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Arabinosyl and glucosyl residues as structural features of acetylated galactomannans from green and roasted coffee infusions

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Abstract—A good yield mild fractionation procedure was developed for the purification of mannans from green and roasted coffee infusions that included anion-exchange chromatography and phenylboronic acid immobilized Sepharose chromatography of the dialyzed and ethanol precipitated material. Enzymatic hydrolysis with *endo*-β-mannanase and ESIMS allowed finding that the mannans from roasted coffee infusions, as well as those from green coffee, are acetylated (8 mol % and 11 mol %, respectively). Fragmentation pattern obtained by ESIMS/MS analysis of the acetylated oligosaccharide ions indicates that the acetylation also occurs at O-2 of the mannose residues. Doubly acetylated and contiguously acetylated hexose residues were also found. Arabinose residues, as side chains, were also found as structural features of hot water soluble green (2%) and roasted (<0.9 mol) coffee galactomannans. Methylation analysis, hydrolysis with specific glycosidases and GC–EIMS analysis of the reduced and methylated oligosaccharides allowed to conclude that β-(1→4)-linked glucose residues are also structural features of green and roasted coffee galactomannans (6 and 1 mol %, respectively). In hot water soluble green coffee mannans, glucose residues are a constituent of the mannan backbone, and in the roasted coffee they were detected only at the reducing end of the mannan backbone.

Keywords: Coffee polysaccharides; Acetylation; Galactomannans; Roasting; ESIMS/MS

1. Introduction

Coffee is one of the most widely consumed beverages. Hot water extractable polysaccharides are the main components of the coffee infusions high molecular weight material. They are mainly responsible for the viscosity of coffee extracts[1] (and, thus, for the creamy sensation perceived in the mouth known as 'body'[2]), for the foam stability of espresso coffee,[3] and also for the retention of volatile substances.[4] Galactomannans comprise nearly 70% of roasted coffee infusion polysaccharides and, with type II arabinogalactans, are the main polysaccharides of roasted coffee infusions.[5–7] The roasting process improves the hot water extractability of coffee galactomannans and is also responsible for a change in their structural features, such as the decrease of the molecular weight and degree of branching.[5–7] These structural changes were also observed for the mannan fractions that remained in the residue after aqueous extraction.[8–10]

The galactomannans of green and roasted coffee are composed by β-(1→4)-linked D-mannopyranosyl residues substituted at O-6 by single α-D-galactopyranosyl residues. Strong evidence that in roasted coffee galactomannans single terminally-linked arabinose residues are also structural elements of galactomannans was shown by Navarini et al.[11]

The detection of acetate after alkaline hydrolysis of the hot water extractable high molecular weight material of green and roasted coffees was the stimuli to study in more depth the structure of green and roasted coffee mannans. As hot water extractable high molecular weight mannans are present in mixture with arabinogalactans, proteins, and, additionally, melanoidins in

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roasted coffee, a soft fractionation procedure was developed in order to obtain pure, hot water soluble mannans and also to keep intact their structural characteristics. The mannans purified from green and roasted coffee were submitted to an enzymatic hydrolysis with specific *endo*-β-mannanase and were analyzed by ESIMS. The new structural features were confirmed by ESIMS/MS and methylation analysis.

2. Material and methods

2.1. Samples and general procedures

Green and roasted Arabica coffee from Brazil with (8% dry matter loss) infusions, high molecular weight material (HMWM) and 50% ethanol precipitated fractions (Et50) were obtained as previously described.[5] The sugars were determined by gas chromatography as alditol acetates after hydrolysis with 1 M sulfuric acid at 100 °C or by hydrolysis with TFA 2 M at 120 °C. The methylation analysis was performed as described previously.[5]

2.2. Alkaline hydrolysis

Samples (10 mg) were dissolved in distilled water (2 mL) and, after solubilization, 0.4 mL of NaOH 2 M were added. The reaction proceeded at room temperature for 1 h. The solution was neutralized by addition of 0.4 mL of 2 M HCl.[12] The acetic acid content of the reaction mixture was quantified by gas chromatography[13] and by enzymatic determination of acetic acid (Boehringer Mannheim, Germany).

2.3. 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 Na-phosphate 100 mM buffer containing 3 M urea and 0.02% sodium azide. Et50 fractions (1 mg/mL) were applied to the column and, after application, the column was flushed with a minimum of four column vol 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[14] and continuously monitored at 280 nm. The appropriated fractions were polled, dialyzed (12 kDa cutoff) and freeze-dried.

2.4. Phenylboronic acid–Sepharose chromatography (PBA–Sepharose)

The PBA–Sepharose 6B (Sigma) medium was packed on a C10/20 column (Pharmacia) after being diluted with 50 mM sodium-2-(*N*-cyclohexylamino)ethanesulfonate (Na-CHES) buffer at pH 9.5 containing 3 M urea. The pH of the buffer was adjusted just before the runs. The fractions not retained in the anion-exchange chromatography (1 mg/mL) were applied in the initial buffer and, after all the samples entered, the column was washed with four column vol 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, and after rinsing with a minimum of four column vol, the column was flushed with 100 mM Na-acetate buffer at pH 5. Fractions of 1 mL were collected on 1 mL of 250 mM Na-phosphate buffer at pH 6.5 containing 0.02% sodium azide. The fractions were assayed for sugars with the phenol–H_2SO_4 acid method[14] and continuously monitored for absorbance at 280 nm. The appropriate fractions were polled, dialyzed (12 kDa cutoff), concentrated to approximately 10 mL, dialyzed again and freeze-dried.

2.5. Enzymatic hydrolysis

Samples (14 mg) retained on PBA–Sepharose and selectively eluted with mannitol were hydrolyzed with pure *endo*-β-(1→4)-mannanase preparation (Megazyme, EC 3.2.1.78) during 48 h at 37 °C with continuous shacking in a 100 mM Na-acetate buffer pH 5.5 containing 0.02% sodium azide. No activity was detected by the *endo*-β-(1→4)-mannanase preparation on gum arabic and carboxymethylcellulose under the working conditions, by determination of the reducing sugars released by the Nelson–Somogyi procedure[15] using glucose as the standard. The freeze-dried material was dissolved in pyridine-acetate 100 mM buffer pH 5.25, and loaded on a XK 1.6/100 column containing Biogel P-2 (Bio-Rad) previously equilibrated with loading buffer, and calibrated with DP3 (raffinose), DP2 (lactose) and monosaccharide (glucose). Fractions (1 mL) were collected and assayed for sugars with the phenol–H_2SO_4 method.[14] The appropriated fractions were pooled and rotary evaporated until all the buffer was removed by repeated additions of distilled water and freeze-dried.

2.6. β-(1→4)-Mannosidase hydrolysis

The DP 2 and DP 3 fractions obtained after size-exclusion chromatography of the *endo*-β-(1→4)-mannanase hydrolysis were further enzymatically hydrolyzed with a β-(1→4)-mannosidase (from Snail, Sigma, E.C. 3.2.1.25). The hydrolysis was performed by adding 0.25 U of enzyme for the dissolved material in 100 mM Na-acetate buffer at pH 4.5 and incubated with continuous shacking during 48 h at 25 °C. After hydrolysis, the material was freeze-dried. No activity was detected on cellobiose.

2.7. β-(1→4)-Glucosidase and α-(1→4)-glucosidase hydrolysis

The DP 2 and DP 3 fractions obtained after size-exclusion chromatography of the *endo*-β-mannanase hydrolysis were further enzymatically hydrolyzed with a β-(1→4)-glucosidase (from Almonds, Sigma, E.C. 3.2.1.21) and α-(1→4)-glucosidase (Type I from Baker Yeast, Sigma, 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)-glucosidase and 100 mM Na-phosphate buffer pH 6.5 for α-(1→4)-glucosidase and incubated with continuous shacking during 48 h at 25 °C.

2.8. GC–EIMS identification of reduced and methylated oligosaccharides

The freeze-dried fractions containing the DP 2 and DP 3 oligosaccharides from green and roasted coffee samples were reduced with 0.5 mL borohydride 2 M. After destroying the excess borohydride with 1 mL acetone, borate was removed as methyl ester by centrifugal evaporation by five additions of 10:1 MeOH–AcOH. To the dried residue 2 mL of dry Me_2SO was added followed by 50 mg of powdered NaOH and 2 mL of methyl iodide. After 20 min, $CHCl_3$ (3 mL) was added followed by 4 mL of water. After extraction, the aq phase was removed and the organic phase was washed four times with water (3 mL). The organic phase was removed by a stream of nitrogen and the residue was dissolved in $CHCl_3$ (0.3 mL) and injected at 285 °C in a GC–EIMS apparatus. The column was a DB1 and the carrier gas was set at 50 cm/s. Initial temperature was 100 °C, 1 min, with an increase to 325 °C at 6 °C/min. The transfer line was set at 290 °C, the EIMS source was set at 230 °C and the energy of 70 eV was used. Retention time of Man–Man*ol* and Glc–Man*ol* disaccharides and Man–Man–Man*ol*, Gal–Man–Man*ol* and Glc–Man–Man*ol* trisaccharides were determined by subjecting pure konjac glucomannan and locust bean gum to *endo*-β-(1→4)-mannanase hydrolysis and analyzing dimers and trimers under the same conditions. The presence of Glc–Man*ol* and Glc–Man–Man*ol* oligosaccharides was confirmed by β-mannosidase hydrolysis of the oligomers obtained from konjac glucomannan. Cellobiose was also used as the standard for the possible presence of Glc–Glc disaccharides.

2.9. Electrospray ionization mass spectrometry (ESIMS and ESIMS/MS)

The freeze-dried fraction obtained after the Biogel P2 column 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. Samples were introduced into the mass spectrometer using a flow rate of 10 μL/min. Positive-ion ESIMS and MS/MS spectra were acquired using a Q-TOF 2 instrument (Micromass, Manchester, UK), setting the needle voltage at 3000 V with the ion source at 80 °C and cone voltage at 35 V. Each spectrum was produced by accumulating data for approximately 1–2 min. MS/MS spectra of pseudo-molecular ions were obtained by collision induced dissociation (CID), using argon as the collision gas and varying collision energy between 40 and 50 eV. In MS and MS/MS experiments, TOF resolution was set at approximately 10,000 (full width between half-maximum peaks-FWHM definition). In MS/MS experiments, Q1 peak width (FWHM) was set at approximately 0.7 Th.

3. Results and discussion

3.1. Purification of mannans from green and roasted coffee infusions

Mannans from green and roasted coffee infusions were purified from the high molecular weight material (HMWM) by precipitation in 50% ethanol solutions,[5] followed by the recovery of the non-retained fraction (QSA) from the anion-exchange chromatography on Q-Sepharose.[16] For both coffees, the non-retained material accounted for 60% of the applied material (Table 1). On the basis of the sugar composition (Table 1) and methylation analysis of the QSA polysaccharides (Table 2) and of the knowledge of the type of polysaccharides present in green and roasted coffee infusions,[5–7,11] the amount of galactomannan, arabinogalactan and glucan can be estimated as 37%, 24% and 28%, respectively, for green coffee QSA fraction, and 95%, 4% and 1% for roasted coffee QSA fraction.

To separate mannan from arabinogalactan and glucan, the QSA material was further fractionated on a Sepharose medium containing immobilized phenylboronic acid. The boronic functionality interacts reversibly with *cis*-diol groups.[17] Likely candidates to link to phenylboronic groups were terminally-linked Gal residues through their O-3–O-4 hydroxyl groups, and 4-linked and terminally-linked Man residues, through O-2–O-3. The 3- and 3,6-linked Gal residues and terminally- and 5-linked Ara residues do not have free *cis*-diol groups, so were not expected to link to the phenylboronic acid stationary phase. With this fractionation procedure, from both coffees, a fraction (PB2) was retained and selectively eluted with mannitol (Fig. 1).

For green coffee fraction, 82% of the applied material and 94% of total sugars were recovered (Table 1); the amount of mannose polymers not retained was only 1%. PB2 fraction was almost exclusively composed by 4- and 4,6-linked mannose and terminally-linked galactose residues, but 6% of 4-linked glucose and 2%

Table 1. Sugar composition (mg anhydrosugars/100 mg of sample) of the non-retained fraction from anion-exchange chromatography (QSA) and fractions obtained from phenylboronic acid immobilized Sepharose (PB1 and PB2)

	Yield (%)	Acetyl[b]	Rha	Ara	Man	Gal	Glc	Total sugars
Green								
QSA	60[a]	nd	0.76 (0.09)[d]	4.23 (0.18)	16.02 (0.64)	11.44 (0.52)	16.74 (1.49)	49.19 (2.92)
PB1	50[c]	nd	0.46 (0.38)	4.38 (0.12)	0.36 (0.01)	9.85 (0.43)	25.09 (0.42)	40.15 (2.61)
PB2	32[c]	3.9% (0.31)	—	2.16 (0.09)	59.5 (2.14)	12.9 (0.61)	6.5 (0.37)	81.12 (3.12)
Roasted								
QSA	59[a]	nd	0.20 (0.02)	0.93 (0.06)	52.48 (2.40)	3.53 (0.15)	1.77 (0.04)	58.17 (2.63)
PB1	19[c]	nd	0.40 (0.10)	1.05 (0.08)	34.09 (1.91)	3.53 (0.17)	2.16 (0.10)	41.23 (1.98)
PB2	58[c]	2.1% (0.14)	0.24 (0.02)	1.03 (0.05)	63.32 (3.13)	3.69 (0.13)	0.91 (0.04)	69.19 (3.36)

nd—not determined.
[a] Percentage (w/w) in relation to the applied material.
[b] Percentage (w/w) of acetyl groups in the sample.
[c] Percentage (w/w) in relation to QSA fraction.
[d] Standard deviation.

Table 2. Glycosidic-linkage composition (mol %) of the non-retained fraction from anion-exchange chromatography (QSA) and fractions obtained from phenylboronic acid immobilized Sepharose (PB1 and PB2)

Linkage	Green						Roasted					
	QSA		PB1		PB2		QSA		PB1		PB2	
T-Rha*p*	1.7		1.7		—		0.2		—		—	
Total Rha	1.7	(2)[a]	1.7	(2)	—	(0)	0.2	(0)	—	(1)	—	(0)
T-Ara*f*	8.4		8.9		2.0		1.1		0.3		0.9	
5-Ara*f*	4.6		4.0		—		0.2		0.3		0.2	
Total Ara	13.0	(11)	12.9	(13)	2.0	(3)	1.3	(2)	0.6	(3)	1.1	(2)
T-Man*p*	1.5		—		2.6		5.5		6.9		5.7	
4-Man*p*	27.9		1.2		67.8		85.1		83.3		83.6	
4,6-Man*p*	3.7		—		11.8		2.8		1.3		2.9	
Total Man	33.9	(32)	1.2	(1)	82.0	(74)	91.4	(90)	91.5	(83)	92.2	(91)
T-Gal*p*	6.2		4.6		10.2		3.0		2.6		3.5	
6-Gal*p*	1.3		1.5		—		0.6		0.3		0.3	
3-Gal*p*	6.3		10.6		—		0.3		2.3		1.5	
3,6-Gal*p*	5.3		12.3		—		0.3		0.5		0.3	
Total Gal	19.1	(21)	28.9	(24)	10.2	(15)	4.2	(6)	5.7	(8)	5.6	(6)
4-Glc*p*	6.3		18.2		5.7		1.0		2.2		1.0	
6-Glc*p*	27.5		37.9		—		—		—		—	
Total Glc	33.8	(34)	56.1	(60)	5.7	(8)	1.0	(2)	2.2	(5)	1.0	(1)

[a] Sugar composition determined by alditol acetates.

of terminally-linked arabinose residues were also found (Table 2). The percentage of mannan residues recovered in this fraction corresponded to 0.76% and 43% of the total mannose residues present in the green coffee beans[8] and hot water soluble coffee fraction,[5] respectively. For roasted coffee, 77% of the applied material and 82% of sugars were recovered (Table 1). Fifty eight percent of the applied material was bound to the PBA–Sepharose, corresponding to 69% of the applied sugars and it was observed that 13% of mannans were not retained in the column. The non-retained fraction (PB1) was run again on freshly activated PBA–Sepharose but no additional material was bound. From the methylation analysis it was observed that PB1 was composed by lower substituted mannans [100*(1→4,6)-Man/total Man] when compared with the mannans of the bound fraction (1.4% and 3.2%, respectively). Since the branching residues of mannans of roasted coffee infusions are composed by terminally-linked Gal residues that contain *cis*-diol groups, this result suggests that the terminally-linked Gal residues of these polysaccharides are responsible for the chromatographic behaviour of the two mannan-rich samples. The percentage of mannan residues recovered in PB2 fraction corresponds to 2.3% and 27% of the total mannose residues present in the roasted coffee beans[8] and in the hot water soluble coffee fraction,[5] respectively. The amount of terminally-linked mannose residues obtained by methylation analysis allowed estimating a degree of polymerization (DP) of only 32 residues for the green coffee galactomannan and 16 the roasted coffee, considering the usual linear mannan backbone. However, these values were similar

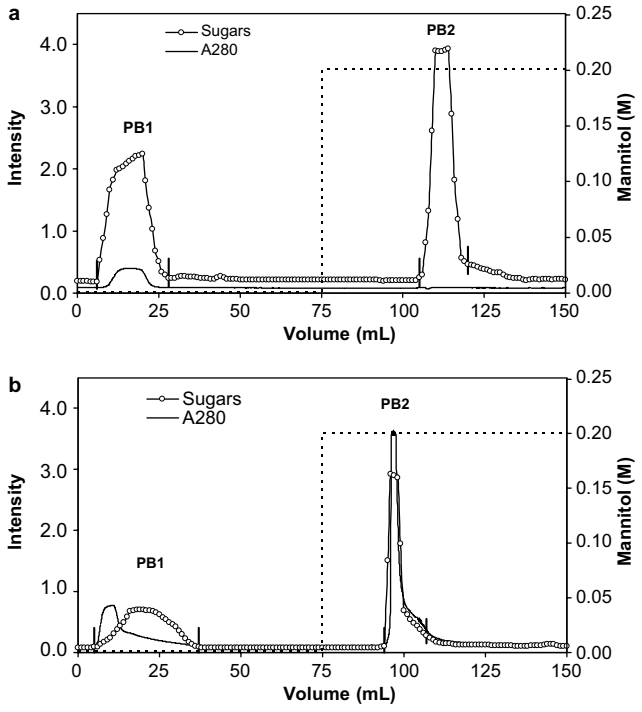


Figure 1. Chromatographic profile on PBA–Sepharose of QSA fraction of green (a) and roasted (b) coffees.

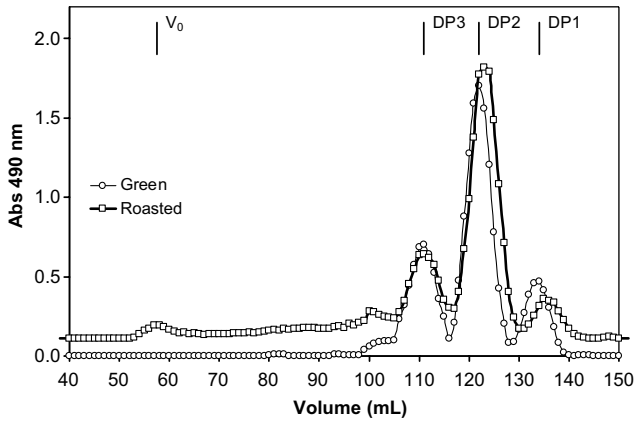


Figure 2. Size-exclusion chromatography of PB2 Fraction of green and roasted coffees obtained after enzymatic hydrolysis with an *endo*-β-mannanase. V_0—void volume, DP 2 and DP 3 correspond to the elution volume of DP 2 and DP 3 standard oligosaccharides, and DP 1 correspond to the elution volume of monomers.

to those found by other authors, as for green coffee water soluble galactomannans, for which DPs of 8[9] and 17–18[18] have been reported, and for water soluble galactomannans from roasted coffee where DPs of 15–26,[19] and 31[9] were estimated. These values, when compared with the DP values available in the literature for the green and roasted bean of 69[18] and 70,[8] respectively, allows inferring that the coffee galactomannans extracted with hot water from green and roasted coffee beans are smaller.

Contrarily to the green coffee, in the roasted coffee PB2 fraction, arabinogalactans (2%) were present, as denoted by the presence of 3-, 6- and 3,6-Gal*p* and 5-Ara*f* residues. These arabinogalactans could have been retained by PBA–Sepharose due to the presence of terminally-linked Gal residues that was reported to increase during the roasting process.[6] As observed for green coffee, in PB2 fraction of roasted coffee, 4-linked Glc and terminally-linked Ara residues were present. The consistent presence of these residues in green and roasted coffee PB2 fractions strongly suggests that these residues could be an integral part of hot water extractable coffee mannans.

3.2. Enzymatic hydrolysis of mannans and ESIMS analysis

For a detailed molecular structural analysis of PB2 galactomannans from green and roasted coffees, samples were submitted to an *endo*-β-mannanase hydrolysis. The material obtained was subjected to size-exclusion chromatography on Biogel-P2 (Fig. 2). The chromatogram patterns of the two samples, according to the known enzymatic mechanism of *Aspergillus niger endo*-β-mannanase,[20] where the hydrolysis of the β-(1→4) linked mannan backbone is hindered by the presence of Gal residues, were in agreement with a low galactose substituted mannan backbone. This was shown by the high abundance of DP 2 and DP 3 saccharides and a low abundance of higher molecular weight oligosaccharides that were absent in the green coffee galactomannans.

The ESIMS spectra of DP 3 fractions obtained from green (Fig. 3a) and roasted (Fig. 3b) coffees presented similar features. Predominant oligosaccharide $[M+Na]^+$ ions were observed at *m/z* 365 and 527, for di- and trihexoses, respectively. The corresponding potassium adduct ions ($[M+K]^+$) were observed at *m/z* 381 and 543. The ion at *m/z* 497, with a mass increase of 132 Da relative to *m/z* 365, was identified as $[M+Na]^+$ ion of the pentose–dihexose trisaccharide. Figure 3 also shows intense peaks at *m/z* 407 and 569, with a mass 42 Da higher relative to the di- and trihexoses, corresponding, possibly, to $[M+Na]^+$ ions of the acetylated di- and trisaccharides. The ion at *m/z* 423 was attributed to the $[M+K]^+$ ions of acetylated dihexoses. The ion at *m/z* 449 was attributed to the $[M+Na]^+$ of diacetylated-dihexoses. The ions attributed to the acetylated and diacetylated saccharides were not detected in the ESIMS spectra of the samples when they were submitted to an alkali hydrolysis (result not shown). In order to confirm that these samples could be acetylated, the analysis of acetyl groups was performed in PB2 samples by gas chromatography and enzymatic analysis after alkaline hydrolysis. Table 1 shows that the weight of acetyl groups accounted for 3.9% of PB2 fraction of green coffee and for 2.1% in roasted coffee, meaning an average

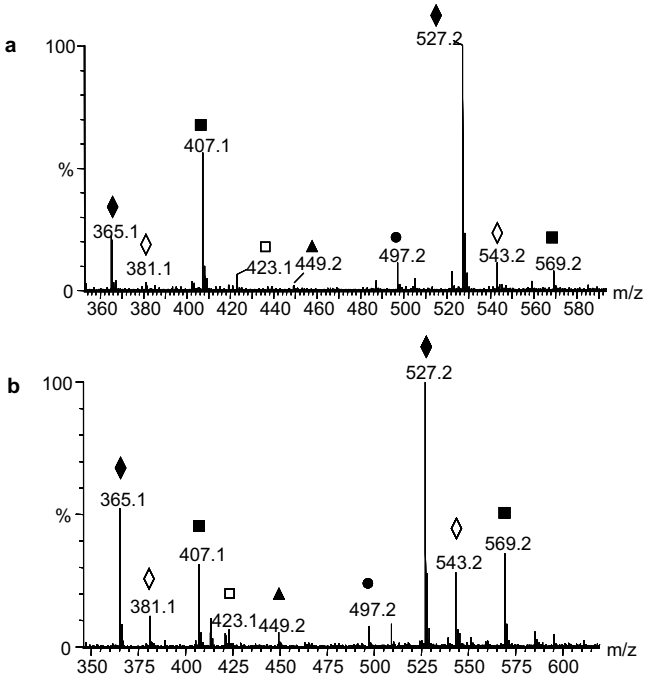


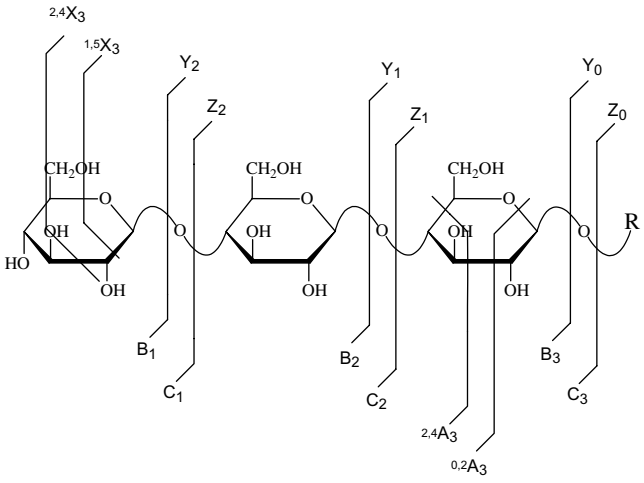
Figure 3. Positive ESIMS of DP 3 fractions obtained after enzymatic hydrolysis of PB2 from green (a) and roasted (b) coffees. ●: $[M+Na]^+$ adduct of $PentHex_2$; ■: $[M+Na]^+$ adduct of $AcHex_{2-3}$; □: $[M+K]^+$ adduct of $AcHex_{2-3}$; ◆: $[M+Na]^+$ adduct of Hex_{2-3}; ◇: $[M+K]^+$ adduct of Hex_{2-3}; ▲: $[M+Na]^+$ adduct of Ac_2Hex_2.

of 11 and 8 mol % of acetylated Man residues in green and roasted coffee, respectively. This decrease in the abundance of acetyl groups in the mannans may be caused by the roasting process due to the lability of the ester group. Nevertheless, the increase in the amount of extractable mannans with the roasting procedure[5–7,9,10] could also promote the extraction of less or non-acetylated mannans already present in the green coffee beans.

In order to confirm the proposed structures attributed to the $[M+Na]^+$ ions observed in the ESIMS spectra of green and roasted coffees, these ions were further analyzed by MS/MS.

3.3. ESIMS/MS spectra of acetyl and pentose substituted oligosaccharides

Oligosaccharide fragmentation under ESIMS/MS conditions are the result of glycosidic cleavages between two sugar residues 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[21] (Scheme 1). 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 non-reducing 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, or the Greek letters (e.g., α) for branched oligosaccharides in the order of the size of branching.



Scheme 1. Nomenclature of fragment ions from carbohydrates according to Domon and Costello.[21]

The ESIMS/MS spectrum of $[Hex_3+Na]^+$ (*m*/*z* 527) is shown in Figure 4a. The predominant ions observed were attributed to C/Y-type glycosidic bond cleavage yielding the ion at *m*/*z* 365 and the ion at *m*/*z* 203 corresponding to the loss of one and two Hex_{res} (162 Da), respectively. Ions at *m*/*z* 467 and *m*/*z* 305 were attributed to a $^{0,2}A_3$ and $^{0,2}A_2$ (−60 Da) cross-ring fragments. $^{2,4}X_1$ cross-ring cleavage could also explain the product ion at *m*/*z* 305. However, since the low energy CID ion spectrum showed predominant fragmentation from the reducing end,[22] it allowed this fragmentation to be attributed to $^{0,2}A$ cross-ring fragmentation. The loss of a neutral fragment with 60 Da and the absence of fragmentations due to losses of 90 Da and 120 Da, is a characteristic of the MS/MS of (1→4)-linked hexoses.[23]

The ESIMS/MS spectrum of $[AcHex_2+Na]^+$ (*m*/*z* 407), showed the presence of ions at *m*/*z* 245, identified as $[AcHex+Na]^+$, due to loss of 162 Da (Hex_{res}) by a C/Y-type cleavage (Fig. 4b). Also, the abundant ion at *m*/*z* 305, corresponding to a loss of 102 Da, was attributed to an $^{0,2}A_2$ cross-ring fragment of a hexose containing one acetyl group at O-2. Another feature of the MS/MS spectra of $[AcHex_2+Na]^+$ is the occurrence of fragment ion at *m*/*z* 364, due to a loss of 43 Da that can be attributed to a homolytic cleavage of the acetate group. The ion at *m*/*z* 347 (loss of a neutral of 60 Da), can be due to cross-ring cleavage of hexose residue and also to loss of acetic acid, from fragmentation of an acetyl substituent. However the higher relative abundance of this ion when compared with the MS/MS of the non-acetylated disaccharides, suggests that this fragment

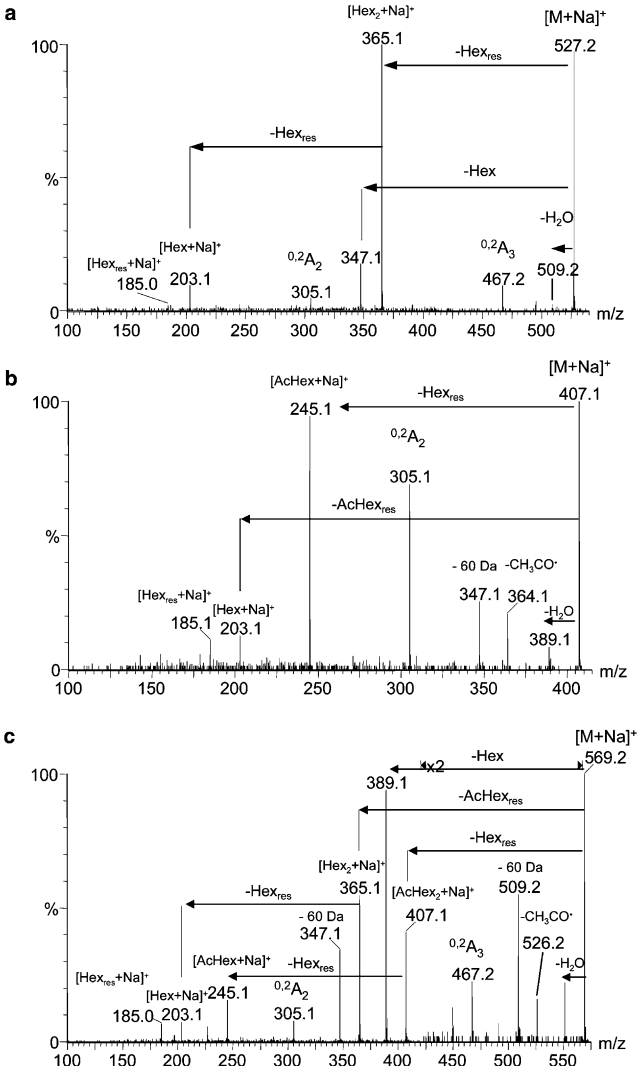


Figure 4. ESIMS/MS spectra of $[M+Na]^+$ adducts of (a) Hex_3, (b) $AcHex_2$ and (c) $AcHex_3$.

ion is also due to the loss of acetic acid, which reinforces the suggestion of the presence of acetyl groups.

The ESIMS/MS spectrum of $[AcHex_3+Na]^+$ (*m*/*z* 569), showed the presence of ions at *m*/*z* 407 and 245, identified as $[AcHex_2+Na]^+$ and $[AcHex+Na]^+$, resulting of loss of one and two Hex_{res} (162 Da), respectively (Fig. 4c). Also observed with a relatively high abundance was ion *m*/*z* 365, identified as $[Hex_2+Na]^+$, resulting from loss of $AcHex_{res}$ (204 Da). The base ion at *m*/*z* 389 was attributed to a B/Z-type cleavage with the loss of a Hex unit (180 Da). Ions at *m*/*z* 467 and at *m*/*z* 305 are resulting from loss of 102 Da from the parent ion of the fragment due to loss of Hex_{res} (162 Da). These fragment ions were attributed to $^{0,2}A_3$ and $^{0,2}A_2$ cross-ring fragment containing the acetyl group at O-2 position. The ESIMS/MS spectrum of the acetylated di- and trisaccharides from green and roasted coffee were similar to those of the acetylated glucomannan from *Amorphophalus konjac* (results not shown).

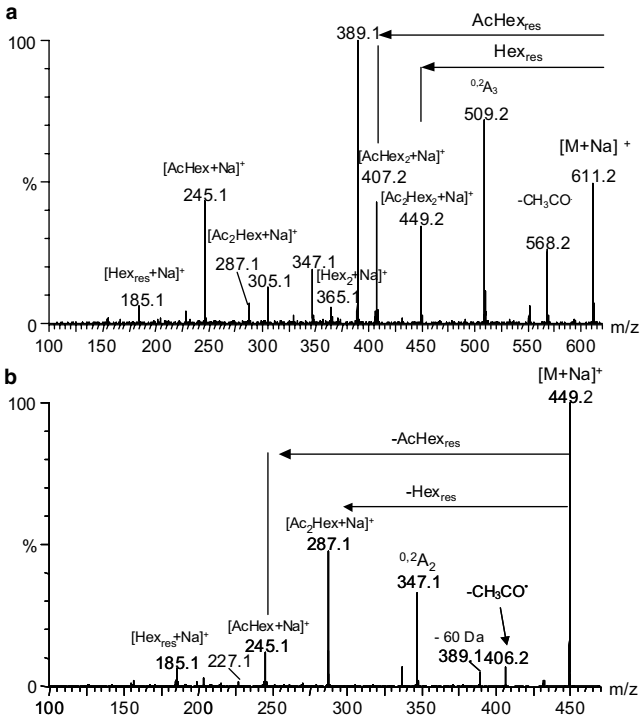


Figure 5. ESIMS/MS spectra of $[M+Na]^+$ adduct of (a) Ac_2Hex_3 and (b) Ac_2Hex_2.

Figure 5a shows the ESIMS/MS spectrum of $[Ac_2Hex_3+Na]^+$ (*m*/*z* 611). Fragment ion at *m*/*z* 449 can be identified as $[Ac_2Hex_2+Na]^+$, resulting from the loss of a Hex_{res} by a C/Y-type cleavage. Fragment ion at *m*/*z* 287 can be identified as $[Ac_2Hex+Na]^+$, resulting from loss of an Hex_{res} by a C/Y-type cleavage from $[Ac_2Hex_2+Na]^+$. Fragment ion at *m*/*z* 245 can be identified as $[AcHex+Na]^+$, resulting from loss of an $AcHex_{res}$ by a C/Y-type cleavage. The presence of the fragment ions at *m*/*z* 449, *m*/*z* 287 and *m*/*z* 245 in the MS/MS spectrum of this trisaccharide indicates that the mannans isolated from green and roasted coffee infusions contain hexose disaccharide residues bearing two acetyl groups that can occur in a single diacetylated residue or in two consecutive acetylated residues. However, the ion at *m*/*z* 245, formed by loss of $AcHex_{res}$ through a C/Y-type cleavage from a diacetylated disaccharide (*m*/*z* 449), obtained by *endo*-β-(1→4)-mannanase hydrolysis (Fig. 5b), is not compatible with the known mechanism of the hydrolysis by this enzyme,[24] due to the fact that the presence of acetyl groups hinders the enzyme hydrolysis of those linkages. The detection of acetyl groups in the non-reducing end of these oligosaccharides can be attributed to acetyl migration during the freeze drying process as was observed in acetylated xylo-oligosaccharides.[25] Anyway, the abundance of the ion at *m*/*z* 245 in Figure 5a is much higher than ion at *m*/*z* 287, identified as $[Ac_2Hex+Na]^+$, contrarily to what was observed in Figure 5b, where the ion at *m*/*z* 245 has much lower relative abundance than the ion at *m*/*z* 287. This shows that the

extent of acetyl migration should not explain the occurrence of all consecutive acetylated residues, but allows inferring their presence in coffee galactomannans. Fragment ions at *m/z* 509 and 568 were identified, respectively, as $^{0,2}A_2$ type fragment ion and the fragment was due to homolytic cleavage of the acetyl ester group (loss of 43 Da). The occurrence of fragmentation pathway by loss of Hex, AcHex and Ac_2Hex residues in the MS/MS spectra and the absence of fragments due to loss of 90 and 120 Da (characteristic of (1→6)-linked Hex residues)[23] shows that the majority of the acetyl groups, if not all, when present are substituent of the (1→4)-linked Hex residues. The fragment ions resulting from loss of 102 Da, observed in the MS/MS spectrum of the acetylated and diacetylated oligosaccharides, suggests that 2-*O*-acetylhexose residues are present. Nevertheless, it does not allow to exclude the presence of other isomeric structures with substitution at O-3 and O-6 position of the (1→4)-linked Hex residues.

Although several glucomannan and galactoglucomannan from other sources were shown to be acetylated,[26–30] only very recently[24] this structural feature was described for the green coffee. However, it was never reported for mannans of roasted coffee infusions. As the alkaline treatment of a mannan rich fraction isolated from roasted coffee infusions resulted in mannan precipitate on dialysis[11] and, in parallelism with the observation that the degree of water solubility of glucomannans from *A. konjac* is dependent on the amount of acetyl groups,[26] the occurrence of acetyl groups could be an important structural feature related to the solubility of roasted coffee mannans.

The MS/MS spectrum of $[Pent.Hex_2+Na]^+$ (*m/z* 497) is shown in Figure 6. The major fragment ion at *m/z* 335, attributed to $[Pent.Hex+Na]^+$, results from the loss of a hexose residue (Hex_{res}). Also observed with a high intensity was the ion at *m/z* 365, attributed to $[Hex_2+Na]^+$, resulting from loss of 132 Da that can be attributed to the loss of a pentose residue ($Pent_{res}$). The ions at *m/z* 173 $[Pent+Na]^+$ and 155 $[Pent_{res}+Na]^+$, resultant from B_α- and C_α-type cleavages, respectively, confirmed

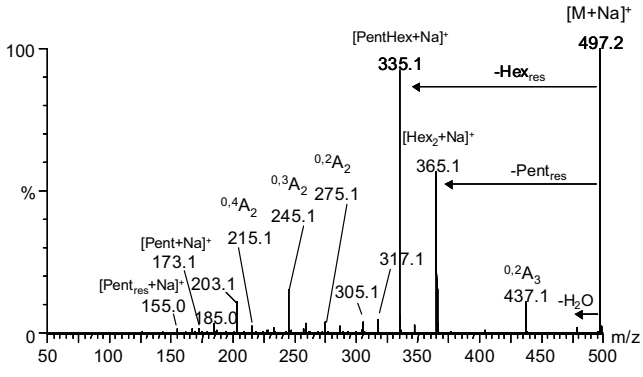


Figure 6. ESIMS/MS spectra of $[M+Na]^+$ adduct of $Pent.Hex_2$.

the presence of the pentose residues in the structure of this oligosaccharide. The loss of 60, 90 and 120 Da from $[Pent.Hex+Na]^+$, at *m/z* 335, leading to the ions, respectively, at *m/z* 275, 245 and 215, confirms the (1→6)-linkage of the pentose residue to the Hex. The ESIMS/MS spectra of $[Pent.Hex_2+Na]^+$ ions obtained after partial acid hydrolysis of coffee arabinogalactans showed a fragmentation pattern completely different from the ions obtained from galactomannans (data not shown), which supported the conclusion that this ion was not from arabinogalactans. These results, together with sugar composition and methylation analysis, allow to unambiguously conclude that the hot water extractable green and roasted coffee mannans bear terminally-linked pentose residues O-6 linked to Man, supporting the data from Navarini et al.[11] The presence of arabinose residues in mannans has been previously described in galactoglucomannans isolated from suspension-cultured cells of *Nicotiana plumbaginifolia*[29] and in a 2-*O*-acetylglucomannan from *Dendobrium officinale* stem.[30] However, this is the first time that these residues were unequivocally detected in mannans from green and roasted coffee. The much lower amount of arabinose residues present in roasted coffee galactomannans can be the result of debranching of the mannans during coffee roasting, as was observed for the arabinose residues in coffee arabinogalactans.[5–10] This decrease can be also due to the extraction from the roasted coffee bean of galactomannans containing a lower amount of arabinose residues.

3.4. Glucose as a structural feature of coffee mannans

In order to evaluate if the presence of glucose is a structural feature of the mannans of green and roasted coffee infusions, the DP 3 and DP 2 oligosaccharide fractions from green and roasted coffees were subjected to acid hydrolysis and, after derivatization to alditol acetates, were analyzed by GC–EIMS. Retention time and EIMS spectra of glucitol hexaacetate (data not shown) confirmed the presence of glucose residues in these green and roasted coffee *endo*-β-mannanase hydrolyzed fractions.

In order to determine the anomeric configuration of the mannose and glucose residues in the oligosaccharide fractions, the DP 2 and DP 3 fractions were submitted to three enzymatic assays with β-(1→4)-specific D-mannosidase, β-(1→4)-D-glucosidase and α- (1→4)-D-glucosidase. The β-(1→4)-D-mannosidase released glucose from the roasted coffee fractions but not from the green coffee. This observation indicates the occurrence of glucose residues at the reducing end of the oligosaccharides from roasted coffee and that no glucose residue was present at the reducing end of the oligosaccharides from green coffee mannans. The enzymatic treatment of fractions from green and roasted coffee with α-(1→4)-D-glucosidase did not yield any glucose, and the

treatment of the same fractions with β-(1→4)-D-glucosidase yielded glucose only for the green coffee fractions. These results suggest different positions of the glucose residues in the oligosaccharides obtained from the green and roasted coffees. In order to verify these different locations, the green and roasted coffee DP 2 and DP 3 fractions were subjected to methylation analysis (Table 3). The results of methylation were in accordance with the results of the enzymatic hydrolysis. In green coffee fractions, the glucose residues were found exclusively in the non-reducing terminal (T-Glc*p*), and in the roasted coffee fractions no glucose residues was detected in the non-reducing terminal. The glucose residues in the roasted coffee oligosaccharide fractions were (1→4)-linked and, in the green coffee oligosaccharide fraction, no (1→4)-linked glucose residues was found. In the oligosaccharides generated from roasted coffees, these glucose residues, if not all, were at the reducing end of β-(1→4)-linked mannose residues. The absence of (1→4)-Glc residues in green coffee oligosaccharide fractions indicates that these galactomannans did not contain contiguous glucose residues.

The pattern of the glucose residues in the oligosaccharides obtained after enzymatic hydrolysis from green coffee mannans with *A. niger endo*-β-mannanase was in accordance with the known mechanism of this enzyme on glucomannans,[29,31] where it was found that the di- and trisaccharides contain glucose residues almost exclusively at the non-reducing end.

In order to establish the structures suggested by methylation and enzymatic analysis for the oligosaccharides released from green and roasted coffee mannans, the oligosaccharide fractions were reduced, methylated and analyzed by GC–EIMS. The retention time and MS spectra showed that Glc–Man was present in green coffee DP 2 fractions together with a Man–Man disaccharide (Fig. 7). In DP 3 fractions from green coffee, Glc–Man–Man could be detected together with Gal–Man–Man and Man–Man–Man (data no shown). No Glc–Man dimer was detected in DP 2 fraction obtained from roasted coffee mannans. This result, together with the information given by enzymatic analysis of the oligomeric fractions obtained, indicates that the mannans extracted with hot water from roasted coffee and recovered by precipitation in 50% ethanol solution, did not contain glucose residues in the mannan backbone, as was observed in the same fraction obtained from green coffee hot water soluble mannans. As the roasting process increases four times the amount of galactomannans extracted with hot water from the coffee beans,[5] this could indicate that only a limited amount of coffee bean galactomannans contain glucose residues in the mannan backbone or, alternatively that other glucomannans, if present, cannot be extracted from roasted coffees by

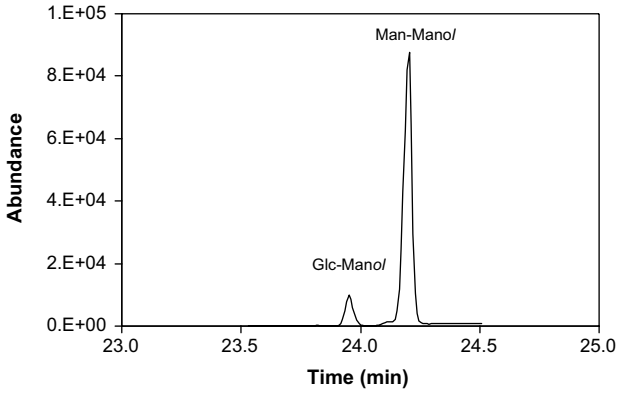


Figure 7. GC–EIMS detection of Glc–Man dimers released from *endo*-β-mannanase hydrolysis of green coffee mannan.

Table 3. Glycosidic-linkage composition (mol %) of the DP2 and DP3 fractions obtained after enzymatic hydrolysis with *endo*-β-mannanase of PB2 samples from green and roasted coffees

Linkage	Green		Roasted	
	DP3	DP2	DP3	DP2
T-Rha*p*	—	—	—	—
T-Ara*f*	1.4	0.1	0.4	0.2
T-Ara*p*	d	d	d	d
5-Ara*f*	—	—	—	—
4-Man*p*$_{red}$[a]	20.0	23.0	12.6	8.3
T-Man*p*	19.9	39.5	49.8	58.5
4-Man*p*	37.6	28.3	29.1	29.3
4,6-Man*p*	4.4	1.8	3.6	1.4
T-Gal*p*	3.8	1.2	3.3	1.1
6-Gal*p*	—	—	—	—
3-Gal*p*	—	—	—	—
3,6-Gal*p*	—	—	—	—
T-Glc*p*	12.9	6.0	—	—
4-Glc*p*	—	—	1.2	1.2

d—detected by GC–EIMS but not quantified by GC-FID.

[a] Reducing terminal residue detected as 1,4-di-*O*-acetyl-1-deuterio-2,3,5,6-tetra-*O*-methyl-D-mannitol.

hot water. Also, as roasting causes loss of coffee bean polysaccharides,[8,10] a loss of this low amount of glucomannan could also be expected. Another hypothesis that cannot be excluded to explain the lack of glucomannans found in roasted coffee infusions is that roasting could change the pattern of oligosaccharide released by *endo*-β-mannanase hydrolysis.

Methylation analysis of the DP 2 and DP 3 oligosaccharides showed that, in these fractions, arabinose residues are the pentose residues detected by ESIMS/MS. Although terminally-linked arabinofuranosyl and arabinopyranosyl residues were present, the latter were only detected in trace amounts.

4. Conclusions

The hot water extractable green coffee mannans, estimated as 43% of the total mannose residues of green coffee bean hot water extract, are acetylated (11 mol %) in the main backbone at O-2 of the mannose residues. Also present in lower amounts are doubly acetylated hexose residues and contiguously acetylated hexose residues. It was also possible to detect arabinose (2 mol %) and glucose (6 mol %) residues as structural features of acetylated galactomannans extracted from green coffee with hot water. The arabinose residues were present as side chains at O-6 of the mannose residues, and the β-(1→4) glucose residues were components of the mannan backbone.

The mannans extracted with hot water from roasted coffee, estimated as 27% of the total mannose residues of roasted coffee bean hot water extract, are, as in green coffee, also acetylated (8 mol %), containing also doubly acetylated hexose residues, and contiguously acetylated hexose residues. Galactose (3.2 mol %) and arabinose (<0.9 mol %) residues are present as side chains. The glucose residues (1.1 mol %), contrarily to what was found in green coffee are, apparently, mainly located at the reducing terminal.

Acknowledgements

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