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Influence of the Degree of Roasting on the Antioxidant Capacity and Genoprotective Effect of Instant Coffee: Contribution of the Melanoidin Fraction

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ABSTRACT: The roasting process induces chemical changes in coffee beans that strongly affect the antioxidant activity of coffee. In this study, the polyphenol and melanoidin contents and the antioxidant activity of three instant coffees with different roasting degrees (light, medium, and dark) were assessed. Coffee brews were separated into fractions, and the potential biological activity of the melanoidins was evaluated by simulating their gastrointestinal digestion. Total antioxidant capacity, hydroxyl radical scavenger activity, lipid peroxidation inhibition capacity, and protection against DNA oxidative damage (*in vitro* and *ex vivo* genoprotective effects) were determined. We report that instant coffee has a high total antioxidant capacity and protective effect against certain oxidative stress biomarkers (lipids and DNA), although this capacity decreases with the roasting degree. Our study confirms the hypothesis that several of the polyphenols present in coffee may become part of the melanoidins generated during roasting. Furthermore, the elevated genoprotective effect of melanoidin-digested fractions is noteworthy.

KEYWORDS: *Antioxidant activity, DNA protection, lipid peroxidation, MRPs, polyphenols, roasting process, soluble coffee*

INTRODUCTION

Coffee, which is one of the most widely consumed beverages in the world, is appreciated for its pleasant flavor and aroma as well as the stimulatory properties arising from its caffeine content. During the past few years, evidence of the health benefits and the important contribution of brewed coffee to the intake of dietary antioxidants has helped to increase coffee consumption.[1] Several factors, including the species (*arabica* or *robusta*) and cultivars,[2,3] the origin,[4] the kind of roasting process (conventional or torrefacto),[5] the degree of roasting,[6−8] and the brewing procedure,[1,9] can influence the antioxidant activity of coffee beverages.

Therefore, the antioxidant activity of coffee is known to be strongly affected by the roasting process. In fact, although various phenolic compounds (i.e., chlorogenic acid) originally found in coffee are partially lost during this process,[10] the antioxidant content can be maintained by the formation of new antioxidants, such as the melanoidins generated via the Maillard reactions (MR) that take place during roasting.[7,8,11−13] Melanoidins are brown, water-soluble, nitrogen-containing macromolecular compounds that are responsible for the color and aroma of roasted coffee. Although their structure is extremely complex and complete knowledge about it is still lacking, it has been proposed that the incorporation of phenolic compounds plays an important role in melanoidin formation.[14] It has also been suggested that part of the antioxidant activity of the melanoidins found in coffee could be due to low-molecular-weight compounds noncovalently linked to them.[15]

Although the effect of coffee roasting on the antioxidant activity of coffee brews has been investigated previously, these studies produced contradictory results: (i) an increase in the antioxidant capacity of brews from medium-roasted coffee and a decrease in those from dark coffee,[6−8] (ii) an increase in the antioxidant activity of brews with the degree of roasting,[16,17] (iii) a decrease of antioxidant capacity of brews with the roasting degree,[3,18−20] and (iv) antioxidant activity of coffee unaffected by the degree of roasting.[21] Several factors, including the use of different methods to determine the antioxidant capacity,[19] the type of coffee employed for the experiments,[17] the use of different time−temperature profiles to reach the same degree of roasting,[16] and the lack of a standard definition of the roasting degree,[6] could explain these literature discrepancies. Furthermore, it must be taken into account that, for instant coffee, after the roasting process, there is an additional thermal extraction treatment at high temperatures and a drying process that also affects the composition and antioxidant activity of coffee.

Despite the great economic importance of instant coffee, there have been few reports concerning its antioxidant potential,[15,21] because most studies in the literature refer to the antioxidant activity of brewing roasted coffee. The objective of this study was therefore to evaluate the effect of the degree of roasting of instant coffee on its antioxidant activity as well as on the biomarkers of oxidative stress. In addition, this study attempts to determine the contribution of the different compounds found in roasted coffee on its overall antioxidant capacity. To this end, the coffee was separated into fractions and the potential biological activity of melanoidins was evaluated by simulating their gastrointestinal digestion. The main contribution to new knowledge in the field is the elevated genoprotective effect found in the instant coffee brews after melanoidin digestion.

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MATERIALS AND METHODS

Chemicals. 2,2′-Azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-diazobis-(2-aminodinopropane)-dihydrochloride (ABAP), 6-hydroxyl-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox), 2,4,6-Tris(2-pyridyl)-*S*-triazine (TPTZ), thiobarbituric acid (TBA), 2-deoxy-D-ribose, gallic acid (GA), low-melting agarose, normal-melting agarose, menadione, Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline (PBS), fetal calf serum (FCS), Triton X-100, bovine serum albumin (BSA), pancreatin, pepsin, bile salts, and calf thymus DNA were purchased from Sigma-Aldrich Co. (St. Louis, MO). Butanol, ethanol, methanol, potassium persulfate ($K_2O_8S_2$), ferric(III)-chloride acid ($FeCl_3$), ferrous(II)-sulfate ($FeSO_4$), hydrogen peroxide (H_2O_2), L-ascorbic acid, citric acid, ethylenediaminetetraacetic acid (EDTA), trichloroacetic acid (TCA), and Folin−Ciocalteau reagent were obtained from Panreac (Barcelona, Spain). Dimethyl sulfoxide (DMSO) and copper(II)-sulfate pentahydrate ($SO_4Cu{\cdot}5H_2O$) were purchased from Merck (Darmastadt, Germany).

Samples. To investigate the effects of roasting independently from the coffee source, three instant coffees produced from the same blend of coffee beans (80% *arabica* and 20% *robusta*) were supplied by the Nestlé Research Center (Lausanne, Switzerland) in three different roasting degrees. Green coffee beans were roasted in a pilot plant for 6 min at different levels, and they were characterized by a color test number (CTn) value of 110, 85, and 60, corresponding to light (water loss of 14.2%), medium (water loss of 16.2%), and dark (water loss of 18.9%) roasted coffee, respectively. Despite differences in the roasting process, all coffees were given the same post-treatments to obtain the three final soluble powder coffees.

Preparation of Coffee Samples (C, F, M, FMD, and MD). A total of 1.000 g of the different instant coffees was resuspended in 100 mL of hot Milli-Q water (55 °C) for 3 min while stirring continuously. The coffee brews (C) were then filtered and stored at 4 °C for a short time until analysis (C-110, C-85, and C-60 samples).

An aliquot of each sample was subjected to ultrafiltration using an Amicon ultrafiltration cell (model 8050, Amicon, Beverly, MA) equipped with a 10 kDa cutoff membrane (Millipore Corp., Vedford, MA).[15] The filtrate and retentate fractions were freeze-dried, weighed, and stored at −80 °C until analysis. Freeze-dried filtrate samples (F) containing the low-molecular-weight compounds present in coffee were identified as F-110, F-85, and F-60. Melanoidin-containing freeze-dried retentate samples (M) were identified as M-110, M-85, and M-60.

Simulated gastrointestinal digestion of samples containing coffee melanoidins (M) was performed following a slight modification of the method described by Rufián-Henares and Morales.[15] Briefly, 0.4 g of each M sample was diluted with Milli-Q water (8 mL), and the pH was adjusted to 2.0 using 6 M HCl. A 125 μL aliquot of freshly prepared pepsin (0.16 g/mL in 0.1 M HCl) was then added, and the mixture was incubated at 37 °C for 3 h in a shaking water bath. After the gastric digestion step, the pH was adjusted to 7.0 with 0.5 M $NaHCO_3$ and 2 mL of freshly prepared pancreatinin−bile mixture (0.004 g of pancreatinin and 0.025 g of bile salts in 1 mL of 0.1 M $NaHCO_3$) was added before incubating for 1 h at 37 °C in a shaking water bath. Then, enzymes were deactivated by thermal treatment at 100 °C for 4 min in a water bath to suppress their contribution from the further studies. Finally, the digested solutions were ultrafiltered and freeze-dried following the same procedure as described above. The freeze-dried filtrate extracts containing low-molecular-weight compounds noncovalently linked to melanoidins were identified as FMD-110, FMD-85, and FMD-60. The freeze-dried retentate extracts containing digested melanoidins were identified as MD-110, MD-85, and MD-60.

Determination of Total Polyphenols (TPPs). The total phenol content was evaluated using the Folin−Ciocalteau reaction,[22] with GA as the standard. Thus, 100 μL of diluted coffee sample was added to 500 μL of Folin−Ciocalteau reagent and 400 μL of sodium carbonate solution (75 g/L). The results were expressed in micrograms of GA equivalent per milligram of sample (absolute approach) or per milligram of coffee (relative approach).

Browning Measurement. The absorbance of the coffee samples at 360 and 420 nm was recorded using an ultraviolet−visible (UV−vis) spectrophotometer (U-2000 Hitachi) to estimate the intermediate and final Maillard reaction products (MRPs) content based on the browning associated with the formation of melanoidins.[23]

Determination of Total Antioxidant Capacity (TAC). *ABTS Method.* This assay is based on the decoloration that occurs when the radical cation $ABTS^{\bullet+}$ is reduced to ABTS.[24] The assay consisted of 980 μL of $ABTS^{\bullet+}$ solution and 20 μL of the sample. The absorbance at 734 nm was measured after 20 min of reaction, and the results are expressed in micrograms of Trolox equivalent per milligram of sample (absolute approach) or per milligram of coffee (relative approach).

DPPH Method. This method is based on the reduction of the radical $DPPH^{\bullet}$.[25] The reaction was started by adding 20 μL of each sample solution to 980 μL of $DPPH^{\bullet}$ (60 μM in methanol). The absorbance at 517 nm was measured after 2 h, and the results are expressed in micrograms of Trolox equivalent per milligram of sample (absolute approach) or per milligram of coffee (relative approach).

Ferric Reducing/Antioxidant Power (FRAP) Method. This method is based on the increase in absorbance at 593 nm because of the formation of TPTZ−Fe^{II} complexes in the presence of a reducing agent.[26] The reactive mixture was prepared by mixing 25 mL of sodium acetate buffer solution (0.3 M, pH 3.6), 2.5 mL of TPTZ (10 mM), 2.5 mL of $FeCl_3$ (20 mM), and 3 mL of Milli-Q water. Then, 30 μL of each sample was added to 970 μL of the latter reactive mixture and incubated at 37 °C for 30 min. The results were expressed in millimolar Fe^{II} per milligram of sample (absolute approach) or per milligram of coffee (relative approach).

Determination of Hydroxyl Radical Scavenger Activity (HRSA). Deoxyribose (2-deoxy-D-ribose) decays when exposed to hydroxyl radicals generated by the Fenton reaction.[27] The reaction mixture containing 1 mM deoxyribose, 5 mM phosphate buffer (pH 7.4), 0.1 mM ascorbic acid, 0.1 mM $FeCl_3$, 1 mM H_2O_2, 0.1 mM EDTA, and 100 μL of the samples, in a total volume of 1 mL, was incubated for 60 min at 37 °C. The samples were then mixed with 1.5 mL of TCA (28%, w/v) and 1 mL of TBA (1%, w/v) and heated at 100 °C for 15 min. The absorbance was recorded at 532 nm, and the results were expressed as percent oxidation inhibition with respect to a control test, following an absolute (per milligram of sample) or a relative (per milligram of coffee) approach.

Lipid Peroxidation Inhibition. The experiments were carried out in rat liver microsomal preparations [28] using peroxyl radicals as an oxidant. The total microsomal protein content was determined using the Bradford method.[29] The microsomal fraction (1 mg/mL of protein) was incubated with a solution of 10 mM ABAP in the presence of 20 μL of sample (diluted to 0.5 mg/mL in Milli-Q water). The incubation temperature was set at 37 °C for a period of 90 min. Lipid peroxidation was evaluated using the thiobarbituric acid reactive substances (TBARS) assay to quantify malondialdehyde. The absorbance measured at 532 nm was proportional to the quantity of peroxyl radicals generated, and the results were expressed as percent oxidation inhibition with respect to a control test, following an absolute (per milligram of sample) or a relative (per milligram of coffee) approach.

DNA Oxidative Damage: Agarose Gels. Oxidative DNA damage was measured following the method described by Rivero et al.,[30] with a reaction mixture containing calf thymus DNA (100 μg), ascorbic acid (10 mM), copper(II) sulfate (0.1 mM), and 300 μL of the coffee samples (diluted to 10 mg/mL in Milli-Q water) in a total volume of 500 μL. The mixture was incubated in a shaking water bath at 37 °C for 60 min. The resulting fragments were separated by electrophoresis on 1% agarose gels using an electrophoresis system (Hoefer-PS500XT, San Francisco, CA). The gel was run at 100 V, 400 mA, and 100 W for 45 min.

Genoprotective Effect: Comet Assay. This assay, also known as single-cell gel electrophoresis under alkaline conditions, was performed in the cell line HT-29 (human colon carcinoma cell line), according to a previously reported method.[31] Cells were grown in DMEM supplemented with 10% FCS at 37 °C and 5% CO_2. After incubation with 50 μL of coffee samples (diluted to 1 mg/mL in DMSO) and 15

Table 1. Amount of Freeze-Dried Fraction of Each Ultrafiltrated Samples (F, M, FMD, and MD) Obtained Per Gram of Coffee or Gram of M Sample[a]

samples	g of sample/g of coffee	samples	g of sample/g of coffee	g of sample/g of M sample
F-110	0.560 ± 0.005 c	FMD-110	0.217 ± 0.005 c	0.567 ± 0.012 c
F-85	0.553 ± 0.003 b	FMD-85	0.199 ± 0.006 b	0.504 ± 0.013 b
F-60	0.545 ± 0.004 a	FMD-60	0.180 ± 0.005 a	0.446 ± 0.011 a
M-110	0.383 ± 0.003 a	MD-110	0.157 ± 0.006 a	0.409 ± 0.013 a
M-85	0.394 ± 0.003 b	MD-85	0.182 ± 0.007 b	0.461 ± 0.015 b
M-60	0.404 ± 0.004 c	MD-60	0.206 ± 0.004 c	0.510 ± 0.010 c

[a]Data are expressed as mean values ± standard error (n = 3). Different letters indicate significant differences as a function of the degree of roasting (CTn-110, CTn-85, and CTn-60) between the values obtained for each sample (F, M, FMD, and MD).

Table 2. TPP Content and Browning Measurement of Coffee Samples

	TPP[a]		browning measurement[b]	
samples	absolute approach (μg of GA equiv/mg of sample)	relative approach (μg of GA equiv/mg of coffee)	absorbance at 360 nm (intermediate MRPs)	absorbance at 420 nm (final MRPs)
C-110	222 ± 3 c/ε	222 ± 3 c/δ	0.793 ± 0.033 c/$\beta\gamma$	0.169 ± 0.018 a/β
C-85	203 ± 3 b/ε	203 ± 3 b/ε	0.708 ± 0.015 b/β	0.205 ± 0.009 b/γ
C-60	176 ± 4 a/ε	176 ± 4 a/ε	0.664 ± 0.011 a/γ	0.247 ± 0.013 c/γ
F-110	174 ± 3 c/γ	97.2 ± 1.9 c/γ	0.633 ± 0.014 c/α	0.086 ± 0.015 a/α
F-85	148 ± 4 b/γ	81.8 ± 2.3 b/δ	0.574 ± 0.026 b/α	0.119 ± 0.009 b/α
F-60	132 ± 2 a/γ	72.0 ± 0.9 a/δ	0.509 ± 0.019 a/α	0.143 ± 0.009 c/α
M-110	183 ± 2 b/δ	70.2 ± 0.8 b/β	0.761 ± 0.013 c/β	0.202 ± 0.011 a/γ
M-85	166 ± 5 a/δ	65.4 ± 1.9 a/γ	0.723 ± 0.016 b/β	0.234 ± 0.008 b/δ
M-60	159 ± 7 a/δ	64.4 ± 2.7 a/γ	0.693 ± 0.005 a/γ	0.281 ± 0.024 c/δ
FMD-110	100 ± 3 c/α	21.7 ± 0.6 c/α	0.835 ± 0.048 c/γ	0.159 ± 0.007 a/β
FMD-85	85.9 ± 4.7 b/α	17.1 ± 0.9 b/α	0.689 ± 0.019 b/β	0.177 ± 0.009 a/β
FMD-60	76.2 ± 3.1 a/α	13.7 ± 0.6 a/α	0.585 ± 0.042 a/β	0.202 ± 0.011 b/β
MD-110	120 ± 2 a/β	18.8 ± 0.4 a/α	0.987 ± 0.019 c/δ	0.396 ± 0.023 a/δ
MD-85	116 ± 5 a/β	21.1 ± 1.0 b/β	0.918 ± 0.036 b/γ	0.445 ± 0.010 b/ε
MD-60	114 ± 4 a/β	23.5 ± 0.9 c/β	0.849 ± 0.025 a/δ	0.477 ± 0.007 c/ε

[a]TPP values are expressed in micrograms of GA equivalent per milligram of sample (absolute approach) or per milligram of coffee (relative approach). [b]Browning is expressed as the absorbance values obtained at 360 and 420 nm. Data are expressed as mean values ± standard error (n = 3). Different letters indicate significant differences as a function of the degree of roasting (CTn-110, CTn-85, and CTn-60) between the values obtained for each sample (F, M, FMD, and MD). Different Greek letters refer to significant differences between the samples (C, F, M, FMD, and MD) obtained from each coffee with a determined degree of roasting (CTn-110, CTn-85, and CTn-60).

μL of catalase (100 units/mL) for 24 h at 37 °C and 5% CO_2, oxidation was induced with 50 μL of menadione (20 μM, diluted in DMEM). The resulting cell suspensions were collected, centrifuged, resuspended in preheated 1% low-melting-point agarose, and added to frosted microscope slides precoated with 1% normal-melting-point agarose, which were then immersed in cold lysing solution (2.5 M NaCl, 100 mM EDTA, 100 mM Tris, 1% sodium-lauryl-sarcosineate, 1% Triton X-100, and 10% DMSO at pH 10) overnight at 4 °C. The microscope slides were then placed in an electrophoresis tank, and the DNA was allowed to uncoil for 40 min in alkaline electrophoresis buffer (1 mM EDTA and 0.3 N NaOH at pH 10). Electrophoresis was conducted at 4 °C, 25 V, 500 mA, and 150 W. The slides were subsequently neutralized with Tris buffer (0.4 M, pH 7.5), stained with ethidium bromide, then observed using a fluorescent microscope, and photographed with a digital camera. The photographs were analyzed using the CometScore freeware, version 1.5.2.6, software (TriTek Corp., Sumerduck, VA). For each analysis, 60 individual cells were calculated and their tail length was evaluated. DNA damage was expressed as a percentage of the relative tail length in relation to the oxidized control ([T/C2] %) using an absolute approach (per 50 μg of sample).

Statistical Procedure. Statistical analysis of the data was carried out using one-way analysis of variance (ANOVA). The least significant difference (LSD) test was applied to determine the statistical significance between various groups. A minimum significance level of $p < 0.05$ was considered. Linear regression was used to study the possible correlations between the parameters studied. The Statgraphics Centurion, version 16.1.11.0, software (Statpoint Technologies, Inc., Warranton, VA) was used.

RESULTS AND DISCUSSION

Coffee brews (C) and derived ultrafiltrated fractions (F, M, FMD, and MD) obtained from three instant coffees that differed in their degree of roasting (light, CTn-110; medium, CTn-85; and dark, CTn-60) were characterized, and their antioxidant activities were assessed using different methodologies. Data from several of these assays were analyzed in two different ways, following an absolute or a relative approach. The absolute approach gives information regarding the net activity of each coffee sample, irrespective of its concentration, as the experiments were carried out with the same amount of each sample resuspended in Milli-Q water, and the results are expressed per milligram of sample. A prior weighing of the amount of ultrafiltrated fractions obtained from each sample also allowed us to follow a relative approach (results expressed per milligram of coffee), which describes the contribution of each fraction to the overall antioxidant activity of the parent coffee. In Table 1, the amount of freeze-dried fraction of each ultrafiltrated sample (F, M, FMD, and MD) obtained per gram of coffee is specified. For samples FMD and MD, the recovery for this fraction expressed per gram of M sample is also indicated. The amount in the filtrate samples (F and FMD)

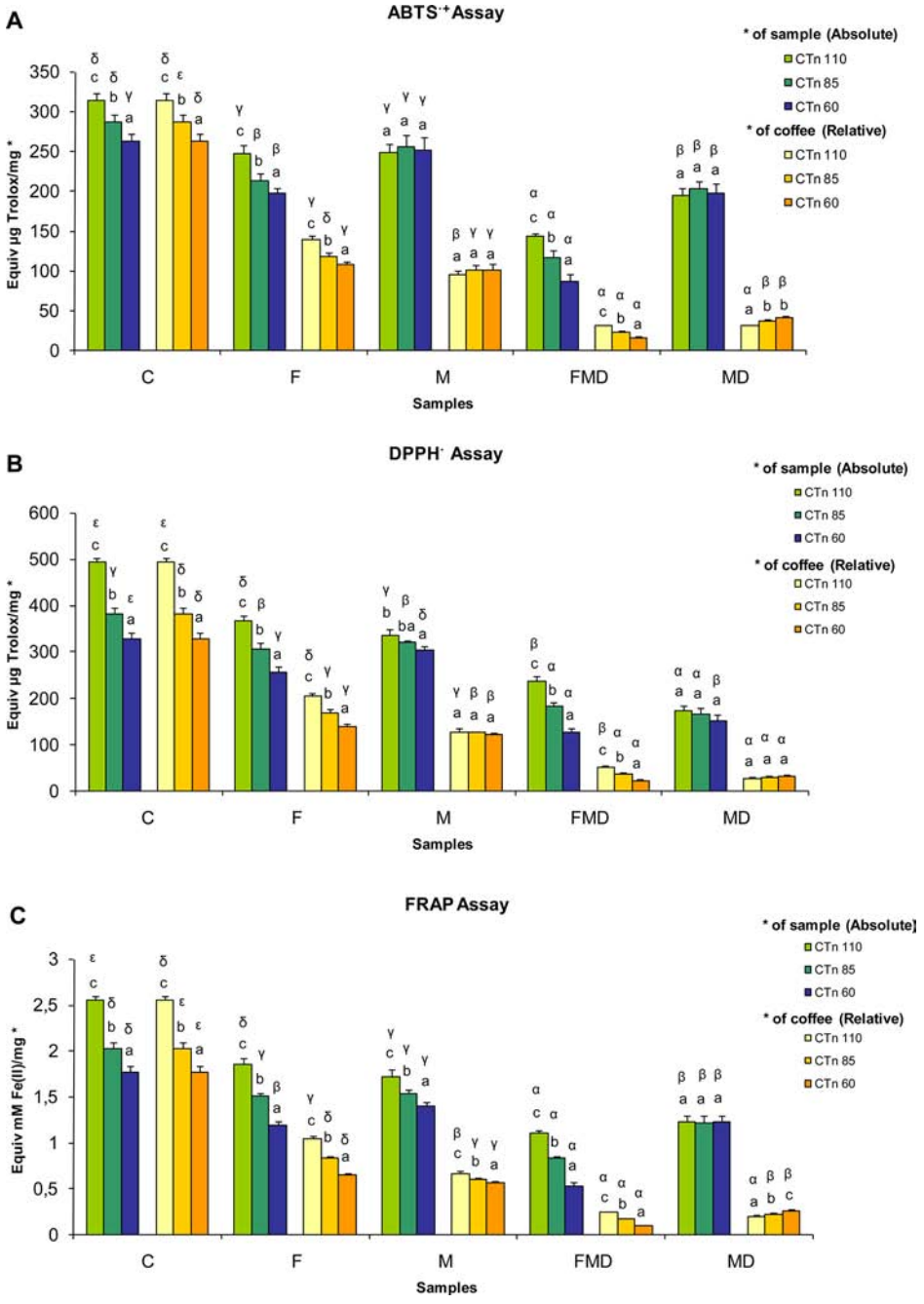

Figure 1. TAC determined using the (A) ABTS, (B) DPPH, and (C) FRAP assays. The ABTS and DPPH values are expressed in micrograms of Trolox equivalent per milligram of sample (absolute approach) or per milligram of coffee (relative approach). The FRAP results are expressed in millimolars Fe^{II} per milligram of sample (absolute approach) or per milligram of coffee (relative approach). Data are expressed as mean values ± standard error (n = 3). Different letters indicate significant differences as a function of the roasting degree (CTn-110, CTn-85, and CTn-60). Different Greek letters refer to significant differences between the samples (C, F, M, FMD, and MD) obtained from each coffee.

decreased with the degree of roasting, whereas an increase in the amount of sample weighed with the roasting degree was observed for the retentate samples (M and MD). These results are in line with those reported by other studies, which found that melanoidins represented around 38−40% of the compounds in roasted coffee.[15]

Characterization of Instant Coffee Samples: TPP Content and Browning Measurement. The TPP content and the browning related to the presence of melanoidins were determined in the coffee samples (Table 2) to attempt to elucidate the important changes that the roasting process induces in the chemical composition of coffee beans.

Coffee is one of the beverages with the highest content in phenolic compounds (between 200 and 550 mg per cup of coffee).[4] Indeed, they constitute approximately 16% of total solids in instant coffee, although this content varies with the degree of roasting.[32] As shown in Table 2, the TPP content in C samples decreased significantly as the degree of roasting increased [a 20.7% decrease of micrograms of GA equivalent per milligram of sample from light (C-110) to dark (C-60) roasted coffee], thereby supporting the theory that the roasting

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process results in a decrease in the polyphenol content of coffee.[6,16] In the absolute approach, this trend was maintained for the remaining samples (F, M, FMD, and MD), especially in the filtrate samples. In the relative approach, the decrease as a function of the degree of roasting was similar in all samples, except for the MD samples. Chlorogenic acids are the most important polyphenols in coffee, but the high temperatures used during the roasting process result in their lactonization and polymerization, either becoming new structures (some of which may participate in the formation of melanoidins) or leading to their degradation.[32] In the relative approach, the filtrate samples (F) contributed more than retentate samples (M) to the TPP of coffees. However, the values obtained in the absolute approach for TPP in M were higher than those obtained in the respective F. This could support the hypothesis proposed by several authors, whereby phenolic compounds are incorporated into the structure of coffee melanoidins generated during the roasting process;[15,33,34] thus, the compounds in the melanoidin structure could be measured by the TPP approach.

Browning is considered a non-specific marker of MR development and has usually been measured spectrophotometrically and expressed in absorbance units.[23,35−37] Some specific markers, such as high-performance liquid chromatography (HPLC) determination of furosine, hydroxymethylfurfural (HMF), and furfural or liquid chromatography−electrospray ionization single quadrupole mass spectrometry (LC−ESI−MS) determination of acrylamide,[23] as well as techniques like high-performance gel-permeation chromatography (HPGPC) and capillary zone electrophoresis (CZE),[38] have been used for the determination of the MR development or melanoidin content in food analysis. However, previous studies have shown that color can be directly related to the concentration of melanoidins using an external standard method, because the relationship between browning and the formation of melanoidins is linear between a limited range of absorbances at both 405 nm[39] and 420 nm,[37] with the value of the molar extinction coefficient of coffee melanoidins remaining constant over the roasting process. However, in this study, we have not calculated concentrations but have just focused on the brown color of coffee samples as an indirect parameter of their amount of melanoidins. In the first stages of the MR, reducing sugars react with amino acids, giving rise to noncolor compounds, which do not absorb in the visible spectra. The formation of these early MR compounds can be monitored at 280 nm, and a pool of more advanced compounds (intermediate MRPs) can be monitored at 360 nm. The progress of the reaction involves the production of high-molecular-weight compounds (final MRPs) that contain chromophore groups with a characteristic absorbance maximum at 420 nm.[23] The results of the absorbance values of the coffee samples at 360 and 420 nm are presented in Table 2. The amount of compounds formed during intermediate steps of MR was higher in light coffee (CTn-110) for all samples (C, F, M, FMD, and MD) and decreased with the degree of roasting. However, roasting induced an increase in the amount of final MRPs. This finding confirms that melanoidins are formed continuously upon the coffee-roasting process and is in accordance with previous reports, where it has been proposed that the initial roasting process predominantly leads to the formation of intermediate-molecular-weight melanoidins when compared to high-molecular-weight melanoidins, while prolonged roasting especially leads to the formation of high-molecular-weight melanoidins.[7,12,14] The results of this study also showed that, for the three instant coffees, the highest amounts of both intermediate and final MRPs were obtained for the MD samples, while the F samples presented the lowest values.

TAC of Instant Coffee Samples. Initially, we assessed the TAC of samples using three methods: ABTS, DPPH, and FRAP. It can be seen from Figure 1 that all samples behave similarly in these three assays, as confirmed by a study of the correlations between them: ABTS/DPPH (r = 0.8388), ABTS/FRAP (r = 0.9311), and DPPH/FRAP (r = 0.9219). All C samples presented an elevated antioxidant capacity, with the TAC decreasing significantly with the increased degree of roasting. These findings are consistent with those reported in other studies involving the ABTS[3,19,20] and FRAP[18] assays. In contrast, previous results from other groups in the field, who found a higher antioxidant capacity for the medium-roasted coffee using the ABTS method[6,7] and the dark-roasted coffee using the DPPH assay[16] or similar TAC values for light- and dark-roasted coffees using ABTS, FRAP, and DPPH methods,[21] differ notably from those obtained in this study. The antioxidant potential of coffee is mainly attributed to the presence of phenolic compounds and melanoidins. However, dependent upon the intensity of the roasting process, the contribution of each group of compounds to the TAC of the resulting coffee varies markedly and there are broad discrepancies in the results obtained by different authors concerning the effects of roasting on the antioxidant activity of coffee. Thus, Del Castillo et al.[7] suggested that, under mild roasting conditions, polyphenols are mainly responsible for the free radical scavenging activity of coffee brews, whereas MRPs are more important for this activity in more strongly roasted coffees. In contrast, Delgado-Andrade and Morales[10] reported that the contribution of the melanoidin fraction to the TAC of coffee was very modest, a finding that is consistent with the study by Sacchetti et al.[10] and with our research. Vignoli et al.[21] found that both compounds, chlorogenic acids and melanoidins, contributed equally to the antioxidant activity of soluble coffee, because the degradation of one was balanced by the formation of the other during the roasting process. Therefore, they concluded that the antioxidant activity depends more upon the initial coffee composition (concentration of phenolic compounds, caffeine, and melanoidins in the raw materials) than the roasting degree. In our study, the antioxidant potential obtained for the F and M samples (Figure 1) and the study of correlations between TAC assays and the composition of coffee, where we found high positive correlations between TPP/ABTS (r = 0.9465), TPP/DPPH (r = 0.9335), and TPP/FRAP (r = 0.9553), lead us to conclude that the decrease in the TAC of coffee upon roasting may be related to the contribution of the low-molecular-weight compounds present in coffee, because F samples always present a lower antioxidant capacity and TPP content as the degree of roasting increases. Moreover, F samples make a much more important contribution to the overall antioxidant activity of coffee (relative approach) than the M samples, especially in light (CTn-110) and medium (CTn-85) roasted coffee. The differences resulting from the degree of roasting in samples enriched in melanoidins (M) were lower than those for the F samples. However, after the gastrointestinal digestion of M samples and their separation into fractions, the results from the absolute approach showed that the antioxidant capacity of those samples corresponding to the low-molecular-weight compounds noncovalently linked to melanoidins (FMD) decreased with the degree of roasting, whereas there were no significant differences for those samples

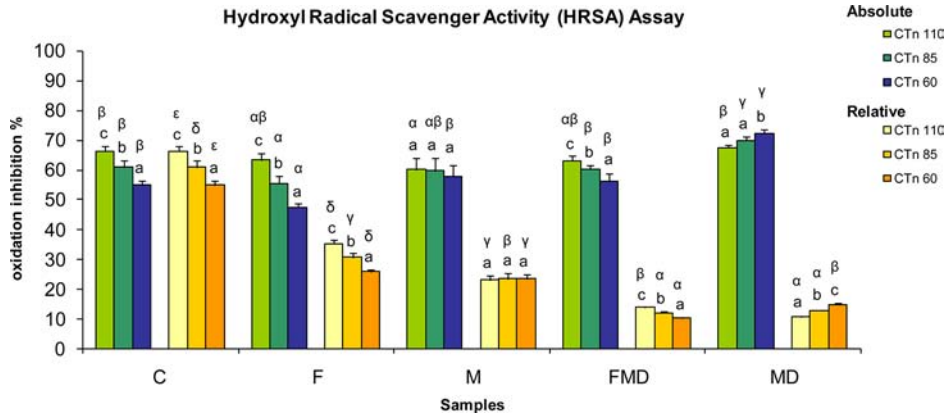

Figure 2. Determination of HRSA using the deoxyribose assay. The results are expressed as percent oxidation inhibition with respect to a control oxidation test using an absolute (per milligram of sample) or a relative (per milligram of coffee) approach. Data are expressed as mean values ± standard error (n = 3). Different letters indicate significant differences as a function of the roasting degree (CTn-110, CTn-85, and CTn-60). Different Greek letters refer to significant differences between the samples (C, F, M, FMD, and MD) obtained from each coffee.

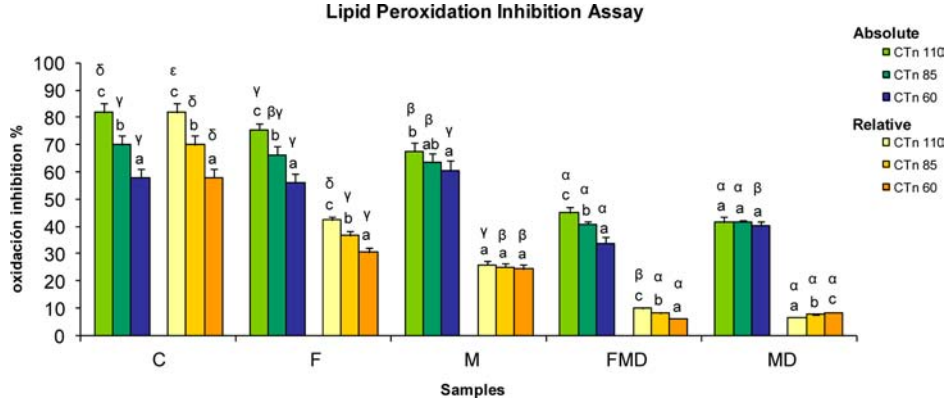

Figure 3. Determination of lipid peroxidation inhibition. The results are expressed as percent oxidation inhibition with respect to a control oxidation test using an absolute (per 10 μg of sample) or a relative (per 10 μg of coffee) approach. Data are expressed as mean values ± standard deviation (n = 3). Different letters indicate significant differences as a function of the roasting degree (CTn-110, CTn-85, and CTn-60). Different Greek letters refer to significant differences between the samples (C, F, M, FMD, and MD) obtained from each coffee.

enriched in purified melanoidins (MD). However, in the relative approach, the contribution to the overall TAC of coffee increased with degree of roasting for MD samples because of the increase in purified melanoidin content with the increase in the roasting degree.

It must be mentioned that phenolic compounds and melanoidins are not the only components that affect the antioxidant activity of instant coffee or coffee brews. Non-phenolic compounds, such as caffeine and derivatives, trigonelline or HMF, are also of interest because of their antioxidant properties and present different mechanisms of antioxidant activity.[36] The content of trigonelline and HMF in coffee is dependent upon the roasting degree, whereas caffeine remains stable.[40] Therefore, these compounds can influence the results of antioxidant activity observed, but their contribution has not been extensively discussed in this study.

HRSA of Instant Coffee Samples. Hydroxyl radicals are extremely reactive and may be generated under physiological conditions in the human body, where they can react with proteins, unsaturated fatty acids, DNA, etc.[4] We used the deoxyribose assay to detect possible scavengers of hydroxyl radicals. This assay had already been employed in a study that showed that coffee was able to inhibit hydroxyl-radical-induced oxidation by acting as a secondary antioxidant that prevent the generation of these radicals.[4] Other authors[2] studied the HRSA in green and roasted coffee and reported that the dialysates (molecular weight < 3500 Da) either from green or roasted coffee showed antiradical activity, while only the retentates (molecular weight > 3500 Da) from the roasted coffee samples were active. However, to the best of our knowledge, there are no previous studies regarding the effect of the degree of roasting or the gastrointestinal digestion of coffee on its HRSA. Our results, which are presented in Figure 2 as percent oxidation inhibition, show a decrease in the scavenger activity of C samples with an increase in the degree of roasting, specifically a 17.4% decrease of HRSA from CTn-110 to CTn-60. A similar behavior was also observed for filtrate samples (F and FMD). The M samples did not present significant differences between the three coffees, whereas an increase of HRSA from light (CTn-110) to dark (CTn-60) coffee was obtained for MD samples. It should be noted that, in contrast to the TAC results, the samples obtained after the gastrointestinal digestion of M samples (FMD and MD) present high HRSA values. Furthermore, the absolute approach shows that MD samples have the highest HRSA for all three coffees. The contribution of FMD and MD samples to the overall HRSA of coffee (relative approach) was dependent upon the degree of roasting. Thus, the FMD was more important in light-roasted

coffee. Both fractions made a similar contribution in medium-roasted coffee. MD presented a higher inhibition value in dark-roasted coffee. It should also be noted that a positive correlation was found between HRSA/intermediate MRPs content (r = 0.8107) and HRSA/final MRPs content (r = 0.5923).

Lipid Peroxidation Inhibition of Instant Coffee Samples. Lipid peroxidation inhibition was evaluated as a biomarker of oxidative lipid stress by determining the ability of samples to scavenge peroxyl radicals generated in the chain reactions that lead to microsome oxidation. As shown in Figure 3, the C-110 sample presented a high lipid peroxidation inhibition of 81.9% per 10 μg of coffee (with respect to a control oxidized test). Inhibition decreased from light- to dark-roasted coffee until 57.9%. This trend was maintained for the remaining samples (F, M, and FMD), except for the MD samples, in which no significant differences were observed. F samples contributed in a higher extend than the M samples to the protection against lipid oxidation (relative approach), whereas we found rather slight differences between the lipid peroxidation inhibition of FMD and MD samples. This results were rather similar to those obtained for the TAC, especially with regard to the DPPH assay (correlation between lipid peroxidation inhibition/DPPH, r = 0.9528). Taking into account the TPP and intermediate and final MRPs composition of the samples, these results agree with previous papers that have shown the importance of polyphenols in peroxidation inhibition[4] and the greater influence of compounds formed in the intermediate steps of the MR than the final products of these reactions on the peroxyl radical scavenging capacity.[7,8]

Protection of Instant Coffee Samples against DNA Oxidation (*in Vitro* and *ex Vivo* Genoprotective Effects). DNA protection against oxidative damage, which may lead to single- and double-strand breaks of DNA,[30] was the last oxidative stress biomarker to be assessed in this study. To evaluate the protection of DNA against oxidation, we carried out two determinations: the first determination *in vitro* (on calf thymus DNA) in agarose gels and the other determination *ex vivo* (on cell cultures), to assess the genoprotective effect of coffee.

Hydroxyl radicals induce DNA chain breakages, thereby generating smaller DNA fragments that can be separated by electrophoresis in agarose gels. Samples may avoid DNA damage as a result of their free radical scavenging properties, their copper(II)-chelating ability, thereby avoiding the generation of hydroxyl radicals in the presence of ascorbic acid, or direct DNA protection because of the interaction between DNA and the compounds present in the samples.[30] Figure 4 shows a photograph of one of the agarose gels obtained. The same amount of each sample (300 μL of a 10 mg/mL dilution) was used for incubation with DNA, and the degree of DNA protection was determined by comparing the DNA bands obtained in the gel for the different samples to those for a non-oxidized (C) and an oxidized (O) control. As far as the influence of the roasting degree of coffee on DNA protection is concerned, a slightly higher protection of CTn-110 coffee in samples C, F, and FMD was observed, whereas M samples protected DNA in a similar way, and DNA protection increased with the degree of roasting for the MD samples. The high protection provided by retentate fractions (M and MD), which was much higher than that provided by C samples and filtrates (F and especially FMD), was surprising. We have been unable to find any similar assay in coffee in the literature to make a comparison. However, because the HRSA assay did not provide as many differences between samples, it is possible that DNA protection is due to both the ability of coffee samples to scavenge hydroxyl radicals and the ability of melanoidins and other compounds with a high molecular weight present in coffee to prevent hydroxyl radical generation [by acting as efficient copper(II)-chelating agents], as well as their direct interaction with DNA to protect against oxidation.

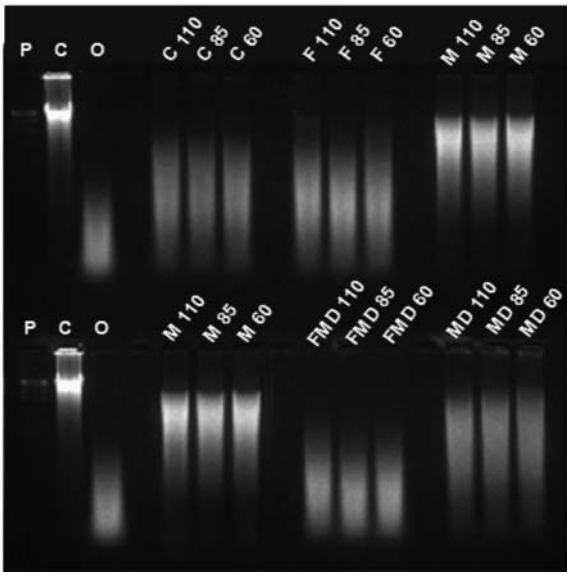

Figure 4. Agarose gel electrophoresis separation of DNA (protection of coffee samples against DNA oxidation). P, molecular weight standard from 23 100 to 125 pb; C, non-oxidized DNA control; and O, oxidized DNA control.

Finally, we researched the *ex vivo* genoprotective effect of instant coffee samples in the HT-29 cell line (human colon carcinoma cell line). DNA damage was evaluated using the "comet assay" or single-cell gel electrophoresis under alkaline conditions, where DNA fragments form the tail of the comet, as seen in the photographs of Figure 5. The comet assay had been previously used to study the *ex vivo* protective effects of coffee on the DNA of human lymphocytes after H_2O_2 oxidation, observing pronounced protective effects.[41] We therefore tried to determine whether the degree of roasting or the gastrointestinal digestion of melanoidins could influence the genoprotective effect of instant coffee. The results of this study are presented in Figure 5D and expressed as a percentage of the relative tail length with respect to the oxidized control ([T/C2] %) using the absolute approach. Every sample was found to protect DNA against oxidation, although a higher degree of DNA protection was found for the C and F samples of light-roasted coffee (CTn-110), with M samples showing no significant differences in terms of the degree of roasting. However, the most remarkable finding of the comet assay was that the samples obtained after melanoidin digestion (FMD and MD) provided the highest degree of DNA protection, thereby suggesting that digestion of the original melanoidins (M) induces modifications in their structures that lead to an increased genoprotective effect. After gastrointestinal digestion of M samples, FMD was found to present a higher genoprotective effect than MD for CTn-110 coffee, whereas MD provided a greater degree of inhibition of DNA oxidation than FMD for CTn-60 coffee. No significant differences were found between FMD and MD fractions for CTn-85 coffee,

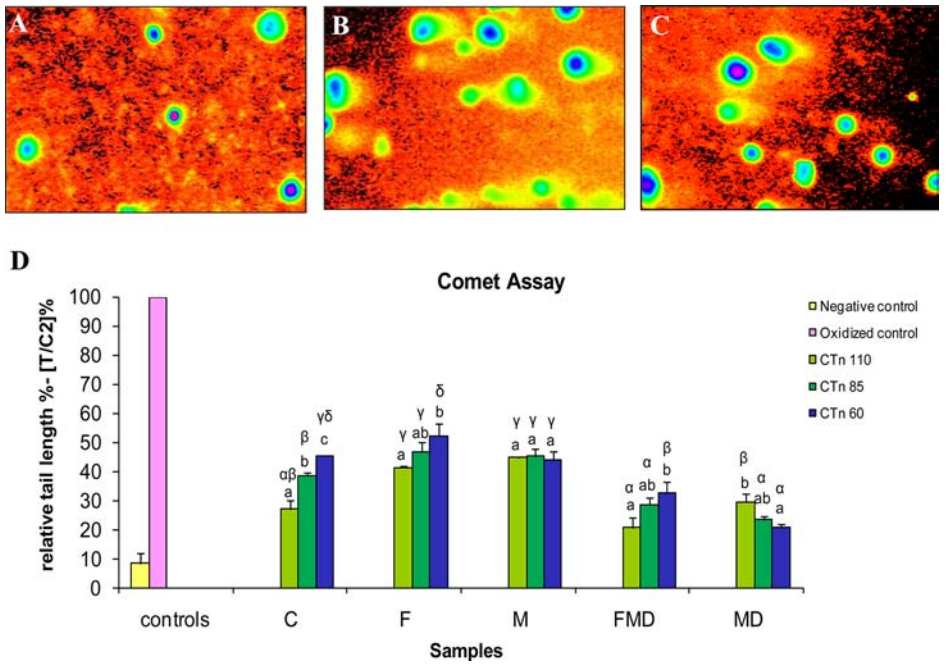

Figure 5. Comet assay in the HT-29 cell line. Photographs analyzed using the CometScore program: (A) non-oxidized control, (B) oxidized control (cells treated with menadione), and (C) cells incubated in the presence of 50 μg of sample CTn-110 and treated with menadione. (D) DNA migration evaluated by the comet tail length (n = 60 cells for each sample). The results are expressed as [T/C2] % = percentage of the relative tail length with respect to the oxidized control, following an absolute approach (per 50 μg of sample). Data are expressed as mean values ± standard error (n = 3). Different letters indicate significant differences as a function of the roasting degree (CTn-110, CTn-85, and CTn-60). Different Greek letters refer to significant differences between the samples (C, F, M, FMD, and MD) obtained from each coffee.

although both fractions presented a higher genoprotective effect than the original melanoidins of this instant coffee. Therefore, the digestion of the melanoidins from light-roasted instant coffee seems to result in the release of compounds from the melanoidin structure that present a higher protection against oxidation of DNA *in vivo* than the melanoidin core. Conversely, in dark-roasted coffee, the melanoidin core retained a higher genoprotective effect than the bound compounds released during the digestion of the original melanoidins. A high positive correlation was observed between this assay and the HRSA assay (r = 0.8218), where FMD and MD fractions also presented a high activity.

In conclusion, instant coffee has a high total antioxidant capacity and protective effect against certain oxidative stress biomarkers (lipids and DNA), although this capacity decreases with the roasting degree because of the lower antioxidant activity and lesser contribution of the low-molecular-weight compounds present in coffee as the degree of roasting increases. However, melanoidin-enriched fractions contribute considerably and in a similar manner to the overall antioxidant activity of coffee, irrespective of the degree of roasting. After gastrointestinal digestion of melanoidins, the compounds noncovalently linked to melanoidins in light-roasted coffee are the main contributors to the antioxidant activity, whereas in dark-roasted coffee, the purified melanoidin fractions contribute to a greater extent. Likewise, whereas phenolic compounds contribute more than melanoidins to the TAC and lipid peroxidation inhibition ability of coffee, HRSA and DNA protection against oxidative damage are mainly determined by its intermediate and final MRPs content. The main contribution to new knowledge in the field is the elevated genoprotective effect found in the fractions obtained after the digestion of original melanoidins present in instant coffee. In addition, our study confirms the hypothesis that several of the phenolic compounds originally present in coffee may form part of the melanoidins generated during the roasting process.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

C, coffee brews; CTn, color test number; F, filtrate samples from coffee brews; FMD, filtrate samples from digested melanoidins; FRAP, ferric reducing/antioxidant power; HRSA, hydroxyl radical scavenger activity; M, melanoidins retentated samples from coffee brews; MD, melanoidins retentated samples from digested melanoidins; MR, Maillard reactions; MRP, Maillard reaction product; TPP, total polyphenol; TAC, total antioxidant capacity

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