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Arabinogalactan Proteins Are Incorporated in Negatively Charged Coffee Brew Melanoidins

E. KOEN BEKEDAM,[†] MARIEKE P. F. C. DE LAAT,[†] HENK A. SCHOLS,*[,†] MARTINUS A. J. S. VAN BOEKEL,[‡] AND GERRIT SMIT[†,§]

Laboratory of Food Chemistry and Product Design and Quality Management Group, Department of Agrotechnology and Food Science, Wageningen University, P.O. Box 8129, 6700 EV Wageningen, The Netherlands, and Unilever Food and Health Research Institute, P.O. Box 114, 3130 AC Vlaardingen, The Netherlands

The charge properties of melanoidins in high molecular weight (HMw) coffee brew fractions, isolated by diafiltration and membrane dialysis, were studied. Ion exchange chromatography experiments with the HMw fractions showed that coffee brew melanoidins were negatively charged whereas these molecules did not expose any positive charge at the pH of coffee brew. Fractions with different ionic charges were isolated and subsequently characterized by means of the specific extinction coefficient ($K_{mix\ 405nm}$), sugar composition, phenolic group content, nitrogen content, and the arabinogalactan protein (AGP) specific Yariv gel-diffusion assay. The isolated fractions were different in composition and AGP was found to be present in one of the HMw fractions. The AGP accounted for 6% of the coffee brew dry matter and had a moderate negative charge, probably caused by the presence of uronic acids. As the fraction that precipitated with Yariv was brown ($K_{mix\ 405nm} = 1.2$), compared to a white color in the green bean, it was concluded that these AGPs had undergone Maillard reaction resulting in an AGP−melanoidin complex. The presence of mannose (presumably from galactomannan) indicates the incorporation of galactomannans in the AGP−melanoidin complex. As the uronic acid content in the more negatively charged melanoidin-rich, AGP-poor HMw fractions decreased, it was hypothesized that acidic groups are formed or incorporated during melanoidin formation.

KEYWORDS: Coffee brew; melanoidins; arabinogalactan proteins; Yariv; anion exchange chromatography

INTRODUCTION

Coffee is a popular beverage that is consumed worldwide by many people every day. In 2004, the total coffee consumption was estimated to be almost 7 billion kilograms of coffee beans (*1*). Many studies have dealt with coffee in relation to growing the crop, its composition, and its effects on health. Also, much research is focused on the formation of aroma, taste, and color during the roasting process. During the roasting of coffee beans, flavors and colored compounds are formed because of the Maillard reaction (*2*) that takes place between carbohydrates or degraded carbohydrates (*3*) and proteins (*4*). Brown-colored compounds are formed, and these compounds are referred to as melanoidins. It has been suggested that, next to the Maillard reaction, autoxidation of polyphenols contributes to the formation of melanoidins as well (*5*). These melanoidins make up to 25% of a coffee brew (*6*).

Melanoidins are not only of interest because of their contribution to color formation but also for their flavor binding properties (*7*−*13*), antioxidative capacity (*6*, *14*−*16*), metal-chelating properties (*16*, *17*), and reactivity in coffee brew (i.e., aging of coffee) (*18*). Thus, melanoidins have been studied in recent years because of their nutritional, biological, and health implications. Melanoidin chemical structures and properties are very complex and largely remain unknown. The complexity of the structure of melanoidins is due to the fact that many green coffee bean constituents play a role in melanoidin formation (*19*). This is also the reason that most research on melanoidins is conducted on melanoidins prepared by model systems. To date, three proposals for the structure of melanoidins have been suggested (*20*). Structural information obtained from melanoidins prepared by model reactions, often heating a reducing sugar and an amino acid, might contribute to understanding what occurs in melanoidin formation in real foods. However, this structural information is quite limited because the Maillard reaction in real foods is far more complicated, since many more possible reactants are present. As the molecular structure of melanoidins is largely unknown, these compounds are generically defined as brown, nitrogenous macromolecular material (*6*, *21*). Melanoidins can be detected by measuring their absorbance at 405 nm, which is an arbitrary chosen wavelength at which the intensity of the brown color is measured (*6*, *9*, *15*, *22*).

* To whom correspondence should be addressed. Tel: +31-317-482239; fax: +31-317-484893; e-mail: Henk.Schols@wur.nl.
[†] Laboratory of Food Chemistry, Wageningen University.
[‡] Product Design and Quality Management Group, Wageningen University.
[§] Unilever Food and Health Research Institute.

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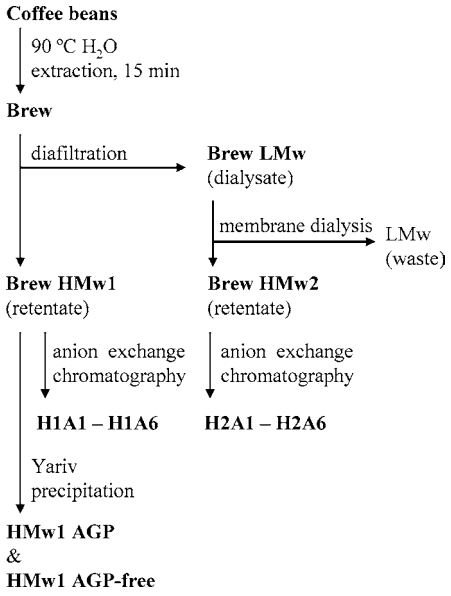

Figure 1. Scheme for the isolation of various melanoidin coffee fractions from roasted Arabica coffee beans.

As many possible structures for melanoidins might occur within coffee, it is interesting to determine whether melanoidins possess charged groups, since this could affect their physical behavior in coffee brew. However, only limited attention has been given to the possible charge aspects of melanoidins from coffee. Tomlinson et al. (*23*) have shown, using capillary zone electrophoresis, that melanoidins generated by the reaction of 5-hydroxymethylfurfural with glycine possess a partially anionic character. More recently, Morales (*24*) showed that high molecular weight coffee material also showed an anionic character. The high molecular weight coffee melanoidins fraction is rich in carbohydrates (*22*), and in green coffee beans, the most abundant carbohydrates are cellulose, galactomannans, and arabinogalactan type II (*25*). Redgwell et al. (*26*) reported that these arabinogalactans type II (arabinogalactan proteins) are acidic in the cell wall of coffee beans.

The objective of the present study was to investigate the charge properties of coffee melanoidins. Coffee brew melanoidin populations were isolated and characterized, and the melanoidin chemical and chromatographic characteristics were investigated with emphasis on the carbohydrate part.

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. Green (frozen with liquid nitrogen) and roasted coffee beans were ground and an extract was prepared as described previously by Bekedam et al. (*22*). For characterization purposes, part of the roasted bean brew was lyophilized, yielding "Brew" (**Figure 1**). The major part of the roasted bean brew was used for further isolation. The green bean extract was lyophilized and was not used for further isolation, yielding "green bean extract".

Defatting of Coffee Samples. The lyophilized coffee samples Brew, Brew HMw1, and Brew LMw were defatted by Soxhlet extraction using a Soxtherm, which was connected to a Multistat system (Gerhardt, Königswinter, Germany). The solvent used for extraction was dichloromethane (Sigma Chemical Co., St. Louis, MO). The extraction procedure comprised boiling in dichloromethane (40 °C) for 15 min followed by refluxing with dichloromethane for 2 h. After defatting, the dichloromethane was evaporated in an oven at 40 °C for approximately 30 min.

Isolation of Two High Molecular Weight Coffee Fractions from Coffee Brew. The first high molecular weight fraction of the coffee brew was obtained by diafiltration according to the procedure previously described Bekedam et al. (*22*). The retentate and dialysate were lyophilized, yielding a high molecular weight brew fraction ("Brew HMw1") and a fraction with a lower molecular weight ("Brew LMw"), respectively (**Figure 1**).

The second high molecular weight fraction was obtained from defatted Brew LMw by using membrane dialysis. The Brew LMw was dialyzed using a Visking size 9 dialysis membrane with a cutoff of 12−14 kDa (Medicell International Ltd, London, United Kingdom) for 2 days against running tap water and 1 day against demineralized water with two water renewals. The retentate of the Brew LMw was lyophilized, yielding a second high molecular weight brew fraction ("Brew HMw2") (**Figure 1**).

Anion Exchange Chromatography of the High Molecular Weight Coffee Fractions. Anion exchange chromatography was performed on a 5-mL HiTrap Q Fast-Flow column (Amersham Biosciences, Uppsala, Sweden) using 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. Samples were dissolved in eluent A (25 mg/mL), and 500 μL was applied onto the column at a flow rate of 4 mL/min. The elution profile consisted of 75 mL isocratic elution with eluent A, in 0.75 mL to 15% eluent B followed by 50 mL isocratic elution, in 0.75 mL to 30% eluent B followed by 50 mL isocratic elution, in 3.5 mL to 100% eluent B followed by 50 mL isocratic elution, in 7.5 mL back to 100% eluent A followed by 35 mL isocratic elution, in 2.5 mL to 50% eluent C followed by 50 mL isocratic elution, in 2.5 mL to 100% eluent C followed by 50 mL isocratic elution. The column was regenerated by elution with 0.5 M NaOH (50 mL), eluent B (100 mL), and eluent A (100 mL).

Cation Exchange Chromatography of the High Molecular Weight Coffee Fractions. Cation exchange chromatography was performed on a 5-mL HiTrap SP Fast-Flow column (Amersham Biosciences, Uppsala, Sweden) using 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. Two eluents were used: (A) 5 mM NaOAc, pH 5.1, and (B) 1 M NaCl in 5 mM NaOAc, pH 5.1. Samples were dissolved in eluent A (12.5 mg/mL) and 50 μL was applied onto the column at a flow rate of 3 mL/min. The elution profile consisted of 10 mL isocratic elution with eluent A, in 5 mL to 100% eluent B followed by 10 mL isocratic elution, in 5 mL to 100% eluent A. The column was reconditioned by elution with eluent A (25 mL) to prepare the column for the next run.

Preparative Anion Exchange Chromatography of the High Molecular Weight Coffee Fractions. Preparative anion exchange chromatography was performed on 5 × 5 mL HiTrap Q Fast-Flow columns (Amersham Biosciences, Uppsala, Sweden) connected in series using an ÄKTA Explorer system (Pharmacia Biotech, Uppsala, Sweden), and the absorbance of the eluate was measured at 280, 325, and 405 nm using the UV-900 detector. Brew HMw1 and Brew HMw2 were dissolved in eluent A (4 mg/mL) and were applied (50 mL) onto the columns at a flow rate of 6 mL/min using eluent A. 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. The elution profile was 100 mL isocratic elution with eluent A (fraction A1), in 12 mL to 15% eluent B followed by 100 mL isocratic elution (fraction A2), in 12 mL to 30% eluent B followed by 100 mL isocratic elution (fraction A3), in 60 mL to 100% eluent B followed by 150 mL isocratic elution (fraction A4), in 12 mL back to 100% eluent A followed by 60 mL isocratic elution (fraction A4), in 30 mL to 50% eluent C followed by 140 mL isocratic elution (fraction A5), in 30 mL to 100% eluent C followed by 100 mL isocratic elution (fraction A6). The column was regenerated by elution with 0.5 M NaOH, eluent B, and eluent A. The six fractions were dialyzed for 2 days against running tap water and 1 day against demineralized water, with two water renewals. The dialyzed fractions were lyophilized, yielding "H1A1−A6" for Brew HMw1 and "H2A1−A6" for Brew HMw2 (**Figure 1**).

Yariv Assay for Arabinogalactan Proteins. The Yariv assay was based on the procedure as described by Van Holst and Clarke (*27*). The Yariv phenyl glycoside (1,3,5-tri[4-β-D-glucopyranosyl-oxyphenylazo]2,4,6-trihydroxybenzene) was prepared as described by Yariv et al. (*28*). A 1% (w/v) agarose solution containing 0.15 M NaCl, 0.02% (w/v) sodium azide, and 0.002% (w/v) Yariv reagent was heated to boiling and was subsequently poured onto Petri dishes to give a layer of approximately 3-mm thick. Wells were made (3 mm in width) and 20 μL sample solution was pipetted into a well. The Petri dishes were sealed with Parafilm and were left for 2 days at ambient temperature to allow the colored halo to develop. Lyophilized coffee sample was dissolved in water (2 mg/mL) and was pipetted into a well. Gum arabic (Sigma Chemical Co., St. Louis, MO) (1, 2, 3, 4 mg/mL), an arabinogalactan (Novo Industries, Bagsværd, Denmark) (2 mg/mL), and a galactomannan (Diamalt GmbH, München, Germany) (2 mg/mL) were used as test polysaccharides. Water was used as blank.

Arabinogalactan Protein (AGP) Isolation. AGP was isolated from coffee in duplicate on the basis of a procedure described by Immerzeel et al. (*29*). Brew HMw1 (50 mg) was dissolved in 42.5 mL 0.176 M NaCl and was centrifuged at 10000*g* for 25 min at 5 °C. To the supernatant, 7.5 mL of Yariv reagent (10 mg/mL in water) was added and the Yariv−AGP complex was precipitated overnight at 4 °C. Next, the solution was centrifuged at 10000*g* for 25 min at 5 °C, yielding the AGP pellet and the AGP-free supernatant. The pellet was washed three times with 75 mL 0.15 M NaCl and was subsequently dissolved in 50 mL water. Sodium dithionite (1 g) was added to the AGP and AGP-free fractions to decompose the Yariv phenyl glycoside, followed by heating at 50 °C (15 min) until the red color disappeared. The solutions were dialyzed at 4 °C for 2 days against running tap water and 1 day against demineralized water, with two water renewals. The dialyzed AGP and AGP-free fractions were lyophilized, yielding "Brew HMw1 AGP" and "Brew HMw1 AGP-free" (**Figure 1**).

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 as described by Bekedam et al. (*22*) on two 300 × 7.8 mm i.d. TSKgel columns in series (G4000 PWXL and G2500 PWXL) (TosoHaas, Stuttgart, Germany), in combination with a PWXL-guard column (TosoHaas, Stuttgart, Germany). 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 (Thermo Electron Company, Waltham, MA), and the absorbance was measured at 280, 325, and 405 nm using a Spectra System UV2000 (Thermo Electron Company, Waltham, MA). 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 (*30*) 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 neutral sugar content was also determined by the phenol sulfuric acid assay according to Dubois et al. (*31*). The uronide content was determined by the automated colorimetric *m*-hydroxydiphenyl method (*32, 33*).

Total Phenolic Groups Content. The total phenolic groups 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 1 mL of the coffee sample solution, 500 μL of Folin−Ciocalteu (Merck, Darmstadt, Germany) reagent was added. After mixing, 1 mL 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 (Sigma Chemical Co., St. Louis, MO) was used as reference phenolic compound.

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 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 $K_{mix\ 405nm}$ (L/g/cm) were calculated as was previously described by Bekedam et al. (*22*).

Table 1. Yield, $K_{mix\ 405nm}$ Value, Nitrogen and Phenolic Groups Level of Coffee Fractions[a]

coffee sample	yield[b] (%, w/w)	$K_{mix\ 405nm}$ (L/cm/g)	nitrogen (%, w/w)	phenolic groups[c] (%, w/w)	total sugar (%, w/w)
Brew	100[d]	0.7	3.13	21	26
Brew HMw1	15.5[d]	1.1	1.42	11	69
Brew HMw2	16.2[d]	1.5	2.81	18	43
H1A1	5	0.2	0.93	2	39
H1A2	30	0.3	0.55	4	74
H1A3	16	0.9	1.17	6	57
H1A4	25	1.2	2.75	9	33
H1A5	9	1.4	3.34	10	16
H1A6	10	0.7	2.91	5	10
H2A1	11	0.3	1.15	2	60
H2A2	21	0.9	4.37	8	41
H2A3	9	1.6	4.57	12	20
H2A4	18	2.6	3.34	17	16
H2A5	7	2.7	2.66	12	13
H2A6	5	1.0	2.27	4	7
HMw1 AGP	41	1.2	1.30	7	50
HMw1 AGP-free	45	1.0	1.48	7	56

[a] The standard deviation of the various parameters was 2.5% on average. [b] From starting HMw material. [c] As chlorogenic acid equivalents. [d] From Brew.

Table 2. Sugar Composition (Mol %) of Brew, Brew HMw1, Brew HMw2, HMw1 AGP, and HMw1 AGP-free

coffee sample	Rha	Ara	Xyl	Man	Gal	Glc	uronic acid	total sugar (%, w/w)
Brew	2	11	0	33	34	6	14	26
Brew HMw1	2	10	1	41	37	1	8	69
Brew HMw2	1	11	0	46	31	1	9	43
HMw1 AGP	0	11	1	9	68	2	9	50
HMw1 AGP-free	3	11	1	54	19	1	10	56

RESULTS AND DISCUSSION

Characterization of the Brew HMw Fractions. The isolation of the Brew, Brew HMw1, and Brew HMw2 was reported previously (*22*). Brew HMw1 was isolated from Brew by diafiltration (cutoff 3 kDa), and the dialysate of this filtration step was subsequently subjected to membrane dialysis (cutoff 12−14 kDa), yielding Brew HMw2. In this previous research, the Brew and Brew HMw1 were characterized, while no characteristics on Brew HMw2 were given. To be able to compare the characteristics for Brew HMw2 with the Brew and Brew HMw1, part of the characteristics on the Brew and Brew HMw1 is shown again in this study. The Brew, Brew HMw1, and Brew HMw2 were analyzed for their composition (**Table 1** and **2**). The yield of Brew HMw1 and Brew HMw2 were both 16% (w/w). Both the HMw fractions were analyzed by size exclusion chromatography, and **Figure 2** shows that Brew HMw1 and HMw2 are two distinct populations in the brew with respect to their molecular weight distribution, with Brew HMw1 having the largest molecular weight (Mw) distribution and Brew HMw2 having the lower Mw distribution. The fact that Brew HMw2 elutes slightly earlier compared to the second HMw peak

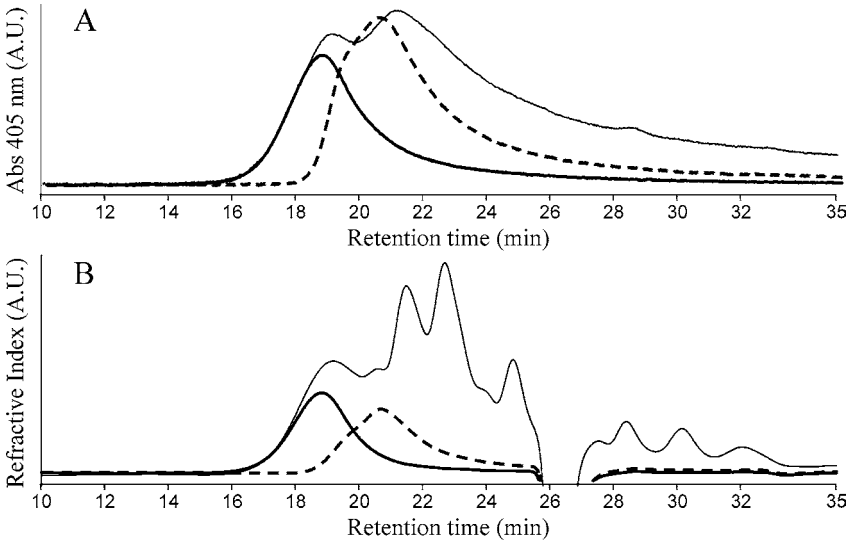

Figure 2. Size exclusion patterns of Brew (solid thin line), Brew HMw1 (solid thick line), and Brew HMw2 (dashed line). **A**: 405-nm absorption patterns; **B**: refractive index patterns.

of the Brew is possibly due to an interaction of the polymers with other coffee compounds. The $K_{\text{mix 405nm}}$ value, a parameter for the melanoidin content (*22*), was 1.1 for Brew HMw1 and 1.5 for Brew HMw2, indicating that Brew HMw2 contains 1.4 times more melanoidins than Brew HMw1. Brew HMw1 (24%) and Brew HMw2 (35%) together account for 59% of the melanoidins present in the Brew while they represent 32% of the Brew dry matter. Thus, a large part of the melanoidins was isolated by the procedures used. The nitrogen and phenolic groups contents were also found to be higher for Brew HMw2, being 2 and 1.5 times higher for Brew HMw2 compared to Brew HMw1, respectively. These results suggest that there is a relationship between the melanoidin content and the nitrogen and phenolic groups level in coffee brew, as previously suggested on the basis of ethanol precipitation (*22*). With respect to the sugar content, it is clear that Brew HMw2 contained less sugars (43%) compared to Brew HMw1 (69%). However, the sugar composition was found to be quite similar in these two fractions, although Brew HMw2 contained relatively more mannose and slightly less galactose. The lower sugar content in Brew HMw2 can be explained by the higher protein/nitrogen and phenolic group content. Taken together, two melanoidin-rich high molecular weight fractions were isolated from coffee Brew, and both fractions had a similar sugar composition but differed in sugar, nitrogen level, and phenolic group content. Both fractions were subjected to further analysis.

Ion Exchange Chromatography of Brew Fractions. Coffee material was analyzed for its charge by ion exchange chromatography. The Brew, Brew HMw1, and Brew HMw2 were applied on a strong cation exchange column using an eluent with a low ionic strength. For all coffee fractions, all the material eluted directly under the conditions chosen and no material was retained on the column (data not shown) as judged on 280, 325, and 405 nm absorption. Since no binding was observed, it was concluded that coffee melanoidins do not expose positively charged groups at the pH of coffee brew.

When coffee fractions were applied on a strong anion exchange column, totally different results were obtained. Brew HMw1 and Brew HMw2 showed strong binding to the anion exchange column and almost no material eluted in the unbound fraction, on the basis of the absorption at 280, 325, and 405 nm. By increasing the ionic strength gradually, no distinct populations could be observed and all material was eluted in a very broad peak (data not shown). However, by increasing stepwise the ionic strength of the eluent, six fractions were obtained and collected for further analysis. The elution patterns at 405 nm of both Brew HMw1 and Brew HMw2 are shown in **Figure 3**. The elution patterns at 280 and 325 nm were quite similar although the peak area at 280 and 325 nm decreased slightly with increasing ionic strength compared to the 405-nm peak areas. Brew HMw1 and Brew HMw2 showed similar elution patterns. From **Figure 3**, it can be concluded that melanoidins in both HMw fractions possessed a negative charge at the pH of coffee brew.

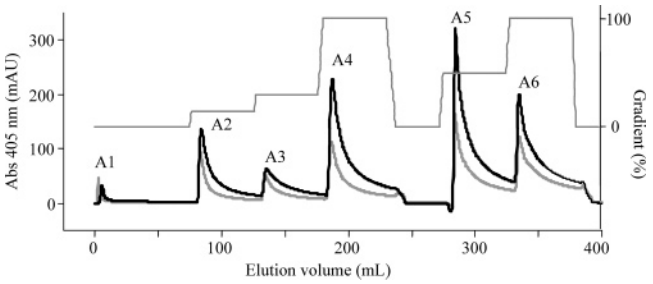

Figure 3. Anion exchange chromatography elution patterns of Brew HMw1 (gray line) and Brew HMw2 (black line).

Characterization of Negatively Charged Brew HMw Fractions. Brew HMw1 and Brew HMw2 were applied on preparative scale on anion exchange columns and the fractions were collected for analysis. The elution patterns obtained were identical to the pattern shown in **Figure 3** (data not shown). Re-elution of the fractions obtained resulted in elution of the fraction at the same ionic strength as that at which it was collected, from which it was concluded that the observed elution behavior was reproducible and that the different fractions have different charges. Characteristics of the fractions obtained are shown in **Tables 1** and **3**. The yield of the unbound fraction of HMw1 and HMw2 was only 6 and 16% (w/w), respectively (**Table 1**), proving that indeed most of the material present in the Brew HMw was negatively charged. On the basis of the 405-nm absorption, only 1% and 3% of the melanoidins eluted in the unbound fraction of HMw1 and HMw2, respectively, from which it was concluded that almost all coffee melanoidins are negatively charged. For both HMw fractions, it was observed that the melanoidin content ($K_{\text{mix 405nm}}$) increased with increasing retention on the anion exchange material (**Table 1**), except for

Table 3. Sugar Composition (w/w%/(Mol %)) of Anion Exchange Chromatography Fractions of Brew HMw1, and Brew HMw2

coffee sample	Rha	Ara	Xyl	Man	Gal	Glc	uronic acids	total sugar (%, w/w)
H1A1	<1/(0)	1/(2)	<1/(1)	34/(85)	3/(7)	1/(2)	1/(2)	39
H1A2	2/(3)	8/(13)	<1/(1)	21/(27)	37/(48)	1/(1)	6/(7)	74
H1A3	2/(3)	7/(14)	<1/(1)	7/(12)	32/(56)	<1/(1)	8/(13)	57
H1A4	1/(3)	4/(14)	<1/(1)	5/(14)	15/(45)	1/(2)	8/(21)	33
H1A5	<1/(3)	2/(16)	<1/(3)	3/(22)	6/(40)	1/(4)	2/(12)	16
H1A6	<1/(2)	1/(9)	<1/(4)	4/(35)	2/(20)	1/(9)	2/(20)	10
H2A1	1/(2)	7/(13)	<1/(1)	23/(37)	25/(42)	1/(2)	2/(4)	60
H2A2	1/(2)	5/(15)	<1/(1)	6/(14)	22/(54)	1/(1)	6/(12)	41
H2A3	<1/(2)	2/(13)	<1/(1)	2/(12)	8/(38)	1/(5)	6/(28)	20
H2A4	<1/(2)	2/(12)	<1/(3)	2/(12)	5/(31)	1/(6)	6/(35)	16
H2A5	<1/(2)	1/(14)	<1/(4)	2/(12)	4/(29)	1/(10)	4/(29)	13
H2A6	<1/(2)	1/(12)	1/(8)	1/(16)	2/(22)	2/(20)	2/(20)	7

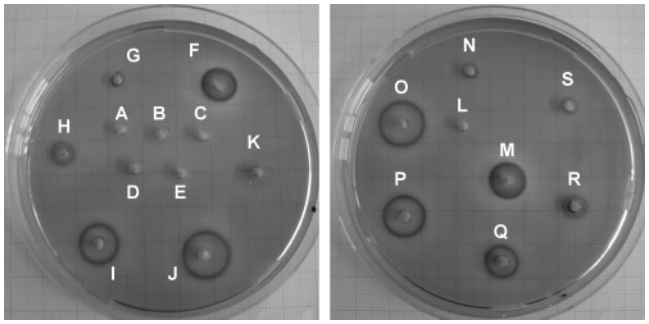

Figure 4. Yariv-gel diffusion assay of water (**A** and **L**), 2 mg/mL arabinogalactan (**B**), galactomannan (**C**), Brew HMw2 (**D**), Brew LMw (**E**), gum arabic (**F** and **M**), green bean extract (**G**), Brew (**H**), Brew HMw1 (**I**), Brew HMw1 AGP (**J**), Brew HMw1 AGP-free (**K**), H1A1 (**N**), H1A2 (**O**), H1A3 (**P**), H1A4 (**Q**), H1A5 (**R**), and H1A6 (**S**).

the last fraction A6 in which the melanoidin content decreased again. The nitrogen level was found to be low in the unbound fractions and varied over the fractions A2−A6. The phenolic group content correlated with the melanoidin content. A positive correlation for the nitrogen and phenolic group content with the melanoidins content was seen for Brew HMw1. The phenolic group content and the melanoidin content in Brew HMw2 showed a positive correlation as well, although no correlation between the nitrogen content and the melanoidin content was found. Thus, it seems that phenolic groups are present within the melanoidin complex. From the results obtained by the Folin−Ciocalteu reagent, no statement about the nature of the phenolic groups in melanoidins can be made. Incorporation of low Mw phenolics might be possible, e.g., ester-linked chlorogenic acid. Formation of neoformed phenolic-like compounds out of nonphenolics during the Maillard reaction is possible as well since Tressl et al. (*34*) mentioned the presence of furans and pyrrole-like structures in melanoidins, which might in the close vicinity of hydroxyl groups behave as phenolic groups.

The sugar composition of each anion exchange fraction is shown in **Table 3**. It is clear that the sugar content in the fractions decreased with increasing ionic strength and that mannose-rich polymers (galactomannans) especially were eluted at low ionic strength (A1 and A2). Elution of galactomannans in the unbound fractions may not be surprising because galactomannans from green beans did not bind to an ion exchange column either (*35*). The uronic acid content initially increased with increasing ionic strength (A1→A2→A3) up to maximally 8% (w/w) of the Brew HMw. This increase in uronic acid content might explain the difference in charge in the different fractions (A1→A3). It might even explain the anionic character of the melanoidins, but in that case, the uronic acid containing carbohydrate should be part of the melanoidin complex. For the populations with the highest melanoidin content (A4 and A5), it is clear that the uronic acid content remained constant (A3→A4) and then decreased (A4→A5). This shows that the anionic character of the material in fractions A5 and A6 cannot be explained by uronic acids only, indicating that another characteristic negative-charged group should also be present to be able to explain the further increase in negative charge. Incorporation of negatively charged low molecular weight coffee compounds (e.g., acids) might be an explanation, as is the possible formation of newly formed acid groups upon roasting.

The recovery of arabinose, galactose, and uronic acids was good (>80%) for both fractions, but the mannose recovery was relatively low, 37 and 31% for Brew HMw1 and Brew HMw2, respectively. Determination of the neutral sugar content of the effluent fractions by the phenol sulfuric acid assay according to Dubois (*31*) showed that the sugars were not lost during the chromatography isolation step. Therefore, it seems that part of the HMw material is lost during dialysis, and as the HMw2 fraction was previously dialyzed thoroughly, it seems that dialysis conditions affect the final yield. In other words, the HMw fraction size will decrease after conducting a second dialysis step using another membrane.

From the results, it can be concluded that coffee melanoidins are negatively charged molecules which are heterogeneous with respect to their polyanionic behavior since melanoidins eluted at different ionic strengths. Furthermore, it was shown that characteristic groups other than uronic acids are likely to be present to account for the total negative charge. The presence of these negative charges on melanoidins probably contributes to the biological and functional properties of melanoidins in coffee brew. For example, covalent or ionic binding of chlorogenic acid with negative-charged groups on melanoidins might explain the antioxidative properties observed for melanoidins (*6*, *14*−*16*). Binding of coffee flavor compounds to melanoidins as suggested by others (*7*−*13*) might occur via the anionic groups of melanoidins and might affect the sensorial perception of coffee. Also, positively charged metal ions might also bind to the negatively charged melanoidins, resulting in the metal-chelating properties observed by others (*16*, *17*). Another option is that melanoidins might contribute to the foaming properties of coffee brews (*36*, *37*) since they might consist partly of hydrophobic sections with mainly furan and pyrrole-like structures (*34*) and partly of hydrophilic sections with the negative-charged groups, resulting in a surface-active molecule. Overall, the anionic character of melanoidins might be responsible for quite some biological and functional properties that are ascribed to melanoidins in the literature.

Identification of Arabinogalactan Proteins (AGPs) in Coffee Brew. Arabinogalactan is, together with galactomannan, the most abundant carbohydrate in coffee brew. The presence of acidic AGPs (∼15%, w/w) in the cell wall of green coffee beans was reported by Redgwell and co-workers (*26*, *38*). Since these acidic coffee bean AGPs are extracted into the coffee brew, it was questioned whether these AGPs are also present in the negatively charged polymer fractions that were isolated, as described above. Therefore, the presence of AGPs in coffee brew fractions was investigated by a Yariv gel-diffusion assay, in which the Yariv phenyl glycoside specifically binds to AGPs resulting in a colored halo (**Figure 4**). Water, arabinogalactan, and galactomannan solutions were used as controls. Gum arabic,

an AGP-rich compound, was used as a positive control, and the development of a clear halo was observed. A linear correlation between the applied amount of gum arabic and the square of the diameter of the halo was obtained (data not shown) as mentioned by Van Holst and Clarke (*27*). The control samples showed that the Yariv reagent very specifically reacts with AGPs and not with carbohydrates (galactomannan and arabinogalactan) that are not bound to protein. A green coffee bean extract was prepared and applied on the Yariv-plate to determine whether the green coffee bean cell wall AGPs (*26*, *38*) are extracted when heated in water at 90 °C. This green bean extract (**Figure 4G**) showed only slight color development in the well area, while no distinct halo could be observed, indicating that AGPs in green beans are not readily extracted. This is in agreement with findings by Redgwell et al. (*26*) who reported that harsh conditions are needed to isolate the majority of the coffee arabinogalactans (8 M KOH and enzymatic degradation of other cell wall components). The Brew (**Figure 4H**) gave a distinct halo, and it can be concluded that the Brew contains AGPs. In addition to the findings reported by Redgwell et al. (*26*), who stated that roasted coffee cell wall material contains AGPs, it is now found that at least part of these AGPs end up in coffee brew as well. Since the Brew showed a halo and since the green bean extract did not show a halo, it can be stated that the roasting process improves the extractability of AGPs. The AGPs present in the green bean are likely entrapped in the cell wall of green coffee beans and the roasting process affects the cell wall in such an extent that they become more readily extractable, resulting in their presence in coffee brew. **Figure 4** also shows that all AGPs from the coffee Brew end up in the Brew HMw1 fraction and that Brew LMw and Brew HMw2 are free of AGPs. Obviously, all AGPs are retained by the diafiltration membrane which was used to isolate Brew HMw1 from Brew. AGP was isolated to investigate their content in the Brew and to determine whether they are included in the melanoidin formation.

Characterization of the AGP Fraction of Brew HMw1. The AGPs were isolated from Brew HMw1 by precipitation with the Yariv reagent on a preparative scale. Isolation of the AGPs from Brew HMw1 yielded an AGP (41%, w/w) and AGP-free (45%, w/w) fraction (**Table 1**), and these AGPs make up ~6% of the Brew's dry matter. Both the isolated fractions were recovered as a brown-colored material. Redgwell et al. (*38*) reported that AGPs in green coffee beans were white and gave a colorless clear solution in water. As the Yariv reagent is very specific for AGPs, and as the isolated fraction was brown instead of white, it allowed us to infer that coffee bean AGPs undergo chemical reactions during roasting and become part of the melanoidin complex. This material is referred to as the AGP−melanoidin complex. The composition of the Brew HMw1 AGP and AGP-free fraction was determined and the results are shown in **Tables 1** and **2**. The $K_{\text{mix }405\text{nm}}$ values, the parameter for the melanoidin content, of the AGP and AGP-free fraction were 1.2 and 1.0, respectively, showing that both fractions were rich in melanoidins. The AGP fraction accounted for 45% of the melanoidins present in the Brew HMw1. With respect to the Brew, this AGP fraction (~6%, w/w) accounted for 11% of all the melanoidins present in the Brew. Because of the specificity of the Yariv reagent and the intensity of the washing steps, it was concluded that coprecipitation of melanoidin molecules and compounds other than AGPs is very unlikely. Thus, the isolated AGP fraction is a melanoidin-rich population from coffee brew that consisted of only one type of compounds, namely, the AGP−melanoidin complex.

The nitrogen levels in the AGP and AGP-free fractions were 1.30% and 1.48%, respectively. The nitrogen content of the AGP fraction is quite in line with the literature. Redgwell et al. (*38*) reported a nitrogen content of 1.88% for green coffee bean AGPs. The nitrogen in the melanoidin-rich AGP fraction can probably largely be explained by the presence of intact amino acids from the protein moiety in the AGPs as they occur in green beans. However, degradation of part of these amino acids upon roasting cannot be ruled out.

The phenolic group content for both the AGP and AGP-free fraction was found to be 7%. Thus, phenolic groups were present in the AGP fraction but the phenolic groups were not specifically related to the AGPs or AGP-free fraction. The relative low phenolic group content in the AGP and AGP-free fractions (7%), compared to the Brew HMw1 fraction (11%), might be explained by incomplete dissolving of the fractions or by the loss of compounds upon dialysis.

The sugar content for the AGP (50%, w/w) and AGP-free (56%, w/w) fractions was similar, but these fractions differed with respect to the sugar composition. The AGP-free fraction contained mainly mannose (54 mol %), galactose (19 mol %), arabinose (11 mol %), and uronic acids (10 mol %) indicating that galactomannans were the most abundant sugars in this fraction and that arabinogalactans are present in a lesser extent. The galactose/arabinose ratio in the AGP-free fraction was only 1.7 and part of this galactose is probably part of the galactomannans, therefore, the galactose/arabinose ratio for the arabinogalactans is probably even lower. The uronic acids are expected to be part of the arabinogalactans as well. On the basis of these findings, it can be concluded that the arabinogalactans in the AGP-free fraction are highly branched polymers. For the AGP fraction, it was found that this fraction contained mainly galactose (68 mol %), arabinose (11 mol %), mannose (9 mol %), and uronic acids (9 mol %), indicating that arabinogalactan was the most abundant sugar, as expected (*38*). Comparing the galactose/arabinose ratio of 6.2 with data found in the literature, it was found that this value was rather high as the AGP fraction of green coffee beans had a value of 3.0 (*38*) and the ratio of gum arabic was 1.8 or lower (*39*). The high galactose/arabinose ratio can be explained by the fact that arabinose is the monosaccharide that is most prone to degradation upon roasting, and losses up to 65% (w/w) were reported (*3*). The presence of 9 mol % mannose in the AGP fraction was unexpected since no mannose was found to be present in AGP from green coffee beans (*38*) and AGP from gum arabic contains only trace amounts of mannose. Since galactomannans do not show precipitation with the Yariv reagent, it allowed us to infer that mannose, probably as galactomannan, is incorporated into the AGP−melanoidin complex.

Redgwell et al. (*26*) reported that green Arabica coffee beans (e.g., var. Yellow Caturra) contained 45% (w/w) carbohydrates, of which 15% (w/w) were from galactose (10%), arabinose (3%), and uronic acids (2%). On the basis of the data that green coffee beans contain ~15% (w/w) AGPs and that 85% (w/w) of these AGPs are carbohydrates (galactose, arabinose, and uronic acids) (*38*), it can be stated that almost all arabinogalactan (85%) in green coffee beans should be present in AGPs. The AGP fraction isolated from green coffee beans by Redgwell et al. (*38*) was positive for the Yariv gel-diffusion assay as well, although a different isolation procedure was applied. It was calculated from the sugar composition and yield of the two Brew HMw fractions that only 60% (w/w) of the arabinose, galactose, and uronic acids ended up in Brew HMw1. From the sugar composition and yield of the two fractions isolated from Brew

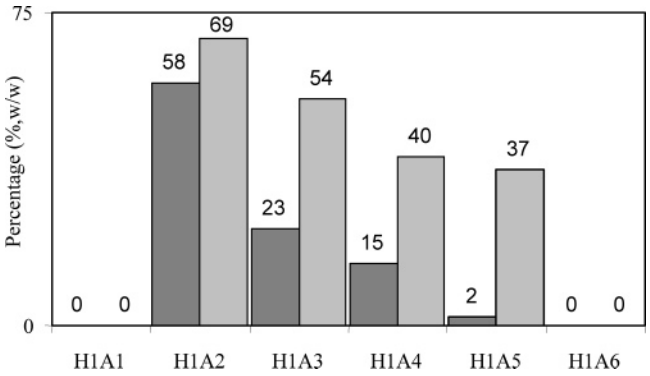

Figure 5. Distribution (%, w/w) of the AGPs over the six Brew HMw1 anion exchange fractions (dark gray columns) and the percentage (%, w/w) of (galactose, arabinose, and uronic acids) that is part of the AGP (light gray columns).

HMw1, the AGP and AGP-free fraction, it was calculated that only 67% (w/w) of the arabinose, galactose, and uronic acids ended up in the AGP fraction. Overall, only 40% (w/w) of the arabinose, galactose, and uronic acids present in the two HMw fractions ended up in the AGP fraction. The remaining 60% of the arabinose, galactose, and uronic acids were not part of the AGP fraction, while most of the arabinose, galactose, and uronic acids were present in AGPs prior to roasting (*26*, *38*). This implies that AGPs present in green coffee beans are chemically modified or degraded during roasting to such an extent that the AGP loses its specific characteristic of binding to the Yariv reagent. It is likely that the protein backbone of the AGPs is degraded by the Maillard reaction, resulting in molecules with a lower Mw. This could then explain the presence of "non-AGP" arabinose, galactose, and uronic acids in the Brew HMw2 fraction. It can be reasoned that the AGPs first react into brown-colored compounds (AGP−melanoidins) which still bind to the Yariv reagent, followed by further reaction in which the AGP−melanoidins lose their specific Yariv binding characteristics. AGPs seem to be very prone to (Maillard) reaction upon roasting of the coffee bean, resulting in brown-colored molecules.

Relation between AGPs and Charge Properties of Melanoidins. The six anion exchange fractions of Brew HMw1 were analyzed by the Yariv gel-diffusion assay to determine which fractions contained most of the AGPs. From **Figure 4**, it is clear that H1A2 contained most of the AGPs and that the AGP content decreased from H1A2 to H1A6. Using the diameter of the halo and the yields of the anion exchange fraction for Brew HMw1, the distribution of the AGPs over the Brew HMw1 anion exchange samples was calculated and is shown in **Figure 5**. The anion exchange fraction H1A2 contained 58% (w/w) of AGPs present in Brew HMw1. Thus, the majority of AGPs had a relatively low charge, probably the result of the uronic acids and possibly negatively charged groups on amino acids. As the AGPs are part of melanoidins, it can be stated that the AGP−melanoidins are slightly negatively charged molecules.

The percentage of galactose + arabinose + uronic acids present in AGP relative to the total galactose + arabinose + uronic acids content was calculated and is shown in **Figure 5**. It is clear that the galactose + arabinose + uronic acids content present in AGPs decreased from H1A2 to H1A5. This might again be explained by chemical modification of the AGPs, resulting in more negatively charged groups being bound/formed leading to less precipitation with the Yariv reagent.

The fractions H1A4 and H1A5 were melanoidin-rich (**Table 1**), had a strong negative charge, and were found to be poor in AGPs. These fractions contained 33% and 16% sugars, respectively, of which mannose was the most abundant monosaccharide (**Table 3**), suggesting that galactomannan is present in these fractions. These two fractions contained less uronic acids (%, w/w) than the fractions with a lower negative charge. It can be reasoned that these melanoidin-rich fractions had to contain negatively charged groups not originating from uronic acids or from amino acids from AGPs. The origin of these negative-charged groups remains unknown. A possible explanation could be that other acidic molecules are incorporated into the melanoidin complex or that newly formed acid groups are formed within the HMw coffee melanoidins upon roasting because of the Maillard reaction.

In this study, it was shown that coffee brew melanoidins were found to be negatively charged molecules, which are heterogeneous with respect to their polyanionic behavior. Arabinogalactan proteins were present in the HMw fraction that was retained by diafiltration, whereas the remainder of the coffee was free of AGP. The AGP had a brown color, as opposed to their white color in green coffee beans, allowing us to conclude that they are incorporated into the melanoidin complex upon roasting. This AGP−melanoidin complex is a distinct melanoidin population that can be specifically isolated from coffee brew. The negative charge of the AGP−melanoidin complex is probably due to the presence of uronic acids, which are known to be bound to arabinogalactans. The uronic acid content in the highly negative, melanoidin-rich fractions could not explain the total negative charge of these fractions. This led to the conclusion that other negatively charged groups are probably also incorporated in HMw melanoidins. Future research should focus on the structural characterization of the AGP−melanoidin complex and also on the fate of AGPs during roasting.

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