilution was performed with distilled water.

Statistical Analysis. Statistical analysis of data was performed by analysis of variance and tested for significance by the Duncan test, which allowed a multiple comparison among the data to individualize the significant differences. Differences were considered significant if $p < 0.05$. At least two independent analyses were carried out per sample.

RESULTS AND DISCUSSION

Insoluble matters of foods, food ingredients, and Maillard reaction products were shown to act as free radical quenchers in preliminary tests. It was hypothesized that when free functional groups on the surface of insoluble solid particles came into contact with the radicals, they were able to quench them as schematically drawn in **Figure 1**.

Before measurement, the samples were extensively washed with water and methanol in order to completely remove their soluble fractions. Each washing volume was subjected to antioxidant activity measurement by using the ABTS method. The washing procedure applied for each sample ensured the absence of any soluble matter that has antioxidant activity in the remaining fraction of the sample. Remaining insoluble

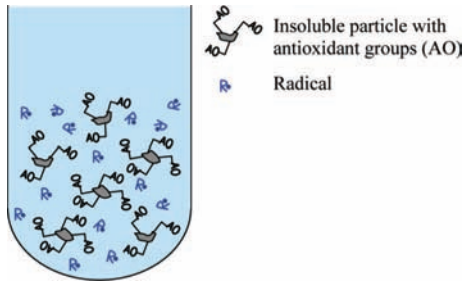

Figure 1. Scheme of the interaction between radical molecules and antioxidant groups of solid particles.

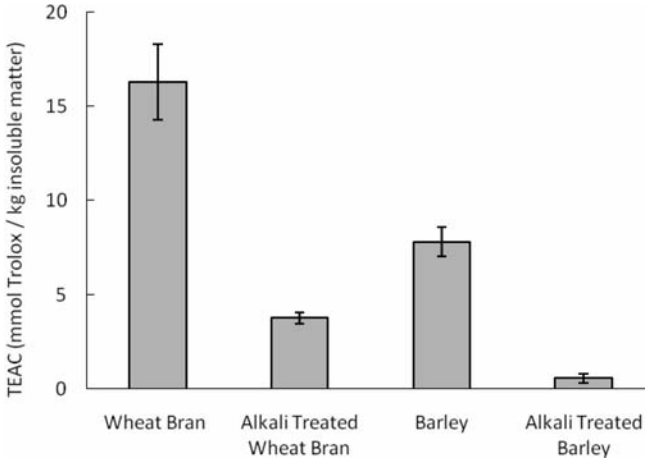

Figure 2. Antioxidant activities of the insoluble matters of dietary-fiber-rich ingredients derived from durum wheat and barley before and after alkali treatment.

matters were mixed with $ABTS^{\bullet+}$ or $DPPH^{\bullet}$ solution to allow contact of the reactive surface with free radicals. After the scheduled reaction time, solid particles were separated from the mixture by centrifugation and the absorbance measurement was performed on the optically clear supernatants. If required, cellulose powder, which was found to be inert toward ABTS and DPPH radicals, was used as the dilution solid. The assay gave reproducible results with an inter-assay coefficient of variation of 10% or less.

Cereal dietary fiber is a suitable material to test the developed procedure: it has ortho diphenolic compounds with high antioxidant capacity that may be free or bound to an insoluble polysaccharide component (*23*). Phenolic acids may form cross-links with cell wall macromolecules such as starch, cellulose, β-glucan, and pentosans (*28*), and they can be released by alkali, acid, or enzymatic treatment (*29–31*). Therefore, two dietary-fiber-rich ingredients obtained from durum wheat bran and barley (*25*) were used as the matrices to test our hypothesis.

Figure 2 shows the TEAC values of the insoluble matters of durum wheat bran and barley before and after alkali treatment as determined by the procedure using ABTS radical cation solution. The insoluble matters of both ingredients showed high antioxidant capacities, and alkali treatment reduced their antioxidant activities by 76.8% and 92.7% for wheat bran and barley, respectively. The strong reduction of the antioxidant activity of these matrices after alkali treatment represented an experimental proof of the working hypothesis; in fact, it confirmed that phenolic compounds are able to quench the ABTS radical when they are bound to the insoluble components. Similar results were obtained also when the same samples were measured using DPPH (data not shown), thus confirming that this surface reaction phenomenon, namely, the scavenging of soluble radicals by antioxidants present in the solid matter, also occurs with a different, widely used radical.

Table 1. Antioxidant Activities of Insoluble Matters of Fruits, Vegetables, and Coffee[a]

	TEAC (mmol Trolox/kg insoluble matter)	
sample	ABTS	DPPH
lettuce	0.84 ± 0.01^{a}	2.08 ± 0.32^{a}
tomato	0.36 ± 0.06^{a}	0.42 ± 0.00^{b}
orange	0.59 ± 0.14^{a}	2.83 ± 0.13^{a}
coffee silverskin	82.24 ± 4.09^{b}	73.00 ± 1.06^{c}
cocoa husk	2.95 ± 0.04^{a}	16.18 ± 0.27^{d}
lemon	0.61 ± 0.04^{a}	3.16 ± 0.02^{a}

[a] Values represent means ± SE. Different letters within the same factor indicate statistical differences (one-way Anova and Duncan test, $p < 0.05$).

Table 2. Antioxidant Activities of Insoluble Matters of Cereal and Cereal-Based Products Determined by ABTS and DPPH Methods[a]

	TEAC (mmol Trolox/kg insoluble matter)	
sample	ABTS	DPPH
breakfast cereals	1.47 ± 0.09^{a}	0.50 ± 0.07^{a}
biscuits	0.11 ± 0.01^{b}	0.52 ± 0.17^{a}
snacks	0.07 ± 0.00^{b}	0.37 ± 0.01^{a}
bread crust	0.37 ± 0.03^{c}	0.34 ± 0.05^{a}
oat flour	1.10 ± 0.08^{d}	0.90 ± 0.01^{b}

[a] Values represent means ± SE. Different letters within the same factor indicate statistical differences (one-way Anova and Duncan test, $p < 0.05$).

The relevance of this finding was expanded by investigating different types of food and food ingredients. Various fruits and vegetables were selected, and the antioxidant activity of their insoluble matters was measured using the developed procedure with both ABTS and DPPH radicals. **Table 1** shows that the antioxidant activity recovered in the insoluble fraction of lettuce, tomato, orange, and lemon was quite low and that there was a significant difference between the TEAC values determined by the ABTS and DPPH methods for lettuce, orange, lemon, and cocoa samples. The byproducts of coffee roasting, namely, the coffee silverskin, showed a very high antioxidant activity, which is likely due to the presence of phenolic compounds as well as MRPs formed during processing (*32*).

Table 2 gives the value of antioxidant activities measured for insoluble fractions of some cereal products. The sampling included products obtained with different ingredients and different processing conditions that could promote the formation of MRPs with antioxidant activity. These samples have relatively low TEAC values as determined by both procedures using ABTS or DPPH solutions. It should be noted that MRPs formed in bread crust, biscuits, and snacks are mainly soluble and thus the overall contribution of their insoluble matters to the total antioxidant activity was limited. The use of refined wheat may be one of the reasons for low antioxidant activity in wheat-based cereal products. In this respect, it should be noted that breakfast cereal containing whole meal had higher antioxidant activity with respect to the other products listed in the table.

The high molecular weight polymers, melanoidins or melanoproteins, formed in processed foods through Maillard reaction possess a relevant antioxidant activity (*33–35*). These polymers are mainly insoluble, and because they largely not digestible by humans, it was proposed that in some cases they behave as dietary fiber (*36*, *37*). Several parameters determine the rate of Maillard reaction in each system, leading to the formation of different chemical species that have different antioxidant properties. In this context, it is worth noting that

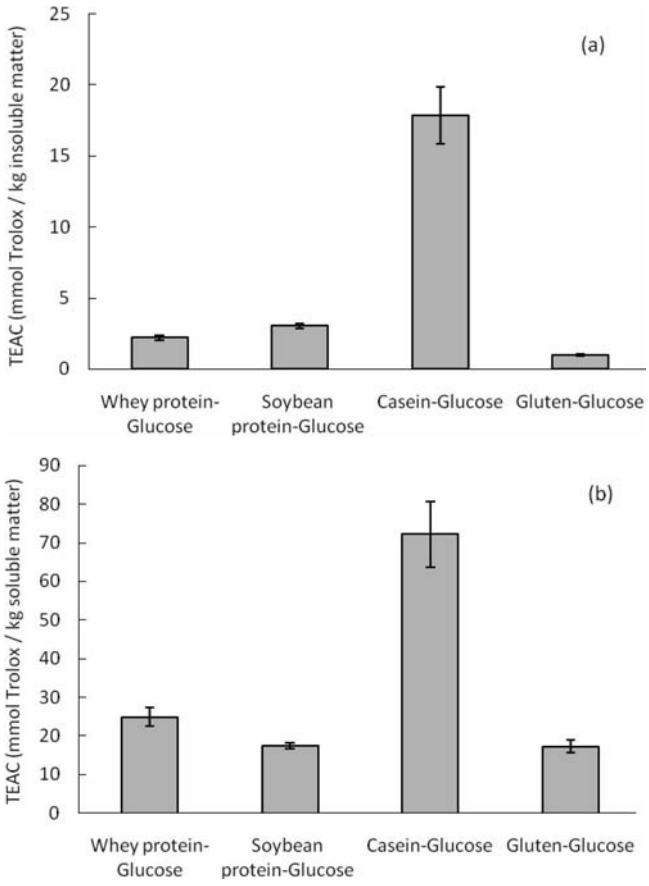

Figure 3. Antioxidant activities of melanoprotein insoluble fraction (a) and soluble fraction (b) determined by the procedure using ABTS radical.

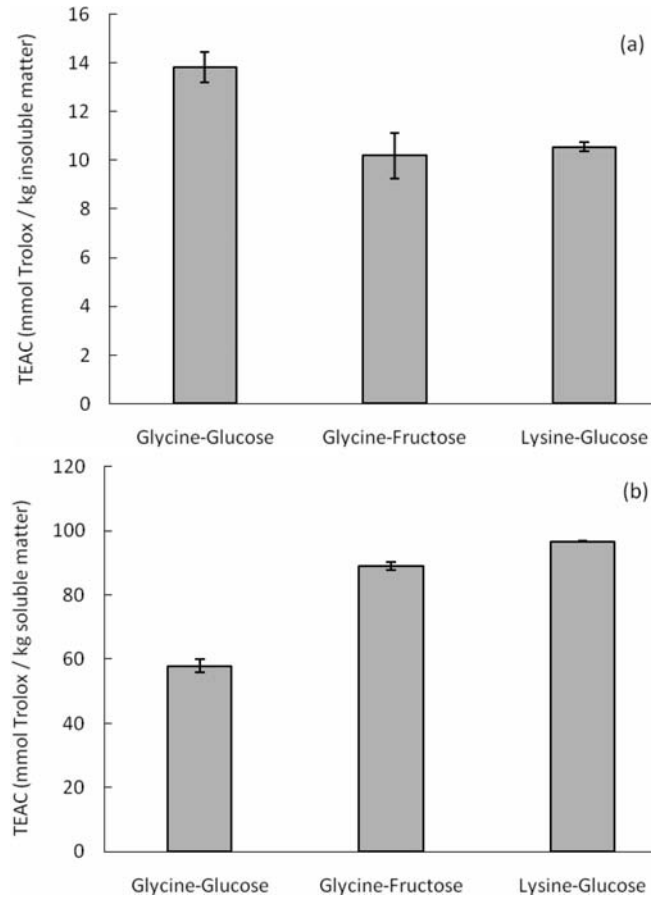

Figure 4. Antioxidant activities of melanoidin insoluble fraction (a) and soluble fraction (b) determined by the procedure using ABTS radical.

Table 3. Antioxidant Activities of Soluble and Insoluble Fractions of Melanoproteins Obtained at 130 °C for 1 h Determined by the Procedure Using DPPH Radical[a]

	TEAC (mmol Trolox/kg)	
melanoproteins	insoluble matter	soluble matter
whey protein/glucose	20.74 ± 1.82[a]	21.36 ± 0.202[a]
soybean protein/glucose	5.40 ± 0.23[b]	7.64 ± 0.38[b]
casein/glucose	79.80 ± 4.24[c]	39.71 ± 0.09[c]
gluten/glucose	1.23 ± 0.06[d]	14.46 ± 0.22[d]

[a] Values represent means ± SE. Different letters within the same factor indicate statistical differences (one-way Anova and Duncan test, $p < 0.05$).

Table 4. Antioxidant Activities of Soluble and Insoluble Fractions of Melanoidins Determined by the Procedure Using ABTS Radical[a]

	TEAC (mmol Trolox/kg)	
glucose/glysine	insoluble matter	soluble matter
130 °C × 5 min	8.60 ± 0.32[b]	67.00 ± 1.04[b]
130 °C × 30 min	9.72 ± 1.60[b]	64.32 ± 4.48[b]
130 °C × 60 min	13.84 ± 0.64[a]	57.88 ± 2.12[a]
110 °C × 30 min	10.88 ± 0.76[b]	78.96 ± 0.96[c]
110 °C × 60 min	10.04 ± 0.64[b]	73.72 ± 1.04[c]

[a] Values represent means ± SE. Different letters within the same factor indicate statistical differences (one-way Anova and Duncan test, $p < 0.05$).

Maillard reaction leads to both soluble and insoluble melanoidins, both having antioxidant activity.

MRPs were prepared in a controlled manner using binary mixtures of protein/sugar (melanoproteins) and amino acid/sugar (melanoidins). **Figure 3** shows the antioxidant activities of melanoproteins determined by the procedure using ABTS radical and expressed as TEAC. Melanoproteins prepared by heating the casein/glucose model system at 130 °C for 1 h had the highest antioxidant activity. In general, the soluble fractions of melanoproteins were found to have antioxidant activity higher than that of the insoluble fractions as determined by both the ABTS and DPPH methods. The ratio between insoluble and soluble MRPs depends on the heating treatment: more severe treatment leads to a higher amount of insoluble matter. It should be noted that the gluten/glucose model system yielded the melanoproteins having the lowest antioxidant activity, and approximately 95% of these melanoidins were water-soluble. This finding could explain why the thermally processed cereal products such as bread crusts and biscuits had a relatively low antioxidant activity in their insoluble fractions. As shown in **Table 3**, the procedure with DPPH radical was in general more sensitive to the insoluble fractions of melanoproteins.

Figure 4 shows the effects of some reducing sugars and amino acids on the antioxidant activities of insoluble and soluble fractions of the MRPs prepared by heating different binary mixtures in the same conditions, namely, 130 °C for 1 h. Individual contributions of the reactants to the antioxidant activity in insoluble fractions of Maillard reaction products were found as follows: glycine > lysine, and glucose > fructose (**Figure 4a**). The opposite was true for the soluble fractions (**Figure 4b**).

The effects of temperature and time were determined in a model system by heating the binary mixtures of glucose and glycine at 110 and 130 °C for up to 1 h. Results showed that increasing the time/temperature treatment also increased the antioxidant activity of insoluble matters in the MRPs (**Table**

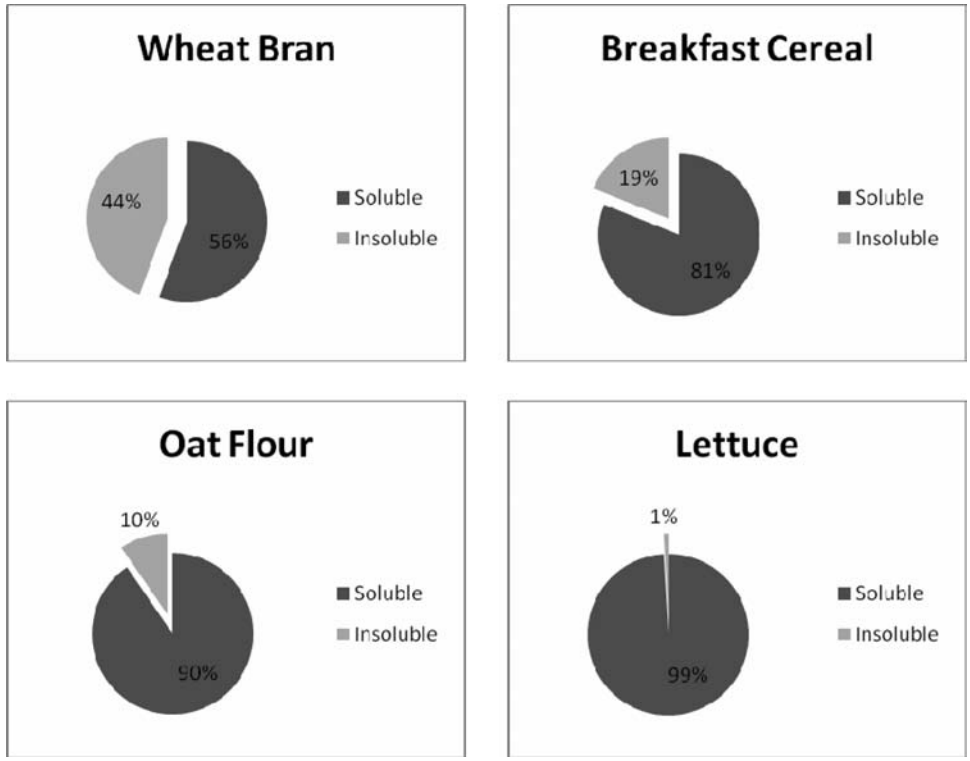

Figure 5. Percentage contributions of insoluble and soluble matters to the antioxidant capacities of some foods and food ingredients.

4). Time-dependent changes in the antioxidant activities of soluble and insoluble matters in the glucose/glycine model system were concordant each other. During the Maillard reaction, when the antioxidant activity of soluble matter decreased, the antioxidant activity of insoluble matter increased. This was most probably due to an increase in the molecular size of melanoidins, which contributed more to the amounts of insoluble matters, with an increase in thermal energy load.

The results of this paper indicated that an accurate measure of the overall antioxidant activity of foods should take into account also the contribution of the insoluble matter by modifying the existing analytical procedures. Recently the criticism has been raised that literature data may be underestimating the actual antioxidant capacities of some foods such as cereals (*38*, *39*). Because foods consist of both soluble and insoluble matters, they both should be taken into account. In fact, in many cases the insoluble matters may contain certain functional groups such as phenolic acids or reductones that may be able to quench free radicals. Previous studies have focused on the determination of antioxidant activity in extracts after alkali, acid, or enzymatic hydrolysis of the antioxidative functional groups bound to the insoluble fractions of foods (*23*,*25*, *39*, *40*). This approach could have little biological relevance, as not all the esterified compounds will be released and remain intact in vivo.

According to the hypothesis described in this paper, antioxidant functional groups bound to the insoluble components of foods are still reactive toward radicals as confirmed by using ABTS and DPPH. Interestingly, as already shown for the soluble antioxidants, TEAC values of the various samples are higher in some cases when measured with ABTS and in others with DPPH. It can be observed that ABTS is usually more sensitive to phenolic-containing compounds, whereas DPPH is more sensitive to MRPs.

Insoluble matters of foods, which were shown to have different levels of antioxidant capacity, remain in the gastrointestinal tract for a long time and may help in quenching the soluble radicals that are continuously formed in the intestinal tract and that could be involved in the etiology of colon cancer (*41*).

As shown in **Figure 5**, the nutritional relevance of the antioxidant activity of the insoluble matter depends on the food considered: it is negligible for lettuce, where the insoluble matters have weak antioxidant power and it represent only 1% of the food. The contribution of insoluble matter rose to about 10% for oat flour and almost to 20% for breakfast cereals where the insoluble components represented about 10% of the food. Finally, for dietary-fiber-rich ingredients the contribution of the insoluble matter became comparable to that of the soluble one.

LITERATURE CITED

(1) Wu, X.; Beecher, G. R.; Holden, J. M.; Haytowitz, D. B.; Gebhardt, S. E.; Prior, R. L. Lipophilic and hydrophilic antioxidant capacities of common foods in the United States. *J. Agric. Food Chem.* **2004**, *52*, 4026–4037.

(2) Pellegrini, N.; Serafini, M.; Colombi, B.; Del Rio, D.; Salvatore, S.; Bianchi, M.; Brighenti, F. Total antioxidant capacity of plant foods, beverages and oils consumed in Italy assessed by three different in vitro assays. *J. Nutr.* **2003**, *133*, 2812–2819.

(3) Pellegrini, N.; Serafini, M.; Salvatore, S.; Del Rio, D.; Bianchi, M.; Brighenti, F. Total antioxidant capacity of spices, dried fruits, nuts, pulses, cereals and sweets consumed in Italy assessed by three different in vitro assays. *Mol. Nutr. Food Res.* **2006**, *50*, 1030–1038.

(4) Serafini, M.; Bellocco, R.; Wolk, A.; Ekstrom, A. M. Total antioxidant potential of fruit and vegetables and risk of gastric cancer. *Gastroenterology* **2002**, *123*, 985–999.

(5) Brighenti, F.; Valtuena, S.; Pellegrini, N.; Ardigo, D.; DelRio, D.; Salvatore, S.; Piatti, P.; Serafini, M.; Zavaroni, I. Total antioxidant capacity of the diet is inversely and independently related to plasma concentration of high-sensitivity C-reactive protein in adult Italian subjects. *Br. J. Nutr.* **2005**, *93*, 619–625.

(6) Cao, G.; Verdon, C. P.; Wu, A. H. B.; Wang, H.; Prior, R. L. Automated assay of oxygen radical absorbance capacity with the Cobas Fara II. *Clin. Chem.* **1995**, *41*, 1738–1744.

(7) Ghiselli, A.; Serafini, M.; Maiani, G.; Azzini, E.; Ferro-Luzzi, A. A fluorescence-based method for measuring total plasma antioxidant capability. *Free Radical Biol. Med.* **1995**, *18*, 29–36.
(8) Trujillo, M.; Mateos, R.; Collantes de Teran, L.; Espartero, J. L.; Cert, R.; Jover, M.; Alcudia, F.; Bautista, J.; Cert, A.; Parrado, J. Lipophilic hydroxytyrosyl esters. Antioxidant activity in lipid matrices and biological systems. *J. Agric. Food Chem.* **2006**, *31*, 3779–3785.
(9) Benzie, I. F. F.; Strain, J. J. Ferric reducing antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. *Methods Enzymol.* **1999**, *299*, 15–27.
(10) Ou, B. X.; 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.
(11) Frankel, E. N. In search for the better methods to evaluate natural antioxidants and oxidative stability in food lipids. *Trends Food Sci. Technol.* **1993**, *4*, 220–225.
(12) Pryor, W. A.; Cornicelli, J. A.; Devall, L. J.; Tait, B.; Trivedi, B. K.; Witiak, D. T.; Wu, M. A rapid screening test to determine the antioxidant potencies of natural and synthetic antioxidants. *J. Org. Chem.* **1993**, *58*, 3521–3522.
(13) Mitsuda, H.; Yasumoto, K.; Iwami, K. Antioxidative action of indole compounds during the autoxidation of linoleic acid. *Agric. Biol. Chem.* **1981**, *45*, 735–739.
(14) Brand-Williams, W.; Cuvelier, M. E.; Berset, C. Use of a free radical method to evaluate antioxidant activity. *Lebensm.–Wiss. Technol.* **1995**, *28*, 25–30.
(15) 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.
(16) Pellegrini, N.; Yang, M.; Rice-Evans, C. Screening of dietary carotenoids and carotenoids-rich frits extracts for the antioxidant activities applying ABTS radical cation decolorization assay. *Methods Enzymol.* **1999**, *299*, 379–389.
(17) Fogliano, V.; Verde, V.; Randazzo, G.; Ritieni, A. Method for measuring antioxidant activity and its application to monitoring the antioxidant capacity of wines. *J. Agric. Food Chem.* **1999**, *47*, 1035–1040.
(18) Emmons, C.; Peterson, D.; Paul, G. Antioxidant capacity of oat (Avena sativa L.) extracts. In vitro antioxidant activity and contents of phenolic and tocol antioxidants. *J. Agric. Food Chem.* **1999**, *47*, 4894–4898.
(19) Zielinski, H.; Kozłowska, H. Antioxidant activity and total phenolics in selected cereal grains and their different morphological fractions. *J. Agric. Food Chem.* **2000**, *48*, 2008–2016.
(20) Martinez-Tome, M.; Murcia, M.; Frega, N.; Ruggieri, S.; Jimenez, A. M.; Roses, F. Evaluation of antioxidant capacity of cereal brans. *J. Agric. Food Chem.* **2004**, *52*, 4690–4699.
(21) Zhou, K.; Yu, L. Antioxidant properties of bran extracts from Trego wheat grown at different locations. *J. Agric. Food Chem.* **2004**, *52*, 1112–1117.
(22) Kroon, P. A.; Faulds, C. B.; Ryden, P.; Robertson, J. A.; Williamson, G. Release of covalently bound ferulic acid from fiber in the human colon. *J. Agric. Food Chem.* **1997**, *45*, 661–667.
(23) Adom, K.; Sorrells, M.; Liu, R. Phytochemical profiles and antioxidant activity of wheat varieties. *J. Agric. Food Chem.* **2003**, *51*, 7825–7834.
(24) Bonoli, M.; Verardo, V.; Marconi, E.; Caboni, M. Antioxidant phenols in barley (Hordeum vulgare L.) flour: comparative spectrophotometric study among extraction methods of free and bound phenolic compounds. *J. Agric. Food Chem.* **2004**, *52*, 5195–5200.
(25) Napolitano, A.; Lanzuise, S.; Ruocco, M.; Arlotti, G.; Ranieri, R.; Knutsen, S. H.; Lorito, M.; Fogliano, V. Treatment of cereal products with a tailored preparation of trichoderma enzymes increases the amount of soluble dietary fiber. *J. Agric. Food. Chem.* **2006**, *54*, 7863–7869.
(26) Surface catalyst. In *University Chemistry*; Mahan, B. H., Ed.; Addison-Wesley Publishing Company: London, 1975.
(27) Kim, J. H; Roy, S.; Kellis, J. T., Jr.; Poulose, A. J.; Gast, A. P.; Robertson, C. Protease adsorption and reaction on an immobilized substrate surface. *Langmuir* **2002**, *18*, 6312–6318.
(28) Yu, J.; Vasanthan, T.; Tamelli, F. Analysis of phenolic acids in barley by high-performance liquid chromatography. *J. Agric. Food Chem.* **2001**, *49*, 4352–4360.
(29) Sosulski, F.; Krzysztof, K.; Lawrence, H. 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.
(30) Krygier, K.; Sosulski, F.; Hodge, L. Free, esterified, and insoluble bound phenolic acids. 1. Extraction and purification procedure. *J. Agric. Food Chem.* **1982**, *30*, 330–334.
(31) Andreasen, M. F.; Landbo, A. K.; Christensen, L. P.; Hansen, A.; Meyer, A. S. Antioxidant effects of phenolic rye (Secale cereale L.) extracts monomeric hydroxycinnamates, and ferulic acid dehydrodimers on human low-density lipoprotein. *J. Agric. Food Chem.* **2001**, *49*, 4090–4096.
(32) Borrelli, R. C.; Visconti, A.; Mennella, C.; Anese, M.; Fogliano, V. Chemical characterization and antioxidant properties of coffee melanoidins. *J. Agric. Food Chem.* **2002**, *50*, 6527–6533.
(33) Anese, M. Role of the Maillard reaction products in affecting the overall antioxidant properties of food processed foods. In *Melanoidins in Food and Health*; Ames, J. M., Ed.; Office for Official Publications of European Communities: Luxembourg, 2000; *Vol. 1*, pp 61–63.
(34) Manzocco, L.; Calligaris, S.; Mastrocola, D.; Nicoli, M. C.; Lereci, C. R. Review of non-enzymatic browning and antioxidant capacity in processed foods. *Trends Food Sci. Technol.* **2000**, *11*, 340–346.
(35) Adams, A.; Borrelli, R. C.; Fogliano, V.; De Kimpe, N. Thermal degradation studies of food melanoidins. *J. Agric. Food Chem.* **2005**, *53*, 4136–4142.
(36) Ames, J. M.; Wynne, A.; Hofmann, A.; Plos, S.; Gibson, G. R. The effect of a model melanoidin mixture on faecal bacterial populations in vitro. *Br. J. Nutr.* **1999**, *82*, 489–495.
(37) Borrelli, R. C.; Fogliano, V. Bread crust melanoidins as potential prebiotic ingredients. *Mol. Nutr. Food Res.* **2005**, *49*, 673–678.
(38) Pérez-Jiménez, J.; Saura-Calixto, F. Literature data may underestimate the actual antioxidant capacity of cereals. *J. Agric. Food Chem.* **2005**, *53*, 5036–5040.
(39) Liyana-Pathirana, C.M.; Shahidi, F. Importance of insoluble-bound phenolics to antioxidant properties of wheat. *J. Agric. Food Chem.* **2006**, *54*, 1256–64.
(40) Esposito, F.; Arlotti, G.; Napolitano, A.; Vitale, D.; Fogliano, V. Durum wheat bran by-products: a novel functional ingredients. *Food Res. Int.* **2005**, *38*, 1167–1173.
(41) Babbs, C.F. Free radicals and the etiology of colon cancer. *Free Radical Biol. Med.* **1990**, (8), 191–200.

Received for review May 2, 2007. Revised manuscript received July 10, 2007. Accepted July 13, 2007. This study has been carried out with the support of the Commission of the European Communities, COST Action 927 "Thermally processed foods: possible health implications".

JF071291Z