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Structural features of acetylated galactomannans from green *Coffea arabica* beans

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Abstract

Polysaccharides were extracted from green *Coffea arabica* beans with water (90 °C, 1 h). Galactomannans were isolated from the water extract using preparative anion-exchange chromatography. Almost all of the galactomannans eluted in two neutral populations, while almost all of the arabinogalactans bound to the column, indicating that these arabinogalactans contain charged groups. Analysis of the molecular weight distribution of the two neutral populations showed that they differ in their molecular weight. Further characterization of these neutral populations by NMR and by MALDI-TOF MS after enzymatic degradation with an endo-mannanase, showed the presence of acetyl groups linked to the galactomannans, a feature not previously described for this type of polysaccharides from coffee beans. It was found that the high molecular weight (ca. 2000 kDa) neutral fraction was highly substituted both with galactose residues and acetyl groups, while the low molecular weight (ca. 20 kDa) population was much less substituted. Based on these results it can be concluded that at least two distinctly different populations of galactomannans are present in green coffee beans. It was also shown that the degradation of the galactomannans from green coffee beans with an endo-mannanase from *A. niger* is hindered by the presence of acetyl groups.

Keywords: Arabica; Green coffee beans; Galactomannans; Acetyl; Branching

1. Introduction

Green Arabica coffee beans consist for 48–60% of polysaccharides (Clifford, 1985; Wolfrom and Patin, 1965; Wolfrom, Plunkett and Laver, 1960). These polysaccharides play an important role in the formation of flavour compounds during roasting and also play a role in the foam stability of espresso coffee (Nunes and Coimbra, 1998).

Galactomannans are the predominating polysaccharides in the green coffee bean besides cellulose, and arabinogalactan type II (Fischer, Reimann, Trovato and Redgwell, 2001; Nunes and Coimbra, 2001, 2002; Wolfrom et al., 1960). These galactomannans are composed of a backbone of 1→4-linked mannans with single unit galactose side-chains at O-6 (Fischer, Reimann, Trovato and Redgwell, 1999). In some studies these polysaccharides were described as mannans based on the low degree of branching of these polysaccharides (Bradbury and Halliday, 1990). On the other hand, it was reported that galactomannans might be substituted with low amounts of arabinose (Navarini, Gilli, Gombac, Abatangelo, Bosco and Toffanin, 1999). The molecular weight range of water extractable polysaccharides (predominantly galactomannans and arabinogalactans) from green Arabica beans was found to be in the range of 200–200,000 Da (Leloup and Liardon, 1993). The extractability of the galactomannans was found to be very low (Fischer et al.; Oosterveld, Harmsen, Voragen and Schols, 2002), however, this was greatly improved after roasting (Nunes and Coimbra; Oosterveld et al., 2002; Oosterveld, Voragen and Schols, 2003).

In the current investigation galactomannans were purified from a hot water extract from green coffee beans.

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The subsequent characterization of the various fractions obtained gave new insights about the substitution of the galactomannans present in green coffee beans.

2. Experimental

2.1. Materials

Green *Coffea arabica* beans (Columbia) were used for the experiments.

2.2. Extraction of polysaccharides from green coffee beans

Ground coffee beans (green) were Soxhlet-extracted for 6 h with petroleum ether to remove the lipids. Defatted ground coffee beans (100 g) was extracted with 2000 ml of water (90 °C, 1 h) as described previously (Oosterveld et al., 2002). The extract was dialyzed using a 10 kDa dialysis membrane and freeze-dried prior to analysis.

2.3. Analytical methods

The uronic acid content was determined by the automated m-hydroxy biphenyl assay (Thibault, 1979). The neutral sugar composition was determined after hydrolysis with sulphuric acid and conversion into alditol acetates as described previously (Oosterveld, Beldman, Schols and Voragen, 1996). The sugar linkage composition of the neutral sugars was determined using the methylation analysis as described previously (Oosterveld et al., 1996), using hydrolysis with 90% (v/v) formic acid (5 h, 100 °C). The methylation step was repeated two times in order to ensure full methylation of the sugar residues. The partially methylated alditol acetates were identified by GC-MS and quantified by GLC. The acetyl contents of the fractions were determined using an Acetic acid test kit (Boehringer Mannheim).

2.4. Chromatography

High-performance size-exclusion chromatography (HPSEC) was performed on three TSK gel columns in series (PW_{XL} G4000, G3000, G2500; Tosohaas) as described (Oosterveld et al., 1996), using 0.2 M $NaNO_3$ as eluent.

Mannose, mannan oligomers and galactosyl-substituted mannan oligomers were detected with high-performance anion-exchange chromatography (HPAEC) as described before (Daas, Schols and de Jongh, 2000).

Preparative anion-exchange chromatography was performed on a column (75×2.6 cm) of DEAE Sepharose Fast Flow using a Hiload System (Ammersham Biotech). Samples (0.5 g) were dissolved in 0.005 M NaOAc buffer (5 ml, pH 5.0) and applied onto the column at a flow rate of 2.5 ml/min (0.005 M NaOAc, pH 5.0). After 120 min, the flow rate was increased to 12.5 ml/min and a gradient was applied as indicated in the corresponding figure (Fig. 1).

The fractions obtained by preparative anion-exchange chromatography were assayed for total neutral sugar (Tollier and Robin, 1979) and uronic acid (Thibault, 1979) content, using arabinose and galacturonic acid as standards, respectively. A correction was made for the response of uronic acids in the neutral sugar test. Pooled fractions were dialyzed and freeze-dried.

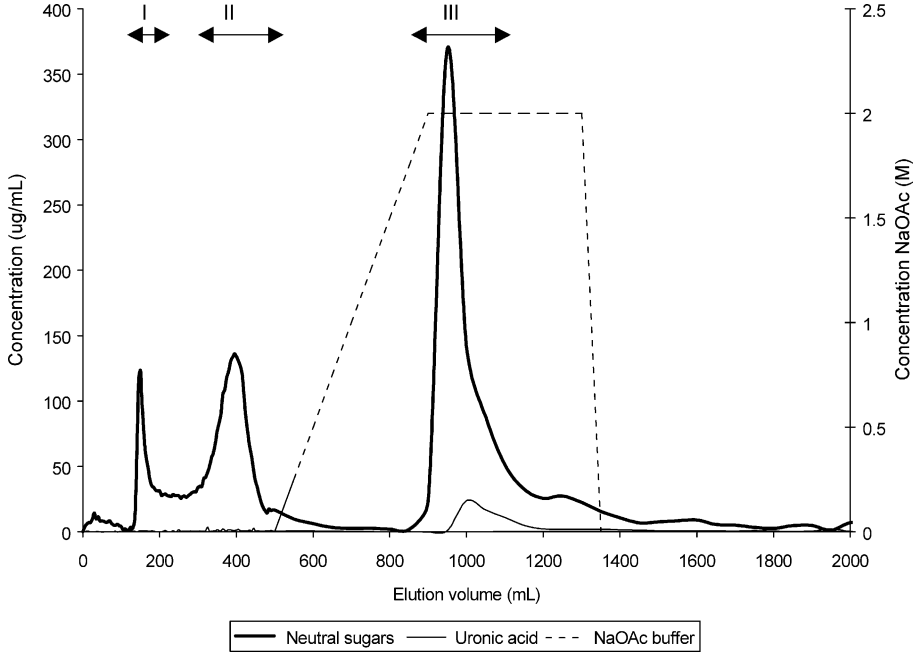

Fig. 1. Anion-exchange chromatography (DEAE Sepharose) of a hot water extract from green coffee beans.

2.5. MALDI-TOF mass spectrometry

For matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) a Voyager-DE RP Biospectrometry workstation (PerSeptive Biosystems Inc., Framingham, MA, USA) was used, operated as described (Daas, Meyer-Hansen, Schols, de Ruiter and Voragen, 1999). The mass spectrometer was calibrated with a mixture of maltodextrines (mass range 365–2309).

The samples were mixed with a matrix solution (1 μl of sample in 9 μl of matrix), after desalting the samples using H^+-Dowex AG 50W X8 (Biorad). The matrix solution was prepared by dissolving 9 mg of 2,5-dihydroxybenzoic acid and 3 mg 1-hydroxyisoquinoline in a 1-ml mixture of acetonitrile:water (300:700 μl). 1 μl of the prepared solutions (sample + matrix) was put on a gold plate and allowed to dry at room temperature.

2.6. ^{1}H-NMR

Prior to NMR analyses, the samples were exchanged in 99.96% D_2O (Cambridge Isotope Laboratories, USA) and dissolved in 99.996% D_2O after freeze-drying. NMR spectra were recorded at a probe temperature of 70 °C on a Bruker AMX-500 spectrometer located at the Wageningen NMR Center. Chemical shifts were expressed in parts per million relative to internal acetone: $\delta = 2.225$ ppm for ^{1}H.

The 1D ^{1}H proton spectra were recorded at 500.13 MHz using 8–200 scans of 8192 data points and a sweep width of 3000 Hz.

2.7. Enzymatic degradation of galactomannan fractions

The galactomannan fractions (5 mg/ml) were dissolved in 50 mM sodium acetate buffer (pH 5.0) and treated with purified enzyme endo-mannanase from *A. niger* (0.1 U/mg substrate; EC 3.2.1.78) (Düsterhöft, Bonte and Voragen, 1993). Incubations were carried out at 30 °C for 24 h. The digests were analyzed using HPSEC as well as HPAEC. The galactomannan fractions (2 mg) were saponified after enzymatic degradation by treatment with 0.5 ml 0.05 M NaOH for 20 h at 4 °C. Afterwards the samples were neutralized with 0.1 M HCl and subsequently analyzed.

3. Results and discussion

3.1. Polysaccharide composition of a hot water extract from green coffee beans

Polysaccharides were extracted from green coffee beans with water (90 °C, 1 h). The polysaccharide content of the water extract was 34% (Table 1), which was higher than was found by Nunes and Coimbra (2001) for a similar extract. Most of the water extract consisted of proteins. However, the protein content was calculated from the nitrogen content of the samples. This means that the protein content may have been somewhat overestimated due to the presence of other nitrogen containing substances. Besides polysaccharides and proteins, the extract also contained a low amount of acetyl (0.3%), which is probably ester-linked to the polysaccharides. Arabinose and galactose were the predominant sugars in the water extract of the green coffee beans (26 and 36 mol%, respectively) indicating that the extract contained mainly arabinogalactans. Mannose represented 18 mol% of the sugars in this extract indicating that also galactomannans were present in reasonable amounts. The sugar composition was somewhat different to what was found by Nunes and Coimbra, who found higher levels of arabinose and especially glucose. The sugar composition was also comparable to that found by Fischer et al. (2001) for a water extract of green coffee beans, except for the galactose content, which was lower in our water extract.

3.2. Isolation of galactomannans from a hot water extract of green coffee beans by anion-exchange chromatography

In order to separate the galactomannans from charged polysaccharides, the water extract was further fractionated using preparative anion-exchange chromatography. Before applying the extract onto the column it was first dissolved in 0.005 M NaOAc pH 5 (90 °C, 10 min). Only 50% of the polysaccharides (with a sugar composition comparable to the water extract; Table 1) dissolved in the buffer. The pellet had a relatively low sugar content and is expected to have a high protein content, based on

Table 1
Sugar composition (mol%) of a hot water extract obtained from green coffee beans and of pools obtained by preparative anion-exchange chromatography (DEAE) of this extract

	Rha	Ara	Xyl	Man	Gal	Glc	UA	Sugars (%)	Proteins (%)	Acetyl (%)	Mass (g)	Sugar recovery (%)
Water	7	26	1	18	36	4	10	34	59	0.3	522	100
Applied	4	26	1	20	38	6	5	55	10	–	161	50
Pool I	0	1	0	73	22	4	0	48	13	1.2	8	2
Pool II	0	5	1	70	8	16	0	45	8	0.5	32	8
Pool III	5	33	1	5	48	3	6	18	13	–	222	23
Pellet	11	36	9	9	20	7	8	24	–	–	361	49

–, not determined.

the protein contents of the applied fraction and of the water extract. Apparently, the protein rich pellet did not redissolve as a result of denaturation of the proteins in this fraction. Anion-exchange chromatography of the soluble fraction revealed the presence of three populations: two neutral populations and a charged population eluting with NaOAc, representing most of the polysaccharides (Fig. 1). The fact that two neutral populations were found after anion-exchange chromatography of the water extract, indicates that these populations were not only separated by charge but also by another mechanism. Most probably, the column used also gave some separation according to the molecular weight.

After elution the fractions were pooled as indicated in Fig. 1. Note that not all fractions were collected in the pools, which explains that only 66% of the carbohydrates, which were applied onto the column were collected in the pools I, II and III. From the sugar composition of these pools (Table 1) it can be calculated that 86% w/w of the mannose recovered from the extract applied to the column eluted in pool I and II. Since most of the arabinogalactans were found in the charged fraction a good separation was obtained between galactomannans and arabinogalactans: approximately 92% w/w of the arabinose and galactose recovered eluted in the charged pool III. From this it can be concluded that the arabinogalactans must include charges. The presence of small amounts of glucuronic acid in the arabinogalactans as described by Redgwell et al. (Redgwell, Trovato, Curti and Fischer, 2002) may explain this phenomena. The total mass of pool III was found to be very high, while the total amount of polysaccharides and proteins was rather low. This may indicate that not all NaOAc salts were removed by dialysis. The sugar composition of the neutral galactomannan pools obtained after anion-exchange chromatography shows that pool I had a higher galactose content than pool II. The ratio mannose: galactose in pool I was 3.3, while it was as much as 8.8 in pool II (Table 1). From this a degree of substitution (DS) of the galactomannans with galactose was calculated of 30% for pool I and of 11% for pool II. Mannose plus galactose made up 78–95 mol% of the sugars present in these pools. These values are comparable with data published before (Nunes and Coimbra, 2002), although arabinogalactans and galactomannans were not always separated sufficiently to compare precise gal:man ratio's for the galactomannan population.

Additional structural information of the galactomannans present in pools I and II was obtained by their sugar linkage composition (data not shown). In spite of the three methylation steps which were applied, a significant percentage of the sugar residues was not methylated, which reduces the accuracy of the measurement. Nevertheless, the results were confirming the different galactose substitution levels of the mannan backbone in the different hot water extractable mannan populations. All types of linkages found were in agreement with linkage analysis of green coffee mannans published before (Nunes and Coimbra, 2002; Oosterveld et al., 2003).

3.3. Molecular weight distribution of galactomannans from a hot water extract of green coffee beans by HPSEC

In order to investigate whether the galactomannan populations obtained after anion-exchange chromatography were homogeneous according to molecular weight, the molecular weight distribution was determined by HPSEC (Fig. 2). Comparison with dextran standards showed that pool I had a molecular weight of approximately 2×10^6 Da, whereas the molecular weight of pool II was a factor 100 lower. Also, it was found that both populations were rather homogeneous according to their molecular weights. From these results it can be concluded that galactomannan populations with different molecular weights are present in green coffee beans, and that the neutral galactomannan pools were indeed separated on the anion-exchange column as a result of an unintentional size-exclusion effect.

3.4. Presence of acetyl groups in galactomannans from a hot water extract of green coffee

Since acetyl was detected in the water extract of green coffee beans, the amount of acetyl groups present in the pools was analyzed using a commercial acetyl kit (Table 1). Pool I contained 1.2% of acetyl, while pool II contained only 0.5% of acetyl. Also the molar ratio Ac:Man was much higher in this pool (1:10 vs. 1:23; Table 2).

In order to confirm that the acetyl groups were linked to galactomannans, pool I and II were degraded with an endo-mannanase, able to degrade (galacto)mannans (Daas et al., 2000), and the reaction products thus obtained were characterized subsequently by HPSEC, HPAEC, and MALDI-TOF MS.

The molecular weight distributions of pool I and II after treatment with an endo-mannanase are shown in Fig. 3. Both pools were almost completely degraded to lower molecular weights by the enzyme. This confirms that both

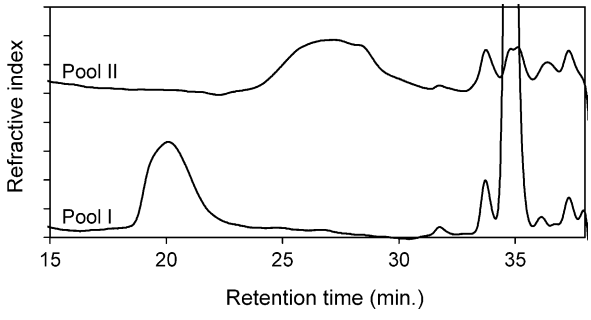

Fig. 2. Molecular weight distribution (HPSEC) of the neutral pools obtained by preparative anion-exchange chromatography (DEAE) of a hot water extract from green coffee beans.

Table 2
Properties of the neutral pools obtained by preparative anion-exchange chromatography (DEAE) of a hot water extract from green coffee beans

	Pool I	Pool II
Mannose (mol%)	73	73
Galactose (mol%)	22	8
Mannose: galactose	3.3	8.8
Degree of substitution (%)	21	5
Substituted vs. non-substituted area ratio	1.69	0.21
Acetyl (w/w%)	1.2	0.5
Ac:Man	1:10	1:23
Ac:Gal	1:3	1:3

pools consisted predominantly of galactomannans, and that other polysaccharides were hardly present.

More detailed information about the degradation products present in the digest was obtained using MALDI-TOF MS (Figs. 4 and 5).

For both pool I and II the molecular masses of the Na- and the K-adducts of the hexose dimer (Na adduct: 365, K adduct: 381), trimer (Na adduct: 527, K adduct: 543), and tetramer (Na adduct: 689, K adduct: 705) were found after degradation with an endo-mannanase. Additionally, the molecular masses of acetylated hexose dimers (Na adduct: 407), trimers (Na adduct: 569), and tetramers (Na adduct: 731) were found as well. This feature was not previously reported for galactomannans from coffee beans. However, acetylation was reported previously for galactoglucomannans from softwood (Tenkanen, Puls, Ratto and Viikari, 1993). In order to prove that the molecular masses found really belonged to acetylated hexose oligomers, both samples were saponified. As shown in Fig. 6 saponification of pool I and II lead to the disappearance of the acetylated hexose oligomers in favour of non-esterified hexose oligomers, thus confirming the presence of acetyl groups to the galactomannans. We also looked for the presence of molecular masses corresponding with oligomers both containing hexoses and pentoses, in order to find evidence for the presence of arabinose substituted to the galactomannans, as described by Navarini et al. (1999). However, we did not find these structural features. Therefore, we were not able to confirm that arabinose is a common residue of the galactomannans present in a hot water extract of green coffee beans.

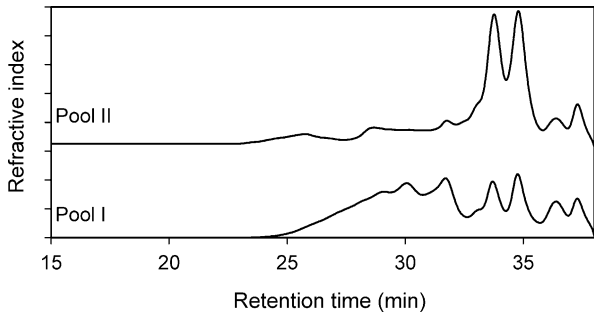

Fig. 3. Molecular weight distribution (HPSEC) of the neutral pools obtained by preparative anion-exchange chromatography (DEAE) of a hot water extract from green coffee beans after degradation by an endo-mannanase.

The presence of acetyl groups on the galactomannans from pool I and II was additionally confirmed with ^{1}H-NMR. The 1D proton NMR spectrum of both pools (not shown) clearly indicated the characteristic signal for acetyl groups at 2.20–2.15 ppm. Pool I appears to have two acetyl signals. This may indicate that there is a difference in the position of the acetyl groups in this pool: at O-2 and/or O-3 and/or O-6. However, it is unclear whether this difference also existed in the original green beans, since evidence has been found for xylo-oligosaccharides obtained from hydrothermally treated *Eucalyptus* wood that acetyl groups may migrate within sugar residues (Kabel, de Waard, Schols and Voragen, 2003). In pool II only one signal is visible. Unfortunately the resolution of the 1D proton NMR experiment was very poor. Therefore it was not possible to obtain more information through 2D NMR experiments.

3.5. Structural characteristics of the galactomannans from a hot water extract of green coffee

In order to obtain more detailed information about the structure of the galactomannans present in the neutral pools, the oligomer composition after enzymatic degradation with the endo-mannanase was determined by HPAEC. Next, the oligomer compositions obtained were compared with a standard containing mono-, di-, tri-, and tetramannose, and with the literature. Using this information an estimation was made of the distribution of the galactose residues in terms of the DS, and the substituted vs. non-substituted area ratio (Table 2), as was also described by Daas et al. (2000) for commercial galactomannans.

Figs. 7 and 8 show the oligomer composition of pools I and II after degradation with an endo-mannanase after separation on HPAEC. Degradation of pool I resulted in the release of mannobiose, mannotriose, and relatively high amounts of galactose substituted galactomannan oligomers, such as $GalMan_2$, $GalMan_3$, and Gal_2Man_5. Only a very low amount of mannose was released, which was attributed to the mechanism of the endo-mannanase used (Daas et al., 2000; McCleary, Clarke, Dea and Rees, 1985). Degradation of pool II with the endo-mannanase mainly lead to the formation of mannobiose and mannotriose, while the relative release of galactose substituted galactomannan oligomers was much lower. Thus, the substituted vs. non-substituted area ratio was much higher for pool I than for pool II, which confirms the higher DS of pool I.

3.6. Effect of acetylation of galactomannans from green coffee beans on the action of an endo-mannanase

In order to investigate the effect of the acetyl substitution of the galactomannans in pool I and II on the action of an endo-mannanase, pools I and II were degraded by

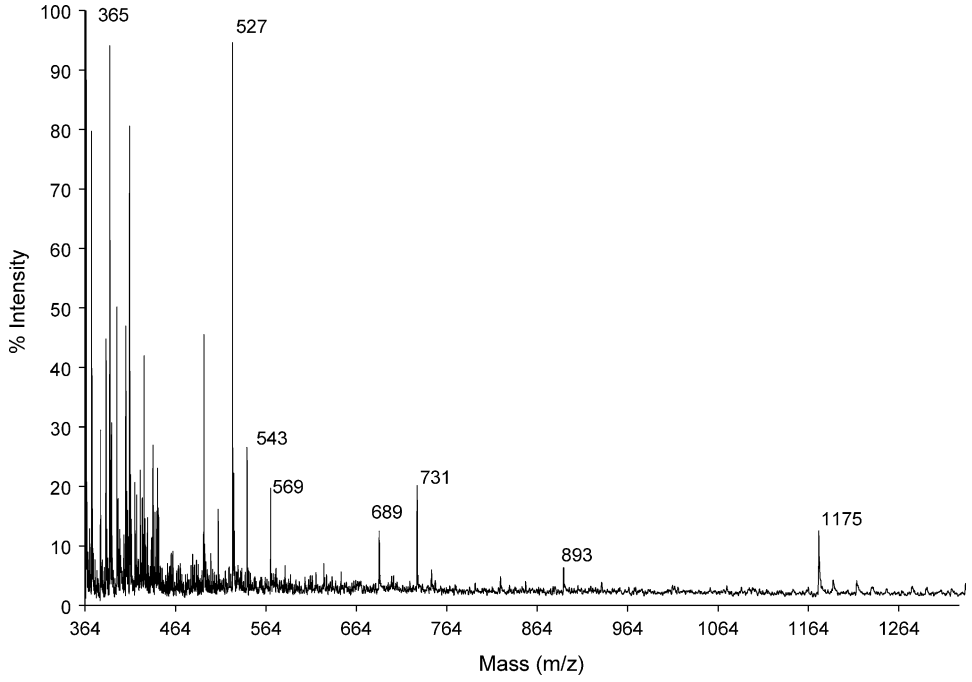

Fig. 4. MALDI-TOF mass spectra of pool I obtained by preparative anion-exchange chromatography (DEAE) of a hot water extract from green coffee beans after degradation by an endo-mannanase.

the enzyme after saponification. Afterwards, the reaction products were analyzed by HPAEC (Fig. 7) and MALDI-TOF MS (data not shown). Strikingly, after enzymatic degradation of the saponified pool I with an endo-mannanase a huge increase of the oligomer Gal_2Man_5 was observed. Additionally, the production of the $GalMan_2$ and $GalMan_3$ also increased. Also, for the saponified pool II an increase of the Gal_2Man_5 oligomer was observed after treatment with an endo-mannanase. However, the relative amount of Gal_2Man_5 produced was much lower than in pool I, thus confirming the lower DS of pool II. These results show that the degradation of the galactomannans from green coffee beans with an endo-mannanase is hindered by the presence of acetyl groups. Our results also indicate that the acetyl groups in pool I are present in clusters with galactose, which hinder the action of the endo-mannanase.

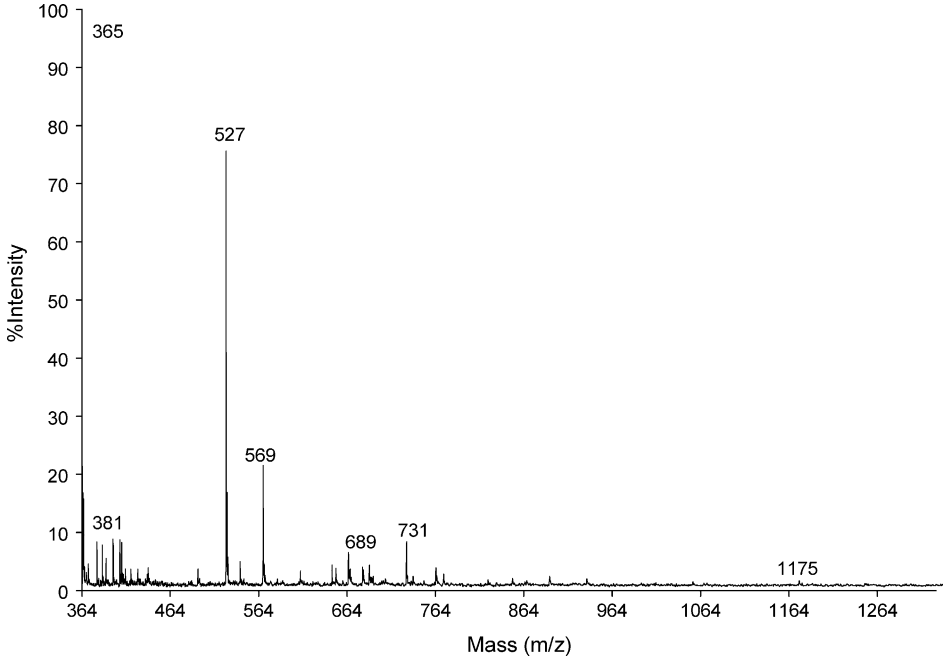

Fig. 5. MALDI-TOF mass spectra of pool II obtained by preparative anion-exchange chromatography (DEAE) of a hot water extract from green coffee beans after degradation by an endo-mannanase.

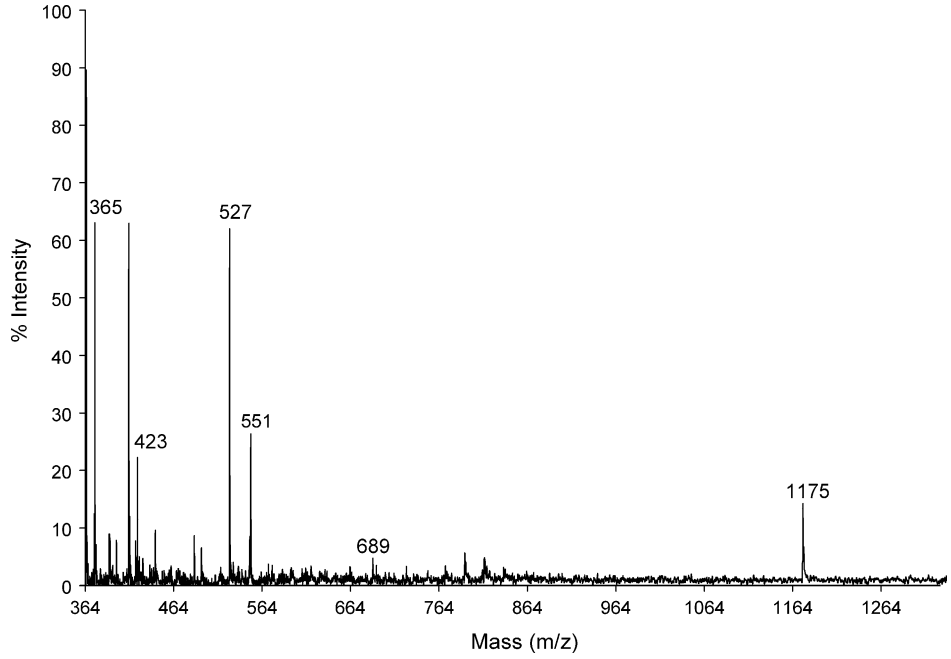

Fig. 6. MALDI-TOF mass spectra of pool I obtained by preparative anion-exchange chromatography (DEAE) of a hot water extract from green coffee beans after degradation by an endo-mannanase and subsequent saponification.

4. Concluding remarks

Galactomannans were isolated from the water extract using preparative anion-exchange chromatography. Almost all of the galactomannans eluted in two neutral populations, while almost all of the arabinogalactans eluted in the charged population, indicating that almost all arabinogalactans from green coffee beans contain charged groups. The first neutral population had a molecular weight of approximately 2×10^6 kDa based on it's HPSEC pattern. The degree of galactose substitution of the galactomannans in this population was 30%. This result was much higher than the value of 5%, which was reported in literature (Fischer et al., 1999). Additionally, 9% of the mannose residues in this population was substituted with acetyl groups, a feature not previously described for galactomannans from coffee beans.

The second population had a much lower molecular weight ($\sim2\times10^4$ kDa) than pool I. The DS of the mannan backbone with acetyl groups (4%) and galactose residues (11%) was also (much) lower than in pool I.

Our results show that at least two types of galactomannans are present in green coffee beans with totally different chemical characteristics. This diversity in structural characteristics is probably related with the function of these galactomannans in the cell wall of the coffee beans (Fischer et al., 1999). The recognition of these different structures opens the way to investigate the role of the different galactomannans in the cell wall metabolism. Also, it reasonable to assume that acetyl groups may play an

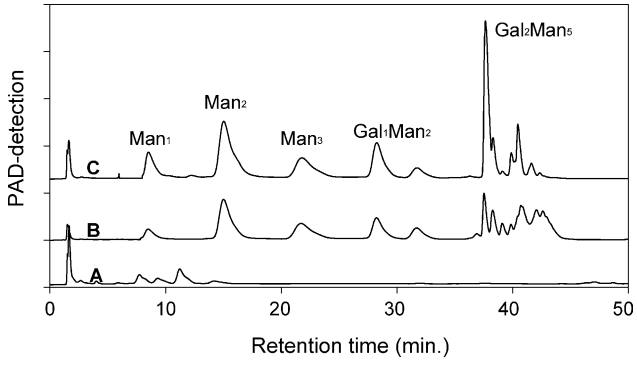

Fig. 7. High-performance anion-exchange chromatography (HPAEC) of pool I obtained by preparative anion-exchange chromatography (DEAE) of a hot water extract from green coffee beans. A: pool I; B: pool I after degradation by an endo-mannanase; C: pool I after saponification and subsequent degradation by an endo-mannanase.

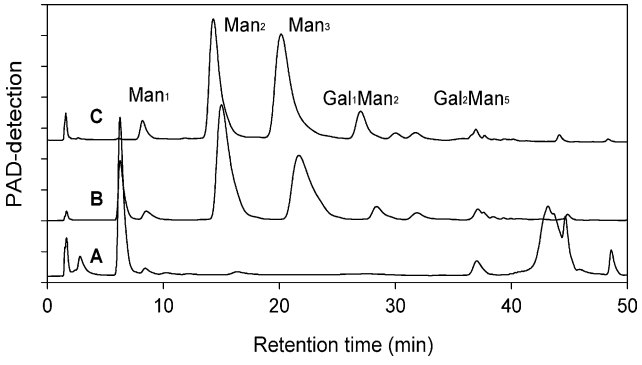

Fig. 8. High-performance anion-exchange chromatography (HPAEC) of pool II obtained by preparative anion-exchange chromatography (DEAE) of a hot water extract from green coffee beans. A: pool II; B: pool II after degradation by an endo-mannanase; C: pool II after saponification and subsequent degradation by an endo-mannanase.

important role in the reactions occurring during roasting and extraction of the coffee bean. For instance, Kabel et al. (2002) suggested that released acetic acid may stimulate hydrolysis of e.g. glycosidic linkages during hydrothermal treatment of acetylated xylan rich wood material (Kabel et al.). However, it is difficult to predict in which way the acetyl groups or their reaction products will participate in the complex reactions that occur during roasting.

Future research will be directed towards the precise distribution of acetyl groups over the mannan backbone and the precise location of substitution of mannose by acetyl groups (O-2 and/or O-3).

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