JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

High Molecular Weight Melanoidins from Coffee Brew

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The composition of high molecular weight (HMw) coffee melanoidin populations, obtained after ethanol precipitation, was studied. The specific extinction coefficient (K_{mix}) at 280, 325, 405 nm, sugar composition, phenolic group content, nitrogen content, amino acid composition, and non-protein nitrogen (NPN) content were investigated. Results show that most HMw coffee melanoidins are soluble at high ethanol concentrations. The amino acid composition of the HMw fractions was similar, while 17% (w/w) of the nitrogen was NPN, probably originating from degraded amino acids/proteins and now part of melanoidins. A strong correlation between the melanoidin content, the NPN, and protein content was found. It was concluded that proteins are incorporated into the melanoidins and that the degree of chemical modification, for example, by phenolic groups, determines the solubility of melanoidins in ethanol. Although the existence of covalent interaction between melanoidins and polysaccharides were not proven in this study, the findings suggest that especially arabinogalactan is likely involved in melanoidin formation. Finally, phenolic groups were present in the HMw fraction of coffee, and a correlation was found with the melanoidin concentration.

KEYWORDS: Coffee brew; melanoidins; Maillard reaction; amino acid analysis; ethanol precipitation

INTRODUCTION

The chemical composition of a coffee brew is strongly influenced by the composition of the green bean, the roasting procedure, and the extraction conditions (*1*). Green coffee beans are rich in carbohydrates, proteins, and phenolic compounds, and these compounds are transformed and/or degraded upon roasting (*2*−*5*). During the roasting process polysaccharides (*6*) and proteins (*7*) are degraded, and the Maillard reaction takes place between amino acids/proteins and reducing sugars, which results in the formation of flavor and colored compounds (*8*). The water-soluble brown colored compounds that are formed are referred to as melanoidins. The formed melanoidins make up a large part of a coffee brew and might be up to 25% (w/w) of the brew's dry matter (*9*).

Melanoidins are of interest not only due to their contribution to color formation but also for their flavor binding properties (*10*−*14*), antioxidative capacity (*9*, *15*−*17*), metal chelating properties (*17*, *18*), and reactivity in coffee brew (i.e., aging of coffee) (*19*). Melanoidins have been studied in recent years because of their nutritional, biological, and health implications.

Although more knowledge about the structure of melanoidins has been revealed in recent years, the chemical structure of melanoidins has not been elucidated yet. At the moment, there are three main proposals for the structure of melanoidins (*20*, *21*): Heyns and Hauber (*22*) and Tressl et al. (*23*) suggest that melanoidins are a polymer built up of repeating units of furans and/or pyrroles, formed during the advanced stages of the Maillard reaction, linked by polycondensation reactions. Hofmann (*24*) detected low-molecular-weight colored substances, which were able to crosslink proteins via ϵ-amino groups of lysine or arginine to produce high-molecular-weight colored melanoidins. And Kato and Tsuchida (*25*), and more recently the group of Cämmerer (*20*, *21*), suggest that the melanoidin skeleton is mainly built up of sugar degradation products, formed in the early stages of a Maillard reaction, polymerized through aldol-type condensation, and possibly linked by amino compounds. Although these proposals provide valuable information on what melanoidins might look like, it is important to realize that these suggested melanoidin structures are mostly based on model studies. In food systems, like coffee beans, the composition of melanoidins is likely to be far more complex due to the presence of many more possible reactants. Therefore, it is likely that all the proposed structures for melanoidins can be found in coffee melanoidins, and they may even occur within the same melanoidin complex.

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10.1021/jf0615449 CCC: $33.50
Published on Web 09/07/2006

As the molecular structure of melanoidins is largely unknown, these compounds are generically defined as brown, nitrogenous macromolecular material (*9*, *26*). The quantity of melanoidins is often determined "by difference", which is the quantity that remains after subtraction of the known compounds (carbohydrates, proteins, caffeine, etc.) from the starting material. Melanoidins are often measured by their absorbance at 405 nm, which is an arbitrary chosen wavelength at which the intensity of the brown color is measured.

The objective of the present investigation was to isolate and characterize different melanoidin populations present in a coffee brew. While the primary focus was on coffee melanoidins, attention was also given to the quantity and composition of carbohydrates, proteins, non-protein nitrogen (NPN), and phenolics in the coffee brew.

MATERIALS AND METHODS

Materials. Roasted coffee beans (*Coffea arabica*), originating from Colombia, were provided by a local factory. The degree of roast, which represents the weight reduction upon roasting, was 16.4% (w/w) and was 8.0% (w/w) on a dry matter basis.

Preparation of Coffee Brew. The roasted beans were ground using a Retsch ZM200 mill equipped with a 0.4-mm sieve and operating at maximum speed (18 000 rpm). Coffee brew was prepared as described by Borrelli et al. (*9*). The coffee brew was obtained from the milled beans by adding 200 g of ground coffee to 1200 g of filtered demineralized water (Millipore Corp, Billerica, MA) at 90 °C. Subsequently, this coffee suspension was kept at 90 °C for 15 min while stirring continuously. The extract was filtered over a Büchner funnel using a S&S 595 filter (Whatman, Maidstone, UK). For characterization purposes, part of the coffee brew and the residue were lyophilized, yielding "Brew" and "Brew Residue", respectively. The major part of the coffee brew was used for further isolation.

Isolation of High Molecular Weight Material from Coffee Brew Using Diafiltration. High molecular weight material of the coffee brew was obtained by diafiltration using a 0.7 square meter hollow fiber filter with a MW cutoff of 3 kDa (A/G Technology Corp., Needham Heights, MA). The flow was applied by a KBl CD/SF 1200 pump, and the pressure over the system was not allowed to exceed 1 bar. A freshly prepared brew was first concentrated 3 times using the diafiltration system and subsequently diafiltered until the conductivity of the dialysate became lower than 10 μS/cm. For characterization purposes, part of the retentate and the dialysate were lyophilized, yielding "Brew HMw" and "Brew LMw", respectively. The major part of the retentate was used for further isolation.

Isolation of High Molecular Weight Material from Coffee Brew Using a Dialysis Membrane. Fresh coffee brew, Brew HMw, and Brew LMw were dialyzed using a dialysis membrane (MW cutoff 12−14 kDa, Visking size 9, Medicell International Ltd, London, UK) for 2 days against running tap water and 1 day against demineralized water with two water renewals. The retentates of the coffee brew, the Brew HMw and Brew LMw were lyophilized, yielding "Brew-Dial", "Brew HMw-Dial", and "Brew LMw-Dial", respectively.

Ethanol Precipitation of the Brew HMw. The Brew HMw obtained directly after diafiltration was subjected to ethanol precipitation. Absolute ethanol (Merck, Darmstadt, Germany) was slowly added to the Brew HMw under continuous stirring until an ethanol concentration of 20% (w/w) was reached. The solution was left for precipitation (8−15 h) at 4 °C. This solution was then centrifuged for 20 min at 18900*g*. The supernatant and the residue were separated, and the residue was washed again using an ethanol concentration of 20% (w/w). The first supernatant was subjected to further ethanol precipitation steps in which the ethanol concentration was increased in steps to 40, 60, and a final concentration of 80% (w/w) following the same procedure. The ethanol, present in the residues of the ethanol precipitation steps at 20, 40, 60, and 80% (w/w) ethanol and in the supernatant of 80% (w/w) ethanol, was removed by flushing under a stream of dry nitrogen at room temperature; nitrogen was used to prevent oxidation of the coffee compounds. Subsequently, the samples were lyophilized, yielding "EP20", "EP40", "EP60", and "EP80" for the coffee fractions that precipitated at 20, 40, 60, and 80% (w/w) ethanol, respectively. The lyophilized supernatant of 80% (w/w) ethanol was coded "ES80".

Defatting of Coffee Samples. All the lyophilized coffee samples were defatted by Soxlet extraction using a Soxtherm, which was connected to a Multistat system (Gerhardt, Königswinter, Germany). The solvent used for extraction was dichloromethane. After defatting, dichloromethane was evaporated in an oven at 40 °C for approximately 30 min.

Analysis of Nitrogen Content. The nitrogen content of various samples was estimated according to the Dumas method using an NA2100 nitrogen and protein analyzer (Carlo Erba Instruments, Milan, Italy) according to the manufacturer's instructions. Methionine was used as a standard.

Molecular Weight Distribution. High-performance size-exclusion chromatography was performed on two 300 × 7.8 mm i.d. TSKgel columns in series (G4000 PWXL and G2500 PWXL) (TosoHaas, Stuttgart, Germany), in combination with a PWX-guard column. Elution took place at 40 °C with 0.2 M sodium nitrate at 0.8 mL/min. The eluate was monitored by refractive index detection using a Spectra System RI-150 detector, and the absorbance was measured at 280, 325, and 405 nm using a Spectra System UV2000. The sample was dissolved in the eluent and was centrifuged prior to injection (100 μL).

Sugar Analysis. The neutral sugar composition was determined by gas chromatography according to Englyst and Cummings (*27*) using inositol as an internal standard. The samples were prehydrolyzed with 72% (w/w) H_2SO_4 for 1 h at 30 °C, followed by hydrolysis with 1 M H_2SO_4 for 3 h at 100 °C, and the constituent sugars released were analyzed as their alditol acetates. The uronide content was determined by the automated colorimetric *m*-hydroxydiphenyl method (*28*, *29*).

Total Phenolic Groups Content. The total phenolic group content of the coffee samples was determined with the Folin-Ciocalteu reagent. For all coffee samples, a 0.33 or 1 mg/mL solution was prepared. To 1000 μL of the coffee sample solution, 500 μL of Folin-Ciocalteu (Merck, Darmstadt, Germany) reagent was added. After mixing of the sample, 1000 μL of a saturated Na_2CO_3 solution was added and filtered demineralized water was added until the total volume was 10 mL. After mixing, followed by 1 h of reaction, the absorbance of the sample was measured at 725 nm on a UV-mini 1240 spectrophotometer (Shimadzu, Kyoto, Japan). Chlorogenic acid was used as reference phenolic compound.

Spectroscopic Analysis. The absorption spectra of coffee solutions were determined in the range 200−700 nm. The coffee solutions were prepared (0.1 mg/mL) by dissolving 1 mg of the lyophilized coffee material in 10 mL of filtrated demineralized water. Each solution was prepared just before measurement. Absorption spectra were recorded on a Hitachi U-3000 spectrophotometer (Hitachi, Tokyo, Japan).

Specific Extinction Coefficient of Coffee Material at 280, 325, and 405 nm. A 1.5 mg/mL sample solution was prepared by dissolving 45 mg of lyophilized coffee sample in 30 g of water. Subsequently, various dilutions were prepared from this coffee solution. To minimize errors that do occur during dilution based on volumes, preparation of these coffee solutions was performed by weighing. The quartz cuvette used for absorption measurements was rinsed thoroughly with the coffee dilution prior to measuring the absorption. The absorption at 280, 325, and 405 nm was measured using a Hitachi U-3000 spectrophotometer (Hitachi, Tokyo, Japan). To guarantee linearity, the value of the absorbance at all three wavelengths had to be between 0.1 and 1.3. In practice, this meant that one dilution had to be prepared for measurement of the absorption at 405 nm and another dilution with a concentration around 5 times lower had to be prepared for measurements at 280 and 325 nm. The measurements were conducted in triplicate. The specific extinction coefficient (K_{mix}) was calculated using the law of Lambert−Beer: $E\,(-) = K_{mix}\,(L\ g^{-1}\ cm^{-1}) \times$ concentration ($g\ L^{-1}$) × length of light path (cm). The use of the specific extinction coefficient (K) was preferred over the molar extinction coefficient (ϵ) since the molecular weight of melanoidins is unknown and is probably variable. By using K, the concentration parameter used in the law of Lambert−Beer is expressed in $L\ g^{-1}\ cm^{-1}$, which makes it applicable for coffee.

Amino Acid Analysis. Samples for amino acid analysis were hydrolyzed using liquid-phase hydrolysis in 6 M HCl at 110 °C for 24

Table 1. Yields of High Molecular Weight Coffee Fractions Using Different Dialysis Techniques

	diafiltration		membrane dialysis	
coffee sample	HMw fraction (%, w/w) brew[a]	HMw fraction (%, w/w) dry[b]	HMw fraction (%, w/w) brew[a]	HMw fraction (%, w/w) dry[b]
Brew	15.5	3.6	31.7	7.4
Brew HMw[c]			95.5	3.5
Brew LMw[c]			18.7	3.6

[a] % (w/w) of the dialyzed coffee fraction. [b] % (w/w) of the dry and defatted coffee beans. [c] The Brew LMw and HMw fraction after diafiltration were dialyzed using membrane dialysis.

h. Amino acid analyses were performed using a Hewlett-Packard Aminoquant 1090M using an automated two-step precolumn derivatization with two different reagents, *o*-phthalaldehyde for primary and 9-fluorenylmethylchloroformate for secondary amino acids (*30*).

RESULTS AND DISCUSSION

Characteristics of the Brew, Brew HMw, and Brew LMw. The obtained Brew consisted of 4.4% (w/w) dry matter. The yield of the coffee brew preparation was 20% (w/w) of the roasted beans, which is 23.5% (w/w) of the dry and defatted roasted bean. This extraction yield is in line with yields reported by Borrelli et al. (17%, w/w) (*9*), Nunes et al. (19%, w/w) (*31*), and Clarke and Vitzthum (24%, w/w) (*32*).

The diafiltration step yielded 16% (w/w) Brew HMw and 82% (w/w) Brew LMw. The Brew HMw is 3.6% (w/w) of the dry and defatted beans (**Table 1**). Nunes and Coimbra (*33*) reported a HMw coffee fraction that was 7.6% (w/w) of the dry and defatted beans, using membrane dialysis (MW cutoff 12−14 kDa). It was expected that the yield of the Brew HMw after diafiltration would be larger than 7.6% (w/w) since the MW cutoff was smaller (3 kDa) compared to the membrane dialysis and should therefore retain more molecules. On the other hand, Hofmann (*34*) suggested that such observed differences might be explained by the fact that coffee compounds react during dialysis, yielding more HMw molecules, resulting in larger amounts of the HMw fraction. To investigate this hypothesis, fresh coffee brew, the Brew HMw, and Brew LMw were also dialyzed using a membrane dialysis. **Table 1** shows that the yield of the Brew-Dial (7.4%, w/w) is in line with the yield reported by Nunes and Coimbra (*33*). The fractions Brew HMw-Dial and Brew LMw-Dial are 3.5 and 3.6% (w/w) of the dry and defatted beans, respectively. It is unlikely that the LMw coffee compounds (Mw < 3 kDa) react and form a high molecular weight (Mw > 12 kDa) fraction that is as large as the Brew HMw. This suggests that the reactivity of the coffee compounds is not responsible for the larger yield and appears that the observed differences should be explained by differences in separation techniques. It is likely that the difference is caused by intrinsic properties of the membranes (cellulose acetate vs polysulfone) and the separation principle (diffusion vs diffusion and pressure). Moreover, the dialysis technique strongly affects the yield of the high molecular weight fraction of coffee. The Brew HMw, obtained after diafiltration, was used for further analysis.

Spectroscopic Analysis of the Brew, Brew HMw, and Brew LMw. Absorption spectra of Brew, Brew HMw, and Brew LMw fractions were recorded (**Figure 1**), and it appeared that coffee Brew shows two absorption maxima, one at 280 nm and one at 325 nm. The absorption maximum at 280 nm

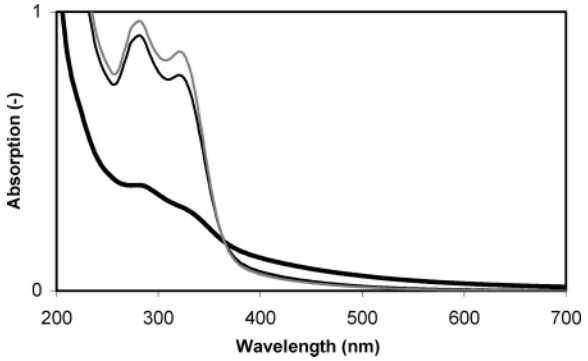

Figure 1. Absorption spectra of 0.1 mg/mL Brew (thin black line), 0.1 mg/mL Brew HMw (thick black line), and 0.1 mg/mL Brew LMw (thin gray line).

Table 2. K_{mix} Values for the Brew, Brew HMw, and Brew LMw[a]

coffee sample	$K_{mix\ 280nm}$ (L cm^{-1} g^{-1})	$K_{mix\ 325nm}$ (L cm^{-1} g^{-1})	$K_{mix\ 405nm}$ (L cm^{-1} g^{-1})
Brew	9.1	7.7	0.7
Brew HMw	3.9	3.0	1.1
Brew LMw	9.7	8.4	0.6

[a] The average and maximal standard deviation were 0.5 and 1.0% of the K_{mix} values, respectively.

can be explained by the presence of proteins, caffeine, chlorogenic acid, and caffeic acid. The absorption maximum at 325 nm can be explained by the presence of chlorogenic acid and caffeic acid. It is generally accepted that melanoidins contain conjugated systems which result in light absorption throughout the whole spectrum. The wavelength selected for measuring melanoidin is most often chosen to be 405 nm (*9*), since other coffee compounds do not absorb light at this wavelength. On the basis of these absorption spectra, it can be stated that measurement of the absorption at 280, 325, and 405 nm provides useful information on the relative amount of melanoidins and other compounds in a specific coffee fraction. To be able to compare absorption values at a specific wavelength of different coffee samples, or to compare absorption values at different wavelengths within one coffee fraction, the absorption of a coffee fraction at a specific wavelength was expressed as *K* values. Since coffee samples used in this study are most likely a mix of various compounds, the specific extinction coefficient was defined as K_{mix}. The value of K_{mix} provides information on the relative amount of melanoidins ($K_{mix\ 405nm}$) and other coffee compounds like proteins ($K_{mix\ 280nm}$) and chlorogenic acid ($K_{mix\ 280,\ 325nm}$) present within a coffee fraction. By using K_{mix}, it becomes possible to determine which coffee fraction has the highest melanoidin concentration, by comparing $K_{mix\ 405nm}$ values. Furthermore, by calculating the $K_{mix\ 280nm}$ to $K_{mix\ 405nm}$ and $K_{mix\ 325nm}$ to $K_{mix\ 405nm}$ ratios, by which the absorption of melanoidins at these wavelengths is taken into account, it is also possible to estimate the relative amount of chlorogenic acid and/or proteins compared to the amount of melanoidins. In **Table 2**, the K_{mix} values and the corresponding ratios for the Brew, the Brew HMw and Brew LMw are given. From the $K_{mix\ 280nm}$ and $K_{mix\ 325nm}$ values it is clear that the most of the compounds responsible for the absorption at 280 and 325 nm end up in the Brew LMw. This can be explained by the fact that coffee compounds like degraded proteins, peptides, chlorogenic acid, and caffeine end up in the low molecular weight fraction. However, the $K_{mix\ 405nm}$ is the highest for the Brew HMw, suggesting that the Brew HMw is relatively rich in

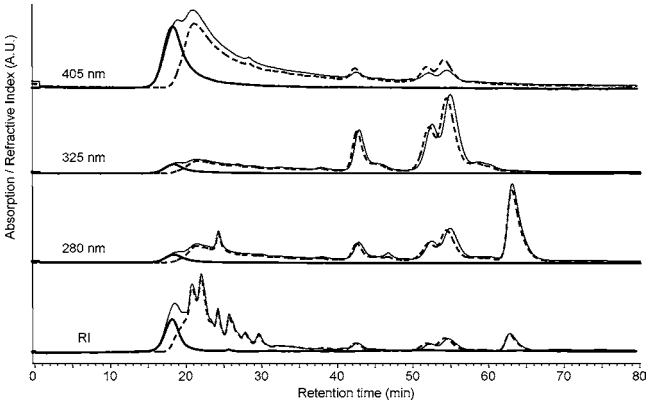

Figure 2. Size exclusion chromatography patterns of the Brew (solid thin line), Brew HMw (solid thick line), and Brew LMw (dashed line) using RI and spectrometric detection.

melanoidins. In addition, the Brew HMw also shows absorption maxima at 280 and 325 nm (**Figure 1**), indicating that proteins and/or chlorogenic acid are present in the Brew HMw. The fact that an absorption maximum at 325 nm is present in the Brew HMw suggests that the low molecular weight chlorogenic acid is incorporated into the HMw material, as was also reported in other studies (*33*, *35*−*37*). The question remains whether these compounds are incorporated into the melanoidin complex or not.

Although the highest $K_{\text{mix 405nm}}$ is observed for the Brew HMw, it is worth mentioning that a large fraction (71%) of the total amount of melanoidins from the Brew, as calculated from the $K_{\text{mix 405nm}}$ values, ends up in the Brew LMw since the Brew LMw represents 82% (w/w) of the Brew. Since the Brew HMw is rich in melanoidins, and since this fraction is free of low molecular weight compounds like caffeine and free chlorogenic acid, it was decided that the Brew HMw was most interesting for further studies on melanoidins.

Molecular Weight Distribution of the Brew, Brew HMw, and Brew LMw. Initially, the molecular weight distributions of the Brew, Brew HMw, and Brew LMw were investigated by size exclusion chromatography using Sephadex G-25 as column material since this material is frequently used for coffee (*5*, *9*, *21*, *38*−*41*). However, after extensive research it was found that the method was not reproducible enough to use results obtained for comparison of different coffee fractions. It is expected that certain coffee compounds interact with the column material and thereby influence subsequent runs, even after thorough regeneration steps. Buffered eluents were used, in order to suppress ionic interactions, which resulted in very poor separation; therefore, this was not an option. It was found that interaction of amino acids with Sephadex as column material was previously reported by Eaker and Porath (*42*). Therefore, it is suggested that the separation of coffee material on Sephadex G-25 is not only based on size exclusion but that some kind of interaction with the column material is also involved. Since results with Sephadex G-25 were not satisfying, other size exclusion techniques were tested.

TSKgel size exclusion columns on an HPLC system were found to give reproducible results, and the elution patterns of the Brew, Brew HMw, and Brew LMw are shown in **Figure 2**. In addition to measuring the absorbance at 280, 325, and 405 nm, the effluent was monitored using a RI detector to be able to also detect carbohydrates. The 405 nm absorbance pattern of the Brew shows that two melanoidin populations were present in the Brew; the first population ended up in the Brew HMw, while the second melanoidin population ended up in the Brew LMw. This is in agreement with findings reported by Hoffmann, who stated that melanoidins can be LMw colored compounds (*24*). The fact that two populations are observed in the elution pattern of the Brew (**Figure 2**) suggests that the molecular weight distribution of melanoidins is not totally heterogeneous. When examining the RI, 280 and 325 nm patterns, it is noteworthy that part of the compounds present in the Brew and the Brew LMw elute after the included volume (∼26 min). From the elution behavior of reference compounds, the compounds eluting at 55 and 64 min were identified as chlorogenic acid and caffeine, respectively. From these results, it was concluded that the TSKgel size exclusion columns do not separate molecules based on size only but that some kind of interaction with the column material occurs. Therefore, the HPSEC elution patterns cannot be interpreted solely as molecular weight information. However, the HPSEC method was found to give reproducible results, and the elution patterns do provide an insight into the diversity of the composition of coffee fractions. It was concluded that the Brew HMw was free of low molecular weight compounds like chlorogenic acid and caffeine and contained the melanoidin population with the highest molecular weight present in the Brew.

Ethanol Precipitation of the Brew HMw. As mentioned above, the Brew HMw was rich in melanoidins and free of LMw compounds that could have disturbed other measurements. Fractionation of this Brew HMw in several fractions was conducted by ethanol precipitation. The reason for choosing ethanol precipitation over preparative gel filtration is based on the observation that coffee compounds like galactomannans and arabinogalactans show different precipitation behavior and can be selectively precipitated by this methodology (*33*, *40*, *41*, *43*); however, in these studies only minor attention was given to melanoidins (*33*, *43*). Therefore, we conducted ethanol precipitation with the main focus on melanoidins, while carbohydrates, proteins, and phenolic groups were also investigated. The yields of the HMw coffee fractions and the corresponding K_{mix} values and ratios obtained by ethanol precipitation are shown in **Table 3**. All five fractions from the ethanol precipitation were obtained in significant amounts, the smallest fraction still being larger than 10% (w/w) of the starting material. The recovery of the ethanol precipitation experiments was 87% (w/w), probably because the supernatant of the wash step of the precipitates was not included. The lyophilized EP20 fraction was poorly soluble (∼33% (w/w)) in water, while the other fractions were completely soluble in water. Because of the partial solubility of EP20, the determined K_{mix} values will be an underestimation. The $K_{\text{mix 405nm}}$ increases with increasing ethanol concentration, the ES80 showed a $K_{\text{mix 405nm}}$ of 2.5, twice as high as the K_{mix} of the Brew HMw. Taking the yield into account, it can be stated that EP80 and ES80 together account for 60% of the absorption at 405 nm present in the Brew HMw, indicating that these two fractions, especially ES80, are rich in melanoidins. The $K_{\text{mix 280, 325nm}}$ values (data not shown) of the ethanol precipitation fractions showed the same trend as $K_{\text{mix 405nm}}$, the higher the ethanol concentration, the higher the K_{mix} value. This indicates that melanoidins themselves appear to show absorption at 280 and 325 nm. The ratio of the K_{mix} values of 280:325:405 nm is around 3.4:2.6:1 for all fractions, except for EP60, which show a ratio of 6:5:1. An explanation for this might be that this EP60 contains relatively much UV 280 and 325 nm absorbing compounds, which might be due to a higher bound chlorogenic acid content in this fraction.

Molecular Weight Distribution of the Ethanol Precipitation Coffee Fractions. The fractions obtained from the ethanol

Table 3. Yield, $K_{mix\ 405nm}$ Value, $K_{mix\ 280,\ 325nm}$ to $K_{mix\ 405nm}$ Ratios, Level of Phenolic Groups, and Phenolic Groups to $K_{mix\ 405nm}$ Ratio of Coffee Fractions[a]

coffee sample	yield (%, w/w)	$K_{mix\ 405nm}$ (L cm^{-1} g^{-1})	ratio $K_{mix\ 280:405nm}$ (–)	ratio $K_{mix\ 325:405nm}$ (–)	phenolic groups (%, w/w)	phenolic groups/ $K_{mix\ 405nm}$ (% g cm L^{-1})
Brew		0.7			29	41
Brew HMw	100	1.1	3.5	2.8	15	14
Brew LMw		0.6			31	52
EP20	19	0.5	3.1	2.5	6	12
EP40	11	0.7	3.6	2.8	8	11
EP60	28	0.6	6.0	5.0	11	18
EP80	17	1.2	3.7	2.9	17	14
ES80	13	2.5	3.3	2.5	26	10

[a] The average and maximal standard deviation were 0.5 and 1.0% of the K_{mix} values, respectively.

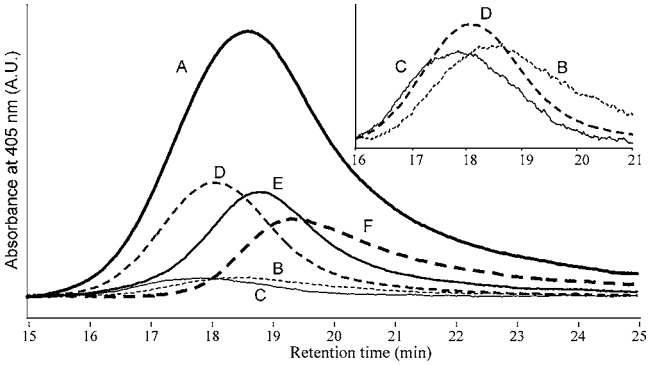

Figure 3. Size exclusion chromatography elution patterns of 0.8 mg/mL Brew HMw (A), 0.15 mg/mL EP20 (B), 0.08 mg/mL EP40 (C), 0.21 mg/mL EP60 (D), 0.13 mg/mL EP80 (E), and 0.10 mg/mL ES80 (F). The inset shows the EP20, EP40 (both 5 times enlarged), and EP60 patterns.

precipitation were analyzed by HPSEC with the aim to see whether any differences in elution patterns could be observed. Differences in elution time were observed for the ethanol precipitation fractions, when the effluent was monitored at 405 nm (**Figure 3**). The RI, and absorbance at 280 and 325 nm elution patterns were found to be similar to the absorbance at 405 nm elution pattern. Especially for the EP60, EP80, and ES80 fractions, differences in elution time were observed, EP60 eluting first and ES80 eluting last. The differences might be explained by the fact that there is a difference in molecular weight, with the EP60 having the highest and the ES80 having the lowest molecular weight. On the other hand, it might also be that the differences are caused by molecular properties and not so much by the molecular weight. It was previously shown in this study that the separation of coffee on this SEC column was not only molecular weight based. It is possible that a higher protein or nitrogen content might result in a delay in elution time since the TSKgel column is primarily designed for the analysis of carbohydrates. Whatever the true explanation may be, differences between the fractions were observed. Further analysis of the fractions is required to characterize the fractions on a more molecular basis.

Sugar Analysis of the Coffee Fractions. The sugar composition of the coffee fractions was determined, and the results are shown in **Table 4**. The quantity as well as the composition of the beans, Brew Residue and Brew, are in accordance with literature (*44*). The sugar content of the Brew HMw was found to be 70% (w/w), and this fraction represents 2.5% (w/w) of the dry and defatted beans. Nunes and Coimbra (*33*) report a sugar content of the Brew HMw of ~30% (w/w), which was 2.4% (w/w) of the dry and defatted beans. This suggests that the amount of carbohydrates retained during diafiltration and membrane dialysis is the same although the overall Brew HMw yield is twice as low for diafiltration. The most abundant sugars in the Brew HMw are mannose and galactose. The Brew LMw contained 16% (w/w) sugars, with also mannose and galactose being the most abundant sugars. It is important to note that 100 g of Brew dry matter contained 26 g of carbohydrates, of which 13 g ended up in the Brew LMw. Thus, only half of the carbohydrates in the Brew were polymeric (>3 kDa), while the other half was present as LMw carbohydrates. At low ethanol concentrations (EP20 and EP40), mainly galactomannans precipitate and at high ethanol concentrations mainly arabinogalactans precipitate, as was also reported (*33*). Since the ratio Ara/Gal increased dramatically when the ethanol concentration was increased, it can be stated that the higher the ethanol concentration, the higher the degree of substitution of arabinose on the galactan backbone. The fractions obtained by ethanol precipitation all contained significant amounts of carbohydrates. However, EP20, EP40, EP60, and EP80 contained higher amounts (70+%, w/w) than did ES80, which contained only 30% (w/w) carbohydrates. Obviously, most of the sugar precipitated before 80% ethanol, and highly substituted arabinogalactans were the most abundant sugars present in ES80.

Combining results from **Tables 3** and **4**, it can be stated that arabinogalactans are the most abundant sugars present in the melanoidin-rich fractions (EP80 and ES80). This could be the result of incorporation of arabinogalactans in melanoidins or due to coprecipitation. Since sugars are required for the Maillard reaction to occur and since Oosterveld et al. (*6*) showed that coffee polysaccharides are degraded upon roasting, it is likely that polysaccharides are part of melanoidins. With increasing ethanol concentration, the galactomannan content decreased gradually and the arabinogalactan content increased gradually, as did the melanoidin content. Combining this observation with the fact that Nunes et al. (*45*) also found that, after enzymatic degradation of galactomannans, an arabinogalactan-rich and galactomannan-poor fraction coeluted with the majority of the melanoidins it can be suggested that arabinogalactans are involved in melanoidin formation. Concerning the galactomannans, it was previously shown that galactomannans might be involved in melanoidin formation (*45*). However, the presence of galactomannans at higher ethanol concentrations (7−10% in ES80) was expected since the presence of galactomannans in a 75% ethanol solution of green bean extract, which is melanoidin free, was reported (*41*). Therefore, it is possible that galactomannans are involved in melanoidin formation, although the inverse correlation between melanoidin and galactomannan content suggests that galactomannans are not the most important carbohydrate involved in melanoidin formation.

Phenolic Groups in the Coffee Fractions. The phenolic group levels of the coffee fractions are shown in **Table 3**. It was found that the Brew and Brew LMw were rich in phenolic

Table 4. Sugar Composition of Coffee Fractions

coffee sample	Rha (%, w/w)	Ara (%, w/w)	Xyl (%, w/w)	Man (%, w/w)	Gal (%, w/w)	Glc (%, w/w)	uronic acid (%, w/w)	total sugar (%, w/w)
roasted beans	<1	2	<1	25	11	8	4	50
Brew residue	<1	2	<1	29	11	12	3	57
Brew	<1	2	<1	9	9	2	4	26
Brew HMw	1	6	<1	29	26	1	7	70
Brew LMw	<1	2	<1	5	5	1	3	16
EP20	<1	1	<1	71	6	<1	3	81
EP40	<1	2	<1	53	12	nd[a]	10	77
EP60	<1	5	<1	13	47	<1	8	73
EP80	3	12	<1	6	40	<1	8	70
ES80	4	10	<1	2	10	<1	4	30

[a] nd = not determined.

groups, 29 and 31% (w/w), respectively. This was no surprise since chlorogenic acid, which is the most abundant "native" phenolic in coffee beans, is present in these fractions (**Figure 2**). The level of 29% (w/w) in the Brew is in agreement with findings reported in literature since green Arabica coffee beans contain around 5−7.5% chlorogenic acid (*2*), resulting a theoretical phenolic groups level of 21−32% in the brew. However, it is important to mention that chlorogenic acid is susceptible to degradation upon roasting. Since the fate of caffeic acid, the phenolic moiety in chlorogenic acid is unknown, the theoretical phenolic group level as calculated from literature is likely an overestimation.

The phenolic group content of the Brew HMw was found to be 15% (w/w). Since the protein content was rather low in the Brew HMw, the phenolic group content cannot be explained by the presence of phenolic amino acids like tyrosine. The presence of these phenolic groups in the HMw fraction might be a result of binding of coffee phenolics to high molecular weight material and/or formation of phenolic groups from non-phenolics upon roasting.

As mentioned previously, the ratio of the K_{mix} values at 325 nm compared to 405 nm in the EP60 fraction was higher than the ratio of the other ethanol precipitation fractions. It was suggested that the amount of chlorogenic acid or caffeic acid in this fraction was relatively high compared to the melanoidin content. By expressing the amount of phenolic groups as determined by the Folin-Ciocalteu color assay relative to the melanoidin content (**Table 3**), it was found that again the EP60 fraction had the highest ratio of phenolic groups to melanoidins. Both the $K_{mix\ 325nm}$ and the color assay point to an increased presence of phenolic groups in EP60 compared to other ethanol precipitation fractions.

Borrelli et al. (*9*) and Nunes and Coimbra (*33*) showed that the HMw fraction of coffee brews were rich in phenolic groups. On the basis of Curie point pyrolysis, Adams et al. (*46*) suggested that chlorogenic acid might be incorporated into the Brew HMw. Rawel et al. (*37*) proved that chlorogenic acid can be covalently bound to the 11S storage protein of coffee beans. Montavon et al. (*36*) even suggested that the polyphenols might bind to the 11S coffee protein and contribute to the behavior and formation of melanoidins. Our results are in line with the findings of these authors and suggest that Brew HMw contains significant amounts of phenolic groups. Additionally, it was found that the phenolic group content increased with increasing ethanol solubility, just like the melanoidin content. It is worth mentioning that 62% of the total amount of phenolic groups present in Brew HMw ended up in the EP80 and ES80 fractions. Previously, it was mentioned that 60% of the melanoidin content ended up in these EP80 and ES80 fractions. This suggests that phenols are not only present in the Brew HMw but most likely are incorporated in the melanoidin complex.

Table 5. Nitrogen and Protein Content According to Dumas and Amino Acid Analysis, and the NPN Content for the Coffee Fractions

coffee sample	nitrogen[a] (%, w/w)	protein[a] (%, w/w)	protein[b] (%, w/w)	nitrogen[b] (%, w/w)	NPN[c] (%, w/w)
green beans	nd	nd	12.0	2.18	nd
roasted beans	2.75	15.1[d]	10.1	1.84	0.91
Brew	3.13	17.2[d]	6.4	1.16	1.97
Brew HMw	1.44	7.9	6.0	1.09	0.35
Brew LMw	3.33	18.3[d]	6.0	1.09	2.24
EP20	1.05	5.8	4.4	0.80	0.25
EP40	0.72	4.0	2.7	0.49	0.23
EP60	0.70	3.9	2.4	0.44	0.26
EP80	1.39	7.6	5.6	1.02	0.37
ES80	4.44	24.4	19.6	3.56	0.88

[a] Nitrogen and protein (% N*5.5) content according to Dumas. [b] Protein and nitrogen (% protein/5.5) content according to the amino acid analysis. [c] Non-protein nitrogen. [d] Overestimation of the protein content due to non-protein LMw material.

Nitrogen, Protein, and Amino Acid Content and Composition. Proteins make up 8.7−12.2% (w/w) of green coffee beans and can be divided into the water-soluble (50%) and the water-insoluble proteins (50%) (*7*). The water-soluble coffee proteins will end up in the brew, and they consist of 85% of globulins (*47*), and these globulins were shown to be 11S storage proteins by Rogers et al. (*48*). The protein content of the coffee fractions was calculated by multiplying the nitrogen by a factor of 5.5 (**Table 5**). Although a nitrogen to protein factor of 6.25 is frequently used in literature (*7*, *9*, *33*), a factor of 5.5 for the 11S storage protein was preferred since this value was calculated for 11S storage protein from coffee (*48*, *49*).

The protein content of the green beans, roasted beans, Brew, and Brew LMw are overestimations of the real protein content since no corrections were made for the nitrogen originating from caffeine and trigonelline and other non-protein nitrogen containing compounds. The nitrogen content of the Brew HMw and ethanol precipitation fractions are expected to give more accurate estimations of the protein content since the LMw nitrogen containing compounds are not present in these fractions. However, no distinction could be made between nitrogen originating from amino acids/proteins and non-protein nitrogen (NPN). The protein content of the Brew HMw (8%) is consistent with Nunes and Coimbra (*33*) who report an average protein content of 9.3% (w/w). From the nitrogen content of the ethanol precipitation fractions, it is clear that the higher the ethanol concentration, the higher the nitrogen content and presumably the higher the protein content. However, it should be realized that not all the nitrogen present in the HMw fractions has to be

Table 6. Amino Acid Composition (Mol %) of Coffee Fractions

amino acid	green bean	roasted bean	Brew	Brew LMw	Brew HMw	EP20	EP40	EP60	EP80	ES80
Ala	7	9	8	7	8	7	7	10	9	8
Cys	nd[a]	nd	nd	nd	nd	nd	nd	nd	nd	nd
Asx	10	10	10	9	9	10	11	11	9	8
Glx	18	22	29	30	29	29	29	29	29	29
Phe	4	5	4	4	4	4	3	3	4	4
Gly	11	12	13	12	13	14	15	15	14	12
His	2	2	2	2	1	2	2	2	2	1
Ile	4	5	4	4	4	4	3	3	3	4
Lys	6	1	0	0	1	1	1	0	0	1
Leu	9	11	9	9	9	10	8	7	8	10
Met	1	1	1	1	0	0	0	0	0	0
Pro	6	7	8	8	7	7	7	6	7	7
Arg	4	0	0	0	0	0	0	0	0	0
Ser	5	3	3	3	3	3	3	3	3	3
Thr	4	3	2	3	3	3	3	3	3	4
Val	6	8	5	5	6	6	5	6	6	7
Trp	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Tyr	2	2	2	2	1	1	2	1	1	2
total % (w/w)[b]	12.0	10.1	6.4	6.0	6.0	4.4	2.7	2.4	5.6	19.6

[a] nd = not determined. [b] Total amount of amino acids in % (w/w).

present in intact amino acids or proteins. It is likely that part of the amino acids or proteins are degraded upon roasting and that the nitrogen from these amino acids may end up in pyrrole-like structures within the melanoidin complex.

To be able to make a distinction between nitrogen from intact amino acids and non-amino acid nitrogen, referred to as non-protein nitrogen (NPN), the protein content was also determined by amino acid analysis, which is known to be an accurate technique for the determination of the protein content in coffee (*7*). Results of the amino acid analysis are shown in **Table 6**. The roasting process lowered the protein content in the beans from 12% in the green beans to 10% in the roasted beans. Because of the roasting process, 21% of the proteins are lost, taking the weight loss of the beans into account. These results are in agreement with literature (*7*). Arginine (−93%, w/w), lysine (−87%), serine (−58%), threonine (−36%), histidine (−35%), and asparagines/aspartic acid (−24%) especially were significantly reduced during roasting. It is remarkable that all hydrophilic amino acids containing amine (Arg, Lys, His) or amide (Asp) side groups showed a reduction of 24% (w/w) or more upon roasting. Obviously, this can be explained by the fact that ϵ-amino groups on amino acids are reactive and were shown to be involved in nonenzymatic browning reactions during thermal processing of foods (*50*−*52*). Rizzi (*53*) suggested that proteins play a key role in melanoidin formation because of the high reactivity of ϵ-amino, thiol, or methylthio groups. The hydrophilic amino acids serine and threonine showed losses of 36% or more upon roasting. In the literature, no information was found on the losses of these amino acids upon roasting. The fact that tyrosine, which also contains a hydroxyl group does not show such a decrease (−7%) might be because the oxygen molecule from the hydroxyl group is stabilized by the phenyl group.

The concentration of proteins in the Brew was found to be 6.4%. Although half of the proteins in green beans are water soluble (*47*), only 13% of the proteins present in the roasted beans are extracted into the Brew. This can be explained by the fact that proteins become less water soluble due to denaturation; that proteins might become less soluble due to chemical reactions occurring during roasting; and/or that proteins (amino acids) are degraded and converted into aroma compounds. The amino acid composition of the roasted beans and the Brew were quite similar, except for the fact that glutamine/glutamic acid increases from 22 to 29 mol %. The major amino acids in the Brew were glutamine/glutamic acid (29 mol %), glycine (13 mol %), and asparagine/aspartic acid (10 mol %), which corresponds with earlier data (*47*). Both the Brew LMw and HMw consisted of 6% of amino acids or proteins, and the amino acid composition of both fractions was similar to the amino acid composition of the Brew. The fact that the amino acid composition of the Brew LMw and HMw are similar suggests that the same protein was present in both fractions, although degraded to different extents. It was expected that the amino acid content in the Brew LMw would be negligible since the free amino acid content in green beans is only 0.3−0.6% (*54*) and are easily transformed upon roasting due to their reactivity toward the Maillard reaction (*32*). When examining the ethanol precipitation fractions, it is remarkable that the protein content first decreased from 4.4% in EP20 to 2.4% in EP60 and than increased again to 19.6% in ES80. The proteins from coffee beans, albumins and the 11S storage protein (a globulin), are reported not to be soluble in alcohol (*55*). Albumins and globulins readily precipitate at 25% ethanol (*56*). Therefore, it was not expected that half of the proteins from the Brew HMw would be soluble in 80% ethanol. This suggests that the proteins indeed did undergo a chemical reaction upon roasting, thereby changing their physical properties and thereby becoming soluble in alcohol. Incorporation of these proteins into a melanoidin complex could be a likely explanation for this. Another remarkable aspect is that the amino acid composition of the different Brew LMw, HMw, and ethanol precipitation fractions was similar for all fractions. This suggests that the proteins in the fractions are of the same type and that the fact whether they precipitate at a certain ethanol concentration only depends on the type and degree of chemical modification or degradation during roasting. It is hypothesized that proteins that were modified or degraded to a low extent, precipitate at low ethanol concentrations (20%); when they have undergone more chemical modification or degradation they become more alcohol soluble.

Comparison of the Nitrogen and Amino Acid Content. When the nitrogen levels and the amino acid analysis are compared, it is important to mention that the protein content based on the nitrogen content multiplied by a factor 5.5 is higher for all the HMw fractions when compared to the actual protein content (**Table 5**). Hence, in all HMw fractions nitrogen is

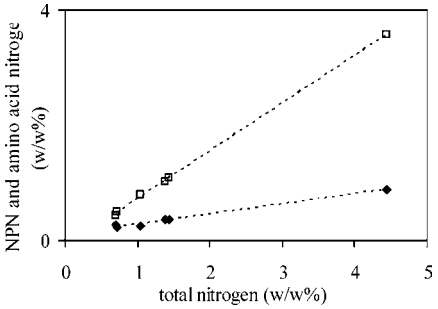

Figure 4. Amount of amino acid nitrogen and NPN from the HMw fractions as a function of the total amount of nitrogen: (open squares) nitrogen from amino acids; (solid diamonds) NPN.

present that cannot be ascribed to amino acids/proteins. The amino acid nitrogen and NPN contents of the HMw fractions, Brew HMw and ethanol precipitation fractions are plotted as a function of the total nitrogen content in **Figure 4**. It was calculated that the NPN was more or less a constant percentage (17%) of the total amount of nitrogen. At very low nitrogen contents, there was relatively more nitrogen originating from non-amino acids than from amino acids. Proteins are the most abundant nitrogen-containing HMw compounds in green beans, and amino acids from these proteins are probably the source for this NPN. The relatively large NPN content suggests that one out of six nitrogen molecules from amino acids were chemically modified or degraded and remained present in the HMw components while losing their amino acid characteristics. This finding is in line with the observation that 21% of the amino acids was lost upon roasting. From these results, it can be concluded that proteins are greatly affected by the roasting process and that the chemically modified or degraded proteins possess totally different solubility properties.

Plotting the $K_{\text{mix 405nm}}$ values of all the HMw fractions against the corresponding nitrogen contents (figure not shown) showed a clear correlation ($R^2 = 0.989$) between the nitrogen content and the $K_{\text{mix 405nm}}$ values. One measurement seems to have a divergent $K_{\text{mix 405nm}}$ value; this measurement corresponds to the EP20 fraction that was not completely soluble, explaining the underestimation of the $K_{\text{mix 405nm}}$ value. Since the $K_{\text{mix 405nm}}$ value represents the melanoidin content, there appears to be a correlation between the melanoidin content and the nitrogen content. Thus, the suggestion is made that the nitrogen present in the Brew HMw indeed is incorporated into the melanoidin complex. Since the nitrogen contents from amino acids/proteins and the NPN were proportional to the total nitrogen content, it can be suggested that all the NPN and proteins were incorporated into the melanoidin complex.

In this research, it was shown that ethanol precipitation led to unique melanoidin populations differing in carbohydrate content and composition. The introduction of K_{mix} allowed us to recognize the presence of possible substituents on melanoidins. From these ratios, it was concluded that, in addition to proteins, also other UV absorbing compounds like phenolic groups (chlorogenic acid) are present in the high molecular weight melanoidins. This is further emphasized by the fact that relatively more UV_{280} absorbance was found for fractions with low protein content. Although the existence of covalent interaction between melanoidins and polysaccharides were not proven in this study, the findings suggest that arabinogalactan is likely involved in melanoidin formation. Furthermore, proteins, which were shown to be partly degraded, seem to be part of melanoidins as well.

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Received for review June 2, 2006. Revised manuscript received July 28, 2006. Accepted August 3, 2006.

JF0615449