Lignin Polysaccharide Networks in Softwood and Chemical Pulps:
Characterisation, Structure and Reactivity

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KTH Chemical Science
and Engineering

Doctoral Dissertation

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Stockholm 2005
Akademisk avhandling som med tillstånd av Kungliga Tekniska Högskolan i Stockholm framlägges till offentlig granskning för avläggande av teknologie doktorsexamen fredagen den 28 oktober 2005 kl. 10.00 i STFI-salen, STFI-Packforsk, Drottning Kristinas Väg 61. Avhandlingen försvaras på engelska.

Martin Lawoko 2005

TRITA-FPT-Report 2005:30
ISSN 1652-2443
ISRN KTH/FPT/R-2005/30-SE
ABSTRACT

The chemical interactions between the main wood components i.e., cellulose, hemicelluloses and lignin are of fundamental importance for understanding the chemical aspects of wood formation and its reactivity during fibre processing e.g during chemical pulping of wood. Future progress in the development of new high value products from wood will greatly depend on a detailed knowledge of how the fibre elements interact with each other in the biological material.

The existence of covalent bonds between lignin and carbohydrates (LCC) has been one of the most controversial issues in the field of wood chemistry. Only until recently, the existence of such bonds has in its entirety been shown by way of indirect analyses, normally suffering from low yields obtained at rather drastic conditions. Furthermore, previous studies on LCC have been targeted on studying the specific lignin-carbohydrate linkage and less emphasis has been put on the whole LCC networks. Detailed structural studies of entire LCC are therefore of importance in understanding the chemistry involved in wood formation and wood reactivity.

The aim of this study was to isolate intact LCC from wood and corresponding chemical pulps made from it in quantitative yield and to clarify their detailed chemical structure.

For the first time, a method for the quantitative analysis of lignin-carbohydrate complexes (LCCs) in softwood is presented and it could be concluded that no carbohydrate-free lignin was present in these wood fibres. From mildly ball-milled wood, all lignin was isolated as LCCs in a sequence involving a partial enzymatic hydrolysis of cellulose, subsequent swelling and quantitative dissolution, into 4 major fractions; a galactoglucomannan-lignin-pectin LCC (GalGlcMan-L-P) containing ~8% of the wood lignin, a glucane LCC (Glc-L) containing ~4% of the wood lignin, a xylan-lignin-glucomannan network LCC (Xyl-L-GlcMan) (with a predominance of xylan over glucomannan) containing ~40% of the wood and a glucomannan-lignin-xylan network LCC (GlcMan-L-Xyl) (with a predominance of glucomannan over xylan) containing ~48% of the wood lignin.

From unbleached kraft pulps, 85 - 90% of residual lignin was found to be chemically bonded to carbohydrates. The effect of the degree of delignification on the LCC types during kraft pulping and during subsequent oxygen stage was studied in order to understand the role of LCC for the stability of residual lignin. For both processes, high delignification rates were observed for the xylan-rich LCC and cellulose-rich LCC fractions, whereas the glucomannan-rich LCC was relatively stable. After a severe oxygen stage, almost all the residual lignin was isolated in the latter complex.

Thioacidolysis in combination with gas chromatography was used to determine the content of β-O-4 structures in the lignin. Periodate oxidation and methanol determinations were used to quantify the phenolic hydroxyl groups, whereas size exclusion chromatography (SEC) of the thioacidolysis fractions was used to monitor any differences between the original molecular size distribution and that after the delignification processes. Major differences between the various LCC fractions were observed, clearly indicating that two different forms of lignin are present in the wood fibre wall. These forms are linked to glucomannan and xylan respectively. The xylan linked lignin was found to consist largely of β-O-4 structures indicating a rather linear coupling mode, whereas the glucomannan linked lignin was more heterogeneous with respect to the known lignin inter-unit linkage types. Based on these findings, a modified arrangement of the fibre wall polymers is suggested.

From acid sulfite pulp (Kappa number 11) residual lignin was isolated at ~80% yield on LCC basis. About 60% was linked to xylan, 30% to glucomannan and 10% to glucans. These values differ greatly from those obtained for softwood pulped to a similar kappa number by the Kraft method. Model compound studies indicated that the benzyl ether type of LC linkage were likely to survive cleavage at the acidic sulfite pulping conditions.
Abbreviations and terms

BMW: Ball milled wood
DC: Degree of condensation
DDQ: 2,3 Dichloro- 5,6- dicyano- 1,4- benzoquinone
DP: Degree of polymerisation
DHP: Dehydrogenation polymer (Synthetic lignin)
EMWL: Enzyme treated milled wood lignin
GC-FID, GC-MS: Gas chromatography (GC) combined with flame ionisation detection (FID) or mass spectrometer (MS)
GalGlcMan-L-P: Galactoglucomannan-lignin-pectin network
Gl-L: Glucan-lignin complex
GlcMan-L-Xyl: Glucomannan-lignin-xylan network with a predominance of glucomannan over xylan
HPLC: High performance liquid chromatography
LC bond: Lignin carbohydrate bond
LCC: Lignin carbohydrate complexes
ML: Middle lamella
MMD: Molar mass distributions
MWEL: Milled wood enzyme lignin
MWL: Mild wood lignin
NMR: Nuclear magnetic resonance
P: Primary cell wall
RI: Refractive index
RL: Residual lignin
SEC: Size exclusion chromatography
SW (S1, S2, S3): Secondary cell wall
UV: Ultraviolet
Xyl-L-GlcMan: Xylan-lignin-glucomannan network with a predominance of xylan over glucomannan
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6. ACKNOWLEDGEMENTS

7. REFERENCES
1. LIST OF PUBLICATIONS

This thesis is based on the papers listed below, which are referred to in the text using their roman numerals.

I. New method for the quantitative preparation of lignin-carbohydrate complex from unbleached softwood kraft pulp: Lignin-polysaccharide networks I
   Martin Lawoko, Gunnar Henriksson and Göran Gellerstedt

II. Changes in the lignin carbohydrate complex in softwood during kraft and oxygen delignification: Lignin-polysaccharide networks II
    Martin Lawoko, Rickard Berggren, Fredrik Berthold, Gunnar Henriksson and Göran Gellerstedt
    Holzforschung 58(6) 603-610 (2004)

III. Characterization of Lignin-Carbohydrate Complexes from Spruce Sulfite Pulp: Lignin-polysaccharide networks III
     Martin Lawoko, Gunnar Henriksson and Göran Gellerstedt
     Submitted to Holzforschung.

IV. Characterisation of Lignin Carbohydrate Complexes (LCCs) in Softwood
    Martin Lawoko, Gunnar Henriksson and Göran Gellerstedt
    Submitted to Holzforschung

V. Analysis of Phenylglycosidic bonds
    Martin Lawoko, Göran Gellerstedt and Gunnar Henriksson
    Manuscript

VI. Structural differences between lignin-carbohydrate complexes present in wood and chemical pulp
    Martin Lawoko, Gunnar Henriksson and Göran Gellerstedt
    Submitted to Biomacromolecules

Reprints are published with the kind permission of the journals concerned.

Other related publications:

Hemicellulase activity of aerobic fungal cellulases
M. Lawoko, A. Nutt, H. Henriksson, G. Gellerstedt and G. Henriksson
Holzforschung 54 497-500 (2000)


Structural changes in the residual kraft pulp lignins. Effects of kappa number and degree of oxygen delignification.
Camilla Rööst, Martin Lawoko and Göran Gellerstedt

Monocomponent endoglucanases- an excellent tool in wood chemistry and pulp processing.
Gunnar Henriksson, Martin Lawoko, Maria Christiernin and Marielle Henriksson
2. INTRODUCTION

2.1 Chemical composition of wood

Wood is a heterogeneous biological material consisting of different types of cells. The cell walls are composed mainly of cellulose, hemicelluloses, lignin and small amounts of extractives, proteins and inorganic components, all in different proportions which vary from hardwood to softwood species. The term fibre is frequently used for most types of wood cells.

Cellulose

Cellulose is the most abundant natural polymer known. It consists of β-D-glucopyranose units linked together by (1-4)-glycosidic bonds. The degree of polymerisation (DP) of wood cellulose is somewhere between 8000-10 000. The molecule is linear, and the linearity leads to a strong tendency for cellulose molecules to interact together by intra and intermolecular hydrogen bonding, whereby the molecules bundle together to form microfibrils consisting of both highly ordered crystalline- and less ordered amorphous regions. Approximately 40-50% of the dry substance in most wood is cellulose.

Softwood hemicelluloses

Unlike cellulose, hemicelluloses are a group of branched heteropolysaccharides. The hemicelluloses in softwoods differ slightly in structure from those in hardwoods. The average degree of polymerisation of the hemicelluloses is approximately 200.

Galactoglucomannans: These are the principal hemicelluloses in softwood, constituting about 20% of the dry material. The backbone is a linear chain of (1-4)-linked β-D-glucopyranose and β-D-mannopyranose units. The α-D galactopyranose residue is (1-6)-linked to the mannopyranoside unit as a single unit. An important structural feature is that the C-2 and C-3 positions in the chain units are partially O-acetylated, on the average of one group per 3-4 hexose residues. These acetyl groups are easily cleaved by alkali. The galactoglucomannan of softwood are divided into 2 types differing in galactose content. The fraction with a low galactose content has a galactose:glucose:mannose ratio of 0.1:1:4, whereas the galactose-rich fraction has a ratio of 1:1:3. The former fraction is normally simply referred to as glucomannan.

Arabinoglucuronoxylan: These constitute 5-10% of the dry material in softwood. They are composed of a framework containing (1-4)-linked β-D-xylopyranose units which are partially substituted at C-2 by 4-O-methyl- α-D-glucuronic acid groups, on the average, two residues per ten xylose units. The framework also contains 1.3 residues of α-L-arabinofuranose per ten xylose units. Due to the furanosidic structure of the arabinose side chains, they are easily hydrolysed by acids. On the other hand, both arabinose and uronic acid substituents stabilize xylan toward alkali-catalyzed degradation.

Pectin: Although the pectic substance is usually not classified as hemicelluloses, the distinction is often difficult and more or less arbitrary. Pectic polysaccharides consist mainly of D-galactosyluronic acid, D-galactose, L-arabinose and L-rhamnose residues. Pectic substances include galacturonans, rhamnogalacturonans, arabinans and galactans and are mainly located in the primary cell wall and the middle lamella.
Hardwood hemicelluloses

Glucuronoxylan: This is the main hemicellulose in hardwood species and its content varies within the limits 15-30% of the dry wood, depending on the hardwood species. The backbone consists of (1-4)-linked β-D-xlyopyranose units, with about seven out of ten of the xylose residues containing O-acetyl groups at the C-2 and C-3 positions. One of ten xylose units carries a (1-2)-linked 4-O-methyl-α-D-glucuronic acid group.

Glucomannan: Around 2-5% of the dry wood is glucomannan material. These are composed of β-D-glucopyranose and β-D-mannopyranose units linked by (1-4)-bonds. The glucose:mannose ratio varies between 1:2 and 1:1 depending on the wood species.

Lignin

Lignin is a polymeric natural product arising from an enzyme-initiated dehydrogenative polymerisation of the three primary precursors shown in Figure 1; coniferyl- (1), sinapyl- (2) and coumaryl- (3) alcohols (Freudenburg 1968, Adler 1977). Softwood lignin structure is largely a result of the polymerisation of coniferyl alcohol, whereas hardwood lignin structure consists mainly of guaiacyl and sinapyl structures and grass lignin of coumaryl structures in addition to the former two types.

\[
\begin{align*}
(1) & \quad R_1 = H, R_2 = OCH_3 \\
(2) & \quad R_1 = R_2 = OCH_3 \\
(3) & \quad R_1, R_2 = H
\end{align*}
\]

Figure 1. Phenyl propanoid units as building blocks for lignin

The ρ-position of the aromatic ring is either etherified or of the phenolic type, whereas the α-position has either none, one or two methoxy groups. Alternatively, the former position may be C-substituted. The structural elements in lignin are not linked to each other in any particular order, although some of the linkage types seem to be more thermodynamically favoured (Table 1, Figure 2). The polymerisation results in a highly branched three-dimensional crosslinked polymer of hitherto unknown molecular mass (Figure 3). About 27-30% of the dry material in softwoods and between 20-26% in hardwoods consists of lignin.

Table 1. Proportions of the main lignin inter-unit linkages in softwood

<table>
<thead>
<tr>
<th>Structure</th>
<th>Linkage type</th>
<th>% of linkage in Softwood lignin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Arylglycerol β ary1 ether unit (β-O-4)</td>
<td>36&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>Phenylcoumaran unit (β-5)</td>
<td>12&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>5-5 units</td>
<td>11&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>Diaryl ether units (4-O-5)</td>
<td>4&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>β-1 units</td>
<td>2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>6</td>
<td>β-β units</td>
<td>3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>7</td>
<td>Dibenzodioxin (5-5-O-4)</td>
<td>5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>The digits represent the structural types in Figure 2.
<sup>b</sup>Determined using spruce MWL (Zhang and Gellerstedt 2000)
<sup>c</sup>According to Brunow et al. 1999
Figure 2. The main types of lignin inter-unit linkages in Softwood (Numbers 1-7 represent the linkage types in Table 1).

Figure 3. A hypothetical structure of the polymerised softwood lignin (The Ljungberg Textbook, Wood Chemistry and Wood Biotechnology).
2.2 Wood morphology

Organisation of fibre elements in the cell wall

The fibre cell wall consists of different layers, namely the primary- and the secondary walls in which the wood components are distributed. The chemical compositions of these layers differ.

![Cell wall structure of softwood tracheid](image)

**Figure 4.** The cell wall structure of softwood tracheid. S1 = secondary cell wall 1\textsuperscript{st} layer, S2 = secondary cell wall 2\textsuperscript{nd} layer, S3 = secondary wall 3\textsuperscript{rd} layer.

The primary cell wall is very thin (0.1-0.2µm) and contains pectin, lignin hemicelluloses and cellulose. Some of the hemicelluloses found in this part are distinct in structure from the hemicelluloses found in the secondary wall, such as xyloglucan.

The secondary cell wall consists of a thin outer layer (S1), a thick middle layer (S2) and a thin inner layer (S3). The S1 layer is between 0.1 and 0.2µm thick (Booker and Sell 1998) and S2 layer is about 1µm thick in early wood and 5µm thick in late wood (Sjöström 1993). Between adjacent cells is the middle lamella, which holds the cell together. The middle lamella contains mainly lignin and higher pectin levels than the secondary cell wall.

![Ultrastructural arrangement of wood polymers](image)

**Figure 5.** A proposed model for the ultrastructural arrangement of wood polymers in the secondary cell wall (Salmen and Olsson 1998). Reprinted with permission.

Studies of the softening behaviour of glucomannan and xylan have led to the proposal that xylan is more associated with lignin and glucomannan is more associated with cellulose (Salmen and Olsson 1998, Figure 5) in the secondary cell wall. This proposition was further supported by a FT-IR spectroscopy study (Åkerholm and Salmen 2001).
2.3 Processing of wood to pulp

Wood is converted into pulp in one of two ways. In mechanical treatment, the wood is disintegrated into fibre by grinding or refining. The resulting pulp is obtained in high yield. The process is however demanding in terms of the mechanical energy needed to produce the pulps. Alternatively, pulp is produced by chemical processing of wood, whereby the lignin is degraded and dissolved to release free fibres. For this purpose, two methods are commonly used; namely, kraft or sulphate pulping and sulfite pulping.

General aspects of Kraft pulping

Kraft pulping is nowadays the dominating technology used to produce unbleached pulp. The pulp produced is superior in strength properties to that from the other pulping processes. The wood chips are pulped at 150 - 170 °C in an alkaline solution with hydroxide- and hydrosulphide ions as the active delignifying agents. The selectivity of delignification is, however, observed to change during the kraft pulping (Gellerstedt and Lindfors 1984a, Lindgren and Lindström 1996). Delignification is thus divided into 3 phases, namely initial, bulk and residual delignification (Figure 6). The yield losses during the kraft pulping have been found to be substantial, especially in the case of the hemicelluloses. The chemical reactions occurring during the different phases have been studied. The main lignin reactions occur during the first two phases. The residual delignification phase is unique in that very little lignin is dissolved and the carbohydrate losses become substantial. Therefore, the kraft cook has to be terminated at this stage. The degree of delignification at termination is often around 90%, but may depend on the pulping conditions. The remaining lignin is removed in the more selective oxygen delignification process. However, even in this case a slow delignification rate is observed in the residual phase, which occurs when ~ 50% delignification has been achieved (Olm and Teder 1979).

![Dissolved carbohydrates, % on wood](image)

**Figure 6.** The selectivity of delignification in the three phases of kraft pulping (Gellerstedt and Lindfors 1984a)

A lot of research, past and ongoing, focuses on trying to understand the reasons for the retarded delignification observed during the final phases of the kraft pulping and oxygen delignification. Three
major conclusions have been derived; 1) The presence of alkaline-stable native lignin structures, 2) condensation reactions occur in lignin, 3) The presence of alkaline stable covalent linkages between lignin and carbohydrates. It has not been possible to determine the relative importance of the three reasons given above, however, experimental support for all three has been presented.

*Acid sulfite pulping*

Although kraft pulping has superseded the sulfite process as the predominant technology used worldwide to produce chemical pulps from wood, the acid sulfite process is still important for the production of dissolving pulps used to produce cellulose derivatives and regenerated cellulose, such as viscose. Acid sulfite pulp will therefore continue to be produced during the foreseeable future at a number of mills around the world.

The effectiveness of delignification during acid sulfite pulping has been attributed to both sulfonation and hydrolysis reactions. The former reaction type makes the lignin more hydrophilic by introducing sulfonic groups, and the latter breaks ether bonds and creates new phenolic groups, thereby increasing the hydrophilicity of lignin as well as lowering its molecular weight. The reaction mechanisms involved in delignification during acid sulfite pulping have been studied using model compounds (Gellerstedt and Gierer 1971), who showed that the sulfonation of lignin occurred mainly at the Cα-position of lignin whereas the β-aryl ethers remained stable. It has also been proposed that the dissolution of lignin is retarded by lignin condensation reactions taking place during the pulping (Gellerstedt 1976).

### 2.4 Background and aims of this study

Although the chemical structure and ultrastructure of wood has been well studied, the interactions between the fibres are less understood. Evidence for covalent linkages between lignin and carbohydrates, in the so-called lignin carbohydrate complex (LCC), have been reviewed in a number of articles, which will be discussed in more detail later on. The existence of covalent bonds between lignin and carbohydrates is of considerable interest in connection with a number of issues in wood chemistry, such as the reactions taking place during the formation of wood, the natural molecular weight distribution of lignin and carbohydrates, swelling and accessibility properties and the reactivity of wood during its processing, e.g., during chemical pulping.

It has been argued that such linkages may be responsible for the retardation of delignification in the final phases of chemical pulping as discussed above. Different linkage types have been proposed, some of which may resist cleavage under the pulping conditions. Yet the LCC issues remain controversial for a number of reasons. The isolation yield of LCC from wood and pulps has been low, making it impossible to quantify them. The reason for this is inaccessibility both in the wood and pulp, making degradation techniques necessary to access them. However, during such degradations some of the lignin carbohydrate linkages may be cleaved, or alternatively, artificial linkages may be formed. On the other hand, if such degradations are controlled, selectively protecting the lignin-carbohydrate linkages or selectively cleaving them in order to analyse the new functional groups formed upon their cleavage, then the data obtained can be valid. This has been done in many studies, although the results are not free from criticism. The method used to isolate LCC is critical, since it is desired that the LCC isolates are representative of their native state in the fibre material. Another problem is that the analytical methods used to study the LCCs have been limited to indirect methods.

The aim of this work has therefore been the following:

1) **To develop a method for quantitatively isolating LCC from wood and chemical pulp**
2) **To study the linkages between lignin and carbohydrate in LCC**
3) **To investigate the importance of LCC for the retardation of delignification in the residual phase of kraft pulping**
To study the chemical structure of the different LCC types in softwood and corresponding kraft- and oxygen-delignified pulps made from it, in order to obtain relevant information on wood formation and on the delignification process from an LCC perspective.

2.5 The chemistry of kraft pulping and oxygen delignification

Carbohydrate reactions

Kraft pulping
The main general reactions of carbohydrates during kraft pulping are of two types; peeling reactions and alkaline hydrolysis. In the former, an alkaline-induced stepwise elimination of monomeric sugars occurs, beginning from the reducing end of the carbohydrate. This reaction rate is substantial already at around 100 °C and a depolymerisation of the polysaccharide occurs (Kondo and Sarkanen 1984). Such depolymerisation facilitates the dissolution of polysaccharides of short chain length such as the galactoglucomannans of softwoods, which become dissolved to a large extent in the initial phase of kraft pulping (Kondo and Sarkanen 1984). In soft- and hard wood xylans however, a less efficient peeling occurs due to their C2- substituents. Generally, the peeling reaction involves the elimination of isosaccharinic acid, which is formed after a series of intermediate reactions (Sjöström 1977). At higher temperatures however, the formation of a metasaccharinic acid occurs, which stabilises the carbohydrate against further peeling in a so-called stopping reaction. In particular, the stabilisation of softwood arabinoglucomannans is observed due to the presence of arabinose on the 3-position and the presence of 4-O-methylglucuronic acid on the 2-position.

In the bulk phase of pulping a second type of reaction, i.e., alkaline hydrolysis of the carbohydrates begins. This reaction begins at temperatures over 150 °C in kraft pulping, and is responsible for the significant decreases observed in the degree of polymerisation of carbohydrates. Furthermore, since it creates new reducing ends, it promotes secondary peeling.

Although the xylan is more stabilized against peeling reactions, significant amounts of it actually become dissolved in the cooking liquor as polysaccharides (Sjöström 1977). However, the readsorption of some of the xylan onto the fibres during kraft pulping has also been observed (Yllner and Enström 1956). Significant loss in yield of polysaccharides occurs during kraft pulping of softwood, and the main contributor to this is glucomannan (present as galactoglucomannan in original wood). During pulping, an important reaction occurs in the 4-O-methylglucuronic moiety in xylan side chain, whereby an unsaturated structure is formed by the loss of methanol (Johansson and Samuelson 1977), leading to the formation of hexenuronic (HexA) groups which remain attached to the xylan backbone (Buchert et al. 1995) and contribute substantially to the acidic groups in pulp. The presence of hexenuronic acid groups has also been found to substantially contribute to the kappa number of pulp (Gellerstedt and Li 1996, Li and Gellerstedt 2000).

Oxygen delignification
During oxygen delignification, carbohydrate reactions also occur. Oxygen is itself a biradical, and therefore initial reactions with electron rich structures, such as phenolate anions formed from phenolic hydroxyl groups in lignin at alkaline conditions, lead to four one-electron reduction steps from oxygen to water, involving the formation of hydroxide radical. The hydroxide radicals can attack polysaccharides leading to chain scissions (Dence 1996) and thus to viscosity loss.
Kraft pulping

The main reaction in lignin during kraft pulping is the cleavage of β-O-4 linkages resulting in the formation of new phenolic hydroxyl groups (Figure 7a). This lignin fragmentation reaction not only cause substantial decrease in the molecular mass of residual lignin, but also gives it a more hydrophilic character by introducing new phenolic groups. Under alkaline conditions, the phenolate anions formed upon protolysis of phenolic β-O-4 structures are converted into a quinone methide intermediate. A nucleophilic attack by a hydrosulfide ion at the α-carbon of the quinone methide leads to the formation of a benzylthiol structure. The cleavage of β-O-4 bonds leading to the fragmentation of lignin then occurs through another nucleophilic attack by the thiol group at the α-carbon, or by another hydrosulfide ion. However, the desirable lignin fragmentation can be restricted by the low availability of hydrosulfide ions.

Figure 7a. The reactions of β-O-4 structures during kraft pulping

In undesirable reactions, the quinone methide also undergoes lignin condensation reactions (Gierer 1970), side chain reduction reactions (Gellerstedt and Robert 1987) and conversion into enol ether structures (Gellerstedt and Lindfors 1987a). The condensation of the quinone methide with polysaccharide peeling intermediate has also been proposed (Gierer and Wännström, 1984) forming lignin carbohydrate bonds (LCC) and could lead to restriction of dissolution of lignin since a higher molecular weight is achieved. Recently, a new mode of radical-initiated lignin condensation reaction has emerged and been proposed to restrict delignification (Gellerstedt et al., 2004).

Oxygen delignification

The main reaction of lignin involves a partial oxidation of the phenolic structures. The phenolate anions formed in the alkaline environment transfer an electron to the oxygen biradical leading to the formation of superoxide and phenoxy radicals. In subsequent reaction steps, the phenoxy radical is converted into a hydroperoxide which in turn reacts to form oxidized products (Kratzl et al. 1966, Gierer and Imsgard 1977). In line with the decrease in the phenolic content of lignin during the oxygen stage, an increase in
the carboxyl groups has been observed (Gellerstedt et al. 1986, Gellerstedt and Lindfors 1987b). Furthermore, structures of the biphenolic type have been found in kraft pulp and their content observed to increase during the oxygen stage (Gellerstedt et al. 1986). Conjugated structures, such as stilbenes, and enol ethers react rapidly whereas non-conjugated types such as propylguaiacol and β-aryl ethers react more slowly (Ljunggren and Johansson 1990a, Ljunggren and Johansson 1990b).

The pulps obtained after the two delignification processes discussed above still contain small amounts of lignin. Such lignins are normally oxidized (bleached) using bleaching sequences which may include the use of ozone, chlorine dioxide, peracetic acid and hydrogen peroxide.

2.6 A review on pioneering- and recent works on Lignin Carbohydrate Complex (LCC)

The question of whether lignin is chemically bound to polysaccharides in the plant cell or whether it is present in free state has been one of the most frequently debated issues in the history of wood chemistry. Earlier results on the existence of LCC in wood were reviewed by Merewether (1957). In 1866 Erdmann hypothesized that covalent bonds occurred between lignin and carbohydrates in wood, basing his hypothesis on the observation that it was difficult to separate the two components. This material he called “glycolignose”. Many decades latter, several works in support of Erdmann's hypothesis emerged. Traynard et al (1953) observed that when poplar was hydrolysed with water buffered at four different pH levels (range 2.2 to 4.2), the ratio of the percentage of lignin dissolved to the percentage of pentosans dissolved was constant. The interpretation of this was that their existed covalent linkages between pentosans and lignin. Earlier on, Sarkar and coworkers (1952) had observed that the treatment of jute with weak alkali doubled its acid value. The additional free acid was interpreted to be a result of the cleavage of ester linkages between lignin and polyuronide. Tachi and Yamamori (1951) made the observation that the carboxyl content of holocellulose was higher than that of original wood, and concluded that the cleavage of an ester linkage between lignin and polyuronide had occurred. The concept of a lignin-polyuronide linkage was thus further substantiated. From finely divided wood, Brauns (1952) was able to extract 2 - 3% of the total wood lignin with cold ethanol. The soluble fraction was named native lignin, also commonly referred to as Brauns native lignin. This fraction was free from carbohydrates. The low yield of soluble lignin was interpreted to mean that the remaining lignin in wood was highly polymerised or that at least a part of it was bound to carbohydrate that restricted its extraction. It was reported that the degree of milling did not lead to an increase in the yield of Brauns native lignin. In support of Brauns, Björkman observed no increase in the alcohol solubility when wood was ground in a vibratory mill for 48 hours.

Björkman (1956) developed a method for isolating lignin from wood after ball milling. The lignin preparation, globally referred to as milled wood lignin (MWL), was obtained by extracting the ball milled wood with dioxane: water mixtures. Furthermore, Björkman found an “inseparable mixture” of lignin and carbohydrates and introduced the term “lignin-carbohydrate complexes” (LCC), a term which has claimed global recognition. From his works on spruce wood, Björkman concluded that ~25% of the lignin was extracted as MWL, ~ 20% as lignin carbohydrate complexes (LCC) with other organic solvents (such as dimethyl sulfoxide (DMSO), dimethyl formamide (DMF), and acetic acid:water mixtures), ~9 % as “intermediate” fractions on purification of the crude LCC, and ~42% remained in the residue. The LCC obtained in the Björkman preparation have been referred to as Björkman LCCs.

Benzylether LC-bond  
Benzylester LC-bond  
Phenyglycoside LC-bond  
Acetal type LC-bond  

**Figure 7b.** Proposed types of lignin carbohydrate linkages.

The biosynthetic pathway for the ether and ester types of lignin-polysaccharide covalent bonds has been proposed to involve a nucleophilic addition of the hemicelluloses to the quinone methide intermediate formed from the dehydrogenative polymerization of coniferyl alcohol during lignin biosynthesis (Freudenberg and Groin 1959, Freudenburg and Harkin 1960), and has been substantiated by model experiments in aqueous solutions (Tanaka et al. 1976, Leary et al. 1983). In another model study, however, it was shown that the addition of hydroxyl groups in carbohydrates could not compete with the addition of water or phenols (Sipilä and Brunow 1991), leading to the proposal that if the formation of the benzyl ether type of lignin-carbohydrate bond takes place in lignin biosynthesis, it does so in the hydrophobic regions of the cell wall.

The analytical methods for LC bonds are summarised below.

**Acid degradation methods** (Eriksson and Lindgren 1977, Eriksson and Goring 1980) have been applied to selectively cleave the ether bonds and further analyse the new functional groups formed on hydrolysis.

**Alkali degradation methods** have been applied to study ester linkages by saponification of the linkage and analysis of the carboxyl function (Eriksson and Goring 1980, Obst 1982, Takakashi and Koshijima 1988). Lundquist et al. (1983) showed that the release of xylan from a mild wood lignin (MWL) preparation occurred on a mild alkaline treatment, and a concomitant decrease in the molar mass of the MWL was observed by size exclusion chromatography, suggesting that linkages between xylan and lignin were cleaved by the alkaline treatment.

**Smith degradation method** was first described by Goldstein et al. (1965). The method involves the transformation of glycosidic linkages to acyclic acetal linkages, which are more susceptible to acid hydrolysis and will be cleaved under appropriate conditions. The method has been applied to study LC bonds (Eriksson and Goring 1980, Yaku et al. 1981) whereby, the sugar remaining on the lignin after the smith degradation and acid hydrolysis procedures were proposed to be linked to the lignin.
Methylation analysis has been employed extensively to study the linkage sequences in carbohydrates (Hakamori 1964, Prehm 1980). The method has also been employed to study LC ether bonds in wood (Koshijima et al. 1976, Minor 1982) and in pulps (Minor 1986), in work up procedures normally involving borohydride reduction, permethylation, acid hydrolysis of glycosidic bonds, reduction with labelled borodeuteride (indicates chain units) and finally, an acetylation of the new hydroxyl group formed upon cleavage of the LC ether bonds. The linkage analysis is often performed by GC FID and GC MS.

The methods discussed above do not include analyses of the lignin skeleton. Therefore, the possibility of the carbohydrate being bonded to some other substance than lignin cannot be excluded. By the following methods, information on both the lignin- and carbohydrate parts of the LC bond was obtained.

DDQ oxidation involves the selective oxidation of α-ether LC bonds and α-ester LC bonds in acetylated LCC, to α-carbonyls by 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) (Koshijima et al. 1984, Watanabe and Koshijima 1989). The carbonyl groups are analysed by 13C NMR methods, whereas the new hydroxyl groups on the polysaccharide are analysed by employing the methylation sequence discussed above. The method may however, result in an underestimation the content of α-ester LC bonds due to uronosyl group migration occurring between the Cα and Cγ positions of lignin (Li and Helm 1995).

Ozonation of LCC in acetic acid: water: methanol 16:3:1 at 0 °C yielded α- etherified erythronic- and α-etherified threonic acids (Karlsson et al 2000, Ikeda et al 2005), which were evaluated by GC MS.

13C labelling techniques in combination with 13C NMR
New approaches involving the administration of 13C-enriched lignin precursors into differentiating xylem followed by NMR analysis are useful tools in the direct analysis of LC bonds. By administering lignin precursor 13C-enriched at the α, β or γ position of coniferin into ginko shoots, a 13C-enriched ginko wood was obtained, from which 13C-enriched Björkmann LCC was isolated (Xie et al 2000). 13C NMR studies indicated the presence of LC bonds of ethers- esters and ketal types at the α-C position of side chain phenyl propane units in the lignin. By employing similar techniques on Eucalyptus globulus, (Etvuguin et al 2005), α and γ-ester linkages between lignin and heteroxylan and pectin were detected and proposed to be the predominant LCC.

The analytical techniques discussed above apply mainly to LC ether- and ester bonds.

Formation of lignin carbohydrate (LC) bonds in kraft pulp

A number of data have indicated the presence of LC-bonds in wood, as discussed above. A few works using model compounds, have, however, indicated the possibility of LC bonds being formed during kraft pulping. The LCC types formed include LC ethers bonds (Gierer and Wännström 1986, Iversen and Wännström 1986) and LC carbon-carbon bonds (Fullerton and Wilkins 1985, Fullerton 1987). On the other hand, based on the similarities in the linkage types and their quantities when LC bonds in pine wood and kraft pulp made from it (Minor 1982, Minor 1986) were compared, it was concluded that an enrichment rather than a formation of such bonds occurs during kraft pulping.
3. MATERIALS AND METHODS

3.1 Isolation of Lignin Carbohydrate Complexes (LCCs)

LCCs were isolated in quantitative yield after a complete dissolution of spruce wood and the corresponding unbleached and oxygen-delignified kraft pulps, respectively. The fractionation and work-up procedure was a new method developed as part of this work and the analytical protocol is shown in Figures 8a and 8b. Detailed accounts on the fractionation are described in Papers I, II and IV. The method is discussed in detail in the results and discussion section.

3.2 Preparation of kraft pulps

Unbleached kraft pulps were prepared from industrial chips of Scandinavian softwood Norway spruce (Picea abies) 70% and Scots pine (Pinus sylvestris) 30% at Södra Cell, Research and Development, Mörrum, Sweden. The chips were cooked in stainless steel autoclaves at a liquor:wood ratio of 3.5:1. A homogeneous temperature profile was achieved by rotating the autoclaves in a polyethylene glycol bath. In all the experiments, the cooking temperature was 170 °C with a heating rate of 0.8 °C/min starting at 70 °C. The cooking conditions are shown in Table 2.

Oxygen-delignification was carried out in teflonised rotating, stainless steel autoclaves. All pulps were treated using a single stage O- or two stage O(1)O(2) process in order to reach a similar lignin content, irrespective of the unbleached kappa number, using the conditions in Table 3.

Table 2. Kraft cooking conditions and kappa numbers of pulps

<table>
<thead>
<tr>
<th>Sample</th>
<th>Kappa no.</th>
<th>Time at max. temp., (min)</th>
<th>Effective alkali (charge, %)</th>
<th>Residual [OH], M</th>
</tr>
</thead>
<tbody>
<tr>
<td>K25</td>
<td>23</td>
<td>75</td>
<td>21.5</td>
<td>0.27</td>
</tr>
<tr>
<td>K35</td>
<td>34</td>
<td>55</td>
<td>20</td>
<td>0.25</td>
</tr>
<tr>
<td>K55</td>
<td>49</td>
<td>35</td>
<td>20</td>
<td>0.28</td>
</tr>
</tbody>
</table>

Table 3. Conditions used in the oxygen delignification stage(s)

<table>
<thead>
<tr>
<th>Conditions</th>
<th>O or O(1)</th>
<th>O(2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Po2, MPa</td>
<td>1.6</td>
<td>0.6</td>
</tr>
<tr>
<td>Temperature, °C</td>
<td>100</td>
<td>110</td>
</tr>
<tr>
<td>Time, min</td>
<td>20, 30</td>
<td>0-170</td>
</tr>
<tr>
<td>Pulp consistency, %</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>NaOH, %</td>
<td>1.5-7.7</td>
<td>-</td>
</tr>
</tbody>
</table>
3.3 Chlorite delignification

Holocellulose was prepared from 10 g of spruce wood meal by repeated treatment with aqueous sodium chlorite at pH 5 and 70 °C (Ahlgren and Goring 1970). The almost lignin-free holocellulose was further fractionated into xylan and galactoglucamannan fractions using the work-up scheme published by Timell (1961).

In another case, the above procedure was optimised so that only 50% delignification was achieved. Thus, after the first treatment with aqueous sodium chlorite (24h), a fresh dose of reagent mixture was added and the reaction was allowed to proceed for 2h at the above conditions.

3.4 Kinetics for the Acid Hydrolysis of Polysaccharides and Model Compounds

The kinetics for acid hydrolysis of xylan and galactoglucamannan was studied and compared to that for cellulose (Avicel) at 80 °C and pH 1.5. The degree of hydrolysis was monitored by the increase in the number of reducing end groups as a function of hydrolysis time using the dinitrosalicylic acid (DNS) assay method (Miller 1959).

For model compounds of the phenylglucoside and benzyl ether types, the kinetics for acid hydrolysis was followed at pH = 1 and 75 °C. In each case, 10 mg of sample in 5 ml of 0.1 M hydrochloric acid was used. A pH = 1 was chosen since, at pH = 1.3, the benzyl ether model was found to be quite stable and no reaction could be observed after 2h.

3.5 Preparation of residual lignins

Residual lignin was prepared according to Gellerstedt et al (1994). Acetone-extracted pulps were treated with 0.1M HCl in dioxane:water 82:18 for two hours at reflux temperature. The dissolved lignin in the dioxane-water extract was precipitated by evaporation of the dioxane. Small portions of water were added repeatedly during the evaporation to maintain a constant volume so as to avoid strong acidity. The precipitated lignin was washed with ice cold water and freeze dried.

3.6 Preparation and fractionation of Milled Wood Lignin (MWL)

MWL was prepared in accordance with the procedure of Björkman (1956), as described briefly below. The acetone extracted wood meal was ball milled for 48 hours. Lignin was extracted from the obtained milled wood upon treatment with dioxane:water 96:4 for 48 hours. The precipitated lignin, formed in the aqueous solution upon the evaporation of the dioxane was freeze dried to obtain a crude milled wood lignin product (MWL-crude) that was purified according to Lundquist (1977) to obtain a pure part (MWL-pure) and a carbohydrate-containing part (MWL-impure).

3.7 Enzymatic hydrolysis of hemicelluloses in Milled Wood Lignin (MWL)

Twenty five millilitres of a 10 mM bistris buffer at pH 7 was added to 50 mg of Impure MWL, and 20 µL of the hemcellulase ecopulp XM was added and the whole mixture was incubated at 50 °C for 2 hours. The enzyme hydrolysate was removed by centrifugation and the pellet was washed copiously with deionised water, before the lignin was freeze dried. The lignin was then acetylated according to a described method (Gellerstedt et al. 1992).

3.8 Control of tranfglycosylation activity of endoglucanase (Novozyme 476)

The endoglucanase was controled for transglycosylation activity of enzyme. 0.5g of vanillyl alcohol and 0.3g of cellulbiose were incubated with endoglucanase for 48 hours. The products were studied by
reverse phase HPLC and the retention times compared with that of the reactants, and that of phenylglucoside model compound. The HPLC system was an acetonitrile/water system.

3.9 Quantification of lignin and carbohydrates

The Klason lignin was determined according to TAPPI test Method T222 om-83 with a slight modification in that, instead of boiling to accomplish complete hydrolysis of polysaccharides, autoclaving at elevated temperature (125 °C) and pressure (1.4 bar) was adopted. Carbohydrate analysis was performed as described previously (Theander and Westerlund 1986). The GC-FID analysis was performed using a Hewlett Packard 6890 instrument equipped with a BPX 70 column (12m, 0.32mm, 0.25µm film thickness). Split injection was used. The injector temperature was set at 230 °C, the detector at 250 °C and the oven at 215 °C, with helium as carrier gas at a flow rate of 0.9 ml/min.
The lignin related kappa number of the pulps was obtained by determining the hexenuronic acid related kappa number, and subtracting it from the kappa number of cook (Li and Gellerstedt 1997). For the oxygen-delignified pulps, the contribution of other oxidizable non-lignin structures to kappa number was also determined and subtracted (Li and Gellerstedt 2000).

3.10 Hydrolysis of phenylglycosidic bonds in LCC

For the determination of glycosidic bonds between lignin and carbohydrate, the original fractions were treated with a pH 1.3 solution (0.05M HCl) for 12 hours at 25 °C. 4% sodium hydroxide/ 0.8% boric acid was added dropwise until the pH was around 14. A complete dissolution was attained. As reference, the original isolates were first dissolved in 1% sodium hydroxide /0.1% boric acid. A half of each of the alkaline solutions above was neutralised with hydrochloric acid, followed by dilution with a 30mM pH 5 sodium acetate buffer. To the other half i.e., the alkaline fraction, more alkali was added so that the total volume was equivalent to that of the pH 5 fraction. The samples were analysed for phenolic content by a UV method as described below.

3.11 Determination of phenolic hydroxyl groups

Periodate oxidation method

The content of phenolic hydroxyl groups in the isolated native- and pulp lignin carbohydrate complexes (LCCs) was determined by periodate oxidation and determination of the liberated methanol (Lai 1992). The methanol determination was performed by GC FID using a Hewlett-Packard 5790 gas chromatograph. The injector and detector temperatures were kept at 150 and 250 °C respectively. The column was an Agilent technologies packed column (Porapak S 80/100 mesh, 1.2 m length, 0.32 cm outer diameter) with helium as carrier gas at a flow rate of 60 ml/min.

Ionisation difference spectroscopy method

The determination of phenolic hydroxyl groups by ionisation difference spectroscopy was employed to analyse phenyl glycoside bonds in lignin carbohydrate complexes. A Varian Cary 1E UV-Visible spectrophotometer was used to obtain the ionisation difference spectrum for the determination of the phenolic content, whereby the spectrum of the dissolved samples in neutral solution was subtracted from that in alkaline solution (Aulin-Erdtman 1954, Goldschmid 1954). From the obtained ionisation difference spectrum, the absorbance peaks at 300nm and 350nm were used to calculate the phenolic content according to Gärtner et al. (1999).
3.12 Thioacidolysis

Thioacidolysis was performed in accordance with Rolando et al. (1992) on wood, on LCCs from wood and on the LCCs isolated from the kraft and oxygen-delignified pulps. Portions of the respective product mixtures were silylated and then analysed by gas chromatography to quantify the uncondensed β-O-4 structures present in the lignin fraction, as described below. Acetylation of thioacidolysis products was performed as described earlier for lignin samples in pyridine: acetic anhydride (1:1, v/v) and allowing the reaction to proceed overnight (Gellerstedt 1992). The excess acetic anhydride was eliminated by adding methanol and cooling the mixture in an ice bath. The pyridine was removed by addition of toluene followed by rotary evaporation. Products were dissolved in tetrahydrofuran (THF) prior to SEC analysis.

3.13 Raney Nickel Desulphuration

The thioacidolysis products, dissolved in dichloromethane were introduced into glass tubes containing 5ml methanol and 1ml of a Raney nickel slurry in water. The reaction was allowed to proceed at 80 °C for 4 h. The experiments and work up procedures were carried out as described by Lapierre et al. (1991). The final product was acetylated as described above prior to GC MS analysis.

3.14 Size Exclusion Chromatography (SEC)

SEC analysis was performed on a system of three Ultra styragel columns (Waters, Milford, MA, 100Å, 500Å and 1000Å, respectively) connected in series, with a Waters 2487 UV-light detector set at 280 nm. Acetylated thioacidolysis products and MWL fractions were dissolved in THF and analysed at a flow rate of 0.8 ml/min.

3.15 Reverse Phase HPLC

The reverse phase HPLC system consisted of a column of type 300SB-CN (4.6mm x 25cm). An isocratic acetonitrile/water (acetonitrile:water 10:90) system was applied operating at a flow rate of 1.0 ml/min and detection was performed by UV at 280 nm.

3.16 Gas Chromatography

The GC FID analysis was performed with a Hewlett-Packard 6890 instrument. A DB 5MS column was used (30 m, 0.32 µm i.d., 0.25 µm film thickness). The following parameters were set for the quantification of the uncondensed β-O-4 structures in the studied samples. The detector and injector temperatures were maintained at 250 °C. A temperature program was set for the oven as follows; from 120 °C to 200 °C at a rate of 15 °C per min, from 200 °C to 260 °C at a rate of 5 °C per min, at 260 °C for 15 min, from 260 °C to 300 °C at a rate of 20 °C per min, and at 300 for 5 min. Split injection was used. Quantitative calculations were done as described by Rolando et al. (1992), Önnerud and Gellerstedt (2003).

The acetylated products from the raney nickel desulphuration were analysed by GC-MS. The same column type as that used in the GC FID experiments was used for the GC MS study. The injector, the interface and the ion source were maintained at 250 °C. The oven temperature program was similar to the GC FID study. The mass spectrometer was a Finnigan Trace GC-MS device, 2000 series, operated at 70 eV.
4. RESULTS AND DISCUSSIONS

4.1 Isolation and characterisation of lignin and lignin carbohydrate complex (LCC)

4.1.1. A survey on the isolation and characterisation of lignin carbohydrate complex from wood

In general, the isolation of wood polymers is complicated by the compact structure of the woody material. Therefore, to increase accessibility in the substrate, a mechanical treatment is often adopted. In almost all cases, the wood chips are converted into wood meal using a Wiley-mill equipped with a 40 mesh screen. At this stage, different alternatives have been used by the wood chemist to achieve a more accessible substrate;

Vibratory ball milling

The isolation of milled wood lignin and lignin carbohydrate complex by the Björkman procedure (Björkman 1956, 1957) was mentioned briefly in the introductory part. Since this method has been widely adopted to prepare both MWL and LCC, a more detailed account of the main reasons for its choice is given. Previously, large amounts of lignin have only been isolated with solvents containing acid or alkaline under rather drastic conditions. Such conditions may result in lignin degradation or condensation reactions. Björkman was able to extract lignin in relatively high yield (25 % as MWL, 20% as LCC) using neutral solvent and relatively mild conditions, after vibratory ball milling for 48 hours. 96% aqueous dioxane was found to give the best yield of MWL, and dimethylsulfoxide (DMSO), dimethylformamide (DMF) and acetic acid: water mixtures were used to extract LCC from the residue left after MWL extraction. Using these solvents, the yields of LCC were within the range of 20-33%. Dry grinding was not recommended since it was found that the wood particles agglomerated on the balls and that a temperature rise due to friction was inevitable. Hence, suspension of the meal in a low viscosity liquid, e.g., toluene was adopted. Importantly, the liquid used should not cause substantial swelling, since this was observed to lead to low yields in the subsequent extraction steps (Björkman, 1956). When Björkman LCC was ball milled, free lignin was released. It would appear that initially, vibratory ball milling reduced the particle size of wood to such an extent that organic solvents with negligible chemical action on the dust were able to extract lignin carbohydrate complexes. From Björkmans experiments, the data strongly indicated that the lignin in LCC was chemically linked to the carbohydrate. It was reasoned that a physical union would require that the LCC be composed of colloidal particles with the lignin inaccessible to solvent or in a highly polymerised state rendering it insoluble. However, Björkman observed that the molecular weight of the liberated lignin was in the same range as that of the LCC. Björkmans hypothesis on LC linkages being present in the isolated material received support from an electrophoretic study performed on Björkman LCC and MWL (Lindgren 1958), whereby the former fraction was observed to have a higher mobility. Pew and coworkers (1957) observed that the removal of carbohydrates during treatment of ball milled spruce wood meal with purified glycosidases stopped totally when about 95% of the carbohydrates were dissolved, and it was proposed that 5% of the carbohydrates were firmly anchored to lignin. On acid hydrolysis, the sugars detected were glucose (42%), mannose (23%), xylene (19%), galactose (13%) and arabinose (3%). Electrophoretic studies on acid- and alkaline- hydrolysis products of Björkman LCC led to the proposal that only the cleavage of acid-labile bonds served to separate the lignin from carbohydrates (Bolker and Wang, 1969).

Although neutral solvents were employed for the extraction of Björkman LCC, there was a possibility that since they were high boiling point solvents, modifications in the isolated LCC may occur during purification. When column chromatography emerged in the late 1960s- early 1970s, improvements in the fractionation techniques for LCC were achieved. Koshijima and Tanaka (1971) extracted the residue left after MWL extraction, first with hot water and then with DMSO. The combined extracts gave a yield of ~13% on a lignin basis. The LCC were fractionated by ion exchange chromatography using a
sephadex column, into acidic-, neutral- and lignin rich LCC fractions with 3.2%, 6.1% and 0.4% yields respectively on lignin basis. The neutral fraction was found to consist largely of acetylglucomannan. Later on it was shown by gel permeation chromatography that the latter fraction consisted of two subfractions on the basis of molecular mass and the sugar composition of each fraction was determined (Koshijima et al. 1976) (Table 4).

**Table 4. Sugar compositions of neutral LCC fraction (acetylglucomannan) of Bjorkman LCC (Koshijima et al. 1976)**

<table>
<thead>
<tr>
<th>Sugar type</th>
<th>Higher molecular mass part Composition, (%)</th>
<th>Lower molecular mass part Composition, (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannose</td>
<td>28.7</td>
<td>75.79</td>
</tr>
<tr>
<td>Glucose</td>
<td>8.19</td>
<td>18.49</td>
</tr>
<tr>
<td>Galactose</td>
<td>3.01</td>
<td>2.14</td>
</tr>
<tr>
<td>Arabinose</td>
<td>1.74</td>
<td>-</td>
</tr>
<tr>
<td>Xylose</td>
<td>5.05</td>
<td>0.37</td>
</tr>
<tr>
<td><strong>Lignin</strong></td>
<td><strong>35.23</strong></td>
<td><strong>2.31</strong></td>
</tr>
</tbody>
</table>

When the acidic LCC fractions of the Björkman LCC (Koshijima et al. 1972) were treated with β-glucosidases (Yaku et al. 1976), four fractions of lignin with lower molecular weight, with or without carbohydrates were formed. The release of free lignin was proposed to be due to the enzymatic hydrolysis of glycosidic linkages between lignin and carbohydrates.

**Chlorite delignification**

Lignin Carbohydrate Complexes (LCC) have also been observed to be present in chlorite holocellulose. After the extraction of the hemicellulose component of the spruce holocellulose with alkaline borate solution, and selectively precipitating the glucomannan with barium hydroxide (Meier 1958), it was speculated that the xylan and galactan impurities found in the precipitate were crosslinked to the glucomannan through lignin. From holocellulose with a lignin content of 8% it was observed that a major part of the lignin accompanied the hemicelluloses on alkaline extraction (Kringstad 1965). Gel filtration studies indicated that the lignin was eluted together with the hemicelluloses, indicating a chemical linkage between the two components (Kringstad and Cheng 1969). Upon treatment of the isolates with pure enzyme with mainly mannanase activity and some xylanase activity, the lignin and hemicelluloses were still eluted together, providing further support for the existence of a chemical linkage between them. Linnell et al. (1966) studied the glucomannan isolated by selective precipitation using aqueous barium hydroxide (Meier 1958) from the alkaline extract of holocelluloses. From the relationship between the lignin content and the viscosity, it was proposed that the lignin crosslinked the glucomannan forming compact molecular configurations of higher molecular weight fractions when compared to linear β-1,4 linked hexosans.

**Methylation**

In another approach to the isolation of LCC, spruce wood meal was methylated with diazomethane and extracted with methanol and chloroform respectively (Kosikova et al. 1969), from which a lignin carbohydrate complex (lignin:carbohydrate ratio 2:1) fraction was obtained. The alkali and acid reactions of the LCC were studied and it was concluded that the lignin carbohydrate bonds were largely of the phenylglycoside type, but that benzylation linkages were also present (Kosikova et al. 1972).
Most of the methods discussed above have provided useful information on the presence of lignin carbohydrate complexes in wood and it seems likely that all types of carbohydrates are involved in chemical bonding to lignin. Although most of the studies discussed above indicate the existence of native LCC, the yields obtained were low and cannot be representative of the whole material. Furthermore, the application of drastic conditions may create artificial LCC or degrade existing LCC. Information about the entire LCC networks may be lost by applying uncontrolled degradation techniques.

The ideal method for preparing native LCC should therefore fulfil the following criteria;
- A quantitative protocol in order to allow evaluation.
- Mild preparation conditions, including a proper control for possible modifications occurring in every step of the preparation.

4.1.2 Quantitative characterisation of Lignin-Carbohydrate Complex (LCC) from Norway Spruce (Paper IV)

For the first time, the wood cell components were quantitatively fractionated and analysed with respect to lignin-polysaccharide networks. The first step in the preparation was a mild ball milling procedure (Figure 8a), performed in order to increase the accessibility of the substrate in subsequent work-up steps. Direct treatment of wood without milling gives no effect since wood is a compact material and the cellulases cannot penetrate it (Blanchette et al. 1997). Importantly, the milling was performed using a laboratory vibratory mill for a short time (3 hours) in comparison to the traditional milling according to Björkman procedure discussed above (i.e. 48 hours).

The ball milled wood was treated with a commercial endoglucanase (Novozyme 476) obtained by DNA methods. A carbohydrate analysis performed on the enzymatic hydrolysate after treatment of the wood fibres with the endoglucanase, revealed the presence of significant amounts of galactose and mannose components (Table 5) in addition to the expected glucose (Table 6). This enzyme had been controlled for hemicellulase activities, which were found to be minimal.

Since some enzyme preparations contain sugars, a carbohydrate analysis was performed on the enzyme and revealed the presence of both galactose and mannose, probably bound to the enzyme. When the amounts of the two sugars present in the enzyme were compared with those in the enzymatic hydrolysate, however, it was evident that a large part of the sugars present in the latter were derived from the wood fibres (Table 5). From the enzyme hydrolysate, the glucomannan-containing fraction was selectively precipitated with aqueous barium hydroxide (P1, Figure 8b). The latter solution has previously been used to precipitate pure galactoglucomannan from holocellulose (Meier, 1958). In this work, however, lignin-containing material was observed to precipitate with the galactoglucomannan, suggesting that the lignin in this fraction was somewhere linked to it. Two types of galactoglucomannans have been reported in literature on the basis of their sugar ratios. The sugar ratios in the low and high galactose-containing fractions have been reported to be galactose:mannose 0.1:1:4 and 1:1:3, respectively (Sjöström 1993). On observation (Table 5), it seems likely that the precipitate (P1, Table 5) is an LCC within the high galactose containing fraction. Furthermore, the galactose:mannose ratio and the arabinose:xylan ratios in the P1 fraction (Table 5) are higher than those reported in the literature for softwood galactoglucomannans and glucuronoxylans, respectively (Sjöström 1993), indicating the presence of pectin in it. In softwood arabinoglucuronoxylans, there are about 1.3 arabinose units per 10 xylose units (Sjöström 1993). The presence of these sugars in the galactoglucomannan precipitate is explained by the crosslinking of the former through lignin to pectin. The presence of covalent linkages between galactoglucomannan and lignin in spruce has previously been reported as discussed above (Kringstad 1965, Linnell et al. 1966 Koshijima et al. 1976) and the presence of pectin-lignin bonds has also been proposed (Meshitsuka et al. 1982, Minor 1982, Westermark et al. 1986, Minor 1991). Lignin forming crosslinks from galactoglucomannan to pectin has however, not been reported before. Yet it seems evident here that the partial enzymatic hydrolysis of cellulose caused a concomitant solubilization of a galactoglucomannan-lignin-pectin complex. This
LCC fraction (P1, Figure 8a) was found to contain around 8% of the lignin present in spruce (P1, Table 5).

![Diagram of lignin-carbohydrate complex (LCC) characterisation process]

Treatment of the partially enzyme hydrolysed fibres with a urea solution (Figure 8a) demonstrated the multi-function of the latter; 1) It swelled up the fibres into a “fibre gel” 2) It removed the residual enzyme adsorbed on the fibres during the hydrolysis step 3) It dissolved any fraction with a lignin content of around 50% and more (P2 and S4, Table 5). The dissolution of pure lignin samples in this solvent was investigated by treating Milled Wood Lignin (MWL) prepared by the Björkman procedure (Björkman 1956) and pulp lignin prepared by acidolysis in dioxane/water system (Gellerstedt et al 1994), and a total dissolution of the two lignins was observed. A selective precipitation of glucomannan from the urea solution (S3, Figure 8a) gave a concomitant precipitation of some xylan and lignin (P2, Table 5), indicating that part of the lignin linked xylan to glucomannan to form network structures. The un-precipitated fraction (S4, Figure 8a) was found to contain predominantly xylan and lignin, but some glucomannan was also present, again indicating the presence of network structures. Although the lignin in the latter fraction was not quantified due to interference from the enzyme, a rough estimate was made from the mass balances on all fractions and accounted for at most 15% of the lignin in wood.

The “fibre gels” (R2, Figure 8a) were washed thoroughly with water. The urea-free fibre gels were completely dissolved in an alkaline borate solvent system at room temperature upon stirring for one
hour. The complete dissolution of wood fibres in a sequence involving three key steps; namely, partial enzymatic hydrolysis of cellulose, subsequent fibre swelling and alkaline dissolution, was thus achieved. The alkaline borate solution (S5, Figure 8a) was selectively precipitated by pH reduction, and at pH 12, a glucane fraction containing 4% of the lignin in wood was precipitated (P3, Table 5). On further acidification to pH 7, all remaining components i.e., hemicelluloses and a large portion of the wood lignin, remained in solution (S6, Figure 8b). Again, upon precipitation of the glucomannan in the latter solution with barium hydroxide, concomitant precipitation of some xylan and lignin components was observed (P4, Table 5), indicating the presence of network structures. This fraction contained 28% of the lignin present in wood (P4, Table 5). The other part, still in solution, was found to contain mainly xylan, although a significant amount of glucomannan was also present. This network contained 25% of the lignin in wood (S7, Table 5).

### Table 5. Sugar and lignin analyses on spruce wood, LCC fractions made from it.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Arabinan (g)</th>
<th>Xylan (g)</th>
<th>Mannan (g)</th>
<th>Galactan (g)</th>
<th>Glucan (g)</th>
<th>Klassen lignin (g)</th>
<th>Absolute amount (%)</th>
<th>Lignin yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMW</td>
<td>1.3</td>
<td>5.9</td>
<td>12.2</td>
<td>1.8</td>
<td>46.7</td>
<td>26.7</td>
<td>94.6</td>
<td>100</td>
</tr>
<tr>
<td>Enzyme</td>
<td>-</td>
<td>-</td>
<td>0.2</td>
<td>0.18</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Enzyme hydrolysate,</td>
<td>-</td>
<td>-</td>
<td>13.7</td>
<td>7.7</td>
<td>78.6</td>
<td>-</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>P1</td>
<td>3.2</td>
<td>2.4</td>
<td>33.5</td>
<td>12.6</td>
<td>5.2</td>
<td>39</td>
<td>95.9</td>
<td>8</td>
</tr>
<tr>
<td>P2</td>
<td>1.2</td>
<td>3.1</td>
<td>19.2</td>
<td>4.0</td>
<td>9.3</td>
<td>56</td>
<td>92.8</td>
<td>20</td>
</tr>
<tr>
<td>S4</td>
<td>3.1</td>
<td>18.7</td>
<td>4.3</td>
<td>2.3</td>
<td>3.6</td>
<td>65</td>
<td>97</td>
<td>≤15</td>
</tr>
<tr>
<td>P3</td>
<td>1.0</td>
<td>0.9</td>
<td>2.5</td>
<td>0.2</td>
<td>85.2</td>
<td>7</td>
<td>96.8</td>
<td>4</td>
</tr>
<tr>
<td>P4</td>
<td>0.7</td>
<td>3.2</td>
<td>33.6</td>
<td>1.8</td>
<td>12.5</td>
<td>41</td>
<td>92.8</td>
<td>28</td>
</tr>
<tr>
<td>S7</td>
<td>4.1</td>
<td>34.9</td>
<td>14.2</td>
<td>4.2</td>
<td>1.7</td>
<td>29</td>
<td>88</td>
<td>25</td>
</tr>
<tr>
<td>Chlorite pulp</td>
<td>1.2</td>
<td>6.8</td>
<td>13.3</td>
<td>1.7</td>
<td>53.8</td>
<td>14</td>
<td>90.8</td>
<td>49.8</td>
</tr>
<tr>
<td>P3-C</td>
<td>0.3</td>
<td>0.8</td>
<td>3.3</td>
<td>0.6</td>
<td>84.8</td>
<td>3</td>
<td>92.2</td>
<td>1.3</td>
</tr>
<tr>
<td>P4-C</td>
<td>0.7</td>
<td>2.6</td>
<td>41.4</td>
<td>3.6</td>
<td>11.6</td>
<td>28</td>
<td>87.9</td>
<td>15</td>
</tr>
<tr>
<td>S7-C</td>
<td>7.3</td>
<td>42.6</td>
<td>6</td>
<td>1.3</td>
<td>2.7</td>
<td>25</td>
<td>84.9</td>
<td>14</td>
</tr>
</tbody>
</table>

*See Figure 8a for abbreviations. Amount of component present in 100 grams of sample. Amount of sample analysed. Wood lignin as reference. ND = Not determined. Amount of component present in the enzyme when 100 grams of sample is incubated with it at time =0. The contribution from enzyme to the klassen lignin may be included.

### Table 6. Substrate specificity of endoglucanase (Novozyme 476)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Activity (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carboxymethyl Cellulose</td>
<td>112</td>
</tr>
<tr>
<td>Birch wood xylan</td>
<td>1.2</td>
</tr>
<tr>
<td>Locust bean gum mannan</td>
<td>4.2</td>
</tr>
</tbody>
</table>

*measured as catalysis per minute. Dinitrosalicylic acid (DNS) assay (Miller 1959) used for detection.

On all purified and freeze-dried fractions containing LCC, attempts were made to extract pure lignin fractions with dioxane:water (96:4). This solvent system dissolves milled wood lignin completely (Björkmann, 1956), and any pure lignin present in the isolates should be extracted. Lignin-containing material could, however, only be extracted from the urea fraction (S3, Figure 8a) using the dioxane:water (96:4) system, with the amount of lignin extracted corresponding to 4% of the total wood lignin. The carbohydrate content of this extract was found to be around 7%. Only trace amounts of pure lignin could be obtained from this fraction by extraction with chloroform according to a described method (Lundquist et al. 1977). The small amount of pure lignin that was found might be the result of the cleavage of lignin inter-unit linkages during the milling step and we propose that no free lignin is present in spruce wood. It is therefore concluded that in spruce wood, the lignin crosslinks parts of all the different polysaccharides to form network structures. The linkages seem to be more abundant to xylan and glucomannan. This is in line with the close proximity of lignin to the xylan and glucomannan in the cell wall observed by Salmen and Olsson (1998) (Figure 5). Several other reports have proposed
close association between lignin and hemicelluloses. This is, however, the first report on a quantitative LCC isolation involving the fractionation of intact network structures. The possibility of LC bonds being formed as artefacts of the grafting of carbohydrates onto lignin as a result of the ball milling procedure (Iversen 1985) could, however, not be dismissed. Therefore, as a control, another approach was adopted to prepare LCCs from wood with the aim of comparing the results of the two methods on an LCC basis. In this approach, a partial chlorite delignification of wood meal under mild conditions (pH 5, 70°C) was performed in order to improve the accessibility of the substrate. The process was optimised so that only around 50% delignification was achieved. The obtained “pulp” was treated in a similar sequence as the isolation of LCC from ball milled wood (Figure 8a). The results obtained were similar to those for the ball milled wood on the basis of LCC fractionation. Most of the LCCs were found in the alkaline borate fraction (S5-C, Figure 8a) and only this fraction was further analysed. Upon fractionation by selective pH precipitation, nearly all of the lignin present was found in the hemicellulose fraction (S6-C, Figure 8a), and the lignin was almost evenly distributed between the barium hydroxide precipitated (P4-C, Figure 8a) and un-precipitated (S7-C, Figure 8a) parts (Table 5). From the analysed fractions, no pure lignin fractions could be isolated by dioxane: water 96:4. Considering that the partial chlorite delignification of wood was performed under mild conditions, the presence of LCCs in such a material was not likely to be an artefact. On the contrary, the results suggested that the LC bonds are native. In previous work on highly chlorite delignified wood, the presence of lignin carbohydrate bonds have been proposed (Meier 1958, Kringstad 1965). In the present work, the striking similarities with regard to the types and quantities of the LCCs, although the approaches adopted to obtain them were quite different, are testimony to their origin in wood.

Previous attempts to create LCC by milling MWL together with a mixture of hemicelluloses (Björkman, 1957) or by milling dioxane acidolysis lignins with holocellulose (Brownell and West, 1961) were unsuccessful. On the contrary, ball milling of isolated LCC from wood liberated MWL (Björkman, 1957). The behaviour of a mixture of lignin and polysaccharides on milling seem therefore to be different from that of some other polymer types that have been shown to be converted to mixed graft- and block copolymers when milled together (Immergut and Mark 1956).

Another artificial source of LCC might be a transglycosylation effect caused by the enzyme and resulting into the formation of phenylglycosidic bonds. Such effects have been observed in impure enzymes with β glucosidase activity (Kondo et al. 1990, Amano et al. 1993). However, in other work, we have tested the endoglucanase for transglycosylation activity. Vanillyl alcohol was incubated with cellobiose in the presence of the endoglucanase, and no addition products were detected upon HPLC analysis.

4.1.3 Isolation and characterisation of residual lignin and residual lignin carbohydrate complex from kraft pulp

Although chemical pulps are relatively more accessible to extractions than is the woody material, due to their high level of delignification, the isolation of lignin is problematic due to its low content in pulps, around 2-5% for bleachable grade pulps. For analytical purposes, residual lignin thus has to be isolated or enriched. Nowadays, the techniques widely used involve preparative acid hydrolysis in a dioxane: water system (Gellerstedt et al. 1994), acidolysis in acetic acid- water- zinc chloride (Lachenal et al. 2004), alkaline extraction (Gellerstedt and Li 1994, Al-Dajani and Gellerstedt 2002), enzymatic degradation techniques (Yamasaki et al. 1981, Hortling et al. 1990, Hortling et al. 1992) and a combination of enzymatic- and acid- hydrolyses (Argyropoulos et al. 2002).

When lignin is prepared by acid hydrolysis, a pure lignin fraction in moderate yield (50-60%) can be obtained rather rapidly. However, the cleavage of some acid-sensitive aryl ether linkages has been reported (Lundquist 1973). On the other hand, when the lignin is isolated by alkaline hydrolysis, most of it is obtained as lignin carbohydrate complexes (LCC), but the yields are low and can only be increased by applying more drastic conditions, such as elevated temperatures. For instance, the treatment
of a kappa 18 softwood kraft pulp with a 2.5M potassium hydroxide solution at 140 °C dissolved 50% of the klason lignin (Al-Dajani and Gellerstedt 2002). The remaining lignin in the pulp was probably prevented from solubilization by bonding to carbohydrates. However, the dissolved lignin was fractionated as LCC and xylan was found to be the most dominant sugar whereas arabinose was enriched in the LCC. The data indicated strongly the existence of chemical linkages between lignin and xylan via arabinose, in line with previous observations (Gellerstedt and Lindfors 1984a, Iversen and Wännström 1986, Tamminen et al. 1994).

When residual lignin is prepared by enzymatic hydrolysis, an unmodified lignin is obtained, but it is contaminated with 6-12% protein (Minor 1986, Iversen and Wännström 1986) and carbohydrates (Yamasaki et al. 1981, Hortling et al. 1990). Enzymatic hydrolysis of cellulose in pine kraft pulp yielded a residue containing 77% klason lignin corresponding to 96% of the pulp lignin (Yamasaki et al. 1981). The solid residue contained 10% carbohydrates, which could not be separated from the lignin. The molecular weight of the residual enzyme lignin was found to be higher than that of MWL, but it was completely soluble in an alkaline solution. The lower reactivity and the resistance to delignification were therefore attributed to the presence of alkaline-stable lignin-carbohydrate bonds. Hortling et al. (1990) used similar enzymatic techniques to study characteristics of residual lignin as a function of kappa number. The carbohydrate content of the residual enzyme lignin was found to be ~8% and consisted mainly of glucose. The molecular mass distributions of the residual enzyme lignins were observed to decrease with the kappa number, and it was proposed that the resistance to the removal of residual lignin was due to its insolubility and to the presence of alkali-resistant LC bonds rather than to its molecular mass. The characteristics of residual lignin have also been studied by using purified enzymes with specific activities. Tenkanen et al. (1999) used pure cellulases, mannanases and xylanases separately, or in combination, and studied the molecular weight profiles by size exclusion chromatography. The results suggested that in birch kraft pulp a major part of the residual lignin was linked to xylan and a minor part to cellulose. In pine kraft pulp, however, residual lignin was linked to cellulose, xylan and glucomannan, in line with other studies (Karlsson et al. 2001a).

The collected data on the studies discussed present strong evidence for the presence of lignin carbohydrate bonds in chemical pulps. These methods are limited in that no quantification of the LCC has been afforded. In this present study, the method adopted to isolate LCC was carefully chosen considering that a large portion of the lignin was associated with the hemicelluloses. Thus the application of an endoglucanase without hemicellulase activity serves a number of advantages. The selective partial degradation of cellulose allows the extraction of intact hemicellulose LCC. In the case of a crude enzyme mixture, degradation of hemicelluloses occurs with the effect that the LCCs may partly be solubilised and will constitute part of the enzyme hydrolysate. Purification of such fractions may be problematic. Furthermore, evaluation of possible network patterns cannot be achieved from degraded substrates.

Commercial enzymes with specific activities can be obtained in large amounts. Such enzymes are made by DNA techniques, whereby the gene encoding for an interesting enzyme is transferred into the chromosomes of a fungus, which in turn produces the protein the gene encodes for. In this work, the application of a commercial endocellulase (Novozyme 476) in combination with a subsequent urea treatment of the partially degraded fibres, were found to be superior to other previous applications for the isolation of LCC from wood and pulp fibres.

4.1.4 Characterisation of lignin-carbohydrate complex in chemical pulp (Papers I, II)

Lignin Carbohydrate Complex (LCC) was also prepared in quantitative yield from softwood unbleached and oxygen bleached kraft pulps by employing a dissolution-precipitation scheme quite similar to that for wood but with the following differences.

1) After acetone extraction, the pulp was first swollen in water and then directly treated with enzyme without prior milling.

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2) The urea treatment was done for 12 hours (48 hours in the case of wood), and after the alkaline borate treatment an un-dissolved residue was present. This residue was washed to neutral pH and treated in a similar sequence as the original pulp.

3) The products dissolved during the enzymatic hydrolysis and during the urea treatment were not studied in detail, since, no lignin was detected in the hydrolysate and only an estimated 10% of free lignin had been found in the urea solution when unbleached kraft pulps were treated according to the dissolution-precipitation scheme in Figure 8b. In the case of oxygen bleached kraft pulps, no free lignin was found to be present in the dissolved fractions.

The method was originally developed using an unbleached kraft pulp, pulped to kappa 15, based on the assumption that such pulp was more accessible to the enzyme and were therefore suitable for preliminary studies. It was shown that the different LCCs varied in lignin content. The glucomannan-rich LCC contained the main portion of the residual lignin, followed by the xylan-rich LCC, and cellulose with the least portion (Table 7). At most, 10% of the residual lignin was estimated to exist as free lignin.

**Table 7.** The distribution of lignin in carbohydrate fractions from kraft pulp at kappa number 15.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Main pulp components in fraction</th>
<th>% of total pulp lignin in fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea soluble fraction</td>
<td>Lignin</td>
<td>10</td>
</tr>
<tr>
<td>pH12 precipitate, P3-P</td>
<td>Cellulose</td>
<td>8</td>
</tr>
<tr>
<td>Ba(OH)₂ precipitate, P4-P</td>
<td>Glucomannan</td>
<td>42</td>
</tr>
<tr>
<td>Ethanol precipitate, S7-P</td>
<td>Xylan and Glucomannan</td>
<td>28</td>
</tr>
<tr>
<td>Ethanol soluble part</td>
<td>Xylan and Glucomannan</td>
<td>12</td>
</tr>
</tbody>
</table>

*a*See Figure 8b for fraction identity.

*b*represents the dominant component in fraction for the cases where two components are given.

In a following study, however, it was shown that the dissolution-precipitation protocol adopted for the kappa 15 pulp (Figure 8b) worked as well for pulps with higher lignin contents, but an extra loop (Loop B, Figure 8b) was required for the quantitative LCC extraction. Three unbleached kraft pulps with different lignin contents (kappa numbers 49, 34, 23) and their oxygen-bleached equivalents to the same lignin content were thus studied for LCC patterns in order to obtain insight into the effects of the degrees of pulping and oxygen delignification on their reactivity.

The contribution of hexenuronic acid and other non-lignin oxidizable structures to the kappa number of pulp have been determined (Dahlman et al. 1997, Li and Gellerstedt 2002) and subtracted to obtain the true lignin content. Hexenuronic acid formed earlier during the cook was observed to be degraded during kraft cooking (Table 8), in line with earlier observations (Buchert et al. 1995). However, its contribution to the kappa number at the end of the cook was still significant and was not affected by oxygen delignification.
Figure 8b. Protocol for the isolation of LCC from kraft- and sulfite pulps.

The contributions to kappa number from oxidizable non-lignin structures (Table 8) were found to be significant for the oxygen-delignified pulps. This was likely to be a result of the formation of unsaturated structures during the process (Li and Gellerstedt 2002). The kraft pulps were subjected to quantitative preparation and fractionation of LCC, both directly and after oxygen delignification to similar lignin content.

Table 8. Contributors to the kappa number (K) in the pulps

<table>
<thead>
<tr>
<th>Sample</th>
<th>$K_{COOK}$</th>
<th>$K_{HEXENURONIC}$</th>
<th>$K_{NON-LIGNIN}$</th>
<th>$K_{LIGNIN}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cooked pulps</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K49</td>
<td>49.2</td>
<td>3.7</td>
<td>NA</td>
<td>45.5</td>
</tr>
<tr>
<td>K34</td>
<td>34.1</td>
<td>3.4</td>
<td>NA</td>
<td>30.7</td>
</tr>
<tr>
<td>K23</td>
<td>22.8</td>
<td>2.4</td>
<td>NA</td>
<td>20.4</td>
</tr>
<tr>
<td>$O_2$-delignified</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K49→K10</td>
<td>11.0</td>
<td>3.7</td>
<td>1.8</td>
<td>5.5</td>
</tr>
<tr>
<td>K34→K10</td>
<td>10.8</td>
<td>3.4</td>
<td>1.6</td>
<td>5.8</td>
</tr>
<tr>
<td>K23→K10</td>
<td>9.4</td>
<td>2.4</td>
<td>1.6</td>
<td>5.4</td>
</tr>
</tbody>
</table>

NA = Not Analysed

After a kraft cook, only three different LCC fractions could be isolated by adopting the scheme shown in Figure 8b. Thus, the well known comprehensive degradation of galactoglucomannan during the initial phase of a kraft cook, together with the changes in lignin and xylan content during the cook seems to affect the resulting LCCs such that the lignin-rich fractions P2 and S4 together with the galactose-rich fraction P1 are completely absent (cf Figure 8a, 8b). It has been shown that a significant portion of the glucomannan is rapidly dissolved whereas the dissolution of xylan becomes more extensive at higher alkali charge (Aurell and Hartler 1965). Obviously, the lignin bound to such glucomannan by alkali-stable bond should be co-dissolved.
Table 9. Klason lignin and carbohydrate analyses of LCC.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ara (g)</th>
<th>Xyl (g)</th>
<th>Man (g)</th>
<th>Gal (g)</th>
<th>Glu (g)</th>
<th>Klason lignin (g)</th>
<th>Abs. amount⁵</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Unbleached</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K23-pulp</td>
<td>0.9</td>
<td>7.9</td>
<td>6.6</td>
<td>0.5</td>
<td>70</td>
<td>3.4</td>
<td>89.3</td>
</tr>
<tr>
<td>P3-P</td>
<td>-</td>
<td>1.9</td>
<td>1.5</td>
<td>-</td>
<td>91.3</td>
<td>0.8</td>
<td>95.7</td>
</tr>
<tr>
<td>P4-P</td>
<td>1.6</td>
<td>9.3</td>
<td>55</td>
<td>2.6</td>
<td>17.9</td>
<td>13.2</td>
<td>99.6</td>
</tr>
<tr>
<td>S7-P</td>
<td>4.3</td>
<td>58</td>
<td>7.2</td>
<td>0.6</td>
<td>9.8</td>
<td>12.4</td>
<td>92.4</td>
</tr>
<tr>
<td>K34-pulp</td>
<td>0.7</td>
<td>7.3</td>
<td>5.7</td>
<td>0.4</td>
<td>75</td>
<td>4.5</td>
<td>93.6</td>
</tr>
<tr>
<td>P3-P</td>
<td>0.5</td>
<td>3.5</td>
<td>4.0</td>
<td>-</td>
<td>89.4</td>
<td>1.4</td>
<td>98.9</td>
</tr>
<tr>
<td>P4-P</td>
<td>1.2</td>
<td>11.2</td>
<td>20.2</td>
<td>1.7</td>
<td>7.4</td>
<td>35</td>
<td>76.8</td>
</tr>
<tr>
<td>S7-P</td>
<td>4.4</td>
<td>56.8</td>
<td>6.3</td>
<td>0.4</td>
<td>7.7</td>
<td>23</td>
<td>98</td>
</tr>
<tr>
<td>K49-pulp</td>
<td>0.4</td>
<td>5.9</td>
<td>5.3</td>
<td>0.4</td>
<td>75</td>
<td>7.3</td>
<td>94.3</td>
</tr>
<tr>
<td>P3-P</td>
<td>0.3</td>
<td>1.0</td>
<td>1.6</td>
<td>-</td>
<td>88.0</td>
<td>2.4</td>
<td>93.4</td>
</tr>
<tr>
<td>P4-P</td>
<td>1.9</td>
<td>23.9</td>
<td>21.5</td>
<td>2.1</td>
<td>12.8</td>
<td>28</td>
<td>90.4</td>
</tr>
<tr>
<td>S7-P</td>
<td>4.5</td>
<td>45.4</td>
<td>6.3</td>
<td>0.5</td>
<td>2.8</td>
<td>25</td>
<td>84.7</td>
</tr>
<tr>
<td><strong>O₂- bleached</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K23→K10-pulp</td>
<td>0.3</td>
<td>6.9</td>
<td>5.1</td>
<td>0.4</td>
<td>79</td>
<td>0.8</td>
<td>92.5</td>
</tr>
<tr>
<td>P3-P</td>
<td>0.5</td>
<td>1.9</td>
<td>1.9</td>
<td>-</td>
<td>89.8</td>
<td>0.3</td>
<td>94.6</td>
</tr>
<tr>
<td>P4-P</td>
<td>2.1</td>
<td>24.6</td>
<td>42.7</td>
<td>1.3</td>
<td>10.7</td>
<td>5.1</td>
<td>86.7</td>
</tr>
<tr>
<td>S7-P</td>
<td>4.7</td>
<td>61</td>
<td>23</td>
<td>1.3</td>
<td>6.3</td>
<td>1.6</td>
<td>97</td>
</tr>
<tr>
<td>K34→K10-pulp</td>
<td>0.5</td>
<td>6.7</td>
<td>5.0</td>
<td>0.3</td>
<td>80</td>
<td>0.9</td>
<td>93</td>
</tr>
<tr>
<td>P3-P</td>
<td>0.4</td>
<td>1.2</td>
<td>1.5</td>
<td>-</td>
<td>87.6</td>
<td>0.3</td>
<td>91.2</td>
</tr>
<tr>
<td>P4-P</td>
<td>1.0</td>
<td>10.7</td>
<td>50.3</td>
<td>2</td>
<td>11.5</td>
<td>8.6</td>
<td>84.2</td>
</tr>
<tr>
<td>S7-P</td>
<td>4.6</td>
<td>53.5</td>
<td>25.3</td>
<td>1.5</td>
<td>6</td>
<td>1.3</td>
<td>92.4</td>
</tr>
<tr>
<td>K49→K10-pulp</td>
<td>0.4</td>
<td>6.5</td>
<td>4.7</td>
<td>0.4</td>
<td>82</td>
<td>0.9</td>
<td>94</td>
</tr>
<tr>
<td>P3-P</td>
<td>0.5</td>
<td>1.0</td>
<td>1.3</td>
<td>-</td>
<td>90.4</td>
<td>0.1</td>
<td>93.4</td>
</tr>
<tr>
<td>P4-P</td>
<td>0.9</td>
<td>7.6</td>
<td>41.6</td>
<td>2.8</td>
<td>13.3</td>
<td>19.6</td>
<td>86</td>
</tr>
<tr>
<td>S7-P</td>
<td>2.3</td>
<td>18.5</td>
<td>54</td>
<td>3</td>
<td>14</td>
<td>4.8</td>
<td>93.7</td>
</tr>
</tbody>
</table>

⁴See figure 8b for abbreviations in brackets ⁵Amount analysed/100g of sample

The results of the carbohydrate analyses and klason lignin content of the LCC fractions are presented in Table 9. The P3-P fraction of all the samples consisted predominantly of cellulose material, whereas the S7-P was predominantly xylan, and the P4-P was predominantly glucomannan. In the latter two LCC, network structures similar to those proposed for wood LCC (c.f. Figure 8a, 8b) were found. The observation that the glucomannan-rich fraction still contained significant amounts of xylan, together with lignin, and the reverse for the xylan-rich LCC, substantiated the idea that lignin acted as a cross link between glucomannan and xylan to form network structures. Therefore, three types of lignin carbohydrate complexes were proposed to be present in kraft pulps; namely cellulose-lignin (Glc-L)-, xylan-lignin-glucomannan (Xyl-L-GlcMan)- (with a predominance of xylan over glucomannan) and glucomannan-lignin-xylan (GlcMan-L-Xyl)- (with a predominance of glucomannan over xylan) complexes. These fractions were also present in wood (cf Figure 8a, 8b).

The effects of the degree of delignification on the lignin carbohydrate complexes during kraft pulping and oxygen delignification were studied (Figure 9, 10). For the high kappa pulp (kappa 49), most of the residual lignin was isolated in the Xyl-L-GlcMan LCC (S7-P Figure 8b, Figure 9) network. In the final portion of the cook, a preferential delignification and /or dissolution of the Xyl-L-GlcMan LCC network
and the Glc-L LCC (P3-P Figure 8b) were observed, whereas the GlcMan-L-Xyl (P4-P Figure 8b) network was more resistant to delignification (Figure 9). Most of the residual lignin after oxygen delignification was isolated in the latter LCC fraction. With an increasing degree of oxygen delignification, favorable delignification and/or dissolution of the Xyl-L-GlcMan LCC network and the Glc-L LCC were observed, with the effect that nearly all of the residual lignin was found in the GlcMan-L-Xyl fraction. Again, the stability of the latter LCC towards delignification was demonstrated.

**Figure 9.** Effect of the degree of delignification on lignin-carbohydrate complexes during kraft cook.

**Figure 10.** Effect of the degree of delignification on LCC during oxygen delignification.

This is the first time that differences in the delignification/dissolution rates of lignin carbohydrate complexes (LCC) have been reported. The above differences are important, since they provide new insights into delignification. The glucomannan-rich LCC fraction (GlcMan-L-Xyl) seemed to be relatively more stable than the other LCC types towards the end of a kraft cook. Several reasons may be responsible for the differences in delignification rates observed above;
1) Structural differences may exist in the lignin part of the different LCC types. From the known chemistry of delignification, certain types of lignin structures are more reactive than others under the pulping conditions.

2) The different LCC types may have different morphological origins in the native fibre and this could affect their relative accessibilities during pulping.

3) The well known dissolution of hemicellulose during kraft pulping may be partly responsible for delignification, since a concomitant dissolution of the lignin bound to it should occur.

Furthermore, the GlcMan-L-Xyl network isolated from the unbleached kraft pulp appeared to be darker than the Xyl-L-GlcMan network, although the difference in their klason lignin contents was rather small. This may have been due to structural differences in the lignin part of the two LCC types and was investigated by UV spectroscopy. The characteristic electronic absorption spectra of lignin may be interpreted, in principle, on the basis of the UV absorption of polysubstituted benzene. The bands in the spectra are grouped into three regions, 203-211nm (E1 band), 224-234nm (E2 band) and 270-280nm (B band). A typical lignin spectrum decreases from a maximum of 205nm to a pronounced shoulder near 230nm and a characteristic lower maximum near 280nm (Lin 1992). However, the nature of substituents in the aromatic side chain cause changes in the transition energies as well as absorption intensities. Therefore, by plotting the specific absorptivity ((absorbance/ (concentration of lignin x light path length)), as a function of wavelength, two spectra can be compared, from which structural differences/similarities can be deduced.

On selected pulps, the specific absorptivity of the two hemicellulose LCC types (GlcMan-L-Xyl and Xyl-L-GlcMan) were plotted against wavelength and compared with each other (Figure 11). The lignin concentration was determined from the Klason lignin content in the different fractions. A distinct difference between the two spectra (Figure 11), indicative of structural differences, was observed. The GlcMan-L-Xyl network showed a higher specific absorptivity than the Xyl-L-GlcMan in the interval studied.

Residual lignin was prepared from the two hemicellulose LCC types by acidolysis in dioxane: water (82:18) (Gellerstedt et al. 1994) in order to study the effect of the cleavage of LC bond on the specific absorptivity spectrum. The yield of pure lignin recovered after the evaporation of dioxane, washing of the precipitated lignin isolate to neutral pH and freeze drying was in both cases between 50 and 60%. The specific absorptivity spectra of the isolated residual lignins were measured in aqueous alkaline borate (0.1M NaOH, 0.6mM H₃BO₃), (Figure 12). The cleavage of LC bonds seemed to cause some modifications on the lignin structure that led to strongly increased absorptivity values (cf. Figure 11, Figure 12).

The above study suggested that the lignin structure in the different LCC were somewhat different. The origin of such differences in the structure of the residual LCCs may be native or a result of differences in their morphological location in the fibre.
Specific absorptivity, l/g cm

![Graph showing specific absorptivity spectrum of Glucomannan rich LCC and Xylan rich LCC fractions from a kappa 49 unbleached kraft pulp.]

**Figure 11.** Specific absorptivity spectrum of Glucomannan rich LCC and Xylan rich LCC fractions from a kappa 49 unbleached kraft pulp. aSee Figure 8b for abbreviations.

Specific absorptivity, l/g cm

![Graph showing specific absorptivity spectrum of the acid dioxane/water lignin isolated from Glucomannan rich LCC and Xylan rich LCC fractions from a kappa 49 unbleached kraft pulp.]

**Figure 12.** Specific absorptivity spectrum of the acid dioxane/water lignin isolated from Glucomannan rich LCC and Xylan rich LCC fractions from a kappa 49 unbleached kraft pulp. aSee Figure 8b for abbreviations.

In the latter case, the differences in accessibilities may allow some lignin molecules to undergo more reaction than the others, although their basic structures in wood may be relatively similar. To investigate this further, a more detailed study on the structure of lignin in the native LCC and in the corresponding LCC from unbleached and oxygen bleached kraft pulps was performed and is discussed in section 4.2.
4.1.5 Isolation and characterisation of LCC from acid sulfite pulp (Paper III)

The effects of the alkaline conditions on the reactivity of the different LCC types present in spruce have been discussed above. Knowledge of how acid pulping conditions affect the LCC structures is, however, scarce. The presence of lignin carbohydrate complexes in unbleached bisulphite pulps has been proposed (Karlsson et al. 2001b). Size exclusion chromatography (SEC) employing a method based on simultaneous detection of lignin and carbohydrates, led to the proposal that the residual lignin in bisulphite pulps was at least in part linked to xylan. From bleached softwood dissolving pulp, the presence of xylan-lignin and galactoglucomannan-lignin complexes have been proposed to restrict the hemicellulase hydrolysis of mannan and xylan (Gubitz et al. 1998).

An unbleached industrial Norway spruce acid sulfite pulp (kappa number 11) was used in this study. The kappa number, traditionally used as a measure of residual lignin content in chemical pulps, has recently been shown to contain contributions not only from lignin but also from a variety of other oxidized structures present in kraft pulps (Li and Gellerstedt 2002). Therefore, the sulfite pulp used in this work was subjected to a similar analysis in order to get a reliable value for the true lignin content. Thus, the “non-lignin” structures were found to make a significant contribution to the overall kappa number (~3 kappa units) giving a lignin-related kappa number of 8 in the pulp.

By employing the dissolution-precipitation scheme in Figure 8b, lignin-carbohydrate complexes (LCC) were prepared in 80% yield on a lignin basis. The residue (R 3, Figure 8b), left after 2 loops (two repetitions of the dissolution-precipitation sequence) was highly resistant to the enzymatic hydrolysis for unknown reasons. This residue contained mainly cellulose, and about 20% of the original lignin and some glucomannan and xylan. By subjecting lignosulfonate isolated from the acid sulfite liquor to a urea treatment, a complete dissolution was observed. Any free lignosulfonates present in the acid sulfite pulp should, therefore, be dissolved upon exposure to urea solution. However, for the acid sulfite pulp, only trace amounts of lignosulfonates were detected in the urea solution. Furthermore, no lignin was extracted from the purified and freeze dried LCC fractions by the urea solution. Therefore, it was likely that the residual lignin was linked to carbohydrate by covalent bonds and resisted extraction. The fractions were subjected to carbohydrate analysis (Table 10), and LCC fractions similar in composition to those obtained in kraft pulps were isolated, but the LCC pattern was different from that in kraft pulp with respect to lignin yield.

Table 10. Carbohydrate and lignin analyses of acid sulfite pulp and LCC fractions.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Arabinan</th>
<th>Xylan</th>
<th>Mannan</th>
<th>Galactan</th>
<th>Glucan</th>
<th>Lignin</th>
<th>Lignin yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated pulp</td>
<td>0.1</td>
<td>3.9</td>
<td>11.2</td>
<td>-</td>
<td>78.4</td>
<td>1.3</td>
<td>100</td>
</tr>
<tr>
<td>Glc-L, P1-P</td>
<td>0.4</td>
<td>1.3</td>
<td>4.0</td>
<td>-</td>
<td>88.3</td>
<td>0.1</td>
<td>8</td>
</tr>
<tr>
<td>S6</td>
<td>0.6</td>
<td>21.5</td>
<td>52.1</td>
<td>0.3</td>
<td>13.2</td>
<td>2.2</td>
<td>72²</td>
</tr>
<tr>
<td>GlcMan-L-Xyl,</td>
<td>0.3</td>
<td>3.0</td>
<td>74</td>
<td>2.0</td>
<td>17.8</td>
<td>2.1</td>
<td>22</td>
</tr>
<tr>
<td>P4-P</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xyl-L-GlcMan,</td>
<td>0.6</td>
<td>64.4</td>
<td>12.8</td>
<td>-</td>
<td>4.3</td>
<td>11.0</td>
<td>43</td>
</tr>
<tr>
<td>S7-P</td>
<td></td>
<td>1.0</td>
<td>3.2</td>
<td>-</td>
<td>85</td>
<td>1.2</td>
<td>19</td>
</tr>
</tbody>
</table>

1See Figure 8b for identity of the fractions
2Amount in 100grams of sample
3Determined as Klason lignin.
4About 10% of the lignin in the hemicellulose fraction (S6 Figure 8b) was not recovered after the fractionation into P4 and S7.
About 60% of the residual sulfite pulp lignin was isolated in the S7-P (Xyl-L-GlcMan fraction), 30% in P4-P (GlcMan-L-Xyl fraction) and 10% to P1-P (Glc-L fraction) (Table 10). In contrast, after a severe kraft cook, most of the residual lignin was isolated in the glucomannan-rich fraction (cf Table 7, Table 10). These differences in the LCC patterns of kraft pulp (kappa 15) and acid sulfite pulp (kappa 11) are most likely to be due to reactivity differences of the LCC types under the different pulping conditions. Such reactivity differences may result from a number of factors:

1) Differences in the lignin structures of the native LCC.
2) Morphological aspects leading to different accessibilities.
3) Differences in the acid hydrolysis rates of the carbohydrates.
4) Stability of the lignin carbohydrate bond type towards the acidic conditions employed.
5) Differences in lignin chemistry in alkaline and acid medium.

We have investigated points 3) and 4) stated above, by studying the acid hydrolysis kinetics of lignin-carbohydrate- and carbohydrate-carbohydrate linkages. In wood, three main types of lignin-carbohydrate linkages have been suggested, viz. linkages of the benzyl ester, benzyl ether and phenylglucosidic types (Fengel and Wegener 1984). All of these are susceptible to acid hydrolysis and, therefore, model compounds representing the latter two structures (Figure 13), were subjected to acid hydrolysis in order to find out the conditions required for cleavage of the lignin-carbohydrate linkage.

![Figure 13. Model compounds used to study acidic conditions for the cleavage of lignin carbohydrate bonds](image)

The data in Figure 14 shows that both types of structures can be hydrolysed in reactions requiring rather acidic conditions. The phenyl glucoside was completely cleaved within 90 min under the conditions applied whereas the benzyl ether model only gave a partial hydrolysis. The latter result is comparable to studies on the hydrolysis of 4-O-anisyl-α-D-glucopyranoside (Kosikova et al 1979).

Since the sulfite pulp used in this work was produced in a two-stage process with only the latter half being conducted at a low pH (pH ~2) and at a temperature of around 140 °C, a substantial portion of the benzyl ethers present in the wood may well survive the pulping process, whereas the phenyl glycosides may be more extensively cleaved.
Figure 14. Disappearance of the starting compound on acid hydrolysis of phenyl β-D-glucopyranoside and veratrylglycerol- α-(6-O-glucopyranoside)- β-guaiacyl ether at pH = 1 and 75 °C.

The behaviour of xylan, glucomannan and cellulose toward hydrolysis under acidic conditions were investigated, based on the reasoning that delignification may in part be a result of the acid catalysed dissolution of the carbohydrates to which lignin is bonded. For this purpose, pure xylan and galactoglucomannan were prepared from spruce holocellulose and subjected to acid hydrolysis at pH = 2 and 100 °C. For each hemicellulose, the number of reducing end groups formed as a function of time was recorded. Microcrystalline cellulose (Avicel) was included as a model for cellulose. The results, shown in Figure 15, clearly demonstrated that xylan has a much higher stability as compared to galactoglucomannan. The higher degree of lignin dissolution from galactoglucomannan during acidic conditions may thus result from a faster degradation of this hemicellulose.

Figure 15. Acid hydrolysis of galactoglucomannan, xylan and cellulose (avicel) measured as the increase of reducing end-groups.
4.1.6 Conclusions 1

Lignin-carbohydrate complexes (LCC) were systematically prepared. Unique to this new method, is the partial enzyme hydrolysis of cellulose and subsequent swelling which facilitates the complete dissolution of mild ball milled wood. For pulps, the dissolution is obtained in high yield, leaving a residue consisting entirely of cellulose. The LCC isolation procedure involved is non-modifying to the fibres and the fractionated components are nearly free from contamination (at most 1.2% contamination from enzyme). Intact LCC networks are obtained from the ball milled wood or chemical pulp.

This study has demonstrated that lignin is linked through covalent bonds to all the major polysaccharides in the woody cell wall, viz. arabinoglucuronoxylan, galactoglucomannan, glucomannan, pectin and cellulose. It is also evident that the lignin polymer crosslinks various polysaccharides to each other. Such a network structure may play an important mechanical role for the "woody" properties of the secondary xylem. Furthermore, the progressive degradation of lignin-carbohydrate networks may be important in technical processes involving wood, such as chemical pulping.

In kraft pulps, 85-90% of the residual lignin molecules were somehow linked to carbohydrates, and mainly to glucomannan and xylan in network structures. During the end of a kraft cook and during oxygen delignification, the residual lignins in LCC were observed to be degraded at different rates. The glucomannan-rich LCC was found to be the dominant LCC after a kraft cook and after oxygen delignification.

Lignin-carbohydrate complexes (LCC) could be prepared from acid sulfite pulp in 80% yield on lignin basis. In this case, the xylan-rich LCC was found to be dominant.

In the next section, a more detailed study on the structure of the different lignin carbohydrate complexes, both in wood and corresponding kraft- and oxygen-delignified pulps, is performed in order to get an insight into the different reactivities observed above.

4.2 Structural variations in lignin carbohydrate complex

4.2.1 Structure and ultrastructural relationship in native lignin

The most common lignin inter-unit linkages and their relative abundances in softwood were presented in the introduction (Figure 2, Table 1 respectively). More detailed studies have however, shown that the lignin structure may be different depending on its morphological origin.

The concentration of lignin in the middle lamella (ML) has been proposed to be over 50%, whereas that in the secondary cell wall (SW) is estimated at less than 25% (Fergus et al. 1969, Westermark et al. 1988). Regarding lignin structure, the ML has been proposed to have a lower phenolic content and lower methoxy content than the SW (Whiting and Goring 1982, Sorvari et al. 1986, Önnerud and Gellerstedt 2003). Furthermore, the ML has been proposed to have a lower content of non-condensed β-O-4 structures than SW (Westermark 1985, Önnerud and Gellerstedt 2003) and a higher content of guaiacyl type lignin (Sorvari et al. 1986).

By administering $^3$H-labelled monolignol glucosides to differentiating xylem of pine, it has been shown that p-hydroxyphenyl lignin was formed mainly in the middle lamella (ML) and cell corner in the early stages of cell differentiation (Terashima et al. 1988). In the same study, it was also shown that the content of condensed guaiacyl units was higher in the ML than in the SW, and that syringyl lignin was formed in the inner layer of the SW in a late state as a minor moiety. Furthermore, by administration of both $^3$H and $^{14}$C in lignin precursor, the dehydrogenative polymerisation of coniferyl alcohol was studied under various conditions (Terashima and Seguchi 1987). The degrees of condensation (DC) in the dehydrogenative polymers (DHPs) were found to be affected by the presence or absence of carbohydrates, the type of carbohydrate as well as the mode of polymerisation. The DC of the DHP prepared in the presence of carbohydrates was higher than that of DHP prepared in the absence of it. In
the case of the presence of carbohydrates, the DC of DHP prepared in pectin was higher than that of the DHP prepared in mannan. Furthermore, the DC of the DHP prepared by bulk polymerisation (zutropf method) was higher than that of the DHP prepared by endwise polymerisation (zulauf method). A number of factors thus seem to be responsible for formation of different structures in lignin.

4.2.2 Structure and ultrastructural relationship in residual lignin in kraft pulp

A series of articles on the structure of residual lignin were published in the 1980s (Gellerstedt and Lindfors 1984a, Gellerstedt and Lindfors 1984b, Roberts et al. 1984, Gellerstedt and Gustafsson 1987, Gellerstedt and Roberts 1987). The results of these papers show that significant changes in the lignin structure take place in the transition from initial- to bulk phases and from bulk- to terminal phases of a kraft cook. The residual lignin has a lower content of β-O-4 structures and an increased phenolic content. Alkali-stable enolether structures are formed from the β-O-4 structures. The residual lignin has an increased amount of biphenyl (5-5) structures and biphenyl ether (4-O-5) structures. Furthermore, condensation reactions have been proposed to occur in the residual lignin (Gierer et al. 1970, Gellerstedt et al. 2004) and these have been proposed to contribute to the stability of residual lignin against delignification.

The topochemistry of delignification has also been investigated. By subjecting wood fractions enriched in middle lamella (ML) and secondary cell wall (SW) to kraft pulping-, acid sulfite pulping- and chlorite delignification conditions, it was demonstrated that the SW lignin reacted faster than the ML lignin in all three cases, the effect being largest for kraft pulp, followed by acid sulphite pulp and smallest for chlorite pulp (Whiting and Goring 1981). The above observation was speculated to be due to differences in the lignin structure in the two regions, but the effect of the dissolution of hemicelluloses, which was observed to be highest in kraft pulp, followed by acid sulphite pulp and smallest in chlorite pulp, on the observed reactivity differences could not be excluded. By UV microscopic studies (Wood et al. 1972), it was shown that the dissolution of lignin from the secondary wall correlated well with the quantity of hemicelluloses removed early in the cook. Therefore, it may be concluded that both physical and chemical effects were involved in the topochemical behavior and that the observed topochemistry was the result of a combination of these factors. Furthermore, UV microscopy and average molecular weight studies demonstrated that the exposed ML lignin was removed as macromolecules of similar size as lignin from the enclosed ML, indicating that the primary and secondary cell walls do not act as physical barriers to diffusion of dissolved lignin macromolecules from the ML (Kerr and Goring 1976).

The lignin structure in the different native LCC may also be different, explaining the different reactivities of LCC observed in Figures 9 and 10. Alternatively, the morphological origin of the LCC types may offer an explanation. We have therefore studied the structure of lignin in native LCCs and the effects of the degree of kraft pulping and oxygen delignification on the structure of lignin in isolated LCC.

4.2.3 Structural differences in lignin carbohydrate complex in wood and kraft pulps (Paper VI)

The main LCCs in wood viz. the glucomannan-rich fractions (P2 and P4), the xylan-rich fraction (S7) and the glucan-rich fraction (P3), together with the ones isolated from kraft pulp viz. glucan-rich fraction (P3-P) and the glucomannan- and xylan-rich fractions (P4-P and S7-P respectively) (Table 11) were studied for the contents of β-O-4 structures and phenolic hydroxyl groups. The former was determined by thioacidolysis in combination with GC FID analysis of the silylated products. During thioacidolysis, a quantitative cleavage of all β-O-4 structures present in lignin is achieved, whereas diaryl ether structures and condensed lignin structures such as 5-5, β-5, β- β and β-1 structures are not degraded (Rolando et al. 1992). In consequence, the lignin degradation products of thioacidolysis will consist of monomeric, dimeric and oligomeric ethylthio derivatives. The monomeric products can
be quantified, thus providing an indirect measure of the content of uncondensed β-O-4 structures in lignin. The cleavage of a β-O-4 linkage in lignin structure during kraft pulping will result in the formation of a new phenolic hydroxyl group. Therefore, such groups were quantified using the periodate oxidation method (Lai 1992). The analytical data, based on thioacidolysis and periodate oxidations respectively are collected in Table 12.

Different values for the content of the β-O-4 structures in the different LCC types are reported. The values are especially low for the xylan- and the cellulose- rich LCC (S7 and P3 respectively). In the case of cellulose LCC, the low value may be explained by effect of the mechanical grinding on the crystallinity of the cellulose. It is reasonable to assume that the mechanical energy is absorbed by the crystalline structure with the effect that the lignin in close proximity to the cellulose will be more degraded. For the same reason, the low content of the β-O-4 structures observed in the xylan LCC may be a result of the morphological location of this LCC relative to cellulose. The glucomannan-rich LCC (P1, P2 and P4) were observed to have higher contents of β-O-4 structures. The differences in the content of β-O-4 structures between the P1, P2 and P4 fractions may also be explained by their morphological locations relative to cellulose. Since the cleavage of the β-O-4 linkage results in concomitant formation of new phenolic groups, a comparison of the β-O-4 and phenolic contents between the different LCC types can be useful to predict the extent of lignin degradation occurring during the ball milling. From the results (Table 12), it is clearly observed that the LCC with a high content of β-O-4 had a lower content of phenolic hydroxyls, and the reverse for the LCC with a lower content of β-O-4 structures. It can therefore be concluded that even a mild ball milling procedure leads to a certain degree of cleavage of the β-O-4 linkage.

Table 11. Sugar and lignin analyses of spruce ball milled wood (BMW), of enzyme solution, of hydrolysate after enzyme treatment and of the various LCC fractions isolated from wood and pulps.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Arabinan (g)</th>
<th>Xylan (g)</th>
<th>Mannan (g)</th>
<th>Galactan (g)</th>
<th>Glucan (g)</th>
<th>Klason lign (g)</th>
<th>Mass balance (g)</th>
<th>Lignin yield, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMW</td>
<td>1.3</td>
<td>5.9</td>
<td>12.2</td>
<td>1.8</td>
<td>46.7</td>
<td>26.7</td>
<td>94.6</td>
<td>100</td>
</tr>
<tr>
<td>Enzyme</td>
<td>0</td>
<td>0</td>
<td>0.2</td>
<td>0.2</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hydrolysate</td>
<td>0</td>
<td>0</td>
<td>13.7</td>
<td>7.7</td>
<td>78.6</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fraction</td>
<td>Arabinan (g)</td>
<td>Xylan (g)</td>
<td>Mannan (g)</td>
<td>Galactan (g)</td>
<td>Glucan (g)</td>
<td>Klason lign (g)</td>
<td>Mass balance (g)</td>
<td>Lignin yield, %</td>
</tr>
<tr>
<td>----------</td>
<td>--------------</td>
<td>-----------</td>
<td>------------</td>
<td>-------------</td>
<td>-----------</td>
<td>----------------</td>
<td>----------------</td>
<td>----------------</td>
</tr>
<tr>
<td>P1/5.0</td>
<td>3.2</td>
<td>2.4</td>
<td>33.5</td>
<td>12.6</td>
<td>5.2</td>
<td>39</td>
<td>95.9</td>
<td>8</td>
</tr>
<tr>
<td>P3/15</td>
<td>1.0</td>
<td>0.9</td>
<td>2.5</td>
<td>0.2</td>
<td>85.2</td>
<td>7</td>
<td>96.8</td>
<td>4</td>
</tr>
<tr>
<td>P2/9.3</td>
<td>1.2</td>
<td>3.1</td>
<td>19.2</td>
<td>4.0</td>
<td>9.3</td>
<td>56</td>
<td>92.8</td>
<td>20</td>
</tr>
<tr>
<td>P4/18</td>
<td>0.7</td>
<td>3.2</td>
<td>33.6</td>
<td>1.8</td>
<td>12.5</td>
<td>41</td>
<td>92.8</td>
<td>28</td>
</tr>
<tr>
<td>S4/6.0</td>
<td>3.1</td>
<td>18.7</td>
<td>4.3</td>
<td>2.3</td>
<td>3.6</td>
<td>65</td>
<td>97.0</td>
<td>15</td>
</tr>
<tr>
<td>S7/22</td>
<td>4.1</td>
<td>34.9</td>
<td>14.2</td>
<td>4.2</td>
<td>1.7</td>
<td>29</td>
<td>88.1</td>
<td>25</td>
</tr>
<tr>
<td>Kraft pulp</td>
<td>0.7</td>
<td>7.3</td>
<td>5.7</td>
<td>0.4</td>
<td>75</td>
<td>4.5</td>
<td>93.2</td>
<td>100</td>
</tr>
<tr>
<td>P3-P/38</td>
<td>0.5</td>
<td>3.5</td>
<td>4.0</td>
<td>0</td>
<td>88</td>
<td>2.4</td>
<td>98.9</td>
<td>12</td>
</tr>
<tr>
<td>P4-P/5.8</td>
<td>1.2</td>
<td>11.2</td>
<td>20.2</td>
<td>1.7</td>
<td>7.4</td>
<td>35</td>
<td>76.8</td>
<td>45</td>
</tr>
<tr>
<td>S7-P/5.3</td>
<td>4.4</td>
<td>56.8</td>
<td>6.3</td>
<td>0.4</td>
<td>7.7</td>
<td>23</td>
<td>98</td>
<td>27</td>
</tr>
<tr>
<td>O-delig. Pulp</td>
<td>0.5</td>
<td>6.7</td>
<td>5.0</td>
<td>0.3</td>
<td>80</td>
<td>0.9</td>
<td>93</td>
<td>100</td>
</tr>
<tr>
<td>P4-PO/18</td>
<td>0.9</td>
<td>7.6</td>
<td>41.6</td>
<td>2.8</td>
<td>13.3</td>
<td>19.6</td>
<td>86</td>
<td>80</td>
</tr>
</tbody>
</table>

a) Abbreviations according to Figures 8a, 8b. b) Amount in 100 g of sample. c) Amount found in the volume of enzyme solution used for 100 g of sample. d) Designation/amount of fraction (g) obtained from 100 g of wood meal or pulp. e) May include a small amount of enzyme. f) Contains cellulose in addition to the glucane-lignin complex.
From kraft pulp, only three of the LCC fractions from wood were present (c.f. Figures 8a, 8b). These LCC fractions denoted P3-P, P4-P and S7-P were subjected to a similar study as described above, for the determination of the β-O-4 and phenolic contents (Table 12). For all three types, the content of the β-O-4 structures were significantly lower than that in the corresponding fractions from wood, whereas the reverse was the case for phenolic hydroxyl groups, in line with previous studies on residual lignin from kraft pulps, whereby the cleavage of β-O-4 linkage has been correlated with the increase in phenolic content of residual lignin (Gellerstedt et al. 1984a, Gellerstedt et al. 1984b). Furthermore, the extremely low content of the arylether structures in the xylan-rich LCC (S4-P) and the cellulose-rich LCC (P3-P) are in agreement with the high rates of delignification of these two LCC types observed in Figure 9, supporting the idea that the decrease in the lignin content of these LCCs occurs by way of established delignification chemistry. The glucomannan-rich LCC on the other hand gave a significant amount of residual β-O-4 structures, again in line with the observation visualised in Figure 9. The resistance of this LCC type towards delignification may be explained by the occurrence of lignin condensation reactions it. Recently, a new mode of lignin condensation reactions, blocking the normal mode of cleavage of the β-O-4, has emerged (Gellerstedt et al. 2004) and this may offer a reasonable explanation for the stability of residual lignin towards delignification.

After a severe oxygen treatment of the kraft pulp, from a lignin-related kappa number of 34 down to 7, one main LCC viz. the glucomannan-rich fraction, denoted P4-PO, and containing ~80% of the residual lignin could be isolated. The content of β-O-4 structures in it, was still found to be significant, substantiating the results visualised in Figure 10.

**Table 12.** Content of uncondensed β-O-4 structures and phenolic hydroxyl groups present in the lignin fractions of wood and LCCs.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>β-O-4 structures, µmol/g lignin</th>
<th>Phenolic OH, µmol/g lignin</th>
<th>Phenolic OH/100 C9, contribution to tot. lignin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ball Milled Wood (BMW)</td>
<td>1313</td>
<td>730&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Wood LCC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P1</td>
<td>1169</td>
<td>NA&lt;sup&gt;d&lt;/sup&gt;</td>
<td>NA&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>P3</td>
<td>658</td>
<td>1558</td>
<td>1.1</td>
</tr>
<tr>
<td>P2</td>
<td>1247</td>
<td>1257</td>
<td>4.6</td>
</tr>
<tr>
<td>P4</td>
<td>1300</td>
<td>717</td>
<td>3.7</td>
</tr>
<tr>
<td>S4</td>
<td>NA&lt;sup&gt;d&lt;/sup&gt;</td>
<td>NA&lt;sup&gt;d&lt;/sup&gt;</td>
<td>NA&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>S7</td>
<td>974</td>
<td>1300</td>
<td>5.9</td>
</tr>
<tr>
<td>Unbl. Pulp LCC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P3-P</td>
<td>50</td>
<td>2129</td>
<td>5.8</td>
</tr>
<tr>
<td>P4-P</td>
<td>292</td>
<td>1450</td>
<td>13.2</td>
</tr>
<tr>
<td>S7-P</td>
<td>37</td>
<td>1230</td>
<td>4.5</td>
</tr>
<tr>
<td>O-delig. Pulp LCC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P4-PO</td>
<td>90</td>
<td>680</td>
<td>10</td>
</tr>
</tbody>
</table>

<sup>a</sup>Designation according to figures 8a, 8b
<sup>b</sup>Based on the yield of lignin in the various fractions as given in Table 11.
<sup>c</sup>Value for spruce wood (Gellerstedt and Lindfors 1984b).<sup>d</sup>NA = not analysed

**Size Exclusion Chromatography (SEC) Analysis of Thioacidolysis Products from Wood and Pulps**

By studying the molecular mass distributions (MMDs) of the thioacidolysis products by SEC, it is possible to obtain information on lignin structure on a relative scale. Such MMDs have previously been studied in tetrahydrofuran (THF) system (Suckling et al. 1994) and in dioxane:water solvent system (Önnerud and Gellerstedt 2003). The distributions observed can be divided into three parts representative of oligomeric, dimeric and monomeric products, the former two attributable to thioacidolysis products of condensed lignin structures and biphenyl ether type structures, whereas the
latter to thioacidolysis products of non condensed β-O-4 structures. All these products are ethylthiolated in the side chain of the aromatic ring. Thus, by comparing the MMDs of LCC types with each other, information on the lignin structure can be obtained on a relative scale. Furthermore, useful information on the reactivity of lignin in the various LCC can be extracted by comparing the chromatograms of the native- and kraft pulp- LCC.

Accurate retention times for the dimeric and monomeric ethylthio derivatives could be obtained from the analytical data derived from the MMDs of thioacidolysed pinoresinol and guaiacylglycerol-β-guaiacyl ether respectively (Figure 16). The peak denoted “a” represents the retention time of the ethylthio derivative of pinoresinol. The peak denoted “b” represents the ethylthio derivative derived from the degradation of the dimeric β-O-4 model, whereas one of the peaks at longer retention times is likely to arise from guaiacol resulting from degradation of the dimer, and the other may originate from some other reagent from the acetylation procedure.

![Figure 16](image-url)

**Figure 16.** Size exclusion chromatograph of a mixture of acetylated thioacidolysis products from pinoresinol and guaiacylglycerol-β-guaiacyl ether. The dimeric and monomeric products are denoted “a” and “b” respectively.

The MMD pattern resulting from the ethylthio derivatives of the reference wood sample (Figure 17a) is in agreement with that of previous work (Suckling et al. 1994).

![Figure 17a](image-url)

**Figure 17a.** Size exclusion chromatograph of acetylated thioacidolysis products from spruce wood

The MMD of the Glucomannan-rich LCC (P2 +P4) from wood was observed to have a similar pattern to that of wood (cf. Figure 17a, 17b). These two fractions (P2 +P4) together contained a main portion of the lignin present in wood (Table 11).
Figure 17b. Size exclusion chromatograph of acetylated thioacidolysis products of the glucomannan-lignin-xylan LCCs (mixture P2 + P4) from wood.

The MMD pattern of the xylan-rich LCC (S4 and S7, Figure 17c) showed a completely different pattern from that of wood. This pattern strongly indicated that this LCC type consisted largely of $\beta$-O-4 structures. These two LCC fractions together contained a significant portion of the wood lignin (Table 11).

Figure 17c. Size exclusion chromatograph of acetylated thioacidolysis products of the xylan-lignin-glucosylmannan LCCs (S4, dotted line, and S7, full line) from wood.

The fact that the glucosylmannan-rich LCC fractions and the xylan-rich LCC fractions contain a main portion of the lignin in wood, together with the large differences in the lignin structures between the two LCC types, introduces the major finding that two types of lignin are present in the fibre wall. One of these consists largely of $\beta$-O-4 structures and is chemically linked to xylan, whereas the other type is more heterogeneous with respect to lignin inter-unit linkages and is covalently linked to glucosylmannan.

To obtain more detailed structural information on the lignin structure in the LCC, a raney nickel desulphuration of the ethylthio derivatives in combination with GC MS studies was employed. During the raney nickel desulphuration, the ethylthio groups are replaced by hydrogen atoms yielding simple reduced side chain structures. The fragmentation patterns of the acetates of these structures have been analysed in detail by GC MS (Lapière et al. 1991, Önnerud and Gellerstedt 2003), and have been used as references in this study. The glucosylmannan-rich LCC were found to yield fragments similar to those
observed when wood was analysed. The xylan-rich fraction on the other hand yielded very small amounts of 5-5, β-1 and β-5 coupled dimers (structures 1, 2 and 3 respectively, Figure 18), apart from the expected predominant monomer i.e., 2-methoxy-4-n-propylphenol (acetate).

![Structures 1, 2, and 3](image)

**Figure 18.** The major dimeric (acetylated) products found after thioacidolysis and Raney-nickel reduction of the fraction S7. The origin of 1 = 5-5, 2 = β-1 and 3 = β-5 structure in lignin.

The close association of lignin with xylan and glucomannan is in agreement with the ultrastructural model of the fibre wall proposed by Salmén and Olsson (1998) and visualised in Figure 5. Based on the above results however, the ultrastructural arrangement seems to be more complex than previously thought. A modified arrangement of the fibre wall polymers is therefore proposed, and a schematic view of this is presented in Figure 19, with one type of lignin surrounded by xylan and the other by glucomannan.

![Schematic view of fibre wall segment](image)

**Figure 19.** Schematic view of a fibre wall segment in spruce wood based on the model of Salmén and Olsson (1998).

The reasons for the large differences in lignin structure between the two main LCC types in wood are still not clear. One reasonable explanation, however, may be the effect of the local pH on lignification. The local pH near xylan, which is an acidic polysaccharide should be lower than that near the neutral polysaccharides. In consequence, lignification occurring in close proximity to the acidic polysaccharide may be different from that occurring in close proximity to the neutral polysaccharides. In support of this theory, previous work on synthetic lignin (DHP) has shown that a low pH enhances the formation of ether linkages in endwise polymers (Grabber et al. 2003). In other work, when lignin was synthethised in different polysaccharide gels, differences in the degrees of lignin condensation were observed (Terashima and Seguchi 1987). Furthermore, the structure of DHP can differ significantly by slight changes in the reaction conditions e.g., in the presence of manganese-(III), DHP devoid of phenolic hydroxyl groups or a normal DHP can be formed depending on the exact reaction conditions (Landucci et al. 2000, Önnerud et al. 2002)
A more conclusive explanation for the above observation however awaits further experimental evidence. The reactivity of lignin in the various LCC types during kraft pulping and subsequent oxygen stage was investigated. The main LCC type in kraft pulp viz., the glucomannan-rich LCC (P4-P, Table 11) was observed to contain some higher molecular mass distributions that were not present in the native LCC fraction (cf. Figure 17b, Figure 20a). This data, together with the fact that the β-O-4 content of this fraction was significant (Table 12) and the sluggish delignification of this fraction observed in Figure 9, provide pieces of evidence strongly indicating that condensation reactions occur in this fraction during kraft pulping. The occurrence of condensation reactions in lignin has recently been shown by thioacidolysis (Önnerud and Gellerstedt 2003, Gellerstedt et al. 2004). The localisation of such reactions to the glucomannan-rich LCC, however, presents an interesting insight into kraft delignification.

Figure 20a. Size exclusion chromatograph of acetylated thioacidolysis product of the glucomannan-lignin-xylan LCC (P4-P) from unbleached kraft pulp.

The MMD of the xylan-rich LCC on the other hand showed a predominance of low molecular mass material (Figure 20b), indicating that the abundant β-O-4 structures present in the native LCC fraction (Figure 17c) had been highly degraded during kraft pulping. This result, together with the high rate of delignification of this LCC type (visualised in Figure 9) provide evidence leading to the conclusion that the native xylan LCC consist largely of β-O-4 that are to a large extent degraded during kraft pulping. The MMD of the cellulose-rich fraction also indicated a high degree of lignin degradation occurring during kraft pulping (Figure 20b).

Figure 20b. Size exclusion chromatograph of acetylated thioacidolysis products of the glucan-lignin LCC (P3-P, dotted line) and xylan-lignin-glucomannan LCC (S7-P, full line) from unbleached kraft pulp.
From the oxygen delignified pulp, the glucomannan-rich fraction was found to contain a predominant amount of the residual lignin and only this fraction was analysed. The results indicated that even after a harsh oxygen treatment, the MMD pattern resembles that of the similar fraction in the unbleached pulp (cf. Figure 20a, Figure 21).

![Figure 21](image)

**Figure 21.** Size exclusion chromatograph of acetylated thioacidolysis products from glucomannan-lignin-xylan LCC (P4-PO) from oxygen-delignified kraft pulp.

### 4.2.4 Conclusions II

Significant differences in the lignin structure between the two main LCC types in wood viz., the glucomannan-rich fraction (GlcMan-L-Xyl) and the xylan-rich fraction (Xyl-L-GlcMan) have been found. The latter type of lignin was found to have rather linearly coupled β-O-4 structures whereas the former gave a chromatographic pattern similar to that obtained from wood. Based on these differences, a modified arrangement of the fiber wall polymers is proposed.

By fractionating residual lignin into LCC fractions and studying the lignin structure per LCC, pieces of information have been obtained to provide new insights into the reactions of lignin in LCC. Undesirable lignin condensation reactions occurring during kraft pulping have been found to be localised to the glucomannan-rich LCC. The differences in the reactivities of the LCC types have been found to originate from structural differences in the native LCC.

### 4.3 On the Molecular Mass Distributions of lignin and the analysis of phenylglycoside bond

Studies on the molecular mass distributions (MMDs) of lignin developed from the use of Sephadex gels (Jensen et al. 1962). Later, Sephadex gels were modified by alkylation to allow the use of organic solvents and were applied to lignin samples (Connors et al. 1978). Polystyrene gels in combination with tetrahydrofuran (THF) solvent were later found to be reliable for the analysis of a variety of acetylated lignin samples (Kringstad et al. 1981, Yamasaki et al. 1981, Gellerstedt and Lindfors 1984a, Gellerstedt and Lindfors 1987, Roberts et al. 1984) and are nowadays commonly used to analyze the molecular mass profiles of lignin samples.
4.3.1 Characterisation of Milled Wood Lignin (Paper IV)

Milled wood lignin (MWL) isolated according to Björkman (1956) contains some carbohydrates. These carbohydrates have often been referred to as contaminants. However, the crude MWL can be further fractionated into pure- and carbohydrate-containing lignin fractions (Lundquist, 1977). From previous work (Björkman 1957, Lundquist 1977), the presences of LC-bonds have been suggested to be a possible reason for the impurity of MWL. In the present work, MWL was studied in a little more detail to clarify the reasons for the presence of carbohydrates in it.

Milled wood lignin was therefore isolated from spruce for this study. The lignin yield of the crude product upon extraction with dioxane: water, 96:4 was ~20%. The crude MWL product was purified as described elsewhere (Lundquist 1977) into an almost pure fraction (MWL-pure) and one enriched in carbohydrates (MWL-impure). The carbohydrate composition of the crude and the carbohydrate enriched fractions are presented in Table 13, whereas only trace amounts of carbohydrates, hardly detectable by our GC system, were present in the purified part.

### Table 13. Carbohydrate and lignin analysis of the impure fractions of milled wood lignin

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Arabinan (g)</th>
<th>Xylan (g)</th>
<th>Mannan (g)</th>
<th>Galactan (g)</th>
<th>Glucan (g)</th>
<th>Klason Lignin (g)</th>
<th>Absolute Amount (%)</th>
<th>Lignin Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude MWL</td>
<td>0.53</td>
<td>0.42</td>
<td>0.67</td>
<td>-</td>
<td>0.47</td>
<td>90</td>
<td>92.1</td>
<td>19</td>
</tr>
<tr>
<td>MWL-impure</td>
<td>1.1</td>
<td>0.7</td>
<td>1.3</td>
<td>-</td>
<td>0.7</td>
<td>91</td>
<td>94.8</td>
<td>7</td>
</tr>
</tbody>
</table>

*a* Amount of component present in 100 grams of sample. *b* Amount of starting material analysed. *c* Wood lignin as reference.

The MWL-impure fraction was especially enriched in arabinan and mannan, although glucane and xylan were also present. In contrast, the content of arabinose in softwood is significantly lower than that of some of the sugars present in the MWL. This suggested that arabinose may link xylan to lignin by bonds that were resistant to cleavage under the prevailing conditions for the MWL preparation. The involvement of arabinose in direct linkage to wood lignin has previously been reported (Minor, 1991). The presence of mannan and glucane in MWL also indicated direct linkage of these sugar moieties to lignin. Galactose was, however, absent. The absence of galactose was most probably a result of cleavage of the galactose linkage to lignin during the milling, since direct linkage of galactose to lignin has been reported (Minor 1991).

The variety of sugars present in MWL further substantiated the results on the variety of LCC types isolated according to Figure 8a, after the relatively milder milling procedure. Therefore, to investigate the existence of LC bonds in MWL, a systematic approach was adopted. The presence of oligosaccharide chains bound to lignin should contribute to the molecular mass of MWL. To confirm this, the molecular mass distributions (MMDs) of the acetylated pure- and impure MWL fractions were first compared in the tetrahydrofuran solvent system with UV detection performed at 280 nm. The carbohydrate-containing fraction had a higher MMD than the pure lignin fraction (cf. MWL-impure and MWL-pure, Figure 22).

The effect of hydrolysis of the hemicelluloses in the MWL-impure on the MMD was then examined to test the above hypothesis by treating the MWL-impure fraction with a commercial enzyme exhibiting only xylanase and mannanase activities. Upon analysis, the MMD of the acetylated enzyme-treated MWL-impure fraction (EMWL-impure) was found to be intermediate between that of the MWL-pure and the MWL-impure fractions (Figure 22).

Obviously, the enzymatic hydrolysis of the xylan and glucomannan caused a lower MMD of lignin. Since the enzyme does not have ligninase activity, the xylan and mannan present in MWL are proposed...
to be chemically linked to lignin such that the enzymatic hydrolysis of the polysaccharide chains leads to a decrease in the MMD of the MWL-impure fraction (LCC). Since we have proposed that all lignin in wood is linked to carbohydrates, we extend our discussion to include the effects of prolonged and harsh ball milling conditions (as in MWL preparation) on native LCC. The free lignin fraction found in milled wood lignin is therefore proposed to be a result of the cleavage of lignin inter-unit linkages and/or cleavage of lignin carbohydrate linkages under harsher conditions. During milling, the lignin carbohydrate networks present in wood may also be broken down into smaller lignin carbohydrate complex aggregates such as those found in the crude MWL preparation.

Detector response

![Graph showing Molar Mass Distribution](image)

**Figure 22.** Molar Mass Distribution of pure MWL (MWL-pure), carbohydrate containing MWL fraction (MWL-impure) and enzyme treated fraction (EMWL-impure).

4.3.2 Analysis of phenylglycosidic bonds in wood (Paper V)

The literature review on LC bonds, discussed in the introduction part, showed clearly that ether and ester type LC bonds have been studied quite extensively by applying different techniques. Furthermore, the mechanisms proposed for their formations have been supported by model experiments. The phenyl glycosidic bonds, however, have not been studied in detail and only a few works have indicated their presence. When an LCC fraction from ball milled wood was treated with a purified β-glucosidase, the lignin moiety was degraded into four lower molecular weight fractions with or without carbohydrates. Since carbohydrate-free lignin was detected, it was concluded that such lignins were originally bound to carbohydrates glycosidically and were released by the action of the enzyme (Yaku et al 1976). The formation of phenyl glycosides during in vitro synthesis of dehydrogenation polymer (DHP) has also been proposed (Joseleau and Kesraoui 1986). Due to the scarce knowledge available on the analysis of phenyl glycosidic linkages in wood fibres, more reliable analytical data are required to confirm their presence.

In this work, a new approach to investigate the presence of phenyl glycosidic type of LC-linkage has been developed, basing its determination on the yield of new phenolic hydroxyl groups in lignin formed as a result of the LC-bond cleavage. The approach takes into consideration the fact that lignin also contains linkages with different relative stabilities to acidic conditions, and may, upon cleavage, yield new phenolic hydroxyl groups under such conditions. Therefore, a carbohydrate free lignin reference, i.e., pure milled wood lignin (MWL-pure) was used to control for any changes in the phenolic content that may occur as a result of cleavage of bonds within lignin.
For this study, the MWL fractions discussed in the previous section, viz. MWL-pure and MWL-impure were used. The carbohydrate composition and lignin yield on wood of the MWL-impure fraction are presented in Table 13.

The MWL-impure fraction contained ~35% of the lignin found in the crude MWL, and the other part of the lignin was found in the pure fraction (MWL-pure).

The phenolic contents of the LCC fractions and that of pure MWL fraction were determined from the ionisation difference spectrum as described previously (Gärtner et al. 1999). In this work, optimal conditions for the selective cleavage of glycosidic bonds between lignin and carbohydrates, for which changes in the phenolic content were observed for the LCC fractions and not for the MWL-pure fraction, were found to be incubation at pH 1.3 and 25 °C for 12 hours. The fractions studied are shown in Table 14.

Table 14. Determination of phenyl glycosidic type of linkage performed at pH 1.3, 25 °C, 12 hours.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Lignin Yield %b</th>
<th>PhOH ref mmol/g (/100C9 units)c</th>
<th>PhOH After H+ mmol/g (/100C9 units)c</th>
<th>ΔPhOH Phenylg. /100 C9 lignin units</th>
<th>Lignin released from fraction on hydrolysis (%)f</th>
</tr>
</thead>
<tbody>
<tr>
<td>Network LCC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-wood P4+P2</td>
<td>48</td>
<td>0.62 (11)</td>
<td>1.0 (18)</td>
<td>7</td>
<td>3.1d</td>
</tr>
<tr>
<td>MWL-impure</td>
<td>7</td>
<td>0.65 (12)</td>
<td>0.95 (17)</td>
<td>5</td>
<td>4e</td>
</tr>
<tr>
<td>MWL-pure</td>
<td>12</td>
<td>1.07 (19)</td>
<td>1.1 (20)</td>
<td>0.4</td>
<td>-</td>
</tr>
</tbody>
</table>

*See Figure 8a for fraction abbreviation. bYield based on total wood lignin. cContent based on total lignin in the fraction. dContent based total wood lignin. eContent based on total MWL. fDetermined from the UV absorbance at 280nm

The results indicated that the changes in the phenolic content observed on treatment of the LCC under the acidic conditions above were due to cleavage of LC linkage; most probably a phenyl glycoside type of linkage.

A minimum of 3% of the C₄ lignin inter-unit linkages in spruce (Table 14) were proposed to be glycosidically bound to carbohydrate.

When the glucomannan fraction was selectively precipitated by an aqueous barium hydroxide solution (Meier 1958) from the acid hydrolysis products of the native LCC, it was observed that ~70% of the lignin in the original fraction had been liberated from the carbohydrate during the mild acid treatment step (Table 14). This observation was reasonable on the account that the cleavage of the phenyl glycoside linkage should release some lignin. Furthermore, since the other portion of the lignin was precipitated together with the glucomannan fraction, it was most probably bound to the polysaccharide by more acid stable bonds. This is in line with our studies on the acid stability of LC bonds in model compounds (visualised in Figure 14). The acid conditions applied in this present study are simply too weak to cleave other types of LC bonds e.g. non phenolic α-ether LC bonds.

The MWL-impure fraction (Table 14) also showed an increase in the phenolic content corresponding to ~2% of the C₄ lignin inter-unit linkages in spruce MWL. A selective precipitation of the glucomannan from the hydrolysis products showed that a main part of the lignin had been released from it by the acid treatment, but that about 40% of the lignin was elsewhere still linked to the polysaccharide, again indicating the presence of another type of a more acid stable LC bond between the carbohydrate and lignin. Therefore, it is proposed that part of the carbohydrates found in MWL were chemically linked to the lignin by glycosidic bonds and the other part was linked by more acid-stable LC bonds. This observation substantiated the results indicated in Figure 22, whereby the decrease in molecular mass of the MWL-impure fraction on treatment with hemicellulase was ascribed to the enzymatic hydrolysis of hemicelluloses that were chemically bound to the lignin.
4.3.3 Conclusions III

The above study has clearly shown that the carbohydrates present in MWL preparation are chemically linked to it. Furthermore, a new method for the analysis of phenylglycoside bonds in lignin carbohydrate complexes (LCC) has been developed. The presence of these linkages in native LCC and in MWL has been demonstrated.
5. GENERAL CONCLUSIONS

For the first time, lignin-carbohydrate complexes (LCC) were prepared in quantitative yield from softwood and corresponding kraft- and oxygen- delignified pulps made from it. From acid sulfite pulp, the procedure facilitated isolation of LCC in ~80% yield. Unique to this new method, is the partial enzyme hydrolysis of cellulose and subsequent swelling which facilitates the complete dissolution of mild ball milled wood. For pulps, the dissolution is obtained in high yield, leaving a residue consisting entirely of cellulose. The LCC isolation procedure involved is non-modifying to the fibres and the fractionated components are nearly free from contamination (at most 1.2% contamination from enzyme). Intact LCC networks are obtained from the ball milled wood or chemical pulp.

This study has demonstrated that lignin is linked through covalent bonds to all the major polysaccharides in the woody cell wall, viz. arabinoglucuronoxylan, galactoglucomannan, glucomannan and cellulose. It is also evident that the lignin polymer crosslinks various polysaccharides to each other. Such a network structure may play an important mechanical role for the "woody" properties of the secondary xylem in contrast to non-lignified plant cells as for instance the seed hairs of cotton. Furthermore, the progressive degradation of lignin-carbohydrate networks may be important in technical processes involving wood, such as chemical pulping.

In kraft pulps, 85-90% of the residual lignin molecules were somewhere linked to carbohydrates, and mainly to glucomannan and xylan in network structures.

During the end of a kraft cook and during oxygen delignification, LCC were observed to be degraded at different rates.

The chemical structure of the two main LCC fractions in wood viz., the glucomannan-rich fraction (GlcMan-L-Xyl) and the xylan-rich fraction (Xyl-L-GlcMan), have been found to differ significantly. The lignin in the latter fraction was found to have a rather linear coupling mode of β-O-4 structures whereas the former was more heterogeneous with respect to the known lignin inter-unit linkage types. Based on this, a modified arrangement of the fibre wall polymers is proposed.

From kraft pulp, condensation reactions seemed to be localised to the glucomannan-rich LCC fraction, explaining the retardation of delignification observed in this LCC type. On the other hand, the relatively faster delignification of the xylan-rich LCC could be explained by the hydrolysis of the predominant β-O-4 structures found in the native LCC fraction.

A new method for the analysis of the phenylglycosidic linkage in LCC has been developed and the presence of such linkages in wood fibres, and in MWL has been demonstrated.
6. ACKNOWLEDGEMENTS

I would like to express my deep gratitude to my supervisors Professor Göran Gellerstedt and Dr. Gunnar Henriksson for skillful guidance, encouragement and valuable discussions throughout this work. It has been a lot of fun working on this subject with your endless support.

I would like to thank my co-authors Rickard Berggren and Fredrik Berthold from STFI-Packfors for good cooperation.

Many thanks to Miss Inga Persson for her kind help throughout these years.

Many thanks to my colleagues from the department for all the encouragement and the fun we have had both at work and elsewhere, the valuable discussions during lunch, the interesting sports and recreational activities and so on. It has been a pleasure.

I also wish to acknowledge the Swedish Research Council (VR) for financial support throughout these years.

Finally, I would like to thank my family for their understanding, support and encouragement.
7. REFERENCES


