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Carbohydrate Polymers 53 (2003) 101–107

Carbohydrate Polymers

www.elsevier.com/locate/carbpol

Structural characterisation by MALDI-MS of olive xylo-oligosaccharides obtained by partial acid hydrolysis

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Received 11 October 2002; revised 17 December 2002; accepted 18 December 2002

Abstract

Alkali extractable olive pulp and olive seed hull glucuronoxylans were submitted to mild acid hydrolysis originating low molecular weight xylo-oligosaccharides. The xylo-oligosaccharides thus formed were partially separated by size exclusion chromatography (Biogel P6) and several elution fractions were identified by MALDI-MS. The MALDI mass spectra obtained revealed the presence of several xylo-oligosaccharides ions in each fraction between the m/z ranges 500–2500. Based on the sugar and methylation analyses of the glucuronoxylans, the proposed structures are the neutral xylo-oligosaccharides (Xyl$_n$, $n = 4$–18) and acidic xylo-oligosaccharides substituted by glucuronic acid residues (Xyl$_n$GlcA, $n = 3$–16 and Xyl$_n$GlcA$_2$, $n = 2$–14), by 4-*O*-methyl-glucuronic acid residues (Xyl$_n$MeGlcA, $n = 3$–14 and Xyl$_2$MeGlcA$_2$) and by both acid residues (Xyl$_n$GlcAMeGlcA, $n = 4$–7). The identification of ions corresponding to long blocks of neutral and acidic oligosaccharides suggested that the acidic residues were irregularly distributed along the xylan backbone in both olive pulp and olive seed hull. In addition, MALDI mass spectra showed that the acidic xylo-oligosaccharides from the pulp were predominantly constituted by Xyl$_n$MeGlcA oligosaccharides, while the acidic xylo-oligosaccharides from the seed hull were predominantly constituted by Xyl$_n$GlcA and Xyl$_n$GlcA$_2$ oligosaccharides.

Keywords: Glucuronoxylan; MALDI-MS; Xylo-oligosaccharides; Olive fruit; Partial acid hydrolysis

1. Introduction

Glucuronoxylans (GX) are one of the major polysaccharides present in plant cell walls (Selvendran, 1985). These polysaccharides are composed of β-(1 → 4)-linked xylopyranose (Xyl) residues forming linear chains. The xylan chains are frequently substituted by terminally linked glucuronic acid (GlcA) residue or by its 4-*O*-methyl derivative (MeGlcA); arabinofuranosyl (Ara) residues and/or acetyl groups are also found as substituents (Shimizu, 1991). The type of substituting residues and their degree and pattern of substitution along the xylan backbone are structural features that determine their properties. The extraction procedure with alkali solutions readily removes the acetyl groups. On the other hand, upon acid treatment, the disaccharide residues of the type Uronic acid-Neutral sugar (aldobiouronic acid residues) remain intact while Ara residues are removed (Fry, 1988). The higher acid lability of the (1 → 4)-linked β-D-Xyl*p* glycosidic linkages than the Glc*p*A-(1 → 2)-β-D-Xyl*p* allows to obtain, by controlling the extent of acid hydrolysis, neutral and acidic xylo-oligosaccharides, from which the pattern of the substitution GlcA residues in glucuronoxylans can be estimated.

The approach commonly reported to determine the distribution pattern in xylans involves acidic degradation of the polysaccharide and have been conducted on wood GX (hardwood and softwood). The various studies allowed the identification of neutral oligosaccharides composed of unbranched xylosyl residues of varied chain length (DP < 18) (Havlicek & Samuelson, 1972) along with acidic oligosaccharides substituted by one and two MeGlcA residues (Jacobs, Larsson, & Dahlman, 2001). Other authors have even identified an acidic oligosaccharide bearing MeGlcA residues in vicinal positions (Shimizu, Hashi, & Sakurai, 1978). The results obtained so far gave evidence for a regular distribution in softwood GX while the hardwood GX appear to contain an irregular distribution of acidic residues (Jacobs et al., 2001). Another approach

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0144-8617/03/$ - see front matter
doi:10.1016/S0144-8617(03)00007-9

used for the estimation of the pattern of substitution in GX, used on beech sawdust (Kohn, Hromádková, & Ebringerová, 1986), *Rudbeckia fulgida*, a medicinal plant (Kardosová, Matulová, & Malovíková, 1998) and shrub (*Mahonia aquifolium*) (Kardosová, Malovíková, Pätoprstý, Nosál'ová, & Matáková, 2002) is based on the evaluation of the linear charge density of GX by determination of the activity coefficients of added calcium counter-ions that establish electrostatic bonds to the carboxyl groups. In view of the results obtained, depending of the sample, these authors suggested the presence of a random (Kohn et al., 1986; Kardosová et al., 2002) or regular distribution (Kardosová et al., 1998) of acidic residues along the xylan backbone. The distribution pattern of acidic residues in fruit xylans, and particularly in olive fruit is, to our knowledge, not yet available.

In the last decade, matrix-assisted laser desorption/ionisation (MALDI) mass spectrometry has become widely applied to the analysis of several classes of oligosaccharides, both from polysaccharides (Körner, Limberg, Mikkelsen, & Roepstorff, 1998; Garozzo, Spina, Cozzolino, Cescutti, & Fett, 2000; Mele & Malpezzi, 2000, and from glycoproteins (Finke, Stahl, Pfenninger, Karas, Daniel, & Sawatzki, 1999; Harvey, 1993; Harvey, Bateman, & Green, 1997). This technique has revealed to be a valuable tool for the structural characterisation of carbohydrates mainly due to salt tolerance, low fragmentation and wide mass range. These advantages have allowed determining the molecular weight and mass distribution of complex mixtures as is often encountered in poly- and oligosaccharide fractions (Finke et al., 1999; Huisman, Schols, & Voragen, 2000; Jacobs, Lundqvist, Stalbrand, Tjerneld, & Dahlman, 2002; Kabel et al., 2002).

The use of MALDI-MS, Electrospray Mass Spectrometry (ESI-MS) and Electrospray Tandem Mass Spectrometry (ESI-MS/MS) for the structural characterisation of low molecular weight xylo-oligosaccharides (400–1000 Da) obtained by partial acid hydrolysis from olive pulp glucuronoxylans allowed to observe the presence of neutral (Xyl_n, $n = 4-8$) and acidic xylo-oligosaccharides substituted with GlcA ($n = 3-5$) and 4-*O*-Me-GlcA ($n = 3-4$) residues (Reis, Coimbra, Domingues, Ferrer-Correia, & Domingues, 2002). Based on these results, this work will extend the knowledge about the structure of higher molecular weight xylo-oligosaccharides obtained by partial acid hydrolysis from the olive pulp and olive seed hull glucuronoxylans.

2. Experimental

2.1. Sample characterisation

Olive pulp glucuronoxylan (K6) sample, with 78 mol% of xylose, 11 mol% of glucuronic acid, 4 mol% of glucose, and 3 mol% of arabinose, in a 82% total sugars, was recovered from the precipitate (5.2% of the CWM) obtained upon neutralisation and dialysis of the 1 M KOH extraction solution from cell wall material (CWM), as described by Coimbra, Waldron, and Selvendran (1994).

Olive seed hull glucuronoxylan (Hp 25) sample, with 88 mol% of xylose, and 10 mol% of glucuronic acid, in a 75% total sugars, was recovered from the precipitate (13.6% of the CWM) obtained upon neutralisation and dialysis of the 0.5 M KOH extraction solution from CWM, as described by Coimbra, Waldron, and Selvendran (1995).

2.2. Size exclusion chromatography of the xylo-oligosaccharides

Xylo-oligosaccharides of both GX samples were obtained by mild acid hydrolysis as described by Coimbra et al. (1995). The supernatant hydrolysate containing the xylo-oligosaccharides was dried under a nitrogen stream and fractionated by size-exclusion chromatography on a Biogel P6 (6000–800 Da; BioRad) gel media column using ammonium formate (100 mM, pH 3.6) as the eluent. The elution profile was monitored using an evaporative light scattering (ELSD) and A_{280} detection modes. The xylo-oligosaccharides separated were analysed by MALDI-MS.

2.3. MALDI analysis

Sample preparation for MALDI analysis was performed by mixing 5 μl of oligosaccharides mixture dissolved in water to 20 μl of 2,5-dihydroxybenzoic acid (DHB) dissolved in a solvent mixture composed by acetonitrile:aqueous TFA (0.1%, v/v) (70:30, v/v). From this mixture, 2 μl were deposited on top of a layer of crystals of 2-cloromercaptobenzothiazole (CMBT) formed by deposition of 1 μl of CMBT solution in tetrahydrofuran:ethanol:water (1:1:1, v/v/v) on the MALDI plate (Pfenninger, Karas, Finke, Stahl & Sawatski, 1999). Positive ion MALDI mass spectra were acquired with MALDI-TOF instrument M@LDI (Micromass, Manchester, UK) equipped with a nitrogen laser emitting at 337 nm operating in linear mode. Each mass spectrum was the accumulating data of approximately 30–50 laser shots. External 3-points calibration was used for mass assignment.

3. Results and discussion

3.1. Size-exclusion chromatography

The ELSD and A_{280} chromatographic profile resultant of the elution of K6 and Hp 25 xylo-oligosaccharides in a Biogel P6 stationary media is shown in Fig. 1. The ELSD detection mode of both samples showed that the elution of the xylo-oligosaccharides occurred predominantly within the exclusion limits of the gel media, since only small peaks were noticed at the void (V_0) and total volumes (V_t).

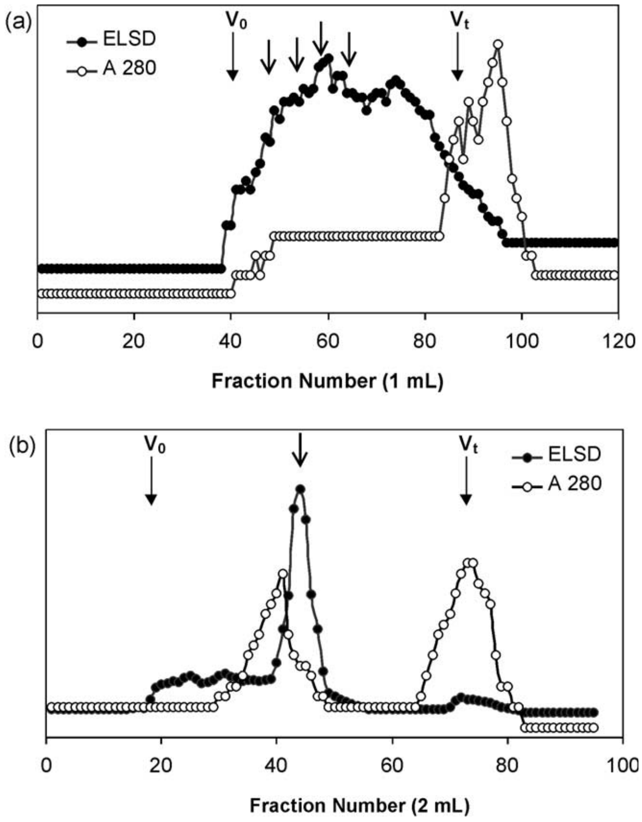

Fig. 1. Size exclusion chromatography elution profile of the xylo-oligosaccharides obtained by mild acid hydrolysis, of a) olive pulp glucuronoxylan and b) olive seed hull glucuronoxylan. (Fractions marked with arrows were collected for further MALDI-MS analyses).

The chromatogram obtained by ELSD detection for the olive pulp (K6) xylo-oligosaccharides (Fig. 1a) showed poor separation, possibly resultant of the high heterogeneity of the samples after acid hydrolysis. The A_{280} signal observed in the chromatogram confirms the presence of lignin-like material in the initial GX sample. This lignin like material is usually found in the olive pulp hemicellulosic extracts (Coimbra et al., 1995; Mafra et al., 2001; Vierhuis, Schols, Beldman, & Voragen, 2001). These compounds eluted in two different peaks (fractions 58 and 75) with elution volumes not coincident with ELSD signal.

The chromatogram obtained by ELSD detection for the seed hull (Hp 25) xylo-oligosaccharides (Fig. 1b) showed the elution of the depolymerised GX in one major and well defined peak showing a maximum of elution for fraction 44. The A_{280} detection gave two peaks, but their maximum was not coincident with the ELSD signal.

In order to determine the structural features in the two xylo-oligosaccharide samples, selected fractions were analysed by MALDI-MS. From olive pulp, considering the low separation obtained, fractions 50, 55, 60, and 65 (arrows in Fig. 1a), all with low A_{280} absorbance, were selected. From olive seed hull, fraction 44 which showed the maximum ELSD signal and a low A_{280} absorbance (arrow in Fig. 1b) was selected.

3.2. Analysis of olive pulp xylo-oligosaccharides by MALDI mass spectrometry

The MALDI mass spectra obtained for the elution fractions 50, 55, 60 and 65 obtained from the chromatogram of K6 sample (Fig. 2) showed the presence of a large number of peaks, distributed in different mass ranges, where the number of peaks decreased with increasing elution volume, from *m/z* 500–1250 for fraction 65 to *m/z* 1250–2250 for fraction 50.

The MALDI spectrum obtained for the low molecular weight fraction (fraction 65) is shown in Fig. 2a. The xylo-oligosaccharide ions present in the mass spectra were identified as sodium adducts, $[M+Na]^+$, corresponding to the following series of oligosaccharides: Xyl_{4-8} (◆), $Xyl_{3-5}GlcA$ (✦), $Xyl_{2-4}GlcA_2$ (✧), and $Xyl_{3-5}MeGlcA$ (■), as described by Reis et al. (2002). The identified xylo-oligosaccharides are summarised in Table 1. The structures of the xylo-oligosaccharides here proposed and identified in the MALDI mass spectrum were confirmed by tandem mass spectrometry using Electrospray Ionisation (Reis et al., 2002). Previous analyses of olive pulp GX by ^{13}C NMR (Coimbra et al., 1994), 4-*O*-methylglucuronidase treatment (Vierhuis, Schols, Beldman, & Voragen, 2000), and MALDI-TOF-MS (Vierhuis et al., 2001) showed the occurrence of MeGlcA and GlcA as substituting residues.

The MALDI spectrum of fraction 60 (Fig. 2b) showed the presence of oligosaccharides identified as $[M+Na]^+$, in the mass range between *m/z* 500 and 1500. The more abundant ions at *m/z* 569, 701, 833, 965, 1097, 1229, 1361, and 1493, showed a mass difference of 132 Da. This mass difference can be assigned to a pentose residue, which allowed attributing the observed ions to neutral xylo-oligosaccharides composed of 4–11 Xyl residues. These ions are identified in the mass spectrum as X_n (◆), where *n* represents the number of Xyl residues. Predominant ions were observed for Xyl_4, at *m/z* 569, and for Xyl_5, at *m/z* 701. The ions at *m/z* 627, 759, 891, 1023, and 1155, with a 58 Da increase relative to Xyl_n, constitute the second most abundant series in the higher mass range of the spectrum. It can be attributed to oligosaccharides substituted with one MeGlcA residue ($Xyl_{3-7}MeGlcA$), identified in the mass spectrum as X_nM (■). Adjacent to the X_nM oligosaccharides were observed ions at *m/z* 649, 781, 914, 1046, and 1179, corresponding to 22 Da increase to X_nM, that can be attributed to the double sodiated ion $[M\text{-}H+2Na]^+$ of $Xyl_{3-7}MeGlcA$ (⇩). These ions resulted from the substitution of one hydrogen atom by a second sodium atom. These species were only observed for xylo-oligosaccharides bearing a MeGlcA residue, which is in accordance with the results obtained for xylo-oligosaccharides from *Eucalyptus* wood (Schols et al., 2000). The third most abundant series of ions, at *m/z* 613, 745, 877, 1009, 1141, 1273, and 1405,

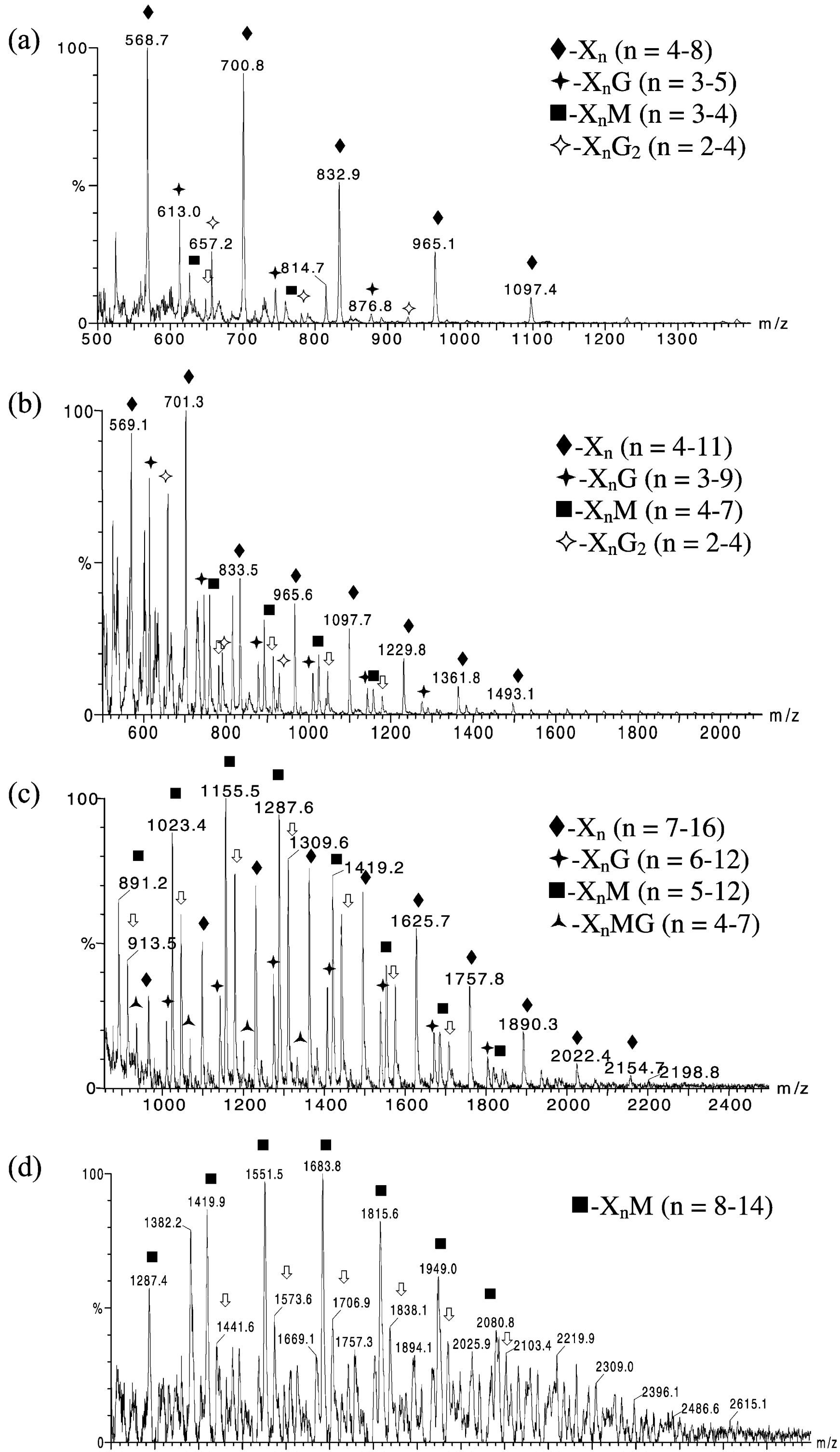

Fig. 2. Comparison of MALDI mass spectra of xylo-oligosaccharides present in fractions a) F-65, b) F-60, c) F-55 and d) F-50 collected from olive pulp size exclusion chromatography.

presenting a 44 Da increase relative to Xyl$_n$, can be attributed to xylo-oligosaccharides substituted with one GlcA residue, corresponding to the structures Xyl$_{3-9}$GlcA. These oligosaccharides are identified in the mass spectrum as X_nG (✦). The ions at *m/z* 657, 789, and 921 presenting an 88 Da increase relative to Xyl$_n$, can be attributed to xylo-oligosaccharides substituted with two GlcA residues, corresponding to the structures Xyl$_{2-4}$GlcA$_2$. These

Table 1
Number of Xyl residues of xylo-oligosaccharides identified by MALDI after partial acid hydrolysis of olive pulp and seed hull glucuronoxylans

Oligosaccharide	Pulp				Seed hull
Fraction number	F-65	F-60	F-55	F-50	F-44
Xyl_n	4–8	4–11	7–16		4–18
Xyl_nGlcA	3–5	3–9	5–14		3–16
$Xyl_nMeGlcA$	3–5	3–7	5–12	8–14	
Xyl_nGlcA_2	2–4	2–4			2–14
$Xyl_nGlcAMeGlcA$			4–8		6
$Xyl_nMeGlcA_2$					2

oligosaccharides are identified in the mass spectrum as X_nG_2 (✧). The ions corresponding to the structure $Xyl_{2-4}GlcA_2$ could also be attributed to the structural isomer containing one 4-*O*-methylglucuronic acid and an additional hexose residue (2-*O*-α-galactopyranosyl-4-*O*-methyl-α-D-glucuronic acid), as was described for GX of *Eucalyptus globulus labill* wood (Shalatov, Evtuguin, & Neto, 1999). However, the absence of Gal in the sample (Coimbra et al., 1994) excludes this possibility. Xylo-oligosaccharides containing acetyl groups were not identified since the initial glucuronoxylan sample was extracted with alkali solutions.

The MALDI spectrum of fraction 55 (Fig. 2c) showed the presence of oligosaccharides identified as $[M + Na]^+$ in the mass range between *m/z* 850 and 2200. The most abundant ions were the xylo-oligosaccharides substituted with one 4-*O*-methylglucuronic acid residue ($Xyl_{5-12}MeGlcA$), at *m/z* 891, 1023, 1155, 1287, 1419, 1551, 1683 and 1816, respectively. The ions of neutral oligosaccharides, at *m/z* 965, 1097, 1230, 1361, 1493, 1625, 1757, 1890, 2023 and 2155, corresponding to Xyl_{7-16}, were only predominant at higher *m/z* values. This can be due to the easy ionisation in positive mode of X_n than X_nM. Present in low relative abundance were identified the ions corresponding to $Xyl_{5-14}GlcA$, at *m/z* 877, 1009, 1141, 1273, 1405, 1538, 1670, 1802, 1934, and 2066. The ions observed at *m/z* 935, 1067, 1199, 1331 and 1463, also with lower relative abundance, showed a series stepped by 132 Da with a 58 mass units higher than the ions of the X_nG series. These ions can be attributed to xylo-oligosaccharides with the structure $Xyl_{4-8}GlcAMeGlcA$. The presence of both GlcA and MeGlcA as substituting residues in the same xylo-oligosaccharides was previously reported in lignified tissues of cotton cake (Matsuo, Yoshida, Kusakabe, & Murakami, 1991), and in maize kernel cell walls (Huisman et al., 2000).

The MALDI mass spectrum obtained for fraction 50 (Fig. 2d) showed the higher molecular weight xylo-oligosaccharides. The predominant series of ions occur at *m/z* 1287, 1420, 1552, 1684, 1816, 1948 and 2081, corresponding to $Xyl_{8-14}MeGlcA$ oligosaccharides. These ions showed the maximum abundance for ion at *m/z* 1684 ($Xyl_{11}MeGlcA$). Adjacent to these ions were noted other series of ions that showed a 22 Da increase, correspondent to the $[M\text{-}H + 2Na]^+$ ions. Other ions observed in the mass spectrum displayed very low S/N ratio, which made *m/z* assignment difficult to any of the structures already proposed.

3.3. Analysis of olive seed hull xylo-oligosaccharides by MALDI mass spectrometry

The MALDI mass spectrum obtained for fraction 44 of olive seed hull GX is shown in Fig. 3a. The spectrum showed the presence of a high number of xylo-oligosaccharides in the *m/z* range of 500–2500. The ions observed in the low mass region (*m/z* 500–1000) showed higher relative abundance than those observed in the high mass region (*m/z* 1000–2500). This behaviour can be attributed to mass discrimination effects well known to occur during MALDI analysis of polydisperse samples (Byrd & McEwen, 2000; Dai, Whittal, & Li, 1996).

The predominant ions observed in the low *m/z* range, at *m/z* 569, 701, 834, and 966, corresponded to neutral xylo-oligosaccharides (Xyl_{4-7}). Present in minor relative abundance were observed $Xyl_{3-5}GlcA$, at *m/z* 613, 745, and 877, and $Xyl_{2-4}GlcA_2$, at *m/z* 657, 789, and 921. The absence of Gal residues in Hp25 fraction (Coimbra et al., 1995), allows excluding the contribution of the oligosaccharide isomers with the structure $Xyl_{2-3}MeGlcAHex$. The ion at m/z 685 (●) can be attributed to $Xyl_2MeGlcA_2$. The occurrence of contiguous MeGlcA residues has been reported to occur in larch and spruce GX (Jacobs et al., 2001; Shimizu et al., 1978). The structures of the ions at *m/z* 538 and 815 were not possible to be assigned, however, the identification of the ion at *m/z* 657, can be attributed to Xyl_2GlcA_2, giving evidence for the occurrence of contiguous GlcA residues which, to our knowledge, has not yet been reported to occur.

In the higher *m/z* range of the spectrum, between 1000 and 2500, several ions were observed with a mass difference of 44 Da between them (Fig. 3b). Three series of xylo-oligosaccharide ions, with differences of 132 Da, can be identified: Xyl_{8-18} (◆), $Xyl_{7-16}GlcA$ (✦), and $Xyl_{5-14}GlcA_2$ (✧). The identification of long Xyl_n blocks, such as Xyl_{18}, is in accordance with the results obtained by partial acid hydrolysis of GX from birch and spruce wood (Havlicek & Samuelson, 1972; Jacobs et al., 2001). The occurrence of series with higher GlcA residues, such as $Xyl_{n-4}GlcA_3$, structural isomers of Xyl_n, should not be excluded. However, as in olive pulp xylo-oligosaccharides containing MeGlcA no $Xyl_{n-4}MeGlcA_3$ oligomers were observed (series with 42 Da relative to Xyl_n), the similarity in the pattern of substitution between the olive pulp and seed hull GX allows to expect that the occurrence of $Xyl_{n-3}GlcA_3$ oligomers in this fraction, if any, do not occur in significant amounts. The xylo-oligosaccharides identified,

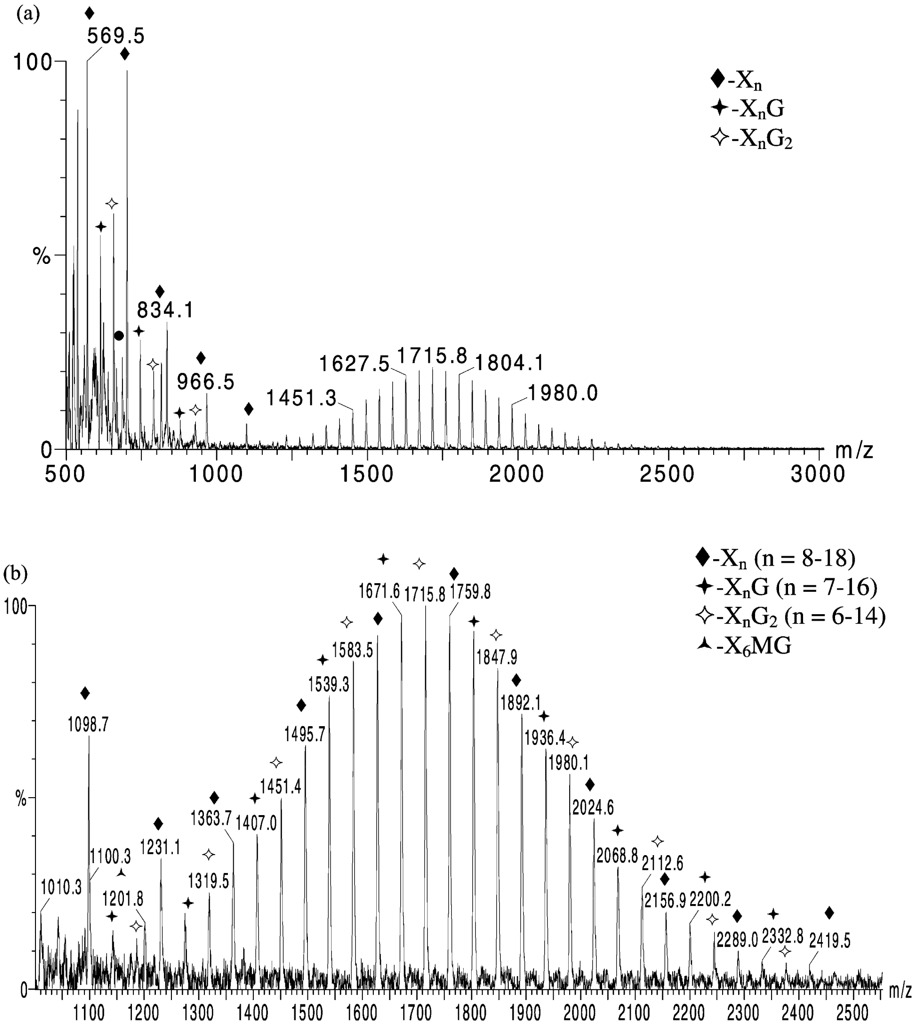

Fig. 3. MALDI mass spectra of fraction 44 of olive seed hull xylo-oligosaccharides, a) *m/z* 500–2500 and b) *m/z* 1000–2500.

including the low relative abundant ion at *m/z* 1201, attributed to Xyl_6GlcAMeGlcA, are summarised in Table 1.

4. Conclusions

The elution of olive pulp and seed hull xylo-oligosaccharides obtained by mild acid hydrolysis and fractionated by size exclusion chromatography showed different chromatographic profiles. The difference of chromatographic behaviour reflects different structural features in the GX chain, showed by MALDI-MS. Blocks of neutral oligosaccharides until 16 Xyl residues (pulp) and 18 Xyl residues (seed hull) were identified. The acidic xylo-oligosaccharides of the olive pulp were predominantly substituted with MeGlcA residues while those of olive seed hull were predominantly substituted with GlcA. Blocks with both substituting residues (Xyl_n-GlcAMeGlcA) were identified in olive GX. The identification of the long Xyl_n blocks and blocks with one and two acid residues suggests an irregular distribution of (Me)GlcA residues along the olive GX backbone.

MALDI-MS revealed to be a valuable tool for the identification of xylo-oligosaccharides obtained by partial acid hydrolysis, since acid hydrolysis of GX occurs in a random manner leading to the formation of a wide variety of oligosaccharides with varied chain length.

Acknowledgements

We gratefully acknowledge Micromass (Manchester, UK) for providing the MALDI instrument used for the analyses here presented and Research Unit 62/94 "Quimica Orgânica, Produtos Naturais e Agro-Alimentares".

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