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Chemical Characterization and Antioxidant Properties of Coffee Melanoidins

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Melanoidins, the brown polymers formed through Maillard reaction during coffee roasting, constitute up to 25% of the coffee beverages' dry matter. In this study chemical characterization of melanoidins obtained from light-, medium-, and dark-roasted coffee beans, manufactured from the same starting material, was performed. Melanoidins were separated by gel filtration chromatography and studied by MALDI-TOF mass spectrometry. Results showed that the amount of melanoidins present in the brews increased as the intensity of the thermal treatment increased, while their molecular weight decreased. The antioxidant activity of melanoidins isolated from the different brews was studied by using different methodologies. Melanoidins antiradical activity determined by $ABTS^{\bullet+}$ and $DMPD^{\bullet+}$ assays decreased as the intensity of roasting increased, but the ability to prevent linoleic acid peroxidation was higher in the dark-roasted samples. Data suggest that melanoidins must be carefully considered when the relevance of coffee intake in human health is studied.

KEYWORDS: Coffee; melanoidins; Maillard reaction; antioxidant activity; MALDI-TOF

INTRODUCTION

Coffee is the second commodity in the rank of international trading after petroleum. Coffee consumption is very popular in Europe, as well as in the United States and Japan, but the types of coffee beverages and the modality of consumption are strictly associated with social habits and cultures of the single countries. Differences in green bean composition, roasting conditions, and extraction procedures adopted for the preparation of coffee brews result in a great diversity of the chemical composition of the final product (*1*). Green coffee beans are rich in phenolic compounds and polysaccharides, which undergo profound molecular changes during roasting (*2*−*6*). Low water activity and high temperature favor the development of the Maillard reaction (MR), with the formation of MR products between proteins and carbohydrates (*7*). During coffee roasting it is likely that phenolic compounds also participate in the reaction, becoming part of the brown, water-soluble polymers called coffee melanoidins (*8*). Melanoidins are one of the major components of coffee beverages, accounting for up to 25% of dry matter (*9*). Several studies suggest that they are responsible for the strong antioxidant properties and metal chelating ability showed by coffee beverages (*10*, *11*), and in turn for the observed antibacterial, antioxidant, and ex vivo protective activities (*12*, *13*). Results of studies on the physiological properties of coffee suggest that some effects which are not due to caffeine (*14*) can be attributed to phenolic compounds, thus also to melanoidins. However, the physiological relevance of coffee melanoidins is so far yet to be elucidated, and the lack of a defined molecular structure greatly hampers the biological studies. As melanoidin molecular structures are largely unknown, these compounds are generically defined as macromolecular materials which are brown and contain nitrogen (*15*). From literature data it is clear that the composition of melanoidins strongly differs with food composition and the technological conditions. Hofmann and collaborators demonstrated that melanoidins are important for staling and antiradical properties of coffee (*16*−*18*). However, the knowledge of coffee melanoidins chemical structure is still in its infancy; in fact, neither the backbone, nor the nature and the amount of phenolic compounds which are incorporated into the brown polymer have been elucidated.

The objective of the present investigation was to chemically characterize melanoidin fractions of light-, medium-, and dark-roasted beans obtained from the same blend of green coffee. Melanoidins were separated by gel filtration chromatography and studied by MALDI-TOF mass spectrometry. Also, their antioxidant properties were determined by using different methodologies.

MATERIALS AND METHODS

Chemicals. Coffee samples were supplied by the Nestlé Research Centre of Lausanne; 2,2′-azo-bis(2-amidinopropane)dihydrochloride (ABAP) was purchased from Wako Chemicals (Germany); N,N-

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dymethyl-*p*-phenylenediamine (DMPD) and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were from Fluka (St. Louis, MO), and 2,2-diphenyl-1-picrylhydrazil (DPPH) and the other chemicals were from Sigma-Aldrich (Steinheim, Germany).

Sample Preparation. Light-, medium-, and dark-roasted coffees were obtained from the same starting blend. The light-, medium-, and dark-roasted coffee samples were characterized by color test Neuhaus (CTN) values of 110, 85, and 60, and weight loss values of 14.2, 16.2, and 18.9%, respectively.

Coffee brews were prepared by solid−liquid extraction with hot tap water (90 °C) of the roasted beans previously ground to a powder in a standard coffee grinder. The ratio between coffee powder and water was 1:6 (w/w). After the aqueous solutions were filtered through Whatman no. 4 filter paper, they were defatted by extraction with dichloromethane. Aliquots of defatted coffee samples were then freeze-dried. The average extraction yields were 17% and 14% for the roasted and green coffees, respectively.

Gel Filtration Chromatography. Freeze-dried coffee material (2 g) was dissolved in 10 mL of water and loaded onto a fine Sephadex G-25 gel filtration chromatography column (75 × 5 cm i.d.; Pharmacia, Uppsala, Sweden). The eluent was monitored at 405 nm with a UV/Vis photometer. The separation afforded four fractions (fractions I−IV), which were pooled, freeze-dried, and stored in a desiccator. Gel filtration was also performed on a coffee brew prepared from green beans taken as a control.

Determination of Protein and Phenol Compounds. Folin Ciocalteu reagent was used to quantify total phenols as indicated by Singleton and Rossi (*19*). Total nitrogen was determined by Kjeldhal automated apparatus.

MALDI-TOF Mass Spectrometry. MALDI-TOF spectra of gel filtration fractions I were recorded on a Kompact MALDI instrument (Kratos Analytical, Shimadzu Group Company, Japan). The instrument was equipped with a nitrogen laser emitting light at 337 nm with a pulse width of 3 ns. Positive ions were recorded in linear mode. For calibration insulin was used as external standard. Sinapinic acid was used as matrix, and ions were accelerated through an acceleration voltage of 70 kV. A saturated solution (10 mg/mL) of sinapinic acid was dissolved in acetonitrile/H_2O (with TFA 0.1%) (2:3 v/v). Samples were dissolved in deionized water at a concentration of 10 mg mL^{-1}. Slides were prepared using a five-step method. At each step, volumes of 0.5 μL were pipetted onto sample plates, and the slides were placed in a fan oven for ca. 30 s to remove solvents. Briefly, matrix, sample, matrix, H_2O/TFA 0.1%, and finally matrix were layered onto slides. A Kompact 1.2 software was used for data analysis.

Antioxidant Activity. *ABTS$^{\bullet+}$ Assay.* The antioxidant activity of whole coffee extracts, as well as that of the four gel filtration fractions, was determined by using the ABTS$^{\bullet+}$ assay as described by Pellegrini and collaborators (*20*) with minor modifications. An aqueous ABTS$^{\bullet+}$ solution was prepared instead of an ethanol one to avoid precipitation of coffee components which would have occurred in the ethanol environment. Volumes of 0.1 μL of coffee brews were used for the ABTS$^{\bullet+}$ color bleaching, and the antioxidant activity was expressed as μM of Trolox. When ABTS assay was performed on gel filtration fractions, 2 μL of fractions I and 100 μL of fractions II, III, and IV were used. In these conditions no absorbance interference due to the coffee brown color was observed. The antioxidant activity was expressed as amount of Trolox equivalents (mg) present in the 2 g of material originally separated by gel filtration chromatography.

DMPD$^{\bullet+}$ Assay. The DMPD$^{\bullet+}$ assay on fractions I was performed according to Fogliano and collaborators (*21*) using 100 μL of melanoidin solution. Calculation was performed as described above for the ABTS$^{\bullet+}$ assay. The antioxidant activity was expressed as mg of Trolox equivalents present in each fraction.

Inhibition of Linoleic Acid Peroxidation. The ability of coffee melanoidins to prevent linoleic acid peroxidation in micellar system was assayed according to the methodology described by Pryor et al. (*22*) and slightly modified by Monti et al. (*23*). A variable volume of each fraction was added to the system, and the experiments giving slope inhibition between 20 and 70% were considered. The antioxidant activity was expressed as mg of Trolox equivalents present in each fraction.

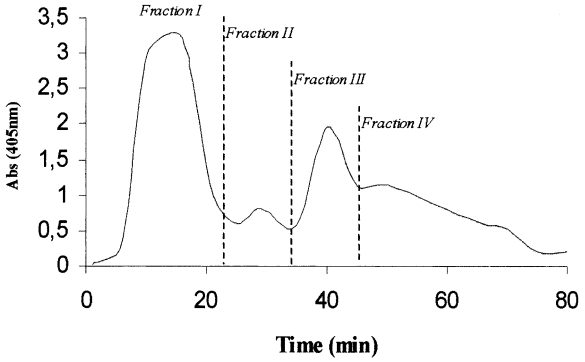

Figure 1. Gel filtration chromatogram of coffee brew obtained from dark-roasted beans, with the indication of the four collected fractions.

DPPH$^{\bullet}$ Assay. The radical scavenging activity of fractions I was measured by means of the DPPH$^{\bullet}$ method (*24*). A volume of 1.9 mL of 6.0 × 10^{-5} M DPPH$^{\bullet}$ methanol solution was used. The reaction was started by the addition of 100 μL of 0.02% (w/v) aqueous solution of fractions I. The bleaching rate was followed at 515 nm (Beckman DU 640; Beckman Instruments, Inc., Fullerton, CA) at 25 °C for at least 20 min. The antioxidant activity was expressed as mg of Trolox equivalents.

Redox Potential. Measurements were made using a platinum indicating electrode and a silver/silver chloride reference electrode, connected by a voltmeter (Hanna Instruments, model 8417, Milano, Italy) according to the methodology proposed by Manzocco and collaborators (*25*). Calibration was performed against a redox standard solution (Reagecon, Shannon, Co. Ireland) having redox potential value of 220 at 25 °C. The electrode was inserted into a 50-mL three-neck flask containing 15 mL of a 0.02% (w/v) aqueous solution of each fraction I. Prior to analysis, oxygen was removed from the system by continuous nitrogen flushing for 10 min. The redox potential was recorded for at least 20 min at 25 °C, until a stable reading was reached (i.e., until the redox potential changed by less than 1 mV over 5 min).

Statistical Analysis. The results reported here are the average of at least three measurements, and the coefficients of variation, expressed as the percentage ratio between the standard deviation (SD) and the mean values, were lower than 5 for dry matter and redox potential, 15 for DPPH and inhibition of linoleic acid peroxidation, and 10 for DMPD and ABTS.

One-way analysis of variance was determined using the Tukey−Krammer test (*26*). Differences between means were considered to be significantly different at $P < 0.05$.

RESULTS

Two grams of the freeze-dried defatted coffee brews, obtained from light-, medium-, and dark-roasted beans, were separated by gel filtration chromatography. The same procedure was run on a coffee brew prepared from green beans taken as a control. For each coffee sample four fractions (I−IV) were collected as illustrated in **Figure 1**. Fractions I contain the high-molecular-weight material, indicated hereinafter as coffee melanoidins; fractions II and III contain compounds having intermediate molecular weight; and fractions IV contain small compounds, including low-molecular-weight phenols.

Table 1 shows the amount of phenols, calculated as equivalents of gallic acid, as well as the total solid content of each gel filtration fraction. The protein content of fractions I is also shown.

As the same amount (2 g) of each freeze-dried sample was loaded on the gel filtration column, the separation of the coffee material into the four fractions depends only on their molecular nature. From the data of **Table 1** the following can be outlined: (i) the more the coffee is roasted, the higher is the amount of total solid content present in fractions I; (ii) a

Table 1. Total Dry Matter and Phenol Concentration (Expressed as Gallic Acid Equivalents, GAE) of Four Gel Filtration Fractions Obtained from Green as Well as Light-, Medium-, and Dark-Roasted Coffee Beans[a]

	fraction I			fraction II		fraction III		fraction IV	
sample	total solids[b] (mg)	phenols[b] (mg GAE)	protein[b] (mg)	total solids (mg)	phenols (mg GAE)	total solids (mg)	phenols (mg GAE)	total solids (mg)	phenols (mg GAE)
dark	550 ± 10a	24 ± 2a	21 ± 2a	486 ± 9a	6 ± 1a	548 ± 35a	15 ± 1a	199 ± 24a	2 ± 0a
medium	495 ± 15a	14 ± 1b	14 ± 3a	445 ± 16a	4 ± 0a	623 ± 18a	18 ± 2a	134 ± 8b	55 ± 11b
light	392 ± 9b	19 ± 3ab	27 ± 1b	366 ± 25b	1 ± 0b	404 ± 24b	10 ± 1b	315 ± 8c	54 ± 5b
green	236 ± 16c	4 ±1c	74 ± 9c	827 ± 36c	nd[c]	312 ± 26c	8 ± 1b	406 ± 33d	67 ± 5b

[a] The protein contents of fraction I samples are also shown. Data are referred to the 2 g of material loaded onto a gel filtration column. [b] All values are presented as the mean ± SD ($n = 3$). Means in a row with different letters are significantly different ($P < 0.05$). [c] Not detected.

Table 2. Antiradical Activity[a], Determined by the $ABTS^{\bullet+}$ Assay and Expressed as mg of Trolox Equivalents (present in the 2 g of material loaded on a gel filtration column) of Four Gel Filtration Fractions Obtained from Green as Well as Light-, Medium-, and Dark-roasted Coffee Beans

sample	fraction I (mg Trolox)	fraction II (mg Trolox)	fraction III (mg Trolox)	fraction IV (mg Trolox)
dark	27a ± 2	15b,c ± 3	18b ± 2	15c ± 1
medium	25a ± 3	16a ± 1	37a,b ± 6	112c ± 18
light	60a ± 3	12b ± 5	11b ± 2	88c ± 4
green	25a ± 1	7b ± 1	64c ± 7	185d ± 32

[a] Data are presented as the mean ± SD ($n = 3$). Means in a row with different letters are significantly different ($P < 0.05$).

Table 3. Antiradical Activity, Determined by the $DPPH^{\bullet}$, and Redox Potential Values of Gel Filtration Fraction I Samples Obtained from Green as Well as Light-, Medium-, and Dark-Roasted Coffee Beans

sample	antiradical activity[a] (mg Trolox)	redox potential[b] (mV)
dark	43a ± 2	131b ± 6
medium	34a ± 6	139b ± 6
light	40a ± 1	144b ± 7
green	64a ± 7	140b ± 6

[a] Antiradical activity values are presented as the mean ± SD ($n = 3$). Means with different letters are significantly different ($P < 0.05$). [b] redox potential values are presented as the mean ± SD ($n = 3$). Means with different letter are significantly different ($P < 0.05$).

significant amount of phenolic material is present in the fraction I of all samples, except in that obtained from the green coffee. In fractions I and II, phenol concentrations are lower in the light-roasted samples than in the dark-roasted ones, whereas low-molecular-weight phenols are almost absent in fraction IV of the dark-roasted coffee. The protein content of fractions I, which was more than 30% in the green coffee, dramatically decreased with roasting.

The fraction I samples from the gel filtration column were analyzed by MALDI-TOF mass spectrometry. All samples showed a broad peak between 1000 and 4000 Da. In **Figure 2** the mass spectra of coffee melanoidins obtained for the light-, medium-, and dark-roasted beans are shown. The widths of the peaks are quite similar, and the average molecular weight is high in the light-roasted coffee (between 1700 and 4200, centered at 2703 Da), intermediate in the medium-roasted coffee (between 1400 and 4000, centered at 2248 Da), and low in the dark-roasted coffee (between 1200 and 3500, centered at 1930 Da). Fraction I obtained from green coffee is also shown in **Figure 2**. In this case several sharp peaks between 3000 and 6000 Da were detected.

Figure 3 shows the antioxidant activity of whole coffee brews measured by means of the $ABTS^{\bullet+}$ assay and expressed as μmol of Trolox per g of dry matter. The antioxidant activity of the brew obtained from the light roasted beans is significantly higher than that of the other two samples.

Table 2 shows the antioxidant activity, determined by the $ABTS^{\bullet+}$ assay and expressed as the amount of Trolox equivalent (mg), of the gel filtration fractions obtained from all the coffee samples. A marked antioxidant activity is present in all fractions, but the melanoidin-containing fractions (I), as well as the low-molecular-weight phenol-rich fractions (IV), are the most active. The antioxidant activity of melanoidins decreased as the roasting severity increased. It can be also noted that fraction IV of the dark-roasted samples showed the lowest antioxidant activity values. This result is in accordance with the content of low-molecular-weight phenolic compounds (**Table 1**).

To further characterize the antioxidant activity of the coffee melanoidins, fractions I were also analyzed by using different antioxidant assays, i.e., $DMPD^{\bullet+}$, $DPPH^{\bullet}$, inhibition of linoleic acid peroxidation, and redox potential. **Figure 4** shows the scavenging activity toward DMPD radical cation (**Figure 4a**) and the inhibition of linoleic acid peroxidation (**Figure 4b**) of the gel filtration fractions I of green and light-, medium-, and dark-roasted coffee extracts. The $DMPD^{\bullet+}$ scavenging ability of these samples decreased as the intensity of the roasting process increased, in agreement with data from the $ABTS^{\bullet+}$ assay (**Table 2**). In contrast, the ability of fractions I to prevent the linoleic acid peroxidation in micelles increased with increasing roasting degree (**Figure 4b**). Furthermore, fraction I from the green coffee brew was the most effective in scavenging $DMPD^{\bullet+}$, but completely ineffective in inhibiting linoleic acid peroxidation.

No significant differences in the scavenging activity toward the DPPH radical were observed among the fractions I of green and light-, medium-, and dark-roasted coffee extracts (**Table 3**).

DISCUSSION

The formation of melanoidins and the degradation of low-molecular-weight phenolic compounds are two major events during roasting of coffee beans (*2*, *8*, *27*). The aim of this study was to shed some light on the molecular changes, particularly melanoidin formation, occurring during roasting of coffee beans. To this purpose a comparative characterization of coffee samples manufactured from the same blend, having different roasting degrees, was carried out. To separate coffee melanoidins, gel filtration chromatography was used. This technique gave satisfactory results in the past (*6*, *5*), and more recently it has been shown that 12% of total coffee dry mass obtained by ultrafiltration is constituted by polymers above 100 kDa (*28*). In our experiments the separation was carried out on the same amount of starting material for all brews. Hence, the different

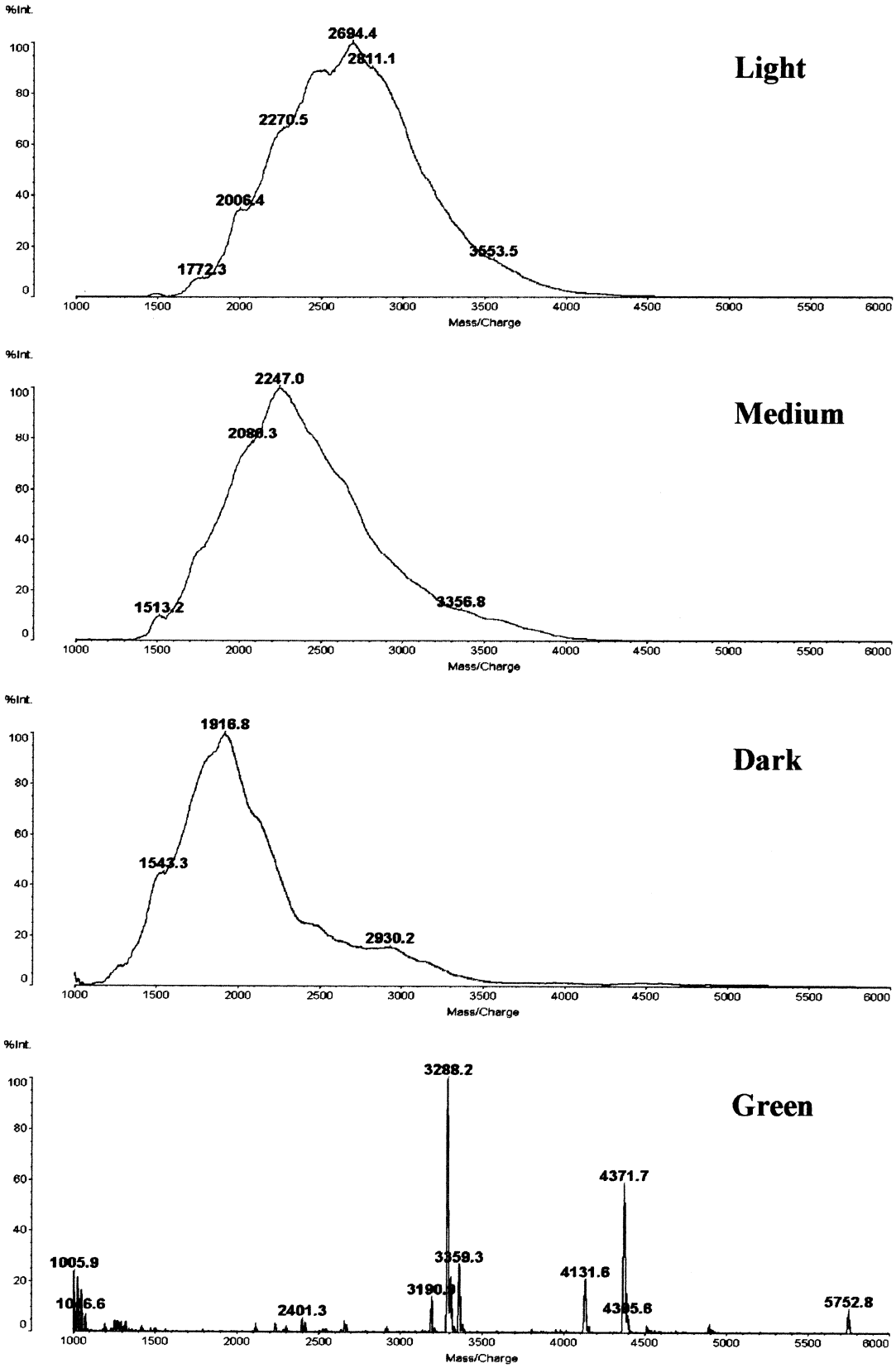

Figure 2. MALDI-TOF mass spectra of gel filtration chromatography fraction I obtained from raw and light-, medium-, and dark-roasted coffee beans.

partition into four fractions was only attributable to the molecular nature of the soluble solids. An irreversible binding of colored material on the gel filtration resin was observed and it determined a loss of about 15% inside the column. The high-molecular-weight containing fractions (I) averaged out at 12−27% of the material loaded on the gel filtration column. Fractions I peak area progressively increased with roasting, and data in **Table 1** showed that the total solids recovered in fractions I also increased with roasting. These findings confirm that melanoidins formation parallels the thermal treatment. Comparing the gel filtration chromatography profile of **Figure 1** with that obtained by Lindedmaier et al. (*28*), using the same

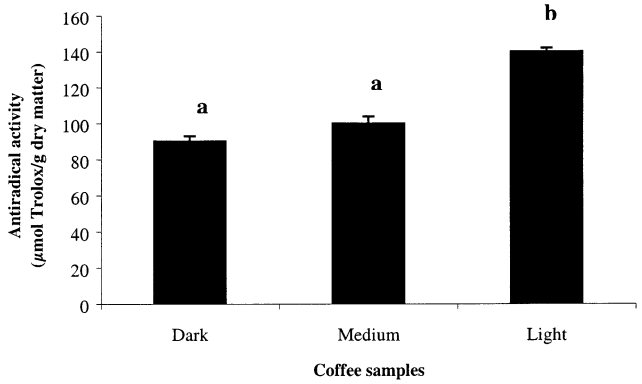

Figure 3. Antiradical activity, measured by ABTS$^{\bullet+}$ assay and expressed as μmol of Trolox per g of dry matter, of whole coffee brews obtained from light-, medium-, and dark-roasted coffee beans. Bars with different letters are significantly different ($P < 0.05$).

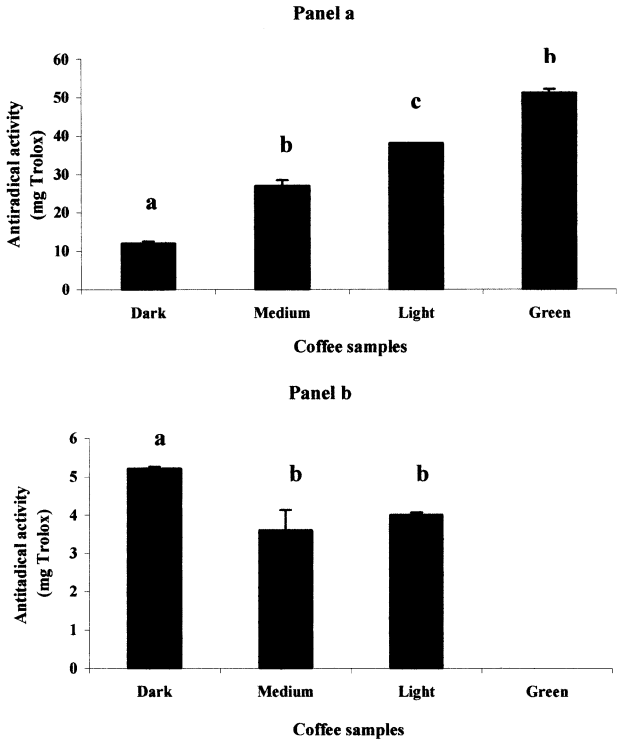

Figure 4. Scavenging of DMPD radical cation (a) and inhibition of linoleic acid peroxidation (b) of gel filtration fractions I obtained from green as well as light-, medium-, and dark-roasted coffee beans. Data are expressed as mg of Trolox equivalents present in the 2 g of material loaded onto a gel filtration column. Bars with different letters are significantly different ($P < 0.05$).

separation procedure, it was possible to estimate the molecular weight ranges of the four fractions. Fraction I corresponded to molecular mass over 100 kDa, fraction II corresponded to 60−15 kDa, fraction III corresponded 6−2 kDa, and fraction IV corresponded to 3−1 kDa. Actually, the determination of melanoidin molecular weight by conventional techniques, such as gel filtration, ultrafiltration, and dialysis, is questionable because of the absence of standards which are structurally similar to melanoidins.

MALDI-TOF mass spectrometry represents a powerful tool to estimate the molecular weight of biological and food polymers (*29*). Despite the good results obtained with other food components, the application of this technique to melanoidins did not give satisfactory results (*30*), and only reacting *N*-methylpyrrole and furan-2-carboxyaldeyde systems informative spectra were achieved (*31*). To our knowledge, data reported in **Figure 2** are the first example of MALDI-TOF mass spectra obtained with intact melanoidins from a food system. The observed molecular weight (between 2000 and 4000 Da) is much lower than that expected. It is very likely that nonionizable polymers having higher molecular weight are also present in the samples. On the other hand, it cannot be ruled out that aggregation phenomena may cause an overestimation of the molecular weight when gel filtration or ultrafiltration are used. The observed downshift of melanoidin molecular weight occurring for the dark-roasted sample could be an indication that, upon severe heat treatments, polymer fragmentation also occurred. This evidence suggests that the ideal model of polymer formation, with a molecular weight that progressively increases with the severity of the heat treatment, should not be correct. The dramatic decrease of protein content in fractions I as a consequence of roasting, which is in agreement with previous data (*8*), also suggests that protein breakdown into small fragments is a major phenomena occurring during coffee processing.

The observations on structural features of coffee melanoidins are strictly related to the antioxidant activity of coffee brews, which is a major property that can influence the stability of coffee products, as well as many physiological parameters of coffee drinkers. It is well-known that coffee brews have a very strong antioxidant activity and that melanoidins greatly contribute to the overall antioxidant activity of certain foods, such as roasted coffee (*10*, *32*). Different assays have been developed to assess the antioxidant activity of foods and each of them is driven by one or more of the different properties which can contribute to the whole antioxidant effect. In fact, it is likely that in complex systems such as foods, mixtures of chemically different compounds, either naturally occurring or formed as a consequence of processing, can act simultaneously with different mechanisms. In addition, melanoidins may exhibit antioxidant capacity through different mechanisms, i.e., chain breaking, oxygen scavenging, or metal chelating (*10*, *12*, *33*). Therefore, the choice of the assay is crucial, particularly for the food systems, and the use of different methodologies could help to give a more complete picture (*34*).

As the ABTS$^{\bullet+}$ assay is a widely used methodology for determining the ability of phenol-rich foods to scavenge the ABTS radical cation (*25*, *35*), the antioxidant activity of the whole coffee brews, as well as that of the four gel filtration fractions, was compared by using this methodology.

Results of **Figure 3** showed that coffee brews have a high antioxidant efficiency, which corresponds to about 30 mg of Trolox per g of dry matter. The high antioxidant activity of coffee brews is in line with the recent finding that they are more efficient than cocoa or black tea in delaying LDL oxidation (*36*). Different results have been reported on the effect of roasting on the antioxidant activity. For instance, Richelle and collaborators (*36*) reported that coffee antioxidant activity decreased with roasting, whereas in other studies a maximum antioxidant activity at intermediate roasting conditions was observed (*11*, *37*).

Comparing the ABTS$^{\bullet+}$ scavenging ability of all the gel filtration fractions (**Table 2**), it can be suggested that melanoidins greatly contribute to the whole coffee antiradical activity. Different results were obtained by Lindenmeier et al. (*28*) when measuring the inhibition of linoleic acid peroxidation by different gel filtration fractions. The low-molecular-weight fractions, presumably containing low-molecular-weight phenolic

compounds, were the most active. It is very likely that this discrepancy is due to the different assays used. The sum of the equivalent antioxidant activity of fractions recovered after gel filtration chromatography was in all cases between 100 and 300 mg of Trolox. The high antioxidant activity of fractions I is probably attributable to the fact that some phenolic compounds are incorporated into melanoidins. The simultaneous presence of phenolic hydroxyl groups, reductons, enaminol, and other potential active scavenging groups, which are typical of MR products (*15*), could explain the strong antioxidant activity exerted by these polymers. Thus, a simultaneous antioxidant action of antioxidants having different mechanisms of action can be hypothesized (*34*).

As already pointed out, by analyzing melanoidin antioxidant activity by means of different assays, different results were obtained. For instance, fractions I showed the same high radical scavenging capacity toward the $DPPH^{\bullet}$, whereas the ability of melanoidins to scavenge the $DMPD^{\bullet+}$ decreased with the increasing of the roasting degree (**Figure 4b**). The latter result is agreement with that found for the $ABTS^{\bullet+}$ (**Table 2**); in fact, the two radical cations have similar chemical properties. Opposite data were obtained by evaluating the ability of coffee melanoidins to prevent linoleic acid peroxidation. The mechanism of inhibition of linoleic acid peroxidation is quite different from that of scavenging a colored radical cation and it is possible that melanoidin polarity and solubility play a major role in affecting the surface tension of linoleic-acid-containing micelles dispersed in the water phase, thus influencing linoleic acid oxidation.

Similarly, chain-breaking activity and redox potential measurements give conceptually different information: the former is a kinetic measure, the latter is a thermodynamic one. Hence, while the assessment of the chain-breaking activity allows estimation of the quenching rate of the most reactive coffee compounds toward a reference radical, the redox potential gives indication on the effective oxidation/reduction efficiency of all the antioxidants present, including the "slow" ones, which cannot be detected by the kinetic method (*38*).

In conclusion, the data presented here suggest that the high antioxidant efficiency of coffee brews must be carefully considered when the relevance of coffee intake in human health is studied. Increasing evidence deriving from nutritional and epidemiological studies concurrently suggest that not all the physiological effects detectable upon coffee consumption can be ascribed to caffeine or to the bioactive terpenes (*14*, *39*, *40*).

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