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Assessing the Antioxidant Activity of Melanoidins from Coffee Brews by Different Antioxidant Methods

CRISTINA DELGADO-ANDRADE, JOSÉ A. RUFIÁN-HENARES, AND FRANCISCO J. MORALES*

Consejo Superior de Investigaciones Científicas, Instituto del Frío, 28040 Madrid, Spain

Antioxidant activity of instant coffees produced from the same green coffee beans roasted at three different degrees was analyzed. Coffee melanoidins were obtained by ultrafiltration (10 kDa cutoff) and subsequent diafiltration. Pure melanoidins were isolated from melanoidins after overnight incubation in 2 M NaCl and then ultrafiltered. Filtrates, corresponding to the low molecular weight (LMW) fraction noncovalently linked to the melanoidin skeleton, were also preserved. Antioxidant activity of coffee brews (CB), melanoidins (M), pure melanoidins (PM), and bounded melanoidin compounds (BMC) were tested using the 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and ferric reducing power (FRAP) methods. The correlation between the different methods was studied. The higher contribution of melanoidins to the total antioxidant activity of coffees was shown to be caused by the LMW compounds linked noncovalently to the melanoidin skeleton, as data from BMC confirmed. CB, M, and BMC fractions exert the highest antioxidant activity in aqueous media, whereas PM was not dependent on the reaction media. The highest correlation was found between DPPH and FRAP methods.

KEYWORDS: Coffee brew; melanoidin; DPPH; ABTS; FRAP

INTRODUCTION

Coffee brews, one of the most consumed beverages throughout the world, are prepared from roasted coffee beans. The roasting process adds value to raw beans, providing color, texture, and flavor highly appreciated by consumers (*1*).

Some physiological effects have been tested in this beverage; the most extended is its antioxidant activity, generally attributed to Maillard reaction products (MRPs) formed during roasting in addition to certain natural phenolic compounds (chlorogenic acid (CGA), caffeic acid, ferulic acid, or p-coumaric acid) present in the green beans (*1*, *2*). The presence of these acids is high in the green coffee, but after the thermal heating applied during roasting, their concentration decreases. On the contrary, MRPs, especially melanoidins, increase in this step and are the prevailing contributors to the maintenance of antioxidant activity of coffee brew (*3*). Some authors have confirmed that this effect is due to the ability to break the radical chain by donation of a hydrogen (*4*, *5*), their affectivity as metal chelating agents (*6*), their capacity to reduce hydroperoxide to nonradical products (*7*), or scavenging hydroxyl radical (*8*). According to these researchers the oxygen scavenging activity was higher for the intermediate MRP formed throughout the reaction and for those produced in alkaline conditions (*9*). Participation of phenolic compounds to the antioxidant properties of coffee is still unclear, although CGA contribution has been recently established (*5*).

Melanoidins have been studied in recent years due to their nutritional, biological, and health implications. They have become subject of growing interest for the scientific community as the creation of a specific COST (Co-Operation in Science and Technology) action entitled “Melanoidins in Food and Health” shows (*10*). Their contribution to the total antioxidant activity of a foodstuff is especially interesting since these products are naturally formed during processing and storage and may influence the oxidative stability and shelf life of several foods. Thus, the use of melanoidins as an oxidative stabilizer in foods is being studied in depth since nowadays they have acquired an important role in the protection and stabilization of natural antioxidants or those chemically formed during processing (*9*, *10*).

The aim of the present work is to evaluate the antioxidant properties of melanoidins in coffee brews from different roasting degrees by applying different antioxidant methods, which are compared. The specific antioxidant activity associated with bonded melanoidin compounds will be tested.

MATERIAL AND METHODS

Chemicals. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and potassium persulfate were purchased from Sigma (St. Louis, MO). 2,2′-Azobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) were from Fluka Chemicals (Madrid, Spain). $FeCl_3 \cdot H_2O$, chloroform, and NaCl were purchased from Panreac.

* Corresponding author. Phone: +34 91 549 23 00. Fax: +34 91 549 36 27. E-mail fjmorales@if.csic.es.

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Samples. Instant coffees produced from roasted coffee beans were supplied by the Nestlé Research Center (Lausanne, CH) in three different roasting degrees (roasting color, roasting loss). They were named with the initials CTn (Color Test number) followed by a number: light (CTn 110, 14.5%), medium (CTn 85, 16.2%), and dark (CTn 60, 18.9%).

Preparation of Coffee Brews. One gram of the different instant coffees was resuspended in 100 mL of hot water (50−60 °C) for 3 min while continuously stirring. The coffee brews (CB) obtained were then filtered (Whatman Filter Paper no 40, ashless, Whatman, U.K.) and stored at 4 °C until analyses were shortly performed (CB110, CB85, and CB60 samples, respectively).

Preparation of Melanoidins Extract and Pure Melanoidins Extract from Coffee Brews. An aliquot of each above-described sample was subjected to ultrafiltration using an Amicon ultrafiltration cell model 8400 (Amicon, Beverly, MA), equipped with a 10 000 Da nominal molecular mass cutoff membrane. The retentate was filled up to 200 mL with water and washed again. This washing procedure (diafiltration) was repeated at least three times. The high molecular weight fraction corresponding to melanoidins was freeze-dried and stored in a desiccator until analysis. Melanoidins (M) isolated from these systems were identified as M110, M85, and M60, respectively.

Obtention of pure melanoidins (PM) was performed preparing solutions containing 5 mg (to obtain a representative amount of product) of different melanoidins per milliliter in 2 M NaCl. NaCl was used to release low molecular weight (LMW) compounds ionically attached to the melanoidin skeleton, such as CGA. After overnight incubation, solutions were again ultrafiltered (Microcon YM-10, regenerated cellulose 10 000 Da, Bedford, MA) at 14 000 × *g* for 50 min. Retentates, containing PM, were resuspended in water and then freeze-dried and stored in a desiccator at 4 °C until analysis. PM obtained were named PM110, PM85, and PM60, respectively. Filtrates containing bonded melanoidin compounds (BMC) were also conserved at 4 °C until antioxidant analysis and named as BMC110, BMC85, and BMC60, respectively.

Antioxidant Assay. *(1) DPPH Assay.* The antiradical activity of different samples was estimated according to the procedure reported by Morales and Jiménez-Pérez (*11*), slightly modified. A 200 μL aliquot of sample (CB, M, PM, or BMC) was added to 1 mL of DPPH·74 mg·L^{-1} in methanol. A daily prepared solution of DPPH· gave a final absorption at 520 nm of 1.8 AU. The mixture was shaken for 1 h, and then absorption was measured at 520 nm. The temperature in the measurement chamber was set at 30 °C. The antiradical activity of the sample was expressed as percentage of disappearance of the initial purple color. The higher the disappearance, the greater the antiradical activity.

Aqueous solutions of trolox and CGA at various concentrations were used for calibration (0.15−1.15 mM). The results were expressed as μmol equivalents of trolox or CGA per gram of sample.

(2) $ABTS^{\cdot+}$ Assay. The antioxidant capacity was estimated in terms of radical scavenging activity following the procedure described by Jiménez-Escrig et al. (*12*). Briefly, $ABTS^{\cdot+}$ was produced by reacting 7 mM ABTS stock solution with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12−16 h before use. The $ABTS^{\cdot+}$ solution (stable for 2 days) was diluted with 5 mM phosphate-buffered saline (pH 7.4) to an absorbance of 0.70 ± 0.02 at 730 nm. After addition of 10 μL of sample (CB, M, PM, or BMC), trolox, or CGA standards to 4 mL of diluted $ABTS^{\cdot+}$ solution, an absorbance reading was taken at 20 min using a Shimadzu UV−visible 1601 spectrophotometer (Duisburg, Germany). Calibration was performed, as described previously, with trolox and CGA stock solution. Results were expressed as μmol equivalents of trolox and CGA per gram of sample.

Table 1. Antioxidant Activity Determined by the DPPH Method[a]

	$TEAC_{DPPH}$[b]				$CEAC_{DPPH}$[c]			
sample	CB	M	PM	BMC	CB	M	PM	BMC
CTn60	428 ± 1	374 ± 3	144 ± 3	196 ± 1	502 ± 2	430 ± 4	125 ± 3	585 ± 1
CTn85	419 ± 5	248 ± 3	157 ± 3	255 ±1	491 ± 7	262 ± 4	142 ± 4	781 ± 3
CTn110	325 ± 3	323 ± 8	160 ± 1	283 ± 1	366 ± 5	362 ± 11	145 ± 1	874 ± 5

[a] CB, coffee brew; M, melanoidin; PM, pure melanoidin; BMC, bonded melanoidin compound. Values are mean ± S. D. [b] Data expressed as μmol equiv of trolox/g of sample. [c] Data expressed as μmol equiv of CGA/g of sample.

(3) FRAP Assay. The ferric reducing ability of each standard solution was estimated according to the procedure described by Benzie and Strain (*13*) with some modifications. Briefly, 900 μL of FRAP reagent, prepared fresh and warmed at 37 °C, was mixed with 90 μL of distilled water and 30 μL of test sample (CB, M, PM, or BMC) or water as the appropriate reagent blank. The final dilution of the test sample in the reaction mixture was 1/34. The FRAP reagent contained 2.5 mL of a 10 mM TPTZ solution in 40 mM HCl plus 2.5 mL of 20 mM $FeCl_3 \cdot H_2O$ and 25 mL of 0.3 M acetate buffer, pH 3.6 (*14*). Readings at the absorption maximum (595 nm) were taken every 15 s using a Shimadzu UV−visible 1601 spectrophotometer equipped with a thermostated automatic sample positioner. The temperature was maintained at 37 °C, and the reaction was monitored for up to 30 min. Trolox and CGA stock solutions were used to perform the calibration curves. Results were also expressed as μmol equivalents of trolox and CGA per gram of sample.

Statistical Treatment. All of the analyses were performed at least in triplicate. The Statgraphics v5.1 statistical procedures were performed at a significance level of 95%.

RESULTS AND DISCUSION

Tables **1**−**3** show the results obtained after measuring the antioxidant activity of different samples by the three selected methodologies. Data are expressed as μmol equiv of trolox/g of sample, a commonly used unit for antioxidant assays, and as μmol equiv of CGA/g of sample due to the high presence of this phenolic acid in coffee and its important contribution to the overall antioxidant capacity. To make data comparisons, data were referenced to 1 g of sample for the different melanoidins, pure melanoidins, and coffee brews. In the case of bonded melanoidin compounds, results are expressed as BMC present in 1 g of melanoidin. In this sense, data included in tables do not directly represent the contribution of different components within coffee to the overall antioxidant activity.

Comparisons between the same group of samples were made to analyze differences caused by the roasting process. The antioxidant activity of CB 60, 85, and 110 coffee brews manifested the same pattern in the three methods used (**Tables 1**−**3**), with stability of antioxidant activity in CB60 and 85 but decreasing around a 35% in CB110 sample ($P < 0.05$). As the roasting degree decreased from CB60 to CB110, a higher extent of the nonenzymatic browning reaction is expected in more severely roasted coffee beans. Previous work by our research group evaluating the peroxyl radical scavenging properties of similar coffee brews and melanoidins, based on the rate of

Table 2. Antioxidant Activity Determined by the ABTS Method[a]

	$TEAC_{ABTS}$[b]				$CEAC_{ABTS}$[c]			
sample	CB	M	PM	BMC	CB	M	PM	BMC
CTn60	1195 ± 12	699 ± 4	163 ± 21	937 ± 8	1361 ± 12	868 ± 4	335 ± 21	1104 ± 8
CTn85	1206 ± 7	530 ± 8	140 ± 28	978 ± 11	1372 ± 7	699 ± 8	312 ± 28	1145 ± 11
CTn110	616 ± 11	690 ± 13	137 ± 17	969 ± 9	770 ± 11	859 ± 13	310 ± 16	1137 ± 9

[a] CB, coffee brew; M, melanoidin; PM, pure melanoidin; BMC, bonded melanoidin compound. Values are mean ± S. D. [b] Data expressed as μmol equiv of trolox/g of sample. [c] Data expressed as μmol equiv of CGA/g of sample.

Table 3. Antioxidant Activity Determined by the FRAP Method[a]

	$TEAC_{FRAP}$[b]				$CEAC_{FRAP}$[c]			
sample	CB	M	PM	BMC	CB	M	PM	BMC
CTn60	314 ± 2	273 ± 6	54 ± 1	384 ± 24	211 ± 1	187 ± 3	55 ± 1	254 ± 15
CTn85	296 ± 11	176 ± 9	69 ± 4	508 ± 3	201 ± 6	128 ± 6	64 ± 2	328 ± 1
CTn110	225 ± 3	255 ± 8	71 ± 3	598 ± 29	158 ± 1	176 ± 4	62 ± 3	382 ± 18

[a] CB, coffee brew; M, melanoidin; PM, pure melanoidin; BMC, bonded melanoidin compound. Values are mean ± S. D. [b] Data expressed as μmol equiv of trolox/g of sample. [c] Data expressed as μmol equiv of CGA/g of sample.

oxidation of linoleic acid, showed no significant differences between activities of CB60, 85, and 110 samples (*5*). Because methods of measuring antioxidant activity are extremely dependent on the conditions used and the substrates or products monitored, all methods did not give the same results. It must be pointed out that DPPH and ABTS methods analyze the antiradical activity, developed in methanolic and aqueous media, respectively, whereas FRAP estimates the ferric reducing ability of samples.

DPPH and ABTS methods showed a very different quantitative response for all samples, although with the same trend in both. In this sense, Del Castillo et al. (*15*) analyzed coffee brews from several roasting processes and reported higher responses by the ABTS test in aqueous dilution vs an ethanolic one. They attributed the difference to the fact that some components, making an important contribution to the antioxidant activity of the aqueous dilutions, were not soluble in ethanol. Probably the fact that the DPPH method was developed in methanolic media was responsible for the low response found. Data obtained by cited researchers differed from those manifested in this study since they assigned the same scavenging activity to coffees with light and medium roasted degree and a significantly lower capacity for the dark one.

The maintenance of antioxidant activity in CB60 and 85 samples could be explained by the contribution of a higher melanoidin fraction in those coffees, whereas that fraction in CB110 is still low and therefore has a lower contribution to the antioxidant capacity. Development of browning is associated with an increase in the antioxidant properties in systems where the Maillard reaction is the prevalent reaction (*16*, *17*). In this sense, although the major presence of still intact phenolic compounds in CB110 sample could be enough to reach similar values of activity, some authors have described enhancement of the antioxidant properties of coffee brews by the appearance of Maillard reaction products possessing antioxidant capacity (*9*, *18*, *19*).

Results for the antioxidant activity of coffee brews by the FRAP method presented in this study are similar to those obtained by Sanchez-Gonzalez et al. (*20*) in different types of coffee independent of the extraction procedure employed and also in line with those shown by Saura-Calixto and Goñi in soluble coffee (*21*). Other authors working on ready-to-drink coffee brews with different roasting degrees have found maximum antioxidant activity in the darkest coffee brew studied using the chain-breaking activity method (*22*).

Variable amounts of melanoidins were obtained for each coffee according to the degree of roasting: 16.4%, 18.4%, and 19.5% for CTn 110, CTn 85, and CTn 60, respectively. When the antioxidant activity of melanoidins was tested by any of the three methods, the antioxidant capacity of M60 and M110 samples was significantly higher than that of the middle roasted M85 sample ($P < 0.05$) (**Tables 1**−**3**). Our research group observed the same behavior when using the peroxyl radical scavenging activity method based on the linoleic acid oxidation (*5*). In the present study only in the DDPH method were the final values reached in M110 sample significantly lower than in M60 ($P < 0.05$), although higher than data from M85 sample ($P < 0.05$) (**Table 1**). These findings can be supported by the gradual formation of high molecular weight (HMW) melanoidins throughout the roasting process. LMW Maillard reaction products could even be bound to melanoidin skeleton (*23*), as well as other products like CGA, and contribute to the final antioxidant activity. It could be hypothesized that in M110 and M60 samples there was an equilibrium between the LMW compounds still present and the newly formed ones, depending on the roasted degree (including LMW melanoidins up to 10 000 Da and linked phenolic acids). A supposed lower activity of melanoidins present in the M85 melanoidin (with an intermediate molecular weight between those present in M110 and 60 samples) would explain the lower antioxidant capacity demonstrated by this sample. This is in agreement with the hypothesis formulated by Delgado-Andrade and Morales (*5*) about melanoidins existing in a dynamic equilibrium in the food matrix, where LMW compounds are noncovalently linked to the core structure. In any case, the mechanism of the antioxidant effect of melanoidins is still unclear and under study. It is assumed that the mechanism of action is based on the ability for trapping positively charged electrophilic species, scavenge oxygen radicals, or metal chelation to form inactive complexes.

After 2 M NaCl treatment to release LMW compounds ionically attached to the melanoidin skeleton (CGA, LMW compounds including Maillard reaction products, etc.), the activity of pure melanoidins (PM) was also analyzed (**Tables 1**−**3**). Data from the ABTS method showed no significant differences in antioxidant activities of different PM (**Table 2**). However, DPPH and FRAP methods exhibited lower values for the PM60 sample than for PM85 and 110 ($P < 0.05$). These results are in parallel with those described by Delgado-Andrade and Morales (*5*) using the peroxyl radical scavenging activity method based on the linoleic acid oxidation, also in pure melanoidins from the same coffee brews. Although the results presented in that study did not differ significantly between each other, antioxidant activity tended to decrease in PM60 sample, a fact that was statistically significant in this assay when ABTS and FRAP methodologies were employed.

The large difference in the antioxidant activity of M and PM (**Tables 1**−**3**) suggests a high contribution of LMW compounds

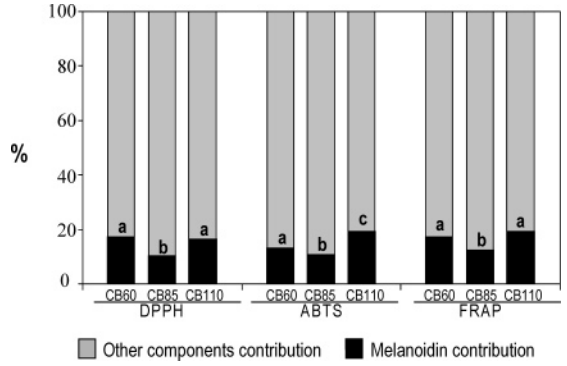

Figure 1. Contribution of melanoidins to the total antioxidant activity of coffee brews determined by the three methods applied. Different letters within the same technique indicate statistically significant differences between melanoidin contributions in each coffee.

linked to the melanoidin core to the antioxidant properties of melanoidins from coffee brews. Although this fact has already been pointed out by previous studies of our group (*5*), the present results encourage that hypothesis, differentiating the contribution on the PM, which is not present in the food itself.

Analyzing BMC samples, containing LMW compounds linked to the melanoidins, it was found that the lower the roasting process, the higher antioxidant activity (**Tables 1**−**3**). The three filtrates obtained, BMC60, BMC85, and BMC110, were significantly different ($P < 0.05$), except in the ABTS method, where no differences were found among BMC85 and BMC110. These data seem logical since destruction of phenolic acids (mainly CGA) gradually takes place during the roasting process (*24*); thus, their contribution to the antioxidant activity decreases.

On the other hand, it must be emphasized that when the samples analyzed were CB, M, or BMC, the response obtained in the ABTS method (aqueous media) was significantly higher than that in the DPPH one (methanolic media). However, the response of both methods was the same in the PM samples. This is indicative of the more hydrophilic character of antioxidant compounds from CB, M, and BMC samples. The soluble properties of antioxidant compounds determine their effective antioxidant activities in aqueous or lipid system (*25*). Then aqueous-based and nonaqueous-based models were chosen in this study to assess the antioxidant activity of coffees and different fractions.

Figure 1 shows percentages of contribution of the overall melanoidins (M) to the total antioxidant activity of coffee brews (assigned 100% of activity) by different methods. Data has been calculated from percentage of melanoidins present in the corresponding coffee (*5*) and the μmol equiv of CGA/g of melanoidin. If data from different methodologies are considered as a pool and medium antioxidant activity values for each melanoidin are compared, only the M85 contribution was lower than that demonstrated by M60 and M110 (15.47%, 10.30%, and 15.59% for M60, M85, and M110, respectively).

Finally, adequate correlation coefficients were found between the three methods used to test the antioxidant activity of samples (**Figure 2**). It must be pointed out that data from BMC were excluded of correlation because these samples were not lyophilized and establishing a quantitative correlation was not possible. Surprisingly, the best relationship was observed among DPPH and FRAP methods ($r^2 = 0.9798$; $P < 0.05$ for CB, M, and PM) despite the different reaction mechanism of each method to test the antioxidant activity.

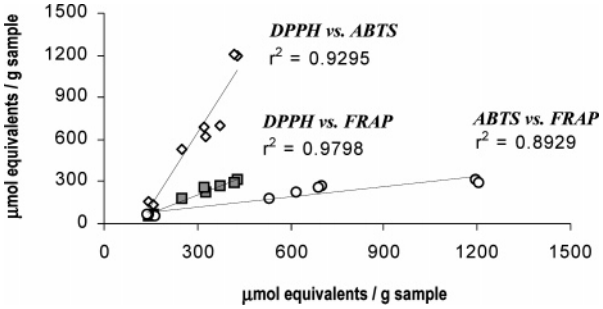

Figure 2. Correlation coefficients between the different antioxidant activity methodologies. Data expressed as TEAC (μmol equiv of trolox/g of sample).

In summary, results from the present assay support those previously obtained by our research group concerning to the important contribution of bound compounds to the melanoidin skeleton to the overall antioxidant activity of this MRP. Probably these kinds of compounds have been generated throughout the roasting process, and they are related to the development and progress of the Maillard reaction. These findings are in accordance with Del Castillo et al. (*15*), who demonstrated that the LMW fraction of coffee brews possesses higher antioxidant activity than the HMW fraction. Moreover, this work demonstrates that BMC are highly active too. On the other hand, the three antioxidant methods used to test different samples gave comparable results, as correlation coefficients exhibited, and conducted samples showed similar behavior in all of them. More investigations must be performed to elucidate the structure of some BMC which could help to gain insight on the mechanism of antioxidant activity of food melanoidins.

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