JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Elucidation of the Structures of Residual and Dissolved Pine Kraft Lignins Using an HMQC NMR Technique

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Comparative studies on the structures of residual and dissolved lignins isolated from pine kraft pulp and pulping liquor have been undertaken using the $^{1}H-^{13}C$ HMQC NMR technique, GPC, and sugar analysis to elucidate the reaction mechanisms in kraft pulping and the lignin reactivity. A modified procedure for the isolation of enzymatic residual lignins has resulted in an appreciable decrease in protein contaminants in the residual lignin preparations (N content < 0.2%). The very high dispersion of HMQC spectra allows identification of different lignin moieties, which signals appear overlapped in 1D ^{13}C NMR spectra. Elucidation of the role of condensation reactions indicates that an increase in the degree of lignin condensation during pulping results from accumulation of original condensed lignin moieties rather than from the formation of new alkyl−aryl structures. Among aryl−vinyl type moieties, only stilbene structures are accumulated in lignin in appreciable amounts. Benzyl ether lignin−carbohydrate bonds involving primary hydroxyl groups of carbohydrates have been detected in residual and dissolved lignin preparations. Structures of the α-hydroxyacid type have been postulated to be among the important lignin degradation products in kraft pulping. The effect of the isolation method on the lignin structure and differences between the residual and dissolved lignins are discussed.

KEYWORDS: Residual lignin; kraft lignin; kraft pulping; NMR; 2D HMQC technique; lignin−carbohydrate complex; condensation reactions; lignin degradation products

INTRODUCTION

Production of pulp for the paper-making and chemical industries is the major means of chemical utilization of renewable wood resources. Among different pulping techniques, kraft pulping is the most important process. It consists of treatment of wood with a solution of sodium hydroxide (NaOH) and sodium sulfide (Na_2S) at high temperature (160−170 °C). This results in wood delignification through degradation of lignin and its dissolution in pulping liquor. Although a major fraction of wood lignin (∼97%) can be removed in kraft pulping, the rest of the lignin is rather resistant under the pulping conditions. Removal of the residual lignin from pulp requires oxidative lignin degradation with bleaching reagents such as oxygen, chlorine, and chlorine dioxide.

Information on the structures of the residual and dissolved lignins, isolated from pulp and the pulping solution, is of primary importance for a better understanding of the underlying mechanisms of kraft delignification and the reactivity of residual lignins in bleaching. Although extensive research efforts on the characterization of residual and dissolved lignins using different analytical techniques have provided valuable information (*1*), their structures are not well-established yet. Further progress in the characterization of technical lignins requires application of advanced techniques.

Two-dimensional (2D) heteronuclear NMR spectroscopy is a very effective tool for the elucidation of the lignin structure (*2*−*4*). The heteronuclear multiple quantum coherence (HMQC) NMR sequence shows correlation between carbons and protons directly bonded to each other (^{1}J correlation). The major advantages of the HMQC technique over 1D NMR techniques are higher dispersion of signals from different moieties, sensitivity enhancement, and more reliable assignment of the signals observed. Particularly, the HMQC sequence provides much better dispersion of lignin and carbohydrate signals than 1D NMR techniques. This is very important in the structural analysis of residual and dissolved lignins, because it eliminates vigorous purification, which usually decreases the yield of lignin preparations and could result in alteration in the lignin structures. In addition, information on carbohydrate constituents and lignin−carbohydrate bonds can be obtained. Although 2D NMR techniques are usually used for qualitative structural investigations, quantitative approaches have been also suggested (*4*, *5*).

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10.1021/jf034372d CCC: $25.00
Published on Web 09/04/2003

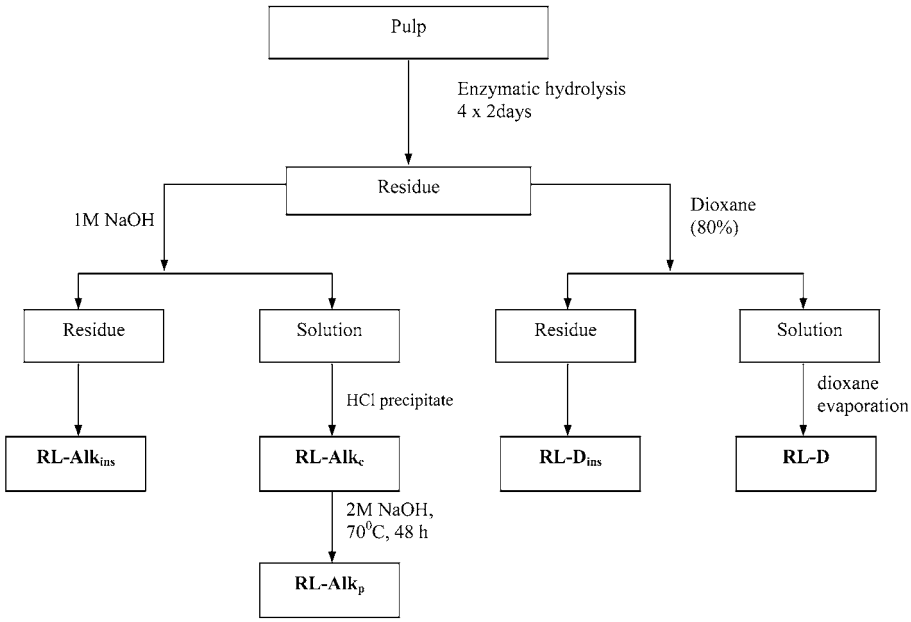

Figure 1. Isolation of residual lignins.

Very recently, several research groups have reported important findings on the structures of different residual and dissolved kraft lignins analyzed by multidimensional NMR techniques (*6*−*13*). However, the information reported is very concise, and there is an obvious lack of detailed reports in this area. In addition, the majority of the publications focused on the disappearance of native lignin structures during the pulping but did not provide significant information on the formation of new lignin moieties.

Our first results from 2D NMR studies (*11*, *12*) on technical lignins originating from different pulping processes have revealed important information on the lignin structures and pulping mechanisms. Particularly, the HMQC NMR technique did not show the presence of diarylmethane structures of the 5-CH_2-5 type, which were commonly believed to be formed in kraft pulping and to hinder further delignification. Stilbene moieties were detected in residual and dissolved lignins in appreciable amounts, whereas vinyl ether structures were found in a small amount only in a eucalypt kraft dissolved lignin. The presence of α-condensed structures and benzyl ether lignin−carbohydrate bonds in a kraft residual lignin has been demonstrated (*13*). However, it was difficult to state from the preliminary research if some specific features established were associated with the wood species used (pine or *Eucalyptus globulus* lignins), lignin type (residual or dissolved), or modified kraft process, such as kraft−anthraquinone (AQ) pulping. In addition, a number of questions have remained for further investigations. The present study continues our investigations via 2D NMR spectroscopy of technical lignins and comparative studies on the structure of pine kraft residual (RL) and dissolved (DL) lignins using the HMQC technique. The corresponding milled wood lignin (MWL) was used for comparison.

MATERIALS AND METHODS

Isolation of Lignin Preparations. Kraft pulping of pinewood was carried out under laboratory conditions at 170 °C for 90 min (time to 170 °C of 120 min) at the active alkali charge of 17.5%, sulfidity of 28%, and liquor-to-wood ratio of 3.5. The resulting pulp was of 45.4% yield (rejects 2.1%) and had a κ no. of 31.5.

Cellulase from *Trichoderma viride* (7.1 units/mg of solid) was purchased from Sigma Corp. The residual lignins were isolated (**Figure 1**) from the pulp by enzymatic hydrolysis with the cellulase in acetate buffer solution (pH 4.5) at 42 °C. Four treatments of 2 days each were applied. The amount of the enzyme was distributed among the treatments in the proportion of 4:2:1:1. The solution after each stage was separated from the residue by centrifugation and then acidified with 1 M HCl solution to pH 2.0 to precipitate lignin dissolved in the buffer solution. However, no precipitates were obtained in these experiments. The residues after the last enzymatic treatments were thoroughly washed with the buffer solution and water and then exhaustively extracted with a solution of ∼80% (v/v) dioxane. The dioxane extracts were combined and concentrated under vacuum. Small portions of water were added, and the rest of the dioxane was evaporated. The suspension obtained was frozen overnight and then carefully thawed and filtered, and the precipitate was washed on the filter with small portions of water. The precipitate was then vacuum-dried at room temperature to obtain the dioxane-soluble residual lignin (RL-D**)**. Alternatively, the residues after the enzymatic hydrolysis were extracted with 1 M NaOH, and the crude alkali-soluble residual lignin (RL-Alk_c) was then isolated by precipitation with dilute HCl. The purification of the alkali-soluble residual lignin was performed as described by Chang (*14*) to obtain the corresponding purified alkali-soluble residual lignin (RL-Alk_p). The dissolved lignin was isolated from the black liquor by precipitation with dilute HCl. The precipitate was thoroughly washed with distilled water and dried. The dried lignin was then exhaustively extracted with hexane in a Soxhlet apparatus to obtain extractive-free crude kraft dissolved lignin (DL_c). For purification of the crude dissolved lignin, 1 g of DL_c was dissolved in 10 mL of 96% (v/v) dioxane, and the insoluble residue was removed by centrifugation. The supernatant was added dropwise into 250 mL of Et_2O under vigorous stirring. The precipitate was collected, washed successively with small portions of Et_2O and water, then vacuum-dried to obtain the purified dissolved lignin (DL_p) preparation.

Carbohydrate Composition and Elemental Analysis. Carbohydrate composition of the lignin preparations was determined by GC analysis (an OV-225 capillary column, initial temperature of 210 °C, final temperature of 220 °C, heating rate of 2 °C/min) of alditol acetates of monosaccharides obtained by Saeman hydrolysis of the samples (*15*). The elemental analysis was performed at E&R Microanalytical Laboratory, Inc., Parsippany, NJ.

NMR Analysis. The NMR spectra were recorded in a Bruker AVANCE 500 MHz spectrometer after ∼40 mg of each lignin preparation had been dissolved in 0.75 mL of DMSO-d_6 containing 0.01% of TMS as internal standard. Conditions for analysis were as follows: temperature, 300 K; 90° pulse; acquisition time, 0.1 s; and 1.0 s acquisition delay (d_1).

Table 1. Characteristics of Residual Lignin Preparations

		isolated lignins			
preparation	enzyme charge (units/g of pulp)	total yield (mg/g of pulp)	carbohydrates (%)	N (%)	corrected yield/ pulp lignin[a] (%)
RL_1-Alk_c	960	45.8	8.3	2.83	75.3
RL_1-Alk_p	960	34.4	3.3	<0.2	73.8
RL_1-Alk_{ins}	960	7.2			
RL_2-Alk_c	600	42.4	10.1	3.23	65.7
RL_2-Alk_{ins}	600	10.6			
RL_2-D	600	33.8	11.2	<0.2	66.7
RL_2-D_{ins}	600	36.0	64.6	4.36	6.5

[a] Calculated as corrected yield = [total yield (100 − carbohydrates − 6.25N)]/(κ no. × 0.15)/10.

Table 2. Sugar and Elemental Composition of Residual and Dissolved Lignin Preparations

	sugars (%	carbohydrate composition (% on sugars)							
preparation	of sample)	Ram	Ara	Xyl	Man	Gal	Glc	C (%)	H (%)
RL_1-Alk_c	8.29		4.3	16.1	31.3	25.1	23.2	60.31	6.14
RL_1-Alk_p	3.32	2.1	9.5	23.3	8.3	31.9	24.9	59.59	5.09
RL_2-Alk_c	10.12	0.7	5.1	19.4	29.0	22.8	23.0	60.42	6.34
RL_2-D	11.17		5.7	25.6	27.0	7.5	34.2	61.58	6.08
RL_2-D_{ins}	64.56	0.4	1.0	4.3	9.7	4.4	80.2	45.82	6.39
DL_c	2.51	2.2	13.2	52.5	2.7	20.4	8.9		
DL_p	2.54	3.4	15.4	43.7	1.2	31.9	4.4		
cellulase	24.86	1.2	13.1	11.8	35.7	18.1	20.1		

Molecular Mass Distribution. The GPC analysis was performed at 70 °C in a PL-GPC 110 system equipped with a 300 mm × 7.5 mm Plgel10 μm MIXED D column (Polymer Laboratories) and a dual (RI and UV (280 nm) detector. A solution of 0.1 M LiCl in DMF was used as eluent at a flow rate of 0.9 mL/min. Two milligrams of a lignin sample was dissolved in 1 mL of the 0.1 M LiCl solution in DMF and then injected into the column. The GPC column was calibrated using lignin preparations previously characterized by ESI/MS (*16*).

RESULTS AND DISCUSSION

Isolation of Residual Lignin. Isolation of lignins by enzymatic hydrolysis is accompanied by minimal structural changes. Although the presence of carbohydrates in the lignin preparations makes analysis of lignins more complicated, it allows investigation of lignin−carbohydrate linkages in kraft pulp. There are a few modifications for the isolation of enzymatic residual lignins (*1*, *14*, *17*, *18*). In most of the procedures, the yield of residual lignins from softwood kraft pulps is high. However, even after different purification procedures, the resulting enzymatic lignin preparations contain ∼5% of protein impurities (= N% × 6.25) (*1*, *18*−*20*), which may interfere with the analysis of the lignin structure, particularly with 2D NMR techniques of high sensitivity (*10*). In contrast, our procedure for the isolation of the enzymatic residual lignins has resulted in preparations with much lower amounts of protein impurities (**Table 1**). The preparations were of rather high yields and were completely soluble in DMSO. The carbohydrate and elemental compositions of the preparations obtained are shown in **Table 2**.

Although the basic procedure was rather similar to traditional methods, some experimental details are apparently important for the reduction of enzyme impurities. One of the major reasons for the success is probably the use of a high-activity cellulase instead of the commonly used commercial enzymes (*1*, *17*−*20*). This allows the amount of the enzyme (g/g of pulp) at the same enzyme charge (units/g of pulp) to be decreased and thus to minimize interaction between cellulase and lignin. In addition, distribution of the same enzyme charge for several treatments instead of only single one (*1*) reduces the enzyme concentration in the multistage treatment compared to that in the one-stage process. The origin of the enzyme could be also important (*1*), and our results indicate that *Trichoderma viride* is a good source for cellulase for the residual lignin isolation. These modifications strongly reduce the amount of protein contaminants in the residual lignin preparations.

After the enzymatic hydrolysis, the residual lignin was extracted from the residue with aqueous dioxane as originally suggested by Yamasaki et al. (*17*) or with a sodium hydroxide solution (*14*, *19*). Analysis of the lignins showed (**Table 1**) that dioxane selectively extracts lignin with low protein contaminants. The contaminants accumulated in the dioxane-insoluble residue, which contains very little lignin. The alkali extraction, in contrast, easily extracted protein contaminants, and the crude lignin preparations required further purification. Although the alkali extraction resulted in a higher total yield of the crude lignins than the extraction with dioxane, the actual yield of the lignin (corrected for protein and carbohydrate contents) was quite close to the yield of actual dioxane-soluble lignin. The purification of the alkali-soluble residual lignins decreased protein contaminants rather effectively without significant loss of the target material. However, this purification under rather severe conditions (alkali concentration, temperature, and time) may result in alterations in the lignin structure.

The conditions for isolation of the residual lignins were not optimized in this work. The enzymatic hydrolysis was terminated when the amount of the residue was quite low. An increase in the enzyme charge from 600 to 960 units/g of pulp resulted in a higher yield of the alkali-soluble residual lignin (RL_1-Alk_c vs RL_2-Alk_c), without increase in protein contamination (**Table 1**). Thus, optimization of the enzyme charge could further improve the isolation procedure.

Identification of Structural Moieties in Residual and Dissolved Lignins by the HMQC Technique. The HMQC spectra of residual and dissolved kraft lignins are present in **Figure 2**. The chemical shifts for lignin signals are listed in **Table 3**. Signal assignments were based on the published data

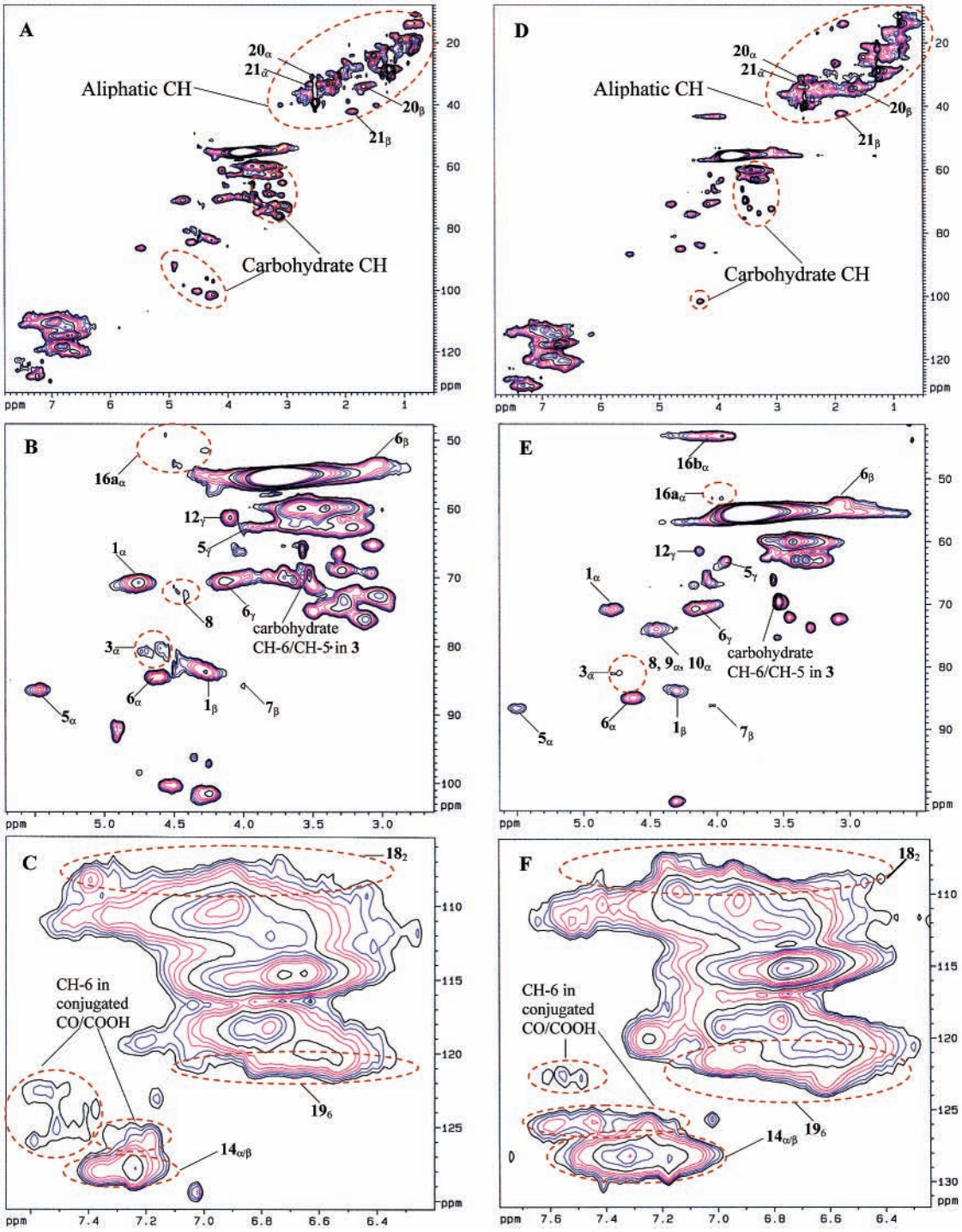

Figure 2. HMQC spectra of the residual dioxane soluble lignin (RL-D**)** (A–C) and dissolved kraft lignin (DL_p) (D–F). The numbers in signal designations correspond to the numbering of lignin moieties in **Figure 3**.

for lignin model compounds (*2*−*4*, *21*−*32*). Although the HMQC spectra are not quantitative, they were obtained under the same conditions, that is, sample concentration, acquisition, and processing parameters. Thus, the intensity of the signals in the spectra can be used as a relative measure of the abundance of specific moieties in different lignin preparations.

Oxygenated Aliphatic Region. Analysis of the lignins by the HMQC technique has shown (**Table 3**; **Figure 2**) that the major structures of native lignin, such as guaiacylglycerol-*β*-guaiacyl ether (*β*-*O*-4) (**1**), phenylcoumaran (*β*-5) (**5**), and pinoresinol (*β*-*β*) (**6**) moieties (**Figure 3**) are still present in lignin after pulping, although their intensities vary depending on the lignin preparation. The amounts of *β*-*O*-4 and *β*-5 structures significantly decrease during pulping. The amount of *β*-*β* moieties apparently did not change, probably due to their resistance to the pulping chemicals. Structures of the *β*-1 type detected in the MWL at δ_C/δ_H 75.0/5.0 were not found in the lignin preparations after pulping.

Signals of *β*-*O*-4 structures with α-CO (**4**), noncyclic α-*O*-4 (**2**), and α-*O*-alkyl (**3**) groups, as well as some carbohydrate signals, appeared overlapped in the 1D ^{13}C NMR spectra at δ_C 79−83. In contrast, the HMQC technique allowed distinguishing these important moieties. A small amount of *β*-*O*-4/α-CO units (**4**) was detected in the MWL but not in any lignin preparations after pulping (**Table 3**). Thus, carbonyl groups present in kraft

Table 3. Assignment of Some Signals in HMQC Spectra of Lignin Preparation

cross-peak (δ_C/δ_H)	DL_c[a]	DL_p[a]	RL-D[a]	$RL\text{-}Alk_p$[a]	MWL[a]	assignment
25.2/1.2–1.3	m	s	m	s	–	CH in extractives and aliphatic lignin moieties
24.3/1.5	m	s	vs	vs	w	
26.2/2.0	s	m	m	w	–	
29.0/1.3	vs	vs	vs	vs	vs	
30.7/1.3	–	vs	vs	–	–	
33.3/2.2–2.3	vs	m	s	vs	w	
26.0/2.5	m	–	–	vw	vw	Ar–CO–$\underline{CH_3}$
26.5/1.5	–	vw	s	w	–	Ar–CHOH–$\underline{CH_3}$
29.4/2.1	vw	w	m	w	–	Ar–CH_2–$\underline{CH_2}$–COOH
31.5/2.5	w	w	w	w	–	Ar–CO–$\underline{CH_2}$–CH_3
31/2.6	s	s	m	m	w	Ar–$\underline{CH_2}$–CH_2–CH_2OH
33.8/2.5	s	s	m	vw	vw	α-CH_2 in **21**
33.8/2.7	s	s	m	vw	vw	
34.3/1.7	vs	vs	s	s	m	Ar–CH_2–$\underline{CH_2}$–CH_2OH
34.2/2.3	m	w	–	–	–	unknown
36.0/2.2	vw	m	m	–	–	unknown
36–37/1.6–1.8	m	vw	w	–	–	unknown
36.5/2.6–2.8	vw	w	vw	vw	–	Ar–$\underline{CH_2}$–CH_2–COOH
39.5/2.4	–	w	w	w	–	Ar – $\underline{CH_2}$ – COOH
39.5/2.7–2.9	w	vw	w	w	–	
42.2/1.9	m	m	m	wv	w	β-CH in **21**
44.5/2.0	m	–	–	–	–	β-Alk
43.5/3.9–4.3	w	m	–	–	–	CH-α in α-5/β-nonoxygenated
48.0/3.2–3.4	w	–	–	w	–	CH-β in β-Ar
47–53.5/3.7–3.8	w	vw	–	m	–	CH-α in α-6
49–53/3.9–4.6	–	vw	vw	vw	–	CH-α in α–5/β-oxygenated
55.6/3.8	vs	vs	vs	vs	vs	Ar–OCH_3
60–61/3.2–3.8	vs	vs	vs	vs	vs	CH_2-γ in **1**–**3**, **7**, **20**, **21**, other γ-$\underline{CH_2}$–OH, CH-6 in hexoses
61.5/4.1	m	m	m	w	m	CH_2-γ in **12**
66.2/4.1	–	m	vw	s	–	γ-ethers?
67.3/4.2	–	m	–	–	–	
67.0/4.3	–	w	–	–	–	
67.0/4.5	–	w	–	–	–	Ar–CHOH–CH_3
69.5/3.5–3.6	w	m	m	w	–	CH-6 of Glc, Gal/CH-5 of Ara in **3**
70.7/4.2	s	s	s	s	s	CH_2-γ in **6**
71.0/4.8	m	s	s	s	vs	CH-α in **1**
72.0/4.7	–	–	vw	vw	–	– $\underline{CH}$OH – COOH in **8**, CH-α in **9**, **10**
72.5/4.6	vw	–	w	w	vw	
74.0/4.5	s	s	–	–	–	
81.0/4.6–4.8	m	w	w	w	w	CH-α in **3**
81.3/5.6	–	–	–	–	vw	CH-β in **4**
83.8/4.3	m	m	m	m	vs	CH-β in **1**
84.7/4.7	s	m	s	s	m	CH-α in **6**
86.5/4.0	–	vw	vw	vw	w	CH-β in **7**
86.5/5.5	m	m	m	m	s	CH-α in **5**
109.5/5.6	w	–	–	–	–	CH-α in **13**
112.5/6.2	m	vw	–	–	–	
123.0/7.3	–	–	–	–	m	CH-6 in **11**
122.5/7.5	m	vw	vw	vw	m	CH-6 in conjugated CO or COOH
125.5/7.0	s	vw	–	–	–	
126–127/7.5–7.6	s	w	w	w	–	
126.5/7.3	m	m	m	w	m	CH-6 in Ar–CH=O
126.5/6.8	–	–	–	–	m	CH-β in **11**
128/6.1	–	–	–	–	w	CH-β in **12**
129.3/6.3	–	–	–	–	vw	CH-α in **12**
128.5/7.1–7.4	s	vs	m	s	–	CH-α/β in **14**
130.0/7.1	–	–	vw	w	–	CH-2,6 in H units
143.0/7.3	vw	–	–	–	–	CH-β in **13**
153.5/7.6	–	–	–	–	m	CH-α in **11**
190.5/9.8	w	w	w	w	m	Ar–$\underline{CH}$=O
193.3/9.6	–	–	–	–	m	CH-γ in **11**

[a] Signal intensity: vw, very weak; w, weak; m, moderate; s, strong; vs, very strong; –, not detected.

lignins are not associated with β-*O*-4 moieties and are probably located in terminal side chains.

Recent studies of acetylated MWL using 2D and 3D techniques (*3*, *33*) have shown that noncyclic α-*O*-4 moieties (**2**) in native softwood lignins are below the detection limit. Our analysis of the nonmodified pine MWL did not show any detectable amount of structures **2** either (no signal for CH-α at δ_C/δ_H 79−80/5.5 ppm). Similarly, these units were not detected in any lignin preparation after pulping. However, signals of α-*O*-4 bonds in *trans*-dibenzodioxocin moieties (**7**) were observed in the MWL, in agreement with previous reports (*2*−*4*, *7*−*9*). A small amount of these structures were detected in the residual and dissolved kraft lignins (**Figure 2**) in contrast to published results (*7*, *9*).

Among new signals that appeared in the oxygenated aliphatic area the most prominent ones were at δ_C/δ_H 74/4.4−4.5 (**Figure 2**; **Table 3**). They could correspond to α-hydroxyacids (**8**), such as Ar−$\underline{CH}$(OH)−COOH, Ar−CH_2−$\underline{CH}$(OH)−COOH (*22*), or

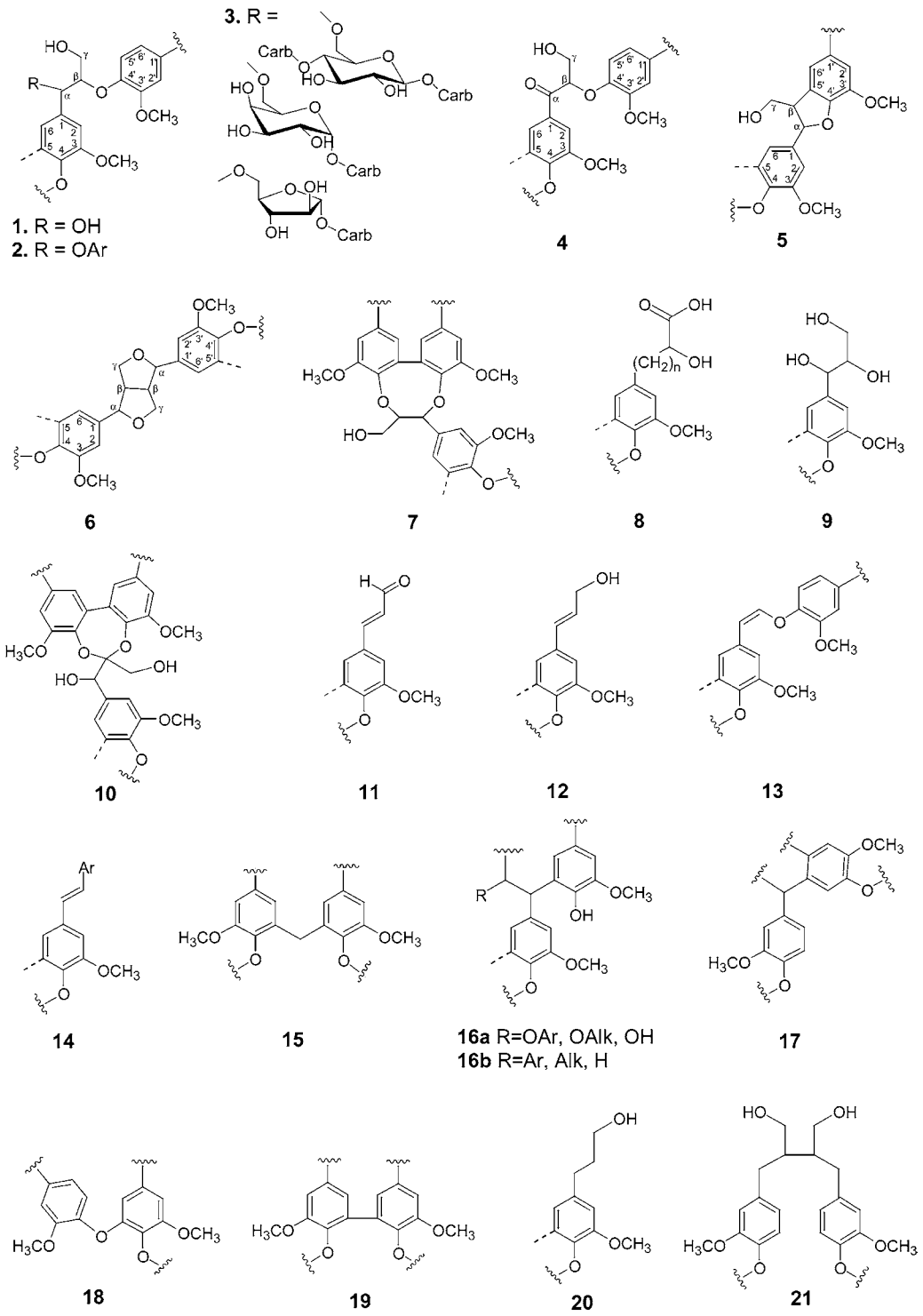

Figure 3. Lignin structures.

homologues. This is consistent with the appreciable amounts of aliphatic acids reported in dissolved and residual lignins (*9*, *18*, *36*, *37*). The chemical shifts of these signals are also rather close to those reported for CH-α in the arylglycerol (**9**) structure (*21*), intermediates in cleavage of nonphenolic *β*-*O*-4 moieties. A relatively strong signal observed at about δ_C/δ_H 63/3.4−3.6 could correspond to CH-*γ* in the arylglycerol structures, whereas the signal for CH-*β* could be overlapped with carbohydrate signals at δ_C 72−74. In addition, the signals at δ_C/δ_H 74/4.4−4.5 could also correspond to CH-α of the structure (**10**) derived from α-aryl ether bond cleavage of dibenzodioxocin structures, according to model compound experiments (*25*). Some signals at δ_C/δ_H 66/4.08 and 67/4.2−4.3, observed in the dissolved and residual kraft lignins (**Figure 2**; **Table 3**), can correspond to *γ*-ether structures formed during pulping. Further studies are required to clarify the nature of these signals.

Lignin—Carbohydrate Complex. All lignin preparations show signals of α-*O*-alkyl/*β*-*O*-4 units (**3**) at δ_C/δ_H 81/4.6−4.8. They were previously assigned (*13*) to lignin−carbohydrate bonds of the benzyl−alkyl ether type according to data for model compounds (*26*). Analysis of published data for various benzyl ether lignin−carbohydrate model compounds (*26*−*29*) allows attribution of these signals to linkages between the α-position of lignin with primary hydroxyl groups of carbohy-

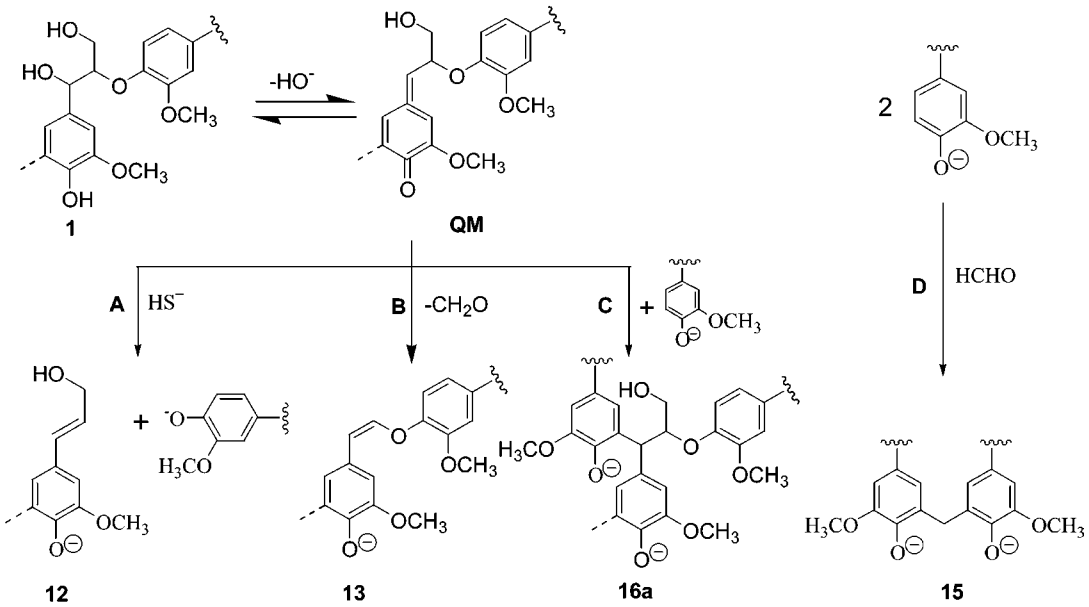

Figure 4. Reactions of phenolic lignin moieties during kraft pulping.

drates, such as those at C-6 in β-D-glucopyranosyl and α-D-galactopyranosyl and C-5 in α-L-arabinofuranosyl units. The CH-α moieties in benzyl ether linkages with secondary hydroxyl groups of carbohydrate give signals at a low proton field, at about δ_C/δ_H 80−82/4.9−5.25 (*27, 28*), which were not detected in the spectra of the lignin investigated. Among various carbohydrate signals, those at δ_C/δ_H 69.5/3.5−3.6 are important. Recently, we tentatively assigned this signal in a *Eucalyptus grandis* kraft residual lignin to glucose CH-6 involved in benzyl ether lignin−carbohydrate bonds (*34*). Moreover, on the basis of model compound data (*27*−*29*), these signals can also be attributed to CH-6 of α-D-galactopyranosyl units and CH-5 of α-L-arabinofuranosyl units linked to lignin by benzyl ether bonds. Softwood lignin preparations contain significant amounts of galactose and arabinose units (**Table 2**) in contrast to eucalypt residual lignin, where glucose and xylose are the main carbohydrate units (*34*). Therefore, the signals at δ_C/δ_H 69.5/3.5−3.6 can be generally assigned to CH of carbohydrates linked to the α-position of lignin via a primary hydroxyl group. This is consistent with the assignment of the signals at δ_C/δ_H 81/4.6−4.8 to CH-α in this type of linkage. Signals at δ_C/δ_H 67/4.2−4.3 assigned to γ-ether moieties (**Table 3**) might particularly belong to lignin−carbohydrate linkages at the γ-position of the side chain of lignin (*30*).

Signals of lignin−carbohydrate linkages of the phenyl glycoside type at about δ_C/δ_H 100−102/4.9−5.1 (*31*), observed earlier in lignin−carbohydrate complexes isolated from wood (*35*), were not detected in the residual and kraft lignin preparations. However, these bonds, if they exist in kraft pulp, can be cleaved during lignin isolation by cellulase treatment.

The HMQC NMR technique, providing high dispersion of carbohydrate signals, gives the general possibility of identification of other possible linkage sites of carbohydrates bonded to lignin. However, this analysis requires precise information on the chemical shifts of the corresponding moieties and the use of equipment with higher sensitivity.

Aryl—Vinyl Moieties. Various aryl−vinyl type structures are important intermediates or products of kraft pulping. These moieties are reactive toward oxidative bleaching chemicals and can contribute appreciably to lignin removal during bleaching. Moreover, they are potential chromophores and may be responsible for a decrease in the pulp brightness. Analysis of vinylic moieties by 1D NMR techniques is difficult because their signals are overlapped by a wide variety of aromatic signals. This problem can be minimized by application of the DEPT technique, which identifies the −CH═CH− group in vinyl ether structures (C-β at δ_C ∼144) (*36*) and coniferyl aldehyde moieties (C-α at δ_C ∼153) (*23*). However, even the DEPT technique does not provide a reliable means of distinguishing vinylic signals in coniferyl alcohol from those in stilbene structures and other possible olefinic moieties because of rather close carbon chemical shifts. In contrast, the HMQC technique offers a better resolution of different aryl−vinyl moieties, allowing identification of cinnamyl aldehyde (**11**), cinnamyl alcohol (**12**), vinyl ether (**13**), and stilbene moieties (**14**) as well as possible olefinic moieties originating from cleavage of the aromatic ring (*13*).

Signals corresponding to a coniferyl aldehyde side chain were clearly detected in the MWL (**Table 3**). In contrast, they were not observed in any lignin after the pulping. All signals (α-, β-, and γ-) of the coniferyl alcohol type end group structures were detected in the MWL preparation. In addition to those originally present in lignin in wood, coniferyl alcohol moieties are the major intermediates of cleavage of phenolic β-*O*-4 units in kraft pulping (**Figure 4**, reaction A) (*37*−*39*). The newly formed noncondensed coniferyl alcohol structures will be eliminated from lignin as monomers. In contrast, these structures of condensed type will be still attached to lignin. Considering an appreciable degree of lignin condensation, ∼40% for native lignins (*23*) and much higher for kraft lignins (*1*), this possibility is rather high. However, the amount of coniferyl alcohol moieties in the residual and dissolved lignins is apparently lower than that in the MWL. No signals for the vinylic −CH═CH− group were detected in the lignins isolated after pulping. Only signals possibly attributable to CH-γ in coniferyl alcohol structures were observed (**Table 3**; **Figure 2B**,**E**), probably due to their higher sensitivity in the HMQC experiment. Thus, coniferyl alcohol moieties do not accumulate in the lignin structures during pulping and undergo further reactions. This observation is consistent with the profile of monomeric coniferyl alcohol in black liquor during the course of kraft pulping (*39*), indicating

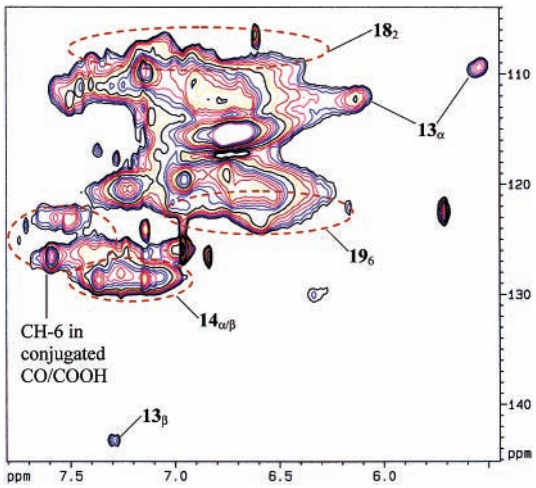

Figure 5. Expansion of the aromatic region of the HMQC spectrum of the DL_c preparation.

its maximum amount at the initial period of delignification and a dramatic decrease as pulping proceeds.

Vinyl ether structures are formed in kraft pulping by formaldehyde elimination from *β-O*-4 moieties in the reverse aldol reaction cleaving the $C_\beta-C_\gamma$ bond (**Figure 4**, reaction B). They are believed to be stable under the conditions of kraft pulping and might be expected to accumulate in lignin. This was confirmed by the analysis of a pine kraft lignin by the DEPT NMR sequence (*36*). Recently, analysis of kraft lignins using the HMQC technique has shown the presence of vinyl ether structures in a *Eucalyptus globulus* dissolved kraft lignin, but not in a pine residual kraft-AQ lignin (*12*). However, this work could not prove if the absence of vinyl ether moieties in the pine residual kraft-AQ lignin was caused by the use of AQ in pulping, a difference in the mechanisms of pine and eucalypt pulping, or a difference in the structure of residual and dissolved lignins. Comparison of the residual and dissolved pine kraft lignins has shown that the vinyl ether structures are present in a detectable amount only in the DL_c preparation, but not in the residual lignins (**Figure 5**; **Table 3**). This is consistent with the analysis of kraft lignins by acidolysis (*40*), which has demonstrated that the amount of vinyl ethers is appreciably higher in dissolved lignins than in the corresponding residual lignins.

Stilbene moieties are also products of the reverse aldol reaction formed from *β*-5 or *β*-1 moieties (*38*). They have been suggested to be present in dissolved (*37*) and residual (*17*) kraft lignins on the basis of their UV spectra. The HMQC spectra clearly show the signals from stilbene vinylic −CH=CH− groups (**Figure 2C**,**F**), which are located in the range of δ_C/δ_H 126−128/7.0−7.4 according to published data for the carbon chemical shifts (*22*) and our 2D NMR analysis of *trans*-stilbene. The stilbene structures were detected in rather appreciable amounts in all preparations isolated after pulping (**Table 3**).

Condensed Structures. For a long time, it was believed that lignin undergoes extensive condensation reactions during kraft pulping. This was suggested on the basis of model compound experiments (*38*) as well as from studies on reactions of isolated lignin preparations under conditions of kraft pulping (*41*) and structural analysis of the residual lignin in kraft pulp by the nuclear exchange degradation technique (*42*). However, other studies (*43*) cast doubt on the occurrence of extensive lignin condensation during kraft pulping. Condensation reactions, if they occur, would have a negative effect on pulping, increasing the molecular weight of lignin and forming moieties stable toward pulping and bleaching reagents. Therefore, examination of the condensed structures in technical lignins is of primary importance. So-called diarylmethane moieties of the 5-CH_2-5 (**15**) and α-5 (**16**) types have been considered to be the main condensed structures formed during alkaline pulping. The former are produced via Lederer−Manasse condensation reactions of formaldehyde, formed in the reverse aldol reaction, with the 5-position in phenolic units (**Figure 3**, reaction D). The latter are postulated to result from condensation reactions between quinone−methide intermediates and other phenolic moieties (**Figure 4**, reaction C).

^{13}C NMR spectra of kraft lignins (*22*, *36*) showed a signal at δ_C ∼29, which was assigned to CH_2 groups in diarylmethane moieties of the 5-CH_2-5 type (**15**). However, the use of the more diagnostic HMQC sequence in lignin structural investigations has revealed (*9*−*12*) that the signal at δ_C 29 usually shows a correlation with proton at δ_H 1.2 instead of δ_H 3.8 for the methylene protons in 5-CH_2-5 moieties (**15**) (*44*). The diarylmethane 5-CH_2-5 moieties have not been detected in any lignin preparation examined in the present work (**Figure 2**). Although this finding contradicts the generally accepted concept of the mechanism of kraft pulping, it is a rather logical consequence of the insignificant role of the reverse aldol reaction for *β-O*-4 structures discussed above. Formaldehyde is released during the formation of stilbene moieties from phenylcoumaran structures. However, the amount of *β*-5 structures in native softwood lignins is ∼10% (*23*), and some of them still survive pulping conditions (**Table 3**). Therefore, because of a low amount of formaldehyde eliminated, the possibility for the formation of 5-CH_2-5 (**15**) structures is also very limited.

Correlations in the range of δ_C 40−55 can be assigned to tertiary aliphatic CH (*21*, *22*, *36*). On the basis of the ^1J correlation between ^1H and ^{13}C in the HMQC spectra, an aliphatic CH linked to an aliphatic carbon can be generally distinguished from that attached to an aromatic carbon (**Table 3**). It is noteworthy that the amounts of the condensed moieties in the lignins investigated (**Figure 2**; **Table 3**) are lower than could be expected from the general concept of kraft pulping (*37*, *38*). However, even insignificant degrees of condensation may result in appreciable changes of polymer properties. Therefore, the elucidation of the structure of condensation products is important. The exact chemical shift for different condensed moieties varies significantly depending on the nature of the substituent. However, on the basis of published data for model compounds (*21*, *22*, *32*) and Advanced Chemistry Development Labs prediction program (ACD/Labs software version 6.0), we can generally divide the signals observed to α-5 (**16**), α-6 (**17**), and *β*-Ar moieties with an oxygenated or nonoxygenated aliphatic vicinal group (**Table 3**). Most of the condensed structures detected have saturated aliphatic substituents, implying that the condensation reactions probably occur after appreciable lignin degradation. Therefore, the role of the condensation reactions presented in **Figure 4** is apparently not so significant to be responsible for the resistance of lignin in pulping and bleaching.

The HMQC technique is not an appropriate method for the direct detection of condensed structures of aryl-*O*-aryl (4-*O*-5; **18**) and biphenyl (5-5; **19**) types because it does not allow observation of quaternary carbon atoms. However, signals for CH-6 in 5-5 structures are downfield-shifted from δ_C 118−120 in noncondensed guaiacyl units to δ_C 120−122, whereas signals for CH-2 in 4-*O*-5 structures are upfield-shifted from δ_C 110−112 in typical guaiacyl units to δ_C <110 (*21*). Although

the signals of 5-5 and 4-*O*-5 moieties are partially overlapped by strong signals for CH-6 and CH-2 in noncondensed guaiacyl units, the spectra of the residual and dissolved lignins (**Figure 2C**,**F**) showed that the amount of 5-5 and 4-*O*-5 moieties appreciably increases during pulping. This can be explained by the stability of these moieties and their accumulation as pulping proceeds. Thus, the analysis of the condensed lignin structures suggests that a higher degree of condensation of residual and dissolved kraft lignins as compared to native lignins is a result of the accumulation of 5-5 and 4-*O*-5 type condensed structures originally present in lignin as well as stilbene moieties formed from β-5 (**5**) structures rather than the formation of new diarylmethane moieties, such as 5-CH_2-5 and α-Ar type structures.

Aliphatic Region. The HMQC spectra of the residual and dissolved lignins showed that β-*O*-4 lignin moieties have been rather extensively degraded. However, immediate products of their cleavage, such as coniferyl alcohol moieties, are not accumulated in the lignins, but undergo further reactions. Their products are not sufficiently elucidated. Moieties with saturated aliphatic groups could be possible reaction products.

All lignin preparations investigated show a wide variety of saturated aliphatic moieties with relatively high intensities (**Figure 2A**,**D**; **Table 3**). Some strong signals in this area correspond to extractives (*9*, *45*). Their amount in lignin increases during the pulping, indicating increases in chemical linkages between lignin and extractives. However, a considerable part of the signals detected in the aliphatic region should belong to lignin moieties formed during pulping. Among the structures identified, the signals of dihydroconiferyl alcohol moieties (**20**) are rather significant (**Table 3**). Signals assigned to β-β moieties of the secoisolariciresinol type (**21**) (*21*, *24*) were observed in all of the lignins at δ_C/δ_H 33.8/2.5, 2.7, and 42.2/1.9. Their amounts are appreciably higher in the lignins after pulping as compared to the MWL (**Table 3**).

The presence of α-hydroxyacids (**9**) with different CH_2 and CH_3 groups, identified earlier in black liquors (*46*), is possible and consistent with the signals at δ_C/δ_H 74/4.5 discussed above. The signals of α-CH_2 in these moieties are in the range of δ_C 35−40 (*22*). Other CH_2 and CH_3 groups generally give signals in a higher field. It was suggested that moieties (**9**) are formed by aldol condensation between an aldehydic lignin fragment and a carbohydrate fragment of the aldol or ketol type, followed by benzylic acid rearrangement (*46*). This reaction could also result in the formation of C−C linkages between lignin and carbohydrate chains.

Conjugated Carbonyl Moieties. All lignins contain α-carbonyl/carboxyl moieties detected by cross signals of CH-6 in the range of δ_C/δ_H 122−126/7−7.6. There were a variety of the signals in this region, indicating some differences in the structures of these moieties. Because α-carbonyl moieties are not associated with β-*O*-4 structures as discussed earlier, they are very likely located at terminal side-chain structures. All lignin preparations showed signals of vanillin structures at δ_C/δ_H 191/9.8, in contrast to a recent publication (*10*), which can originate from the native lignin as well as from oxidation of coniferyl alcohol type intermediates (*37*, *38*). Signals of aliphatic CH_3 and CH_2 groups in Ar−CO−CH_3 and Ar−CO−CH_2−CH_3 structures were detected in the aliphatic region (**Table 3**). Consequently, reactions of carbonyl moieties should not noticeably contribute to lignin fragmentation during bleaching. However, carboxyl groups, which may be formed during the oxidation of conjugated carbonyl moieties, should increase lignin solubility and thus facilitate its removal from pulp.

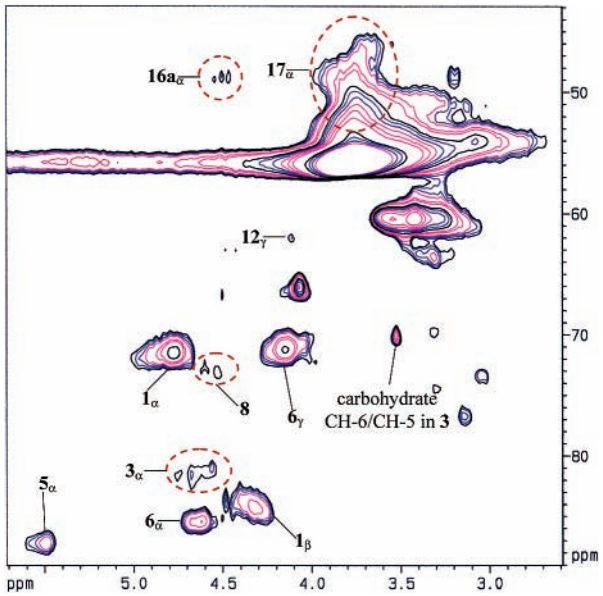

Figure 6. Expansion of the oxygenated aliphatic region of the HMQC spectrum of the RL-Alk_p preparation.

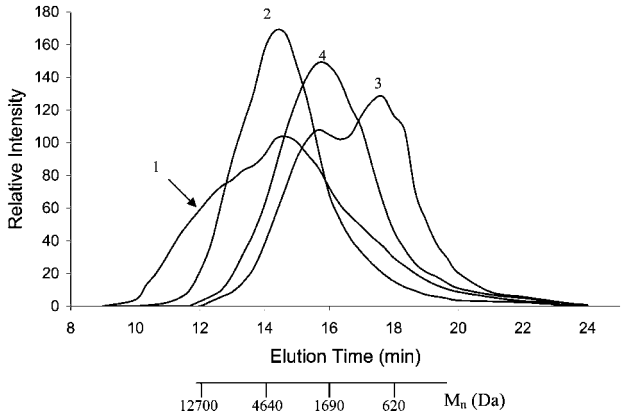

Figure 7. Normalized gel permeation chromatograms of RL-Alk_p (1), RL-D (2), DL_c (3), and DL_p (4) preparations.

Effect of Isolation Method on Lignin Structures. The structure of the kraft residual lignin extracted with sodium hydroxide solution after the enzymatic hydrolysis (RL-Alk) was compared with the structure of the corresponding residual lignin extracted with dioxane (RL-D) in order to elucidate possible differences caused by the isolation procedures. Very weak signals corresponding to α-5 condensed structures with oxygenated β-substituents (**16a**) were observed at δ_C/δ_H 49−53/4.3−4.6 in the spectra of both dioxane- and alkaline-soluble residual lignin preparations (**Table 3**). However, in contrast to the RL-D spectrum **(Figure 2B)**, the spectrum of the RL-Alk_p preparation additionally exhibited a rather prominent group of signals in the range of δ_C/δ_H 54−52/3.8 (**Figure 6**), which were tentatively assigned to α-6 structures (**17**). Moreover, GPC revealed that the alkali-soluble residual lignin contains more fractions with higher molecular mass than the dioxane-soluble lignin (**Figure 7**). Because the yield of the RL_2-Alk_p preparation is slightly higher than that of the RL_1-D, it is difficult to determine if the presence of the structures **17** is a result of more effective extraction or if they are formed during the lignin purification with a rather strong sodium hydroxide solution and relatively long time used. Further experiments are required to verify this important point.

The residual lignins contained 3−11% carbohydrates (**Table 2**). The results obtained in this work and those published earlier (*17*−*20*) indicate that the carbohydrate composition of the residual lignins is dependent on the isolation procedure and probably on the enzyme origin. Comparison of RL-D and RL-Alk preparations showed that the amount of glucose in the dioxane-soluble lignin was higher and the amount of galactose was much lower than in the alkali-soluble lignins (**Table 2**). The amount of mannose appreciably decreased after the purification of the alkali-soluble residual lignin (RL-Alk_c vs RL-Alk_p**)**, probably due to elimination of protein contaminants enriched with mannose. Another cause could be peeling of mannose in fragments of galactoglucomannan chains. Decomposition of carbohydrates during the purification of the alkali-soluble residual lignin was evident from the appreciable decrease in the carbohydrate content in the purified lignin preparations. Accumulation of galactose and arabinose in the RL-Alk_p preparation as compared to the RL-Alk_c sample implies that these carbohydrates are likely involved in lignin−carbohydrate bonds. This is consistent with an appreciable signal at δ_C/δ_H 69.5/3.5 (**Figure 6**) assigned to benzyl ether lignin−carbohydrate linkages involving primary hydroxyl groups of carbohydrates, such as those at CH-6 of α-D-galactopyranosyl units and CH-5 of α-L-arabinofuranosyl units.

GPC analysis did not show any difference between elution curves obtained using RI and UV detectors. The former detected all components, whereas the latter was sensitive only for lignin. Therefore, we can conclude that carbohydrates in the residual lignins were attached evenly to lignin fractions of different molecular masses.

Differences between Residual and Dissolved Kraft Lignins. The structures of residual lignins are much less altered than those of the dissolved lignins (**Figure 2**; **Table 3**). The amounts of original lignin moieties, such as *β*-*O*-4 and phenylcoumaran structures, were higher in the residual lignins than in the dissolved lignins. In contrast, the intensities of newly formed signals such as stilbene structures, saturated aliphatic groups, and signals at δ_C/δ_H 74/4.5 were lower. The concentration of conjugated carbonyl moieties was also lower in the residual lignins than in the dissolved lignins. Some of the moieties identified in the dissolved lignins, for example, vinyl ether structures, were not detected in the residual lignins. These observations imply that chemical reactions of lignin inside the fiber are rather limited. Changes in the residual lignin structures during pulping consist mainly of decreases in the amount of reactive moieties and accumulation of structures with low reactivity, such as *β*-*β*, 5-5, and 4-*O*-5 structures.

There are significant differences in the nature of lignin moieties assigned to alkyl−aryl condensed structures in residual and dissolved lignins. The dissolved lignins contain a noticeable amount of α-5 condensed structures with nonoxygenated *β*-substituents (**16b**) as well as *β*-Ar structures (δ_C/δ_H 48.0/3.2−3.4), which are not detected in the residual lignins. The intensity of signals assigned to α-6 moieties was appreciably higher in the residual lignin (RL-Alk_p) than in the dissolved lignins. These indicate differences in the mechanisms of condensation reactions in pulping liquor vs inside the fiber.

As expected, the molecular mass of the dissolved lignins is appreciably lower than that of the residual lignins (**Figure 7**). The purification of the crude dissolved lignin (DL_c) removed the low molecular mass fraction. This resulted in some differences in the compositions of the DL_c and DL_p lignins (**Table 3**). Vinyl ether structures were detected in the spectrum of the crude dissolved lignin (DL_c) (**Figure 5)**, but only traces of them were observed in the purified preparation (DL_p) (**Table 3**). These moieties are probably present in low molecular mass fractions, which were eliminated during the purification. In contrast, signals of stilbene moieties were apparently more intense in the purified lignin, indicating their presence in high molecular mass fractions. The composition of aliphatic structures is also different in the crude and purified dissolved lignins.

The amounts of carbohydrates in the dissolved lignins were in the range of 2.5−2.8%, much lower than in the residual lignins. In contrast to the residual lignins, xylose was the dominant sugar in the dissolved lignins (**Table 2**). This was also evident from the HMQC spectra, which showed mostly signals corresponding to xylose units in the dissolved lignins, whereas the residual lignins exhibit rather prominent signals corresponding to hexose units in addition to xylose units (**Figure 2**). Another difference between dissolved and residual lignins was an appreciable amount of terminal carbohydrate units, both reducing (δ_C 92−97) and nonreducing (δ_C 65−67) end groups, observed in the spectrum of the RL-D preparation. This indicates that the carbohydrate chains are appreciably shorter in the residual lignin preparation than in the dissolved lignins.

Thus, we can conclude that our modifications in the procedure for the isolation of enzymatic residual lignins gave preparations with rather high yields and with appreciably lower protein contamination than has been reported earlier. The HMQC NMR technique has been shown to be a very useful method in the structural characterization of lignin preparations isolated after kraft pulping, providing important information on the pulping mechanisms. Elucidation of the role of condensation reactions shows that the increase in the degree of lignin condensation after the pulping resulted from accumulation of the native condensed lignin moieties rather than the formation of new alkyl−aryl structures. The formation of vinyl ether via the reverse aldol reaction of *β*-*O*-4 moieties is very limited under the conditions of kraft pulping. However, stilbene structures are accumulated in the lignins in appreciable amounts. All lignins show the presence of ether linkages between the benzylic atom of lignin and primary hydroxyl groups of carbohydrates. Elucidation of the products of *β*-*O*-4 bond cleavage is still a challenge. Structures of α-hydroxyacid type have been postulated to be among the important products of lignin degradation. Saturated aliphatic moieties are accumulated in a great amount during the pulping. Their structure requires further investigation. The residual lignin is in general less altered than the corresponding dissolved lignin, implying that chemical reactions are rather limited inside the fiber.

ABBREVIATIONS USED

HMQC, heteronuclear multiple quantum coherence; GPC, gel permeation chromatography; MWL, milled wood lignin; RL-D, dioxane-soluble residual lignin; RL-Alk_c, alkali-soluble crude residual lignin; RL-Alk_p, alkali-soluble purified residual lignin; DL_c, crude dissolved lignin; DL_p, purified dissolved lignin; DEPT, distortionless enhancement by polarization transfer.

ACKNOWLEDGMENT

We are thankful to the Research Laboratory of Kotlas Pulp and Paper Mill, Russia, for the pulping of pinewood.

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Received for review April 11, 2003. Revised manuscript received August 1, 2003. Accepted August 5, 2003. This research was supported by USDA NRI Competitive Grants under Cooperative Agreements 95-37103-2268 and 98-35504-6781, for which we are grateful.

JF034372D