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Demonstration of the presence of acetylation and arabinose branching as structural features of locust bean gum galactomannans

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ABSTRACT

Galactomannans from locust bean gum (LBG) are polysaccharides containing single Gal*p* residues as side chains in a β-(1 → 4)-Man*p* backbone. Nevertheless, sugars other than mannose and galactose are always detected, namely, arabinose and glucose, although in small amounts. In order to demonstrate that LBG galactomannans contain other structural features than those usually reported in literature, the galactomannans from LBG obtained from two different commercial sources were submitted to a selective degradation by *Aspergillus niger endo*-β-D-mannanase and the oligosaccharides (OS) obtained were fractioned by size exclusion chromatography using a Biogel-P2 (100–1800 Da). *A. niger endo*-β-D-mannanase is able to hydrolyse the β-(1 → 4)-linked mannan backbone when the mannose residues are not hindered by the presence of substituents. This enables collection of fragments of the substituted mannan polysaccharide in the different chromatographic fractions that can be analysed by Electrospray Tandem Mass Spectrometry (ESI-MS^n). Both samples showed the presence of hexose OS (Hex$_{3-6}$), acetylated hexoses (AcHex$_{3-6}$), hexoses substituted with a pentose unit (PentHex$_{3-6}$), and OS composed by acetylated hexoses substituted with a pentose unit (PentAcHex$_{3-6}$). These structures were confirmed by GC–qMS of reduced and permethylated oligosaccharides. Sugar and glycosidic-linkage analysis allowed to assign the pentose unit to a terminally-linked arabinofuranose residue at the O-6 of a (1 → 4)-Man*p* residue. The presence of arabinose substituted OS and acetylated mannoses have never been reported to occur in LBG.

1. Introduction

The locust bean gum (LBG) is a white to yellowish powder obtained by crushing the endosperm of the seeds of the fruit pod of carob tree (*Ceratonia siliqua* L.), a large leguminous evergreen found in Mediterranean regions (Dea & Morrison, 1975). The LBG is widely used in food industry as viscosifier, stabilizer, and gelling agent due to its low cost and wide range of functional properties (Fox, 1997). It has found increasing applications in the pharmaceutical industry (Mu, Tobyn, & Staniforth, 2003; Sujja-areevath, Munday, Cox, & Khan, 1998), as well as in textiles, paper, and cosmetics, among other industries. Its mixture with other polymers, namely carrageenan, agar, or xanthan, form more elastic and stronger gels (Andrade, Azero, Luciano, & Gonçalves, 2000; Bresolin, Milas, Rinaudo, Reicher, & Ganter, 1999; Hernandez, Dolz, Dolz, Delegido, & Pellicer, 2001) allowing to structure the systems, improving the application scale and the consumer acceptability of the products. The galactomannans are the main component of LBG (80–91%), with protein accounting for 5–6%, 1–4% cellulose, and 1% of ashes (Glicksman, 1969).

Galactomannans are linear polysaccharides with a β-(1 → 4)-Man*p* backbone substituted at O-6 with single residues of α-D-Gal*p* (McCleary, Clarck, Dea, & Rees, 1985). This structure has been identified in LBG as well as in the galactomannans from different sources, such as *Aloe vera* (Gowda, Neelisidaiah, & Anjaneyalu, 1979) and the seeds of *Gleditsia ferox* Desf. (Egorov, Mestechkina, & Shcherbukhin, 2003), *Cassia grandis* (Joshi & Kapoor, 2003), coffee (Nunes, Domingues, & Coimbra, 2005; Simões, Nunes, Domingues, & Coimbra, 2010), *Cassia javahikai* (Singh, Srivastava, & Tiwari, 2009), *Cassia pleurocarpa* (Singh, Sethi, & Tiwari, 2009), *Dimorphandra gardneriana* Tul. (Cunha, Vieira, Arriaga, Paula, & Feitosa, 2009), *Adenanthera pavonina*, *Caesalpinia pulcherrima*, *Gleditsia triacanthos*, *Sophora japonica* (Cerqueira, Pinheiro, Souza, Lima, Ribeiro, & Miranda, 2009), and *Caesalpinioideae* and *Faboideae* subfamilies (Pollard, Eder, Fischer, & Windhab, 2010). All these galactomannans present side chains randomly distributed where the segments of the chain composed by unsubstituted β-D-mannopyranosyl units are interspersed with chain segments in which α-D-galactopyranosyl side chains are linked to one mannose unit of the main chain (Daas, Schols, & Jongh, 2000). They

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differ also in their degree of polymerization (number of mannose residues constituent of the main backbone) and degree of branching (ratio of mannose to galactose residues) (Robinson, Ross-Murphy, & Morris, 1982). Beyond the origin, the degree of polymerization as well as the Man/Gal ratio of galactomannans depends on the plant age, the growth conditions, and even the method of extraction of the polysaccharides (Kok, 2007).

Although the structural similarity has been verified in all plant seed galactomannans, the galactomannan of the seeds of *Retama raetam*, a wild plant belonging to the *Fabaceae* family, presents an unusual backbone structure, containing (1 → 3)-linked residues together with a small proportion of β-(1 → 4)-linked D-mannopyranosyl residues with galactopyranosyl units attached at O-6 (Ishurd, Kermagi, Zgheel, Flefla, Elmabruk, & Yalin, 2004). This shows that each species should be analysed for its own specificities and structural features.

The galactomannans of *Coffea arabica* are acetylated polysaccharides (Oosterveld, Coenen, Vermeulen, Voragen, & Schols, 2004), being the acetyl groups substituent of O-2 or O-3 of mannose residues (Nunes et al., 2005; Simões et al., 2010). Also, coffee galactomannans are composed by arabinose residues as side chains (Nunes et al., 2005; Simões et al., 2010) and contain β-(1 → 4)-Glc*p* residues interspersed in the main backbone (Nunes et al., 2005).

Mass spectrometry has been successfully used for the study of the structural details of polysaccharides, namely using soft ionization methods such as electrospray (ESI). ESI-MS gives information about the molecular weight of OS even when present in mixtures, and in combination with the sugar residues composition allows the proposal of a range of structures (Zaia, 2004). Tandem mass spectrometry (MS/MS) proved to be a valuable tool in the structural characterization of carbohydrates, allowing detailed information about their structure to be obtained (Asam & Glish, 1997; Zhou, Ogden, & Leary, 1990). This approach has the advantage over the other commonly used methods due to its feasibility even on complex and in trace amount of samples. The detailed structural features of coffee galactomannans have been disclosed using ESI-MS tools (Nunes et al., 2005; Simões et al., 2010). For the analysis of LBG samples, the utilization of mass spectrometry was performed by Davis, Hoffmann, Russell, and Debet (1995), using MALDI-TOF mass spectrometry. In this study, after enzyme hydrolysis and size-exclusion chromatography, a digalactosylmannopentaose was described. Its structural features, determined by NMR showed that the two Gal residues were contiguously linked to two Man residues in the mannan backbone.

In order to obtain more information about the detailed structural features of LBG galactomannans, in this work, these polysaccharides, obtained from two commercial sources, were submitted to a selective degradation by an *endo*-β-mannanase and selected fractions obtained by size exclusion chromatography were analysed by ESI-MS and ESI-MS/MS.

2. Experimental

2.1. LBG samples

LGB galactomannans were obtained from two different sources. LBG-FI was from a commercial sample, HG M200-INDAL-Faro, Portugal. This sample was purified by suspension in water 0.5% (w/v) at room temperature during 1 h and then by solubilisation of the residue left at 90 °C during 30 min. LBG-FI galactomannan was isolated by precipitation in ethanol 80% (12 h/4 °C) followed by filtration (Tavares, Monteiro, Moreno, & Lopes da Silva, 2005). LBG-S was a commercial sample from Sigma Aldrich, used without any purification.

2.2. Sugar analysis of polysaccharides

Neutral sugar was released from polysaccharides by treatment with 72% (w/w) H_2SO_4 (10 mg/mL) during 3 h at room temperature with occasional stirring followed by hydrolysis for 2.5 h with 1 M sulfuric acid at 100 °C. The sugars were then derivatised to their alditol acetates (Blakeney, Harris, Henry, & Stone, 1983; Harris, Henry, Blackeney, & Stone, 1984) and analysed by gas chromatography (GC–FID), as previously described by Nunes and Coimbra (2001).

2.3. Linkage analysis of polysaccharides

Polysaccharides were activated with powdered NaOH and methylated with CH_3I (Ciucanu & Kerek, 1984; Isogai, Ishizu, & Nakano, 1985) as described by Coimbra et al. (1996), followed by a remethylation to ensure complete methylation of the polysaccharides (Nunes & Coimbra, 2001). The remethylated material was hydrolyzed with 2 M trifluoroacetic acid (Harris et al., 1984), and the partially methylated sugars were reduced with $NaBD_4$ and acetylated with acetic anhydride with 1-methylimidazole as catalyst. The partially methylated alditol acetates (PMAAs) were identified by gas chromatography–mass spectrometry (Nunes & Coimbra, 2001).

2.4. Enzymatic hydrolysis and size-exclusion chromatography (Bio-Gel P2)

Samples (15 mg) were hydrolysed with 1 U of a pure *endo*-β-(1 → 4)-D-mannanase preparation (Megazyme, EC 3.2.1.78) during 48 h at 37 °C with continuous stirring in a 100 mM Na-acetate buffer, pH 5.5, containing 0.02% sodium azide. The freeze-dried material was dissolved in water, and loaded on a XK 1.6/100 column containing Bio-Gel P-2 (Bio-Rad) previously equilibrated with water, and calibrated with DP4 (stachyose), DP2 (cellobiose), and monosaccharide (glucose), using a flow of 0.3 mL/min. Fractions (1 mL) were collected and assayed for sugars with evaporative light scattering detection. Fractions containing oligosaccharides were pooled and evaporated until all the eluent was removed. No lyophilisation was performed since it has been shown that lyophilisation promotes O-acetyl migration on galactomannans (Nunes et al., 2005).

2.5. ESI mass spectrometry of oligosaccharides

The fractions obtained from the Biogel P2 column were dissolved in water and further diluted in methanol/water/formic acid (50:49.9:0.1, v/v/v). Positive ion ESI-MS and MS/MS spectra were acquired using a LXQ linear ion trap mass spectrometer (Finningan) using the following conditions: nitrogen sheath gas 30 psi, spray voltage 5 kV, heated capillary temperature 275 °C, capillary voltage 1 V, and tube lens voltage 40 V. The flow rate was set to 8 μL/min and the voltage applied was 5 kV. Nitrogen was used as nebulising and drying gas. Full scan mass spectra ranging from *m*/*z* 100 to 1500 were acquired. In the MS^2 experiments, collision energy varied between 15 and 25 of normalised collision. Data acquisition was carried out with Xcalibur data system.

2.6. GC–MS of oligosaccharides

The oligosaccharides released by the *endo*-β-(1 → 4)-D-mannanase were reduced with $NaBH_4$ (15% in NH_3 3 M) during 1 h at 30 °C. The excess of BH_4^- was destroyed by additions of several glacial acetic acid aliquots (10 μL) until no bubbles were formed. Methanol portions (3 mL) were added and the solutions were evaporated to remove the borate in the form of methyl borate esters. For methylation, the oligosaccharides were dissolved in 1 mL of dimethylsulfoxide (DMSO) and powdered NaOH (40 mg) was added. The solution was allowed to react during 30 min and then CH_3I (80 μL) was added, allowing to react for 1 h. The solution was diluted with 2 mL of distilled water and neutralized using 1 M HCl. The methylated OS were extracted with 3 mL of dichloromethane, being this phase washed by several additions of distilled water (2 mL). The organic phase was evaporated and the material was re-methylated by the same procedure to guarantee complete methylation. The reduced and permethylated OS were separated and analysed by gas chromatography–mass spectrometry (GC–MS) on an Agilent Technologies 6890N Network. The GC was equipped with a 400-1HT, dimethylpolysiloxan capillary column (25 m length, 0.22 mm of internal diameter, and 0.05 μm of film thickness). The samples were injected in splitless mode (time of splitless 2 min), with the injector operating at 220 °C, and using the following temperature program: start at 100 °C with a linear increase of 15 °C/min up 350 °C, and standing for 10 min at this temperature, The helium carrier gas had a flow rate of 0.2 mL/min and a column head pressure of 13.8 psi. The GC was connected to an Agilent 5973 mass quadrupole selective detector operating with an electron impact mode at 10 eV and scanning the range m/z 50–800 at 2 scans s^{-1}, in a full scan mode acquisition. Ion extraction chromatograms were obtained from the full-scan acquisition mode using the ion at m/z 175, which is a diagnostic fragment ion of permethylated terminally-linked arabinose residue.

Standards of mannose, β-(1 → 4)-D-mannobiose, α-(1 → 6)-D-galactosyl-β-(1 → 4)-D-mannobiose, β-(1 → 4)-D-mannotriose, α-(1 → 6)-D-galactosyl-β-(1 → 4)-D-mannotriose, β-(1 → 4)-D-mannotetraose, and α − (1 → 5)-L-arabinobiose were also reduced and permethylated and then analysed by the same procedure.

3. Results and discussion

3.1. Chemical characterization of LBG extracts

LBG-FI and LBG-S sugars composition is presented in Table 1. Mannose (77 and 73%, respectively) is the main sugar residue, followed by galactose (22 and 23%), glucose (1 and 3%), and arabinose (traces and 2%). This allows to calculate a Man/Gal ratio of 3.5 for LBG-FI and 3.2 for LBG-S. These values are in agreement with the values between 3 and 4 reported in the literature (Dakia, Blecker, Roberta, Watheleta, & Paquota, 2008; Kok, 2007; Lazaridou, Biliaderis, & Izydorczyk, 2000; Sittikijyothin, Torres, & Gonçalves, 2005).

The results of methylation analysis of the galactomannans from LBG samples are shown in Table 2. LBG-FI and LBG-S samples contained 59.2 and 54.6%, respectively, of (1 → 4)-linked mannose residues, 19.3 and 18.4% of (1 → 4,6)-linked, and 1.3 and 10% of terminally linked mannose residues. The terminally linked galactose residues were 19.3 and 16.2%. The sugars composition (Table 1) and methylation analysis of the LGB polysaccharides (Table 2) confirm the presence of galactomannans in both LBG samples. Both polysaccharides have similar ratio of total Man/(1 → 4,6)-linked Man, of 4.1 for LBG-FI and 4.0 for LBG-S, which shows an average of one branched residue in each four Man residues. The ratio of total Man/terminally-linked Man, which allows estimating the average degree of polymerisation of the galactomannans, is 63 for LBG-FI and 73 for LBG-S. In both samples, it was found that all arabinose residues were terminally-linked, presenting LBG-S a higher amount than LBG-FI, in agreement with sugars analysis.

Table 1
Sugar analysis of LBG samples.

	Sugar composition (mol%)				% Total sugars	Man/Gal
	Ara	Man	Gal	Glc		
LBG-FI	t	77	22	1	91.4	3.5
LBG-S	2	73	23	3	74.0	3.2

Table 2
Glycosidic-linkage composition of LBG samples.

Linkage	Area (%)	
	LBG-FI	LBG-S
T-Ara*f*	0.1	1.4
T-Man*p*	1.3	1.0
4-Man*p*	59.2	54.6
4,6-Man*p*	19.5	18.4
T-Gal*p*	19.3	16.2
T-Glc*p*	0.0	1.3
4-Glc*p*	0.6	7.1
Total Man*p*/T-Man*p*	63	73
Total Man*p*/4,6-Man*p*	4.1	4.0

According to Table 1, the LBG-FI sample is richer in sugars (91.4% of total sugar) than LBG-S (74.0%), confirming the purification step reported to be performed on LBG-FI. However, the different purities of the samples used do not interfere in the specific enzymatic hydrolysis with the *endo*-β-(1 → 4)-D-mannanase done. The *endo*-β-(1 → 4)-D-mannanase, cleaving the β-(1 → 4)-Man linkages in the mannan backbone, allows the resultant oligosaccharides to be obtained from the galactomannans under study. If the arabinose found in sugar analysis is a component of the galactomannans it should be present in the low molecular weight material resultant of the enzymatic hydrolysis, as reported to occur in galactomannans of coffee (Nunes et al., 2005; Simões et al., 2010). The occurrence of arabinose in galactomannan-rich extracts has been reported in a crude locust bean gum (Kok, 2007), as well as in extracts of galactomannans from other sources, such as *G. triacanthos* (Navarro, Cerezo, & Stortz, 2002) and *A. pavonina* and *C. pulcherrima* (Cerqueira et al., 2009). It is still uncertain if this is a structural feature of these polysaccharides or if this is an impurity.

3.2. Selective hydrolysis of LBG galactomannans by an *endo*-β-(1 → 4)-D-mannanase

The hydrolysis with the *endo*-β-(1 → 4)-D-mannanase allows a selective cleavage of the galactomannan backbone between adjacent β-(1 → 4)-linked mannose residues, allowing to obtain mannan oligosaccharides that contain structural details on the substituents of the mannan backbone (Dhawan & Kaur, 2007; Moreira & Filho, 2008). According to the known enzymatic mechanism of *Aspergillus niger endo*-β-mannanase, the hydrolysis of the β-(1 → 4) linked mannan backbone is hindered by the presence of substituted residues, resulting in linear and branched di- and trisaccharides (DP2 and DP3) that were separated by size-exclusion chromatography on Biogel-P2 (Fig. 1). Oligosaccharides with a higher degree of polymerisation were also present. These compounds resistant to *endo*-β-mannanase hydrolysis are expected to be made up of higher branched structures and/or by the presence of β-(1 → 4)-Glc, as reported to occur in galactomannans of other sources, such as coffee (Nunes et al., 2005). The presence of (1 → 4)-linked Glc in both samples (Table 2), especially in the purified one, suggests that LBG contains also Glc interspersed in the mannan backbone.

3.3. Characterization of LBG galactomannans by electrospray mass spectrometry

To study the structural features of LBG galactomannans, the oligosaccharides (OS) obtained after selective hydrolysis with an

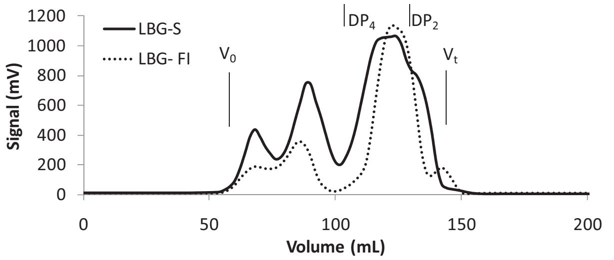

Fig. 1. Size-exclusion chromatography on Bio-Gel P2 of the OS obtained after enzymatic hydrolysis with an *endo*-β-D-mannanase of galactomannans from LBG-S and LBG-FI. V_0 – void volume; V_T – elution volume of monomers.

endo-β-mannanase treatment and further fractionated by size exclusion chromatography were analysed by mass spectrometry (MS) with electrospray ionization (ESI). The OS identified are summarized in Table 3 with indication of the *m*/*z* value of the $[M+Na]^+$ ions. “Hex” represents a hexose, as Man, Gal, and Glc cannot be distinguished because they have the same molecular weight; “Pent” represents a pentose, probably Ara, as this was the only pentose detected by sugar and methylation analysis, and “Ac” represents the acetyl groups.

By ESI-MS, based on the correspondent molecular weight, it were identified, in both samples of LBG galactomannans, $[M+Na]^+$ ions of OS composed by hexose residues (Hex_n, $n=[3–6]$), the ions present in higher relative abundance in the mass spectra (Table 3). It was also observed, although with a small relative abundance, $[M+Na]^+$ ions corresponding to OS with hexoses and a pentose residue ($PentHex_n$, $n=[3–6]$), and also hexose OS with an acetyl group ($AcHex_n$, $n=[3–6]$). Furthermore, OS with a pentose and acetyl groups ($AcPentHex_n$, $n=[3–6]$) were also found in the ESI-MS spectra of both samples. Galactomannans bearing a pentose residue and/or acetyl groups have previously been observed in coffee (Nunes et al., 2005; Simões et al., 2010).

3.4. ESI-MS/MS analysis

In order to confirm the presence of pentose residues and acetyl groups as structural features of LBG, the OS were also studied by ESI-MS/MS. With this method, it is possible to observe product ions formed by glycosidic cleavages between two sugar residues that allow confirmation of the sugar composition and also product ions formed by cross-ring cleavages that can give information about the type of linkages (cleavage of two bonds within the sugar ring) (Domon & Costello, 1988; Zaia, 2004). The analysis of the ESI-MS^n spectra allowed the validation of the structures of OS observed by ESI-MS and proposed in Table 3. To demonstrate this outcome, the product ion spectra (ESI-MS^n) obtained for the ions $[M+Na]^+$ of $AcHex_6$ (*m*/*z* 1055), $PentHex_6$ (*m*/*z* 1145), and $PentAcHex_6$ (*m*/*z* 1187) will be described in detail. Data obtained from the ESI-MS/MS spectra from the other OS identified are summarized in Table 4.

Table 3
Oligosaccharide $[M+Na]^+$ ions observed in the ESI-MS spectra of the several fractions obtained after enzymatic hydrolysis with an *endo*-β-(1 → 4)-D-mannanase of the mannans obtained from LBG-FI and LBG-S.

n	3 (*m*/*z*)	4 (*m*/*z*)	5 (*m*/*z*)	6 (*m*/*z*)
LBG (FI and S)				
Hex_n	527	689	851	1013
$AcHex_n$	569	731	893	1055
$PentHex_n$	659	821	983	1145
$PentAcHex_n$	701	863	1025	1187

3.4.1. ESI-MS^n of $AcHex_6$

Tandem mass spectra of Hex_n have previously been described in detail in other studies (Simões et al., 2010), showing successive losses of Hex_{res} (−162 Da).

The ESI-MS/MS spectrum of the ion $[AcHex_6+Na]^+$, at *m*/*z* 1055, observed for LBG samples, is shown in Fig. 2a. In this spectrum it is possible to observe the product ions formed by loss of one (−162 Da), two (−2 * 162 Da) and three (−3 * 162 Da) hexose residues with formation of sodium adducts of acetylated OS, respectively, $[AcHex_5+Na]^+$ at *m*/*z* 893, $[AcHex_4+Na]^+$ at *m*/*z* 731, and $[AcHex_3+Na]^+$ at *m*/*z* 569, confirming that this is an OS composed by hexose units. It is possible to see the product ions at *m*/*z* 833, identified as $[Hex_{5res}+Na]^+$, formed by loss of one AcHex (loss of 222 Da). This identification confirms the occurrence of acetylation in one hexose, probably at the reducing end position. The presence of one acetyl group is also noticed by other product ions observed in the MS/MS spectrum, namely their combined loss of hexose with an acetyl group plus loss of Hex_{res}, such as loss of $AcHex_2$ (−384 Da, ion at *m*/*z* at 671), loss of $AcHex_3$ (−546 Da, ion at *m*/*z* 509), and loss of $AcHex_4$ (−708 Da, ion at *m*/*z* 347) (Table 4 and Fig. 2a).

Attending to the linear ion trap mass spectrometer potentialities, which come from the ion trap capacity to perform multiple stages mass spectrometry analysis (MS^3), and in order to confirm the proposed feature of acetylated OS, the sequential fragmentation was also studied. Fig. 2b shows the ESI-MS^3 experiment carried out for the ion at *m*/*z* 893, $[AcHex_5+Na]^+$, resultant from the ion at *m*/*z* 1055, $[AcHex_6+Na^+]$. The product ions correspond to loss of one, two and three hexoses residues, with formation of, respectively, ions at *m*/*z* 731 $[AcHex_4+Na]^+$, *m*/*z* 569 $[AcHex_3+Na]^+$ and *m*/*z* 407 $[AcHex_2+Na]^+$. It is also possible to see product ions formed by loss of AcHex, at *m*/*z* 671, and combined loss of AcHex plus one and two Hex_{res} with formation of the ions at *m*/*z* 509 and 347. All the fragmentation pathways reinforce the presence of an acetylated OS.

3.4.2. ESI-MS^n of $PentHex_6$

The ESI-MS/MS spectrum of the ion $[PentHex_6+Na]^+$, at *m*/*z* 1145 (Fig. 3a) shows the product ions at *m*/*z* 1013, formed due to the loss of a $Pent_{res}$ (−132 Da). This fragmentation pathway shows the presence of a pentose residue in this OS. Loss of a neutral 132 Da

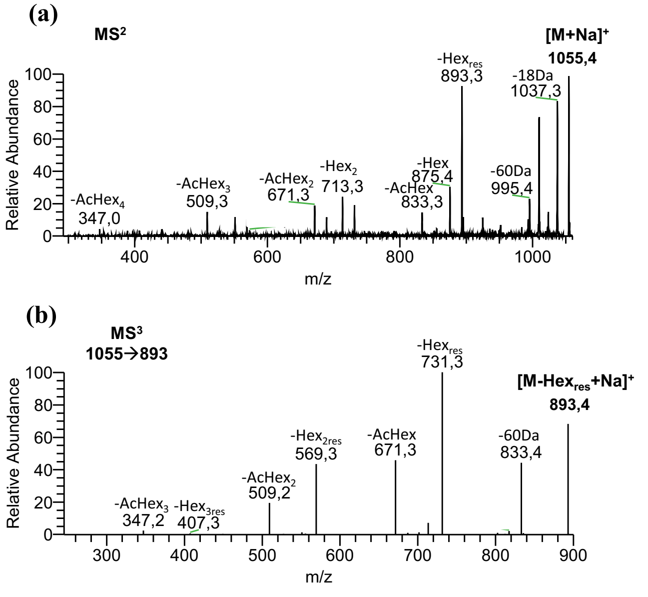

Fig. 2. ESI-MS^n spectra of the ion at *m*/*z* 1055, attributed to $[AcHex_6+Na]^+$. (a) ESI-MS/MS spectrum. (b) ESI-MS^3 spectrum of the product ion $[AcHex_5+Na]^+$ (*m*/*z* 893).

Table 4
Product ions and their corresponding neutral losses identified in the ESI-MS/MS spectra of the LBG OS of the series Pent$_m$Ac$_n$Hex$_6$ and ESI-MS^3 of the LBG OS Pent$_m$Ac$_n$Hex$_5$, $[m,n]=[0,1]$; [1,0]; [1,1] and Ac$_n$Hex$_6$, $n=0$–1, with the indication of the *m*/*z* values.

m/*z*	Fragment ions	MS^2 AcHex$_6$ 1055 *m*/*z*	MS^3 AcHex$_5$ 893 *m*/*z*	MS^2 PentHex$_6$ 1145 *m*/*z*	MS^3 PentHex$_5$ 983 *m*/*z*	MS^3 Hex$_6$ 1013 *m*/*z*	MS^2 PentAcHex$_6$ 1187 *m*/*z*	MS^3 PentAcHex$_5$ 1025 *m*/*z*	MS^3 AcHex$_6$ 1055 *m*/*z*
1055	AcHex$_6$						−Pent$_{\mathrm{res}}$		
1013	Hex$_6$			−Pent$_{\mathrm{res}}$					
1025	PentAcHex$_5$						−Hex$_{\mathrm{res}}$		
1007	PentAcHex$_{5\mathrm{res}}$						−Hex		
983	PentHex$_5$			−Hex$_{\mathrm{res}}$					
965	PentHex$_{5\mathrm{res}}$			−Hex					
893	AcHex$_5$	−Hex$_{\mathrm{res}}$							−Hex$_{\mathrm{res}}$
875	AcHex$_{5\mathrm{res}}$	−Hex							−Hex
851	Hex$_5$				−Pent$_{\mathrm{res}}$	−Hex$_{\mathrm{res}}$			
833	Hex$_{5\mathrm{res}}$	−AcHex				−Hex			−AcHex
863	PentAcHex$_4$							−Hex$_{\mathrm{res}}$	
845	PentAcHex$_{4\mathrm{res}}$							−Hex	
821	PentHex$_4$			−Hex$_{2\mathrm{res}}$	−Hex$_{\mathrm{res}}$		−AcHex$_{2\mathrm{res}}$		
803	PentHex$_{4\mathrm{res}}$			−Hex$_2$	−Hex				
731	AcHex$_4$	−Hex$_{2\mathrm{res}}$	−Hex$_{\mathrm{res}}$						
713	AcHex$_{4\mathrm{res}}$	−Hex$_2$	−Hex					−PentHex$_{\mathrm{res}}$	
689	Hex$_4$	−AcHex$_{2\mathrm{res}}$		−PentHex$_{2\mathrm{res}}$	−PentHex$_{\mathrm{res}}$	−Hex$_{2\mathrm{res}}$	−PentAcHex$_{2\mathrm{res}}$		−AcHex$_{2\mathrm{res}}$
671	Hex$_{4\mathrm{res}}$	−AcHex$_2$	−AcHex	−PentHex$_2$	−PentHex	−Hex$_2$			−AcHex$_2$
683	PentAcHex$_3$							−Hex$_{2\mathrm{res}}$	
659	PentHex$_3$			−Hex$_{3\mathrm{res}}$	−Hex$_{2\mathrm{res}}$				
641	PentHex$_{3\mathrm{res}}$			−Hex$_3$	−Hex$_2$				
569	AcHex$_3$	−Hex$_{3\mathrm{res}}$	−Hex$_{2\mathrm{res}}$						
551	AcHex$_{3\mathrm{res}}$	−Hex$_3$	−Hex$_3$						
527	Hex$_3$	−AcHex$_{3\mathrm{res}}$			−PentHex$_{2\mathrm{res}}$	−Hex$_{3\mathrm{res}}$			
509	Hex$_{3\mathrm{res}}$	−AcHex$_3$	−AcHex$_2$	−PentHex$_3$	−PentHex$_2$	−Hex$_3$			
407	AcHex$_2$		−Hex$_{3\mathrm{res}}$						
389	AcHex$_{2\mathrm{res}}$		−Hex$_3$						
347	Hex$_{2\mathrm{res}}$		−AcHex$_3$	−PentHex$_4$	−PentHex$_3$	−Hex$_4$			

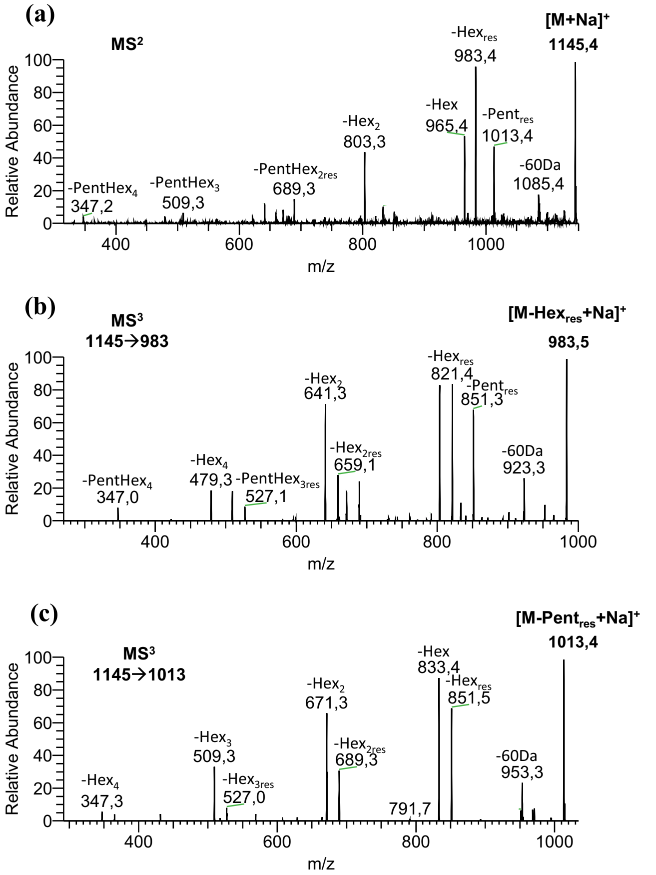

Fig. 3. ESI-MS^n spectra the ion at *m*/*z* 1145, attributed to [PentHex$_6$+Na]$^+$. (a) ESI-MS/MS spectrum. (b) ESI-MS^3 spectrum of the product ion [PentHex$_5$+Na]$^+$ (*m*/*z* 983). (c) ESI-MS^3 spectrum of the product ion [Hex$_6$+Na]$^+$ (*m*/*z* 1013).

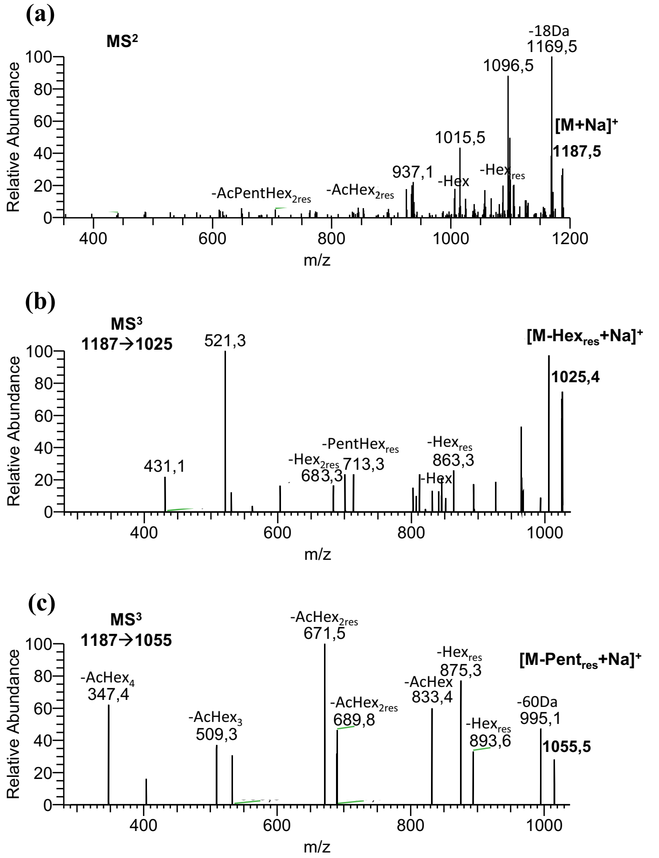

Fig. 4. ESI-MS^n spectra the ion at *m*/*z* 1187, attributed to [PentAcHex$_6$+Na]$^+$. (a) ESI-MS/MS spectrum. (b) ESI-MS^3 spectrum of the product ion [PentAcHex$_5$+Na]$^+$ (*m*/*z* 1025). (c) ESI-MS^3 spectrum of the product ion [Hex$_6$+Na]$^+$ (*m*/*z* 1055).

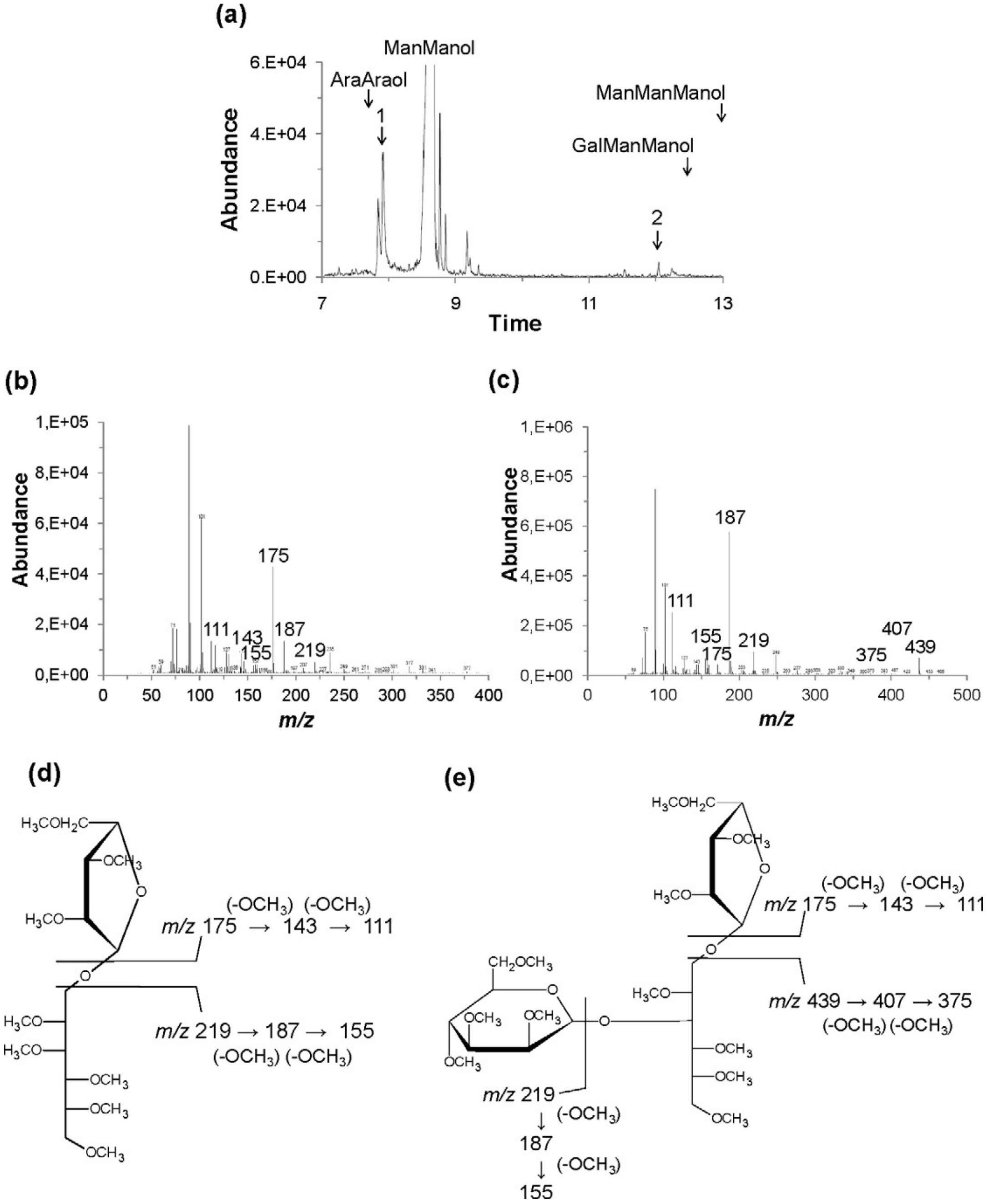

Fig. 5. GC–MS assays of reduced and permethylated oligosaccharides formed by mannanase hydrolysis. (a) Ion extracted chromatogram using the ion at *m/z* 175, diagnostic for permethylated terminally-linked pentose residues, with indication of the elution time of permethylated standards of α-(1 → 5)-L-arabinosyl-L-arabinitol (AraAraol), β-(1 → 4)-D-mannosyl-D-mannitol (ManManol), β-(1 → 4)-D-mannosyl-[α-(1 → 6)-galactosyl]-D-mannitol (GalManManol), and β-(1 → 4)-D-di-mannosyl-D-mannitol (Man_2Manol). (b) EI-mass spectrum of peak 1. (c) EI-mass spectrum of peak 2. (d) Fragmentation pattern and proposed structure for peak 1, permethylated AraManol (*m/z* 426). (e) Fragmentation pattern and proposed structure for peak 2, permethylated AraManManol (*m/z* 630).

fragment, attributed to the loss of a pentose residue, was already observed in the study of pentosyl galactomannans from coffee infusion and coffee residue (Nunes et al., 2005; Simões et al., 2010). Others ions, such as the ion at *m/z* 689 due to loss of $PentHex_2$ (−474 Da), the ion at *m/z* 509 due to loss of $PentHex_3$ (−636 Da), and the ion at *m/z* 347 due to loss of $PentHex_4$ (−798 Da) are also observed in the MS/MS spectrum. All these product ions confirmed the presence of one structure with a pentose residue. The losses of one, two and three hexose residues were also observed, corresponding to formation of the product ions $[PentHex_5+Na]^+$ at *m/z* 983, $[PentHex_4+Na]^+$ at *m/z* 821, and $[PentHex_3+Na]^+$ at *m/z* 659 (Table 4), as well as loss of one to three Hex, leading to the ions $[PentHex_{5res}+Na]^+$ at *m/z* 965, $[PentHex_{4res}+Na]^+$ at *m/z* 803 and $[PentHex_{3res}+Na]^+$ at *m/z* 641, all confirming the proposed structure.

The ESI-MS^3 spectrum of the ion $[PentHex_5+Na]^+$, at *m/z* 983 (Fig. 3b), shows the product ions corresponding to losses of Hex residues: $[PentHex_4+Na^+]$ at *m/z* 821, $[PentHex_{4res}+Na^+]$ at *m/z* 803, $[PentHex_3+Na^+]$ at *m/z* 659, and $[PentHex_{3res}+Na^+]$ at *m/z* 641. The loss of a pentosyl group (−132 Da) was also observed with formation of ion $[Hex_5+Na^+]$ at *m/z* 851, as well as the loss of a pentosyl group combined with loss of hexose: loss of $Pent_{res}$Hex (−294 Da) with formation of ion $[PentHex_4+Na^+]$ at *m/z* 689, loss of PentHex (−312 Da) with formation of ion $[Hex_{4res}+Na^+]$ at *m/z* 671, loss of

PentHex$_{2res}$ (−456 Da) with formation of ion [Hex$_3$+Na$^+$] at m/z 527, and loss of PentHex$_2$ (−474 Da) with formation of ion [Hex$_{3res}$+Na$^+$] at m/z 509 (Table 4). These product ions indicate the presence of a pentose residue group as ramification in the LBG galactomannans structure.

The ESI-MS^3 spectrum of the ion [Hex$_6$+Na]$^+$ at m/z 1013 (Fig. 3c) confirms the fragmentation of a mannan OS.

3.4.3. ESI-MS^n of PentAcHex$_6$

The ESI-MS/MS spectrum of the ion at m/z 1187, attributed to [PentAcHex$_6$+Na]$^+$, is shown in Fig. 4a. Although the low abundance of these ions, the formation of the product ion [AcHex$_6$+Na]$^+$ at m/z 1055 due to loss of Pent$_{res}$ was observed, confirming the presence of a pentosyl group. The formation of the product ion [PentHex$_4$+Na]$^+$ at m/z 821, due to loss of AcHex$_2$, was also identified, showing that the acetyl group was present in the galactomannans structure of LBG and it was linked to the hexose residue and not linked to the pentose residue. The product ion formed by loss of AcPentHex$_{2res}$ (m/z 689) and the product ions formed by loss of Hex$_{res}$ and Hex (m/z 1025 and 1007, respectively) were also observed (Table 4). The presence of the acetyl groups linked to the galactomannan backbone was also confirmed by the analysis of the ESI-MS^3 spectrum of the ion [(PentAcHex$_6$−Hex$_{res}$+Na)]$^+$ at m/z 1025 (Fig. 3b) and the ESI-MS^3 spectrum of the ion [(PentAcHex$_6$−Pent$_{res}$+Na)]$^+$ at m/z 1055 (Fig. 3c and Table 4). The presence of the product ions [Hex$_{5res}$+Na]$^+$ at m/z 833, [Hex$_4$+Na]$^+$ at m/z 689, and [Hex$_{4res}$+Na]$^+$ at m/z 671, formed due to loss of AcHex, AcHex$_{2res}$, and AcHex$_2$, respectively, reinforced the presence of acetyl groups.

3.5. Identification of reduced permethylated arabinose containing oligosaccharides

In order to confirm the presence of arabinose residues as side chains of LBG galactomannans by an alternative independent method, GC–MS analysis of reduced and permethylated oligosaccharides formed by *endo*-β-mannanase hydrolysis were performed. The presence of a permethylated terminally-linked arabinose residue can be identified by the presence in the EI-MS spectra of a fragment ion at m/z 175. The extracted m/z 175 ion chromatogram is shown in Fig. 5a. The main abundant peak, with a Rt of 8.5 min showed a fragmentation pattern and Rt compatible with a permethylated ManManol. The EI-MS spectrum of this compound shows that the ion at m/z 175 occurs in small amount. However, because this is the most abundant compound, even using the extraction ion mode it shows the highest peak. Nevertheless, the full EI mass spectrum of peak 1 (Fig. 5b), with a Rt of 7.7 min, allows to observe a fragmentation pattern compatible with a permethylated AraManol (Fig. 5d). Also, the full EI mass spectrum of peak 2 (Fig. 5c), with a Rt of 12.0 min, allows to observe a fragmentation pattern compatible with a permethylated AraManManol (Fig. 5e). The fragmentations and retention times of these compounds confirm that arabinose is linked to the mannan backbone of LBG.

4. Concluding remarks

The detection of arabinose residues linked to the oligosaccharides formed by the specific hydrolysis of the mannan backbone of two LBG by an *endo*-β-mannanase, using independent methods, namely ESI-MS/MS and GC–qMS, clearly shows that LBG galactomannan contains arabinose residues as side chains, linked at O-6 to the mannan backbone. ESI-MS/MS analysis also shows that LBG galactomannan contains acetyl groups linked to the mannan backbone. These structural features are similar to those previously reported to occur in coffee products resultant from coffee beans (Nunes et al., 2005; Simões et al., 2010). This is the first report demonstrating these structural features on galactomannan isolated from LBG.

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