STRUCTURES OF HEMICELLULOSES AND PECTINS IN WOOD AND PULP

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Dissertation for the degree of Doctor of Science in Technology to be presented with due permission of the Department of Chemical Technology for public examination and debate in the Auditorium Ke2 (Komppa Auditorium) at Helsinki University of Technology (Espoo, Finland) on the 10th of June, 2005, at 12 noon.

Keywords: analytical method, carbohydrate, delignification, hemicellulose, kraft pulp, methylation, *Picea abies*, *Pinus silvestris*, polysaccharide, residual lignin, TMP, wood

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ISSN 1457-6252

ABSTRACT

This work aims to fill gaps in the present knowledge of the structures of wood polysaccharides of potential importance in mechanical and chemical pulping. The detailed structures of the polysaccharide components from wood that dissolve during mechanical pulping were determined. The structures of polysaccharide components linked to residual lignin in chemical pulp were also determined. Methylation analysis as linkage analysis was further developed and verified for this purpose using the procedure methylation, methanolysis, silylation and GC/MS analysis.

Water-soluble arabinogalactans obtained from spruce and pine heartwood were analyzed using methylation analysis, NMR spectroscopy and carbohydrate composition analysis. The arabinogalactans isolated were acidic and had a backbone of β-(1→3)-linked D-galactopyranose units, highly branched at C6. Water-soluble galactoglucomannans were extracted from spruce wood and TMP. Based on both methylation analysis and NMR spectroscopic analysis, the main C6-branched unit was D-mannopyranose. Some C6-branched D-glucopyranose units were also detected by methylation analysis.

The polysaccharides that dissolved during oxygen delignification of pine kraft pulp comprised mainly xylan, together with galactans, 1,3-linked glucan, arabinan and glucomannan. This indicated that the glucose units present in the dissolved polysaccharides did not originate from cellulose but from a 1,3-linked glucan. This was a new and unexpected finding indicating that the content of dissolved glucose is not related to the degree of cellulose degradation. Polysaccharides in the corresponding filtrates from birch kraft pulp also contained mainly xylan. An indication was found for the presence of bonds between lignin and position C3 of the backbone units in birch xylan.

Residual lignin-carbohydrate complexes (RLCCs) isolated from spruce and pine kraft pulps contained oligosaccharides representing the main polysaccharides in softwood. In addition, 1,4-linked and 1,3/6-linked galactans were present in considerable amounts, together with smaller amounts of 1,3-linked glucan and 1,5-linked arabinan, and possibly also xyloglucan. 1,4-linked galactan was enriched in the RLCC of spruce fiber surface material. Towards the inner part of the fiber, 1,3/6-linked galactan became the major lignin-bound galactan structure. Some differences related to the alkaline cooking method used were detected in the carbohydrate component of pine pulp RLCCs. Oxygen delignification removed 1,3/6-linked galactan from the inner parts of fiber in preference to 1,4-linked galactan.

CONTENTS

PREFACE

This thesis is based on experimental work carried out during the years 1998-2001 at Oy Keskuslaboratorio, KCL, Espoo, Finland. I am grateful to professor Jorma Sundquist and Dr. Kristiina Poppius-Levlin from KCL for the opportunity to perform this work and for their support.

I am deeply indebted to the supervisor of this thesis, professor Reija Jokela, for encouraging me to continue for the doctoral degree and for discussions during the work. I warmly thank my advisor Dr. Tarja Tamminen for initiating this work and for her guidance and support throughout. Special thanks go to my co-authors, in particular to Dr. Stefan Willför, Dr. Bo Hortling, professor Bjarne Holmbom and professor Tapani Vuorinen. I also want to thank my colleagues at KCL in general for creating such a stimulating working atmosphere and, in particular, Seija Salo, Eija Ylinen and Tarja Tapanila for their contributions to this work. Thanks are also due to Philip Mason for checking the language of this paper.

Financial support from the EU (FAIR CT 98 3460 and QLK5-CT-1999-01277), the Technology Agency of Finland (TEKES), the Emil Aaltonen Foundation, Finland, the Foundation of Technology, Finland, and the Kemira Foundation, Finland is gratefully acknowledged.

Finally my warmest thanks to my husband Petri for his support and love.

LIST OF PUBLICATIONS

This work is based on the following papers (Appendices I-V). In the text, the papers are referred to by their Roman numerals.

I Laine, C., Tamminen, T., Vikkula, A.,Vuorinen, T. (2002) Methylation analysis as a tool for structural analysis of wood polysaccharides, *Holzforschung 56:6*, 607-614.

II Willför, S., Sjöholm, R., Laine, C., Holmbom, B. (2002) Structural features of water-soluble arabinogalactans from Norway spruce and Scots pine heartwood, *Wood Sci. Technol. 36:2*, 101-110.

III Willför, S., Sjöholm, R., Laine, C., Roslund, M., Hemming, J., Holmbom, B. (2003) Characterisation of water-soluble galactoglucomannans from Norway spruce wood and thermomechanical pulp, *Carbohydr. Polym. 52:2*, 175-187.

IV Laine, C., Tamminen, T. (2002) Origin of carbohydrates dissolved during oxygen delignification of birch and pine kraft pulp, *Nord. Pulp Pap. Res. J. 17:2*, 168-171.

V Laine, C., Tamminen, T., Hortling, B. (2004) Carbohydrate structures in residual lignin-carbohydrate complexes of spruce and pine pulp, *Holzforschung 58:6*, 611–621.

Author's contribution

The author's role in each of the publications has been the following:

I The author planned and performed the experimental work (except the GC/MS analysis) and wrote the manuscript taking into account the comments of the coauthors.

II The author planned and performed the experimental work for the methylation analyses, and wrote the draft version of the corresponding parts of the paper.

III The author planned and performed the experimental work for the methylation analyses and wrote the draft version of the corresponding parts of the paper.

IV The author planned the experimental work and wrote the manuscript of the paper taking into account the comments of the coauthor.

V The author planned the experimental work for the different carbohydrate analyses, performed part of the experimental work, and wrote the manuscript taking into account the comments of the coauthors.

ABBREVIATIONS

Ac	Acetyl group
AEC	Anion exchange chromatography
AG	Arabinogalactan
AHQ	Anthrahydroquinone
AQ	Anthraquinone
Ara(*f*,*p*)	Arabinose (Arabinofuranosyl, Arabinopyranosyl)
BK	Birch kraft pulp
BKE	Alkaline stage of birch kraft pulp
BKO	Oxygen delignification stage of birch kraft pulp
BSTFA	*N,O*-Bis(trimethylsilyl)-trifluoroacetamide
CE	Capillary electrophoresis
CK	Conventional kraft
CTMP	Chemithermomechanical pulp
DMSO	Dimethylsulfoxide
DP	Degree of polymerization
DP_n	Apparent numerical average degree of polymerization
E	Alkaline extraction stage
Gal(*p*)	Galactose (Galactopyranosyl)
GalA(*p*)	Galacturonic acid (Galacturonic acid unit in pyranose form)
GC	Gas chromatography
GGM	Galactoglucomannan
Glc(*p*)	Glucose (Glucopyranosyl)
GlcA(*p*)	Glucuronic acid (Glucuronic acid unit in pyranose form)
GW	Groundwood
HPAEC	High-performance anion exchange chromatography
HPLC	High-performance liquid chromatography
HPSEC	High-performance size exclusion chromatography
LCC	Lignin-carbohydrate complex
MALDI	Matrix-assisted laser desorption ionization
Man(*p*)	Mannose (Mannopyranosyl)
Me	Methyl group
MeGlcA	4-*O*-Methylglucuronic acid
ML	Middle lamella
MS	Mass spectrometry
MWEL	Milled wood enzyme lignin
MWL	Milled wood lignin
NMR	Nuclear magnetic resonance
O	Oxygen delignification stage
P	Primary wall
PAD	Pulsed amperometric detection
PCK	Pine conventional kraft pulp
PGW	Pressure groundwood
PK	Pine kraft pulp

PKE	Alkaline stage of pine kraft pulp
PKO	Oxygen delignification stage of pine kraft pulp
PPSAQ	Pine polysulfide/anthraquinone pulp
prois	Protease-purified residual lignin, insoluble fraction at pH 9.5
proisis	Protease-purified residual lignin, insoluble fraction at pH 9.5 and in 0.5 M NaOH
proissP	Protease-purified residual lignin, insoluble fraction at pH 9.5, soluble in 0.5 M NaOH, precipitated at pH 2.5
proRL	Protease-purified residual lignin, soluble fraction at pH 9.5, precipitated at pH 2.5
PS	Polysulfide
PSoAQ	Pine soda/anthraquinone pulp
Rha(*p*)	Rhamnose (Rhamnopyranosyl)
RLCC	Residual lignin-carbohydrate complex
S	Secondary wall
SCK	Spruce conventional kraft pulp
So	Soda
T	Terminal, non-reducing unit
TMCS	Trimethylchlorosilane
TMP	Thermomechanical pulp
TMS	Trimethylsilyl group
TOC	Total organic carbon
UV	Ultraviolet
Xyl(*p*)	Xylose (Xylopyranosyl)
W	Warty layer

1 INTRODUCTION

Polysaccharides are widespread in nature. They account for an estimated 66% of all global bound carbon (Gruber 1976). Polysaccharides are macromolecular carbohydrates consisting of a large number of monosaccharides connected to each other by glycosidic bonds (Römpp Chemie Lexikon 1992). Not all the functions of individual polysaccharides are known, but they may act as storage material, structural components and protective substances. Starch, glycogen, some β-glucans, fructans and some galactomannans are examples of storage polysaccharides. Structural polysaccharides are either fibrous polysaccharides – mainly cellulose in higher plants and some algae or chitin in yeast and fungi – or matrix polysaccharides, for example arabinoxylans, galactomannans or pectins in plants. Protective polysaccharides include extracellular polysaccharides from microorganisms or exudate gums from plants (Aspinall 1982).

Polysaccharides are the principal components of wood. Cellulose, the main polysaccharide, has long been studied to elucidate its structure and function. The structure and role of hemicelluloses and pectins, which also represent a large proportion of polysaccharides in wood and pulp fibers, have been studied widely in the 70's and the work is still continuing.

Knowledge of the role and behavior of polysaccharides during wood and pulp processing is essential for understanding and controlling these processes. In addition to the principal components such as cellulose and the major hemicelluloses, minor components also play an important role. Examples are the enrichment of acidic polysaccharides in the circulation water of paper machines, and polysaccharide components that retain lignin in chemical pulp fibers during cooking and bleaching.

This work aims to fill gaps in the present knowledge of the structures of those wood polysaccharides that are of potential importance in mechanical and chemical pulping. The detailed structures of the polysaccharide components from wood that dissolve during mechanical pulping were determined. The acidic components that dissolve have not been studied in detail before. The detailed structures of fractions of the hemicellulose component glucomannan dissolving under various conditions are not known, either. Knowledge of the structure of these polysaccharides will help us to understand their interactions with dissolved and colloidal substances as well as with fibers and other components in the circulation water of paper machines. This knowledge can be used to advantage in choosing the conditions and chemicals for the papermaking process. Second, the aim is to determine the structure of the polysaccharide components linked to residual lignin in chemical pulp. No comparison has yet been made of the structures of carbohydrate residues in residual lignin-carbohydrate complexes (RLCCs) in different pulps before and after oxygen delignification. This information will be useful in developing the cooking and bleaching processes. The pulp production process and the subjects of the present study are outlined in Figure 1.

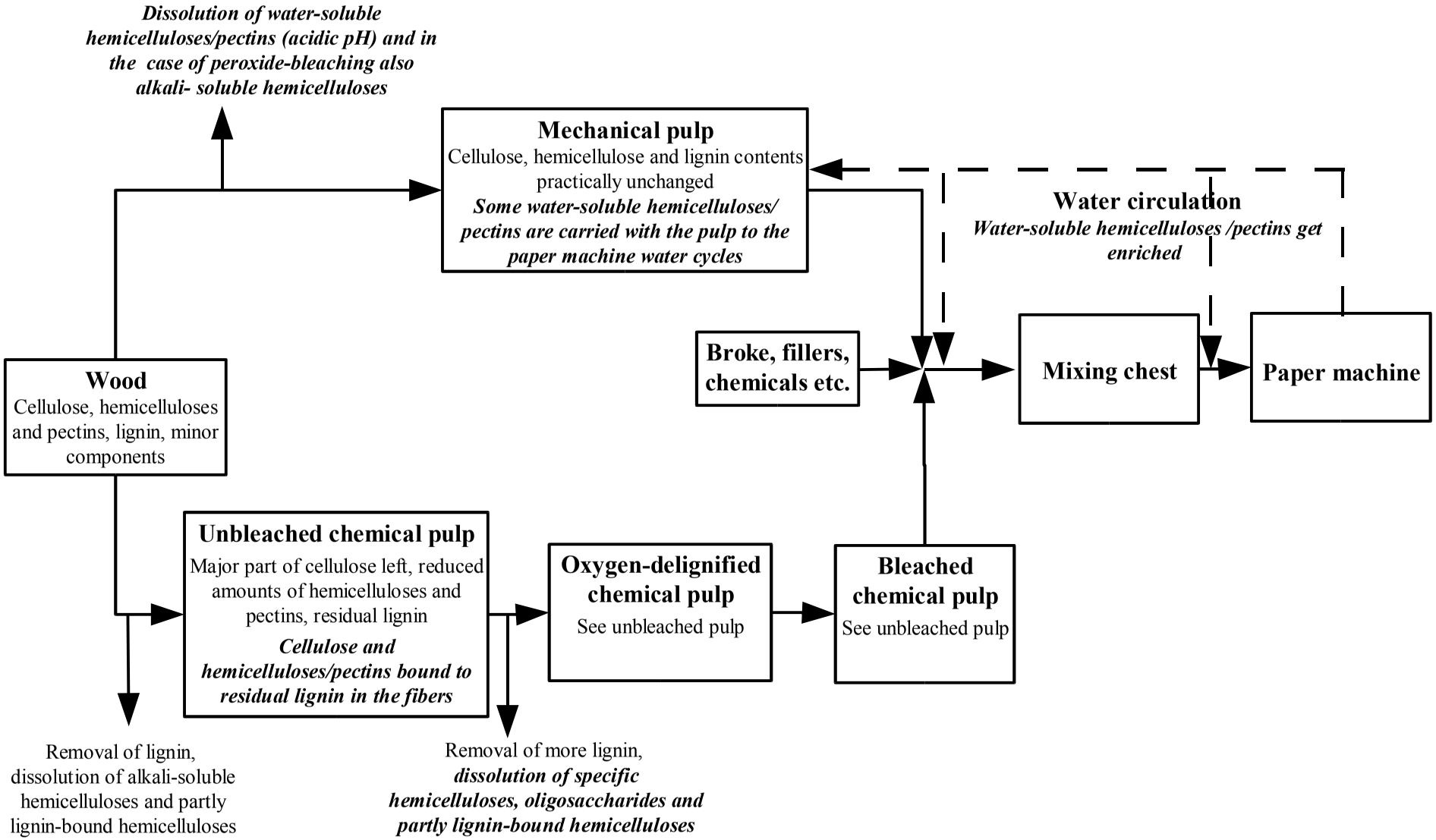


Figure 1. Schematic representation of process stages in pulp production for papermaking. The stages concerning polysaccharides and partly lignin are shown. The parts that play a role in this thesis are printed in bold italics.

2 STRUCTURAL COMPONENTS OF WOOD

The main components of wood are structural components. The structural components are cellulose, hemicelluloses and lignin, together with smaller amounts of pectic substances (Fengel and Wegener 1989, Alén 2000a). For softwood and hardwood in general, the contents of the main components vary in the ranges shown in Table 1. Polysaccharides represent the major part of both wood types.

Table 1. Contents of the main components, % of dry wood, adapted from Sjöström (1993).

	Cellulose	Glucomannan	Xylan	Other polysaccharides	Lignin
Softwood	33-42	14-20	5-11	3-9	27-32
Hardwood	38-51	1-4	14-30	2-4	21-31

2.1 Cellulose

The main polysaccharide in wood is cellulose. Cellulose is a homopolysaccharide composed of D-glucopyranose units linked to each other by β-(1→4) glycosidic bonds (Figure 2) (Sjöström 1993). The molecules are completely linear and have a strong tendency to form intra- and intermolecular hydrogen bonds. This leads to bundling of cellulose molecules into microfibrils, which in turn form fibrils and finally cellulose fibers.

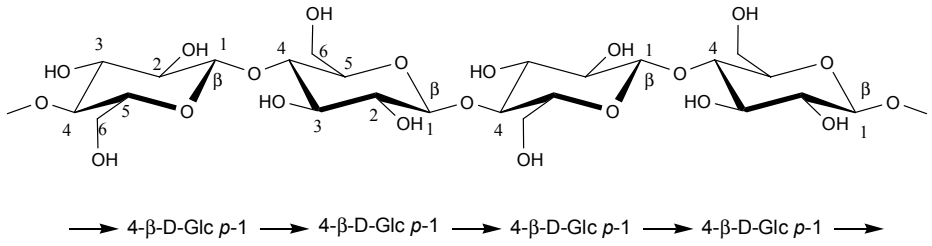


Figure 2. Structure of cellulose.

2.2 Hemicelluloses and pectins

Softwood

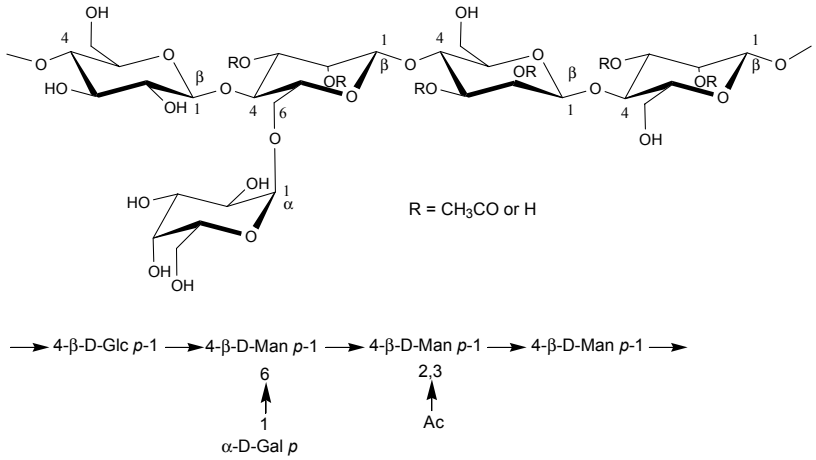
The major hemicelluloses in coniferous wood (softwood) are galactoglucomannan, glucomannan and arabinoglucuronoxylan. Other softwood hemicelluloses are arabinogalactan, xyloglucan and other glucans. Other polysaccharides are pectins. The term pectin or pectic compounds is used either strictly for the component rhamnogalacturonan or more generally for the group of components comprising rhamnogalacturonans, galactans and arabinans.

(Galacto)glucomannans are the principal hemicelluloses in softwood (Table 1, *glucomannan*). The backbone is a linear or slightly branched chain of β-(1→4)-linked D-mannopyranose and D-glucopyranose units (Figure 3) (Fengel and Wegener 1989, Sjöström 1993, Shimizu 2001). D-Galactopyranose residues are linked as single-unit side chains by α-(1→6)-bonds. Galactoglucomannan can be roughly divided into two types: one with a low galactose content – sometimes referred to simply as glucomannan – and the other with a high galactose content.

The ratios of galactose to glucose to mannose are 0.1-0.2:1:3-4 and 1:1:3 in the two types respectively. The hydroxyl groups at position C2 and C3 in the backbone units are partly substituted by *O*-acetyl groups, on average one group per 3-4 hexose units.

Softwood arabinoglucuronoxylan (referred to as *xylan* for short) has a backbone of β-(1→4)-linked xylopyranose units (Figure 3) (Fengel and Wegener 1989, Sjöström 1993, Shimizu 2001). Single-unit side chains are 4-*O*-methyl-D-glucuronic acid units attached by α-(1→2) bonds, on average one unit per 5-6 xylose units, and L-arabinose units attached by α-(1→3) bonds, on average one unit per 5-12 xylose units. Some branching of the otherwise linear backbone at C2 of the xylose units has been reported. No substitution by acetyl groups has been found.

Glucomannan



Xylan

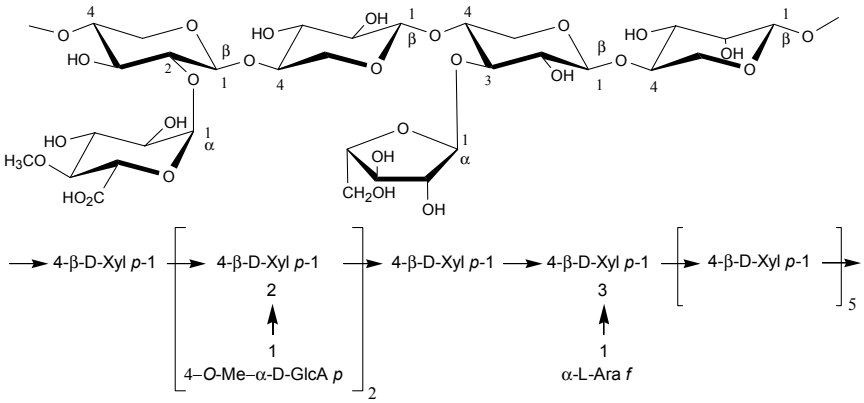
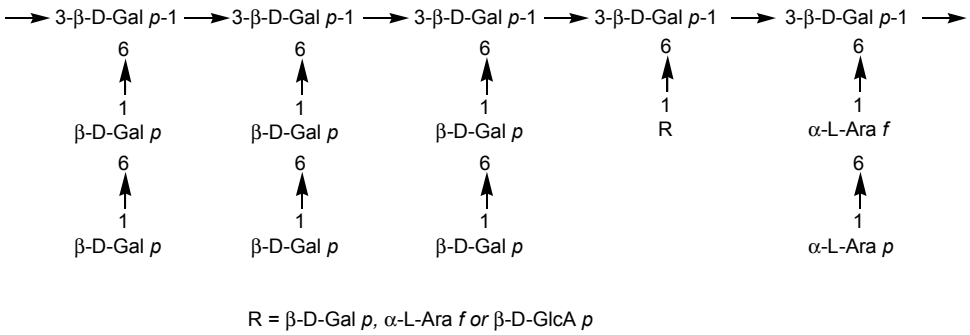


Figure 3. Structures of major softwood hemicelluloses.

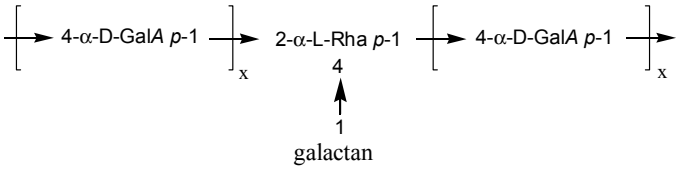
Arabinogalactan is mainly known as a component of the heartwood of larches (Figure 4) (Fengel and Wegener 1989, Sjöström 1993, Shimizu 2001). Larchwood arabinogalactan has a backbone of β-(1→3)-linked D-galactopyranose units and is highly branched at C6. The side chains are composed of β-(1→6)-linked D-galactose units, D-galactose and L-arabinose units or single L-arabinose units and single D-glucuronic acid units. Arabinose is present in furanose and pyranose forms in an approximate ratio of 1:2. Larchwood arabinogalactan is a

water-soluble polysaccharide. Most other softwoods contain only small amounts of arabinogalactan amounting to less than 1% by weight of wood (Shimizu 2001).

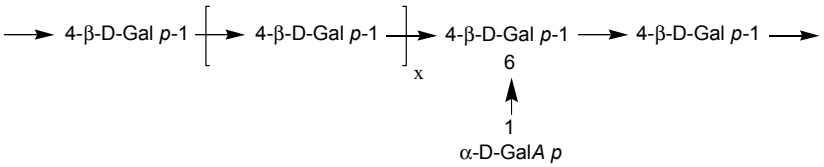
Arabinogalactan of larches



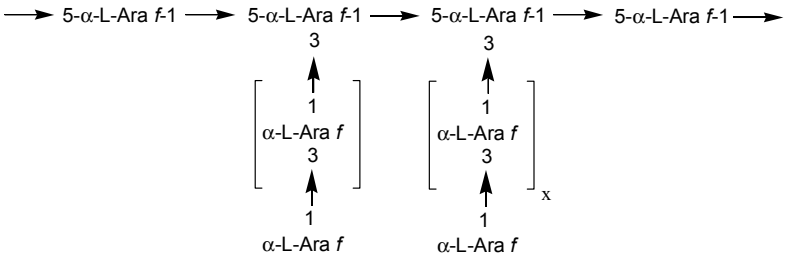
Rhamnogalacturonan



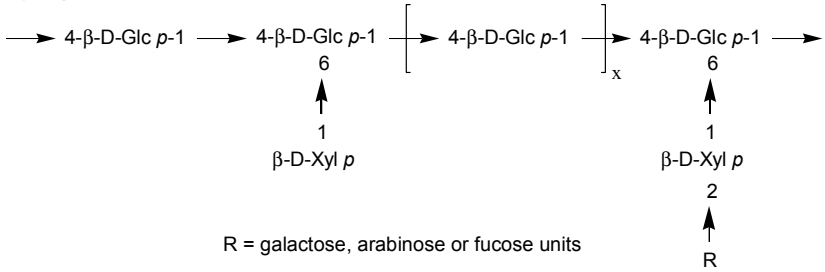
"Pectic" galactan



Arabinan



Xyloglucan



Laricinan (1->3-β-D-glucan)

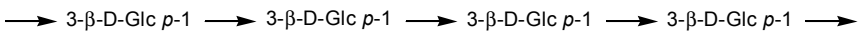


Figure 4. Structures of minor softwood hemicelluloses and pectins.

Cold and hot water extracts of conifers other than larches have been reported to contain arabinogalactans consisting of galactose, arabinose and minor proportions of uronic acids, as reviewed by Timell (1965, and references therein). The studies covered arabinogalactans from different pine and spruce species as well as Douglas fir.

Rhamnogalacturonan has a backbone of α-(1→4)-linked D-galacturonic acid units and α-(1→2) or α-(1→4)-linked L-rhamnose (Figure 4) (Fengel and Wegener 1989). The ratio of galacturonic acid to rhamnose units is about 8:1. Half of the rhamnose units carry a galactan side chain.

A *galactan* sometimes termed pectic galactan (see above) has been described mainly in compression wood (Figure 4) (Fengel and Wegener 1989) but also in enzymatically isolated lignin samples (V, and references therein). This galactan has a backbone of β-(1→4)-linked D-galactose units, partly substituted at the hydroxyl group of C6 with galacturonic acid units.

Arabinan has been isolated from the wood of maritime pine (Figure 4) (Fengel and Wegener 1989). Its backbone consists of α-(1→5)-linked arabinose units with side chains of arabinose units joined by α-(1→3) linkages.

Xyloglucan is known mainly as a polysaccharide in the primary cell wall of higher plants (Figure 4) (Fry 1989). It has the same backbone as cellulose with β-(1→4)-linked D-glucose units (Figure 2). Unlike in cellulose, there are side chains attached at the hydroxyl group of C6 in xyloglucans. The side chains consist either of single xylose units or of galactose, arabinose or fucose units (1→2)-linked to xylose. In softwood, xyloglucan is only a minor compound (Sjöström and Westermark 1998). Evidence for the presence of xyloglucan has been found in studies on oligomeric products of enzymatic hydrolysis from the jack pine holocelluloses (Perilä and Bishop 1961).

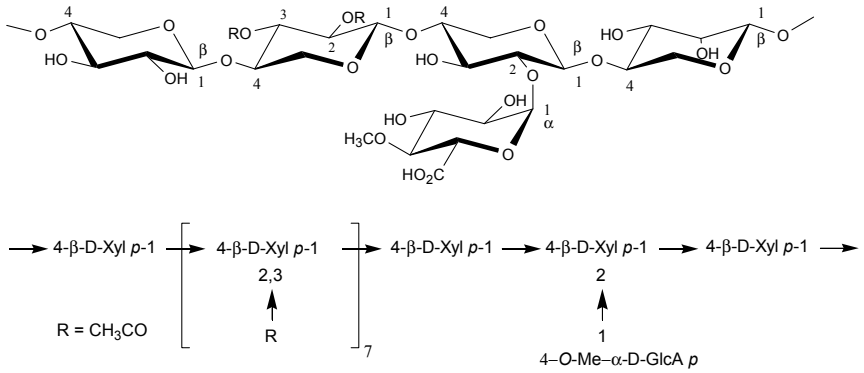
Other glucans include callose – a compound in sieve cells of phloem and parenchyma cells in xylem – and laricinan – known mainly as a component of compression wood, but also found in ray cells of normal wood (Figure 4) (Fengel and Wegener 1989, Hoffmann and Timell 1972a and b). Both components consist of a backbone of β-(1→3)-linked glucose units. Laricinan also has a small number of β-(1→4)-linked glucose units (6-7%) and a few side chains (about 8 per 200 units) of several glucuronic acid units and some galacturonic acid units.

Hardwood

The main hemicellulose in hardwood is a *xylan*, more specifically an *O*-acetyl-4-*O*-methylglucurono-ß-D-xylan (Figure 5) (Fengel and Wegener 1989, Sjöström 1993, Shimizu 2001). As in softwood xylan, the backbone consists of β-(1→4)-linked xylopyranose units. Most of the hydroxyl groups at C2 and/or C3 of the xylose units are substituted with acetyl groups. In addition, xylose units are substituted with α-(1→2)-linked 4-*O*-methylglucuronic acid residues, in most hardwood xylans on average at every 10th xylose unit. Unlike softwood xylan, hardwood xylan does not contain arabinose side chains.

Hardwood also contains *glucomannan* with a backbone of β-(1→4)-linked D-mannopyranose and D-glucopyranose units (Figure 5) (Fengel and Wegener 1989, Sjöström 1993, Shimizu 2001). The ratio of mannose to glucose units is between 1:1 and 2.3:1.

Xylan



Glucomannan

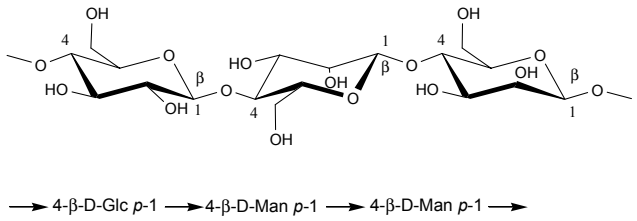


Figure 5. Structures of the major hardwood hemicelluloses.

Some reports on minor wood components describe the occurrence of the following components in hardwood:

Galacturonan is present in the middle lamella and tori of bordered pit membranes (Fengel and Wegener 1989).

The *glucans* described under softwood hemicelluloses, including callose, laricinan and xyloglucan, also occur in hardwood (Fengel and Wegener 1989).

A galactan containing rhamnose (*rhamnoarabinogalactan*) with a backbone of β-(1→3)-linked D-galactose units and a ratio of galactose:arabinose:rhamnose of 1.7:1:0.2 has been reported in sugar maple (*Acer saccharum*) (Fengel and Wegener 1989).

Tension wood of hardwood species has been reported to contain some *galactan* with a backbone of β-(1→4)-linked D-galactopyranose units, substituted to a high degree at the hydroxyl group of C6 with galactose units (Fengel and Wegener 1989, Shimizu 2001). Tension wood galactan also contains 4-*O*-methyl-D-glucuronic acid, D-glucuronic acid, D-galacturonic acid, L-rhamnose and L-arabinofuranosyl residues.

2.3 Lignin

Lignins are polymers consisting of phenylpropane units (Alén 2000a). The main precursor of lignin in softwoods is *trans*-coniferyl alcohol (Figure 6). In hardwoods, *trans*-sinapyl alcohol and *trans -p*-coumaryl alcohol are also lignin precursors. The precursors are polymerized during lignin biosynthesis.

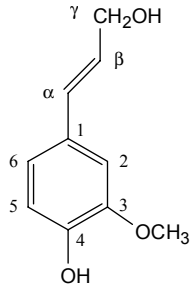


Figure 6. Structure of the softwood lignin precursor *trans*-coniferyl alcohol.

Typical linkages between the subunits are ether bonds and carbon-carbon bonds. The structure of lignin is often described in terms of the frequency of functional groups. Typical functional groups are methoxyl, phenolic hydroxyl, benzyl alcohol and carbonyl groups expressed, for example, per 100 phenylpropane units (Sjöström 1993). Several formulae for lignin have been suggested (Freudenberg 1968, Adler 1977, Brunow et al. 1998) (Figure 7).

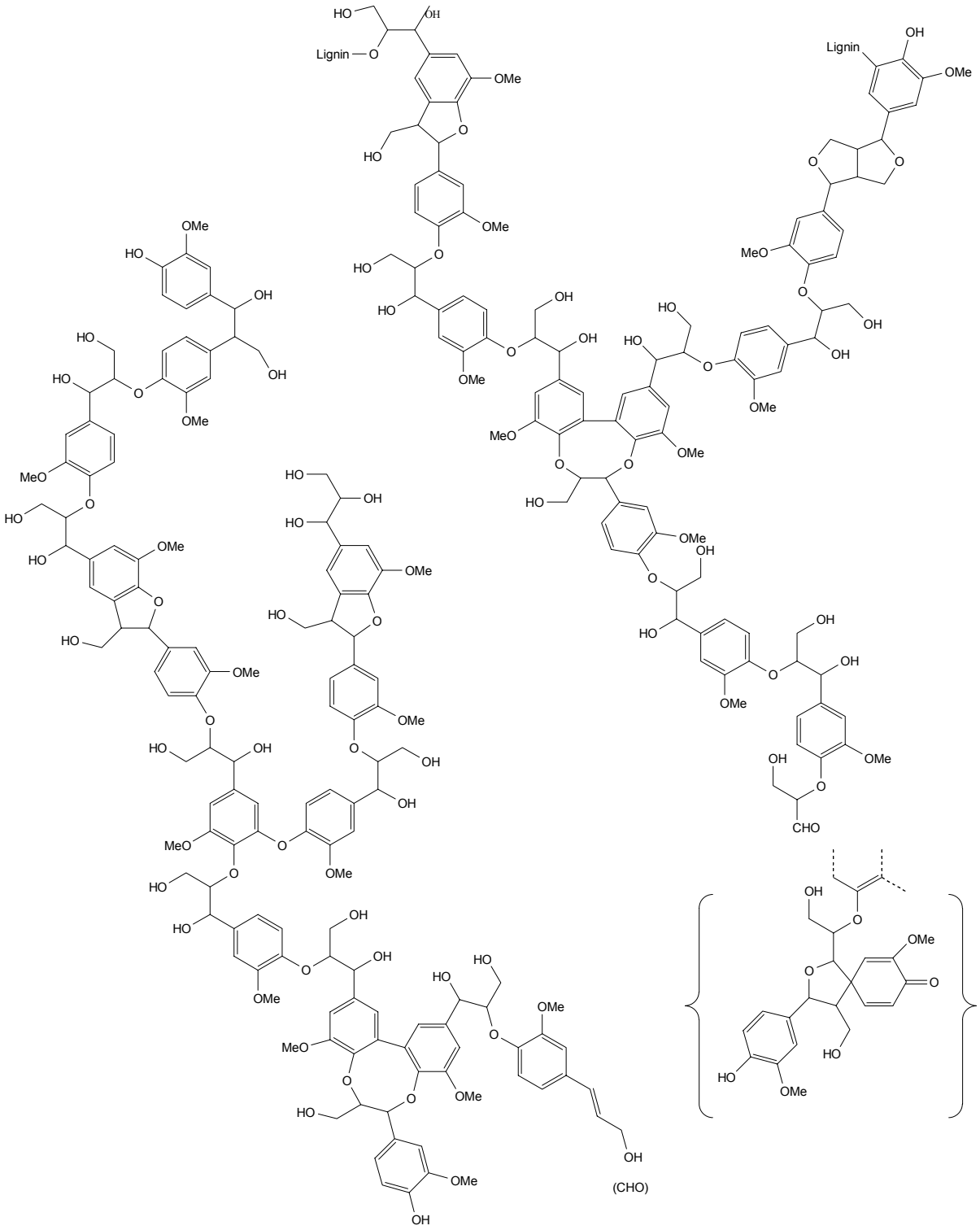


Figure 7. Schematic structure of softwood lignin (Brunow et al. 1998).

3 NON-STRUCTURAL COMPONENTS OF WOOD

Non-structural components of wood are the polysaccharides of starch, wood extractives, proteins, some water-soluble organic compounds and inorganic compounds (Alén 2000a).

Softwood and hardwood contain both *starch* (Fengel and Wegener 1989). Starch consists of both amylose and amylopectin (Figure 8). Linear amylose has a backbone of α-(1→4)-linked glucose units. Amylopectin has the same backbone, but is partly branched *via* α-(1→6) linkages.

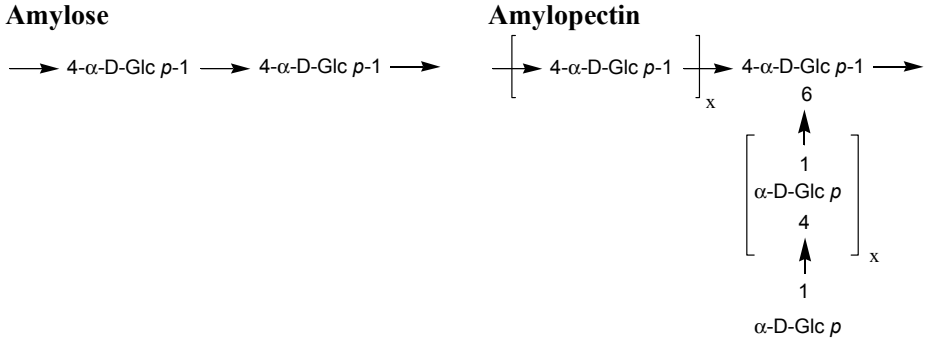


Figure 8. Structures of starch constituents.

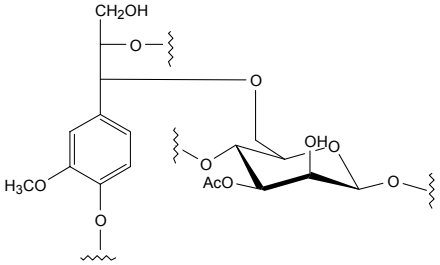
4 LIGNIN-CARBOHYDRATE BONDS

The presence of bonds between lignin and carbohydrates was first proposed in 1866 by Erdmann, who put forward the hypothesis that these constituents combine chemically (Erdmann 1866). The hypothesis is nowadays based on either indirect or direct evidence. Indirect evidence is obtained from efforts to isolate lignin either by extraction of wood (Björkman 1957, Lindgren 1958) or by isolation of residual lignin from milled wood or chemical pulp using enzymatic hydrolysis methods (Yamasaki et al. 1981, Minor 1982, 1986, 1991, Iversen 1985, Iversen and Westermark 1985, Iversen and Wännström 1986, Hortling et al. 1990, 2001). Carbohydrates have been found in the isolated lignin. Some evidence has been obtained using techniques such as alkaline degradation, acid degradation and Smith degradation (Koshijima and Watanabe 2003). Smith degradation includes oxidation with periodate, reduction to a polyalcohol with borohydride followed by hydrolysis with dilute acid under mild conditions. This procedure is used to identify glycol groups (vicinal primary or secondary hydroxyl groups). Reduction, methylation analysis, chromatography, spectroscopy and electron microscopy have also provided some evidence for lignin-carbohydrate bonds, as reviewed by Koshijima and Watanabe (2003). Direct evidence has been obtained using oxidative cleavage of benzyl ether and ester bonds (Watanabe 1989, Choi and Faix 1999, Kosíková and Eberingová 1999, Koshijima and Watanabe 2003) and administration of ^{13}C-enriched lignin precursors to ginkgo shoots (Xie et al. 2000).

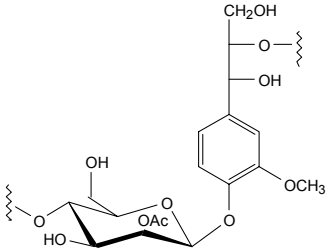
Proposed lignin-carbohydrate bonds have been reviewed recently (Watanabe 2003, and references therein) (Figure 9):

- a benzyl ether type between the α-hydroxyl group of a lignin unit and a hydroxyl group of a carbohydrate
- a benzyl ester type between the α-hydroxyl group of a lignin unit and a carboxylic acid group of a carbohydrate
- a glycoside type between an aliphatic or aromatic hydroxyl group and the reducing end group of carbohydrates
- an acetal type between two hydroxyl groups of carbohydrates and a carbonyl group of lignin

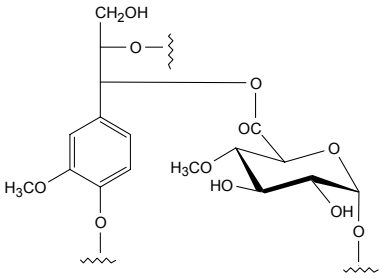
Benzyl ether type



Glycosidic type



Benzyl ester type



Acetal type

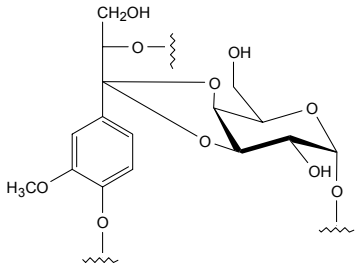


Figure 9. Proposed structures of the different bonds between lignin and hemicelluloses (adapted from Watanabe 2003).

Carbon-carbon bonds between the xylan and lignin in kraft pulp have also been suggested (Vikkula et al. 2001) (Figure 10). The suggestion was based on condensation reactions observed in model compound studies of 4-*O*-methyl glucuronoxylo-oligosaccharides and creosol (lignin model compound) under kraft pulping conditions.

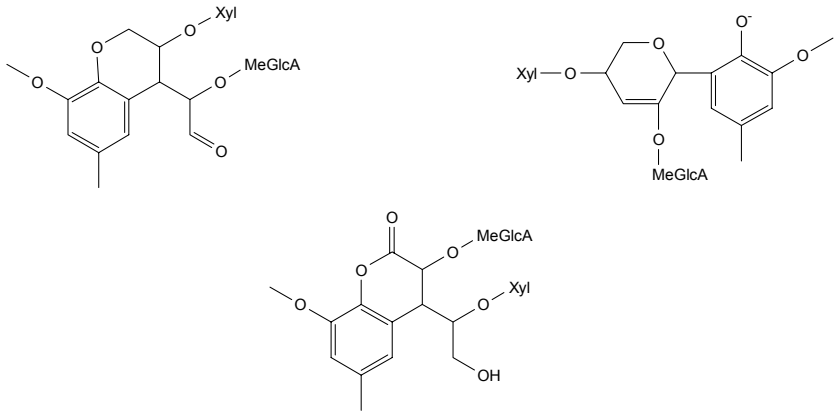


Figure 10. Condensation products of 4-*O*-methyl glucuronoxylo-oligosaccharides and creosol (lignin model compound) (Vikkula et al. 2001).

5 WOOD FIBERS

The wood in softwoods is composed of two different cell types – tracheids (90-95%) and ray cells (5-10%) (Sjöström 1993). The tracheids of Scandinavian softwoods are long and narrow with an average length of 2-4 mm and an average width of 0.02-0.04 mm. The ray cells – parenchyma cells and ray tracheids – are 0.1-0.16 mm long and 0.002-0.050 mm wide. The term fiber is used from this point on to refer to tracheids.

Hardwoods contain several cell types specialized for different functions (Sjöström 1993). These cell types are libriform fibers (supporting tissue), vessel elements (conducting tissue), ray parenchyma cells (storage tissue) and hybrids of these cell types classified as tracheids. The term fiber denotes specifically libriform fibers and tracheids. Libriform fibers of hardwood are shorter than softwood tracheids, averaging 1.1-1.2 mm in length and 0.014-0.040 mm in width. Vessels are even shorter (0.3-0.6 mm) and wider (0.03-0.130 mm).

Wood fibers (wood cells) have a thick fiber wall. The fiber wall comprises several layers (Sjöström 1993). From the outside to the inside the layers are middle lamella (ML), primary wall (P), outer layer of the secondary wall (S1), middle layer of the secondary wall (S2), inner layer of the secondary wall (S3) and warty layer (W) (Figure 11).

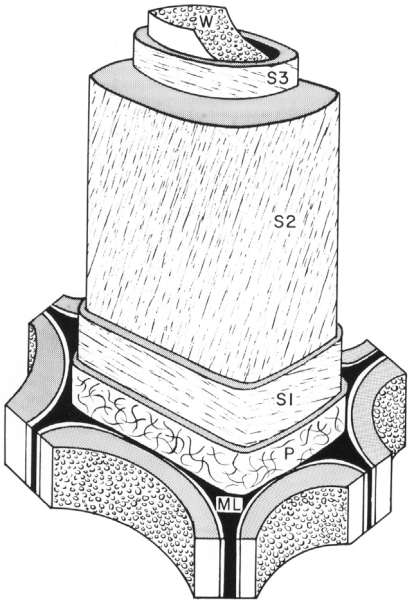


Figure 11. Simplified structure of a woody cell, showing the middle lamella (ML) and the layers of the cell wall (P, S1, S2, S3 and W, see text) (Côté 1967).

6 DEFIBRATION PROCESSES

6.1 Mechanical pulping

In mechanical pulping, the fibers are separated from each other by mechanical forces. Basically, this involves either pressing wood logs against a revolving pulpstone (grinding) or disintegrating wood chips in a disk refiner (refining) (Sundholm 1999). Currently the main types of mechanical pulp are groundwood (GW), pressured groundwood (PGW), thermomechanical pulp (TMP) and chemithermomechanical pulp (CTMP). Mechanical pulping is typified by high yields (97-98%). This implies that the mechanical pulp contains the wood components in a fairly unchanged composition. Water-soluble components dissolve both during mechanical pulping itself and in the water circulation of paper machines. The water-soluble organic components of wood and TMP include carbohydrates (including mono-, oligo- and polysaccharides), lignans, low-molar-mass acids, low-molar-mass lignin and lipophilic wood extractives (Sjöström 1990, Ekman et al. 1990, Thornton 1993).

6.2 Alkaline delignification

6.2.1 Chemical pulping

In chemical pulping, “Wood pulp fibers are manufactured by chemically dissolving those components, mainly lignin, that keep wood cells together to form the original wood structure” (Gullichsen 2000a). Enough lignin from the (lignin-rich) middle lamellae has to be dissolved to free the undamaged wood fibers from each other. Aqueous solutions of several alternative alkaline, neutral or acidic components at elevated temperature and pressure are used to dissolve lignin and some carbohydrates from wood chips. The aqueous solutions of cooking chemicals penetrate from the lumen through the cell wall towards the middle lamellae. (Gullichsen 2000a).

The polysaccharides of wood react during alkaline cooking. Alkali is consumed by the rapid hydrolysis of acetyl groups of (galacto)glucomannans in softwoods and xylan in hardwoods (Sjöström 1993, Gellerstedt 2001). The dissolution of hemicelluloses and the degradation of cellulose are the limiting factors in alkaline cooking. Substantial amounts of xylan and around 75% of glucomannan are lost from softwoods during cooking. The predominant degradation reactions of polysaccharides are alkali-induced stepwise eliminations of monomeric units, starting from the reducing end. These reactions are referred to as peeling reactions. The reactions depend very much on alkali charge and concentration and on cooking time and temperature.

The first industrial alkaline cooking process was soda cooking using sodium carbonate and sodium hydroxide as chemicals (Gullichsen 2000 a, b). Nowadays, the kraft process is the dominant chemical pulping method. It is a strongly alkaline process, in which the active components are the hydroxide (OH^-) and the hydrosulfide (SH^-) ions. The cooking chemicals are recovered and regenerated. The kraft cooking liquor is a mixture of white liquor, water in wood chips, condensed steam and weak black liquor. White liquor is regenerated from spent cooking liquor. Black liquor comes directly from the preceding cook.

The dissolution of lignin in kraft pulping is mainly due to the cleavage of β-aryl ether linkages in β-O-4 structures (Sjöström 1993, Gellerstedt 2001). The first step is the formation of a quinone methide intermediate from free phenolic structures in lignin after the elimination of the α-substituent (hydroxide, alkoxide or phenoxide). In the presence of hydrogen sulfide ions, nucleophilic attack by species such as SH^- at the α-carbon of the quinone methide intermediate is the next step. The thiol reacts further to a cyclic thiirane under cleavage of β-aryl ether bond. The result is lignin fragmentation together with liberation of free phenolic hydroxyl groups. The free phenolic groups are ionized under the strongly alkaline pulping conditions and the phenolates formed improve the solubility of the liberated lignin fragments. Certain complex reactions also occur in the kraft cooking process.

Polysulfide (PS) cooking was introduced in order to retain more carbohydrates, in particular hemicelluloses (Gullichsen 2000b). PS can be prepared by adding elemental sulfur to white liquor. PS stabilizes hemicelluloses at low temperature (100°C-120°C) by oxidizing the reactive end groups (reducing end groups) of polysaccharides to form alkali-stable carboxylic acid end groups (e.g. aldonic acid or metasaccharinic acid end groups). This slows down the peeling reactions. The oxidized carbohydrate end groups reduce the degradation and subsequent dissolution of carbohydrates in cooking.

Anthraquinone (AQ) can be used as an additive in alkaline cooking to stabilize the carbohydrates against alkaline degradation (Gullichsen 2000b). AQ oxidizes the reducing end groups, too. AQ is reduced to anthrahydroquinone (AHQ), which is soluble in alkaline media. AHQ reduces lignin to a more reactive form and AQ is regenerated.

6.2.2 Oxygen delignification

Unbleached chemical pulp still contains some lignin, and this is referred to as residual lignin (Sjöström 1993). The residual lignin cannot be removed during chemical pulping itself, because towards the end the process becomes slow and unselective towards lignin. Degradation of polysaccharides increases towards the residual delignification of cooking. Therefore, pulp strength characteristics suffer if the cooking process is continued. One reason for the difficulty of removing residual lignin lies in the complex fiber matrix. In addition, linkages between lignin and carbohydrates (as described in Chapter 4) have been suggested to limit the delignification.

The delignification of chemical pulp after chemical pulping is nowadays often continued with oxygen delignification as the next step (Gellerstedt 2001). Around 50-65% of the residual lignin can be removed from softwood kraft pulp without detrimentally affecting the pulp strength characteristics.

Oxygen delignification is carried out in alkaline medium (Gellerstedt 2001). Partial oxidation of phenolic structures in lignin takes place. This is accompanied by oxidation of conjugated double bonds, for example in enol ethers and stilbenes. Complex reactions including radical intermediates lead to removal of part of the residual lignin.

Under the conditions used for oxygen delignification hydroxyl radicals are formed (Gellerstedt 2001). These attack the cellulose and can finally lead to cleavage of the cellulose chains. This impairs the strength of the pulp. This undesirable side-reaction can be reduced by the addition of magnesium ions. Yield losses are limited, because the peeling reactions of polysaccharides described above are suppressed in favor of oxidative stabilization reactions. The reducing ends of polysaccharide chains are oxidized to carboxylic acid end groups as described for polysulfide and anthraquinone cooking.

7 STRUCTURE OF RESIDUAL LIGNIN

After the cooking process, chemical pulp typically contains less than 5% lignin. This lignin is referred to as residual lignin (Yamasaki et al 1981, Tamminen and Hortling 1999, Alén 2000b). Cleavage of β-aryl ether bonds during cooking leads to the formation of new free phenolic hydroxyl groups. These groups are also formed *via* partial demethylation of the methoxyl groups of lignin. The resulting phenolic structures are reactive during oxygen delignification and further bleaching of the pulp. Less reactive, typically condensed lignin structures, are enriched during cooking. Some condensation as well as the formation of new lignin-carbohydrate linkages may even take place during cooking.

The molar mass of residual lignin decreases during cooking (Tamminen and Hortling 1999). The molar mass of native lignin cannot be reliably measured, as the yield from its isolation as milled wood lignin (MWL) is only ca. 50% and MWL is thus not fully representative. Some degradation may also take place during the isolation. The molar mass of enzymatically isolated residual lignin is higher than that of MWL.

8 METHODS FOR POLYSACCHARIDE ANALYSIS

8.1 Total carbohydrate content and composition

The total carbohydrate content and composition of polysaccharides-containing samples of different origins can be determined after hydrolysis to the monosaccharides with acids or enzymes (Scherz and Bonn 1998):

- Non-cellulosic polysaccharides containing neutral sugars can be hydrolyzed with dilute sulfuric acid or trifluoroacetic acid at elevated temperature.
- Non-cellulosic polysaccharides containing neutral sugars and uronic acid groups can be hydrolyzed using either methanolic hydrochloric acid at elevated temperature or 72% sulfuric acid at room temperature.
- Cellulose-containing materials can be hydrolyzed by pretreatment with 72% sulfuric acid at room temperature followed by heating of the diluted (1M) solution.
- Enzymes acting on different polysaccharides can be used for hydrolysis. Mixtures of enzyme activities are capable of hydrolyzing cellulose-containing materials and polysaccharides containing neutral sugars and uronic acid groups.

There are several ways to quantify the resulting monosaccharides as total sugars (Scherz and Bonn 1998, Vuorinen and Alén 1999). The presence of reducing sugars can be shown by means of oxidation reactions. Reducing sugars contain an aldehyde group, which can be oxidized to a carboxylic acid group by Cu(II), for example using Fehling's reagent ($CuSO_4$ / tartrate). The resulting precipitate of Cu_2O can be quantified either gravimetrically, titrimetrically or photometrically. Other procedures use hexacyanoferrate(III), 3,5-dinitrosalicylic acid or other aromatic nitro compounds and tetrazolium salts combined with photometric methods. Reducing sugars can also be quantified by reaction with phenylhydrazine to osazones followed by photometric determination. Other detection methods are based on the formation of furfural and 5-hydroxyfurfural and their condensation with a colored reagent or oxidation of 1,2-diol structures.

The liberated monosaccharides can be separated and quantified by chromatographic methods (Vuorinen and Alén 1999). Most common are gas chromatography (GC), high performance liquid chromatography (HPLC) and anion exchange chromatography (AEC). Recently, capillary electrophoresis (CE) has also been used.

8.2 Structural analysis

Methylation analysis is an established method for the structural analysis of wood and pulp polysaccharides (Aspinall 1959, Bouveng and Lindberg 1960, Timell 1964, 1965). The analysis yields information on the substitution of the different monosaccharide building block units

The first step in methylation analysis is the permethylation of non-substituted hydroxyl group units in the intact polysaccharide or carbohydrate residue (Scherz and Bonn 1998). The next steps are cleavage of glycosidic bonds and derivatization of the liberated hydroxyl groups. The most wide used methylation analysis comprises methylation, hydrolysis, reduction, acetylation and gas chromatography.

In methylation analysis, no differentiation can be made between units with or without a glycosidic bond, because the glycosidic bonds are hydrolyzed in the hydrolysis step of the procedure. Aldose units (monosaccharides with an aldehyde group) without a glycosidic bond are referred to as "reducing end units" (reducing sugars, see above). Reducing end units are mainly present in the hemiacetal ring form – the form presented in most figures. In solution, the hemiacetal is in equilibrium with the open-chain aldehyde, the open-chain hydrate and the anomers with different ring sizes (Sjöström 1993). This phenomenon is called mutarotation. Mutarotation of D-glucose is shown in Figure 12. The hemiacetal can be a five or six-membered ring (furanose or pyranose form). The units at the other end of the main chain and the end units of side chains are called "non-reducing end units". The analysis does not distinguish between these two types of end unit.

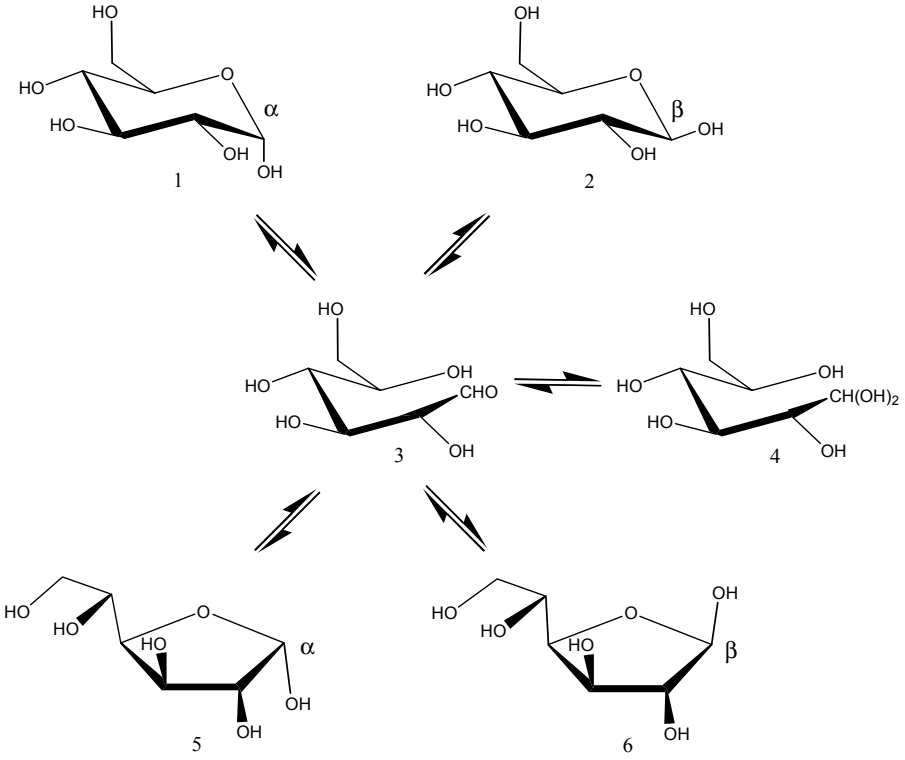


Figure 12. Mutarotation of D-glucose. α-D-Glucopyranose (1), β-D-glucopyranose (2), the open-chain aldehyde form (3), the open-chain hydrate (4), α-D-glucofuranose (5), and β-D-glucofuranose (6).

The structures of polysaccharides can also be studied using partial hydrolysis (acid or enzymatic hydrolysis) combined with chromatographic methods (Aspinall 1959, Bouveng and Lindberg 1960, Timell 1964, 1965, Scherz and Bonn1998). Other methods are selective oxidation, alkaline degradation, acid degradation and Smith degradation. The procedure for the Smith degradation was described in Chapter 4.

Modern techniques have been developed for structural analysis, among them NMR (Vuorinen and Alén 1999 and references therein, Capek et al. 2000, Teleman et al. 2000, 2002, 2003, Marques Domingues and Evtuguin 2001, Lundquist et al. 2002, Hannuksela and Hervé du Penhoat 2004) and matrix-assisted laser desorption ionization (MALDI) mass spectroscopy (Rydlund and Dahlman 1997, Jacobs and Dahlman 1999, 2001, Marques Domingues and Evtuguin 2001, Jacobs et al. 2002, Teleman et al. 2003, Lundquist et al. 2003, Dahlman et al. 2003, 2004, Mais and Sixt 2004). However, these modern 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.

9 MATERIALS AND METHODS

9.1 Separation methods

9.1.1 Water-soluble polysaccharides

Stem cross-sections of fresh and healthy Norway spruce (*Picea abies*), Scots pine (*Pinus silvestris*) and Siberian larch trees (*Larix sibirica*), grown in southern Finland, were used to isolate water-soluble polysaccharides. In addition, TMP from Norway spruce was used (II, III).

The arabinogalactan samples were isolated from knot-free parts of heartwood of all three wood species (II). For the isolation, the wood was splintered, freeze-dried, ground and extracted using methyl *tert*-butyl ether. The extracted wood powder was stirred vigorously in distilled water at pH 7 and 2.5% consistency for 1.5 h at room temperature. The arabinogalactan fractions were obtained by precipitation from the concentrated filtrate.

Glucomannan samples were isolated from knot-free sectors of two Norway spruce trees (III) with no visible reaction wood. The sectors were splintered, freeze-dried, ground and blended to form a composite sample. TMP was obtained from a Finnish mill using two-stage refining of Norway spruce. TMP was stored at 40% consistency at -24ºC before freeze-drying. Both the composite wood meal sample and the TMP sample were extracted with acetone to remove lipophilic extractives. The extracted samples were vigorously stirred in distilled water at 23±2°C for 3 h at 2.9% consistency. The pH was not adjusted. The suspension was filtered first on a paper machine wire (<400 mesh) and the filtrate was further filtered on glass fiber filters and then on a 0.2-µm filter. The final filtrate was concentrated and polysaccharides were precipitated with ethanol. The once water-extracted fibers/wood meals were then vigorously stirred in distilled water at 90°C for 1 h at the same consistency as the first water extraction. Dissolved polysaccharides were isolated as described above. A third water extraction was performed in distilled water at the same consistency as before at 90°C for 12 h followed by polysaccharide isolation as described above.

Galactoglucomannan was also isolated from TMP to a "standard" procedure developed earlier by Sundberg et al. (2000) (II, III). The TMP was extracted using hexane:acetone 9:1. The extracted TMP was repeatedly stirred in distilled water at 60°C and 2% consistency for 3 h. The suspensions were filtered on a paper machine wire and the filtrates were centrifuged. The supernatants were concentrated and filtered on a 0.2-µm filter. The polysaccharides were obtained by precipitation.

9.1.2 Polysaccharides in effluents from oxygen delignification

Conventional laboratory-cooked kraft pine (*Pinus silvestris*) and birch (*Betula pendula/pubescens*) pulps were treated in rotating reactors (1l) in an air bath (IV). The conditions were 9% consistency, 100ºC, 3% NaOH, 0.2% $MgSO_4$ and 8 bar oxygen or nitrogen pressure in oxygen and reference alkaline treatments, respectively. The pulp suspensions were filtered through a plastic wire. Both pulps and filtrates were collected.

Ultrafiltration was performed on the supernatant of the filtrates after pH adjustment to 2.5 and centrifugation. A Prep/Scale™ TFF cartridge (polyethersulfone, membrane size 0.09 m^2, Millipore, USA) with a cut-off value corresponding to a nominal mass limit of 10 kDa was used. The retentate (high-molar-mass fraction) was washed with water adjusted to pH 2.5 and freeze-dried (Liukko and Poppius-Levlin 1999) (IV).

9.1.3 Residual lignin-carbohydrate complexes

Conventional kraft pulp was prepared from spruce chips (*Picea abies*) (V). Two parallel pulps were prepared from the same batch of spruce chips. The kappa number of the first pulp was 35.7 (SCK1) and of the second 37.3 (SCK2). SCK1 (1.3 kg) was refined in a Voith Sulzer laboratory refiner at 4% consistency with a specific edge load of 1.0 Ws/m. The refined pulp was fractionated using a filter with a 200-mesh wire into fines (SCK1 Fine, 4.5% of the refined pulp) and fibers (SCK1 Fiber) as described previously (Liitiä et al. 2000a).

Alkaline chemical pulps were prepared from pine wood chips (*Pinus silvestris*) from southeastern Finland (V). The unbleached chemical pulps produced were conventional kraft pulp (PCK), a polysulfide/anthraquinone pulp (PPSAQ) and a soda/anthraquinone pulp (PSoAQ). The cooking conditions were selected to obtain kappa numbers close to 30 for the different pulps. The unbleached pulps were oxygen-delignified to a target delignification of 50%. The resulting kappa numbers were 17.2 for PCK/O, 18.7 for PPSAQ/O and 16.3 for PSoAQ/O, which was close to the target of 50% delignification (Hortling et al. 2000).

Residual lignin-carbohydrate complexes (RLCCs) were isolated from the pulps and pulp fractions by enzymatic hydrolysis with the cellulolytic enzyme preparations Econase CEP (Röhm Enzymes) and Novozym 188 (Novo Nordisk), followed by protease purification using Subtilisine (Sigma) (V). Two fractions were obtained: the insoluble residue at the pH of the purification (pH 9.5) (prois) and the precipitate after pH adjustment to 2.5 (proRL) (Tamminen and Hortling 1999). Further fractionation of the prois sample was performed by dissolution in 0.5 M NaOH. The fraction insoluble in 0.5 M NaOH was called proisis and the soluble fraction in 0.5 M NaOH obtained after precipitation at pH 2.5 proissP.

The content of protein impurities due to the enzymatic isolation was calculated from the nitrogen content (Analytical Laboratories, Lindlar, Germany) multiplied by 6.25. These protein impurities are known to contain 10% carbohydrates, consisting of 85% mannose, 8.5% glucose and 6.5% galactose (Tamminen and Hortling 1999).

9.2 Structural analysis of carbohydrates

Methylation analysis (I-IV)

About 20 mg of ground NaOH and 0.1 ml of methyl iodide were added to 3-5 mg of the sample in 0.5 ml dimethylsulfoxide (DMSO). The sample was then 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 to dryness (Ciucanu and Kerek 1984, Asres and Perreault 1997).

2 ml of 2 M HCl/methanol was added to the methylated samples in pear-shaped flasks fitted with teflon seals. 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 reduced pressure of 5 kPa and bath temperature of 50°C for 10 min. The samples were transferred to small glass bottles using 0.08 ml dry pyridine.
The samples were silylated with 0.25 ml *N,O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) containing 5% trimethylchlorosilane (TMCS). Analysis was performed using gas chromatography coupled with mass spectroscopy (GC/MS) (I).

Methylation analysis of RLCCs (V)

Methylation was performed as described above using a 20 mg RLCC sample. Water was added after the ultrasonic treatment, pH was adjusted to 2.5 and the sample was centrifuged at 6000g for 20 min. The residue was freeze-dried. Two or three methylation cycles were performed followed by acid methanolysis, silylation and GC/MS analysis.

Analysis of reducing ends (V)

RLCC samples were reduced using $NaBH_4$ in 0.1 M NaOH. The RLCC was precipitated by adjusting the pH to 2.5 using 1 M HCl. The precipitate was obtained by centrifugation and freeze-drying. The reduced RLCC sample was subjected to acid methanolysis. Identification and quantification was performed using GC/MS as described above with the exception that inositol was used as internal standard instead of sorbitol.

9.3 Other determination methods

Carbohydrate content and composition were determined by acid methanolysis and GC according to Sundberg et al. (1996) (II, III, IV, V) and by acid hydrolysis and high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC/PAD) according to Hausalo (1995) (IV, V). The water solubility of wood and pulp (TAPPI T207 cm-99, III), total organic carbon (TOC) (SFS-EN 1484, IV), kappa number (SCAN-C 1:77 / V, ISO 302, IV), viscosity (SCAN-CM 15:99, IV, V) and ISO brightness (ISO 2470, IV) were determined according to standard procedures. Lignin content was determined by UV spectroscopy (Tamminen and Hortling 1999, IV) and as Klason and acid-soluble lignin after acid hydrolysis (Browning 1967). The content of hexenuronic acid groups was determined for the hydrolyzate of the enzymatic hydrolysis before precipitation of lignin using HPAEC/PAD (Tenkanen et al. 1995, V). High-performance size exclusion chromatography (HPSEC) was used to determine the apparent molar mass of the polysaccharides isolated (III). ^{13}C NMR and ^{1}H NMR spectroscopic analyses were performed according to II and III.

10 RESULTS AND DISCUSSION

10.1 Structural analysis of polysaccharides (I)

The main method employed in this thesis is the methylation analysis. Information on the substitution of the different monosaccharide building block units is obtained (see Chapter 8.1).

A fairly new variation of methylation analysis was further developed and verified using model compounds (I). The analysis protocol includes (Figure 13):

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

This variation differs from the more common variations of methylation analysis, which include methylation, hydrolysis, reduction, acetylation and GC analysis. The fact that one stage (reduction) could be omitted was seen as an advantage. In addition, no ion exchange step is necessary after the methanolysis employed here. After the more commonly used acid hydrolysis, removal of ions is necessary before acetylation can be carried out. A third advantage is that more than one peak in the gas chromatogram can be used for identification and quantification. This is due to the fact that the α and β anomers equilibrate during acid methanolysis of the methylated oligo- or polysaccharides. Both anomers are present after this step and information on 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 (Figure 1 in paper I). 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.

This new procedure applied has been described in the literature (Kamerling and Vliegenthart 1989, Vuorinen and Alén 1999), but no covering data have been published on retention times and quantification using mass spectrometric detection in the linkage analysis of polysaccharides common in wood and pulp. This was done in the present work (I).

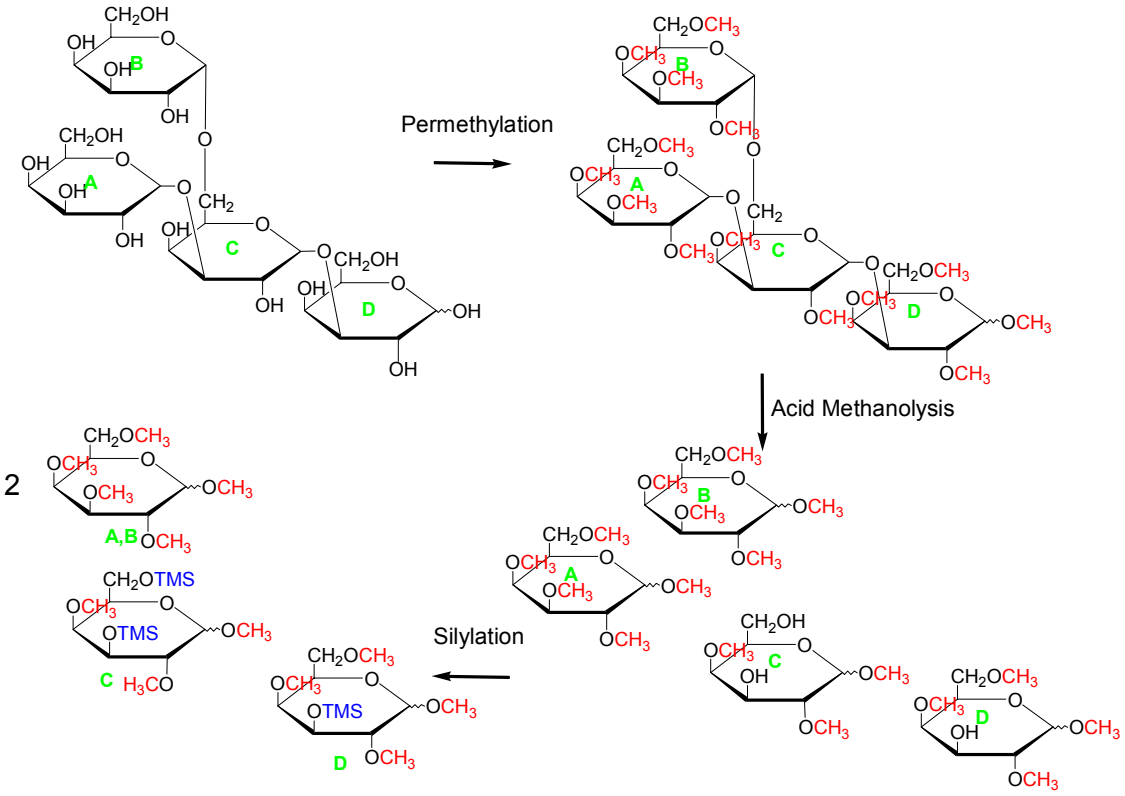


Figure 13. The reactions during methylation analysis are shown for a tetramer of galactose units. Permethylation leads to quantitative methylation of all free hydroxyl groups. Acid methanolysis hydrolyzes the glycosidic bonds and leads to formation of the α and β anomers of the methyl glycosides. Finally, silylation silylates the hydroxyl groups liberated in the acid methanolysis step. A,B = non-reducing end units, C = 3,6-substituted unit and D = 3-substituted and reducing end unit.

The method was tested for model compounds. It was important to find conditions that do not lead to the loss of fully methylated non-reducing end units in the work-up procedure after the methanolysis. Evaporation of methanol is necessary before silylation can be performed. On the other hand, at higher temperatures and low pressures, fully methylated pentoses and to a smaller extent hexoses evaporate, too. Evaporation conditions were found using a rotary evaporator at a pressure of 5 kPa for a short time instead of a vacuum oven at lower pressure. The silylation worked and losses were avoided (Figure 3 in paper I). The optimized conditions yielded good and reproducible results for the oligosaccharides tested (Figure 4 in paper I).

Other problems can be encountered in samples containing uronic acids. The hydrolysis of the glycosidic bond of uronic acid units is not quantitative under conditions suitable for the cleavage of glycosidic bonds of neutral units (Scherz and Bonn 1998). In addition, 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 the rest of the decomposed acid throughout the methylation, and secondly that the acid methanolysis cleaved the remainder. A substituted xylotetraose with one 4-*O*-methylglucuronic acid substituent at the third xylose unit (MeGlcA-Xyl_4) (Teleman et al. 1996) was chosen.

The results showed that the position of uronic acid substituents was only partly determined (Figure 5 in paper I). The problem of incomplete hydrolysis of the glycosidic bond can be solved if reduction of the methylated polysaccharide is performed as suggested for a xylan of *Eucalyptus globulus* (Shatalov et al. 1999). This reduction of the carboxyl group of the methylated 4-*O*-methylglucuronic acid unit leads to partly methylated glucose labeled with deuterium. In the subsequent hydrolysis, the substituted glucose unit is a neutral substituent and the cleavage of the glycosidic bond presents no problem. Without the reduction step, care must be taken when interpreting the substitution of units in xylan or other acidic polysaccharides that contain uronic acids.

The method developed in paper I was applied in later work. A comparison with NMR techniques was performed for isolated water-soluble polysaccharides. This provided the opportunity to evaluate the method with "real samples".

The methylation analysis employed showed limitations in two cases:

- Arabinogalactans did not dissolve in the solvent used in the permethylation step (solid sodium hydroxide-dimethylsulfoxide, NaOH-DMSO). As a result, only a small fraction of the sample was analyzed (II).
- Carbohydrate residues in residual lignin-carbohydrate complexes (RLCC) were not permethylated in one methylation cycle. Even a second methylation cycle was not enough. Only after the third methylation cycle the degree of methylation was close to quantitative for alkali-soluble RLCCs (>90%) (V).

These restrictions have to be considered when choosing a suitable methylation technique. The first problem can be overcome by choosing a technique for water-soluble polysaccharides. The second problem has also been encountered for glucose units using another methylation technique (Minor 1982). Even repeated methylation cycles probably do not produce complete methylation, because some of the carbohydrate residues are not accessible to the reagent for steric reasons. This steric hindrance is probably due to the close interaction of carbohydrate residues with lignin, possibly even the covering of carbohydrate residues by lignin.

The methylation analysis did not distinguish between reducing end group units and units with glycosidic bonds in the carbohydrate chain. Both yield methyl glycosides in the acid methanolysis step (Figure 13). A separate analysis of reducing ends was performed based on reduction using sodium borohydride ($NaBH_4$) before acid methanolysis (V). The reducing end group units are reduced to alditols and can be quantified separately from the methyl glycosides originating from glycosidically bound units (Figure 14).

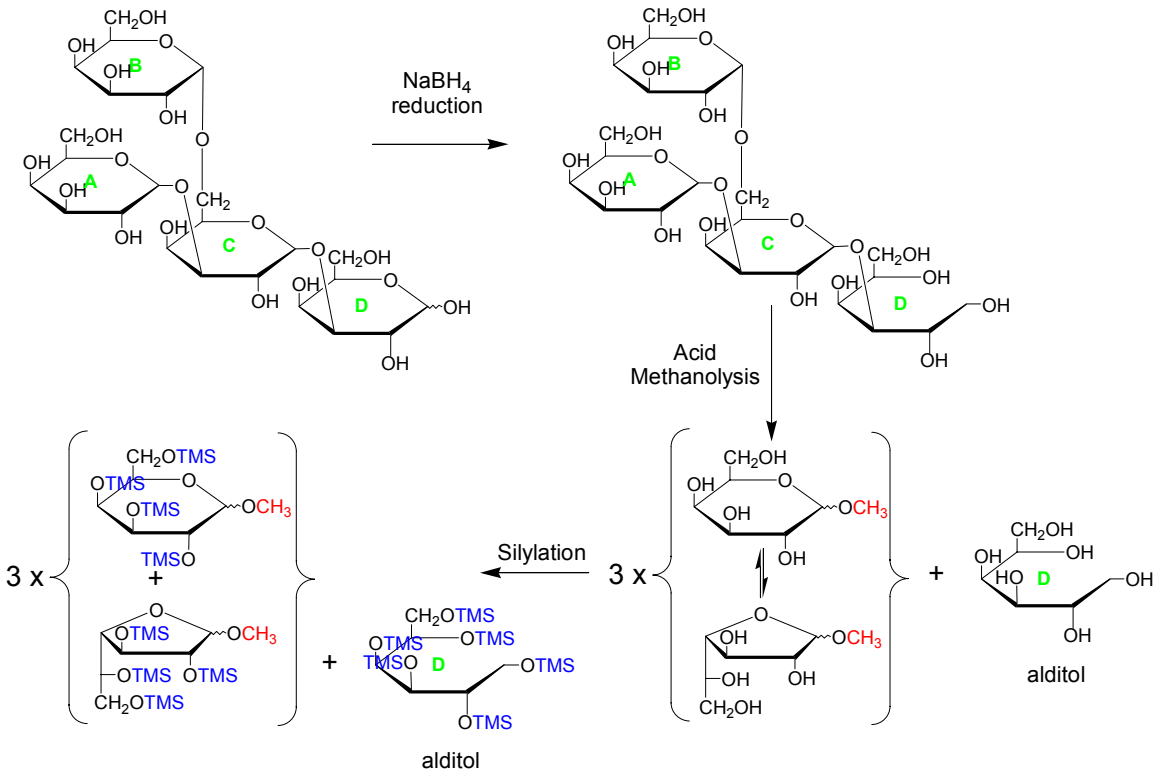


Figure 14. Reduction followed by acid methanolysis is shown for a tetramer of galactose units. A,B = non-reducing end units, C = 3,6-substituted unit and D = 3-substituted and reducing end unit.

The chain length of short polysaccharides and oligosaccharides can be estimated based on the proportion of reducing end units or non-reducing end units. In the case of molecules with linear backbones and unmodified end units, the number of non-reducing end units should equal the number of reducing end units.

Carbohydrate residues in RLCCs have short chains, because the enzymes used for the isolation of the RLCCs hydrolyze the major part of the carbohydrate backbones. Enzymatic hydrolysis produces carbohydrate residues with reducing end units. This differs from carbohydrates in chemical pulp. The strongly alkaline conditions in chemical pulping are known to convert the reducing end units of carbohydrates. Peeling reactions combined with stopping reactions produce mainly metasaccharinic acids end groups (Sjöström 1993).

In RLCCs, an oligomeric or polymeric carbohydrate chain can be bound to the lignin *via* the non-reducing end unit, the reducing end unit, or any backbone unit of the chain. Apparent average degrees of polymerization can be calculated based on the proportion of end units and other backbone unit of the chain. This information was used to suggest possible binding sites in the carbohydrate chains (Chapter 10.3.1, V).

10.2 Structural features of water-soluble polysaccharides

The major water-soluble polysaccharides of Norway spruce and Scots pine in the pH range 4 to 8 are *O*-acetylated galactoglucomannans and acidic arabinogalactans (Aspinall and Wood 1963, Thornton et al. 1994a and b).

10.2.1 Spruce and pine heartwood arabinogalactan (II)

At neutral pH, spruce and pine heartwood released about 0.5% and 0.8% of the dry matter, respectively, of polysaccharides in the water extraction employed here. Larch heartwood, extracted as a reference, released 15% of polysaccharides. The isolated spruce polysaccharides comprised approximately 45% arabinogalactans (AGs), 45-50% galactoglucomannans (GGMs) and 5-10% other polysaccharides. The isolated pine heartwood polysaccharides in turn comprised approximately 80% AGs, 10% GGMs and less than 10% other polysaccharides. As much as 97% of the larch polysaccharides were AGs (Willför and Holmbom 2004). The GGM sample isolated from spruce TMP contained mainly GGM and a small amount of AG (II).

Structural analysis using the methylation analysis was difficult; problems with the solubility of the AGs were experienced in the alkaline solvent (NaOH-DMSO) employed in the methylation. Only a small fraction of the AG was analyzed and consequently the results are not representative of the sample as a whole. This disturbed in particular the analysis of spruce heartwood AGs, because GGMs, present in about the same amounts as AGs, were soluble and methylated without problems.

^{13}C NMR spectroscopic analysis showed the presence of the following galactose and arabinose units: β-D-Galp-(1→, →6)-β-D-Galp-(1→, →3,6)-β-D-Galp-(1→, α-L-Araf-(1→, β-L-Arap-(1→ and →3)-α-L-Araf-(1→. No →3)-β-D-Galp-(1→ was observed. The assignments were based on data published by Ponder and Richards (1997).

The results of the methylation analysis agreed in most respects with the ^{13}C NMR analysis. The following units were identified: Non-reducing end units of galactopyranose (corresponds to β-D-Galp-(1→ of the ^{13}C NMR analysis) and arabinofuranose (corresponds to α-L-Araf-(1→) and 3,6- and 6-substituted galactose units (corresponds to →3,6)-β-D-Galp-(1→ and →6)-β-D-Galp-(1→). Weak signals of non-reducing arabinopyranose were found (corresponds to α- and β-L-Arap-(1→). The methylation analysis also detected a few 3-substituted galactose units.

The presence of glucuronic acid units was shown using acid methanolysis and confirmed using ^{13}C NMR spectroscopic analysis. The acid methanolysis provides information on the carbohydrate composition, including the neutral monosaccharides as well as uronic acids. A higher proportion of glucuronic acid was found in the spruce arabinogalactan (8 w-%) than in the pine arabinogalactan (4 w-%). The spruce AG was thus more acidic. The methylation analysis does not yield information on acidic carbohydrate units.

Structural features were suggested for the spruce and pine heartwood AGs based mainly on the ^{13}C NMR analysis and to a smaller extent on acid methanolysis and methylation analysis (Figures 15 and 16). The presence of 1,3-linked galactose units was concluded from the methylation analysis only.

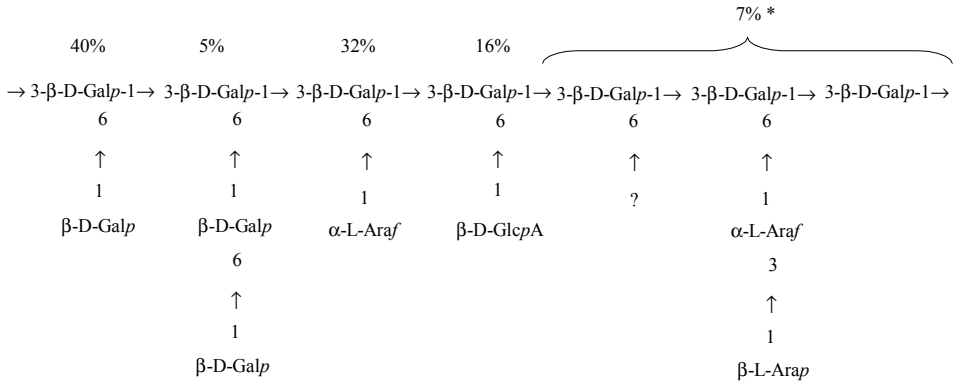


Figure 15. Suggested major structural features of a typical spruce arabinogalactan molecule. The probability and ratio of the different side chains probably varies with the molar mass and between different molecules. *The ^{13}C NMR analysis did not show the presence of any arabinobiosyl groups or unsubstituted galactose units in the main chain. The presence of these groups was shown by methylation analysis. About 7% of the sugar units remain undefined (II).

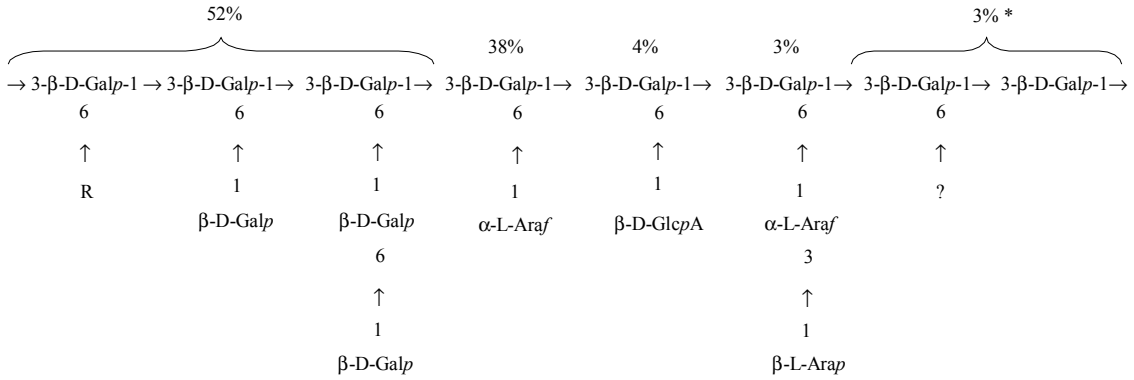


Figure 16. Suggested major structural features of a typical pine arabinogalactan molecule. The probability and ratio of the different side chains probably varies with the molar mass and between different molecules. *The ^{13}C NMR analysis did not show the presence of any unsubstituted galactose units in the main chain. The presence of these groups was shown by methylation analysis. About 3% of the sugar units remain undefined. R = side chains containing three or more β-D-Gal*p* units (II).

The presence of 1,3-linked galactose units has been reported in the literature in studies on water-soluble polysaccharides from Scots pine (*Pinus silvestris*) (Aspinall and Wood 1963), cell wall polysaccharides and isolated arabinogalactan-proteins from *Picea abies* L. (Karácsonyi et al. 1996, 1998) as well as for arabinogalactan from western Larch (*Larix occidentalis*) (Ponder and Richards 1997).

Probably only a small fraction of the isolated AGs contained these 3-substituted galactose units in the backbone. The amount was too small to be detected in the ^{13}C NMR spectroscopic analysis. It has to be remembered that the methylation was successful only for a

minor part of the sample, because it was hardly soluble in the solvent. It is quite possible that the methylation analysis worked preferably for AG fractions enriched in 3-substituted galactose units in the backbone. The presence of these units could improve the solubility of the arabinogalactan in NaOH-DMSO, because the fragments contain fewer side chains. Mostly linear polysaccharides, for example GGMs, xylans, 1,4-linked galactans and glucans, were analyzed quantitatively using the same methylation conditions as in this study (III, IV).

Comparison of water-soluble **spruce** heartwood AG with the corresponding **larch** heartwood AG showed the following differences:

- Spruce heartwood AG has a shorter main chain than larch heartwood AG.
- Spruce heartwood AG has more single unit side chains than larch heartwood AG.
- Spruce heartwood AG has more acidic side chains than larch heartwood AG.

Comparison of water-soluble **pine** heartwood AG with the corresponding **spruce** heartwood AG yielded the following information:

- Pine heartwood AG has a slightly shorter main chain than spruce heartwood AG.
- Pine heartwood AG has fewer acidic side chains than spruce heartwood AG.
- Pine heartwood AG has a higher proportion of 1,6-linked galactose units in side chains and thus a higher probability of side chains with more than two units than spruce heartwood AG.

10.2.2 Spruce wood and TMP galactoglucomannans (III)

Spruce wood and TMP were extracted in three subsequent water extractions. At room temperature, about 0.4% of carbohydrates dissolved from the wood sample (% of dry matter). A further 0.2% was extracted at 90°C for one hour and 0.5% in the final extraction at 90°C for 12 h. The corresponding values for TMP were about 1% (estimate based on cold water extraction according to TAPPI), 0.2% and 0.4% (Table 1 in paper III).

Analysis of the isolated polysaccharides showed that galactoglucomannan (GGM) was the main polysaccharide present in all samples. The purest GGMs were obtained from the second extraction step (90°C, 1 h). This was also the fraction recovered as a pure white powder. The polysaccharides extracted first (23°C, 3 h) had a light-white-brownish color and the polysaccharides obtained in the third extraction (90°C, 12 h) a dark-white-brownish color. The fraction obtained by prolonged extraction at 90°C contained other components than polysaccharides, probably lignin-derived compounds.

The average apparent molar mass was 33 kDa for the polysaccharides extracted from wood at 23°C for 3 h. At elevated temperature and short extraction time (90°C, 1 h), polysaccharides with higher average apparent molar mass (46 kDa) were extracted. Prolonged extraction at high temperature (90°C, 12 h) yielded polysaccharides with lower average apparent molar mass (29 kDa). Recently, similar trends in the molar mass distributions were observed for prolonged heating times in the microwave heat fractionation of spruce chips (Lundqvist et al. 2002). The longer heating probably induced hydrolysis and cleavage of larger molecules in the fiber wall.

The corresponding average molar masses from the TMP extraction were higher (55, 66 and 45 kDa). Again, the highest average molar mass was observed at elevated temperature and short extraction time (90°C, 1 h). The external and internal fibrillation resulting from the TMP process probably enabled the release of larger molecules. It is also possible that a lower molar mass fraction had already dissolved in the TMP process and that fewer small molecules were left before the water extraction of the present study. The molar masses obtained agree with other studies, as discussed in detail in III.

In addition to GGMs, some acidic AGs were released in the first two extractions. The prolonged extraction at elevated temperature also released xylan and pectins (Table 1 in paper III).

The ratio of mannose to glucose was nearly constant at 4.3-4.5:1 in the three GGM samples extracted from wood as determined by acid methanolysis. However, the results of the methylation analysis showed lower ratios of 3.4-3.8:1 for two of these GGM samples. These were the GGMs extracted at 90°C from wood. The reason for the different results is not known. More galactose was present in the GGM extracted from wood at room temperature than at 90°C (Table 3, III). The higher degree of branching with galactose side chains probably explains the good water solubility.

As expected, the methylation analysis showed the presence of 4-substituted mannose and glucose units for all GGM samples (Table 2). In addition, 4,6-substituted mannose and 4,6-substituted glucose were also present. Galactose was present as non-reducing end units. Galactose was thus bound not only to mannose but also to glucose units. NMR spectroscopic analysis showed the presence of 4,6-substituted mannose units, but was not able to detect 4,6-substituted glucose units. Conflicting results on the presence of glucose substituted by galactose in GGMs have been published. GGM isolated from a water extract from Scots pine contained 4,6-substituted glucose as determined by methylation analysis (Aspinall and Wood 1963). 4,6-Substituted glucose has also been reported in GGMs from several softwood species (Timell 1965). On the other hand, NMR spectroscopic analysis of GGM obtained by microwave fractionation of spruce chips did not show the presence of 4,6-substituted glucose (Lundqvist et al. 2002). It is possible that the low content of 4,6-substituted glucose was not detectable by NMR spectroscopy, as found in the present study. Enzymatic treatment of pine kraft pulp using endo-β-D-mannanase released oligosaccharides, some of which contained 4,6-substituted mannose but no 4,6-substituted glucose (Tenkanen et al. 1997). It is possible that the GGM fraction containing 4,6-substituted glucose units was not accessible to the enzyme. It can be concluded from the methylation analysis that 4,6-substituted glucose is present in glucomannans and that this finding is not in contradiction with the literature.

The wood GGM was branched at every 11-14 mannose or glucose unit. A trend for more relative branching at glucose units was found (Table 2). The proportion of non-reducing galactose units is approximately equal to the sum of 4,6-substituted mannose and glucose. The GGMs extracted from TMP at room temperature showed branching similar to that in the wood GGMs. At elevated temperature (90°C), the GGMs from TMP were less branched than the others. Only every 23th and every 17th backbone unit is branched in the GGM after extraction at elevated temperature for 1 and 12 h, respectively. This could be because the thermal treatment and fibrillation of fibers during the TMP process enable the release of

slightly different GGM. Some of this GGM may originate from the secondary fiber wall. Another possibility is that the TMP process induces the cleavage of galactose units from the GGM.

Table 2. Results of methylation analysis of GGM samples isolated from wood and TMP. The data are shown for the units assigned to GGM (III). The positions of substitution are indicated (mol% of all identified units, T = terminal, non-reducing end). In addition, branching is presented separately for mannose and glucose.

	Mannose (mol%)			Glucose (mol%)			Galactose (mol%)	Every xth unit branched	
Position of substitution	T	4	4,6	T	4	4,6	T*	Man	Glc
Wood (23°C, 3 h)	1.0	58.5	4.6		12.3	1.1	5.2	13.7	12.2
Wood (90°C, 1 h)	1.4	56.7	5.3	0.3	16.5	1.6	5.0	11.7	11.3
Wood (90°C, 12 h)	1.2	52.6	4.3	0.3	13.7	1.3	4.1	13.2	11.5
TMP (23°C, 3 h)	1.1	53.4	5.2	0.3	16.1	1.6	4.8	11.3	11.8
TMP (90°C, 1 h)	1.3	58.7	2.7	0.4	19.6	0.9	2.5	22.7	22.8
TMP (90°C, 12 h)	1.1	52.7	3.1	0.3	14.1	0.9	3.1	18.0	16.7

* Some of the terminal galactose units possibly originate from arabinogalactan present in the samples.

Arabinose was present in all GGM samples. Arabinose units other than non-reducing end units were also found, particularly in the samples from prolonged extraction at higher temperature (90°C, 12 h). Non-reducing end units of arabinose are usually assigned to xylan, as explained in the introduction (Figure 3), but substituted arabinose units cannot be assigned to polysaccharides other than pure arabinan. 1,5-Linked arabinan has been reported in enzymatically isolated. Lignins from pine and spruce pulp samples (Iversen and Wännström 1986, Minor 1991) and in carbohydrates isolated from black liquor from kraft pulping (Luonteri et al. 2003). Some of the arabinan in the GGM sample was probably bound to lignin co-extracted under the conditions employed.

In all samples, two structural units were present that could not be identified. These represented a significant proportion (up to 7.5 mol-%) of the sample. One of the units had a mass spectrum similar to that of 2,4,6-substituted glucose but a different retention time. The retention time was comparable to that of 4,6-substituted hexoses. It is possible that the unit was 2,6-substituted. The other unit had a mass spectrum comparable to that of 3,6-substituted glucose or galactose but again differed in retention time. No model compounds providing information on these structural units were available, although a wide choice of model compounds was used to build up a library (I).

^{13}C NMR spectroscopic data in general agreed with those of the methylation analysis.

10.3 Factors affecting carbohydrates in RLCCs in delignification

As stated in the introduction, carbohydrates are partly bound to lignin in both wood and pulp. The carbohydrate units attached to lignin have to be isolated before analysis to distinguish them from the major carbohydrates in the fibers. A convenient method to isolate residual lignin from chemical pulp is enzymatic hydrolysis. Enzyme preparations are used that hydrolyze the cellulose and hemicelluloses of the fibers (Tamminen and Hortling 1999). The mixture of different cellulose- and hemicellulose-hydrolyzing enzymes employed cannot act in close proximity to lignin because of their steric requirements, thus leaving oligomeric residues of carbohydrates bound to lignin. Residual lignin-carbohydrate complexes (RLCCs) are obtained. This method is suitable for studying carbohydrates bound to residual lignin.

Studies reported in the literature have shown that the enzymatically isolated RLCC samples as well as other lignin-carbohydrate complexes contain more galactose and arabinose than would be expected based on the carbohydrate composition of wood and pulp (V). Particular attention was therefore given to the galactan structures bound to lignin. The type of galactan involved in RLCCs is of interest. As described in Chapters 2.2 and 10.2.1, two separate galactans are known in wood, a mostly linear "pectic" β-1,4-linked galactan with substitution mainly at position 6, and a highly branched β-1,3-linked galactan with frequent substitution at position 6 (1,3/6-galactan).

The carbohydrates that dissolved during oxygen delignification were also studied. These carbohydrates are partly bound to lignin.

10.3.1 Fiber morphology in unbleached spruce kraft pulp (V)

RLCCs of the surface and inner layers of spruce kraft were compared. Material enriched in fiber surface matter was released from unbleached spruce kraft pulp (SCK1) by gentle refining. The pulp was then fractionated into fines material (SCK1 Fine) containing the surface matter, and peeled fibers (SCK1 Fiber) (Figure 1 in paper V). SCK1 was used as a reference. Two RLCC fractions were isolated: the insoluble residue at the pH of the purification (pH 9.5) (prois) and the precipitate after pH adjustment to 2.5 (proRL) (Tamminen and Hortling 1999). The RLCCs isolated contained most of the residual lignin (87-93% calculated as pure lignin). They also contained 4.9-7.9 mg carbohydrates /100 mg RLCC sample, mainly galactose, mannose, xylose and glucose (Table 4 in paper V). No significant amounts of uronic acids were detected. The occurrence of high galactose contents in these RLCCs (25-28 w-% of carbohydrates) compared to that of the SCK1 Fine and SCK1 Fiber samples (less than 1 w-% of carbohydrates) confirmed the finding from earlier studies that galactose is enriched in enzymatically isolated RLCCs (Yamasaki 1981, Minor 1986, Iversen and Wännström 1986, Hortling et al. 2001).

The structure of carbohydrate residues in the RLCCs of the SCK1 Fine and SCK1 Fiber samples was studied in more detail for the RLCC fraction proRL. The carbohydrate residues were not completely methylated after the two methylation cycles, but a reasonable degree (53-74%) could be reached to allow interpretation of the results. The prois samples caused some problems in the analysis, because the methylation was less quantitative, in particular for the prois fraction of the SCK1 Fine sample. The extreme unreactivity of the SCK1 Fine prois

sample was possibly due to the presence of cellulose in the RLCC (Liitiä et al. 2000b). In the primary fiber wall, cellulose fibril orientation is less ordered than in the secondary wall. It is suggested that lignin partly surrounds cellulose fibrils on the fiber surface. This would prevent cellulolytic enzymes from hydrolyzing the cellulose completely in fines for steric reasons, although they are able to hydrolyze most of the crystalline cellulose – cellulose of the secondary wall – present in pulp fibers. Minor (1982) has reported similar results in the methylation of glucose units in enzymatically isolated MWEL of loblolly pine. In all cases in the present study, non-methylated carbohydrate residues also included hemicellulose residues. The reason for the unreactivity might be that even hemicelluloses are partly covered by lignin.

The main units of the proRL fractions were 4-substituted xylose, 4- and 3-substituted galactose, 4-substituted glucose and 4- and 4,6-substituted mannose, in agreement with earlier results (Minor 1986, Iversen and Wännström 1986). The units were assigned to cellulose and the major and minor hemicelluloses xylan, glucomannan and galactans as presented above (Figures 2-4). Branching of the galactan with 1,3-linked galactose units in the backbone was observed and for this reason the term 1,3/6-linked galactan is used here. No branching units that could be assigned to the galactan with 1,4-linked galactose units in the backbone were found. Correspondingly, the term 1,4-linked galactan is now used.

Differences in the ratios of 1,4-linked galactan to 1,3/6-linked galactan were observed in the RLCCs of the fiber layers. 1,4-linked galactan was the enriched galactan type in the RLCC of the fiber surface material, both galactan types were present in approximately equal proportions in the RLCC of the original pulp and 1,3/6-galactan was enriched in the RLCC of the inner part of the fiber (Table 3). The backbone of 1,3/6-linked galactan was branched to the same degree in all three RLCC samples with approximately every third backbone unit substituted at position 6.

Table 3. Structural features of carbohydrate residues in the RLCCs (proRL fractions) of fiber surface material (SCK1 Fine) and inner part of fibers (SCK1 Fiber).

RLCC of	Ratio 1,4-linked galactan to 1,3/6-linked galactan	Branched galactose units in 1,3/6-linked galactan, % of backbone units
SCK1 Fiber	0.6	37
SCK1	0.9	36
SCK1 Fine	1.3	35

The enrichment of 1,4-linked galactan units in the residual lignin of the fiber surface has been reported earlier (Minor 1991). However, the enrichment of 1,3/6-linked galactan in the RLCC of the inner part of the fiber has not been shown clearly before. 1,3/6-Linked galactan is known as water-soluble arabinogalactan and is present in small amounts in spruce (*Picea abies*) and pine (*Pinus silvestris*) heartwood (II). Its presence in RLCCs indicates that 1,3/6-linked galactan also has a role in linking residual lignin to carbohydrates in the secondary wall. This linkage may be one reason for the difficulty of achieving selective delignification. Improved bleachability has been shown after endo-β-1,4-galactanase treatment of unbleached spruce kraft pulp (Tamminen et al. 1999). These positive results could probably be further improved by using an endo-β-1,3-galactanase simultaneously (Tenkanen et al. 2002). However, such an enzyme has not been found (Luonteri et al. 2003).

Arabinose was present mainly as its 5-substituted unit or unmethylated in all samples. This is in agreement with reports in the literature that arabinose is present in enzymatically isolated lignin mainly in the form of 1,5-linked arabinan and not as non-reducing end groups in xylan (Minor 1991, Iversen and Wännström 1986). The presence of arabinan in RLCCs supports the finding that arabinan is partly bound to lignin, as discussed above (Chapter 10.2.2).

Xylose and mannose units were substituted in a way that corresponded to the known structure of xylan and glucomannan.

Glucose was present as its 4-substituted unit, and to a small amount also as its 3-substituted unit. 1,3-Linked glucan was found as the major glucose-containing oligo- and polysaccharide in effluents from the alkaline stage and oxygen delignification of pine kraft pulp (*Pinus silvestris*) as reported below (IV, Chapter 10.3.3). 1,3-Linked glucan is therefore linked in small amounts to the residual lignin.

10.3.2 Cooking method (V)

Different alkaline cooking methods are available as described in the introduction (Chapter 6.2). Cooking method affects the yield, carbohydrate composition and bleachability of chemical pulps.

RLCCs were isolated from the unbleached spruce kraft pulp described in the previous chapter and from three unbleached alkaline pine pulps. The pine pulps studied were a conventional kraft pulp (PCK), a polysulfide/anthraquinone pulp (PPSAQ) and a soda/anthraquinone pulp (PSoAQ) all cooked to the same kappa number of approximately 30. The bleachability of the pulps decreased in the order PCK, PPSAQ and PSoAQ (Hortling et al. 2000). A study on the structure of the residual lignin revealed no clear correlation with the different bleachabilities (Tamminen and Hortling 2001). Based on these earlier results, it was hypothesized that the carbohydrate structures linked to the residual lignin differed between the pulps and that this would explain the different bleachabilities. The aim of the present work was to test this hypothesis.

The degree of methylation achieved in the methylation analysis was greater for the RLCCs of the pine pulps than for those of the spruce pulp. A third methylation cycle was included and this raised the proportion of methylated carbohydrate units to over 90%. The difference in the degree of methylation has to be taken into account in the evaluation of spruce compared to pine pulps.

The proRL fractions were subjected to detailed analysis. This fraction contained 52% and 63-77% of the residual lignin in the spruce pulp and pine pulps, respectively.

Mannose and galactose were the major carbohydrate components in the RLCCs of the unbleached pulps (Table 4). The mannose content in the RLCCs of the different pulps showed the highest variation. The high mannose content in the RLCC from PSAQ reflected the higher mannose content of the corresponding pulp (Table 2 in paper V). The RLCC of the spruce pulp had lower mannose and galactose contents than the RLCCs of the pine pulps. This might be because of the different wood raw material or the different kappa number.

Table 4. Carbohydrate content and composition in the RLCCs (proRL fractions) of unbleached pine and spruce pulps analyzed after acid hydrolysis, mg/100 mg. No correction was made for mannose present in protein residues.

RLCC of	Total	Arabinose	Galactose	Glucose	Xylose	Mannose
PCK	7.5	0.4	2.1	1.2	1.6	2.2
PPSAQ	9.4	0.4	2.7	1.6	1.3	3.4
PSoAQ	8.3	0.4	2.1	1.6	1.4	2.8
SCK1	7.0	0.4	1.9	1.2	1.6	1.9

The same substituted monosaccharide units were found in the carbohydrate residues of all unbleached pulps studied, including 4-, 2,4- and 3,4-substituted xylose, 5- and 3,5-substituted arabinose, 3-, 4-, 6- and 3,6-substituted galactose, 3- and 4-substituted glucose, 4- and 4,6-substituted mannose and non-reducing end units of all these monosaccharides (Figures 3 and 4, V).

A more detailed evaluation of the results for the RLCCs of the pine pulps than for the spruce pulp revealed the presence of 4,6-substituted glucose units. These originate either from branching points in glucomannan (III) or from xyloglucan. Xyloglucan is known to be a component of the primary cell wall of higher plants and has a backbone of 1,4-linked glucose units, some of which are branched at position 6 (Fry 1989). 2-Substituted xylose units are present in the side chains of xyloglucan. 2-Substituted xylose units were identified in the pine samples, too. The total content of xyloglucan in pulp is minor, but enrichment in enzymatically isolated RLCC is possible.

Table 5. Ratio of 1,4- to 1,3/6-linked galactan. Branching in 1,3/6-linked galactan (expressed as % of branched units in backbone). Unmethylated units of all identified carbohydrate structural units in RLCCs (proRL fractions) of unbleached pine and spruce pulps in mol%.

RLCC of	Ratio 1,4-linked galactan to 1,3/6-linked galactan	Branched galactose units in 1,3/6-linked galactan, % of backbone units	Non-reducing ends of methylated units, mol%	Unmethylated, mol%
PCK	1.4	41	18	9
PPSAQ	1.4	31	18	7
PSoAQ	1.5	17	14	8
SCK1	0.9	36	23	26

The ratios of 1,4- to 1,3/6-linked galactan were similar for the RLCCs of all unbleached pine pulps (Table 5). Less 1,4-linked galactan was present in the RLCCs of unbleached spruce kraft pulp. The degree of branching in 1,3/6-galactan differed between the RLCCs. The 1,3/6-galactan was clearly less branched in the RLCC of the PSoAQ pulp, while branching was more pronounced in the RLCC of PPSAQ and most pronounced in the RLCCs of CK pulps of pine and spruce. This indicates that SoAQ cooking, and to a lesser extent PSAQ cooking, removes more side chains from 1,3/6-galactan than does CK cooking. The RLCC of PSoAQ contained more 4-substituted glucose than the corresponding other two RLCCs (Figure 4 in paper V). This may indicate an enrichment of cellulose bound to the residual lignin in the PSoAQ proRL. A possible explanation for this is the formation of ether-type bonds between glucose and lignin in sulfur-free SoAQ cooking. The formation of this type of bond has been shown to be enhanced under sulfur-free cooking conditions compared with sulfur-containing cooking methods in model compound studies (Gierer and Wännström 1986).

The methylation analysis yielded information on the non-reducing end units of the different oligosaccharide chains linked to lignin in the RLCCs. The proportion of non-reducing end units in the carbohydrate residues was lower for the RLCC of PSoAQ than for the RLCCs of the other pine pulps. The corresponding proportion was even higher in the RLCC of SCK1 than in the RLCCs of the pine pulps (Table 5). The values are shown separately for each monosaccharide (Table 6). The proportion of non-reducing ends of xylose and glucose units showed a clear difference between the pulps. Assuming that the corresponding carbohydrate residues (from cellulose, 1,3-glucan and xylan) would not be bound to lignin at the non-reducing end unit, this would mean differences in chain length. The xylan and glucan residues in the RLCC of PSoAQ thus had longer chains than in the RLCCs of the other pine pulps, and the chains were shortest in the RLCC of SCK1.

Table 6. Proportion of non-reducing end groups within each individual monosaccharide in RLCCs (proRL fractions) of unbleached pine and spruce pulps, mol%.

RLCC of	Xylose	Galactose	Glucose	Mannose
PCK	22	15	15	20
PPSAQ	19	18	15	21
PSoAQ	15	13	9	19
SCK1	31	16	25	22

The small differences observed between the pine pulps could indicate differences in the linkage between lignin and carbohydrates in the PSoAQ pulp that contribute to its poorer bleachability. However, the differences were smaller than expected and the hypothesis that different carbohydrate structures bound to residual lignin are the reason for differences in bleachability could neither be convincingly proved nor entirely disregarded. A second possible explanation for the different bleachabilities is in the fiber wall ultrastructure. The differences found in the length of the oligosaccharides in RLCCs can be interpreted as differences in the accessibility of the pulp polysaccharides to the enzymes used in the enzymatic hydrolysis for the isolation of RLCCs. The accessibility to these enzymes might differ due to differences in fiber wall ultrastructure. These differences in turn may be due to differences in cooking methods.

The differences between the spruce and pine pulps could be due to the higher kappa of the spruce kraft pulp (36 compared to 30-33), differences between the wood species or the lower degree of methylation resulting from only two methylation cycles for the RLCC of the spruce kraft pulp.

The possible binding modes between carbohydrates and lignin in the RLCCs of the unbleached spruce kraft pulp were studied. An oligomeric or polymeric chain can be bound to lignin *via* the non-reducing end, the reducing end or a backbone unit in the middle of the chain (Figure 17). The different binding positions affect the ratio of non-reducing to reducing ends. For example, if a chain of a certain structural type (e.g. xylan, glucan, glucomannan) is bound to lignin only in the middle, the numbers of reducing and non-reducing end units would be equal. The proportion of non-reducing end units obtained by methylation analysis (see above) and the proportion of reducing ends were used to calculate the apparent numerical average of the degree of polymerization (DP_n) (Table 7).

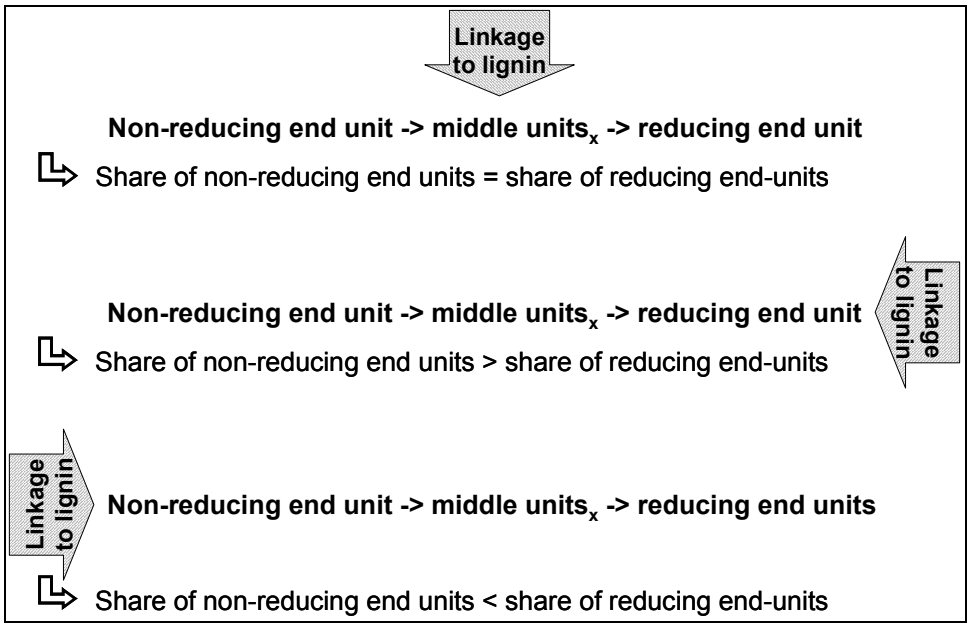


Figure 17. Effect of possible lignin-carbohydrate linkages on the ratio of non-reducing and reducing end units for linear carbohydrates.

Table 7. Apparent numerical average degree of polymerization (DPn) for carbohydrate residues in RLCCs of spruce pulp samples. The calculation is based on either A: ratio of chain units to non-reducing end units or on B: ratio of chain units to reducing end units (V).

Sample	Based on	Galactose	Glucose	Xylose	Mannose
proRL of SCK1	A		4	3	5
proRL of SCK2	B	>20*	5	8	10
prois of SCK1	A		8	5	6
prois of SCK2	B	>20*	10	10	14

* An exact value higher than 20 is unreliable.

For glucose, the DP_ns are equal, indicating bonds between a chain unit of cellulose or glucan and lignin. The DP_ns are approximately 5 and 10 for the proRL and prois fractions, respectively.

For xylose, the estimated DP_ns differ. This could be because some xylo-oligosaccharides are bound to lignin *via* the reducing end group. This is supported by the reported formation of bonds between reducing ends of xylo-oligosaccharides and lignin model compounds under kraft pulping conditions (Vikkula et al. 2001). The DP_n of xylo-oligosaccharides is estimated to be 3-5. The situation is similar for the mannose units, but caution should be exercised because some of the mannose originates from the protein impurity.

The proportion of reducing ends in galactose is very small, possibly indicating bonds to lignin or other oligosaccharides *via* the reducing end. Estimation of DP_n based on the proportion of non-reducing ends could not be performed, because the 1,3-linked galactan chains were branched and thus non-reducing end groups are situated to a large extent at the end of side chains. In addition, the distribution of the non-reducing end groups between residues of 1,3/6- and 1,4-linked galactan and galactoglucomannan was not known.

A report by Minor (1991) also showed a high apparent chain length of galactans in residual lignin from the secondary wall of loblolly pine kraft pulp based on analysis of reducing end groups. Another study reported approximately equal amounts of galactose as non-reducing and reducing ends in residual lignin from pine kraft pulp (Iversen and Wännström 1986). Iversen and Wännström's (1986) study also differed from ours and from that of Minor (1991) in that no presence of any 3- or 3,6-substituted galactose was reported. The question still remains whether the RLCCs differ because of the isolation method or whether differences in the methylation analysis procedure used give rise to the different results.

The results for galactose in the RLCCs in the present study can be interpreted to indicate that chains consisting of galactose units are rather long because of the absence of galactanase activity in the enzyme mixture employed, as suggested in the literature (Iversen et al. 1987). This is supported for 1,4-linked galactan by a report that an endo-β-1,4-galactanase released some galactobiose from an RLCC of softwood kraft pulp (Luonteri et al. 2003). The lack of the necessary enzyme for hydrolyzing 1,3-linked galactan (Luonteri et al. 2003) would also support the theory for long chains of 1,3/6-linked galactan in RLCCs. A second possibility is that shorter chains of galactose units are connected to lignin at the reducing end. However, even if galactan residues in residual lignin had long chains, numerous bonds to lignin would be expected based on the properties of the RLCCs. The galactose residues follow the RLCCs throughout the isolation procedure with several dissolution and precipitation steps (Figure 2 in paper V). The presence of numerous bonds is further supported by a study on the frequency of benzyl ether bonds between carbohydrates and lignin which showed there was a high frequency of bonds between galactose units and residual spruce kraft lignin (Choi and Faix 1999). Co-elution of galactose with high molar mass residual kraft lignin in preparative gel permeation chromatography has also shown that these galactan residues are bound to lignin (Hortling et al. 2001).

The bonding mode of arabinan residues was not evaluated, because the reliability of the values was low due to the low arabinan content.

Above, the structural studies of RLCCs were discussed for proRL fractions. The sparingly soluble RLCC prois fraction was studied in detail only for SCK1 (Figure 3 in paper V). The same substituted units were found as for the proRL fractions. The results differed in that a higher ratio of 1,4- to 1,3/6-linked galactan and fewer non-reducing and reducing end group units were found than in the corresponding proRL fraction. Further fractionation of the prois fraction into a sparingly alkali-soluble (proissP) and alkali-soluble (proisis) fraction further lowered the degree of methylation achieved. The treatment with strong alkali obviously caused changes in the interaction between lignin and carbohydrates in the RLCCs.

10.3.3 Oxygen delignification (IV, V)

The effect of oxygen delignification was studied by analyzing the dissolved carbohydrates in the high-molar-mass fraction of the filtrates and by comparison of carbohydrate residues in RLCCs before and after oxygen delignification. Pine and birch kraft pulps were used in the filtrate study and pine pulps for the RLCC study.

Carbohydrate structures in filtrates of oxygen delignification (IV)

The main hemicellulose component that dissolved during oxygen delignification and during the reference alkaline treatment of pine kraft pulp was xylan. Trace polysaccharides from the pulp also dissolved in relatively large proportions.

Methylation analysis of the samples revealed that 1,4-linked xylan, 1,3-linked glucan, 1,3/6-linked and 1,4-linked galactan, and 1,5-linked arabinan dissolved from the pine pulps (Table 8). These components were probably linked to lignin, because the same components were present in carbohydrate residues of the RLCCs of unbleached and oxygen-delignified pulps (previous chapter and below). The ratio of 1,4- to 1,3/6-linked galactan in the filtrate from oxygen delignification of pine pulp was 1.1. This value lies between the ratios found for the RLCCs of spruce and pine kraft pulps (proRL fraction) as shown above (Table 5).

Table 8. Distribution of structural units in the polysaccharides present in the high molar mass fraction from alkaline treatment (E) and oxygen delignification (O) of pine kraft (PK) and birch kraft (BK) pulps. The positions of substitution are indicated (mol% of identified units, T = terminal, non-reducing end) (IV).

	Xylose					Arabinose		Galactose				Glucose		Mannose
Position of substitution	T	4	2	2,4	3,4	T	5	T	3	3,6	4	3	4	4
PKE	-	70.5	-	8.2	7.5	3.9	-	-	4.8	-	-	5.1	-	-
PKO	-	55.6	-	6.5	4.5	2.5	1.4	1.5	6.2	2.4	7.5	9.4	1.5	1.1
BKE	3.3	83.3	0.3	9.1	1.5	0.2	0.1	-	0.3	0.5	-	-	1.4	-
BKO	3.5	82.3	0.3	9.8	1.7	0.1	0.4	-	0.4	0.5	-	0.1	0.8	-

An interesting result was that the glucose units found in the effluent from the pine pulp did not originate from cellulose (1,4-linked glucan, see Figure 2) but most probably from the 1,3-linked glucan laricinan (Figure 18). The presence of this type of glucan in bleaching effluents has not been reported before. Small amounts were found in the RLCCs of unbleached and oxygen-delignified pulps (previous chapter and below). This finding is important for the future development of oxygen delignification and alkaline stages. The content of carbohydrates in filtrates is normally determined after acid hydrolysis. The content and composition of carbohydrates are obtained, but information on the structure of oligo- and polysaccharides is not. The presence of glucose in filtrates has so far been readily adopted as a measure of the degree of degradation of cellulose. This was proved wrong in the present work, and in the future, other measures of the degree of cellulose degradation will have to be used.

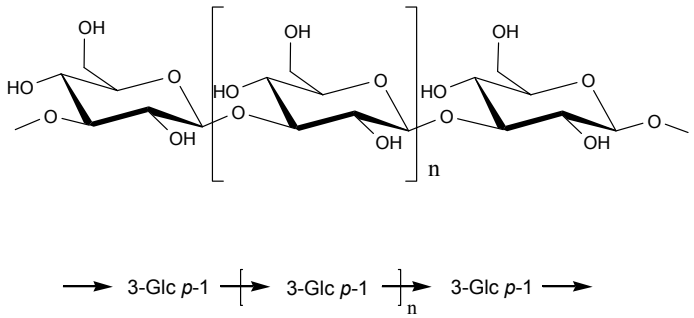


Figure 18. Structure of the main glucose-containing polysaccharide (1,3-glucan) in the effluents from oxygen delignification and alkaline treatment of pine kraft pulp. The probable anomeric configuration (β) is shown.

Similar analysis of the corresponding effluents from birch pulps showed different results. Most of the carbohydrates that dissolved together with lignin during oxygen delignification and the reference alkaline treatment were xylan of typical structure (Table 8). The presence of 3,4-linked xylose units was surprising, because no substituent was expected in this position of birch xylan. This may indicate bonds to lignin (Figure 19). Lignin-carbohydrate bonds at position 3 in xylose units have been reported in residual lignins of beech pulps (Choi and Faix 1999) and in LCC fractions and residual lignins of birch and eucalypt (*Eucalyptus globulus*) (Balakshin et al. 2001). Only traces of 1,3-linked glucan were found in the birch effluents. Instead, small amounts of 1,4-linked glucan were present in the effluent. This glucan may be degraded cellulose or, more likely, non-cellulosic 1,4-β-glucan.

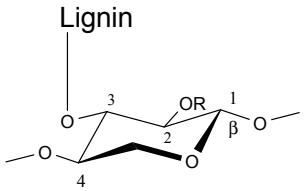


Figure 19. Possible bonds between xylan and lignin in birch kraft pulp.

Carbohydrate structures in RLCCs of oxygen-delignified pulps

RLCCs of oxygen-delignified pulps were studied for the alkaline pine pulps described in the previous chapter. The RLCC fraction proRL contained most of the residual lignin. The total carbohydrate content was similar to that for the corresponding RLCCs of the unbleached pulps (Table 4 in paper V), but the galactose content was lower and the mannose content higher in the RLCCs of the oxygen-delignified pulps. Oxygen delignification is thus effective in cleaving bonds between galactans and residual lignin or in preferentially dissolving galactan-bound lignin. The increased mannose content was partly due to increased protein residues in the RLCCs of the oxygen-delignified pulps.

The methylation analysis showed the presence of the same structural units as in the RLCCs of the unbleached pulps.

The proportion of non-reducing ends showed that the chains of the carbohydrate residues were shorter in RLCCs after oxygen delignification than in the corresponding RLCCs before oxygen delignification (Table 5 in paper V). The proportion of non-reducing ends was 14-18% in the unbleached pulps but as high as 21-22% in the oxygen-delignified pulps. This indicated less steric hindrance for the enzymes used for RLCC isolation in oxygen-delignified pulps compared to unbleached pulps.

A special feature of the RLCCs of the oxygen-delignified PSoAQ pulp was the presence of more non-reducing xylose units compared to the RLCCs of oxygen-delignified PCK and PPSAQ pulps. Oxygen delignification had a greater impact on the PSoAQ pulp in terms of the chain length of carbohydrates (in particular xylan and glucan residues) in the RLCCs.

The ratio of 1,4- to 1,3/6-linked galactan units was higher for the RLCCs of the oxygen-delignified pulps (1.9-2.4) than for those of the unbleached pulps (1.4-1.5). The ratio increased most for the RLCCs of PSoAQ (from 1.5 to 2.4). Thus oxygen delignification again had a greater impact on the RLCCs of SoAQ than on the RLCCs of the other two pulps.

The results for the RLCCs of all three pulps indicated either that 1,4-galactan remains connected to residual lignin to a greater extent than 1,3/6-linked galactan during oxygen delignification or that more lignin is removed from the secondary wall than the primary wall during oxygen delignification. The RLCC originating from the secondary wall has been shown to be richer in 1,3/6-linked galactan than that from fiber surface material (Chapter 10.3.1). Consequently, preferential removal of lignin from the secondary wall would reduce the content of 1,3/6-linked galactan in isolated RLCCs relative to 1,4-linked galactan.

However, this is not supported by the study on the structure of carbohydrates in the filtrates from oxygen delignification. No enhanced dissolution of 1,3/6-linked galactan compared to 1,4-linked galactan has been observed (see above, IV). Possible reasons are the preferential degradation of 1,3/6-linked galactan compared to 1,4-linked galactan during oxygen delignification. Thus, oligosaccharides or monosaccharides and their degradation products would be formed preferentially from 1,3/6-linked galactan in the process. These oligosaccharides would be found in the low molar mass fraction of the filtrate, which was not analyzed in detail (IV). Primary wall galactan (mostly 1,4-linked galactan) in turn was enriched in high molar mass RLCC fractions in an earlier study using preparative gel permeation chromatography (Hortling et al. 2001). It can be expected that the 1,4-linked galactan is also preferentially bound to lignin in the filtrate from oxygen delignification and thus enriched in the high molar mass fraction studied (IV). The good water solubility of 1,3/6-linked arabinogalactans – as shown for the arabinogalactans of spruce and pine heartwood (II) – would also enhance the degradation of this type of galactan during oxygen delignification.

11 CONCLUDING REMARKS

Methods for the structural analysis of polysaccharides and oligosaccharides were adapted for the present work. For the linkage analysis, methylation analysis based on the protocol methylation, acid methanolysis, silylation and GC/MS analysis was developed to allow the identification of the structural units of cellulose and the major and minor hemicelluloses of wood and pulp. The method proved to be powerful for polysaccharides in the solvent used and for the carbohydrate residues of RLCCs. Some problems were encountered with the permethylation of carbohydrate units in RLCCs. Another method – the analysis of reducing ends of oligomeric carbohydrate in RLCCs – was also employed.

Structural features of the water-soluble arabinogalactans (AGs) from pine and spruce heartwood were studied. The AGs investigated had a backbone of 1,3-linked ß-D-galactose units, substituted at nearly all galactose units at position 6 with side chains comprising galactose, arabinose and glucuronic acid units. The backbone also contained 1,3-linked galactose units without substitution at position 6. Both spruce and pine AGs were more acidic than arabinogalactan of larch heartwood. The AGs of spruce heartwood had a higher proportion of acidic side chains, longer backbones and shorter side chains than those of pine heartwood. No 1,4-linked galactan dissolved into water from the wood samples studied. Lignin-bound carbohydrate residues in the RLCCs of spruce and pine pulps were also shown to comprise a 1,3/6-linked galactan. These galactan residues did not contain acidic groups. Obviously, a galactan with the same backbone as arabinogalactan is involved in lignin-carbohydrate bonds. RLCCs also contained 1,4-linked galactan. The proportion of 1,3/6-linked galactan was higher in the RLCC from the inner part of the fiber wall than in the surface of spruce kraft pulp fibers. On the other hand, the fiber surface had a high proportion of 1,4-linked galactan. The high proportion of 1,3/6-linked galactans bound to lignin may be one reason for limited delignification of the secondary wall. Oxygen delignification removed lignin-bound 1,3/6-linked galactan in preference to 1,4-linked galactan structures. The galactans in RLCCs might be bound *via* their reducing end to lignin. The filtrates from the oxygen delignification of pine pulp kraft contained both 1,4- and 1,3/6-linked galactans.

Structural features of the water-soluble galactoglucomannans (GGMs) released from spruce wood and TMP at different temperatures and/or times were investigated. The purest GGM was obtained from both wood and TMP at elevated temperature and short extraction time (90ºC, 1 h). The GGMs had a backbone of 1,4-linked ß-D-mannose and 1,4-linked ß-D-glucose units. Both backbone units were partly substituted at C6 with D-galactose. The substitution was at approximately every 12th unit for all GGMs extracted from wood. The glucomannans extracted from TMP were substituted to a smaller extent. The ratio between mannose, glucose and galactose differed between samples. GGM structures were also present in the RLCCs of spruce and pine pulps. The structure determined corresponded to the known structure of GGM. Only small amounts of glucomannans were present in the filtrates from oxygen delignification.

Xylan dissolved from spruce wood and TMP into water only after prolonged treatment at high temperature (90ºC, 12 h). Under the same conditions, some lignin also dissolved, probably as a result of the degradation of lignin-xylan components. Xylan was present in the RLCCs of spruce and pine pulp. In the RLCC of spruce pulp, the short xylan residues are probably bound partly *via* the reducing end. Xylan was the main component dissolving during oxygen delignification of pine kraft pulp. Xylan also dissolved from birch kraft pulp during oxygen delignification. The birch xylan was probably bound to lignin at position 3.

An overview about the polysaccharides studied in this thesis is given in Figure 20.

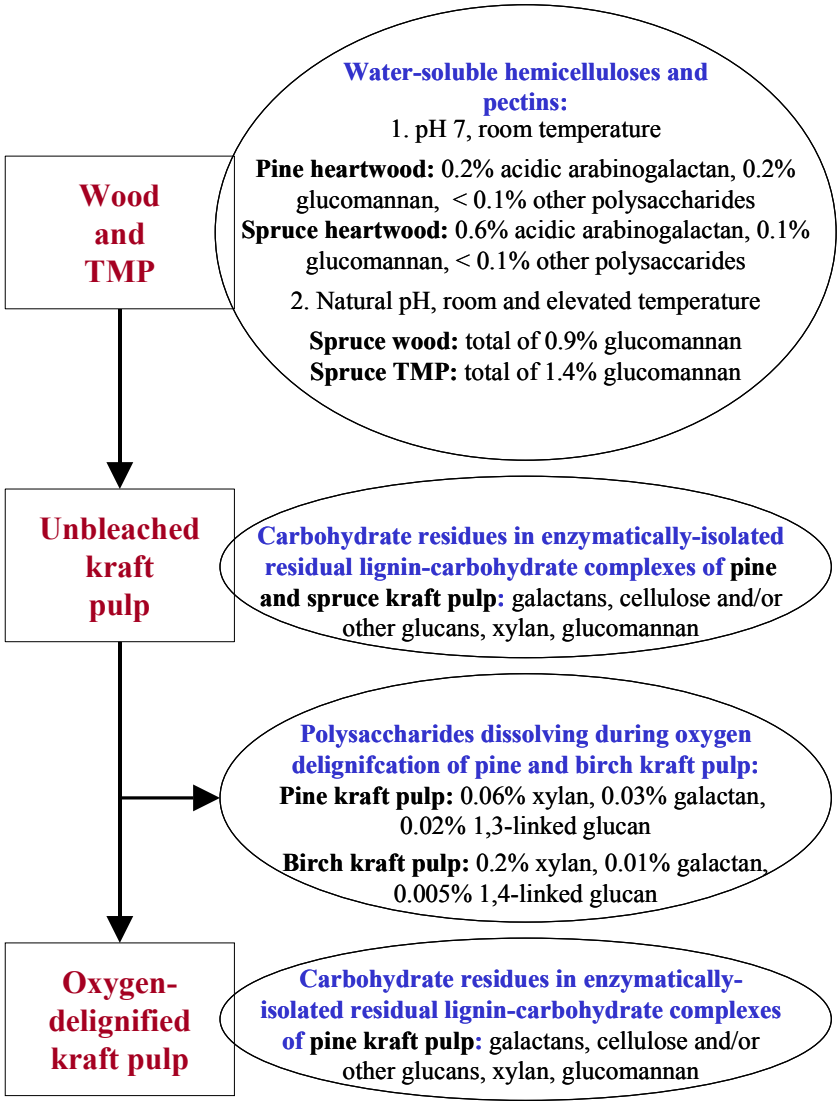


Figure 20. Overview over the polysaccharides studied in this thesis. The isolated amounts of the dissolved components are given as weight-% of wood. Higher contents of the components can be present in wood.

During oxygen delignification, a glucan other than cellulose was dissolved. This was a 1,3-linked glucan. The presence of this type of glucan in the filtrates from oxygen delignification was shown for the first time. Small amounts of 1,3-linked glucose units were also found in the RLCCs of spruce and pine pulps and thus bonds to lignin can be concluded. The 1,3-linked glucan was not detected in the water extracts with glucomannans or arabinogalactans.

The effect of different cooking methods on lignin-bound carbohydrate residues was studied. There were small differences in the structure of carbohydrates in the RLCCs of three unbleached pine pulps. Slightly longer oligosaccharide chains, less branching in 1,3/6-galactan residues and more cellulose residues were found in the RLCC of PSoAQ compared to those of PCK and PPSAQ pulps. Subsequent oxygen delignification shortened the oligosaccharide chains present in RLCCs and removed 1,3/6-linked galactan in preference to 1,4-linked galactan structures connected to residual lignin. The RLCC of PSoAQ/O differed from those of the other two pulps after oxygen delignification in that it had a higher ratio of 1,4- to 1,3/6 linked galactan, and shorter xylan residues.

This work has generated new information about the structure of the water-soluble polysaccharides arabinogalactan and glucomannan present in softwood species. This information can be used to evaluate the solubility of these compounds in the mechanical pulping process.

A new finding is that glucose-containing oligo- and polysaccharides dissolving during the oxygen delignification of pine kraft pulp do not originate from cellulose but from a 1,3-linked glucan. This means that the common interpretation that glucose in the filtrate indicates cellulose degradation is wrong. Other measures will therefore have to be used to evaluate cellulose degradation during oxygen delignification – for example determination of molar mass distribution or drop in viscosity. This knowledge can be used to advantage in developing the oxygen delignification process. The role of the 1,3-linked glucan residues connected to lignin in the fiber remained unclear in this work. This is an interesting question for further work.

Based on the results obtained, two reasons can be suggested for the different bleachabilities of the unbleached pulps. First, the small differences in carbohydrate structures in the RLCCs of the unbleached pulps may reflect differences in the linkages between carbohydrates and lignin in the fibers, which in turn affect pulp bleachability. Second, the chain length of the oligosaccharides in RLCCs can be interpreted as reflecting the accessibility of the pulp polysaccharides to the enzymes used in the enzymatic hydrolysis for isolation of RLCCs due to differences in fiber wall ultrastructure. These differences in turn may be due to the different cooking methods. Further work is necessary to clarify the role of fiber wall ultrastructure in delignification processes.

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