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Characterization of water-soluble hemicelluloses from spruce and aspen employing SEC/MALDI mass spectroscopy

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Abstract

Partly depolymerized hemicelluloses isolated from wood chips of spruce and aspen employing microwave treatment were resolved using size-exclusion chromatography (SEC) into oligo- and polysaccharide fractions containing components with a narrow range of sizes, as determined by MALDI mass spectroscopy. The degree of substitution with acetyl moieties (DS) was also calculated on the basis of the MALDI-MS spectra obtained prior to and following deacetylation. For spruce hemicelluloses, the low molecular mass fraction contained small *arabino*-4-*O*-methylglucuronoxylan oligosaccharides, with DP values ranging from 4 to ~20, separated primarily on the basis of their charge density. The fraction eluted last consisted of an *O*-acetyl-(galacto)glucomannan polysaccharide of peak-average DP value (DP_p) 14. The degree of substitution with acetyl groups (DS) decreased with decreasing DP, a value DS of 0.39 being obtained for the fraction with DP_p 12. For the aspen hemicelluloses, the SEC fractions eluted first contained an acidic *O*-acetyl-4-*O*-methylglucuronoxylan polysaccharide with DP ranging from 10 to ~28 and an average DS of ~0.75. The fractions eluted last consisted of oligosaccharide mixtures composed primarily of small neutral *O*-acetyl-xylooligosaccharides (DP_p 6, DS 0.41), together with minor quantities of an *O*-acetyl-glucomannan.

Keywords: Aspen wood; Spruce wood; Hemicellulose; Xylan; Mannan; O-Acetylation; Size-exclusion Chromatography; Microwave treatment; MALDI-TOF-MS

1. Introduction

Acetylated hemicelluloses are present both in softwood species, such as spruce, and in hardwood species, such as aspen.[1,2] The major constituent of the hemicellulose of softwood is an *O*-acetyl-(galacto)-glucomannan, with a minor proportion of an *arabino*-4-*O*-methylglucuronoxylan. In the case of hardwood, the predominant component of the hemicellulose is an *O*-acetyl-(4-*O*-methylglucurono)-xylan, together with small amounts of a glucomannan[1,2]

Wood hemicelluloses can be extracted in good yield employing alkaline aqueous solutions.[1–3] However, upon extraction of acetylated hemicelluloses under alkaline conditions, the *O*-acetyl substituents are rapidly removed by hydrolysis. Acetylated hemicelluloses can be isolated by extraction of the original or delignified wood with organic solvents such as Me_2SO.[4,5] Partly depolymerized acetylated hemicelluloses can be isolated by water extraction of the wood following steam or microwave treatment.[6,7]

During the past decade, matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) has proven to be an effective tool for the analysis of oligo- and polysaccharides;[8,9] for extensive reviews see Refs. 10 and 11. In combination with size-exclusion chromatography, MALDI-MS has been employed in detailed investigations of complex polysaccharide mixtures,[12] as well as to obtain the average molar masses for polysaccharides.[13–17] We have previously reported the application of MALDI-MS in molar mass analysis of hemicelluloses isolated from wood and chemical pulps.[14,15,18]

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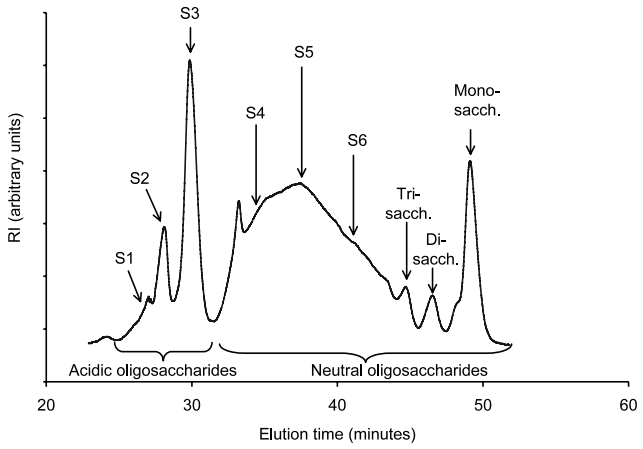

Fig. 1. Separation of the acidic and neutral oligo- and polysaccharides obtained from spruce wood chips pretreated with microwaves using the microscale SEC system A. Six fractions, designated as S1–S6, were collected from the outlet of the system. Oligosaccharides containing 4-*O*-methylglucuronic acid residues are eluted first from this SEC system. Sacch. = saccharides.

In a recent study,[19] we observed that acidic xylooligosaccharides can be separated from the corresponding neutral xylooligosaccharides by size-exclusion chromatography (SEC) involving elution with aqueous solutions of low ionic strength. A similar ionic-exclusion behavior in connection with aqueous SEC has also been observed for certain acidic arabinogalactans.[20,21]

We have previously reported the isolation of partly depolymerized hemicelluloses from aspen and spruce, their fractionation by SEC and subsequent characterization by NMR.[6,7] The present study documents characterization of these SEC fractions with respect to molar mass, molar-mass distribution, and degree of polymerization, as well as degree of substitution with acetyl moieties employing SEC and MALDI-MS.

2. Results and discussion

The partly depolymerized hemicelluloses, extracted with water from wood chips of spruce and aspen wood chips pretreated with microwaves, were fractionated by SEC employing elution with an aqueous solution of low ionic strength. The resulting fractions, containing acidic and neutral oligo- and polysaccharides, were subsequently characterized by MALDI-MS.

SEC/MALDI-MS analysis of spruce hemicelluloses.—The mixture of water-soluble hemicelluloses obtained from spruce wood was fractionated using SEC system A with deionized water as the SEC eluent. Fig. 1 depicts the chromatogram thus obtained, in which the hemicellulose fractions collected (fractions S1–S6) are indicated.

Fraction S1 (Fig. 2), eluted first from the SEC system, contains acidic oligosaccharides with pentose backbones, and three 4-*O*-methylglucuronic acid substituents (DP 9–20). The relative contents of xylose and arabinose residues in these oligomers could not be determined from the MALDI-MS spectra, since these residues have the same molecular mass (i.e., 132 mass units). Approximately 10% of the weight of spruce *arabino*-4-*O*-methylglucuronoxylan normally consists of arabinose residues.[2,17] Fractions S2 and S3 (Fig. 2), respectively, contain acidic oligosaccharides of increasing chain-lengths with two or one 4-*O*-methylglucuronic acid residues, respectively. Thus, these acidic

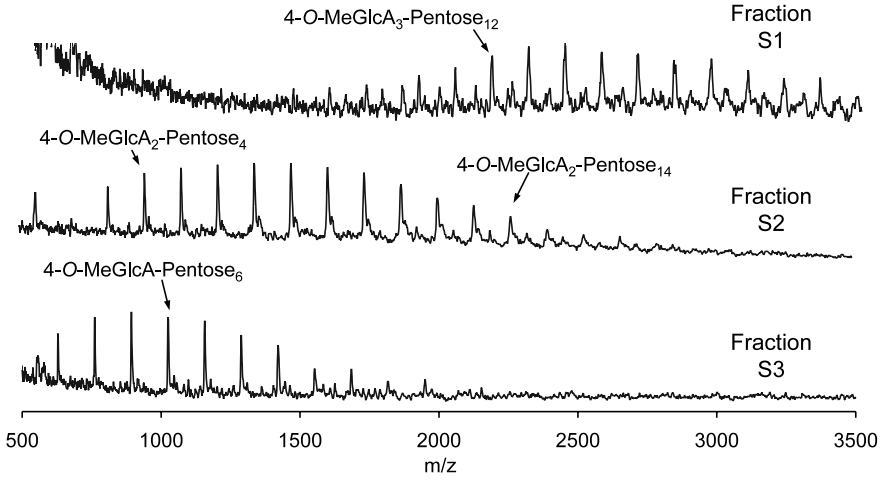

Fig. 2. MALDI-MS spectra of the acidic oligosaccharides in fractions S1–S3 collected in connection with SEC of the water-soluble hemicelluloses from spruce wood (see Fig. 1). Fraction S1 (the uppermost spectrum) consists of acidic *arabino*-4-*O*-methylglucuronoxylan oligosaccharides containing three 4-*O*-methylglucuronic acid residues and with DP values ranging from 9 to as much as ~ 20. Fraction S2 is composed of acidic oligosaccharides containing two 4-*O*-methylglucuronic acid residues and with DP values ranging from 3 to as much as ~ 17. Fraction S3 contains acidic oligosaccharides with one 4-*O*-methylglucuronic acid residue and with DP values ranging from 3 to as much as ~ 12.

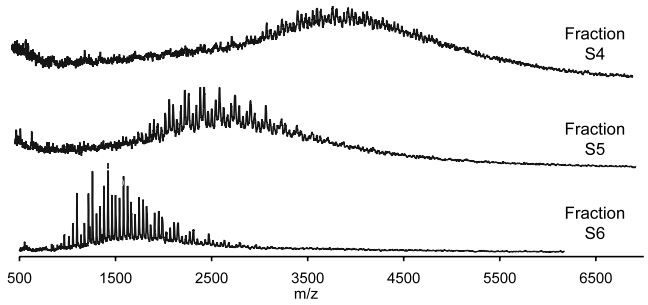

Fig. 3. MALDI-MS spectra of the neutral oligosaccharides in fractions S4–S6 collected in connection with SEC of the water-soluble hemicelluloses from spruce wood (see Fig. 1).

arabino-4-*O*-methylglucuronoxylosaccharides were resolved on the basis of both their charge and their size.

The neutral hemicelluloses were well resolved from the acidic hemicelluloses, being separated on the basis of molecular size alone, and recovered in the fractions S4–S6 (Fig. 3). MALDI-MS analysis of the oligosaccharides in fractions S4–S6 revealed them to be Hexose-oligomers containing different numbers of *O*-acetyl groups. Previous analysis of the composition of these neutral fractions demonstrated the primary constituent to be a (galacto)glucomannan.[7]

The relationship between molar mass and elution time in the SEC system was calibrated using the neutral hemicellulose fractions characterized by MALDI-MS. The average molar masses and molar-mass distributions of the entire (galacto)glucomannan could then be calculated from this relationship. This analysis revealed a M_n value of 1000 and a M_w value of 2500 for the neutral portion (i.e., the (galacto)glucomannan) of the partly depolymerized spruce hemicellulose investigated here.

The SEC fractions S4–S6 were also analyzed by MALDI-MS following alkaline hydrolysis to remove the *O*-acetyl substituents. Fig. 4 illustrates the MALDI-MS spectra of fraction S6 before (upper spectrum) and after (lower spectrum) such deacetylation. The number of Hexose residues and *O*-acetyl groups in the oligosaccharides of the original fraction S6 could be calculated from comparison of the mass numbers obtained from this MALDI-MS analysis.

Mass signals from oligosaccharides with different DP and DS may partially overlap. Magnification of the peak area around $m/z \sim 1830$ in the upper spectrum of Fig. 4 (inset) reveals that this peak consists of two partially resolved mass signals corresponding to the sodium-adduct ions of the non-acetylated oligosaccharide $Hexose_{11}$ (m/z 1824.5) and the *O*-acetylated oligosaccharide $Hexose_{10}$–Ac_4 (m/z 1834.5).

In order to determine the DP value (i.e., the average number of Hexose units) of the spruce (galacto)glucomannan, the fractions were also analyzed by MALDI-MS after removal of the *O*-acetyl groups. The (galacto)glucomannan portion of the spruce hemicellulose exhibited a peak average DP (DP_p) of 15.

In addition, the degree of acetyl substitution (DS) of the (galacto)glucomannan could be examined using the MALDI-MS spectra. For example, the MALDI-MS spectrum of the (galacto)glucomannan in fraction S6 (Fig. 4, upper spectrum) exhibits a considerable heterogeneity with respect to the degree of acetylation. As described earlier, peaks corresponding to $Hexose_{11}$ (m/z 1824.5) and $Hexose_{10}$–Ac_4 (m/z 1834.5) are observed in this spectrum. The former glucomannan oligomer has a DS value of zero, whereas for the latter oligosaccharide, DS 0.4. This is in agreement with earlier findings on (galacto)glucomannan from pine, in which the acetyl groups are irregularly distributed along the glucomannan backbone.[22] The average DS of the oligosaccharides in each fraction could be estimated from the difference in the molecular mass (i.e., M_p) before and after the removal of *O*-acetyl groups.

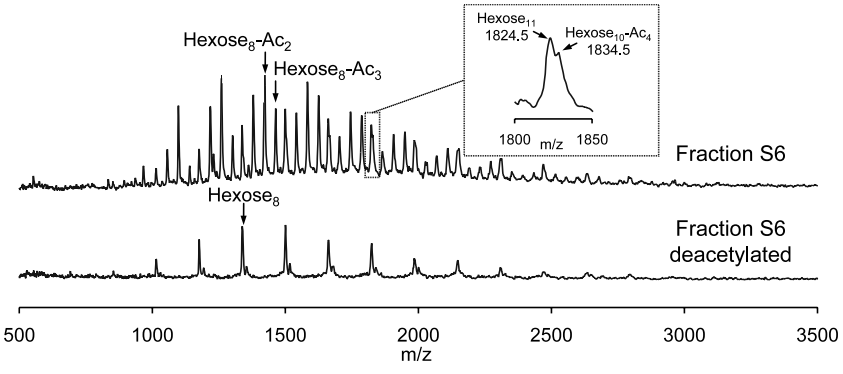

Fig. 4. MALDI-MS spectra of fraction S6 obtained by SEC of the water-soluble hemicelluloses extracted from spruce wood. In the case of analysis of the untreated fraction (upper spectrum), the distance between adjacent peaks in each oligomer cluster corresponds to 42 mass units (i.e., an acetyl unit). Enlargement of this spectrum in the m/z range from 1800 to 1850 (inset) demonstrates that this peak consists of two peaks which are only partially resolved and correspond to $Hexose_{11}$ (m/z 1824.5) and $Hexose_{10}$–Ac_4 (m/z 1834.5). Spectral analysis of the same fraction following alkaline hydrolysis (lower spectrum) revealed peaks originating from glucomannan oligomers with DP values ranging from 5 to 17.

Table 1
Molecular properties of spruce (galacto)glucomannan fractions separated by SEC

	Property							
Fraction	M_p	M_n	M_w	M_w/M_n	DP_p	DS	DP_n [a]	DS [a]
S7	2000	2300	2400	1.06	12	0.39		
S6	1250	1440	1540	1.07	8	0.31		
8	2480	2450	2560	1.04	15	0.32	~20	0.28
9	1670	1580	1662	1.06	10	0.27	~11	0.25

[a] Determined by NMR spectroscopy.[7]

The molecular properties (i.e., molar mass, molar-mass distribution, DP and DS) of four fractions of the spruce (galacto)glucomannan are presented in Table 1. The DP and DS values estimated from the MALDI mass spectra before and after alkaline deacetylation are in good agreement with the values previously estimated from NMR spectra.[7] For all fractions, the degree of acetyl substitution decreased somewhat as the DP of the oligosaccharides decreased.

SEC/MALDI-MS analysis of aspen hemicelluloses.— The mixture of partly depolymerized hemicelluloses was separated into two major series of peaks using SEC system B (Fig. 5).[6] The first series contained the acidic oligosaccharides (i.e., the 4-*O*-methylglucuronoxylan) and the second series of peaks contained the neutral oligosaccharides. Three fractions (designated A2, A3, and A10) eluting at increasing times were collected as indicated in Fig. 5, and subsequently characterized by MALDI-MS.

The MALDI-MS spectrum of fraction A3 is depicted in Fig. 6 (upper spectrum). In the case of aspen *O*-acetyl-4-*O*-methylglucuronoxylan saccharides, which, in addition to *O*-acetyl groups, have different numbers of 4-*O*-methylglucuronic acid residues linked to their xylan chains, the differences in molar mass between different oligosaccharides are smaller than the resolution of the MALDI-MS instrument employed here. This explains the poor resolution of the MALDI-MS spectrum of the aspen fraction A3. However, it is possible to observe a series of poorly resolved signals separated by 42 mass units (designated as Δ in the upper spectrum of Fig. 6). These signals originate from *O*-acetyl-(4-*O*-methylglucurono)xylan oligosaccharides with different DP values and numbers of *O*-acetyl substituents. After deacetylation by alkaline hydrolysis, the MALDI-MS spectrum of the fraction A3 (Fig. 6, lower spectrum) revealed that this fraction contained acidic xylooligosaccharides with one or two 4-*O*-methylglucuronic acid residues (DP 10–28).

MALDI-MS analysis of fraction A2 before and after alkaline deacetylation was also performed. The molecular properties (i.e., average molar masses, degree of polymerization and degree of acetyl substitution) of this oligosaccharide are documented in Table 3. The oligosaccharides recovered in the fractions A2 and A3 exhibited approximately the same DP value. However, the acidic xylan oligomers in fraction A2 contained two (or three) 4-*O*-methylglucuronic acid residues per xylooligosaccharide, indicating that in this case as well, separation was achieved primarily on the basis of the charge density of the acidic acetylated xylooligosaccharides.

Finally, fraction A10, containing neutral oligosaccharides, was also analyzed by MALDI-MS. The major peaks in the MALDI-MS spectrum (Fig. 7) could be assigned on the basis of their mass numbers to the sodium adduct ions of *O*-acetyl-xylooligosaccharides containing different numbers of *O*-acetyl substituents. The peaks corresponding to the xylooligosaccharides with no (Xyl_4), one (Xyl_4–Ac) and two *O*-acetyl groups (Xyl_6–Ac_2) are indicated in Fig. 7.

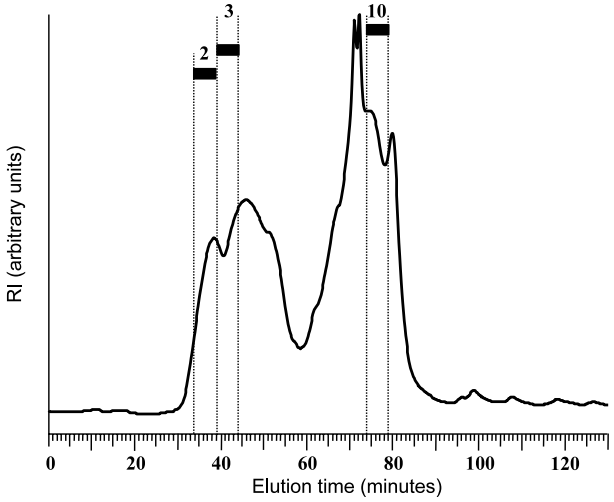

Fig. 5. Preparative scale SEC separation (system B) of the water-soluble acidic and neutral oligo- and polysaccharides obtained utilizing microwave treatment of aspen wood chips.[6] Three fractions, denoted A2, A3, and A10, were collected at the outlet of the system. Oligosaccharides containing 4-*O*-methylglucuronic acid residues were eluted first in this SEC system.

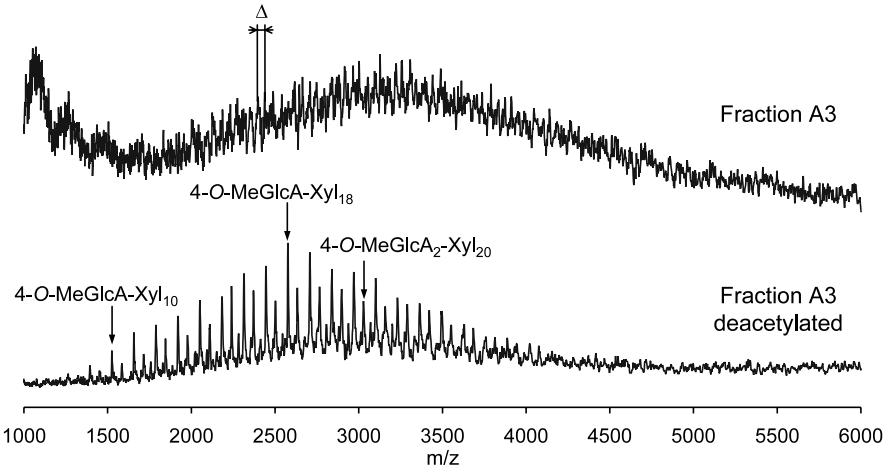

Fig. 6. MALDI-MS spectra of the acidic oligosaccharides in fraction A3 obtained by SEC separation (depicted in Fig. 5) of the water-soluble polysaccharides extracted from aspen wood chips subjected to microwave treatment. Following deacetylation (lower spectrum) fraction A3 contained acidic xylooligosaccharides with one or two 4-*O*-metylglucuronic acid residues. Prior to such deacetylation (upper spectrum), a series of weak signals (Δ) separated by 42 mass units (i.e., the mass of an acetyl group) were observed. The separation Δ corresponds to the mass difference between *O*-acetyl-(4-*O*-methylglucuronoxylan) oligosaccharides with different DP values and degrees of acetylation.

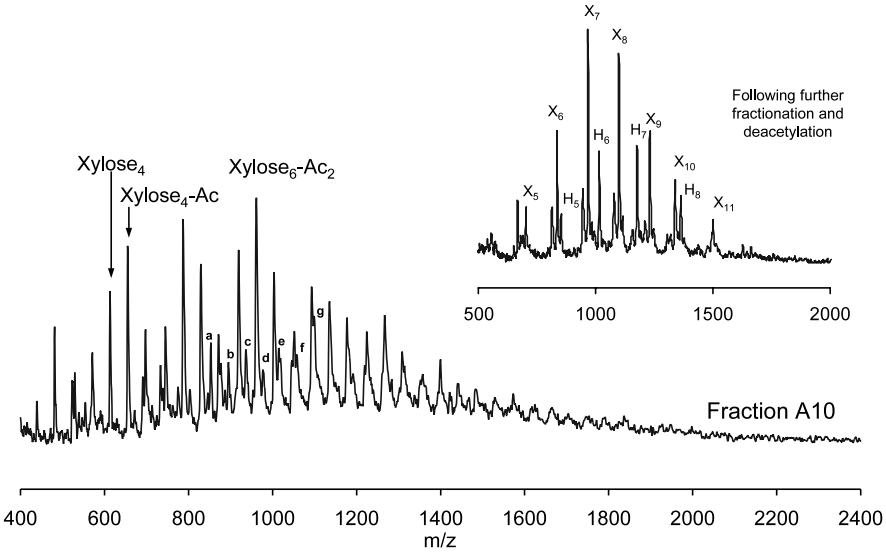

Fig. 7. MALDI-MS spectra of the neutral oligosaccharides in fraction A10 collected in connection with SEC separation of the water-soluble polysaccharides obtained from aspen wood chips subjected to microwave treatment. Fraction A10 consisted primarily of neutral xylooligosaccharides containing different numbers of acetyl substituents and with chain lengths ranging from 3 to ~9. In addition to the xylooligosaccharide peaks, a small series of peaks (designated a–g) tentatively assigned to glucomannan oligomers containing different numbers of acetyl groups is also observed. The compositions of peaks a–g are presented in Table 1. The MALDI-MS spectrum of fraction A10 following further fractionation and deacetylation (inset) exhibits two overlapping series of peaks corresponding to the xylooligosaccharides (designated X_n, where n = the number of residues) and to the postulated glucomannan oligomers (H_5–H_8).

In addition to the signals originating from the *O*-acetyl-xylooligosaccharides, a minor series of mass signals, designated a–g, was also observed in this spectrum. These peaks were tentatively assigned on the basis of their masses to *O*-acetylated glucomannan oligosaccharides containing increasing numbers of Hexose residues (Table 2). This assignment is supported by our earlier NMR study of fraction A10, which also indicated the presence of an *O*-acetyl-glucomannan.[6] The MALDI-MS spectrum of fraction A10 following additional SEC fractionation and alkaline deacetylation (inset in Fig. 7) provided support for this interpretation. After deacetylation, fraction A10 contained neutral xylooligomers (designated X_n, where $n = 5$–11) and neutral Hexose oligosaccharides (designated H_m, where $m = 5$–8). As in the case of spruce (galacto)glucomannan, considerable

Table 2
MALDI-MS characterization of the minor components of fraction A10 obtained by SEC of water-soluble oligosaccharides from aspen

Peak	Experimental MM	Composition	Calculated MM
a	853.0	$Hexose_5$	851.7
b	895.1	$Hexose_5$–Ac_1	894.7
c	936.5	$Hexose_5$–Ac_2	937.8
d	979.0	$Hexose_5$–Ac_3	980.8
e	1014.9	$Hexose_6$	1013.8
f	1057.5	$Hexose_6$–Ac_1	1056.9
g	1099.0	$Hexose_6$–Ac_2	1099.9

Assignment was based on the molar masses (MM) determined.

heterogeneity with respect to the degree of acetyl substitution of the hemicellulose saccharides from aspen can be observed in the MALDI-MS spectrum of fraction A10. For example, peaks originating from xylooligosaccharides with DS values ranging from zero ($Xylose_4$) up to at least 0.5 ($Xylose_6$–Ac_3) are present in the spectrum. For this fraction as well, the average degree of acetyl substitution was calculated from the difference in M_p before and after removal of the acetyl groups. The molecular properties of the xylan component in fraction A10 are presented in Table 3.

In the case of the tentatively identified aspen glucomannan, the saccharides presented in Table 2 exhibited DS values ranging from zero ($Hexose_6$) up to 0.6 ($Hexose_5$–Ac_3). The average degree of acetyl substitution of the glucomannan components in fraction A10 was estimated to be 0.25.

3. Experimental

Materials.—All reagents employed were of analytical grade. The water used in the preparation of reagents and buffer solutions was first purified utilizing a Millipore Milli-Q Plus apparatus (Millipore, Milliford, USA).

Size-exclusion chromatography.—Two different systems for SEC, using deionized water as the eluent, were employed. In the first of these (designated as SEC system A), SEC fractionation was performed on a microscale utilizing three columns of Ultrahydrogel 120, 250 and 500 (Waters Assoc. USA), respectively, linked in series to a device for measuring refractive index (RI) (Waters Assoc. USA). Approximately 1 mg of hemicellulose was injected onto the SEC column system. The signal from the RI detector was processed using the PL Caliber SEC software and interface (Polymer Laboratories Ltd., UK) on a standard PC. At regular intervals during the emergence of peaks from the SEC system, fractions (100 μL) were collected at the outlet of this detector.

The second SEC system (designated SEC system B) consisted of a fast protein liquid chromatography system (Pharmacia Biotech, Uppsala), with three columns; Superdex 75, Superdex 75 (HR 10/30), and Superdex 200 (HR 10/30) coupled in series, as described previously.[6,7]

Molecular mass calibration of the SEC system A.—In the case of the *O*-acetylated galactoglucomannan, eight fractions were collected from the outlet of the refractometer at regular intervals during the emergence of the SEC peaks. The peak-average molar mass (M_p) values of the fractions were determined by MALDI-MS analysis. For each fraction, the logarithm of the M_p value determined by MALDI-MS was graphed as a function of the time required for elution from the SEC system. Analysis of this relationship by least-squares linear regression yielded a regression coefficient of 0.995. This linear relationship between log M_p and elution time was subsequently employed to determine the number and weight-average molar masses (M_n and M_w) of the whole (galacto)glucomannan distribution.

Removal of O-*acetyl substituents.*—The *O*-acetyl moieties of the *O*-acetylated oligo- and polysaccharides were removed by alkaline hydrolysis in the presence of ammonium hydroxide. For this purpose, approximately 50 μL of 25% aq ammonium hydroxide was added to each SEC fraction and this mixture was then maintained at 80 °C for approximately 30 min.

Table 3
Molecular properties of fractions of aspen xylan separated by SEC

	Property							
Fraction	M_p	M_n	M_w	M_w/M_n	DP_p	DS	DP_n [a]	DS [a]
A2	3300	n.a.	n.a.	n.a.	20	0.79		0.60
A3	3150	n.a.	n.a.	n.a.	19	0.73		0.58
A10	850	820	880	1.08	6	0.41	~5	0.32

n.a. = not applicable.
[a] Determined by NMR spectroscopy.[6]

MALDI mass spectrometry.—The MALDI analyses were performed using a Hewlett–Packard G2025 A MALDI-TOF mass spectrometer equipped with a linear detector, employing 1–5 μJ energy pulses of the UV (337 nm) laser beam. The spectra depicted routinely represent the sums of 20–50 laser shots. Both positive- and negative-ion spectra were determined.

A saturated, aqueous solution of the matrix 2,5-dihydroxybenzoic acid (DHB) was added to each fraction collected from the SEC system, and approximately 0.5 μL of the sample–matrix mixture was applied to the MALDI probe. The peak-average molar mass (M_p) of each fraction was determined as the molar mass at the maximal peak intensity in the MALDI-MS spectrum. The weight- and number-average molar masses (M_w and M_n) for the fractions were calculated from the MALDI-MS spectra using the Hewlett–Packard G2025 MALDI-TOF software.

Acknowledgements

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