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Low Molecular Weight Melanoidins in Coffee Brew

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Analysis of low molecular weight (LMw) coffee brew melanoidins is challenging due to the presence of many non-melanoidin components that complicate analysis. This study focused on the isolation of LMw coffee brew melanoidins by separation of melanoidins from non-melanoidin components that are present in LMw coffee brew material. LMw coffee fractions differing in polarity were obtained by reversed-phase solid phase extraction and their melanoidin, sugar, nitrogen, caffeine, trigonelline, 5-caffeoylquinic acid, quinic acid, caffeic acid, and phenolic groups contents were determined. The sugar composition, the charge properties, and the absorbance at various wavelengths were investigated as well. The majority of the LMw melanoidins were found to have an apolar character, whereas most non-melanoidins have a polar character. The three isolated melanoidin-rich fractions represented 56% of the LMw coffee melanoidins and were free from non-melanoidin components. Spectroscopic analysis revealed that the melanoidins isolated showed similar features as high molecular weight coffee melanoidins. All three melanoidin fractions contained ~3% nitrogen, indicating the presence of incorporated amino acids or proteins. Surprisingly, glucose was the main sugar present in these melanoidins, and it was reasoned that sucrose is the most likely source for this glucose within the melanoidin structure. It was also found that LMw melanoidins exposed a negative charge, and this negative charge was inversely proportional to the apolar character of the melanoidins. Phenolic group levels as high as 47% were found, which could be explained by the incorporation of chlorogenic acids in these melanoidins.

KEYWORDS: Coffee; melanoidins; low molecular weight; solid phase extraction; anion exchange chromatography

INTRODUCTION

Coffee melanoidins are an abundant group of compounds that are formed during the roasting process of coffee beans (*1*). Melanoidins are generally defined as nitrogenous, macromolecular, brown-colored, final Maillard reaction products that absorb light at 405 nm (*1–3*). The chemical structure of melanoidins is largely unknown due to its complex composition. This is evident from the fact that heating of one reducing sugar in the presence of one amino acid yields numerous Maillard reaction products (*4*). In real food systems, the chemical structure of melanoidins is further complicated due to the presence of a wide range of reactants. In coffee beans, different reducing sugars are present, and these sugars can be present in various forms (mono-, di-, oligo-, and polysaccharides); the same holds true for the amino acids (*5*). Additionally, other compounds such as chlorogenic acids were shown to be involved in melanoidin formation as well (*6, 7*).

In coffee brew, melanoidins may account for up to 25% of the dry matter (*1*). Most of the research on coffee brew melanoidins has been devoted to macromolecular melanoidins (*8–12*), which account for 59% of all coffee melanoidins (*8*). Even though 41% of the coffee melanoidins have a low molecular weight (LMw), relatively little research has been dedicated to these LMw melanoidins. This might be ascribed to the presence of many other LMw compounds (e.g., caffeine, chlorogenic acids, trigonelline, minerals) in coffee brew. To circumvent this complexity, model systems are often used for the investigation of structural properties of LMw Maillard reaction intermediates (*13–17*). As research in this field is sophisticated, most of it is dedicated toward a single type of reaction intermediate and does not cover the whole range of melanoidin formation mechanisms. However, one should be extremely careful with extrapolating results of model systems to complex foodstuffs such as coffee beans. As a result, no or

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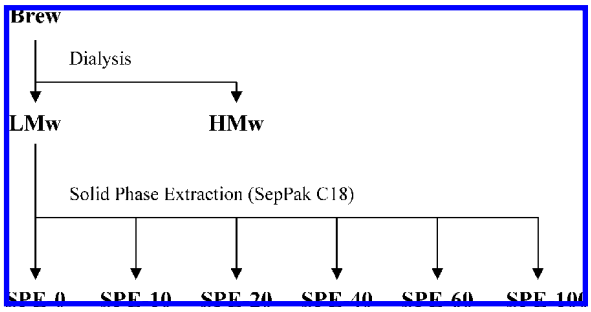

Figure 1. Scheme for the isolation of various LMw melanoidin coffee fractions from brew.

not much information is known on the overall structural properties of low molecular coffee melanoidins.

Previously, it was proposed that melanoidins are polymers built from repeating units of furans and/or pyrroles linked by polycondensation reactions in the advanced stages of the Maillard reaction (*18, 19*). This implies that melanoidins start off as LMw compounds, which polymerize upon prolonged roasting. Thus, even if one does not consider LMw brown-colored compounds as melanoidins, it is important to obtain knowledge on these compounds as they might be Maillard reaction intermediates ending up in the final macromolecular melanoidins. The proposed furan/pyrrole-like structure suggests that LMw melanoidins are apolar compounds.

The aim of this research was to gain insight into the structural properties of LMw coffee brew melanoidins. To this end, LMw coffee brew material was fractionated depending on polarity, and isolated fractions were characterized.

MATERIALS AND METHODS

Materials. Roasted Colombian coffee beans (*Coffea arabica*) were provided by a local factory. The degree of roast was 16.4% (w/w), which was 8.0% (w/w) on a dry matter basis. Chemicals were bought from Sigma Aldrich (Sigma Chemical Corp., St. Louis, MO) in the highest purity available.

Preparation of Coffee Brew. Coffee beans were ground, and a brew was prepared as described previously (*2*). Briefly, 600 g of water was added to 100 g of milled beans, and this mixture was heated for 15 min at 90 °C, followed by filtration to obtain the brew. For characterization purposes, part of the brew was lyophilized, yielding "brew". The major part of the brew was used for further isolation.

Isolation of High and Low Molecular Weight Materials from Brew. High and low molecular weight materials were isolated from 2 L of freshly prepared coffee brew by membrane dialysis (MW cutoff 12−14 kDa, Visking size 9, Medicell International Ltd., London, U.K.) against 6 L of demineralized water with three water renewals at 4 °C for 4 days. In total, four 6 L dialysate batches were used for dialysis to ensure that the retentate was free from LMw material. Small-scale dialysis experiments revealed that the first two 6 L dialysate batches should contain 95% of the LMw material. The last two dialysate batches, which should contain only the last 5% of LMw material, were discarded to minimize possible artifacts induced by prolonged dialysis. Prolonged dialysis was conducted to ensure that the high molecular weight (HMw) fraction would be really free from LMw material. The retentate and the first two dialysates were lyophilized, yielding a HMw fraction and a LMw fraction (**Figure 1**).

Defatting of Coffee Samples. Lyophilized coffee brew samples were defatted by Soxhlet extraction using a Soxtherm, which was connected to a Multistat system (Gerhardt, Königswinter, Germany), as described previously (*8*). The solvent used for extraction was dichloromethane.

Solid Phase Extraction of the LMw Fraction. Defatted LMw material was fractionated by reversed-phase (RP) solid phase extraction (SPE) using a 5 g Sep-Pak Vac 20 cm^3 C18 cartridge (Waters, Milford, MA). The RP material in the cartridges was conditioned prior to usage by rinsing with 50 mL of methanol and subsequently with 50 mL of Millipore water. Aqueous sample solution (10 mL, 10 mg/mL) was loaded on the cartridge, and material that did not bind to the RP matrix was washed from the cartridge by 100 mL of demineralized water. Subsequently, bound material was washed from the column in steps using 100 mL of 10% aqueous methanol, 100 mL of 20% aqueous methanol, 100 mL of 40% aqueous methanol, 100 mL of 60% aqueous methanol, 100 mL of 100% methanol, and 100 mL of water. This procedure was repeated 10 times, after which 1.1 g of LMw material was fractionated. For each methanol concentration, the effluents were collected and pooled, and methanol was evaporated using a rotary evaporator operating at 40 °C. The fractions were subsequently lyophilized, yielding SPE-0, SPE-10, SPE-20, SPE-40, SPE-60, and SPE-100 for the fractions that eluted at 0, 10, 20, 40, 60, and 100% methanol, respectively (**Figure 1**).

Determination of the Content of Various LMw Coffee Components. The levels of 5-caffeoylquinic acid (5-CQA), caffeic acid (CA), caffeine, and trigonelline were determined using RP high-performance liquid chromatography (HPLC). Aqueous sample solution (1 mg/mL) was centrifuged, and the supernatant was analyzed using a 150 × 4.6 mm i.d. XTerra MS C18 3.5 μm column (Waters, Milford, MA) with a 20 × 3.9 mm i.d. XTerra MS C18 3.5 μm guard column (Waters) as described previously (*7*). The absorbance of the eluate was measured at 280, 325, and 405 nm using a Spectra System UV3000 (Thermo Electron Co., Waltham, MA). The levels of trigonelline and caffeine were determined by their absorbance at 210 and 280 nm, respectively, whereas the levels of 5-CQA and CA were determined by their absorbance at 325 nm. The reference compounds, bought from Sigma Aldrich, were dissolved in Millipore water: caffeine (100 μg/mL), trigonelline (100 μg/mL), 5-CQA (100 μg/mL), and CA (25 μg/mL). Experiments were performed at least in duplicate. The coefficient of variation was 1% on average for all experiments using RP chromatography.

Determination of Quinic Acid by Ion-Moderated Partitioning HPLC. The level of quinic acid (QA) was determined using ion-moderated partitioning HPLC. Aqueous sample solution (1 mg/mL) was centrifuged, and the supernatant was analyzed using a 300 × 7.8 mm i.d. Aminex HPX 87H column equipped with a cation H+ guard column filled with AG 50W-X4 (Bio-Rad, Hercules, CA) using the procedure described previously (*7*). An aqueous QA solution was used as reference compound, and experiments were performed at least in duplicate. The coefficient of variation was 3% on average for all experiments using ion-moderated partitioning chromatography.

Sample Preparation for Total CA Determination. For determination of the total CA level, samples were saponified prior to analysis using the procedure described previously (*7*). Analysis was performed by RP-HPLC as described above. Saponified CA was used as reference compounds, and experiments were performed at least in duplicate.

Sample Preparation for Total QA Determination. For determination of the total QA level, samples were saponified prior to analysis using the procedure described previously (*7*). Analysis was performed by ion-moderated partitioning HPLC as described above. Saponified QA was used as reference compound, and experiments were performed at least in duplicate.

Anion Exchange Chromatography (AEC) of LMw Fractions. AEC was performed on the basis of a procedure described previously (*8*). A 1 mL HiTrap Q Fast-Flow column (Amersham Biosciences, Uppsala, Sweden) was connected to an ÄKTA Purifier system (Amersham Pharmacia Biotech, Uppsala, Sweden). The absorbance of the eluate was measured at 280, 325, and 405 nm using the UV-900 detector. Three eluents were used: (A) 5 mM NaOAc, pH 5.1; (B) 2 M NaOAc, pH 5.1; and (C) 2 M NaCl in 5 mM NaOAc, pH 5.1. Sample was dissolved in Millipore water (20 mg/mL), and 500 μL was applied onto the column with a flow rate of 1 mL/min. The elution profile consisted of isocratic elution with 15 mL of eluent A, in 0.15 mL to 7.5% eluent B followed by 15 mL isocratic elution, in 0.15 mL to 30% eluent B followed by 15 mL isocratic elution, in 0.70 mL to 100% eluent B followed by 15 mL isocratic elution, in 1.5 mL back to 100% eluent A followed by 10 mL isocratic elution, in 0.5 mL to 50% eluent C followed by 15 mL isocratic elution, in 0.5 mL to 100% eluent C

Table 1. Yield, K_{mix} Values, Percentage Melanoidins, and K_{mix} Ratios of Brew, HMw, LMw, and SPE-0−SPE-100

	yield[a] (%, w/w)	$K_{mix,405nm}$ (L/cm/g)	$K_{mix,325nm}$ (L/cm/g)	$K_{mix,280nm}$ (L/cm/g)	melanoidin[a] (%)	$K_{mix,325:405nm}$ ratio	$K_{mix,280:405nm}$ ratio
brew	19[b]	0.7	8	9	100[c]	11	13
HMw	37[c]	1.3	5	6	69[c]	4	5
LMw	51[c]	0.4	9	10	29[c]	23	25
SPE-0	60	0.1	5	4	15	50	40
SPE-10	11	0.4	24	17	11	60	43
SPE-20	8	0.6	10	22	12	17	37
SPE-40	11	0.9	16	28	25	18	31
SPE-60	4	3.4	14	18	34	4	5
SPE-100	1	1.8	4	6	5	2	3

[a] Percentage from LMw. [b] Percentage from beans. [c] Percentage from brew.

followed by 15 mL isocratic elution. After each run, the column was regenerated by elution with 0.5 M NaOH (20 mL), eluent B (20 mL), and eluent A (20 mL).

Preparative AEC of SPE-40. AEC was conducted using a 5 mL HiTrap Q Fast-Flow column (Amersham Biosciences) connected to an ÄKTA Purifier system (Amersham Pharmacia Biotech). The absorbance of the eluate was measured at 280, 325, and 405 nm using the UV-900 detector. Dry SPE-40 was dissolved in Millipore water (350 μg/mL), and 30 mL was injected onto the column with a flow rate of 5 m mL/min. Material not interacting with the column was collected by isocratic elution with 100 mL of 5 mM NH_4Ac, pH 5.1. Subsequently, the eluent was changed in 5 mL to 4 M NH_4Ac, pH 5.1, followed by 300 mL isocratic elution while the effluent containing negatively charged components was collected. The column was regenerated by elution with 2 M NaCl (100 mL), 0.5 M NaOH (100 mL), 4 M NH_4Ac, pH 5.1 (100 mL), and 5 mM NH_4Ac, pH 5.1 (100 mL). The fractions collected were subjected to rotary evaporation at 40 °C and freeze-drying, yielding SPE-40 AEC− and SPE-40 AEC+ for the material that eluted at 5 mM and 4 M NH_4Ac, respectively.

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.

Sugar Analysis. The neutral sugar composition was determined by gas chromatography according to the method of Englyst and Cummings (*20*) using inositol as an internal standard. Briefly, 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 total neutral sugar content was also determined by using the phenol−sulfuric acid assay according to Dubois et al. (*21*). The uronide content was determined by the automated colorimetric *m*-hydroxydiphenyl method (*22, 23*).

Total Phenolic Groups Level. The total phenolic groups content of the coffee samples were determined with the Folin−Ciocalteau assay as described previously (*2*). The used reference compound was 5-CQA.

Specific Extinction Coefficient of Coffee Material at 280, 325, and 405 nm. Coffee fractions were dissolved in water (1 g/L), and the absorption was determined at 280, 325, and 405 nm using a Hitachi U-3000 spectrophotometer (Hitachi, Tokyo, Japan). The coffee solutions were further diluted in case the absorption was higher than 1.3. The specific extinction coefficients K_{mix} (L/g/cm) were calculated as was previously described by Bekedam et al. (*2*).

RESULTS AND DISCUSSION

Yield and Melanoidin Levels of Coffee Brew Fractions. The coffee brew extraction procedure yielded 19% dry matter in the brew (**Table 1**), which was in line with previous results (*2*). The membrane dialysis procedure yielded 37% HMw material, which was somewhat higher than the 32% that was expected (*2*). The majority of the brew was found to be LMw material, and 51% of the brew's dry matter was recovered in the LMw fraction. The fact that the recovery of the dialysis procedure was 88% is caused by discarding the last two water renewals, which contained part of the LMw brew material. The $K_{mix,405nm}$ value is an indication for the melanoidin content (*2*) as will be explained below and was 0.4 for LMw and 1.3 for HMw (**Table 1**), showing that the melanoidin content was low in the LMw material and high in the HMw material. The low melanoidin content confirmed that quite some non-melanoidin material was present in the LMw fraction. The percentage melanoidins in a fraction was calculated using both the isolation yield and the $K_{mix,405nm}$ values: $[\text{yield} \times K_{mix,405nm}]_{fraction}/[K_{mix,405nm}]_{basis}$. For example, the percentage melanoidins from brew ending up in the HMw fraction was calculated by $37 \times 1.3/0.7 = 69\%$. Likewise, it was calculated that 29% of the melanoidins present in the coffee brew ended up in the LMw fraction.

The LMw material was fractionated by RP-SPE with the aim to separate the presumed apolar melanoidins from the more polar compounds such as sugars, caffeine, chlorogenic acid, trigonelline, and minerals (**Figure 1**). The majority of the LMw material was highly polar and did not show any interaction with the SPE material. This SPE-0 fraction, being 60% of the applied LMw material, had a $K_{mix,405nm}$ value of only 0.1, showing that the melanoidin content in this fraction was very low. By this first extraction step, it seems that the majority of the LMw compounds are obtained, whereas only 15% of the LMw melanoidins are coextracted. The material in fractions SPE-10 and SPE-20 was slightly less polar than the SPE-0 material as it showed some interaction with the SPE cartridge. These SPE-10 and SPE-20 fractions represented 11 and 8% of the dry matter of LMw. The $K_{mix,405nm}$ values were still relatively low, being 0.4 for SPE-10 and 0.6 for SPE-20, and these fractions together accounted for 23% of the LMw coffee melanoidins. The remaining fractions, SPE-40, SPE-60, and SPE-100, had relatively high $K_{mix,405nm}$ values, being 0.9, 3.4, and 1.8, respectively. Together, they accounted for the majority (64%) of the LMw melanoidins, whereas these fractions represented only 15% of the dry matter of LMw material. Obviously, LMw melanoidins seem to be quite apolar, and the RP-SPE isolation procedure allowed isolation of most of the melanoidins from the multicomponent, LMw coffee brew material into a fraction with a high melanoidin concentration.

***K_{mix}* Ratios of the LMw Coffee Fractions.** LMw coffee components show different light-absorbing properties at specific wavelengths. For example, caffeine absorbs light at 280 nm, whereas chlorogenic acids absorb light at 280 and 325 nm. Melanoidins absorb light throughout the whole wavelength spectrum. They can be exclusively detected at 405 nm as no other coffee components are known to absorb light at this

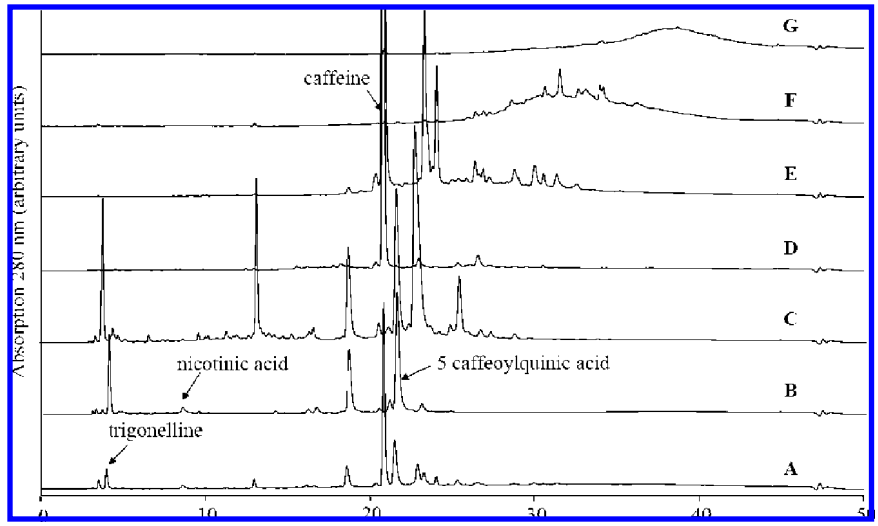

Figure 2. RP chromatography elution patterns of LMw (**A**), SPE-0 (**B**), SPE-10 (**C**), SPE-20 (**D**), SPE-40 (**E**), SPE-60 (**F**), and SPE-100 (**G**).

wavelength. The absorption at a specific wavelength, as expressed by the K_{mix} value, gives an indication of the components that might be present in a specific coffee fraction. The $K_{mix,325:405nm}$and $K_{mix,280:405nm}$ ratios give an indication of the relative purity of the melanoidins in a fraction (*2*). Relatively pure melanoidin populations will show high $K_{mix,405nm}$ values and low K_{mix} ratios, as can be seen for the HMw material (**Table 1**). The opposite, low $K_{mix,405nm}$ values and high K_{mix}ratios indicate that there are many non-melanoidin components present relative to the amount of melanoidins (*2*). However, it should be realized that constituents that do not absorb light at these wavelengths, such as carbohydrates and minerals, are not taken into account in this ratio. The high $K_{mix,325:405nm}$ ratios for SPE-0 and SPE-20 (**Table 1**) indicated higher chlorogenic acids levels than melanoidin levels. The fact that the K_{mix} values at all three wavelengths were low for SPE-0 indicates the presence of many non-light-absorbing compounds such as carbohydrates and minerals. The presence of these compounds in this fraction was also expected as carbohydrate and minerals are rather polar compounds. SPE-20 and SPE-40 show very high $K_{mix,280:405nm}$ ratios (**Table 1**), whereas the $K_{mix,325:405nm}$ ratios were rather low, which indicates that there might be relatively much caffeine present in these fractions. The SPE-60 and SPE-100 fractions have $K_{mix,325:405nm}$ and $K_{mix,280:405nm}$ ratios of 4 and 5 for SPE-60 and 2 and 3 for SPE-100 (**Table 1**), respectively. These values are comparable with the K_{mix} ratios of HMw melanoidins, indicating that these fractions consist of relatively pure melanoidins. Overall, the $K_{mix,405nm}$ values and the K_{mix} ratios gave clear indications on which coffee fractions are enriched in melanoidins and which fractions still contain a lot of other coffee components. The melanoidin-rich fraction SPE-40 was expected to be polluted with a non-melanoidin component such as caffeine, whereas the melanoidin-rich fractions SPE-60 and SPE-100 are expected to be free of non-melanoidin components.

Total, Caffeine, Trigonelline, and Melanoidin Nitrogen Contents of the Coffee Brew Fractions. The most abundant nitrogen sources in green coffee beans are free amino acids, protein, caffeine, and trigonelline. The ~0.5% free amino acids in green coffees are largely transformed upon roasting, and negligible amounts are present in roasted coffee (*6, 24*). Free amino acids take part in the Maillard reaction, resulting in components that contribute to the flavor and color of coffee brew (*24*). Proteins make up 9−12% of green coffee beans (*6*), and they might become incorporated into coffee melanoidins upon roasting (*2, 6, 25*). Green coffees contain about 1% trigonelline, of which 50−80% is degraded upon roasting (*26*). Major trigonelline degradation products are volatiles such as pyridine and pyrazines and nonvolatiles such as nicotinic acid and *N*-methylpyridinium ions (*26–28*). Caffeine is a heat-stable coffee component that makes up ~1.1% in green coffees (*26*). Nitrogen present in the LMw moiety of coffee brew can be partly accounted for by nitrogen from caffeine and trigonelline. The remainder should be accounted for by nitrogen present in melanoidin structures; this nitrogen is probably derived from amino acids and peptides.

RP HPLC was conducted to investigate the distribution of melanoidins and non-melanoidins, such as trigonelline and caffeine, over the various SPE fractions (**Figure 2**). The RP elution patterns of the most polar SPE-0, SPE-10, and SPE-20 fractions, which had low melanoidin levels (**Figure 2B−D**) showed many peaks, of which trigonelline, caffeine, 5-CQA, and CA were identified. The elution pattern of the melanoidin-rich SPE-40 fraction (**Figure 2E**) also showed several peaks, of which the main peak was identified as caffeine (t_R = 35 min). The other peaks were not identified, but they were likely due to chlorogenic acids. Additionally, a broad bump can be observed under the sharp peaks between 32 and 55 min of elution. The 405 nm elution pattern (not shown) showed the same bump at the same place, whereas the sharp peaks were absent. On the basis of these findings, it can be stated that the observed bump should be caused by LMw melanoidins. The RP elution patterns of the two most apolar SPE fractions (**Figure 2F**,**G**) showed even more pronounced bumps, with SPE-60 showing a few minor peaks as well. These bumps and peaks were also visible at 405 nm and are likely caused by LMw melanoidins. Thus, the RP elution patterns showed that both SPE-60 and SPE-100 were essentially free from non-melanoidin contaminants. The other melanoidin-rich fraction, SPE-40, still appeared to contain some non-melanoidin components, which is in line with the K_{mix} values and ratios.

The total nitrogen, caffeine nitrogen, trigonelline nitrogen, and calculated melanoidin nitrogen (N_{Mel}) levels are shown in **Table 2**. It stands out that all fractions contained nitrogenous compounds because all fractions contained at least 1% total nitrogen. The SPE-0 fraction contained all trigonelline from coffee brew; the trigonelline content in this fraction was 7%, which corresponded to 0.7% nitrogen. SPE-20 and SPE-40 contained high caffeine levels, which were already predicted on the basis of the K_{mix} ratios. The SPE-20 and SPE-40 fractions contained 29 and 25% caffeine, corresponding to 8.4 and 7.2%

Table 2. Total Nitrogen, Caffeine Nitrogen, Trigonelline Nitrogen, and Melanoidin Nitrogen Contents of Brew, HMw, LMw, and SPE-0−SPE-100

sample	total N (%, w/w)	caffeine N[a] (%, w/w)	trigonelline N[a] (%, w/w)	melanoidin N (%, w/w)
brew	2.8	1.1	0.3	na
HMw	2.1	0.0	0.0	na
LMw	3.4	1.8	0.4	1.2
SPE-0	1.1	0.0	0.7	0.4
SPE-10	2.3	0.0	0.0	2.3
SPE-20	10.7	8.4	0.0	2.3
SPE-40	9.5	7.2	0.0	2.3
SPE-60	3.4	0.0	0.0	3.4
SPE-100	3.4	0.0	0.0	3.4

[a] Percentage nitrogen is 28.9% in caffeine and 10.2% in trigonelline. na, not available due to the presence of proteins.

Table 3. Phenolic Groups, 5-CQA, and Free and Total QA and CA Contents in Brew, HMw, LMw, and SPE-0−SPE-100

sample	phenolic groups[a] (%, w/w)	5-CQA (%, w/w)	free QA (%, w/w)	total QA (%, w/w)	free CA (%, w/w)	total CA (%, w/w)
brew	23	2.8	2.6	11.2	0.0	4.3
HMw	17	0.2	0.0	3.5	0.0	1.1
LMw	23	4.4	5.2	11.9	0.0	5.7
SPE-0	11	5.6	7.5	21.4	0.0	3.7
SPE-10	47	8.5	0.2	20.2	0.1	13.9
SPE-20	33	0.2	0.0	9.2	0.0	3.0
SPE-40	53	0.0	0.0	10.1	0.0	8.5
SPE-60	41	0.0	0.0	4.9	0.0	1.6
SPE-100	47	0.0	0.0	0.4	0.0	0.2

[a] As 5-CQA equivalents.

caffeine nitrogen, respectively. The high caffeine content is, at least partly, the reason for the observed high K_{mix} ratio for SPE-40. Because both amino acids and proteins undergo chemical reaction upon roasting, yielding flavor and melanoidins, all non-caffeine, non-trigonelline nitrogen is likely to be incorporated in melanoidins. In **Table 2**, the N_{Mel} level was calculated by subtracting the caffeine and trigonelline nitrogen contents from the total nitrogen level. The N_{Mel} levels were 2.3, 3.4, and 3.4% for the melanoidin-rich fractions SPE-40, SPE-60, and SPE-100, respectively (**Table 2**). For SPE-40, the percentage of melanoidin nitrogen is underestimated due to the presence of 25% caffeine in this fraction. Removal of caffeine would lead to a higher N_{Mel} content because the same amount of melanoidin nitrogen would then be present in 25% less material. The calculated N_{Mel} level would then be 3.0%, which is 1.3 times higher than 2.3%. Thus, the melanoidins in SPE-40−SPE-100 have quite similar N_{Mel} contents, indicating that the melanoidins present in these three fractions possess similar nitrogen levels, although the polarity is different.

Phenolic Groups and Chlorogenic Acid Derivatives Level in the Coffee Brew Fractions. It was previously shown that HMw coffee melanoidins contained phenolic groups, which was ascribed to incorporation of chlorogenic acids upon roasting (*7*). The LMw SPE fractions had different phenolic group levels and chlorogenic acid levels compared with each other (**Table 3**). It was found that 5-CQA, CA, and QA ended up in SPE-0 and SPE-10, whereas the other SPE fractions were free of these components. The fractions SPE-20 and SPE-40 showed many large and unidentified peaks in their corresponding RP elution patterns (**Figure 2D**,**E**). Even though no 5-CQA was found to be present in SPE-40, it not unlikely that the observed peaks are due to the presence of other chlorogenic acids that are more polar, such as dicaffeolylquinic acids (*29*). Therefore, it is difficult to draw conclusions on the phenolic properties of the melanoidins present in the melanoidin-rich SPE-40 fraction. However, it was shown that SPE-60 and SPE-100 were free from non-melanoidin components as they did not show any non-melanoidin peak in their corresponding RP elution pattern (**Figure 2F**,**G**). Because the Folin−Ciocalteu phenolic group levels of these latter two fractions were as high as 47%, it can be concluded that the LMw melanoidin complexes should contain high levels of phenolic groups within their structures. As saponification of these latter two fractions yielded both QA and CA, it can be stated that chlorogenic acids are likely incorporated upon roasting in LMw coffee melanoidins. This incorporation of chlorogenic acids in coffee melanoidins was previously found for HMw coffee melanoidins as well (*7*). The total CA levels of the melanoidin-rich fractions SPE-40 to SPE-100 were 6−224 times lower than the phenolic groups level as determined by the Folin−Ciocalteu assay. This indicates that a large part of the phenolics within melanoidins is not caused by the presence of ester-linked CA, but rather by non-ester-linked CA. Very recently, Frank et al. (*30*) reported on the formation of apolar, non-ester-linked caffeic acid oligomers during heating of CA. The non-ester-linked CA molecules in melanoidins, as reported by us, are expected to have structural properties similar to those identified by Frank et al. (*30*). This non-ester-linked CA to coffee melanoidins is in line with findings previously reported for HMw coffee melanoidins (*7*). The ferulic acid levels (data not shown) were found to be negligible. The level of QA released by saponification was higher than the level of CA, as was previously found as well for other coffee melanoidins (*7*). The last remarkable point was that the ratio of phenolic groups over total QA level increased steadily with decreasing polarity of the LMw material. This indicates that LMw melanoidins contain relatively less incorporated QA compared to CA when the apolar character of the melanoidins is higher. This increase in apolar character can be explained by the fact that QA is a polar molecule, which is especially due to its carboxyl group. Thus, the less QA is present, the lower the effect of this QA on the overall polarity, which results in a more apolar melanoidin structure.

Sugar Composition of the Coffee Brew Fractions. The isolated fractions were analyzed for their sugar content and composition, and results are shown in **Table 4**. The majority of the carbohydrates from coffee brew were probably polysaccharides because 83% of the coffee brew carbohydrates ended up in the HMw fraction and only 17% ended up in the LMw fraction. With respect to the SPE fractions, it was found that all fractions contained carbohydrates, with contents ranging between 4 and 13%. It was calculated that 83% of all LMw sugars ended up in the SPE-0 (56%), SPE-10 (18%), and SPE-20 (9%) fractions. Thus, 17% of the sugars did not end up in these polar to moderately apolar fractions, which was unexpected because carbohydrates are polar compounds and should not bind to the reversed-phase media. The fact that 17% of the LMw sugars was retained on the SPE cartridge even after elution with 20% aqueous methanol suggests that these sugars are part of apolar molecules. These findings strongly implicate that intact sugar moieties are present in apolar LMw melanoidins after roasting of coffee beans.

The presence of sugar moieties in HMw melanoidins was reported previously by various groups (*8, 10, 31, 32*). However, no studies reported on the presence of sugar fragments in LMw coffee melanoidins. Presumably, carbohydrates with a DP ≥ 2

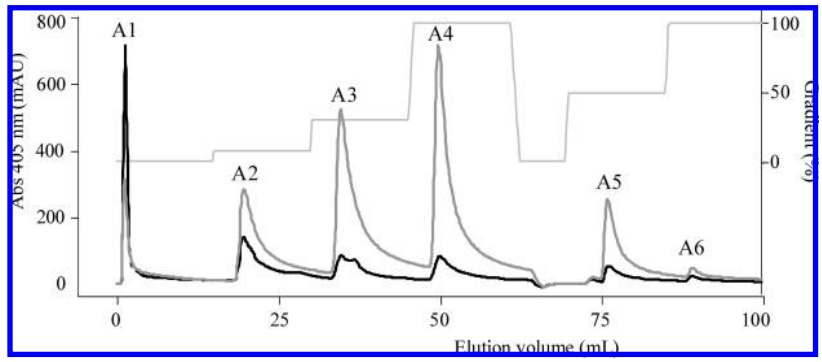

Figure 3. AEC elution patterns of HMw (gray trace) and LMw (black trace) coffee material.

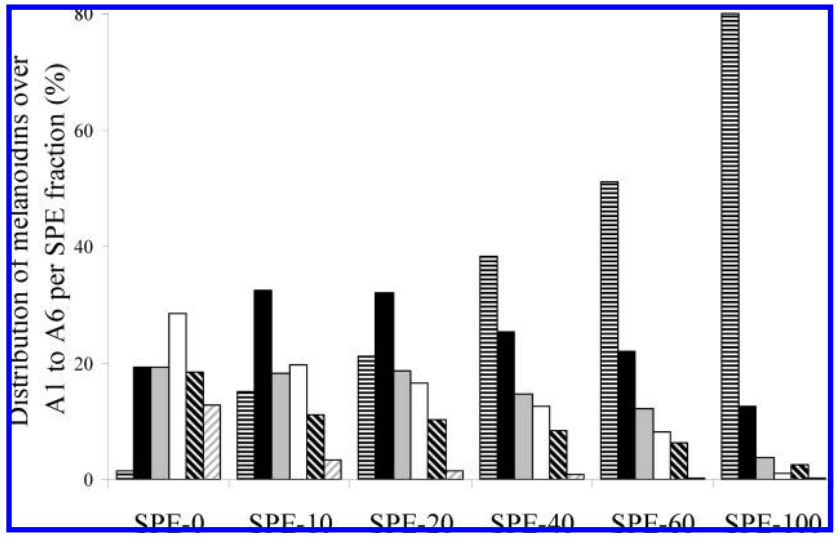

Figure 4. Distribution of melanoidins in the SPE fractions over the AEC peaks A1−A6 (A1, horizontally striped bars; A2, black bars; A3, gray bars; A4, white bars; A5, backslashed bars; A5, slashed bars).

Table 4. Sugar Composition (Mole Percent) of Brew, HMw, LMw, and SPE-0−SPE-100

	Rha	Ara	Man	Gal	Glc	uronic acid	total sugars (%, w/w)
brew	2	13	34	34	5	12	26
HMw	2	11	42	36	1	8	55
LMw	2	21	10	31	14	22	8
SPE-0	2	21	8	32	14	23	7
SPE-10	3	24	15	31	10	17	13
SPE-20	3	24	12	28	9	24	8
SPE-40	3	17	5	21	32	22	8
SPE-60	2	13	7	17	41	20	10
SPE-100	2	10	4	18	46	20	4

take part in the Maillard reaction, resulting in a Maillard reaction product with apolar characteristics and a carbohydrate moiety. The sugar that took part in the Maillard reaction is of course not recognizable as sugar anymore. The nonreducing sugar moiety from the carbohydrate is then attached via a glycosidic linkage to the brown-colored and apolar Maillard reaction product, the melanoidin.

When the sugar composition of the melanoidin-rich fractions SPE-40, SPE-60, and SPE-100 fractions is examined (**Table 4**), it stands out that glucose makes up almost half of all sugars present in these fractions. As glucose-based carbohydrates were not expected to bind to the SPE cartridge and as glucose is especially present in melanoidin-rich fractions, it can be concluded that this glucose is likely incorporated within the LMw melanoidin structures. In green coffee beans, there are three glucose-containing carbohydrates that could serve as sources for glucose-containing melanoidins. The first possible source for glucose is sucrose, which is a disaccharide built from glucose and fructose and which is the principal LMw sugar (6−9%) in green coffee (*33*). Upon roasting, sucrose is degraded rapidly and its content after roasting has decreased ∼100 times, even though sucrose has no reducing end (*33*). Cellulose is the second glucose source present in green coffees. Cellulose is a polysaccharide consisting of β-(1→4) linked glucan accounting for 6−8% of green coffees (*33*). The β-(1→4) linked glucose is highly stable and, therefore, cellulose remains largely undegraded even at prolonged roasting (*34*). The third possible source for glucose is xyloglucan, which was reported to be present in green coffee beans (*35*). As xyloglucan consists of β-(1→4) linked glucose monomers, such as cellulose, it should be stable and remain largely undegraded upon roasting as well. It was reported that the glucose content dropped initially during roasting by around 20% (*36*); this was ascribed to the sucrose degradation and not so much to the cellulose or xyloglucan degradation. Combining the facts that (i) nearly all sucrose is degraded upon roasting, (ii) the β-(1→4) glucose-glucose linkage is heat stable, and (iii) only part of the glucose is degraded upon roasting, it can be stated that sucrose is by far the most likely candidate that provides glucose for LMw, intact glucose-containing melanoidins. Additionally, it can be stated that ring opening of the fructose moiety from sucrose likely occurs prior to further chemical degradation, leading to brown color development; otherwise, it would not be possible to recover intact glucose from melanoidins.

Charge Properties of the Coffee Brew Fractions. It was previously shown that HMw coffee melanoidins expose a negative charge (*8*). Now, LMw coffee material was investigated for its charge properties. It was found that LMw coffee material that absorbs light at 405 nm showed interaction with the anion exchange column as well (**Figure 3**). These 405 nm light absorbing compounds eluted at various ionic strengths of the elution buffer. Therefore, it was concluded that LMw coffee melanoidins expose a negative charge and that they are heterogeneous with respect to this negative charge. HMw and LMw melanoidins show differences in their negative charge properties. First, the LMw melanoidins were less negatively charged than the HMw melanoidins: the peak corresponding to uncharged melanoidins (**Figure 3**, A1) was higher for LMw than for HMw; and the peaks corresponding to negatively charged melanoidin (**Figure 3**, A2−A6) were lower for LMw than for HMw. Second, HMw melanoidins were on average more negatively charged than LMw melanoidins, which is clear from the increase in HMw melanoidin peak area with increasing ionic strength of the eluent (**Figure 3**, A1→A4), whereas the LMw melanoidins showed a decrease in peak area. Overall, it could be concluded that LMw melanoidins expose less negative charge than HMw melanoidins do.

The charge properties of the melanoidins in the SPE fractions over the AEC peaks A1−A6 are shown in **Figure 4**. From this figure, it is clear that the most polar melanoidins, present in SPE-0, expose the highest negative charge and these melanoidins also show the widest distribution of this negative charge over the fractions A1−A6. It was found that the negative charge on melanoidins decreased with increasing apolarity. In SPE-100, 80% of the melanoidins were found to be neutrally charged versus only 1% in SPE-0. Thus, the apolarity is inversely proportional to the negative charge properties of LMw coffee melanoidins.

Chlorogenic Acid Incorporation Mechanism. According to Heyns and Hauber and later Tressl et al. (*18, 19*), it should be expected that LMw coffee melanoidins evolve toward more apolar compounds upon roasting. This implies that LMw melanoidins should evolve toward less negatively charged molecules. Even though results are not conclusive, it might be speculated that LMw melanoidin formation involves (i) incorporation of the whole chlorogenic acid into the melanoidin structure. The CA moiety provides the phenolic and apolar characteristics, whereas the QA moiety provides more polar characteristics and it contributes to the negative charge. (ii) QA is split off upon further roasting, leading to more apolar and more neutrally charged LMw melanoidins. The CA moiety remains present, providing apolarity, and causes the high phenolic groups level of around 50%.

This explanation is in line with previous studies which showed (i) that rather apolar, phenolic CA oligomers were formed during heating of CA (*30*) and (ii) that chlorogenic acids are most probably incorporated to melanoidins via the CA moiety through nonester linkages (*7*).

Isolation of One Pure LMw Melanoidin Population. One of the aims of this research was the development of a rapid procedure for the isolation of the majority of the LMw melanoidins from the multicomponent and complex LMw coffee brew material. Therefore, it would be advantageous to combine melanoidins in SPE-40 (25%) and melanoidins in SPE-60 and SPE-100 (39%). This combined fraction would then account for 64% of the LMw melanoidins, in <16% of the LMw material. However, the non-melanoidin components in SPE-40 had to be removed to obtain pure melanoidins and, therefore, an additional isolation step was needed. Because caffeine is neutrally charged and because 62% of the melanoidins in SPE-40 are negatively charged (**Figure 4**), AEC was performed to separate melanoidins from non-melanoidin components. The material that did not bind (SPE-40 AEC−, non-melanoidins) and the material that eluted at high ionic strength (SPE-40 AEC+, melanoidins) were analyzed by RP-HPLC (data not shown). The RP elution pattern of SPE-40 AEC− showed all peaks that were present in the elution pattern of SPE-40 (**Figure 2E**). The RP elution pattern of melanoidin fraction SPE-40 AEC+ showed only a broad bump corresponding to the melanoidins and no distinct peaks. With respect to the distribution of the melanoidins over the two fractions, it was found that 66% of the melanoidins from SPE-40 ended up in the melanoidin fraction SPE-40 AEC+. The $K_{mix,405:325}$ and $K_{mix,405:280}$ ratios for SPE-40 AEC+ were determined to be 6 in both cases. These values are comparable to the K_{mix} ratios for HMw melanoidins and were also in line with the ratios found for SPE-60 and SPE-100 (**Table 1**). Thus, both the RP elution pattern and the K_{mix} ratios show that the additional purification step performed on SPE-40 results in a "pure" melanoidin fraction. The combination of both RP-SPE and an additional quick purification step on SPE-40 allows the isolation of 56% of the melanoidins into a "pure" melanoidins fraction that was <13% (w/w) from the crude LMw coffee material. The results presented herein open possibilities for further research of individual LMw "pure" fractions as well as giving us the availability of a quick method, when combining these fractions, to screen total melanoidin levels in crude mixtures.

In this study, it was shown that most of the LMw coffee melanoidins had an apolar character and could be successfully separated from non-melanoidin coffee components. The presence of intact glucose as main sugar in fractions rich in melanoidins indicated that sucrose is likely involved in melanoidin formation. The LMw coffee melanoidins exposed a negative charge, and incorporation of chlorogenic acids likely caused the high level of phenolic groups in LMw melanoidins.

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