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Nature of Phenolic Compounds in Coffee Melanoidins

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Supporting Information

ABSTRACT: Phenolic compounds are incorporated into coffee melanoidins during roasting mainly in condensed form (42−62 mmol/100 g) and also in ester-linked form (1.1−1.6 mmol/100 g), with incorporation levels depending on the green coffee chlorogenic acid content. The phenolic compounds are incorporated in different coffee melanoidin populations, but mainly in those soluble in 75% ethanol (82%), a significant correlation between the amount of phenolic compounds and the amount of protein and color characteristics of the different melanoidin populations being observed. The incorporation of phenolic compounds into coffee melanoidins is a significant pathway of chlorogenic acid degradation during roasting, representing 23% of the chlorogenic acids lost. These account for the nearly 26% of the material not accounted for by polysaccharides and proteins present in coffee melanodins. The cleavage mechanism and the efficiency of alkaline fusion used to release condensed phenolics from coffee melanoidins suggest that the phenolic compounds can be linked to the polymeric material by aryl-ether, stilbene type, and/or biphenyl linkages.

KEYWORDS: *coffee, melanoidins, chlorogenic acids, phenolic, roasting, infusions*

INTRODUCTION

Melanoidins are still today one of the most enigmatic food macromolecules present in several heat-processed foods such as coffee, bread, malt, and beef,[1,2] with coffee brew being one of the main sources of melanoidins in the human diet.[3]

The exact structure of coffee melanoidins is still unknown, but recent structural studies allowed a partial elucidation of some of their structural features and highlighted their structural diversity.[4−10] Melanoidins' importance is not limited to their color contribution to heat-processed foods; these molecules are also important for their modulation of flavor release, antioxidant and metal chelating properties, and dietary fiber behavior.[11−13] The involvement of chlorogenic acids or their degradation products in coffee melanoidins can be due to the formation of either ester linkages[14,15] or condensed forms (non-ester covalent linkages) not yet completely structurally disclosed.[6,7,9] The relative amount of these two incorporation forms is also not known, nor is the relevance of phenolic compounds for the different properties of the melanoidin populations present in coffee brews.

The purpose of this work was to profile the different forms of phenolic compounds present in coffee melanoidins to assess their abundance in the different coffee brew melanoidin populations. The influence of green coffee bean chlorogenic acid levels on the amount of phenolic compounds incorporated into coffee melanoidins and in melanoidin color was also studied. For this, a modified "in bean" coffee model[16] enriched with a typical green coffee chlorogenic acid mixture was used.

MATERIALS AND METHODS

Chemicals. 5-Caffeoylquinic acid, caffeic acid, ferulic acid, coumaric acid, verartric acid, 2-deoxyglucose, fucose, galacturonic acid, glucuronic acid, and caffeine were obtained from Sigma (USA). 3,4-Dihydroxybenzoic acid, hydroquinone, catechol, gallic acid, benzoic acid, 2-hydroxybenzoic acid, 4-hydroxybenzoic acid, 2,3-dihydroxybenzoic acid, 3,5-dihydroxybenzoic acid, glucose, mannose, arabinose, and galactose were purchased from Merck (Darmstadt, Germany). 8-*O*-4-Dehydrodiferulic acid, 8,5(noncyclic)-dehydrodiferulic acid, and 5,5-dehydrodiferulic acid were isolated as described in Jilek and Bunzel.[17]

Chlorogenic Acids (CGA) Extraction and Purification. Green coffee beans (200 g) were frozen in liquid nitrogen, ground, and consecutively extracted with petroleum ether (24 h) followed by methanol (24 h) in a Soxhlet apparatus. The petroleum ether extract was discarded, and the CGA were recovered from the methanol extract by rotary evaporation under vacuum at 35 °C, dissolution in water, and freeze-drying. The extract was purified by Sephadex G-25 column chromatography, using water as eluent.[18] The eluted solution containing the chlorogenic acids was then treated with neutral lead acetate (5 mL, 0.5 M), and the initial gray precipitate formed was discarded. CGA were precipitated by further addition of neutral lead acetate until no precipitate was formed.[19] The precipitate was dissolved in 5% sulfuric acid and reprecipitated at pH 8; this precipitate was suspended in water, the remaining lead was removed with the 5% sulfuric acid solution, and the lead-free filtrate was freeze-dried. The composition and purity of the CGA purified from green coffee beans was determined by RP-HPLC as described below.

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Preparation of the Modified "in Bean" Green Coffee Models. Modified "in bean" models enriched with CGA were prepared using a previously developed method[16] with small modifications. Brazil arabica green coffee beans were initially freeze-dried until constant weight (6% weight loss). For the preparation of the chlorogenic acid-enriched coffee model (CGAcoffee), to 100 g of the freeze-dried green coffee beans was added 200 mL of a CGA aqueous solution (1.5 g/200 mL), and the mixture was shaken for 16 h at 4 °C. After incorporation, the coffee beans were washed with distilled water for removal of the surface-adhering solution and freeze-dried. As a control, 100 g of freeze-dried coffee beans was immersed in 200 mL of water under the same conditions.

Roasting of Coffee Bean Model Samples. Prior to roasting, all samples (original freeze-dried coffee, water control, and CGAcoffee) were adjusted to the same moisture of 6% by spraying enough water and checking coffee weight after 1 week. Then, the coffee beans (40 g) were roasted at 200 °C during approximately 5 min to obtain an organic roast loss of 8%.

Preparation of Coffee Brews and Isolation of the High Molecular Weight Material (HMWM). Roasted coffee beans were ground (250−450 μm), and infusions were prepared by extraction of 25 g of powdered coffee with 800 mL of water at 80 °C during 20 min. After filtration and vacuum concentration to 200 mL, 50 mL of coffee infusion was freeze-dried to calculate the total soluble solids. The other 150 mL was extensively dialyzed at 4 °C (six water renewals, MW cutoff 12−14 kDa). After dialysis, the retentate was freeze-dried, giving the HMWM.

Fractionation of the HMWM by Graded Precipitation in Ethanol. For the fractionation of HMWM for each coffee infusion, the method previously developed[20] was used with a modification;[16] for complete solubilization of the freeze-dried HMWM, a solution of 6 M urea was used. The HMWM was then precipitated by the addition of absolute ethanol. The material was recovered in the 50% ethanol solution (Et50) and in the 75% ethanol solution (Et75). It was resolubilized in water and dialyzed (six water renewals, MW cutoff 12−14 kDa). After dialysis, the retentate was freeze-dried. The material soluble in the 75% ethanol (EtSn) fraction was recovered after concentration, dialysis, and freeze-drying.

Chlorogenic Acids and Caffeine Content of Green and Roasted Coffees. CGA were extracted overnight from green and roasted ground coffees (1 g) with 100 mL of a solution of methanol/water (70:30 v/v) containing 0.5% Na_2SO_3 with constant stirring (125 rpm) in the dark. To the solution was added 1 mL of veratric acid as internal standard (100 mg/mL, in methanol). After filtration of the coffee powder, the colloidal material present in the extracts was precipitated by the addition of 1 mL of Carrez I and II solutions.[21] The extracts were analyzed by HPLC (Dionex, Ultimate 3000) by injection of 25 μL of the sample on a reversed-phase HPLC column (C18-ACE; 25 cm length, 0.45 cm internal diameter, and 5 μm particle diameter). Eluent A was a 5% formic acid aqueous solution, and eluent B was methanol. The eluent program was as follows: 0−5 min, 5% eluent B; 5−45 min, 40% B; 45−65 min, 70% B; 65−75 min, 5% B. The column temperature was set at 25 °C, and the flow was 0.8 mL/min. The eluent was continuously monitored from 200 to 600 nm with a photodiode array detector (PDA-100, Dionex). The identification of caffeine and 5-caffeoylquinic acid was made by comparison of their retention times and UV−vis spectra with those of pure standards analyzed under the same conditions. The other caffeoylquinic acids (CQA) and cinnamoyl-1,5-γ-quinolactones (CQL) were tentatively identified by comparison of their retention times and UV−vis spectra with those of the literature.[22] CGA were quantified by the internal standard method using a 5-CQA calibration curve. Caffeine was quantified using the same methodology and a caffeine calibration curve.

Characterization of Coffee Bean Samples and Fractions. Green and roasted coffee and coffee models were characterized regarding their sucrose, glucose, and fructose contents by high-performance anion exchange chromatography.[23] Total sugars content was determined by anion exchange chromatography after Saeman and acid hydrolysis.[20] Protein content was determined according to the Dumas method (PRIMACS, carbon−nitrogen/protein analyzer, Skalar, The Netherlands) by multiplying the nitrogen content by 6.25. The melanoidin content of fractions was determined by the K_{mix} at 405 nm as previously described.[4]

Color of Roasted Coffee Powder. Roasted coffee powder colors obtained for the original, control, and "in bean" CGAcoffee model were directly measured with a Minolta chroma meter (model CR-400, Minolta, Tokyo, Japan). The equipment was set up for illuminant D65 and 10° observer. The equipment was calibrated with a white standard (L^* = 97.71, a^* = −0.59, and b^* = 2.31), and each sample was put on a Petri dish and read in five different locations. Numerical values of chroma (C^*), hue (h), and color difference (ΔE^*) were calculated according to the following formulas:

$$h^* = \tan^{-1}\left(\frac{b^*}{a^*}\right)$$

$$C^* = (a^{*2} + b^{*2})^{1/2}$$

$$\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$$

Total Phenolic Groups Content. The total phenolic groups content of the coffee samples was determined with the Folin−Ciocalteu reagent.[4] For all coffee samples, a 0.33 or 1 mg/mL solution was prepared. To 1 mL of the coffee sample solution was added 0.5 mL of Folin−Ciocalteu reagent. After mixing of the sample, 1 mL of a saturated Na_2CO_3 solution was added, and filtered demineralized water was added to reach a total volume of 10 mL. After 1 h of reaction, the absorbance of the sample was measured at 725 nm. 5-CQA was used as reference.

Adsorbed Phenolic Compounds. For determination of coffee melanoidins adsorbed phenolic compounds, a 5 mg/mL solution of coffee melanoidins was analyzed by direct injection (100 μL) on a RP-HPLC according to the method described previously for CGA determination. For determination of the effect of melanoidins in the determination of free phenolic compounds, melanoidin solutions (5 mg/mL) were spiked with different concentrations of 5-CQA and incubated overnight at room temperature. Each solution was analyzed by RP-HPLC as described previously, and the areas were compared with that of aqueous solutions of 5-CQA with the same concentrations.

Alkaline Saponification. For determination of ester-linked phenolic compounds, melanoidins were subjected to alkaline saponification by using a procedure that prevented oxidation of phenolic compounds.[24] To 750 μL of the melanoidin solution (12 mg/mL) was added 750 μL of a 2 M NaOH solution containing 2% (w/w) ascorbic acid and 20 mM ethylenediaminetetraacetic acid. After incubation for 1 h at 30 °C, the mixture was quenched to pH ≈1 with 330 μL of 5 M HCl to precipitate most of the coffee material, preventing precipitation during further analysis. The mixture was stored for 2 h at 4 °C, the precipitate was removed by centrifugation, and the supernatant was analyzed by reversed-phase HPLC. Experiments were performed at least in duplicate. The identification of caffeic, ferulic, and 4-hydroxycinnamic acids was made by comparison of their retention times and UV−vis spectra with that of pure standards analyzed under the same conditions. Caffeic, ferulic, and 4-hydroxycinnamic acids were quantified by external calibration.

Alkaline Fusion. In a nickel crucible were weighed 1 g of solid NaOH and 100 mg of zinc dust, and, after fusion of the mixture at 350 °C,[6,25] 5 mg of HMWM was added (or 5 mg of melanoidin fractions). After 10 s, the nickel crucible was removed and rapidly cooled on ice. The fusion cake was solubilized by adding 6 M HCl, and 200 μL of internal standard solution was added (3,4-dimethoxybenzoic acid, 1 mg/mL) and acidified to pH 1−2 with 6 M HCl. The acidic mixture was extracted four times with 30 mL of diethyl ether. After evaporation of the organic solvent, carefully and with protection, the residue was derivatized and analyzed by GC-EI/MS. Samples were derivatized with 0.5 mL of pyridine and 0.5 mL of *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide and heated at 70 °C for 30 min. After cooling to room temperature, the silyl derivatives of phenolic compounds were

Table 1. Soluble Sugar, Caffeine, and Protein Contents (Grams per 100 g, Dry Basis) of Original, Control, and "in Bean" Green and Roasted[a] Coffee Models

coffee	glucose	fructose	sucrose	total	caffeine	protein[b]
green						
original	0.018 ± 0.001	0.60 ± 0.00	7.45 ± 1.32	8.07 ± 1.32	1.37 ± 0.01	14.0 ± 0.9
control	0.018 ± 0.001	0.59 ± 0.00	5.78 ± 1.10	6.39 ± 1.10	1.12 ± 0.02	14.1 ± 0.7
CGAcoffee	0.017 ± 0.001	0.58 ± 0.00	5.08 ± 0.98	5.69 ± 0.99	1.07 ± 0.05	14.0 ± 1.1
roasted						
original	0.018 ± 0.001	0.008 ± 0.002	0.64 ± 0.06	0.66 ± 0.07	1.42 ± 0.15	9.5 ± 0.7
control	0.018 ± 0.001	0.019 ± 0.001	0.99 ± 0.02	1.07 ± 0.05	1.23 ± 0.12	12.3 ± 0.9
CGAcoffee	0.017 ± 0.001	0.032 ± 0.025	3.07 ± 0.07	3.16 ± 0.02	1.11 ± 0.07	11.8 ± 0.5

[a]Not corrected for weight loss during roasting. [b]$(N_{total} - N_{caffeine}) \times 6.25$.

Table 2. Chlorogenic Acid (CGA) Composition of Original, Control, and "in Bean" Green and Roasted[a] Coffee Model (Grams per 100 g, Dry Basis)

	green			roasted		
CGA	original	control	CGAcoffee	original	control	CGAcoffee
3-CQA	0.53 ± 0.02	0.44 ± 0.03	0.44 ± 0.01	0.48 ± 0.04	0.43 ± 0.02	0.53 ± 0.00
5-CQA	3.36 ± 0.02	2.92 ± 0.17	3.34 ± 0.20	1.04 ± 0.10	1.12 ± 0.07	1.07 ± 0.02
4-CQA	0.007 ± 0.001	0.001 ± 0.001	0.006 ± 0.002	0.17 ± 0.03	0.20 ± 0.02	0.11 ± 0.00
total CQA	3.90 ± 0.03	3.37 ± 0.20	3.79 ± 0.20	1.70 ± 0.16	1.75 ± 0.11	1.72 ± 0.02
1-FQA	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
3-FQA	0.70 ± 0.05	0.60 ± 0.07	0.63 ± 0.05	0.76 ± 0.02	0.69 ± 0.04	0.88 ± 0.01
5-FQA	0.05 ± 0.01	0.04 ± 0.00	0.04 ± 0.00	nd	nd	nd
4-FQA	0.003 ± 0.005	0.003 ± 0.004	0.003 ± 0.004	nd	nd	nd
total FQA	0.77 ± 0.06	0.65 ± 0.07	0.68 ± 0.06	0.77 ± 0.02	0.69 ± 0.04	0.89 ± 0.01
3-CoQA	nd	nd	nd	nd	nd	nd
5-CoQA	0.007 ± 0.001	0.008 ± 0.003	0.006 ± 0.002	nd	nd	nd
4-CoQA	0.04 ± 0.02	0.03 ± 0.02	0.03 ± 0.02	nd	nd	nd
total CoCQA	0.05 ± 0.02	0.04 ± 0.02	0.03 ± 0.02	nd	nd	nd
3-CQL	nd	nd	nd	0.04 ± 0.02	0.04 ± 0.00	0.04 ± 0.00
4-CQL	nd	nd	nd	0.09 ± 0.03	0.09 ± 0.01	0.09 ± 0.00
3-FQL	nd	nd	nd	0.01 ± 0.00	0.01 ± 0.00	0.04 ± 0.05
4-FQL	nd	nd	nd	0.02 ± 0.01	0.02 ± 0.00	Nd
1-FQL	nd	nd	nd	0.04 ± 0.01	0.06 ± 0.00	0.03 ± 0.03
total FQL	nd	nd	nd	0.08 ± 0.02	0.09 ± 0.01	0.07 ± 0.02
3,4-diCQA	0.18 ± 0.00	0.16 ± 0.02	0.31 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	nd
3,5-diCQA	0.30 ± 0.01	0.26 ± 0.05	0.48 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.06 ± 0.02
total diCQA	0.48 ± 0.01	0.42 ± 0.07	0.79 ± 0.02	0.08 ± 0.01	0.08 ± 0.01	0.06 ± 0.02
total (CGA)	5.19 ± 0.13a	4.48 ± 0.09ab	5.43 ± 0.20bc	2.53 ± 0.19	2.52 ± 0.16	2.67 ± 0.00

[a]Not corrected for weight loss during roasting. Identical letters in the same row are significantly different.

analyzed by GC-MS (Agilent) and quantified by GC-FID (PerkinElmer) using a DB-1 column (30 m length, 0.23 mm internal diameter, and 0.2 μm film thickness) and injecting 1 μL in splitless mode (time of splitless, 0.75 min). Both the injector and the transfer line were set at 250 °C. The initial column temperature was 70 °C, held for 1 min, increasing at 5 °C/min until 250 °C. The MS source temperature was set at 180 °C, and the electron ionization energy was set at 70 eV, with scans from m/z 40 to 600. Retention times and electron impact ionization spectra of pure standards were used to identify the following compounds: 3,4-dihydroxybenzoic acid, hydroquinone, catechol, gallic acid, benzoic acid, 2-hydroxybenzoic acid, 4-hydroxybenzoic acid, 2,3-dihydroxybenzoic acid, and 3,5-dihydroxybenzoic acid. Other phenolic compounds were identified by comparison of their mass spectra with those of the Wiley mass spectra database (Wiley 8). Quantification was performed by the internal standard method using 3,4-dimethoxybenzoic acid as the internal standard.

Statistical Analysis. All chemical analyses of green coffees, roasted coffees, soluble solids, HMWM, and fractions obtained by ethanol fractionation of the HMWM were performed in duplicate unless otherwise stated. Significant differences ($p < 0.05$) in chemical composition were analyzed by one-way ANOVA using the software Statistica 8.0 (StatSoft, Inc., Tulsa, OK, USA). A Newman−Keuls post hoc test was performed for detecting significantly different means ($p < 0.05$). For calculating the correlation between the phenolic compounds determined by the Folin−Ciocalteau method and those released by alkaline saponification, the nonparametric Spearman correlation coefficient (ρ) and the parametric Pearson correlation coefficient (r) were used.

Table 3. Total Sugar[a] Composition (Grams per 100 g, Dry Basis) of Original, Control, and "in Bean" Green and Roasted Coffee Models[b]

	Ara	Man	Gal	Glc	$Glc_{polymeric}$	total	$total_{polymeric}$
green							
original	1.71 ± 0.04	22.7 ± 0.2	12.7 ± 0.2	11.2 ± 1.0	7.61	48.3 ± 1.1	44.7
control	1.86 ± 0.81	26.3 ± 7.9	13.2 ± 2.2	9.81 ± 3.58	6.60	51.2 ± 4.5	48.0
CGAcoffee	2.12 ± 0.44	30.8 ± 2.2	14.7 ± 0.7	10.3 ± 1.3	7.44	57.9 ± 4.6	55.0
ANOVA	0.758	0.359	0.412	0.835		0.169	
roasted							
original	1.62 ± 0.20	18.0 ± 2.5	7.61 ± 1.01	8.41 ± 1.23	8.07	35.7 ± 5.0	35.4
control	1.99 ± 0.27	20.3 ± 2.2	8.86 ± 1.18	9.30 ± 0.80	8.75	40.4 ± 4.4	39.9
CGAcoffee	1.92 ± 0.05	20.6 ± 0.4	9.37 ± 0.07	10.2 ± 0.2	8.60	42.0 ± 1.1	40.4
ANOVA	0.276	0.444	0.276	0.259		0.370	

[a]Values expressed in anhydrosugar residues. [b]Not corrected for weight loss during roasting.

RESULTS AND DISCUSSION

With the purpose of studying the effect of the green coffee CGA content, if any, in the incorporation of phenolic compounds into coffee melanoidins, a modified "in bean" model coffee was produced by enrichment of the freeze-dried coffee beans with a CGA mixture isolated and purified from green coffee beans. The procedure for production of the modified "in bean" coffee model was performed at 4 °C to minimize side reactions. This was a modification from our previous work, in which the incorporation was performed at room temperature.[16]

Chemical Characterization of Green and Roasted Coffee Samples and Model. *Green Coffee.* As can be seen in Table 1, the sucrose hydrolysis was significantly lowered in this new protocol when compared to the previous one, when a significant sucrose hydrolysis was observed.[16] Original green coffee had a total soluble sugar content of 8.1%, mainly composed of sucrose (92%) and low amounts of glucose and fructose (Table 1), which is in accordance with the literature values.[26] For the control coffee there was a decrease of 20% of total soluble sugar content when compared with the original green coffee, only due to the decrease in sucrose. For the CGAcoffee model the amounts of sucrose, fructose, and glucose are similar to those of the control coffee (Table 1). This decrease in the amount of soluble sugars can be due to leaching of compounds during the incorporation process and contrasts with the previous procedure at room temperature,[16] when all of the solution was incorporated into coffee beans.

The amount of caffeine present in the original coffee was significantly higher than that present in control and CGAcoffee model, but the protein content of the coffees, estimated on the basis of the nitrogen content after correction for caffeine nitrogen, was not significantly different (Table 1).

The amount of total CGA in the CGAcoffee model was significantly higher (21% higher) than that present in control coffee, showing that the incorporation process was successful (Table 2). As observed for the soluble sugars, control coffee contained a significantly lower amount of CGA. The CGA profiles were similar in all coffees, with 5-CQA as the most abundant, representing, on average, 64% of the total CGA.

The sugar composition of green coffee polysaccharides was determined by subtracting from the total sugar content the contribution of glucose and sucrose present as soluble sugars (Table 3). The sugar content was in accordance with the known sugar composition of coffee cell wall polysaccharides,[16,27,28] not significantly different for the original, control, and CGAcoffee model.

The results presented showed that this new protocol for production of the modified "in bean" model coffees results in fewer changes in the chemical composition of control coffee in relation to the original coffee when compared with the original protocol,[16] being more suitable for the study of the incorporation of selected compounds into green coffee beans prior to the roasting process.

Roasted Coffee. Original, control, and CGAcoffee model coffees were roasted to 8.0 ± 0.5% of organic matter loss, and their chemical composition was determined. After the roasting process, losses of 92% of the total soluble sugar content of the original coffee, 84% for the control, and only 44% for the CGAcoffee model were observed (Table 1). This difference in total soluble sugar loss may be due to the higher content of CGA in the CGAcoffee model that might be preferentially lost during the roasting process (further discussed below). The decrease in the total soluble sugar in roasted original and control coffees are similar to that described in the literature.[16,29] The caffeine level remained unchanged, and the protein content of roasted coffees decreased between 20 and 38% (Table 1).

The amount of total CGA also decreased during the roasting process in all coffee models (Table 2). For the original and CGAcoffee model, the amount of total CGA decreased 55%, and a decrease of 48% was observed for control coffee. This decrease is in accordance with a coffee submitted to a degree of roast of 8%.[30]

The content of polymeric sugars had also a significant decrease after the roasting process (Table 3). All coffee samples behaved similarly, with an average decrease of 28% in the content of total polymeric sugars, in accordance with the literature.[31,32] For all coffees, when calculated for the equivalent weight of green coffee, the polymeric glucose levels were maintained or decreased slightly. For all other sugars, when calculated for the equivalent weight of green coffee, there was observed a decrease (17% for arabinose, 30% for mannose, and 41% for galactose).

The identical degradation profile of the studied coffee constituents led to the conclusion that the freeze-drying of green coffee beans and their immersion in water did not change significantly the behavior and properties of coffee during the roasting process.

During the roasting process, browning reactions occur, contributing to the coffee brown color formation. By visual inspection of the interior of roasted coffee beans, an even distribution of the brown color could be observed. The CIELAB color space used to characterize the coffee powders

Table 4. Yield and Chemical Composition (Grams per 100 g, Dry Basis) of the High Molecular Weight and Resulting Ethanol-Precipitated Fractions of the Original, Control, and "in Bean" Coffee Models

	g/100 g coffee	g/100 g HMWM	Ara	Man	Gal	Glc	total	protein	$K_{mix405\ nm}$	MBI	phenolics[a]
original											
soluble solids	22.5								0.75 ± 0.03		
HMWM	2.39		11.1 ± 0.3a	20.2 ± 0.3	19.8 ± 0.7a	0.50 ± 0.09a	51.6 ± 1.3	9.9 ± 0.3a	1.40 ± 0.05a	3.63	4.59 ± 0.24a
Et50		34.8	3.9 ± 0.9	30.1 ± 3.0	9.4 ± 2.0	0.53 ± 0.09	44.0 ± 0.3	2.3 ± 0.1	0.85 ± 0.01	1.58	1.16 ± 0.10
Et75		12.5	14.2 ± 0.6	11.6 ± 0.2	32.8 ± 2.6	0.50 ± 0.07	59.1 ± 3.4	12.1 ± 0.5	0.82 ± 0.02	2.85	1.69 ± 0.20
EtSn		52.7	16.8 ± 1.2	5.5 ± 0.4	23.8 ± 1.2	1.63 ± 1.48	47.7 ± 4.4	14.3 ± 0.4	1.37 ± 0.11	3.61	5.36 ± 0.33
control											
soluble solids	14.9								0.74 ± 0.08		
HMWM	3.73		11.5 ± 1.3b	17.1 ± 1.9	17.2 ± 1.9b	0.45 ± 0.15ab	46.2 ± 5.2	14.1 ± 0.8ab	0.89 ± 0.00ab	2.24	4.50 ± 0.61b
Et50		32.7	2.7 ± 0.1	34.9 ± 1.1	4.85 ± 0.22	1.54 ± 1.27	44.0 ± 0.2	5.2 ± 0.2	0.89 ± 0.03	1.75	1.57 ± 0.04
Et75		16.8	19.2 ± 3.8	7.9 ± 1.7	30.4 ± 5.5	0.49 ± 0.08	57.9 ± 11.0	12.3 ± 0.5	0.56 ± 0.02	1.88	1.93 ± 0.13
EtSn		50.5	16.7 ± 1.8	4.0 ± 0.4	20.4 ± 2.2	0.44 ± 0.05	41.6 ± 4.4	14.6 ± 0.3	1.07 ± 0.19	2.44	7.28 ± 0.18
CGAcoffee											
soluble solids	18.4								0.70 ± 0.00		
HMWM	3.86		5.94 ± 0.6ab	20.1 ± 2.0	12.5 ± 1.3ab	−ab	38.6 ± 4.0	18.9 ± 1.0ab	1.27 ± 0.15b	2.99	6.19 ± 0.36ab
Et50	1.44	37.3	1.70 ± 0.4	38.7 ± 9.2	3.5 ± 0.8	1.02 ± 0.28	44.9 ± 10.1	4.8 ± 0.4	0.56 ± 0.09	1.11	1.55 ± 0.16
Et75	0.26	6.7	11.3 ± 1.7	11.1 ± 1.9	25.3 ± 3.7	1.13 ± 0.31	48.7 ± 7.6	14.7 ± 0.5	0.88 ± 0.13	2.40	3.13 ± 0.27
EtSn	2.16	56.0	13.2 ± 0.9	3.5 ± 0.2	14.8 ± 0.3	0.87 ± 0.48	32.3 ± 1.5	27.9 ± 0.7	1.27 ± 0.07	3.19	8.20 ± 0.45
ANOVA			0.0121	0.234	0.0296	0.0277	0.0940	0.0030	0.0234		0.049

[a]Phenolics measured by the Folin−Ciocalteu method and expressed as 5-CQA equivalents. Identical letters in the same column are significantly different.

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showed that the C^* value (chroma) of the CGAcoffee model was significantly higher than that of the control, showing that the incorporation of CGA increased the color intensity of the resulting roasted coffee. Furthermore, this increase is related to a significant increase of the b^* value for the CGAcoffee model, showing that the CGAcoffee model had a yellower color than that presented by the control.

Chemical Composition of Coffee Infusions and HMWM. Coffee infusions were prepared from the original, control, and CGAcoffee model, and the HMWM was isolated from each coffee infusion. The amount of soluble solids extracted from control coffee was lower (33% less) than that extracted for the original coffee (Table 4). For the CGAcoffee model an increase of 23% was observed in the amount of soluble solids in relation to control coffee. However, using the $K_{mix\ 405}$ values for estimating the melanoidins present in soluble solids, it can be observed that the incorporation of CGA did not significantly increase the $K_{mix\ 405}$ value of the soluble solids in relation to the control.

The chemical composition of the HMWM isolated from coffee infusions prepared from the original, control, and CGAcoffee model is shown in Table 4. The main component of all the HMWM was the carbohydrates (39−52%), with no significant differences between the coffees, followed by the protein (10−19%), with CGAcoffee model containing a higher amount of protein when compared to the control (+34%). The amount of phenolic compounds was also significantly higher for the CGAcoffee model when compared to the control (+38%), with the latter presenting a similar amount of phenolic compounds to that present in the original coffee HMWM. From these results it is evident that the incorporation of CGA in the "in bean" model resulted in an increase in the amount of phenolic compounds and protein in the HMWM.

The original coffee HMWM presented a significantly higher $K_{mix\ 405\ nm}$ value when compared to control coffee that also presented a significantly lower $K_{mix\ 405\ nm}$ value than the CGAcoffee model. In fact, the proportion of $K_{mix\ 405\ nm}$, indicative of the brown color of the HMWM, in relation to the $K_{mix\ 405\ nm}$ of the soluble solids was higher for the CGAcoffee model (37%), being the lowest for the control coffee (19%). By subtracting the percentage of carbohydrates and protein present in the HMWM, the amount of unknown material can be calculated. The results show that it is similar for all coffees and has an average value of 60%. Also, and as previously observed,[16] there is no trend between the amount of unknown material and the color of the HMWM determined by the $K_{mix\ 405\ nm}$ value. Using the melanoidin browning index (MBI), calculated by dividing the $K_{mix\ 405\ nm}$ values by the relative amount of unknown material,[16] which corresponds to the absorbance at 405 nm by unit amount of unknown material (Table 4), it can be seen that the enrichment of CGAcoffee with CGA increased by 33% the MBI. These observations allow inferring that the CGA have a significant influence on the color of the HMWM, although not increasing significantly the amount of unknown material.

Profiling Phenolic Compounds in Coffee HMWM. To evaluate the different natures of phenolic compounds present in the HMWM, either adsorbed, ester-linked, or in condensed form, as well the effect of the green coffee CGA levels in the phenolic compounds profile, the HMWM was subjected to alkaline saponification and alkaline fusion.

Adsorbed Phenolic Compounds. The amount of adsorbed phenolic compounds was determined by direct injection of the HMWM solutions on an RP C-18 column. To determine the possible influence of the HMWM matrix on the determination of phenolic compounds, a solution containing 5 mg/L HMWM and different amounts of 5-CQA was compared to the amount determined by injection of the same amounts of 5-CQA in water. The regression line obtained by representing the amount determined in water in relation to the amount determined in the presence of HMWM was $y = 0.9962x + 0.2584$ with $r = 0.9998$, indicating that the presence of HMWM did not interfere in the determination of phenolic compounds by RP-HPLC. The direct injection of HMWM showed the presence of intact CGA acids in all HMWMs (Figure 1a), mainly 5-CQA, although the presence of diCQA and 3CQA could also be detected (not shown). The amount of adsorbed CGA ranged from 0.1 to 0.7 mmol/100 g HMWM.

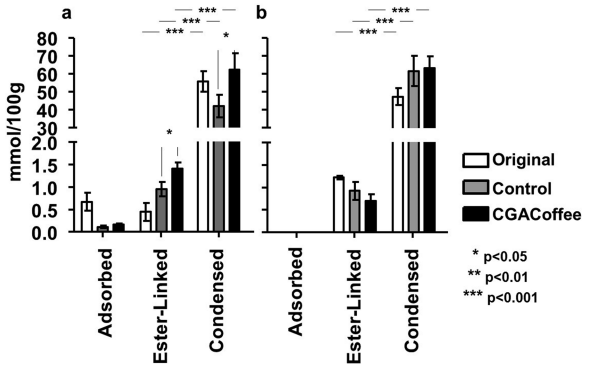

Figure 1. Profile of phenolic compounds present in (a) original, control, and CGAcoffee model HMWM; (b) original, control, and CGAcoffee model 75% ethanol soluble melanoidin population.

Ester-Linked Phenolic Compounds. The presence of ester-linked phenolic compounds in the HMWM from original, control, and CGAcoffee was determined after saponification. This procedure, known to release ester-linked phenolic compounds,[33] allowed the release of simple phenolic compounds from the HMWM, including caffeic, ferulic, and coumaric acids (Figure 2). Caffeic acid was the main cinnamic acid detected in all analyzed HMWMs. These simple phenolic compounds released by alkaline saponification have their origin in the adsorbed CGA present but also, probably, in the CGA incorporated into melanoidins, with structures where the cinnamic acids are esterified and maintain unchanged their structure. The amount of phenolic compounds released by saponification ranged from 1.1 to 1.6 mmol/100 g, which was significantly higher than that found adsorbed in the HMWM. For calculating the amount of ester-linked phenolic compounds in the HMWM, the amount of simple phenolics released by saponification was subtracted from the amount of adsorbed phenolics (Figure 1a). As can be observed, the amount of ester-linked phenolic compounds present in the HMWM ranged from 0.5 to 1.4 mmol/100 g. The values are in the range of those found in a previous study,[15] although lower values have been reported[14] or even not detected after alkaline hydrolysis,[6,9] probably due to their oxidation during extraction.[24] The amount of ester-linked phenolic compounds present in the CGAcoffee model was significantly higher than that present in the HMWM of control, showing that the incorporation of phenolic compounds in the CGAcoffee resulted in a significantly higher amount of ester-linked phenolic compounds in the HMWM.

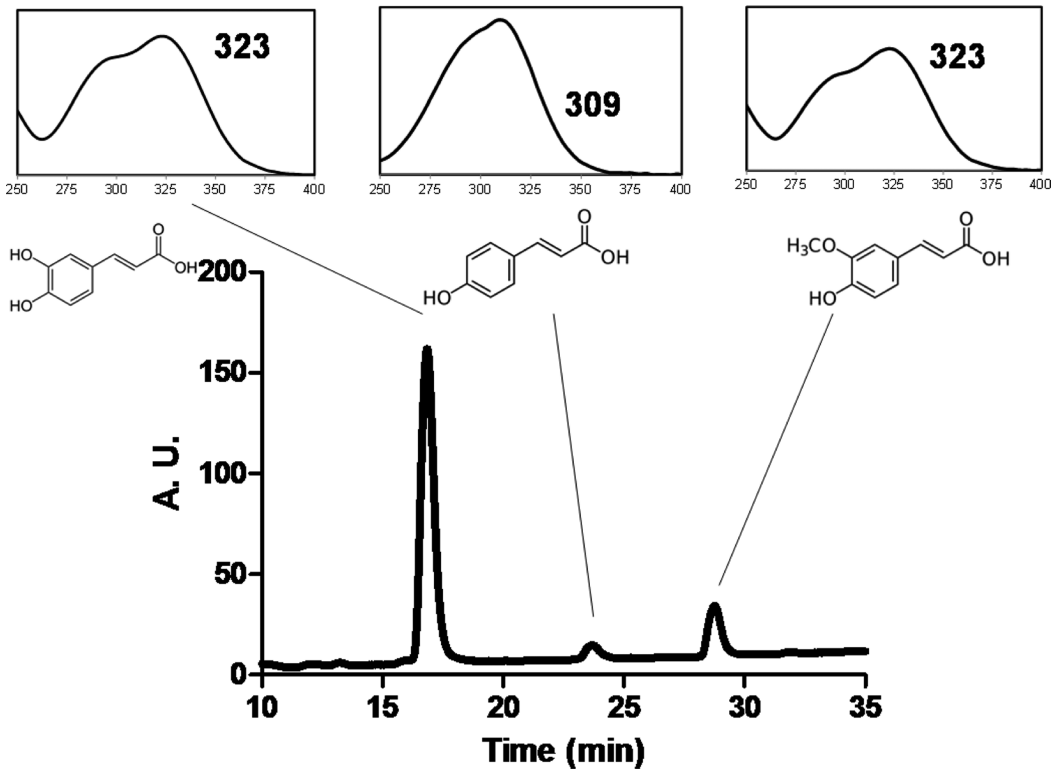

Figure 2. Phenolic compounds ester linked to the HMWM.

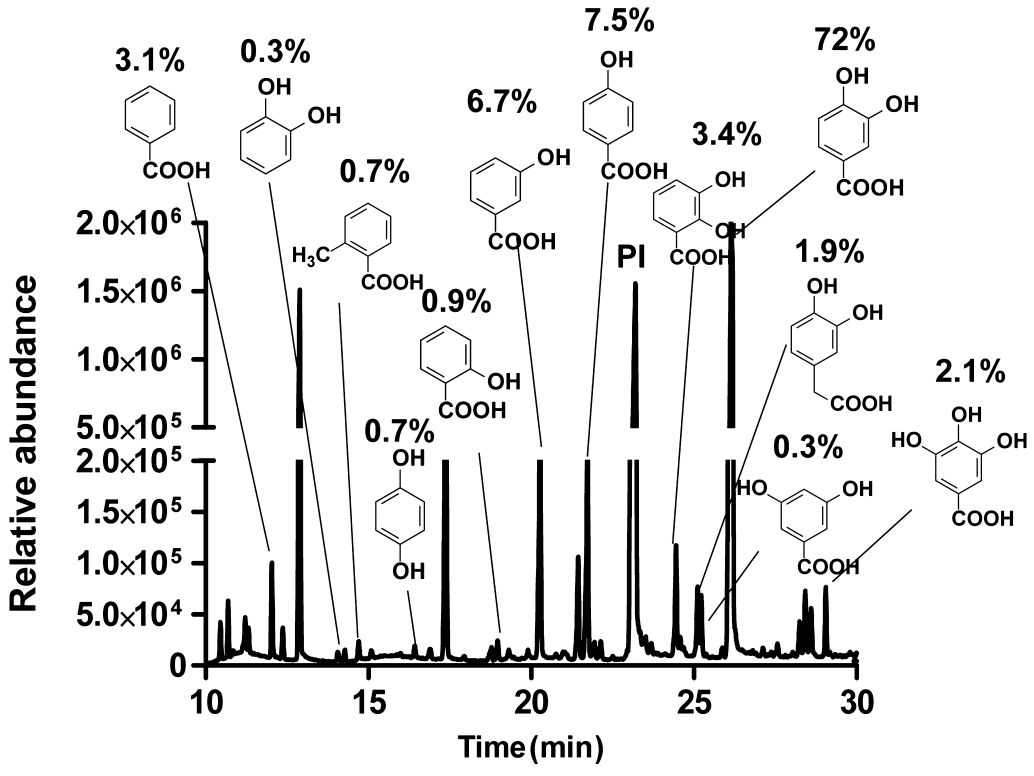

Figure 3. Nature and relative abundance of phenolic compounds released by alkaline fusion of the high molecular weight material recovered from the "in bean" CGAcoffee model.

Condensed Phenolic Compounds. For determining the amount of condensed phenolic compounds present in the HMWM, the samples were subjected to alkaline fusion. As can be observed in Figure 3, more than 10 simple phenolic compounds were released from the HMWM after alkaline fusion. 3,4-Dihydroxybenzoic acid was the most abundant phenolic compound in all samples. The abundance of the different phenolic compounds, expressed as the relative areas,

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was not significantly different for the different HMWMs studied. The amount of phenolic compounds released by alkaline fusion was significantly higher than the amount of phenolic compounds released by alkaline saponification by a factor of approximately 40 times and ranged from 42 to 62 mmol/100 g. These values are in the range of those reported by Nunes et al.[16] and Nunes and Coimbra[6] for HMWMs isolated from coffee infusions and higher than those found by Takanake et al.[9] for a zinc chelating fraction isolated from instant coffee. The low abundance of esterified phenolic compounds if compared to condensed phenolics present in coffee melanoidins is in accordance with the results of Gniechwitz et al.,[10] who performed an NMR analysis of an isolated intact melanoidin fraction and could not observe HSQC signals diagnostic for intact caffeic acid moieties. When the amount of phenolic compounds present in condensed form was calculated by subtracting the amount of phenolic compounds released by alkaline fusion from that released by alkaline saponification, it can be observed that the amount of phenolic compounds present in condensed form in the HMWM recovered from the CGAcoffee was significantly higher than that present in control. As observed for the ester-linked phenolic compounds, the incorporation of phenolic compounds in CGAcoffee model also increased the amount of condensed phenolic structures in the HMWM (Figure 1a). Expressing the amount of condensed phenolic structures in equivalent weight of caffeic acid, it can be concluded that the HMWM contained 7.5 g of condensed phenolics/100 g for control coffee, 10.0 g/100 g for the original coffee, and 11.2 g/100 g for the CGAcoffee model, showing that these condensed phenolic structures are an important component of the HMWM. In fact, the contribution of these condensed phenolic structures to the overall weight of the HMWM can be higher as some of the phenolic compounds bound to the HMWM seem to remain esterified with quinic acid after roasting.[14]

Mechanism and Efficiency of Alkaline Fusion. The alkaline fusion method is known to release phenolic compounds from condensed structures such as lignin and humic acids.[25] Nevertheless, there is a lack of knowledge about the nature of linkages between phenolic compounds cleaved by alkaline fusion and the efficiency of their cleavage. To have a deeper insight into which types of linkages are cleaved by alkaline fusion and to what extent, a series of standard phenolic compounds containing aryl-ether linkages (8-*O*-4-dehydrodiferulic acid), stilbene-type linkages (8,5(noncyclic)-dehydrodiferulic acid), and biphenyl linkages (5,5-dehydrodiferulic acid) between ferulic acids were subjected to alkaline fusion. The phenolic compounds containing aryl-ether linkages and stilbene-type linkages were quantitatively cleaved by alkaline fusion, yielding only 3,4-dihydroxybenzoic acid, catechol, and hydroquinone. Nevertheless, the phenolic compound containing biphenyl type linkage was not quantitatively recovered from the alkaline fusion, being recovered only 19% of the original phenolic compound. This result may show that this type of linkage is more resistant to alkaline fusion. Also, it is possible that the phenolic compounds released were of different nature or that they were lost during the alkaline fusion, not allowing their recovery.

Fractionation of the HMWM by Ethanol Precipitation. The HMWM of roasted coffee infusions is composed by a mixture of thermally transformed galactomannans and arabinogalactans and by a diversity of melanoidin populations presenting different physical properties and chemical composition, including melanogalactomannans, melanoarabinogalactans, and melanoidins containing low amounts of carbohydrates.[6,7] These macromolecules can be roughly separated by ethanol precipitation. Galactomannans and melanogalactomannans can be obtained by precipitation in 50% ethanol solutions (Et50), arabinogalactans and melanoarabinogalactans can be obtained by precipitation in 75% ethanol solutions (Et75), and a second melanoarabinogalactan fraction and the melanoidins with low amounts of carbohydrates remain soluble in 75% ethanol solutions (EtSn). The yield and chemical composition of the fractions obtained by ethanol fractionation are shown in Table 4. For all coffees, the amount of material recovered was highest on the EtSn fraction (53%, on average) followed by the Et50 fraction (35%), and the Et75 fraction accounted for the lowest amount (13%). This distribution of the different melanoidin populations is in accordance with previous ethanol fractionation procedures in Arabica and Robusta coffees.[6,16,20,34,35] The amount of protein recovered in the fractions isolated from the different coffees increased significantly with the increase of the ethanol concentration, in accordance with Bekedam et al.[4] The amount of protein recovered in the EtSn and Et75 fractions from CGAcoffee was significantly higher than that of control. The phenolic compounds present in the each fraction also increased with the increase in ethanol solubility, being also observed a significantly higher amount of total phenolics in the Et75 and EtSn fractions recovered from CGAcoffee when compared to control. A significant correlation between the amount of protein and phenolic compounds present in the ethanol fractions ($r = 0.830$, $p < 0.05$) was also observed. When the percentage of phenolic compounds present in each fraction is calculated in relation to the amount of phenolic compounds present in the HMWM, it can be observed that the phenolic compounds present in the EtSn fraction represent nearly 83% of the HMWM phenolic compounds, followed by the Et50 fraction (15%) and the Et75 (12%). These results show that the melanoidin population soluble in ethanol contained the majority of the phenolic compounds incorporated into the HMWM during roasting, which is in accordance with Nunes et al.[16] and Bekedam et al.[4] The MBI increased for all coffee fractions with the increase of their solubility in ethanol. For the Et75 and EtSn fractions recovered from the CGAcoffee model, an average of 30% increase in the MBI was observed. In these fractions, the amounts of protein and phenolic compounds are significantly higher for CGAcoffee model when compared to the control. Also, a significant correlation between the phenolic compounds and MBI ($r = 0.674$, $p < 0.05$) is observed. The amount of phenolic compounds was also significantly correlated with the color of the different melanoidin populations ($r = 0.782$, $p < 0.05$), a significant correlation between the amount of protein and MBI ($r = 0.749$, $p < 0.05$) being also observed.

As the melanoidin populations soluble in 75% ethanol contained the majority of the phenolic compounds present in the HMWM, the nature of the phenolic compounds present in these melanoidin populations was further studied (Figure 1b). The EtSn fraction did not contain adsorbed phenolic compounds, which can be explained by the experimental procedure used, including dialysis and urea solubilization, probably resulting in the loss of the adsorbed phenolics. The amount of ester-linked phenolic compounds was in the range of that found for the HMWM, and there was no significant difference between the amounts of ester-linked phenolics for

the three coffees studied. The amount of condensed phenolic compounds present in the EtSn fraction was significantly higher, by a factor of 60, than the ester-linked phenolic compounds. The amount of condensed phenolic compounds present in the CGAcoffee model was, on average, higher than that present in the control coffee (Figure 1b). Nevertheless, it was not significantly different, probably due to the higher variability obtained in the analysis, as the total phenolic acids measured by the Folin−Ciocalteu colorimetric method were significantly higher. There was observed a significant correlation between the amount of phenolic compounds released by alkaline fusion and that determined by the colorimetric Folin−Ciocalteu method (ρ = 0.886, p < 0.05), although this correlation was not significantly linear (r = 0.767, p < 0.075). The Folin−Ciocalteu colorimetric method measures the sample's reducing capacity, which includes, among others, phenolic compounds.[36] However, this assay is also sensitive to the presence of proteins, ascorbic acid, and other reducing compounds.[37,38] In fact, melanoidins produced from model systems containing only sugar and amino acids are known to possess reducing capacity,[39] showing that proteins or reducing Maillard reaction products such as reductones present in the material can contribute to the reducing power of the solution, resulting in an overestimation of the amount of phenolic compounds present when measured by the Folin−Ciocalteu method. Nevertheless, as the reducing power of 5-CQA is 21 times higher, on a weight basis, than that of bovine serum albumin (BSA), protein interference should be comparably low (although the actual interference clearly depends on the amino acid composition of the proteins). Also, it is certainly harder to estimate the contribution of Maillard reaction products to the reducing power of the actual coffee sample. Nevertheless, although the application of the Folin−Ciocaultau method can have drawbacks, it is widely used for accessing the presence of phenolic compounds in coffee melanoidins, and the results obtained in this work show that it can be used as an indicator for phenolic compounds present.

Fate of CGA during Roasting, Contribution to Melanoidin Formation, and Potential Health Effects. The amount of phenolic compounds incorporated in coffee melanoidins, determined by the alkaline fusion method, ranged from 10 to 13 mol/100 mol of the CGA content present in green coffee. When the amount of phenolic compounds incorporated in coffee melanoidins was calculated in relation to the amount of phenolic compounds lost during roasting, the values ranged from 18 to 33 mol/100 mol. From these values it can be observed that the incorporation of phenolic compounds into the HMWM is a significant pathway of CGA degradation during roasting. When the amount of phenolic compounds incorporated in the HMWM is expressed as caffeic acid, it represents, on average, 23 g/100 g of the material not accounted for by polysaccharides and proteins. These results show that the phenolic compounds incorporated into coffee melanoidins represent a significant proportion of the previously described unknown material. The correlation found between the amount of phenolic compounds present in the different melanoidin populations and the K_{mix} color as well the MBI shows that the incorporation of phenolic compounds has an impact on the color of melanoidins. Although the structure of phenolic compounds present in the coffee melanoidins cannot be deduced from the data obtained from the methods applied, the data showed that they can be connected by aryl-ether, stilbene-type linkages, and biphenyl linkages, as these can be cleaved by alkaline fusion. The presence of phenolic compounds besides 3,4-dihydroxybenzoic acid, catechol, and hydroquinone in the alkaline fusion may represent different linkages or phenolic compounds transformed during roasting with structural features different from caffeic acid, but not yet available to be determined. The detection of 4-hydroxybenzoic acid in the alkaline fusion medium may represent the incorporation of coumaric acid in coffee melanoidins. The relative contribution of ferulic acid and caffeic acid to the amount of phenolic compounds incorporated into coffee melanoidins cannot be deduced due to the observed demethylation of phenolic compounds during alkaline fusion.[16] Further work is needed to disclose the structural details of the condensed phenolic compounds present in coffee melanoidins. The correlation between proteins and phenolic compounds in the different melanoidin populations suggests that the incorporation of phenolic compounds in coffee melanoidins is related to the amount of proteins. This can be explained by the easy cross-link between phenolic compounds through quinoidal structures and amino acids such as lysine and cysteine present in proteins.[40−42] The condensation of phenolic compounds during roasting has been shown to occur, but only low molecular weight structures have been found.[43−45] These might represent the starting point of the reaction that, on further reaction with proteins and also possibly with polysaccharides, incorporates these compounds into coffee melanoidins. As condensed phenolic structures are present in light-roasted coffee,[6] the incorporation process seems to occur as soon as the roasting starts, and this is in agreement with the data obtained from Smrke et al.[46] The presence of phenolic compounds in coffee melanoidins, mainly in condensed form, may be an explanation for the high antioxidant activity described for coffee melanoidins.[47,48] The association of these condensed phenolic structures with indigestible polysaccharides and melanoidins present in coffee HMWM may also be one of the reasons for the observed association between coffee consumption and the antioxidant capacity of feces from healthy subjects.[49] The presence of these condensed phenolic structures can act as a "radical-sponge" in the gastrointestinal tract (stomach and small and large intestine), as those described for other antioxidant dietary fibers when associated phenolic compounds are present.[50] Studies on the impact of gut microorganisms on the antioxidant activity of melanoidins are rare, but it was demonstrated, for example, that after a 24 h in vitro fermentation of high-molecular weight coffee fractions with gut microorganisms, roughly 25% of the original antioxidant activity was still present.[13] Due to the relatively high amount of phenolic compounds that can reach the gut, especially the large intestine, the fact that they are linked to the coffee dietary fiber deserves further study.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional figures and tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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