JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Literature Data May Underestimate the Actual Antioxidant Capacity of Cereals

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Several recent articles have reported a significant antioxidant capacity of cereal products, determined in methanolic and ethanolic extracts. The aim of this work was to conduct an assessment of the antioxidant capacity of cereals using both chemical and in vitro digestive enzymatic extraction of antioxidants. Ferric reducing power (FRAP) and free radical scavenging capacity (DPPH) methods were used to determine the antioxidant capacity in wheat flour, bread, raw and boiled rice, wheat bran, and oat bran. The most efficient antioxidant extraction was achieved by using successively acidic methanol/water (50:50 v/v, pH 2) and acetone/water (70:30 v/v). The antioxidant capacity in these extracts ranged from 1.1 to 4.4 μmol Trolox/g dw. A significant amount of hydrolyzable phenolics with a high antioxidant capacity (from 5 to 108 μmol Trolox/g dw) was found in the residues of this aqueous−organic extraction. The antioxidant capacities of these nonextractable polyphenols are usually ignored in the literature, although they may have an antioxidant role in the gastrointestinal tract, especially after colonic fermentation, and may be fermentated to active metabolites. On the other hand, in vitro digestive enzymatic extracts obtained by enzymatic treatments that mimic conditions in the gastrointestinal tract showed that the amount of antioxidants released by the cereal matrix into the human intestine may be higher than the one that can be expected from measurements in the usual aqueous−organic extracts.

KEYWORDS: Antioxidant capacity; cereals; in vitro physiological antioxidants

INTRODUCTION

There is growing scientific evidence associating diets rich in antioxidant compounds—which occur particularly in plant foods—with a lower risk of developing cardiovascular disease, certain kinds of cancer, and age-related degenerative processes. The main dietary antioxidants are vitamins C and E, carotenoids, terpenes, and polyphenols, including flavonoids (*1*, *2*).

Several recent articles have reported the antioxidant capacity of different cereal products, such as oats (*3*−*8*), wheat (*9*−*20*), rice (*21*−*25*), and ready-to-eat breakfast cereals (*11*, *26*). These articles conclude that cereals possess significant free radical scavenging capacities and may serve as a potential source of natural antioxidants. Ferulic and caffeic acid, phytic acid, avenanthramides (substituted hydroxycinnamic acid conjugates present in oats), γ-oryzanol (a mixture of 10 ferulate esters of triterpene alcohol present in rice bran), and Maillard compounds are reported to be responsible for this antioxidant capacity (*3*, *7*, *15*, *21*, *26*).

However, most reports on cereal antioxidant capacity may present two limitations. First, the procedure used to extract antioxidants may be incomplete. In most of these experiments, the solvent most commonly employed is absolute ethanol (*11*, *12*) or ethanol:water in different proportions (*6*, *10*, *13*, *20*, *24*), although the extraction of phenolic compounds could be improved by using more polar solvents such as methanol (*9*). Some authors use methanol:water as an extraction solvent (*5*), but it is not often acidified, which has shown to improve the extraction (*23*, *27*). Second, the reported antioxidant capacities of cereals are measured in alcoholic extracts, and the results may differ quantitatively and qualitatively from physiological extracts from the human gastrointestinal tract.

The aim of this work was to conduct an assessment of the antioxidant capacities of cereals using both chemical extraction and in vitro digestive enzymatic extraction of antioxidants. The samples selected for this work were raw rice, boiled rice, wheat flour, French bread, wheat bran, and oat bran. Wheat flour and rice are the chief sources of cereal foods in the diet, while wheat and oat bran are increasingly used in ready-to-eat breakfast cereals and as ingredients in dietary fiber-enriched foods. Boiled rice and French bread (whose main ingredient is wheat flour, plus water, salt, and some additives) are two of the most common ways of consuming cereals.

Two complementary methods were used to determine the antioxidant capacities in these samples: ferric reducing/antioxidant power (FRAP), which measures the sample's ferric reducing power, and 2,2-diphenyl-1-picryhydrazyl (DPPH), which measures free radical scavenging capacity.

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10.1021/jf050049u CCC: $30.25
Published on Web 05/03/2005

MATERIALS AND METHODS

Chemicals. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water soluble analogue of vitamin E and DPPH• were from Sigma-Aldrich Química, S. A. (Madrid, Spain). 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ) was from Fluka Chemicals (Madrid, Spain). L-Tryptophan was from Fisher Scientific Co. (United States). L-Tyrosine was from Merck (Darmstadt, Germany). All reagents used were of analytical grade.

The enzymes used for the digestive enzymatic treatment were as follows: pepsin (Merck), pancreatin, α-amylase, and amyloglucosidase (Sigma-Aldrich Química S. A.).

Materials. Wheat flour (Gallo, S. A., Barcelona, Spain), rice (SOS S. A., Villarejo de Salvanés, Madrid, Spain), wheat bran (Santiveri, S. A., Barcelona, Spain), oat bran (Santiveri S. A.), and French bread were purchased in local supermarkets. Rice and wheat bran were milled to a particle size of less than 0.5 mm in a mill Retsch ZM 200. The same milling was applied to boiled rice (85 g of sample in 200 mL of water) and French bread after they were freeze-dried.

Method. Extractions were performed in three different samples for each cereal product. Determinations were performed by triplicate in each extract and are reported on a dry matter basis. The moisture content was determined by drying at 105 °C in an oven to constant weight. Results are expressed as mean values ± standard deviation.

For chemical extraction, 2 g of rice, wheat flour, oat bran, and wheat bran and 3 g of boiled rice and French bread were placed in a test tube and 20 mL of acidic methanol/water (50:50 v/v, pH 2) was added (10 mL for boiled rice and French bread). The tube was thoroughly shaken at room temperature for 1 h. The tube was centrifuged at 2500*g* for 10 min, and the supernatant was recovered. Twenty milliliters of acetone/water (70:30, v/v) was added to the residue (10 mL for the boiled rice and French bread), and shaking and centrifugation were repeated. Both methanolic and acetonic extracts were combined.

The digestive enaymatic extraction was carried out by using the in vitro procedure previously described by Saura-Calixto et al. (*28*), which allows one to isolate indigestible and digestible food constituents. Samples were successively incubated with digestive enzymes to simulate digestion in the small intestine. Briefly, 900 mg of sample was incubated with pepsin (0.2 mL of a 300 mg/mL solution in a buffer of 0.2 M HCl−KCl, pH 1.5, 40 °C, 1 h, Merck 7190), pancreatin (1 mL of a 5 mg/mL solution in 0.1 M phosphate buffer, pH 7.5, 37 °C, 6 h, SigmaP-1750), and α-amylase (1 mL of a 120 mg/mL solution in 0.1 M tris-maleate buffer, pH 6.9, 37 °C, 16 h, Sigma A-3176). Then, samples were centrifugated (15 min, 3000*g*) and supernatants were removed. Residues were washed twice with 5 mL of distilled water, and all supernatants were combined. Each supernatant was incubated with 100 μL of amyloglucosidase (Roche, 102857) for 45 min at 60 °C. Both aqueous−organic and digestive enzymatic extracts were used as test samples to determine the antioxidant capacity and polyphenols content.

Antioxidant Capacity. For the FRAP assay (*32*, *33*), 900 μL of the FRAP reagent, containing TPTZ, $FeCl_3$, and acetate buffer, was mixed with 90 μL of distilled water and 30 μL of the test sample or the blank (solvents used for extraction). Maximum absorbance values at 595 nm were taken every 15 s and at 37 °C, using a Beckman DU-640 spectrophotometer (Beckman Instruments Inc., Fullerton, CA) equipped with a thermostatic autocell holder. The readings at 30 min were selected for calculation of FRAP values, since at 4 min, the time usually taken, the reaction to form the ferric−TPTZ complex has not finished (*33*).

The radical scavenging capacity of the samples was measured according to the DPPH• method (*34*), modified in our laboratory to measure kinetic parameters (*35*). To avoid interferences, a previous precipitation of polysaccharides and protein in digestive enzymatics extracts was performed by adding methanol. After the blank was adjusted with methanol, different volumes of the extracts (0.5−1.5 mL) were mixed with a DDPH• methanolic solution to a fixed final volume. The absorbance at 515 nm was measured until the reaction reached the plateau. A calibration curve at that wavelength was made to calculate the remaining DDPH•. The parameter EC_{50}, which reflects the depletion of free radical to 50%, was expressed in terms of g dry weight/g DDPH•. The time taken to reach the steady state at EC_{50} ($t_{EC_{50}}$) and the antiradical efficiency ($AE = 1/EC_{50}t_{EC_{50}}$) (*35*) were also calculated. The antioxidant capacity was also measured in methanolic solutions of Trolox in order to obtain a calibration curve to express the results.

To evaluate possible interferences in the digestive enzymatic extracts, the antioxidant capacity was measured in a blank with the different buffers, enzymes, and incubation conditions, producing no results. Under the enzymatic treatments, samples release glucose from starch and amino acids from protein; to find out whether some of these compounds could interfere with the analysis, the antioxidant capacity of glucose and aromatic amino acids (tyrosine, tryptophan) was also determined in the same blank. It was concluded that neither reagents nor sugars and amino acids interfered in the measurement of antioxidant capacity in the samples.

Polyphenols Content. Total phenolics were determined according to the Folin−Ciocalteau procedure (*29*). The test sample (0.5 mL) was mixed with 0.5 mL of Folin−Ciocalteau reagent and swirled. After 3 min, 10 mL of sodium carbonate solution (75 g/L) was added and mixed. Additional distilled water was mixed thoroughly by inverting the tubes several times. After 1 h, the absorbance at 750 nm was recorded. The results were expressed as gallic acid equivalents.

The hydrolyzable phenolics and condensed tannins were measured in the residues of both aqueous−organic and in vitro digestive ezymatic extracts, and calculations were referred to the original amount of sample (dry matter) taken for the extractions. Hydrolyzable phenolics were measured according to the method described by Hartzfeld (*30*). Briefly, 200 mg of the residue was mixed with 20 mL of methanol and 2 mL of concentrated sulfuric acid. Then, the samples were placed in a water bath with constant shaking at 85 °C for 20 h. The samples were then centrifugated (2500*g* for 10 min), and the supernatant was recovered. After two washings with distilled water, the final volume was taken up to 50 mL. The antioxidant capacity (by FRAP method) and total phenolics content (by Folin−Ciocalteau reagent) were measured in this supernatant. As regard to condensed tannins, the residues were treated with HCl/buthanol (5:95, v/v) at 100 °C for 3 h, and the absorbance was read at 538.5 nm (*31*).

RESULTS AND DISCUSSION

Most natural antioxidants are multifunctional, and in complex heterogeneous foods, their activity cannot be evaluated by a single method (*36*, *37*). In the present work, two different methods were used to measure the antioxidant activity, the FRAP, and the free radical scavenging capacity (DPPH•).

Chemical Extraction. A prior test was run to compare the most common solvents applied in the literature (ethanol/water 70:30 v/v, acetone/water 50:50 v/v, and methanol/water 50:50 v/v) to extract cereal antioxidants and those routinely employed in our laboratory to extract polyphenols from vegetable foods, which are acidic methanol/water (50:50 v/v, pH 2), followed by acetone/water (70:30, v/v). The test was applied to two of the samples: wheat flour and wheat bran. The highest yield of phenolics was achieved by using successively acidic methanol/water (50:50 v/v, pH 2) and acetone/water (70:30, v/v). For example, in the case of the wheat flour, these solvents yielded 12% more of phenolics than ethanol:water 70:30 v/v and 33% more than methanol:water 70:30 v/v.

We classify food polyphenols into two groups: extractable and nonextractable polyphenols (*38*, *39*). Extractable polyphenols are low and intermediate molecular mass phenolics that can be extracted using different organic and organic−aqueous solvents. Nonextractable polyphenols are high molecular mass compounds (condensed tannins and hydrolyzable phenolics) or polyphenols bound to dietary fiber and protein and can be found in the residues of the aqueous−organic extracts.

The antioxidant activities and total phenolics contents of the aqueous−organic extracts are shown in **Table 1**. Because

Table 1. Polyphenols Content and Antioxidant Capacity of Cereals in Aqueous–Organic Extracts[a] and Its Residues[b]

	extracts					residues	
		antioxidant capacity					
			DPPH				
	total phenolics[c] (gallic acid equivalents)	FRAP (μmol Trolox/ g dw)	EC_{50} (g/g)	$t_{EC_{50}}$ (min)	AE	hydrolyzable phenolics[c] (gallic acid equivalents)	antioxidant capacity FRAP (μmol Trolox/g dw)
raw rice	1340 ± 20	1.57 ± 0.14	604.85 ± 44.07	21.36 ± 1.78	0.0001	3870 ± 20	17.64 ± 2.73
boiled rice	210 ± 20	1.07 ± 0.07	499.95 ± 63.67	29.91 ± 2.82	0.0001	3490 ± 200	5.09 ± 0.77
wheat flour	3440 ± 330	1.63 ± 0.13	805.77 ± 31.67	8.69 ± 1.47	0.0001	2510 ± 470	8.92 ± 0.47
French bread	760 ± 20	2.86 ± 0.23	129.71 ± 2.33	42.29 ± 2.07	0.0002	4400 ± 190	6.81 ± 0.45
oat bran	1950 ± 80	3.68 ± 0.14	266.70 ± 18.71	21.81 ± 0.56	0.0002	9710 ± 890	30.59 ± 1.71
wheat bran	2800 ± 40	4.41 ± 0.10	347.74 ± 17.35	24.59 ± 2.53	0.0001	16430 ± 1500	108.30 ± 0.46

[a] Acidic methanol/water (50:50 v/v, pH 2) plus acetone/water (70:30 v/v). [b] Treated with methanol and concentrated sulfuric acid. [c] mg/kg.

Table 2. Polyphenols Content and Antioxidant Capacity of Cereals in Digestive Enzymatic Extracts[a] and Its Residues[b]

	extracts					residues	
		antioxidant capacity					
			DPPH				
	total phenolics content[c] (gallic acid equivalents)	FRAP (μmol Trolox/ g dw)	EC_{50} (g/g)	$t_{EC_{50}}$ (min)	AE	hydrolyzable phenolics[c] (gallic acid equivalents)	antioxidant capacity FRAP (μmol trolox/g dw)
raw rice	6470 ± 70	3.77 ± 0.45	182.87 ± 5.49	3.36 ± 2.26	0.0016	1240 ± 60	1.64 ± 0.05
boiled rice	3230 ± 60	4.30 ± 0.35	108.76 ± 8.04	3.39 ± 0.72	0.0027	1640 ± 40	2.07 ± 0.26
wheat flour	7030 ± 200	5.62 ± 0.69	168.28 ± 3.57	1.14 ± 0.05	0.0052	1020 ± 50	2.71 ± 0.18
French bread	3540 ± 110	6.59 ± 0.88	91.31 ± 1.06	7.71 ± 0.97	0.0014	1550 ± 160	4.78 ± 0.83
oat bran	8450 ± 260	11.81 ± 1.90	153.18 ± 2.02	5.59 ± 1.51	0.0012	4340 ± 190	13.22 ± 0.98
wheat bran	8630 ± 250	22.73 ± 1.34	97.63 ± 0.29	11.57 ± 0.49	0.0009	9660 ± 220	47.66 ± 0.47

[a] Treatments with pepsin, pancreatin, amylase, and amyloglucosidase. [b] Treated with methanol and concentrated sulfuric acid. [c] mg/kg.

different extraction solvents are used to determine the antioxidant capacity of cereals, it is not possible to make a direct comparison with literature data (*7*). For example, Yu (*11*, *12*) reported phenolics contents from 0.036 to 0.092% for wheat bran and 0.026% for oat bran, using ethanol as the extraction solvent. Our results were 0.2797% for wheat bran and 0.1946% for oat bran. A higher yield of phenolic compound in wheat bran was reported by using aqueous methanol (0.11%) or acetone (0.26%) (*16*).

It can be seen that brans (both wheat and oat) have more extractable polyphenols and a higher antioxidant capacity than rice and wheat flour as measured by both FRAP and DPPH, excluding the case of wheat flour, which has a value of the extractable polyphenols abnormally high in relation to its antioxidant activity. The presence of some amino acids may interfere with the Folin−Ciocalteau reaction (*15*), affecting the phenolic content values. Other nonphenolic compounds such as phytic acid can also affect the determination of antioxidant capacity (*26*, *40*).

As regards to kinetics, the DPPH results indicate that the antioxidants in wheat flour have the highest kinetics (lowest $t_{EC_{50}}$) and French bread the lowest one. The rest of the samples presented intermediate values of $t_{EC_{50}}$. This $t_{EC_{50}}$, along with EC_{50} values, determine the AE, which is highest in French bread and oat bran and similar in the rest of the samples.

As regards bread, extractable polyphenols decreased by 78.04% with respect to the raw flour. This appears to be a consequence of the thermal effect (250 °C) during baking. However, despite this reduction in the phenolics, the antioxidant capacity was higher than in wheat flour. The formation of Maillard compounds during the baking process (*41*) may account for this fact. The antioxidant activities of some of these compounds have been described (*42*), especially when the amino acid that reacts with the sugar is histidine or cysteine. The antioxidant capacity of Maillard compounds in bread was recently reported (*43*). Our results suggest that the antioxidant effect of Maillard compounds acts more slowly than that of phenolic compounds, resulting in a higher $t_{EC_{50}}$. Anyway, it should be considered the fact that the bread was from the supermarket and it is not known what type and amount of flour was used for its making, so this may also partially explain the differences, along with the possible presence of artificial antioxidants.

There are only minor differences in antioxidant capacities between raw and boiled rice, despite the significant reduction of phenolics in boiled rice (84.16%). This suggests a release of nonphenolic antioxidants from the food matrix during boiling.

However, extractable antioxidants in chemical extracts are only a part of the picture. We also looked for antioxidant phenolics (hydrolyzable phenolics and condensed tannins) in the residue of the aqueous−organic extraction.

Condensed tannins were measured in all of the cereal samples, yielding no result. This was consistent with a previous work, which concluded that oats and rice have no condensed tannins (*44*).

Cereals are rich in cinnamic acids such as ferulic acid, esterified to arabinose residues in primary cell wall and arabinoxylan and arabinogalactan in the aleurone layer and pericarp (*45*). These acids are the constituents of hydrolyzable phenolics. The concentrations of hydrolyzable phenolics in the samples are shown in **Table 1**. Cereals contain significant amounts of hydrolyzable phenolics (from 0.251 to 1.643%). Moreover, brans are richer in these compounds than in extractable polyphenols. We would note here that nonextractable polyphenols are usually ignored in the evaluation of antioxidant capacity of cereals and other foods.

The antioxidant activity of hydrolyzable phenolics was also determined in the corresponding hydrolysates by the FRAP

method (**Table 1**). DPPH was not an option because a precipitate was formed in the acidic methanolic solvent. As the table shows, the hydrolyzable phenolics presented higher ferric reducing powers than extractable polyphenols. It had been previously reported (*33*) that phenolic acids such as gallic acid exhibit higher antioxidant capacities as measured by the FRAP method than flavonoids.

If we exclude the values recorded for the wheat flour, there is a significant correlation ($R^2 = 0.7919$) between phenolics compounds and antioxidant capacity (FRAP assay), which indicate that polyphenols appear to be the main sources of antioxidant capacity. As regards to the nonextractable polyphenols, a high correlation ($R^2 = 0.8939$), with or without wheat flour, was also found between the hydrolyzable phenolics content and antioxidant capacity (FRAP assay).

It can be concluded from this approach that the antioxidant capacity associated with nonextractable polyphenols (indigestible in the small intestine but bioactive in the large intestine) of cereals largely exceeds the antioxidant capacity measured in the usual aqueous alcohol−acetone extracts.

In Vitro Digestive Enzymatic Extraction. The samples were processed by in vitro digestive enzymatic extraction consisting of enzymatic treatments that mimic conditions in the gastrointestinal tract (pH, temperature, incubation times, and solvent). The antioxidant capacity values determined in these in vitro digestive enzymatic extracts may be more useful for nutritional purposes than the values determined in aqueous−organic extracts.

Table 2 shows the results of antioxidant capacity and polyphenols content in the digestive enzymatic extracts. Note that the amounts of polyphenols, and hence the antioxidant capacity of the digestive enzymatic extracts of all of the samples, are significantly higher than in aqueous−organic extracts (**Tables 1** and **2**). This suggests that the amount of antioxidants released by the cereal products matrix into the human intestine, and hence also the antioxidant capacity of these samples, may be higher than it might be expected from the data based on chemical extracts. This is a fact that could be taken into account when evaluating the antioxidant capacity of cereals from a nutritional standpoint. These antioxidants are potentially available in the small gut; the degree to which they produce an antioxidant effect depends on the rate of absorption. Also, the antioxidants that are not released in these digestive enzymatic extracts may enter the colon, where they can be fermented by the microflora, yielding different compounds that may be metabolized and may provide an antioxidant environment.

The sums of the extractable polyphenols (total phenolics content) and nonextractable polyphenols (hydrolyzable phenolics) in the aqueous−organic extracts and their residues (**Table 1**) give values of the same order as the sum of polyphenols in the digestive enzymatic extracts and their residues (**Table 2**). It suggests that the high antioxidant capacity values in the digestive enzymatic extraction may be due to partial hydrolysis of the hydrolyzable phenolics by the enzymatic treatments. The enzymatic treatments hydrolyze starch and protein, which may favor the release of polyphenols. Therefore, the release of antioxidant compounds in the gastrointestinal tract is not only quantitatively but also qualitatively different to the one in the chemical extraction.

It must be pointed out that the antioxidant capacity of wheat bran and oat bran was higher than the one of the rice, wheat flour, and bread. A significant association of whole grain intake with a reduced risk of coronary heart disease was found in a recent prospective cohort study of 42850 males (*46*). The relative contribution of bran antioxidants, dietary fiber, and other constituents to this beneficial effect remain to be elucidated.

In summary, the antioxidant capacity of cereals may be underestimated in the literature because the extraction solvents usually used do not allow a complete release of antioxidant compounds and, additionally, nonextractable polyphenols with a high antioxidant capacity are ignored. On the other hand, the analysis of in vitro digestive enzymatic extracts suggests that the antioxidant activity of cereals in the human gut may be higher than what might be expected from literature data based on measurements of aqueous−organic extracts.

ABBREVIATIONS USED

AE, antiradical efficiency; DPPH, 2,2-diphenyl-1-picryhydrazyl; FRAP, ferric reducing/antioxidant power; TPTZ, 2,4,6-tri(2-pyridyl)-S-tryazine.

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Received for review January 10, 2005. Revised manuscript received April 6, 2005. Accepted April 8, 2005. The present research was performed under the financial support of the Spanish Ministery of Education and Science (Project AGL 2002-04104). J.P.-J. thanks the Consejo Superior de Investigaciones Científicas for granting her an I3P scholarship, financed by the European Social Fund.

JF050049U