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Original

The effect of isolation method on the chemical structure of residual lignin

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Abstract Two methods are used for the isolation of residual lignin: acidolytic and enzymatic hydrolysis. Recently a two-step procedure that is a combination of enzymatic and acidic hydrolyses was proposed. In this paper, the structures of residual lignins isolated by these three methods are compared. Enzymatic hydrolysis gave lignin with the highest yield (83%); however, it contained high amounts of carbohydrates and protein. The molar mass of enzymatic lignin was the highest, indicating that no cleavage of lignin occurred. Acidolysis gave a significantly lower lignin yield (40%), but this lignin was practically free from impurities. The β-aryl ether and lignin-carbohydrate linkages cleaved during the isolation, which was manifested in the decreased molar mass of the lignin as well as in increased phenolic hydroxyl group content. The new two-step isolation procedure gave properties between the preparations of enzymatic and acidolytic hydrolyses. The lignin yield was high (78%), but it contained some impurities, although less than the enzymatic lignin. The lignin-carbohydrate linkages cleaved to some extent, but the β-aryl ether linkages remained intact.

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Introduction

The structural details of residual lignins are of considerable importance in pulp delignification and bleaching studies. The chemical structure of residual lignin supplies information on the reactivity of the lignin in the ensuing bleaching stages. In addition, the reactions taking place during different chemical treatments can be characterized by the changes in residual lignin structure. In order to characterize the residual lignin, it has to be isolated from the pulp matrix. Although several methods are available for lignin isolation, none can be used to quantitatively isolate the residual lignin in pulp without the risk of changing its structure. The most commonly used isolation methods are acidolysis and enzymatic hydrolysis.

During acidolysis, the lignin is isolated by refluxing the pulp under a nitrogen atmosphere with acidic dioxane–water solution and recovering the lignin from the solution (Gellerstedt et al. 1994). The yield of lignin is typically 40% of the lignin in pulp, and the resulting material is of a very high purity. However, it has been proposed that some structural changes in lignin, such as cleavage of α-aryl, α-alkyl ether and β-aryl ether bonds in benzyl alcohol structures, take place during the isolation. Alternatively, condensation reactions are not believed to occur during acidolysis (Gellerstedt et al. 1994).

Enzymatic hydrolysis has been used for lignin isolation since 1981 (Yamaski et al. 1981). In this method, the pulp carbohydrates are depolymerised and dissolved by cellulase (and hemicellulase) enzymes, and the solid residue contains the residual lignin. Enzymatic hydrolysis retains the linkages between lignin and carbohydrates, and therefore these structures can be studied from enzymatically isolated lignin. This method gives high lignin yields, but the isolated fraction also contains significant amounts of carbohydrates and some protein residues from the enzymes.

A new method to isolate the lignin was recently published (Argyropoulos et al. 2000). In this method the afore mentioned two procedures are combined by first removing most of the carbohydrates by a mild enzymatic treatment, and then the lignin is further treated with a mild acidolysis. The first step is believed to remove most of carbohydrates and therefore expose the fiber structure. In the subsequent acidolytic step, diffusion no longer limits the extraction of lignin from the fiber wall, and the acid most likely cleaves remaining lignin–carbohydrate bonds. This method provides lignin with about 70% yield and relatively low amounts of impurities.

The residual lignins isolated by these three different methods, acidolysis, enzymatic and the new two-step isolation procedure, have remarkably different content impurity contents and different yields. This raises the question whether the chemical structure of these lignins is the same, or whether there are discrepancies in their structure. In order to answer this question, the residual lignin of a softwood kraft pulp was isolated by these three methods, and the chemical structures were characterised using several wet chemical, spectroscopic and chromatographic procedures.

Experimental

Initial pulp

Black spruce chips were pulped using conventional kraft pulping procedures to kappa number 30. The total lignin content of the pulp was 4.5% when measured as the sum of Klason lignin and acid soluble lignin.

Residual lignin isolation

Acidolysis was performed for acetone-extracted pulp using 0.1-M HCl in dioxane:water (volume ratio 85:15) as published elsewhere (Gellerstedt et al. 1994). The two-step isolation was performed by treating the pulp by cellulase (Iogen, Canada) for 48 h at 50°C. The residue was freeze-dried and further treated with 0.05-M HCl in dioxane–water (volume ratio 85:15, Yamaski et al. 1981). Enzymatically isolated lignin was obtained by treating the pulp with Econase (Roehn Enzyme, Finland) and Novozym (Novo, Denmark) enzymes for 48 h at 50°C, as described in detail elsewhere (Hortling et al. 1990; Tamminen, Hortling 1999). A protease treatment was also used (Hortling et al. 1990) in an effort to remove as much protein as possible.

The extractives in residual lignins were removed from the residual lignins by treating the lignin three times with excess of dichloromethane at room temperature. The samples were filtered and dried under vacuum. The extractives contents were 5.7%, 6.9% and 1.3% by weight for the acidolysis, two-step method and enzymatic lignins, respectively. All of the following analyses were performed on extracted lignins.

Carbohydrates

The carbohydrate contents of residual lignins were determined by subjecting the samples to acid hydrolysis (3% H_2SO_4, 125°C, 2 h). The carbohydrate content and composition of the solution was determined using the HPLC-PAD method described elsewhere (Hausalo 1995).

Elemental analyses

The elemental analysis (C, H and N) was performed at the Schwarzkopf Microanalytical Laboratories, Woodside, NY. The remaining sample was assumed to be oxygen. The protein content of the enzymatically isolated lignin was calculated from the nitrogen content by multiplying the content by 6.25 (protein contains 16.5% nitrogen).

Quantitative ^{31}P NMR analyses

Aliphatic hydroxyl groups, the phenolic group content and acid group content were determined using ^{31}P NMR (Zia et al. 2001). Duplicates of all samples were analysed. The samples (35 mg) were dissolved in pyridine:$CDCl_3$ (1.6:1) solution followed by the addition of 100 µl of cholesterol solution as an internal standard. Then, 200 µl of 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane was added as the phosphitylation reagent 2 h before the measurement. All samples were fully soluble in the solvent. The spectra were recorded using a NMR instrument (Mercury 200 MHz). Each FID was Fourier transformed and integrated three times, and the data were calculated from the average of these integrations. Integration limits were 149.5–145.5 ppm for aliphatic hydroxyl groups, 144.0–140.1 ppm for condensed phenolic hydroxyl groups, 140.0–137.5 ppm for guaiacyl and parahydroxyphenyl groups and 136.0–134.0 ppm for acid groups.

^{13}C NMR

Quantitative ^{13}C NMR spectra were recorded using a spectrometer (Varian Unity, 500 MHz), as described recently (Zia et al. 2001). For this measurement 450 mg of lignin were dissolved in deuteriated DMSO-d_6, and 30 mg of trioxane (93.0 ppm) were added as internal standard. All samples were fully soluble in the solvent.

Each FID was Fourier transformed and integrated four times. The integration limits were as previously described (Hausalo 1995).

Difference UV

Phenolic hydroxyl groups were measured using the difference UV method described by Argyropoulos et al. (2000).

Molar mass

The molar masses of lignin samples in 0.1-M NaOH solution were determined using GPC as described by Hortling et al. (1999). The preparative column was filled with Superdex 200 resin, and it was externally calibrated using polystyrenesulphonate standards of the range 1,000–100,000 g/mol.

Permanganate oxidation

The content of condensed structures were determined using a permanganate oxidation method described in detail elsewhere (Ericksson et al. 1973; Gellerstedt, Gustafsson 1987) and later modified by Tamminen et al. (1997).

Derivatization followed by reductive cleavage (DFRC)–^{31}P NMR

The derivatization followed by reductive cleavage (DFRC) method followed by ^{31}P NMR measurement was performed according to the procedure first introduced in lignin analysis by Lu and Ralph (1997) and later modified by Tohmura and Argyropoulos (2001a).

Results and discussion

Yield and chemical composition

Residual lignin from a conventionally pulped black spruce kraft pulp was isolated using three different isolation procedures: acidolysis, two-step (a mild enzymatic hydrolysis followed by a mild acidolysis) and enzymatic hydrolysis. The obtained lignins were further extracted with dichloromethane in order to remove extractives. It should be noted that the extractives in the pulp had to be removed from the residual lignin and not from the pulp, because extracting the pulp with acetone before the lignin isolation markedly decreased the yield of acidolysis lignin. This decrease was probably due to the collapse of the fibres in drying (Argyropoulos et al. 2000).

The highest yield of lignin was obtained when the isolation was performed using enzymatic hydrolysis; in this case the yield of pure lignin was 83% on total lignin of pulp (Table 1). In addition to this yield, another lignin fraction (insoluble in protease treatment) was obtained. The yield of this fraction was 6.9% of the lignin in pulp (not corrected with purity), which is typical for this type of pulp (Hortling et al. 1990). This fraction was not included in the structural characterization of lignin.

The yield of acidolysis lignin was less than half the yield of enzymatically isolated lignin. The fairly low yield can partly be explained by the mass transfer limitations. Most of the lignin in kraft pulp is located inside the fiber, and during acidolysis this lignin probably cannot fully diffuse into dioxane–water solution. This mass transfer hindrance was successfully delineated in the two-step isolation procedure, where the pulp was first treated with a low amount of cellulase enzyme, which ruptured the fibrous structure of pulp, and then the solid residue was subjected to a mild acidolysis. Using this procedure it was possible to obtain 78%

Table 1. Yield of lignin (from total lignin content of pulp) using three different isolation methods

	Acidolysis	Two-step	Enzymatic
Yield of impure lignin (%)	40	83.8	93.3
Purity[a](%)	99.6	93.6	89.2
Yield of pure lignin (%)	39.8	78.4	83.2

[a]Purity=100%−protein (%)−carbohydrates (%)

yield of residual lignin, compared to the 40% yield by acidolysis alone. It can be seen that the purity of the lignin decreased in the order: acidolysis, two-step and enzymatic lignin. Acidolysis lignin was practically free from impurities, whereas the two other lignins contained some residues from carbohydrates and proteins (Table 1).

Enzymatically isolated lignin contained as much as 6% carbohydrates (Table 2). This was because cellulase and hemicellulase enzymes are known to retain the bonds between lignin and carbohydrates. The high content of galactose indicates an abundance of lignin–galactan bonds, probably originating from the outer cell wall layers suggested earlier by Hortling et al. (1996).

The carbohydrate content of the two-step lignin, only 1.9% by weight, was remarkably lower than that of enzymatically isolated lignin. This may be explained by the acidic cleavage of linkages between lignin and carbohydrates during the mild acidolysis step, as described by Wang et al. (1997). Nevertheless, the carbohydrate content of the two-step lignin was still relatively high when compared to that of acidolysis lignin, possibly implying that the mild acidolysis performed for enzymatically isolated lignin did not cleave all lignin–carbohydrate linkages.

Acidolysis lignin contained only a minor amount of carbohydrates, only 0.4% by weight. These carbohydrates may be linked to lignin by ether bonds, because it has been shown that in residual spruce soda lignin, isolated by acidolysis, some of these linkages between cellulose and lignin remain intact (Kosikova and Ebringerova 1994).

The nitrogen content of the samples originates from the protein residues. The protein content of the lignins showed that the two-step lignin preparation contained less protein residues (4.4%) than the enzymatically isolated lignin (5.0%), in spite of the protease purification of the enzymatically isolated lignin. The amount of enzyme used in the two-step isolation procedure was significantly smaller than in the enzymatic isolation.

Table 2. Carbohydrate content of lignins isolated by three different procedures

	Acidolysis	Two-step	Enzymatic
Arabinose (mg/g)	+	+	2.7
Galactose (mg/g)	+	1.5	14.5
Glucose (mg/g)	2.3	7.2	10.4
Xylose (mg/g)	1.7	5.1	15.1
Mannose (mg/g)	+	5.9	17.4
Total (mg/g)	4.0	18.7	60.1

+ indicates detected but under quantification limit

Table 3. Carbon, hyrogen, nitrogen and oxygen contents of the lignins isolated by three different procedures. The values (C, H, O and OMe) are corrected with carbohydrates and protein

	Acidolysis	Two-step	Enzymatic
C (%)	62.8	61.2	63.2
H (%)	6.2	5.6	5.6
O_2[a](%)	31.0	33.1	31.2
OMe (%)	13.9	13.2	14.1
N_2 (%)	0	0.7	0.8
Protein[b] (%)	0.0	4.4	5.0

[a]O_2=100%−C (%)−H (%)−N (%)
[b]Protein content=6.25·N

The elemental analysis of acidolysis, two-step and enzymatic lignins showed almost no difference between these lignins (Table 3). The methoxyl group contents of the lignins were also the same in all lignin samples (Table 3). These data agree with that of Lachenal et al. (1999), who did not observe differences in methoxyl group content between acidolysis and enzymatic lignins.

Structural details

Molar mass

The molar mass of residual lignins follow the order: enzymatic>two-step>acidolysis lignin (Table 4), which agrees with the results of Lachenal et al. (1999). The average molar mass (related to polystyrene sulfonates) of two-step lignin was twice as high as that of acidolysis lignin. Enzymatically isolated lignin had a molar mass nearly twice as high as that of two-step lignin.

The molar mass distributions show that in both acidolysis and two-step lignins the most dramatic decrease was in the high molar mass region (Fig. 1). The acidolysis lignin had a notable peak at about 1,000–10,000 g/mol, whereas the molar mass of enzymatically isolated lignin was more or less uniformly distributed between 1,000–100,000 g/mol.

The low molar mass of acidolysis was probably the consequence of the cleavage of β-aryl ether linkages during the acidolysis step. It is also remarkable that the enzymatically isolated lignin contain carbohydrate chains linked in lignin. These chains increase the hydrodynamic volume of lignin and therefore increase the apparent molar mass of the lignin when measured using GPC. The partial (two-step lignin) or total (acidolysis lignin) cleavage of lignin–carbohydrate linkages during the isolation procedure therefore may

Table 4. Weight average (M_w) molar mass and polydispersity of acidolysis, two-step and enzymatic kraft residual lignin. The calculated values are related to the polystyrene sulfonate standards

	M_w (g/mol)	DP
Acidolysis lignin	11,030	3.5
Two-step lignin	21,080	3.9
Enzymatic lignin	35,110	4.2

DP degree of polymerization

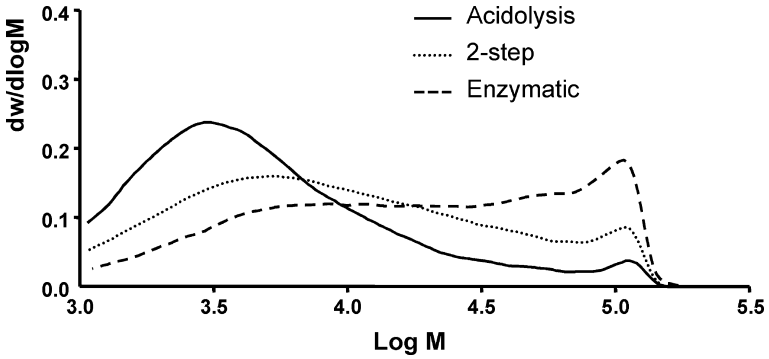

Fig. 1. Molar mass distribution of residual lignins isolated by acidolysis, two-step (enzymatic+acidolysis) and enzymatic isolation procedures. The column was calibrated with monodisperse polystyrene sulfonates

decrease the apparent molar mass of lignin, although lignin itself was not cleaved.

β-aryl ether linkages

The content of *β*-aryl ether linkages in residual lignins were determined using the novel DFRC ^{31}P method recently developed by Tohmura and Argyropoulos (2001b). The results in Fig. 2 show that the content of these bonds in residual lignin was small, because most of them were already cleaved during the kraft pulping. The content of these linkages in acidolysis lignin was lower than in two-step or enzymatic lignins. This result suggests that during the acidolysis step (in 0.1-M HCl), these linkages cleaved markedly, whereas the acidolytic step of the two-step isolation (0.05 M HCl) was too mild to cleave these bonds. The cleavage of these bonds during acidolysis could also be cited as being partly responsible for the low molar mass of the acidolysis lignin (Table 4).

Phenolic hydroxyl group content

The phenolic hydroxyl group contents were measured using quantitative ^{31}P NMR spectroscopy and the difference UV method. The two methods gave almost equal amounts of phenolic OH groups, which confirms that both methods were equally effective in determining the total phenolic content of the lignins.

The phenolic hydroxyl group content decreased in the order of acidolysis>two-step>enzymatic (Table 5). This result can be partly explained by the fact

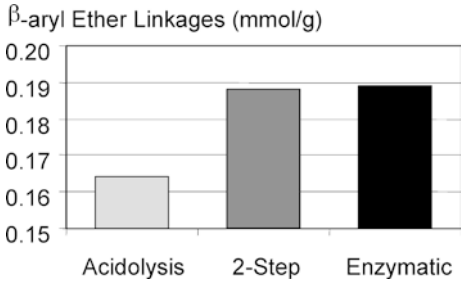

Fig. 2. *β*-aryl ether contents of residual lignins isolated by conventional acidolysis, the new two-step procedure and by enzymatic (cellulose) hydrolysis

Table 5. Phenolic hydroxyl group (PhOH) contents and other functional groups of the lignins as determined by ^{31}P NMR and difference UV methods

	Acidolysis	Two-step	Enzymatic
Total phenolic OH[a] (mmol/g)	2.19	1.81	1.37
Total phenolic OH[b] (mmol/g)	2.20	2.06	1.65
Condensed PhOH[a] (mmol/g)	1.14	0.94	0.70
PhOH in 5–5' biphenols[a] (mmol/g)	0.58	0.49	0.37
Weakly acidic PhOH[b] (mmol/g)	0.31	0.35	0.29
Conjugated PhOH[b] (mmol/g)	0.33	0.31	0.20

[a]Measured using ^{31}P NMR
[b]Measured using difference UV method

that acidolysis cleaved the β-ether bonds, and as such it released new phenolic hydroxyl groups. However, the β-ether bond content in the residual lignins was too small to account for all the difference. It is also probable that, because of its much lower yield, the acidolysis lignin is not as representative as the two other lignins. Consequently, in the acidolysis lignin the phenolic structures, especially the condensed phenols, are accumulated in the isolated fraction. The content of condensed phenolic hydroxyl groups were markedly higher in acidolysis lignin than in enzymatic lignin, as seen in the ^{31}P NMR spectra (144.0–140.2 ppm in Fig. 3). The effect for two-step lignin was not as pronounced due to lower acid concentration and thus less intense degradation of these structures in the acidolysis step. The formation of condensed phenolic groups during acidolysis is not probable, because this reaction has not been observed to occur under these conditions (Gellerstedt et al. 1994).

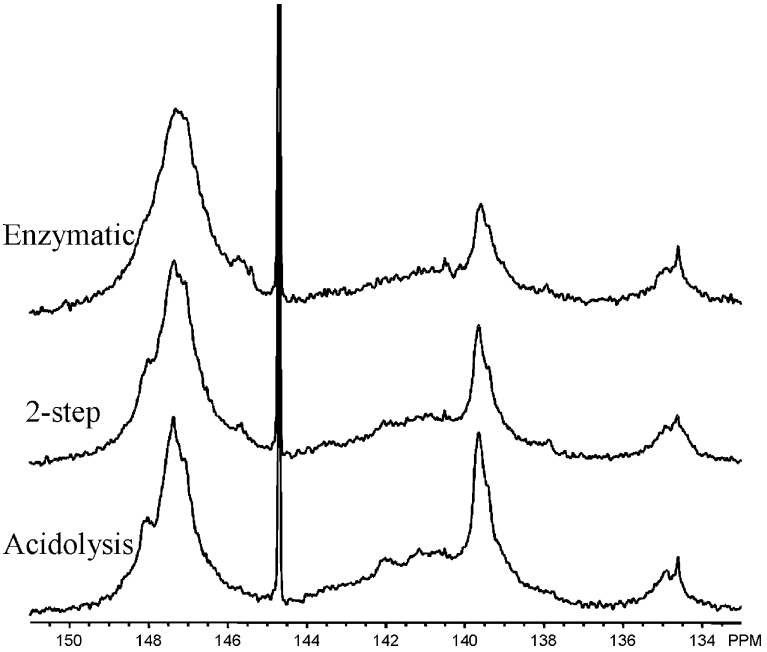

Fig. 3. ^{31}P NMR spectra of residual lignins isolated by acidolysis, two-step and enzymatic isolation procedures

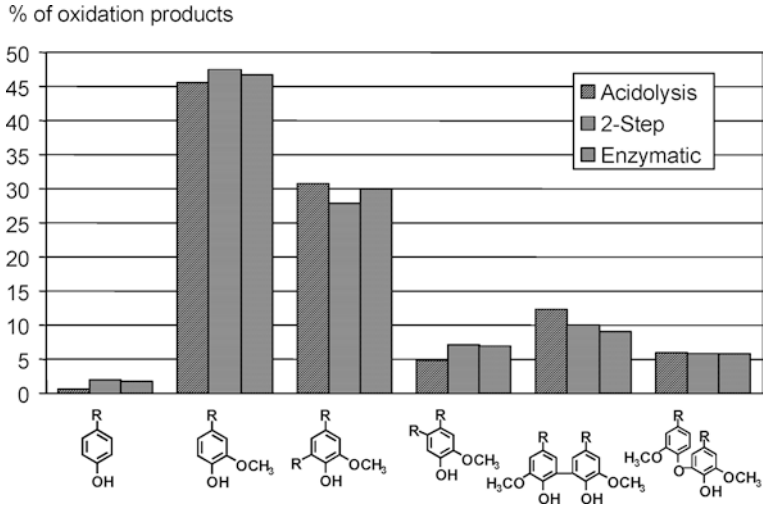

Fig. 4. Distribution (mol %) and structure of the oxidative degradation products of the residual lignins obtained by acidolysis, two-step (enzymatic+acidolysis) and enzymatic isolation procedures

Permanganate oxidation

Permanganate oxidation gives useful information on the structures of the free phenolic groups in lignin. The results of this method in Fig. 4 are in fairly good agreement with the results of the ^{31}P NMR data. The content of 5–5' biphenolic structures is highest in acidolysis lignin, followed by the two-step and enzymatic lignins. The content of *p*-hydroxyphenyl structures was fairly low, being more or less the same in all three lignin samples, as observed by ^{31}P NMR. The content of biphenylether structures was also the same in all three lignin samples.

Aliphatic hydroxyl groups

The aliphatic hydroxyl group content for the three lignins was found to follow the order of the carbohydrate content of the isolated fraction (Table 6). However, the different nature of the aliphatic hydroxyl groups can be seen in the ^{31}P NMR spectra of the lignins (Fig. 3). In the enzymatically isolated lignin, there is a clear signal for 146–145.5 ppm, whereas this signal is missing from the acidolysis lignin. This region is known to originate from carbohydrates and thus may explain some of the difference in the aliphatic hydroxyl group content of these lignins.

Another remarkable difference in the aliphatic hydroxyl group region of the ^{31}P NMR spectra is at about 148 ppm. In enzymatic lignin, there is only a hardly notable shoulder at this position, whereas in acidolysis lignin there is clear signal at 148 ppm. It has been shown (Jiang et al. 1995) that secondary hydroxyl groups

Table 6. Aliphatic hydroxyl groups in the lignins as measured by ^{31}P NMR spectroscopy

	Acidolysis	Two-step	Enzymatic
Total aliphatic OH (mmol/g)	1.94	2.37	3.20

in lignin, such as α-hydroxyl groups in β-aryl ether structures, give signal at this position (*erythro* at 148.2 ppm, and *threo* at 147.8 ppm). The high intensity of the signal at this position in acidolysis lignin can be explained by the cleavage of lignin–carbohydrate linkages. Because the carbohydrates are most probably connected to α-carbon of the lignin phenyl propane unit, the cleavage of this lignin–carbohydrate bond liberates a new α-hydroxyl group.

Acid groups

The content of acid groups was calculated from the ^{31}P NMR spectra. The results showed that the acid group contents were the same (0.25–0.28 mmol/g lignin) in all lignins, regardless of the isolation procedure.

Quantitative ^{13}C NMR

Quantitative ^{13}C NMR spectroscopy can be used to quantify different structures in lignin (Hausalo 1995). However, it has been shown (Hortling et al. 1993) that the protein residues and carbohydrates in enzymatically isolated lignins interfere with some regions of the ^{13}C NMR spectra.

The acid group content measured from the ^{13}C NMR spectra were 0.32, 1.32 and 1.50 mmol/g for the acidolysis, two-step and enzymatic lignins, respectively. The acid group content of acidolysis lignin was thus comparable to the result obtained using ^{31}P NMR spectroscopy (0.28 mmol/g). On the contrary, the values for the two-step and enzymatic lignins were much higher than those obtained by ^{31}P NMR spectroscopy. This confirms that protein residues interfere with the quantification of carboxylic carbons using ^{13}C NMR spectroscopy, as has also been observed earlier by Akim et al. (2001).

The aromatic carbon region (155–105 ppm) in all three lignins was similar (Fig. 3). No significant differences were observed, indicating that no substantial changes in these structures occurred during the isolation procedure. The aliphatic carbon region at 89–58 ppm was significantly affected by the carbohydrate residues. It can be seen that in the enzymatic lignin (Fig. 5) there are significant peaks at this region. Although the impurities made it impossible to accurately quantify this region, the region at 90–80 ppm, which also originated from β-carbon of β-O-4 structure, was higher in enzymatic lignin than in acidolysis lignin. This result thus agrees with the results of DFRC analysis.

The peak at 55.0 ppm originates from the methoxyl group of lignin, and in acidolysis lignin it was possible to quantify the methoxyl groups (4.00 mmol/g lignin), which is close to the figure obtained by elemental analysis (4.52 mmol/g lignin). However, in enzymatic and two-step lignins there was another peak, which did not resolve from the methoxyl peak. Therefore the quantification of methoxyl groups in these lignins was not as accurate as it was for acidolysis lignin.

Conclusions

There is no perfect method to isolate the residual lignin in pulp. Enzymatic hydrolysis gave almost quantitative yield without changes in lignin structure, but the impurities in lignin (protein and carbohydrates) disturbed remarkably some analyses.

Acidolysis lignin gave pure lignin with only traces of impurities; therefore it was also possible to analyse the lignin structure with the most sophisticated analytical techniques. Unfortunately, the yield of lignin was low, and therefore the sample is not as representative. In addition, the lignin structure changed during

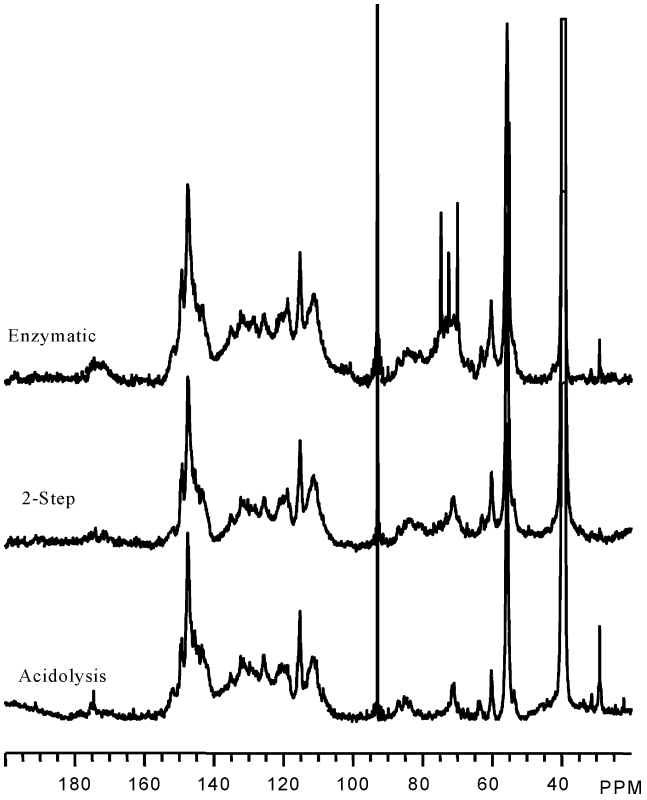

Fig. 5. Quantitative ^{13}C NMR spectra of acidolysis, two-step and enzymatic lignins. Trioxane (at 93 ppm) was used as internal standard

the isolation procedure, and thus some information on lignin structure was misleading.

The novel two-step isolation procedure gave lignin with properties between acidolysis and enzymatic lignins. The yield was fairly high and thus the lignin was considered representative. However, the lignin still contained impurities, which disturbed the performance of a few analytical techniques. The structure of the residual lignin changed only slightly during the isolation procedure.

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