Holzforschung
56 (2002) 607–614

Methylation Analysis as a Tool for Structural Analysis of Wood Polysaccharides

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Keywords

Polysaccharides
Hemicellulose
Structure
Methylation analysis
Retention time
Relative molar response

Summary

In modern structural analysis of complex mixtures of wood polysaccharides, methylation analysis is still a valuable and powerful tool for linkage analysis. In this paper, methylation analysis is described for the procedure methylation, methanolysis, silylation and GC/MS. The retention time indexes for the partly methylated methyl glycosides of the relevant wood polysaccharides are listed together with the ratios of the isomers of the different structural units. A calculation model for relative molar response factors is suggested based on a published model for FID detection and on experimental data. Tested for oligosaccharides of known structure including xylotetraose, mannotriose and $6^3,6^4$-α-D-galactosyl-mannopentaose, the model gives reproducible and sufficiently correct results. The fate of xylose units substituted with 4-*O*-methyl glucuronic acid at position 2 is investigated with a model compound.

Introduction

The first structural studies on polysaccharides present in wood and in kraft and sulfite pulps were performed from the 1930s to the 1960s and are reviewed by several authors (Aspinall 1959; Bouveng and Lindberg 1960; Timell 1964, 1965). The main aim of these early studies was to determine the structure of different wood polysaccharides, and those isolated for this purpose included (arabino)glucuronoxylans, (galacto)glucomannans, (arabino)galactans, pectins, xyloglucans and arabinans. The methods used were designed mainly for linkage position analysis and included methylation analysis, degradation techniques (*e.g.*, Smith degradation, periodate oxidation) and partial acid or enzymatic hydrolysis combined with these methods.

Today, these traditional methods for the structural analysis of wood and pulp polysaccharides have been supplemented by NMR techniques (Shimizu 1991) including novel multidimensional techniques as recently reviewed by Vuorinen and Alén (1999). New information on a xylan isolated from aspen based on NMR studies, including information on acetyl groups, was published recently (Teleman *et al.* 2000). In addition, matrix-assisted laser desorption ionization (MALDI) mass spectroscopy has been used to determine the molar mass distribution of wood polysaccharides. This also gives some structural information, as recently reported for isolated xylans (Dahlman *et al.* 1999; Jacobs and Dahlman 2000). However, these techniques require relatively pure polysaccharides and good solubility of the sample. Thus, linkage positions in oligo- and/or polysaccharides, in particular those in complex mixtures, are still mainly determined using methylation analysis. Some examples are recent studies on isolated hemicelluloses from wood (Shatalov *et al.* 1999; Willför *et al.* 2002) and hemicelluloses isolated from black liquors (Vikkula *et al.* 1997; Laine *et al.* 1999) as well as studies on oligosaccharide residues in lignin-carbohydrate complexes (Minor 1982, 1986, 1991; Iversen and Wännström 1986; Laine *et al.* 1999; Lee *et al.* 1999).

Methylation analysis is often employed in linkage analysis, but little has been published on how the method is actually carried out and how reliable the results are. This work presents in detail the following protocol of methylation analysis: 1) methylation using methyl iodide and powdered NaOH in DMSO, 2) acid methanolysis, 3) silylation, 4) quantification and identification of the totally methylated monosaccharides or partially methylated units after silylation using GC/MS. The protocol differs from the more common variations of methylation analysis, which include methylation, hydrolysis, reduction, acetylation and gas chromatography. The fact that one stage (reduction) could be omitted was seen as an advantage. The possibility of this procedure has been described in the literature (Kamerling and Vliegenthart 1989; Vuorinen and Alén 1999), but no covering data on retention times and quantification using mass spectrometric detection in linkage analysis of polysaccharides common in wood and pulp has been published.

During acid methanolysis of the methylated oligo- or polysaccharides, the α and β-anomers equilibrate. Both anomers are present after this step and information on

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the original anomeric configuration is lost. For liberated methyl glycosides unsubstituted in positions 4 and 5 (originally substituted at position 4 or 5), the pyranoside and furanoside forms also isomerize. The original ring size can thus be determined only for units that were originally unsubstituted at positions 4 and 5. Consequently, up to four isomers can be obtained for one structural unit. Earlier, the separation of the complex mixture of methyl glycosides was a problem. However, modern gas chromatography enables almost complete separation of complex mixtures, and its combination with mass spectroscopy enables the correct interpretation of even small peaks. The occurrence of up to four isomers has even become an advantage, because the ratio of the isomers after acid methanolysis is almost constant. It is thus possible to base the identification on more than a single peak. Any overlapping of one of the peaks can be corrected for based on the other isomer(s) of the structural unit.

In addition to basic data such as retention times, the ratios of different linkage types were determined for oligosaccharides of known structures.

Experimental

The model compounds (mono-, oligo- and polysaccharides) were purchased or obtained as shown in Table 1. The silylation reagents trimethylchlorosilane and *N,O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) were obtained from Pierce, Rockford, Illinois, USA.

Methylation

About 20 mg of ground sodium hydroxide and 0.1 ml of methyl iodide were added to 3–5 mg of the oligo- or polysaccharide sample in 0.5 ml dimethylsulfoxide. The sample was kept for 30 min in an ultrasonic bath at room temperature. Water was added and the aqueous phase was extracted with dichloromethane. The organic phase was extracted with water, dried and evaporated in pear-shaped flasks (see acid methanolysis below) to dryness (Ciucanu and Kerek 1984; Asres and Perreault 1997).

Acid methanolysis

2 ml of 2 M HCl/methanol was added to the methylated samples in the pear-shaped flasks fitted with teflon seals. 2 M HCl/methanol was prepared by slow addition of 16 ml acetylchloride to methanol p.a. so that the final volume of the mixture was 100 ml. The samples were kept in an oven at 100 °C for 3 h (Sundberg *et al.* 1996; Vuorinen and Alén 1999). 0.08 ml of dry pyridine and sorbitol in methanol as internal standard were added and the samples were evaporated to near dryness and kept in a rotary evaporator at a vacuum of 5 kPa and bath temperature of 50 °C for 10 min (optimal procedure) or dried in a vacuum oven over P_2O_5 at 2.5 kPa and 40 °C for 20 to 180 min. The samples were transferred to small glass bottles using 0.08 ml dry pryridine for the silylation.

Silylation

The samples were silylated with 0.25 ml *N,O*-bis(trimethylsilyl)-trifluoroacetamide containing 5 % trimethylchlorosilane.

Gas chromatography

GC/MS: About 1 µl of silylated sample was injected *via* a split injector (HP7683 Series Injector/Autosampler, 260 °C, split ratio 1:50) into a 30 m/0.25 mm HP-5 column (film thickness 0.25 µm) in a HP6890 Series GC System with a HP5973 Series Mass Selective Detector. The temperature program was 100 °C (2 min); 4 °C/min; 220 °C (2 min); 15 °C/min; 300 °C (2 min). The carrier gas was helium (1 ml/min, constant flow). The detector conditions were 70 eV, 40–600 amu.

GC/FID: About 1 µl of silylated sample was injected *via* a split injector (Autosampler, 260 °C, approximate split ratio 1:50) into a HP-5 column in a HP5890 Series II GC System with Flame Ionization Detector. The temperature program was 70 °C; 4 °C/min; 260 °C (10 min). The carrier gas was helium.

Table 1. Origin of model compounds

Substance	Origin
Xylose, mannose, galactose, arabinose, lactose	Merck, Germany
Glucose	BDH Chemicals, UK
Sorbitol	Aldrich Chemicals, UK
Cellobiose, raffinose	Fluka, Switzerland
1,4-β-D-Xylotetraose, debranched arabinan, 1,4-β-D-mannotriose, $6^3,6^4$-α-D-galactosyl-mannopentaose, lichenan (Cetraria islandica), galactan (lupin) and pectic galactan (lupin)	Megazyme, Ireland
Arabinogalactan (Sigma A2012)	Sigma, US
Stachyose, pullulan	Polymer Laboratories, UK
Dextran	Pharmacia, Sweden
Xyloglucan (tamarind)	Prof. Stephen C. Fry, University of Edinburgh, UK
Black liquor arabinan and acid-treated arabinogalactan (Luonteri *et al.* in preparation), MeGlcA-Xyl_4 (Teleman *et al.* 1996)	Dr. Maija Tenkanen VTT Biotechnology, Finland
2-*O*-Methyl xylose, 3-*O*-methyl xylose, 3-*O*-methyl glucose	Jan Janson, KCL, Finland (synthetic products)

Table 2. Retention time index and ratio of different isomers originating from the same linkage types (as methyl glycosides after silylation). The ratios of the isomers were approximate constant for various samples

Linkage	Me	TMS	model compound	retention time index / peak area % of all isomers of the linkage type
xylose T, furanose form	2,3,5		xylose	0.31, 0.32 / ratio not determined
xylose T, pyranose form	2,3,4		xylotetraose	0.27 / 27 %, 0.30 / 73 %
2-linked xylose	3,4	2	xyloglucan	0.40 / 33 %, 0.41 / 67 %
4-linked xylose[1]	2,3	4	xylotetraose	0.40 / 29 %, 0.44 / 48 %
	2,3	5		0.44 / 15 %, 0.45 / 8 %
2,4-linked xylose[1]	3	2,4	3-*O*-methyl-xylose[2]	0.54, 0.54 / not fully separated, 96 %
	3	2,5		0.62 / 2 %, 0.65 / 2 %
3,4-linked xylose[1]	2	3,4	2-*O*-methyl-xylose[2]	0.57 / 33 %,0.58 / 48 %
	2	3,5		0.52 / 12 %, 0.54 / 7 %
2,3,4-linked xylose[1]		2,3,4	xylose[2]	0.71 / 61 %, 0.73 / 32 %
		2, 3, 5		0.62 / 4 %, 0.63 / 3 %
arabinose T, furanose form	2,3,5		arabinose	0.29 / 83 %, 0.32 / 17 %
arabinose T, pyranose form	2,3,4		arabinose	0.35 / not fully separated
5-linked arabinose[1]	2,3	4	debranched arabinan	0.41 / 58 %, 0.45 / 13 %
	2,3	5		0.42 / 10 %, 0.44 / 19 %
3,5-linked arabinose[1]	2	3,4	black liquor arabinan	0.52 / 25 %, 0.54 / 29 %
		3,5		0.51 / 30 %, 0.55 / 16 %
2,3,5-linked arabinose[1]		2,3,4	arabinose[2]	0.61 / 42 %, 0.62 / 22 %
		2,3,5		0.60 / 26 %, 0.65 / 10 %
glucose T, furanose form	2,3,5,6		glucose	0.51, 0.53 / ratio not determined
glucose T, pyranose form	2,4,5,6		pullulan	0.46 / 28 %, 0.51 / 72 %
3-linked glucose	2,4,6	3	lichenan	0.61 / 33 %, 0.64 / 67 %
4-linked glucose[1]	2,3,6	4	pullulan	0.60 / 30 %, 0.64 / 63 %
	2,3,6	5		0.62 / 5 %, 0.64 / 2 %
6-linked glucose	2,3,4	6	pullulan, stachyose	0.57 / 30 %, 0.61 / 70 %
3,6-linked glucose	2,4	3,6	dextran	0.71 / 32 %, 0.72 / 68 %
4,6-linked glucose[1]	2,3	4,6	xyloglucan	0.69 / 30 %, 0.72 / 70 %
	2,3	5,6		covered or minor peaks
2,4,6-linked glucose[1]	3	2,4,6	3-*O*-methyl-glucose[2]	0.79 / 72 %, 0.79 / 23 %
	3	2,5,6		0.79 / 3 %, 0.80 / 2 %
2,3,4,6-linked glucose[1]		2,3,4,6	glucose[2]	0.94 / 62 %, 0.96 / 34 %
		2,3,5,6		0.86 / 2 %, 0.87 / 2 %
mannose T, furanose form	2,3,5,6		mannose	0.56 / only one peak identified
mannose T, pyranose form	2,3,4,6		mannose, mannotriose	0.51 / 95 %, 0.52 / 5 %
4-linked mannose[1]	2,3,6	4	mannotriose	0.62 / 94 %, 0.63 / 4 %
		5		0.63 / 2 %
4,6-linked mannose[1]	2,3	4,6	$6^3,6^4$-α-D-galactosyl-	0.68 / 95 %, 0.70 / 5 %
		5,6	mannopentaose	minor peaks, not identified
2,3,4,6-linked mannose[1]		2,3,4,6	mannose[2]	0.85 / 83 %, 0.87 / 11 %
		2,3,5,6		0.92 / 5 %, 0.93 / 1 %
galactose T, furanose form	2,3,5,6		galactose	0.51, 0.52 / ratio not determined
galactose T, pyranose form	2,3,4,6		galactose	0.51 / 34 %, 0.52 / 66 %
3-linked galactose	2,4,6	3	acid-treated arabinogalactan	0.61 / 67 %, 0.62 / 33 %
4-linked galactose[1]	2,3,6	4	lupin	0.58 / 14 %, 0.61 / 29 %
	2,3,6	5		0.59 / 47 %, 0.62 / 10 %
6-linked galactose	2,3,4	6	stachyose	0.65 / 30 %, 0.66 / 70 %
3,6-linked galactose	2,4	3,6	larchwood arabinogalactan	0.73 / 67 %, 0.74 / 33 %
4,6-linked galactose[1]	2,3	4,6	pectic galactan	0.70 / 12 %, 0.72 / 27 %
		5,6		0.70 / 55 %, 0.73 / 6 %
2,3,4,6-linked galactose[1]		2,3,4,6	galactose[2]	0.89 / 44 %, 0.92 / 21 %
		2,3,5,6		0.86 / 25 %, 0.89 / 9 %
sorbitol (26.30 min)	1,2,3,4,5,6		sorbitol[2]	1.00

T = terminal, non-reducing end, Me = methyl group, TMS = trimethylsilyl group
[1]No distinction can be made between 4- and 5-substitution due to isomerization between the pyranose and furanose forms during acid methanolysis. The linkage information is deduced from knowledge of the model compounds.
[2]Sample preparation started directly from acid methanolysis.

Results and Discussion

Identification of derivatives

The partly or totally methylated derivatives of the neutral monosaccharides xylose, arabinose, mannose, galactose and glucose were identified by their retention time order and their mass spectra using model compounds. The data is presented in Table 2. The mass spectra obtained were compared to published spectra (Petersson and Samuelsson 1968a,b and others as reviewed by Dutton 1974).

Relative molar response factors

The structural units of the starting molecule undergo methylation and silylation to different extents in the course of the reaction chain, depending on the amount of linkages to other units. The resulting monosaccharide derivatives carry different numbers of methyl and silyl substituents. Hence, the relative molar response for detection in the gas chromatographic determination has to be taken into account. In the case of flame ionization detection, molar responses were determined and a sug-

Table 3. Relative molar responses of the partially or totally methylated and trimethylsilylated monosaccharide units

Derivative	FID relative molar response factor calucalted[1]	average ratio of MS/FID response factors (see Table 2)	total relative molar response factor
per-*O*-(trimethylsilylated) sorbitol	1	1	1
methyl tetra-*O*-TMS-hexoside	1.37	1.16	1.59
methyl *O*-methyl-tri-*O*-TMS-hexoside	1.59	1.16	1.84
methyl di-*O*-methyl-di-*O*-TMS-hexoside	1.89	1.16	2.19
methyl tri-*O*-methyl-*O*-TMS-hexoside	2.32	1.16	2.69
per-*O*-methylated hexoside	3.02	1.37	4.14
methyl tri-*O*-TMS-pentoside	1.77	1.16	2.05
methyl *O*-methyl-di-*O*-TMS-pentoside	2.15	1.16	2.49
methyl di-*O*-methyl-*O*-TMS-pentoside	2.73	1.16	3.17
per-*O*-methylated pentose	3.76	1.37	5.15

TMS = trimethylsilyl
[1]model of Verhaar and De Wilt 1969.

Table 4. Ratio of the relative molar responses of the totally or partially methylated and silylated monosaccharides units using FID and MS detection

	Monosaccharide	model compound	ratio of MS/FID relative molar response
permethylated	Arabinose	arabinose	1.57
	Arabinose	arabinose	1.57
	Glucose	glucose	1.57
	Glucose	glucose	1.23
	Glucose	glucose	1.29
	Xylose	xylose	1.55
	Glucose	cellobiose	1.26
	Galactose	lactose	1.31
	Xylose	xylotetraose	1.19
	Mannose	mannotriose	1.30
	Galactose	raffinose	1.35
	Galactose	stachyose	1.33
	Average		1.37
partly methylated, silylated	*O*-methyl 2,3,6-tri-*O*-methyl 4-*O*-TMS glucoside	cellobiose	1.04
	O-methyl 2,3,6-tri-*O*-methyl 4-*O*-TMS glucoside	lactose	1.13
	O-methyl 2,3-di-*O*-methyl 4-*O*-TMS xyloside	xylotetraose	1.19
	O-methyl 2,3,6-tri-*O*-methyl 4-*O*-TMS mannoside	mannotriose	1.13
	O-methyl 2,3,4-tri-*O*-methyl 6-*O*-TMS glucoside	raffinose	1.28
	O-methyl 2,3,4-tri-*O*-methyl 6-*O*-TMS glucoside	stachyose	1.19
	O-methyl 2,3,4-tri-*O*-methyl 6-*O*-TMS galactoside	stachyose	1.11
	O-methyl 2,3-di-*O*-methyl 4-*O*-TMS xyloside	xylan	1.16
	O-methyl 2,4-di-*O*-methyl 3,6-*O*-di-TMS galactoside	arabinogalactan	1.18
	Average		1.16

gested model was employed to calculate relative molar responses (Table 3) (Verhaar and De Wilt 1969). The relative molar responses for mass spectroscopic detection (total ion chromatogram) were obtained by running the same samples on a GC with mass spectroscopic detector and a GC with FID detection. The ratio of the peak areas in mass spectroscopic detection and in FID detection was determined for several samples (Haakana 1997, Table 4) and the total relative molar responses for mass spectroscopic detection were calculated from the data as presented in Table 3.

Development of the procedure

The crucial steps in this procedure are the successful quantitative methylation of all free hydroxyl groups (permethylation), isolation of the permethylated product, quantitative cleavage of glycosidic bonds, and silylation. The evaporation and drying of the partly or totally methylated monosaccharides after acid methanolysis has been reported to be a problem due to the volatility of the totally methylated monosaccharides, in particular those of the pentoses xylose and arabinose (Timell 1964).

The method was first tested for 1,4-β-D-xylotetraose (purity grade > 95 %) with respect to drying method and time after methanolysis. The reactions of the methylation analysis are shown in Figure 1 for this oligosaccharide and the resulting total ion chromatogram of the products is shown in Figure 2. Drying was originally performed in a vacuum oven at 2.5 kPa and 40 °C over P_2O_5 to ensure total moisture removal. As presented for different drying times in Figure 3, the ratio of the 4-linked xylose to the non-reducing end of xylopyranose was too high, indicating loss of the non-reducing unit. This may result in what appears to be too high a degree of polymerization. In the extreme case of 3 h, even the 4-linked xylose units were lost during evaporation and the proportion of the identified impurities (2,4- and 3,4-linked xylose units) increased. Consequently, the evaporation step was reconsidered. Keeping the sample in a rotary evaporator at a pressure of 5 kPa for 10 min after evaporation of most of the methanol was sufficient for final methanol removal, as shown by successful silylation. Some pyridine remained in the sample under the conditions employed. The ratio of the 4-linked xylose to the non-reducing xylopyranose end groups obtained with this procedure was satisfyingly close to 3 : 1 in xylotetraose and the reproducibility was good. Consequently, this drying procedure was employed throughout the following parts.

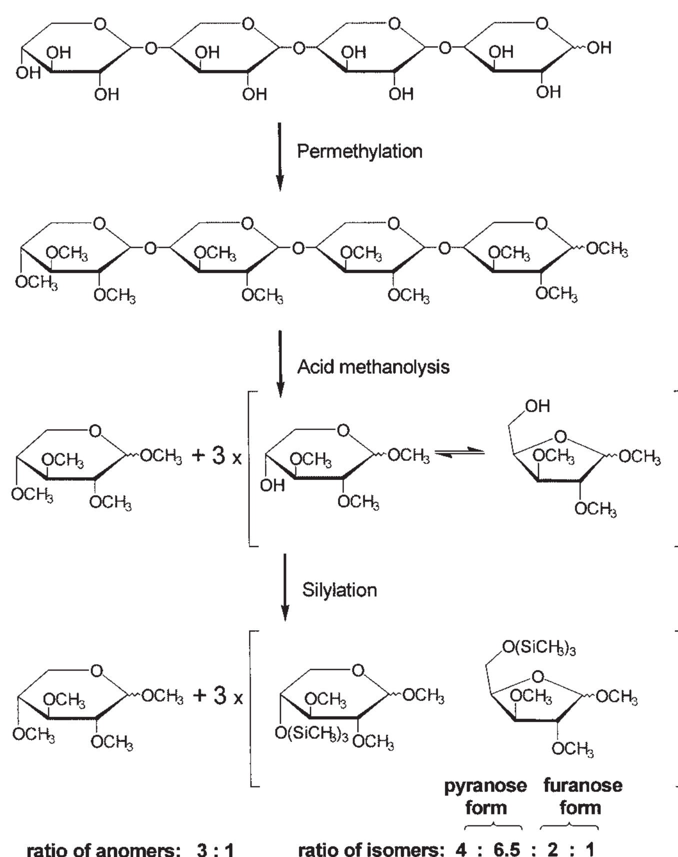

Fig. 1. The reactions during methylation analysis with 1,4-β-xylotetraose as an example. Permethylation leads to quantitative methylation of all free hydroxyl groups. Acid methanolysis hydrolyzes the glycosidic bonds and leads to an equilibration of the α and β anomers of the methyl glycoside. For the liberated methyl glycosides of the 4-substituted xylopyranose, the pyranose and furanose forms also isomerize. Silylation finally silylates the hydroxyl groups liberated in the acid methanolysis step.

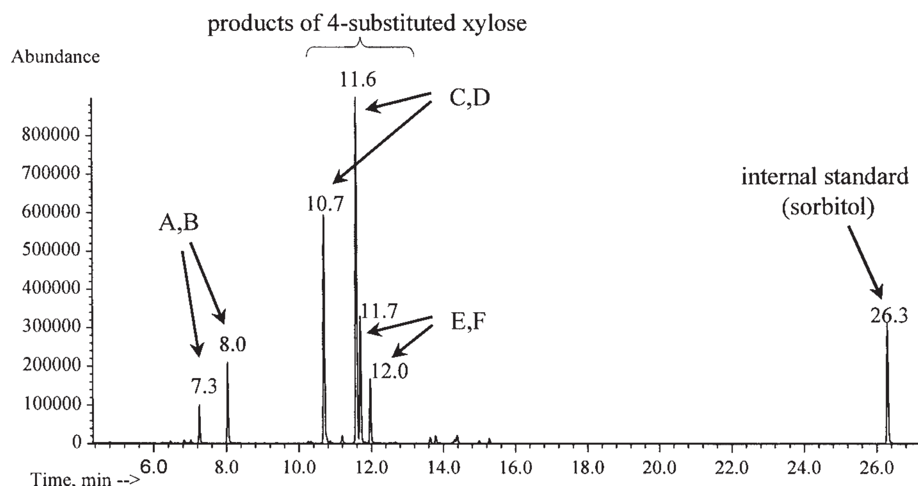

Fig. 2. Total ion chromatogram of the products of the methylation analysis of xylotetraose. The non-reducing xylopyranose end group is analyzed as α- and β-*O*-methyl 2,3,4-tri-*O*-methyl xylopyranoside (A,B) and the 4-substituted unit as α- and β-*O*-methyl di-2,3-*O*-methyl-4-trimethylsilyl xylopyranoside (C,D) and α- and β-*O*-methyl di-2,3-*O*-methyl-5-trimethylsilyl xylofuranoside (E,F).

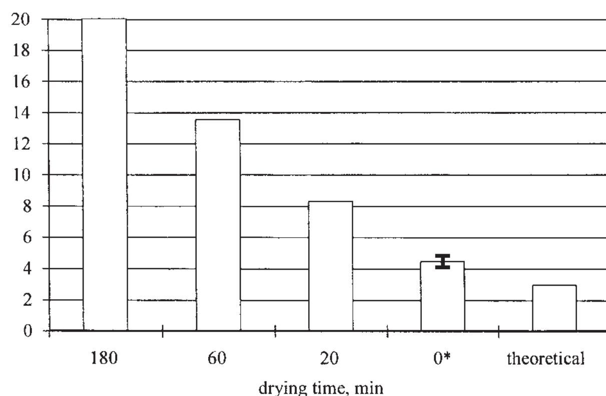

Fig. 3. Ratio of 4-substituted xylose units to non-reducing xylopyranose end groups determined for xylotetraose after different drying times in a vacuum oven (*drying only in a rotary evaporator, average of three determinations, error bar shows the standard deviation).

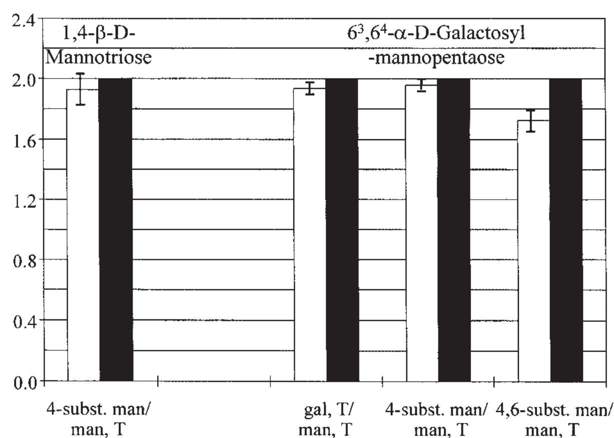

Fig. 4. Experimental (□) and theoretical (■) ratios of the different structural units to the non-reducing mannopyranose end group determined for model oligosaccharides (average of two determinations, error bars show the interval of the determinations).

Oligosaccharides of known structure

The method developed in this work was tested for 2 oligosaccharides of known structure: 1,4-β-D-mannotriose and 6^3,6^4-α-D-galactosyl-mannopentaose. The results are presented as ratios of the different structural units to the non-reducing mannopyranose end group in Figure 4. For mannotriose, the ratio of 4-linked mannose to non-reducing mannopyranose end groups is with 1.9 close to the theoretical of 2. The reproducibility was very good, as indicated by the error bar (interval of two determinations). 6^3,6^4-α-D-galactosyl-mannopentaose contains non-reducing ends of mannopyranose and galactopyranose, and 4-substituted and 4,6-disubstituted mannose. Excellent ratios were obtained for the non-reducing galactose end groups and the 4-substituted mannose units with 1.9 and 2.0, respectively. Only for the 4,6-substituted mannose units was the ratio with 1.7 slightly too low. Again the reproducibility was satisfactory.

These tests with model compounds of known structure show that ratios of structural units can be determined with satisfactory accuracy with the method presented here.

Uronic acid substituents

In the chosen procedure, uronic acids were not determined. Uronic acids may decompose during the methylation or acid methanolysis steps. The question arose as to whether those structural units with uronic acid substituents were detected as a substituted unit. This would mean that the substituted unit carries the intact acid or

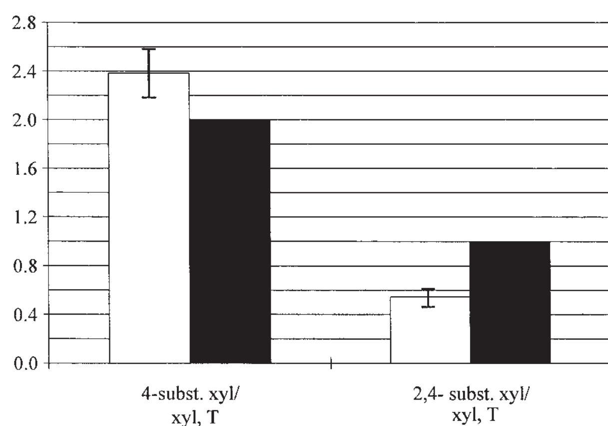

Fig. 5. Experimental (□) and theoretical (■) ratios of the different structural units to the non-reducing xylopyranose end group determined for xylotetraose substituted with one 4-*O*-methyl glucuronic acid substituent at the third xylose unit (average of two determinations, error bars show the interval of the determinations).

the rest of the decomposed acid throughout the methylation, and secondly that the acid methanolysis cleaved the rest.

The ratio of structural units for a substituted xylotetraose with one methyl glucuronic acid substituent at the third xylose unit (MeGlcA-Xyl_4) (Teleman *et al.* 1996) was determined. The results are presented in Figure 5 as the ratio of substituted units to the non-reducing xylopyranose unit. Only trace amounts of 2-linked xylose units were found, showing that the 4-*O*-methyl glucuronic acid substituent was only at units also substituted at position 4 in agreement with the structure of the model compound. When calculating the ratios of differently substituted xylose units, the presence of the terminal units with 2-substitution has therefore been neglected. For the 2,4-linked xylose unit, the ratio was smaller than 1 : 1 as expected in the event that the uronic acid behaves as a neutral monosaccharide substituent. This may partly explain why the ratio of 4-linked units to terminal units was higher than 2 : 1. The uronic acid substituents were thus only partly determined in the results. More correct information on the uronic acid substituents might be obtained if reduction of the methylated polysaccharide were performed as suggested for a xylan of Eucalyptus globulus (Shatalov *et al.* 1995). Without the reduction step, care must be taken when interpreting the substitution of xylose units if the xylan contains uronic acids.

Conclusions

The retention times for structural units of polysaccharides present in wood and pulp derived samples were listed and the resolution of modern gas chromatography was found powerful enough even for complex mixtures of polysaccharides. In some cases overlapping of peaks occurred, but this could be accounted for by the presence of up to four isomers in a constant ratio for an individual linkage type. Tests with the procedure developed here showed that evaporation of the solvent from a mixture of totally or partially methylated methyl glycosides is a crucial step due to the volatility of the permethylated methyl glycosides. A careful evaporation avoiding drying in a vacuum oven was chosen, and this gave reproducible results for model compounds including xylotetraose, mannotriose and $6^3,6^4$-α-D-galactosylmannopentaose. The problem of 4-*O*-methyl glucuronic acid units linked to xylan was shown to cause an underestimation of 2,4-substituted xylose units as present in glucuronoxylan. A reduction step before the methylation would probably solve this problem. Methylation analysis using the combination methylation, acid methanolysis, silylation and GC/MS was shown to be a powerful tool for linkage analysis.

Acknowledgements

We would like to thank Prof. Steven C. Fry, The Edinburgh Cell Wall Group, The University of Edinburgh, for the tamarind xyloglucan and Dr Maija Tenkanen, VTT Biotechnology, for the black liquor arabinan, acid-treated arabinogalactan and MeGlcA-Xyl_4. The authors are grateful to Sami Haakana, Lic. Tech., for the preliminary work. Thanks are also due to Tarja Tapanila, M.Sc., for help with the gas chromatography and to Ms. Seija Salo and Ms. Eija Ylinen for their skilful experimental work.

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Received May 4th 2001

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