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Characterization of Galactomannan Derivatives in Roasted Coffee Beverages

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In this work, the galactomannans from roasted coffee infusions were purified by 50% ethanol precipitation, anion exchange chromatography, and phenylboronic acid-immobilized Sepharose chromatography. Specific enzymatic hydrolysis of the β-(1→4)-D-mannan backbone allowed us to conclude that the galactomannans of roasted coffee infusions are high molecular weight supports of low molecular weight brown compounds. Also, the molecular weight of the brown compounds linked to the galactomannan increases with the increase of the coffee degree of roast. The reaction pathways of galactomannans during the coffee roasting process were inferred from the detection of specific chemical markers by gas chromatography−electron impact mass spectrometry and/or electrospray ionization tandem mass spectrometry. Maillard reaction, caramelization, isomerization, oxidation, and decarboxylation pathways were identified by detection of Amadori compounds, 1,6-β-anhydromannose, fructose, glucose, mannonic acid, 2-ketogluconic acid, and arabinonic acid in the reducing end of the obtained oligosaccharides. The implication of the several competitive reaction pathways is discussed and related to the structural changes of the galactomannans present in the roasted coffee infusions.

KEYWORDS: Coffee; galactomannans; roasting; melanoidins; Maillard reaction; caramelization; mannonic acid; 2-ketogluconic acid; arabinonic acid; ESI-MS/MS

INTRODUCTION

Polysaccharides account for approximately half of the green coffee beans content. Arabinogalactans are the major group of polysaccharides extracted with hot water from green coffee beans (*1*, *2*). However, after roasting, the coffee bean polysaccharide content is rendered more extractable and galactomannans become the major polysaccharide in roasted coffee infusions. Together with arabinogalactans, they are the major components of the hot water extractable material (*1*−*3*). These polysaccharides play an important role in the retention of coffee volatile substances and contribute to the brew viscosity and thus to the creamy sensation perceived in the mouth known as "body" (*4*). They are also related to the foam stability of espresso coffee, an important quality attribute of this coffee brew (*5*, *6*).

The increase of extraction of galactomannans after roasting has been related to the loosening of the cell walls (*7*) and also to the depolymerization of the galactomannans (*1*−*3*). Nevertheless, the mechanism through which depolymerization occurs is not yet known. Furthermore, during the roasting process, 80, 50, and 35% of the arabinose (Ara), galactose (Gal), and mannose (Man) residues, respectively, are degraded (*7*, *8*) and their fates are largely unknown. At present, polysaccharide modifications such as degradation and/or copolymerization caused by the roasting process are still uncertain (*6*, *9*, *10*). Knowledge about the polysaccharides reaction(s) pathway(s) occurring during the roasting process could help to understand their structural changes and, additionally, the structure of the high molecular weight material (HMWM) of roasted coffee infusions, of which the polysaccharides are one of the main components.

This work describes some of the roasting-induced transformations of the galactomannans present in coffee infusions in terms of reaction pathways and highlights the importance of these reaction pathways in the roasting-induced structural transformations.

MATERIALS AND METHODS

Reagents. Glucose (Glc), Man, Gal, Ara, ribose, xylose, rhamnose, and fucose were from Merck (Darmstadt, Germany), and gluconic acid, 2-keto-gluconic acid, 1,6-β-anhydromannose, α-D-glucosidase type I from baker's yeast, β-D-glucosidase from almonds, β-D-mannosidase from snail, and phenylboronic acid (PBA) Sepharose were from Sigma-Aldrich Co. (St. Louis, MO). *endo*-β-(1→4)-D-Mannanase from *Aspergillus niger* was from Megazyme, Ltd. (County Wicklow, Ireland). Sephacryl S-200 HR and Q-Sepharose Fast Flow were from Amersham

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Biosciences (Uppsala, Sweden). Biogel P2 was from Bio-Rad Laboratories Ltd. (Hertfordshire, United Kingdom). Other reagents were analytical grade or higher available purity.

Samples and General Procedures. The infusions were prepared from Arabica Brazil coffee from two degrees of roast (DRs): a light roast, with 5% dry matter loss (DR 5%), and a dark roast, with 10% dry matter loss (DR 10%). From them, the HMWM and the 50% ethanol-precipitated fractions (Et50) were obtained as previously described (*1*, *3*). The sugars were determined by gas chromatography as alditol acetates (gas chromatography−flame ionization detection, GC-FID) after hydrolysis for 2.5 h with 1 M sulfuric acid at 100 °C or by hydrolysis for 1 h with 2 M trifluoroacetic acid at 120 °C. The methylation analysis was performed as described previously (*1*, *3*). The protein content was determined after acid hydrolysis with 6 M HCl during 24 h. After the acid was removed by centrifugal evaporation (Univapo 100 ECH, UniEquip, Munich, Germany) at 40°C under vacuum, the solid residue was dissolved in 3 mL of 0.1 M HCl and filtered through a 45 μm membrane. Amino acids were determined by GC-FID after derivatization as heptaflurobutyl isobutyl derivatives (*11*).

Acetic Acid Content. Samples (10 mg) were dissolved in 2 mL of distilled water, and after solubilization, 0.4 mL of 2 M NaOH was added. The reaction proceeded at room temperature for 1 h. The solution was neutralized by addition of 0.4 mL of 2 M HCl. The acetic acid content was determined by gas chromatography and by enzymatic determination of acetic acid (Boehringer Mannheim, Germany).

Anion Exchange Chromatography. Anion exchange chromatography was performed on a Q-Sepharose FF stationary phase (loaded on a C10/10 column, Pharmacia). The eluent was a pH 6.5, 100 mM Na−phosphate buffer containing 3 M urea and 0.02% sodium azide. Et50 fractions (150 mg) were dissolved in the initial buffer (1 mg/mL) and were applied to the column and eluted at a flow rate of 0.20 mL/min with a minimum of 4 column volumes of the initial buffer or until the absorbance at 280 nm reached the initial level. The retained material was eluted with buffer containing 3 M urea and 1 M NaCl. Fractions (2 mL) were collected and assayed for sugars by the phenol−sulfuric acid method, and the column eluent was continuously monitored at 280 nm for detection of brown compounds. The polysaccharide rich fractions were pooled, concentrated on a rotary evaporator (40 °C), dialyzed (12 kDa cutoff), and freeze-dried.

PBA-Sepharose Chromatography (PBA-Sepharose). The PBA-Sepharose 6B (Sigma) medium was packed on a C10/20 column (Pharmacia) after it was equilibrated with 50 mM sodium−2-(*N*-cyclohexylamine)ethanesulfonate (Na−CHES) buffer at pH 9.5 containing 3 M urea (*12*). The pH of the buffer was adjusted just before the runs. The material not retained in the anion exchange chromatography was dissolved in the initial buffer (25−50 mL) to give an approximate concentration of 1.0 mg/mL and was eluted at a flow rate of 0.10 mL/min with a minimum of 4 column volumes of the initial buffer or until the absorbance at 280 nm reached the initial level. The retained material was selectively eluted with the initial buffer containing 200 mM mannitol, with a minimum of 4 column volumes. To neutralize the eluted material, the eluent was collected in fractions of 1 mL to tubes already containing 1 mL of 250 mM Na-phosphate buffer at pH 6.5 and 0.02% sodium azide. The fractions were assayed for sugars with the phenol−sulfuric acid method and continuously monitored for absorbance at 280 nm. The polysaccharide rich fractions were pooled, dialyzed (12 kDa cutoff), concentrated on a rotary evaporator (40 °C) to approximately 10 mL, dialyzed again, and freeze-dried.

Size-Exclusion Chromatography on Sephacryl S-200 HR. Size-exclusion chromatography on Sephacryl S-200 HR was performed on a 70 cm × 1.6 cm column (XK 16/70, Pharmacia) at a flow rate of 0.2 mL/min. Samples (2 mg) were suspended in 1 mL of 100 mM Na−phosphate buffer, pH 6.5, containing 3 M urea. The same phosphate−urea buffer was used as the eluent. Fractions (1 mL) were collected and assayed for sugars with the phenol−H_2SO_4 method, and the eluent was continuously monitored at 280 nm. Exclusion and total volume were calibrated with Blue Dextran and Glc, respectively.

***endo*-β-(1→4)-D-Mannanase Hydrolysis.** Samples (14 mg) retained on PBA-Sepharose and selectively eluted with mannitol were hydrolyzed with pure *endo*-β-(1→4)-D-mannanase preparation (EC 3.2.1.78; 1 U) during 48 h at 37 °C with continuous shaking in 5 mL of 100 mM Na−acetate buffer, pH 5.5, containing 0.02% sodium azide. No activity was detected by the *endo*-β-(1→4)-D-mannanase preparation on Arabic gum and carboxymethylcellulose under the working conditions, by determination of the reducing sugars released by the Nelson−Somogyi procedure (*13*) using Glc as the standard for construction of the calibration curve. The freeze-dried material was dissolved in 2 mL of 100 mM pyridine−acetate buffer, pH 5.3, and loaded on a XK 1.6/100 column containing Biogel P-2 with a flow rate of 0.1 mL/min, previously equilibrated with loading buffer, and calibrated with DP3 (raffinose), DP2 (lactose), and monosaccharide (Glc). Fractions (1 mL) were collected and assayed for sugars with the phenol−H_2SO_4 method. The appropriated fractions were pooled and rotary evaporated until all of the buffer was removed by repeated additions of distilled water and freeze-dried.

β-(1→4)-D-Mannosidase Hydrolysis. The DP2 and DP3 fractions obtained after size exclusion chromatography of the *endo*-β-(1→4)-mannanase hydrolysis were further enzymatically hydrolyzed with β-(1→4)-mannosidase (E.C. 3.2.1.25). The hydrolysis was performed by adding 0.25 U of enzyme for the dissolved material in 0.2 mL of 100 mM Na−acetate buffer at pH 4.5 and incubating with continuous shaking for 48 h at 25 °C. After hydrolysis, the material was freeze-dried. No activity was detected on cellobiose.

β-(1→4)-D-Glucosidase and α-(1→4)-D-Glucosidase Hydrolysis. The DP2 and DP3 fractions obtained after size exclusion chromatography of the *endo*-β-D-mannanase hydrolysis were further enzymatically hydrolyzed with β-(1→4)-D-glucosidase (E.C. 3.2.1.21) and α-(1→4)-D-glucosidase (E.C. 3.2.1.20). The hydrolysis was performed by adding 0.25 U of enzyme for the dissolved material in 100 mM Na−acetic acid buffer at pH 5.0 for β-(1→4)-D-glucosidase and 100 mM Na−phosphate buffer, pH 6.5, for α-(1→4)-D-glucosidase and incubating with continuous shaking for 48 h at 25 °C.

Oximation Silylation of the β-(1→4)-D-Mannosidase Hydrolysates. The products resulting from β-(1→4)-D-mannosidase hydrolysis were freeze-dried, and 200 μL of a solution of 2.5 g of hydroxylamine in 100 mL of pyridine was added and heated at 70 °C for 30 min. After the mixture was cooled to room temperature, 200 μL of bis-(trimethylsilyl)trifluoroacetamide (BSTFA) was added and the reaction mixture was heated at 70 °C for 30 min. The mixture was analyzed by GC-MS using a DB-1 column (30 m length, 0.23 mm internal diameter, and 0.2 μm film thickness) by injection of 3 μL in splitless mode (time of splitless, 0.75 min). Injector and transfer lines were set at 250 °C, and the initial column temperature was 120 °C and held for 1 min. The column temperature was increased at 2 °C/min until 230 °C. The source temperature was set at 180 °C, the electron ionization energy was set at 70 eV, and the chromatogram was scanned from *m*/*z* 40 to 600. The gas chromatography−electron impact−mass spectrometry (GC-EI-MS) method used was able to resolve the following isomers: gluconic, galactonic and mannonic acids, and arabinonic and ribonic acids.

Synthesis of Mannonic, Galactonic, Arabinonic, and Ribonic Acids. The synthesis of mannonic, galactonic, arabinonic, and ribonic acid was performed by oxidation of the corresponding sugar with bromine water with an excess of calcium carbonate (*14*). The reaction was followed by GC-MS after removing the solvent by centrifugal evaporation at 40 °C under vacuum of 200 μL of the reaction mixture and derivatization by the method of oximation silylation described above, following the disappearance of the sugars and the appearance of the corresponding acids and lactones.

Electrospray Ionization Mass Spectrometry (ESI-MS) and ESI-Tandem Mass Spectrometry (MS/MS). The material of DP2 and DP3 fractions was dissolved in 200 μL of 1:1 MeOH−water containing 1% (v/v) formic acid in a concentration of approximately 0.25 mg/mL (*12*). Samples were introduced into the mass spectrometer using a flow rate of 10 μL/min. Positive ion ESI-MS and MS/MS spectra were acquired using a Q-TOF 2 instrument (Micromass, Manchester, United Kingdom), setting the needle voltage at 3000 V with the ion source at 80 °C and a cone voltage at 35 V. Each spectrum was produced by accumulating data during approximately 1−2 min. MS/MS spectra of pseudo-molecular ions were obtained by collision-induced dissociation, using argon as the collision gas and varying the collision energy between

Table 1. Sugar Composition (mg Anhydrosugars/100 mg of Sample) of the Nonretained Fraction from Anion Exchange Chromatography (QSA) and Fractions Obtained from PBA-Immobilized Sepharose (PB1 and PB2)

sample		yield (%)	Rha	Ara	Man	Gal	Glc	total sugars
DR 5%	QSA	68[a]	0.15 ± 0.01[d]	1.11 ± 0.06	41.76 ± 1.78	3.66 ± 0.16	0.81 ± 0.06	47.50 ± 2.06
	PB1	17[b]	0.41 ± 0.29	0.72 ± 0.09	7.70 ± 0.51	2.38 ± 0.19	0.85 ± 0.03	12.08 ± 0.97
	PB2	61[b]	0.17 ± 0.01	1.32 ± 0.06	68.14 ± 2.78	5.74 ± 0.30	0.94 ± 0.02	76.30 ± 3.17
DR 10%	QSA	57[a]	0.26 ± 0.01	0.84 ± 0.00	52.10 ± 1.88	2.96 ± 0.09	0.94 ± 0.01	57.11 ± 1.97
	PB1	32[b]	0.40 ± 0.03	1.24 ± 0.08	47.07 ± 3.10	6.59 ± 0.21	2.11 ± 0.11	57.41 ± 3.15
	PB2	46[b]	0.23 ± 0.01	0.77 ± 0.01	57.97 ± 2.09	3.61 ± 0.18	0.83 ± 0.04	63.41 ± 2.22

[a] Percentage (w/w) in relation to the applied material. [b] Percentage (w/w) in relation to QSA fraction. [c] Standard deviation.

20 and 30 eV for the $[M + H]^+$ adducts and between 40 and 50 eV for the $[M + Na]^+$ adducts.

Reduction of DP2 and DP3 Oligosaccharide Fraction for ESI-MS and ESI-MS/MS Analysis. The oligosaccharide alditol derivatives were prepared by addition of 50 μL of sodium borohydride solution (15% in NH_4OH, 3 M) to 100 μL of DP2 and DP3 samples (0.25 mg/mL) and left to react for 1 h at 30 °C in a screw cap tube. The excess of reducing agent was removed by addition of 5 μL aliquots of glacial acetic acid until no bubbles were observed. The content of borate salts was decreased in solution by evaporation of methanol under reduced pressure. The addition of methanol was repeated until the absence of salts was observed in the screw cap tube. The dried hydrolyzate was solubilized in 100 μL of MilliQ high purity water, and the samples were prepared as described previously for ESI-MS and ESI-MS/MS analysis.

RESULTS AND DISCUSSION

Isolation, Purification, and Chemical Characterization of Galactomannans from Roasted Coffee Infusions. The galactomannans from light- (DR 5%) and dark (DR 10%)-roasted coffee infusions were isolated and purified from the HMWM by precipitation with 50% ethanol solutions (*3*) and were further purified by anion exchange chromatography on Q-Sepharose (*12*). The nonretained material that contained the majority of the galactomannans accounted for 68 and 57% of the applied material (**Table 1**) for the light- and dark-roasted coffees, respectively. The polysaccharides accounted for 48 and 57 wt % of QSA fractions, containing mainly Man (42 and 52% of QSA fractions).

Further purification of galactomannans recovered on the nonretained fraction from anion exchange chromatography was achieved by PBA affinity chromatography, a methodology that was shown to be specific for the isolation of relatively high-substituted galactomannans (*12*). As can be seen in **Figure 1**, the fractionation of QSA fractions yielded two fractions, one nonretained (PB1) and a second retained and selectively eluted from PBA-Sepharose with 200 mM mannitol (PB2). Approximately 78% of the applied material and 82−100% of the applied sugars were recovered. The amount of Man not retained in the PBA-Sepharose medium was much higher for the dark roast (**Table 1**). Methylation analysis of PB1 and PB2 fractions (**Table 2**) shows that the mannans recovered in PB1 fraction had a lower degree of substitution [100 × (1→4,6)-Man*p*/total Man*p*] (1.5−1.6%) when compared with that of the mannans retained (3.1−3.6%). This result shows that at least two mannan populations with different degrees of substitution are present. Also, these data, complemented with the data obtained from a medium roast (DR 8%, *12*), show that the relative amount of low-substituted mannans recovered in PB1 fraction increases with the increasing DR. For PB2 fractions, polysaccharides accounted for 63−76% of the determined material and this amount decreased with increasing DR and PB2 fractions accounting for the majority of the galactomannans.

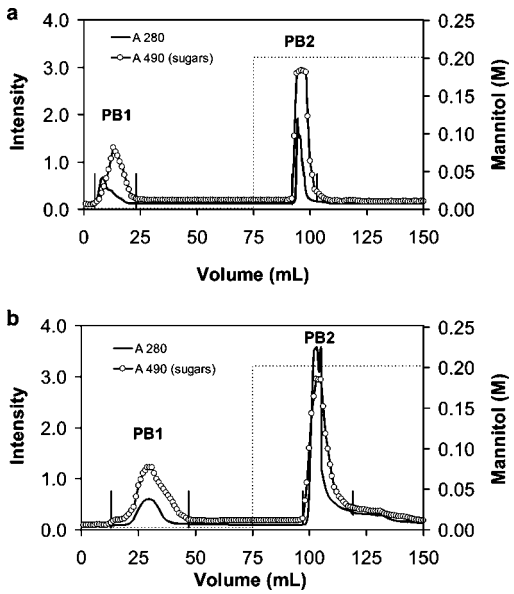


Figure 1. Chromatographic profile on PBA-Sepharose of QSA fractions of light (**a**) and dark (**b**) roasted coffees.

Table 2. Glycosidic Linkage Composition (mol %) of the Fractions Obtained by PBA-Immobilized Chromatography

	DR 5%				DR 10%			
linkage	PB1		PB2		PB1		PB2	
T-Rha*p*	3.1							
total	3.1	(4)[a]		(0)		(1)		(0)
T-Ara*f*	1.4		0.4				0.9	
5-Ara*f*	1.5		0.2					
total	2.9	(7)	0.6	(2)		(2)	0.9	(1)
T-Man*p*	5.7		4.6		7.3		6.2	
4-Man*p*	72.4		83.9		84.4		85.4	
4,6-Man*p*	1.3		3.3		1.4		2.9	
total	79.4	(63)	91.8	(89)	93.1	(83)	94.5	(91)
T-Gal*p*	4.0		4.0		2.9		2.1	
6-Gal*p*	1.1		0.3					
3-Gal*p*	5.2		1.4		3.1		0.8	
3,6-Gal*p*	1.2		0.4		0.4		0.3	
total	11.5	(19)	6.1	(7)	6.4	(11)	1.1	(5)
4-Glc*p*	3.2		1.8		0.6		2.1	
6-Glc*p*								
total	3.2	(7)	1.8	(2)	0.6	(3)	2.1	(2)

[a] Sugar composition determined by alditol acetates.

These results, when compared with values in the literature for coffee infusions and coffee beans, show that the Man residues recovered in PB2 fractions accounted for 47 and 27% of the total Man residues extracted for the light- and dark-roasted coffee infusions (*3*) and between 2.3 and 3.0% of the roasted coffee beans Man residues (*7*). Considering that the galactomannans from green and roasted coffee infusions have been reported to be acetylated (*12*, *15*), the acetic acid content was

Table 3. Amino Acid Composition[a] of the Retained Fractions Obtained by PBA-Immobilized Chromatography

	DR 5%	DR 10%
Ala	0.951 ± 0.117[b]	0.479 ± 0.010
Gly	0.072 ± 0.010	0.308 ± 0.017
Val	0.039 ± 0.002	0.067 ± 0.002
Thr		
Ser	0.069 ± 0.065	
Leu	0.056 ± 0.002	0.062 ± 0.010
Ile	0.172 ± 0.030	0.081 ± 0.038
Pro	0.056 ± 0.005	0.065 ± 0.005
Hyp	0.082 ± 0.063	0.048 ± 0.031
Met		
Asp	0.234 ± 0.008	0.241 ± 0.032
Phe	0.049 ± 0.004	0.062 ± 0.004
Glu	0.271 ± 0.019	0.313 ± 0.012
Lys	0.017 ± 0.003	0.057 ± 0.003
Tyr	0.074 ± 0.019	0.077 ± 0.005
Total	2.14 ± 0.10	1.83 ± 0.12

[a] Percentage (w/w) of PB2 fractions. [b] Standard deviation.

determined after alkaline hydrolysis. For the PB2 fraction of DR 5%, the acetic acid content found was 2.6% (w/w) and was 2.0% for DR 10%. The results of methylation analysis (**Table 2**) showed that arabinogalactans were present in PB2 fractions obtained from light and roasted coffees, as denoted by the presence of 3-, 6-, and 3,6-Gal*p* and 5-Ara*f* residues, and accounted for 2−4% of the polysaccharides of PB2 fractions. Also present were four-linked Glc residues (1.8−2.1 mol %) and terminally linked Ara residues (0.4−0.9 mol %), which can occur as mannan structural elements, as was described recently (*12*).

To determine the protein content of PB2 fractions, amino acids were determined after acid hydrolysis (**Table 3**). The amino acid content was low and ranged from 1.8 to 2.1%. Calculated by the difference between the total mass and the determined amount of sugars, amino acids, and acetic acid, the "chemically noncharacterized material" was estimated to account for 19 and 33% for light and dark roast PB2 fractions, respectively.

Galactomannans as High Molecular Weight Supports of the Low Molecular Weight Brown Compounds. Contrary to what was observed for green coffee fractions obtained in a similar way (*12*), the retained material in the PB-Sepharose column showed absorbance at 280 nm (**Figure 1**) and a yellow-brown color. The relative amount of carbohydrate and material with absorbance at 280 nm, inferred from the areas in the obtained chromatograms, indicates that the amount of this UV-absorbing material increases with the increase of the DR. This observation is in accordance with the amount of "chemically noncharacterized material" and also with the color of the fractions after freeze drying, where the light-roasted fractions presented a yellow color and the dark-roasted fractions presented a brown color. All of these results, together, strongly suggest that the brown material is covalently linked to the polysaccharide material, the main component of these factions. To verify this hypothesis, the PB2 fractions were subjected to size-exclusion chromatography on Sephacryl S-200 HR (**Figure 2**). As can be seen, coelution occurred between the polysaccharide (Abs 490 nm) and the brown material (Abs 280 nm) of the PB2 fractions recovered from light- and dark-roasted coffees, and the polysaccharide material from the dark-roasted coffee presented a lower molecular weight than that of the light-roasted coffee. These results were in accordance with those obtained for the polysaccharides recovered in Et50 fractions of coffee infusions (*3*).

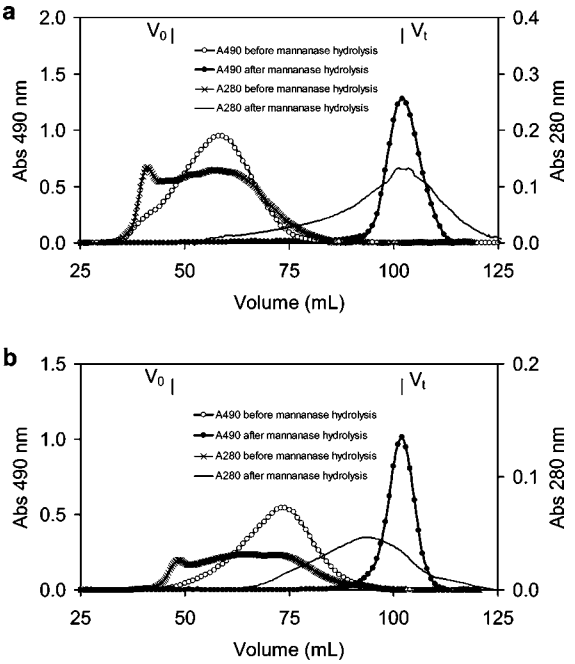


Figure 2. Size exclusion chromatography on Sephacryl S-200 HR of PB2 fraction of light (**a**) and dark (**b**) roasted coffees, before and after enzymatic hydrolysis with *endo*-β-(1→4)-D-mannanase. V_0, void volume; V_t, total volume.

These polysaccharides were hydrolyzed with an *endo*-β-(1→4)-D-mannanase, and the elution profile of the material was compared with the initial fraction (**Figure 2**). As expected from the polysaccharide composition of the PB2 fractions and from the specificity and hydrolysis pattern of the *A. niger* enzyme used (*16*), the majority of the carbohydrate material eluted in the total volume of the gel. For the light-roasted coffee, the elution profile of the brown material followed the elution behavior of the carbohydrate material, eluting in the total volume of the gel. However, for the dark-roasted coffee, although a large decrease in the molecular weight of the brown material was observed, the elution profile allows us to infer that the molecular weight of the brown material linked to the galactomannans was higher in the dark-roasted than in the light-roasted coffee.

The hydrolyzate was further fractionated by size-exclusion chromatography on Biogel P-2 (**Figure 3**). As can be seen, the majority of the sugars eluted as monomers, DP2 or DP3. The UV-absorbing material eluted in all of the volume ranges, although the amount of UV-absorbing material that eluted in the exclusion volume (>1 kDa, F1) increased with the increase of the DR, as was observed for the UV-absorbing material on Sephacryl S-200 HR. This material shows a coeluting carbohydrate material, and its nature was investigated by methylation analysis (**Table 4**). The main carbohydrate material present in F1 was characteristic of type II arabinogalactans as observed from the detection of 3- and 3,6-Gal*p* and T- and 5-Ara*f*, and Man residues, mainly in terminal position, were also present. Although this could be due to the presence of contaminating oligosaccharides, no carbohydrate material was observed when the galactomannans recovered from green coffee infusions were hydrolyzed with the same *endo*-β-(1→4)-D-mannanase (*12*). Also, no oligosaccharide ions were observed when this fraction was analyzed by ESI-MS (data not shown), indicating that there was not a contamination by oligosaccharide material. The marked decreased in the molecular weight of the brown material observed after the specific enzymatic hydrolysis of the galactomannan backbone, together with the presence of a small

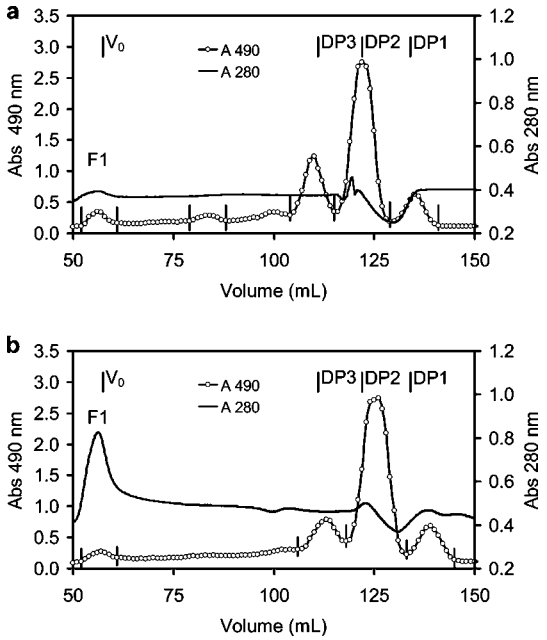


Figure 3. Size exclusion chromatography on Biogel P2 of PB2 fractions of light (**a**) and dark (**b**) roasted coffees obtained after enzymatic hydrolysis with *endo*-β-(1→4)-D-mannanase. V_0, void volume; F1, fraction recovered on the exclusion volume; DP2 and DP3 correspond to the elution volume of DP2 and DP3 standard oligosaccharides, and DP1 corresponds to the elution volume of monomers.

Table 4. Glycosidic Linkage Composition (mol %) of the Fractions Obtained by Size Exclusion Chromatography on Biogel P-2 after Enzymatic Hydrolysis with *endo*-β(1→4)-D-Mannanase of PB2 Fractions[a]

	DR 5%			DR 10%		
linkage	F1	DP3	DP2	F1	DP3	DP2
T-Rha*p*	0.7					
T-Ara*f*	17.4	0.2	0.1	7.9	0.4	0.6
T-Ara*p*	5.2	d	d	1.3	d	d
5-Ara*f*	8.2			3.8		
Man*p*$_{red}$[b]		6.0	20.4		13.1	12.7
T-Man*p*	8.9	55.2	59.6	16.6	49.3	61.1
4-Man*p*	7.1	31.4	17.3	11.5	29.0	23.1
4,6-Man*p*	2.0	2.7	0.5	3.4	3.4	0.6
T-Gal*p*	15.9	2.6	0.4	16.2	3.4	0.1
6-Gal*p*	4.8			11.4		
3-Gal*p*	14.3			13.8		
3,6-Gal*p*	11.4			8.3		
T-Glc*p*						
4-Glc*p*	4.0	1.9	1.8	6.0	1.2	2.0

[a] d, detected by GC-EIMS but not quantified by GC-FID. [b] Reducing terminal residue detected as 1,4-di-O-acetyl-1-deuterio-2,3,5,6-tetra-O-methyl-D-mannitol.

amount of Man residues in the exclusion material (**Table 4**), allows us to infer that the galactomannans recovered in this fraction act as high molecular weight supports of the lower molecular weight brown material.

The high molecular weight nature of the PB2 fraction and its yellow-brown color represent in practical terms a melanoidin molecule (*17*), although not with an appreciable negative charge density as the material was not bound to the anion exchange column under our working conditions. The possibility that food melanoidins can be formed by Maillard reaction of polysaccharides and amino acids has been addressed in model studies (*18*). Although PB2 fractions did not have the darkest shade of brown of all obtained fractions in the fractionation of the HMWM, they are important components in terms of HMWM

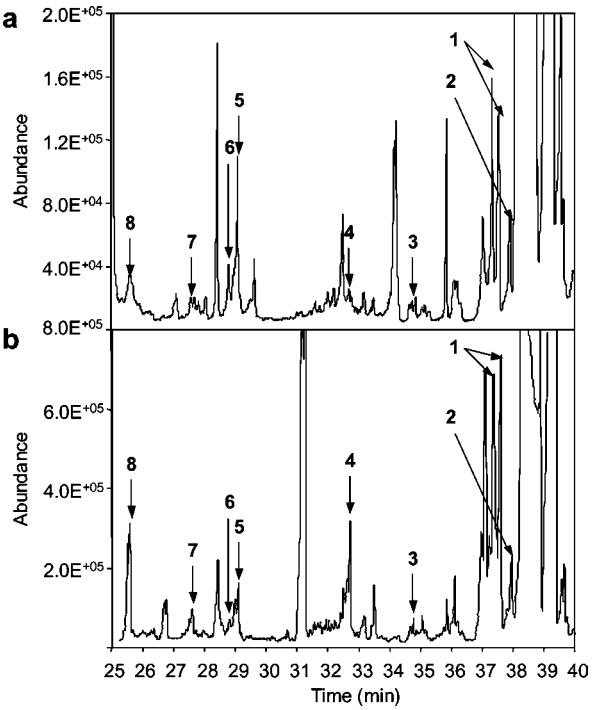


Figure 4. Gas chromatography–electron impact mass spectrometry (GC-EIMS) chromatogram of the products obtained after β-(1→4)-D-mannosidase hydrolysis of DP3 and DP2 oligosaccharides fractions from *endo*-β-(1→4)-D-mannanase-hydrolyzed PB2 fractions from dark roast coffee infusions. Peaks: 1, fructose; 2, mannonic acid; 3, mannonic acid δ-lactone; 4, arabinonic acid; 5, 1,6-β-anhydromannose; 6, 2-ketogluconic acid; 7, arabinonic acid δ-lactone; and 8, 2,3,4-trihydroxybutyric acid.

Table 5. Summary of the Oligosaccharide Ions Observed in the ESI-MS Spectra of Light and Roasted Coffee Attributed to Products Resulting from the Roasting Process

	$n=1$	$n=2$	$n=3$	$n=4$
oligosaccharides with caramelization type modified residues				
$[Hex_nAnHex + Na]^+$	347	509	671	833
$[Hex_ndPent + Na]^+$		481	643	
$[Hex_nAcF + Na]^+$	329	491		
oligosaccharides with oxidation/ decarboxylation modified residues				
$[Hex_nHexA + Na]^+$	381	543		
$[Hex_nPentA + Na]^+$		513		
oligosaccharides with Amadori compounds residues				
$[Hex_nFruPro + H]^+$	440			
$[Hex_nFruVal + H]^+$		604		
$[Hex_nFruLeu + H]^+$		618		
$[Hex_nFruVal–CO_2 + H]^+$		560		

total mass (12.1 and 8.3% of the HMWM for light- and dark-roasted coffee infusions, respectively). Nevertheless, this is the first time that it is shown that melanoidins from coffee infusions can be formed by low molecular weight brown compounds covalently linked to polysaccharides and, particularly, to galactomannans.

GC-EI-MS Characterization of the Modifications in the Coffee Galactomannans Induced by the Roasting. The reducing end sugar residues of polysaccharides are the most reactive units of these polymers. Because of the polymeric nature, the reducing end sugars are present in relatively low amounts. The *endo*-β-(1→4)-D-mannanase enzymatic hydrolysis resulted in a range of low molecular weight oligosaccharides,

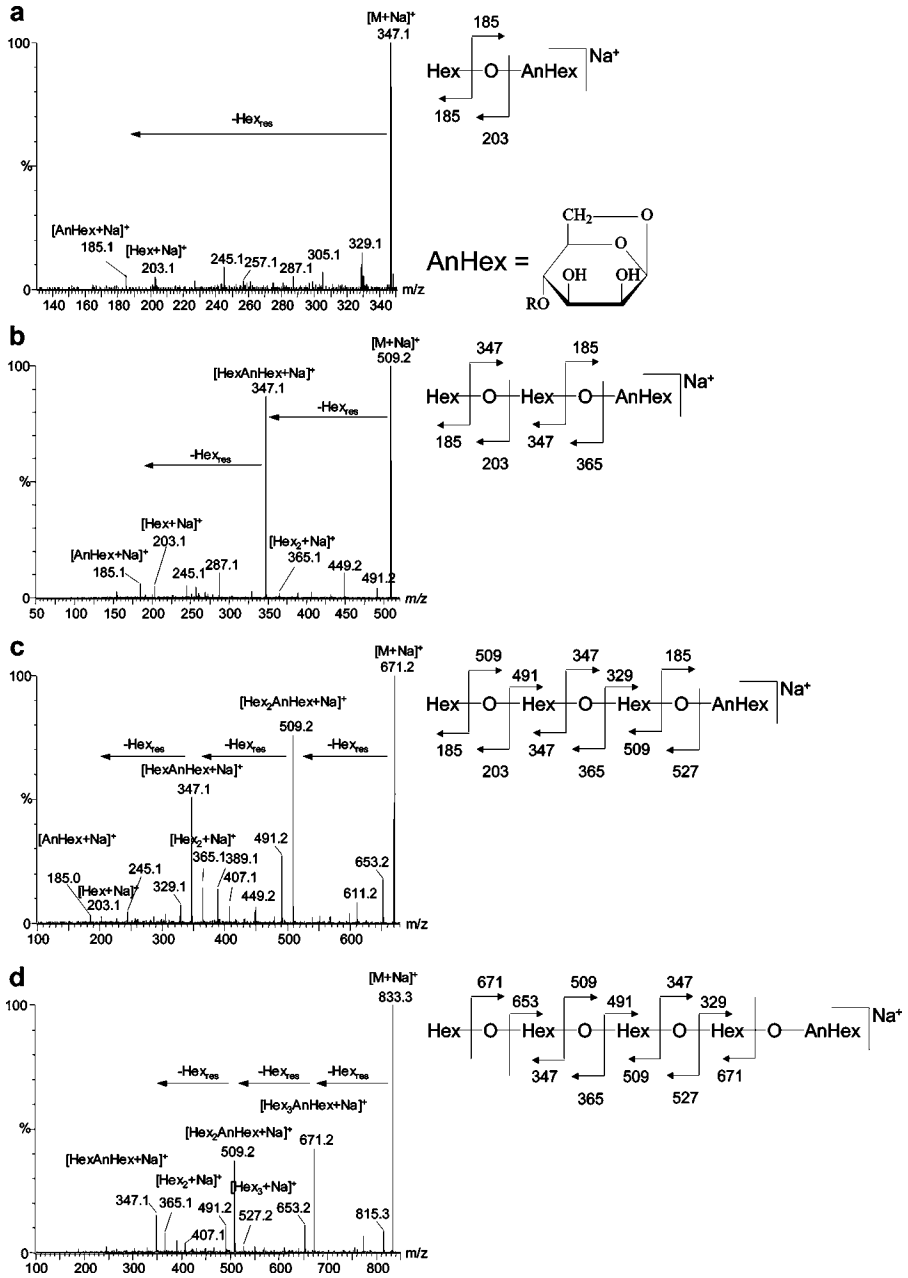


Figure 5. ESI-MS/MS spectra of $[M + Na]^+$ adducts and schematic fragmentation pathways of $Hex_{1-4}AnHex$.

and because of the mild hydrolytic conditions used, some of these oligosaccharides are expected to retain the reducing end of the original polysaccharide intact. In a previous work, it was shown that the oligosaccharides obtained from the galactomannans of the roasted coffee infusions, beyond Man, also contained Glc residues in the reducing end, contrary to what was observed for the green coffee infusions (*12*). Methylation analysis of the polysaccharides present in the PB2 fractions showed the occurrence of (1→4)-linked Glc residues (**Table 2**), indicating that these residues are structural features of galactomannans for these two roasting degrees. Also, acid hydrolysis of DP2 and DP3 fractions and analysis of the resulting monosaccharides as alditol acetates by GC-EI-MS showed that Glc residues were also present in these oligosaccharide fractions (data not shown). Methylation analysis of the oligosaccharides obtained for the light- and dark-roasted coffee infusions (**Table 4**) showed that the Glc residues in these oligosaccharide fractions are also (1→4)-linked. Enzymatic hydrolysis of DP2 and DP3 fractions using β-(1→4)-D-mannosidase, and α- and β-(1→4)-D-glucosidase, showed that only β-(1→4)-D-mannosidase was able to liberate Glc residues from the oligosaccharide fractions, giving more evidence for the occurrence of Glc residues located at the reducing end of these two oligosaccharide populations.

The hydrolysis products resulting from the β-(1→4)-D-mannosidase treatment of the DP2 and DP3 oligosaccharide fractions were further investigated by GC-EI-MS analysis after oximation and silylation (**Figure 4**). A variety of low abundance carbohydrate derivatives could be identified by comparison of the EI-MS spectrum and that of standards and retention time match, which included fructose, 1,6-β-anhydromannose, mannonic acid and mannonic acid δ-lactone, 2-keto-gluconic acid, arabinonic acid and arabinonic acid δ-lactone, and 2,3,4-trihydroxybutyric acid. As these compounds were only detected after β-(1→4)-D-mannosidase hydrolysis, which hydrolyzes β-(1→4)-linked D-Man residues from the nonreducing end of oligosaccharides, these carbohydrate modifications are present in the reducing end of the oligosaccharides. Furthermore, all derivatives identified contained the right *arabino* configuration

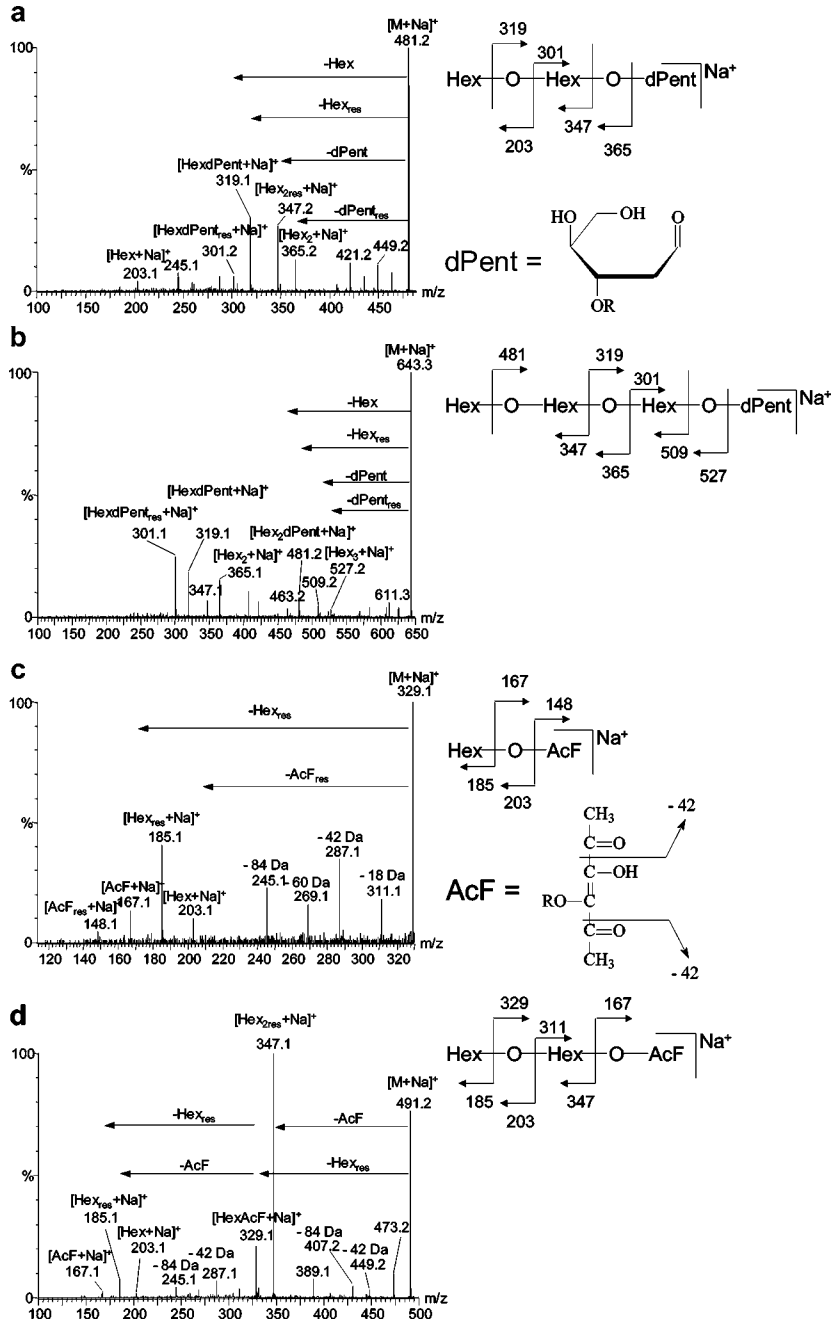


Figure 6. ESI-MS/MS spectra of $[M + Na]^+$ adducts and schematic fragmentation pathways of (**a**,**b**) Hex_{2-3}dPent and (**c**,**d**) Hex_{1-2}AcF.

that allowed us to infer that they were derived from the transformation/degradation of Man residues of the galactomannans.

From the obtained areas in the GC-EI-MS chromatograms, fructose (peak 1) is apparently the main modification observed in the roasted coffee galactomannans detected by this method. The formation of fructose can be explained by ring opening of the reducing end Man residues of polysaccharides through a 1,2-keto−enol tautomery. This isomerization process has been observed in various model roasting experiments for oligosaccharides and polysaccharides (*19*).

The 1,6-β-anhydrosugar residues are characteristic thermal degradation products of oligo- and polysaccharides (*19*, *20*). The formation of oligosaccharides bearing 1,6-β-anhydrosugar residues in the reducing end has been observed in the thermal treatments of polysaccharides (*21*). The detection of 1,6-β-anhydromannose (peak 5) in the reducing end shows that this reaction pathway occurs during coffee roasting. Also, the detection of 1,6-β-anhydromannose and fructose residues, two typical caramelization products (*22*), shows that caramelization reaction pathways occur during coffee roasting.

The oxidation of the reducing end of galactomannans during the roasting process can also be deduced by the presence of mannonic acid (peak 2). Also, the presence of the 2-keto-gluconic acid (peak 6) has been shown to be an intermediate in the oxidative decarboxylation of Glc to arabinonic acid (*23*), which was also found in the GC-EI-MS analysis (peak 4). The 2-keto-gluconic acid and arabinonic acid were most probably formed by consecutive oxidation/decarboxylation of the mannonic acid residues. A similar reasoning can be made for the formation of 2,3,4-trihydroxybutyric acid (peak 8) by decarboxylation/oxidation of the arabinonic acid residues. All of these derivatives were detected in enzyme-treated light- and dark-roasted coffee polymeric material. To confirm that these and

possibly other modified residues are covalently linked to the galactomannan-derived oligosaccharides, these fractions were further analyzed by ESI-MS.

ESI-MS Analysis of Oligosaccharide Fractions. ESI-MS analysis offers the advantage of allowing the study of intact oligosaccharides, even when present in mixtures and with low abundance, without any manipulation/derivatization being required.

The ESI-MS spectra of DP3 fractions obtained by *endo*-β-(1→4)-D-mannanase hydrolysis of the galactomannans from the light- and dark-roasted coffee were similar to those obtained for green and medium-roasted coffee (*12*). The major oligosaccharides of DP3 fraction were identified as the sodium adducts of Hex_2 and Hex_3 ($[M + Na]^+$ at *m*/*z* 365 and 527, respectively), the corresponding acetylated species, $AcHex_2$ and $AcHex_3$ ($[M + Na]^+$ at *m*/*z* 407 and 569), the diacetylated disaccharide Ac_2-Hex_2 ($[M + Na]^+$ at *m*/*z* 449), and the pentosyl-containing trisaccharide Pent-Hex_2 ($[M + Na]^+$ at *m*/*z* 497). Furthermore, some low abundant ions, absent in the ESI-MS spectra obtained for the green coffee galactomannans (*12*), were observed in the ESI-MS spectra of the roasted coffees (**Table 5**).

Three series of ions that could be attributed to oligosaccharides containing modified residues usually found in caramelization reactions were observed as follows: (a) anhydrohexose residues (AnHex), present at *m*/*z* 347, 509, 671, and 833 ($[Hex_{1-4}AnHex + Na]^+$); (b) deoxypentose residues (dPent), present at *m*/*z* 481 and *m*/*z* 643 ($[Hex_{2-3}dPent + Na]^+$); and (c) acetylformoin residues (AcF), present at *m*/*z* 329 and *m*/*z* 491 ($[Hex_{1-2}AcF + Na]^+$). Other two series of ions were identified as modified oligosaccharides by oxidation/decarboxylation: (a) hexonic acid residues (HexA), present at *m*/*z* 381 and 543 ($[Hex_{2-3}HexA + Na]^+$); and (b) pentonic acid residues (PentA), present at *m*/*z* 513 ($[Hex_2PentA + Na]^+$). The detection of the oligosaccharides with HexA, in the ESI-MS spectra, was not easily assigned because its sodium adducts had the same *m*/*z* value as the potassium adducts of di- and trihexose oligosaccharides ($[Hex_{2-3} + K]^+$). To confirm the presence of $[Hex_nHexA + Na]^+$, the oligosaccharides were reduced with sodium borohydride. The alditol derivatives obtained show *m*/*z* values 2 Da higher than the original corresponding oligosaccharides (*27*), but the *m*/*z* value of the oligosaccharides containing HexA residues did not changed. The reduction of the DP3 fractions resulted in the appearance of ions at *m*/*z* 367 and *m*/*z* 529 instead of those at *m*/*z* 365 and *m*/*z* 527 (not shown). Also present were the ions at *m*/*z* 381 and *m*/*z* 543, confirming the presence of the sodium adducts of hexonic acid (HexA)-containing oligosaccharides, $[HexHexA + Na]^+$ and $[Hex_2HexA + Na]^+$, respectively.

Other series of ions were identified in the ESI-MS spectra of DP3 fraction, showing even *m*/*z* values, thus indicating the presence of an odd number of nitrogen atoms in the molecules (MacLafferty−nitrogen rule, *24*). Furthermore, the collision energy used during the MS/MS experiments was much lower than that used for the $[M + Na]^+$ ions, suggesting the presence of $[M + H]^+$ ions (*25*). These two characteristics allowed us to propose these ions as protonated molecules, $[M + H]^+$, of nitrogen-containing oligosaccharides. These ions were observed at *m*/*z* 440, 604, 618, and 560 and were identified as the $[M + H]^+$ ions of Amadori compounds of proline, valine, leucine/isoleucine or 4-hydroxyproline, and decarboxylated valine, respectively.

To confirm the proposed structures observed in the ESI-MS spectra of roasted coffee, these ions were further analyzed by MS/MS. The fragmentation of the oligosaccharide ions under

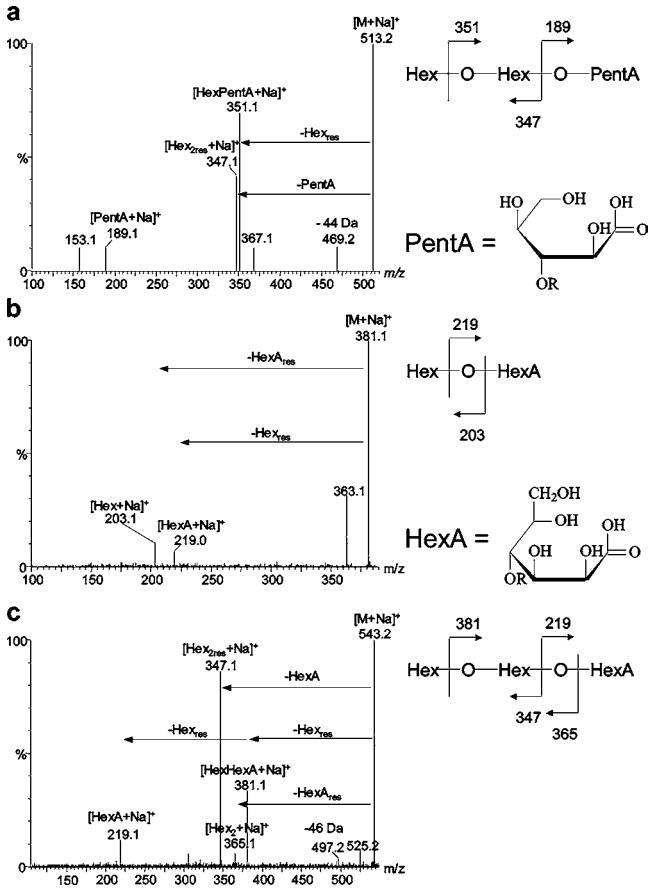


Figure 7. ESI-MS/MS spectra of $[M + Na]^+$ adducts and schematic fragmentation pathways of (**a**) Hex_2PentA and (**b**,**c**) $Hex_{1-2}HexA$.

MS/MS conditions is the result of glycosidic cleavages and of cross-ring cleavages (cleavage of two bonds within the sugar ring). Fragment ions formed are usually named according to the nomenclature proposed by Domon and Costello (*26*). Briefly, oligosaccharide fragment ions that retain the charge at the reducing end are designated X when originated by cross-ring cleavages or Y and Z when originated by glycosidic cleavage. Fragment ions that retain the charge at the nonreducing end are designated A when originated by cross-ring cleavages or B and C when originated by glycosidic cleavage. Following the letter that defines the fragment type, there is a number in superscript that identifies the number of sugar residues in a linear oligosaccharide.

ESI-MS/MS Spectra of $[M + Na]^+$ Oligosaccharide Derivatives Containing Modified Residues Typical of Caramelization Reactions. Figure 5 shows the ESI-MS/MS spectra of $[Hex_{1-4}AnHex + Na]^+$ ions, having a common fragmentation pattern. Predominant Y type ions are observed, formed by glycosidic bond cleavage, with successive loss of one to *n* Hex_{res} (-*n*162 Da), depending on oligosaccharide molecular weight. Loss of anhydrosugar (AnHex, 144 Da) was also observed in all spectra, occurring from either precursor ion or combined with loss of Hex_{res}. Typical fragmentations are exemplified in the schemes presented in **Figure 5**. The ion at *m*/*z* 185 corresponds to the sodium adduct of AnHex. This residue, according to the data obtained by GC-EI-MS, can be attributed to 1,6-β-anhydromannose, present in the reducing end of the galactomannan-derived oligosaccharides.

The ESI-MS/MS spectra of $[Hex_{2-3}dPent + Na]^+$ ions at *m*/*z* 481 and 643 (**Figure 6a**,**b**) showed major fragments that resulted from loss of Hex_{res} (−162 Da), with formation of $[Hex_{1-2}dPent$

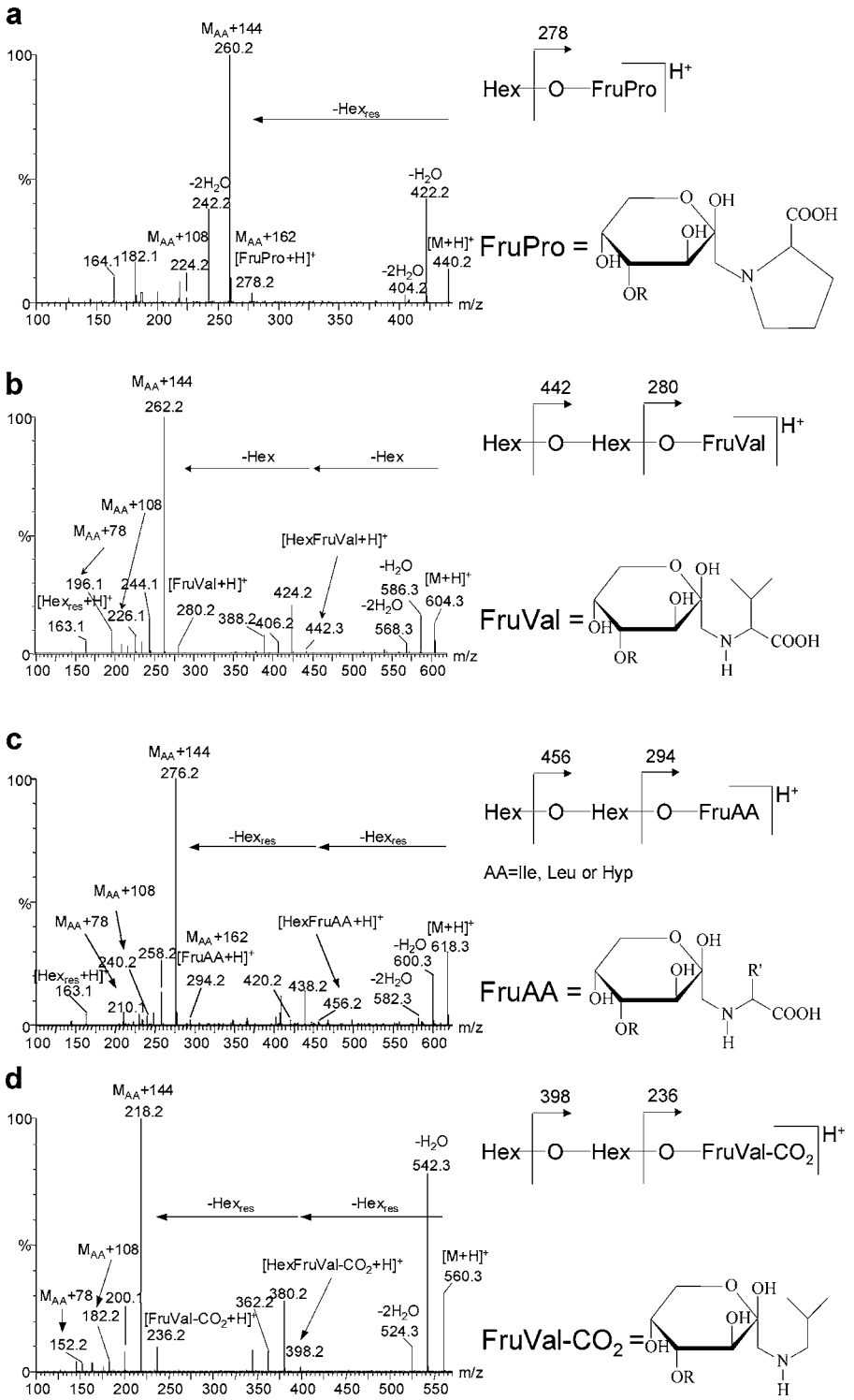
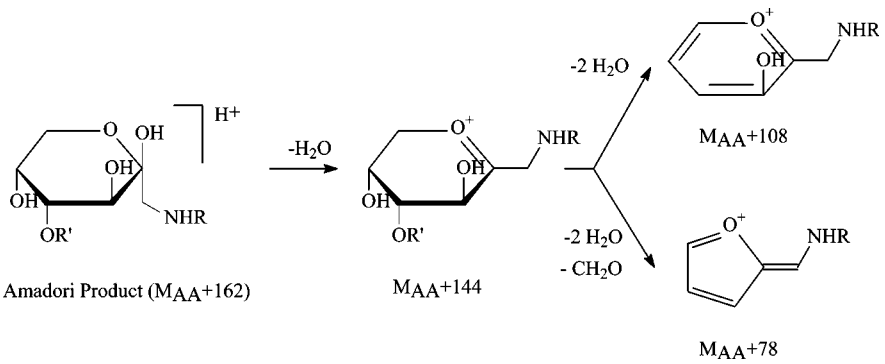


Figure 8. ESI-MS/MS spectra of $[M + H]^+$ adducts and schematic fragmentation pathways of (**a**) HexFruPro, (**b**) Hex_2FruVal, (**c**) Hex_2FruAA (AA = Leu/Ile or Hyp), and (**d**) Hex_2FruVal-CO_2.

$+ Na]^+$ ion at *m*/*z* 319. The loss of $dPent_{res}$ (−116 Da) and dPent (−134 Da) with formation of $[Hex_{2-3} + Na]^+$ and $[Hex_{2-3res} + Na]^+$, respectively, confirmed the presence of dPent in these oligosaccharides. The presence of dPent can be justified by reducing end opening and formation of 3-deoxyglucosone via 1,2-endiole and water elimination followed by α-diketo cleavage yielding 2-deoxyarabinose and formic acid (*27*). The 1,2-endiole intermediate is a common intermediate in the route of fructose formation.

The ESI-MS/MS spectra of the oligosaccharides containing acetylformoin $[Hex_{1-2}AcF + Na]^+$ ions at *m*/*z* 329 and 491 (**Figure 6c**,**d**) showed fragment ions resulting from loss of a neutral with 144 Da, leading to the ion $[Hex_{n-1res} + Na]^+$, confirming the occurrence of a residue with 144 Da covalently linked at the reducing end. These spectra also showed abundant fragment ions due to the loss of 42 Da and loss of 84 Da, either from the precursor ion or from the fragment $[M\text{-}Hex_{res} + Na]^+$. These fragmentation pathways can be explained due to the symmetry of this moiety that permits the loss of one and two H_2C_2O molecules, from C1−C2 and C5−C6 of acetylformoin aglycone. The acetylformoin aglycone can be formed under Maillard (*17*) and caramelization (*22*) reaction conditions.

Scheme 1. Nomenclature of Fragment Ions from Amadori Compounds According to ref *29*[a]



[a] M_{AA}, molecular weight of amino acid involved in the formation of the Amadori compound.

ESI-MS/MS Spectra of $[M + Na]^+$ Oligosaccharide Derivatives Containing Acid Residues. The ESI-MS/MS of the oligosaccharides with pentonic (PentA) and hexonic acids (HexA) are shown in **Figure 7**. The ESI-MS/MS spectrum of $[Hex_2PentA + Na]^+$ ion at *m*/*z* 513 (**Figure 7a**) showed predominant ions formed by Y type glycosidic cleavage, with loss of one and two Hex_{res} leading to $[HexPentA + Na]^+$ and $[PentA + Na]^+$ ions at *m*/*z* 351 and 189, respectively. The fragment at *m*/*z* 469, due to loss of CO_2 (−44 Da), confirms the presence of a carboxylic acid (*28*). Furthermore, the ion due to the loss of −166 Da, with formation of the ion $[Hex_{2res} + Na]^+$, confirms the presence of PentA at the reducing end. This residue, according to the data obtained by GC-EI-MS, can be attributed to arabinonic acid, present in the reducing end of the galactomannan-derived oligosaccharides.

The oligosaccharides with HexA have a fragmentation pattern similar to the oligosaccharides with PentA (**Figure 7b**,**c**). Their ESI-MS/MS spectra showed a main loss of one and two Hex_{res} with formation of $[HexA + Na]^+$ at *m*/*z* 219 and also fragment ions at *m*/*z* 347 due to the loss of HexA (−196 Da), confirming the occurrence of HexA at the reducing end of the galactomannan-derived oligosaccharides. To ensure the attribution of these ions, lactobionic acid was synthesized by oxidation of lactose and the resulting ESI-MS/MS spectrum (*m*/*z* 381, data not shown) was observed to be similar to the one obtained for the $[HexHexA + Na]^+$. For this type of oligosaccharide, it is possible to observe an ion due to loss of HCOOH (46 Da) confirming the presence of a carboxylic acid. The loss of HCOOH in HexA residues occurs probably because the acidity of the C-3 hydrogen, eliminated with the acid group, is higher than the one conserved in the case of PentA.

ESI-MS/MS Spectra of $[M + H]^+$ Oligosaccharide Derivatives Containing Amadori Compounds. Amadori compounds were identified as containing an odd number of nitrogen atoms and ionized as $[M + H]^+$ ions. The ESI-MS/MS spectra obtained for the ions *m*/*z* 440, 604, 618, and 560, identified as oligosaccharide-containing Amadori compounds of proline, valine, leucine/isoleucine, or 4-hydroxyproline and decarboxylated valine, are shown in **Figure 8**. As can be seen in each spectrum, the oligomeric structure is evidenced by the loss of one or two Hex_{res} (162 Da), leading to the fragments at *m*/*z* 278 (**Figure 8a**), *m*/*z* 280 (**Figure 8b**), *m*/*z* 294 (**Figure 8c**), and *m*/*z* 236 (**Figure 8d**) that were identified as the Amadori products of fructosylproline, fructosylvaline, fructosylleucine/isoleucine, or 4-hydroxyproline and fructosyl-decarboxylated valine, respectively. All of the MS/MS spectra of these fructosylamino acid derivatives showed abundant loss of one and two H_2O. Also observed in each ESI-MS/MS spectrum of these derivatives were the diagnostic fragment ions named as $M_{AA} + 144$, $M_{AA} + 108$, and $M_{AA} + 78$ (**Scheme 1**), which were found to specify Amadori compounds, in a previous study using model Amadori compounds (*29*). The detection of oligosaccharide-linked Amadori compounds, a specific indicator of the Maillard reactions (*17*), shows that the galactomannans react through the Maillard reaction during the roasting process.

Reaction Pathways and Implication in the Structure and Reactivity of Roasted Coffee Galactomannans. The various chemical markers covalently linked to the galactomannans purified from coffee infusions, and structurally related to the Man residues, are specific enough to permit us to deduce certain reaction pathways of coffee galactomannans during the roasting procedure, some of them being competitive and others consecutive. Caramelization, oxidation, and Maillard reactions were shown to occur during the roasting process. The Amadori compounds and nitrogen-containing derivatives unequivocally show that during the roasting process high molecular weight coffee galactomannans react through the Maillard reaction despite their low content of reducing end groups and despite other competitive reaction pathways. Although only Amadori compounds of amino acids were detected in this galactomannan fraction, this reaction pathway, if occurring with proteins, could induce a cross-linking between galactomannans and proteins during the roasting process, similar to what is observed during dry heating of several model studies of polysaccharides and proteins (*30*). Nevertheless, this cross-linking should be rather limited due to the low protein content of PB2 fractions. The presence of Amadori compounds opens the possibility of a decrease in molecular weight of coffee galactomannans through their stepwise β-elimination (*31*). Nevertheless, this reaction pathway is not very relevant in molecular weight reduction. The presence of Glc residues in the reducing end of the galactomannans (*12*) can now be explained by the thermal reversion of Amadori compounds on dry heating conditions, yielding Man and Glc residues (*32*).

The 1,6-β-anhydromannose residues present at the reducing end make the galactomannans unreactive by the Maillard reaction (*33*). This reaction pathway may also account for the decrease in the molecular weight observed for the galactomannans during the roasting procedure (*20*). Such mechanism of molecular weight reduction should be more effective as the random nature of the process, if occurring in the middle of the mannan backbone, and may result in an effective molecular weight decrease. The formation of 1,6-β-anhydrosugars occurs via glycosyl ions (*20*) that are also intermediates in the transglycosylation reactions (*34*). Transglycosylation reactions are known to occur during the thermal processing of mono- and oligosaccharides and also 1,6-β-anhydrosugars on dry

heating conditions (*20*, *33*), thus opening a possibility for this reaction pathway during coffee roasting.

The detection of mannonic acid, 2-keto-gluconic acid, arabinonic acid, and 2,3,4-trihydroxybutyric acid shows that oxidation and decarboxylation reactions occur. The chemical mechanism of oxidation cannot be determined from the obtained results; nevertheless, hydroxyl radicals have been implicated in the thermal degradation of polysaccharides (*35*). The occurrence of hydroxyl radical formation and reaction during the roasting process can be inferred from the detection of 8-oxocaffeine (*36*) in roasted coffee powders, resulting from the reaction of hydroxyl radicals with caffeine. The oxidation products detected in this work are also characteristic end products of hydroxyl radical reaction with oligo- and polysaccharides (*37*). This reaction pathway, if operative, could contribute also to the decrease in molecular weight of galactomannans, as previously observed for model studies of polysaccharides (*38*). Anyway, the presence of these oxidation derivatives, mannonic, arabinonic, and 2,3,4-trihydroxybutyric acids, at the reducing end of galactomannans stops the Maillard reaction due to the absence of carbonyl groups needed for reaction.

ABBREVIATIONS USED

ESI, electrospray ionization; MS/MS, tandem mass spectrometry; GC-EIMS, gas chromatography−electron impact mass spectrometry; PBA-Sepharose, aminophenylboronic acid immobilized Sepharose; Ara, arabinose; Man, mannose; Gal, galactose; Glc, glucose; DR, degree of roast; Hex, hexose residue; Pent, pentose residue; AnHex, anhydrohexose residue; dPent, deoxypentose residue; AcF, acetilformoin residue; Pent A, pentonic acid residue; HexA, hexonic acid residue.

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