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A method for isolation of milled-wood lignin involving solvent swelling prior to enzyme treatment

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Summary A new lignin isolation method has been developed. Wood and pulp were subjected to ball milling, swelled in an organic solvent, and then treated with a cellulase. The enzyme digestion time could be shortened to 1 day with this method. The lignin obtained has been named Swelled Enzyme Lignin (SEL). Swelling and enzyme digestion conditions and their effects on lignins were investigated. The SEL's from wood could be directly washed with water, while those from pulp had to be washed with aqueous acetic acid because they were water soluble. The purification of crude SEL's was accomplished by extracting them with dioxane-water, and then precipitating and washing with ethyl ether. Lignin yields were 24–67% based on the total amount of lignin present. The characteristics of the SEL's were further investigated by gel-permeation chromatography (GPC), infrared and ^{13}C nuclear magnetic resonance (NMR) spectroscopy.

Symbols

The symbols used were the following:

SUKP	Softwood Unbleached Kraft Pulp
BUKP	Birch Unbleached Kraft Pulp
TD	Todomatsu (Abies sachalinensis) wood
MWL	Milled Wood Lignin
MWLS (BK, TD)	Milled Wood Lignin of SUKP (BUKP, TD)
SEL-C	Swelled Enzyme Lignin (crude)
SELS-C	SEL-C of SUKP, other materials named in the same way
SELS-90	SEL-C of SUKP and extracted with 90% dioxane-water
SELS-96	SEL-C of SUKP and extracted with 96% dioxane-water, other materials named in the same way

Introduction

In order to study the structure and properties of lignins and their changes they undergo on pulping procedure, the lignin must first to be isolated from wood or pulps with a minimum change in structure. The Björkman or milled-wood-lignin (MWL) (Lin, Dence 1992) method is the standard procedure for isolation of lignin. However, the yield of MWL is often low, (Glassier, Barnett 1979; Obst, Kirk 1988), and it is clear that a lignin isolated in a higher yield would be more representative of the total lignin present.

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To improve the yield, another lignin preparation called Milled Wood Enzyme Lignin (MWEL) was developed in recent years (Chang et al. 1975; Lin, Dence 1992). Here, ball-milled wood or pulp is treated with enzymes to solubilize the carbohydrate components, resulting in a much higher yield of lignin. However, the procedure is tedious, and the enzyme treatment requires 10 or more days (Iversen 1986; Obst, Kirk 1988), because the cellulose is highly crystalline and inaccessible, which impairs the physical contact between cellulose and enzyme. It should also be noted that only an endo-cellulase can initiate the microbial degradation of the crystalline cellulose.

To cope with this problem, a swelled enzyme lignin (SEL) method was developed. Ball-milled wood and pulps were swelled by an organic solvent, regenerated in water, and treated with cellulase. Swelling opens up the cellulose crystalline or destroys them, loosens and reduces the physical connection between lignin and hemicelluloses. As a result, prior swelling should not only shorten the digestion time but also lead to a more complete enzymic degradation of the polysaccharides.

Experimental

The entire SEL procedure is schematically outlined in Fig. 1. Todomatsu wood was first milled in a Wiley mill to pass a 20 mesh screen and then extracted with ethanol: benzene

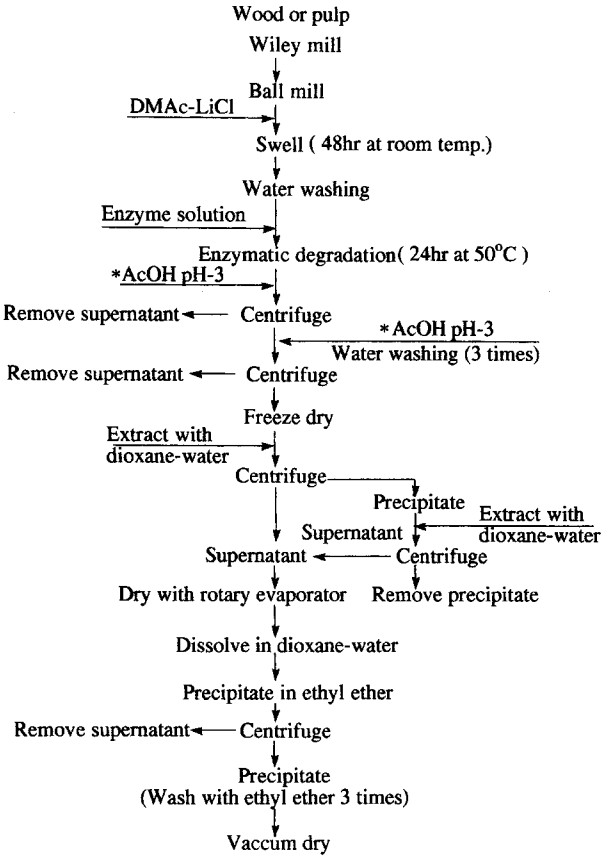

Fig. 1. Outline of the SEL method. *For isolation of wood lignin, an adjustment of the pH value was not necessary

(1 : 2, v : v) and vacuum dried. The extracted wood was milled in a vibratory ball mill for 24 hours.

SUKP was obtained from a plant. It contained 3.90% Klason lignin. BUKP was prepared by cooking birch chips under a laboratory conditions. The pulp contained 8.0% Klason lignin (see the section of Determination of lignin). These air-dried pulps were also milled in a Wiley mill and a ball mill as described above.

Swelling

The swelling solvent consisted of 100 ml *N,N*-Dimethylacetamide (DMAc) and 7.0 g lithium chloride. Solvent, wood and pulps were all completely dry. Swelling was taken place at room temperature for 48 hr after which the wood and pulps were regenerated completely washed with water.

Enzymatic degradation

Cellulase "ONOZUKA" R-10 (YAKULT PHARMACEUTICAL IND. CO. LTD.) was used for enzymatic digestion. The cellulase was dissolved in a Na_2HPO_4-citric acid buffer solution at pH-5.0 (5 g/ml). Wood or pulps (5% w/v) was incubated in the enzyme solution at 50 °C for 1 day with mechanical shaking.

Washing of the residue after enzymatic digestion

The TD was washed three times with water by centrifuging and removing the supernatant. Finally, 1 g of the residue was suspended in 60 ml of water and freeze-dried.

For pulp materials from SUKP and BUKP, the enzyme digested liquor was adjusted to a pH value to 3.0 by dropwise addition of acetic acid to precipitate the lignin. The supernatant was removed by centrifugation. This operation was repeated three times. Finally, 1 g of the precipitate was suspended in 60 ml of water and freeze-dried.

Purification

Residual carbohydrates must be removed from the SEL-C before NMR spectra can be obtained. For purification, the freeze-dried SEL-C, after further drying in vacuo, was twice extracted with 96% (or 90%) dioxane-water solution. The extract was evaporated to dryness on a rotary evaporator at 40 °C. The dried sample was dissolved in a small amount of the dioxane solution (0.5 g/20 ml). The dioxane solution was added dropwise to anhydrous ethyl ether (220 ml) with stirring, and the precipitated lignin was recovered by centrifugation. The lignin was washed twice with fresh ethyl ether and once with petroleum ether, and finally vacuum dried.

Determination of lignin purity

The lignin content of the materials was determined by the Klason lignin method. However, as the acid-soluble lignin in pulp was significant (Kawamura, Higuchi 1964), it was measured by the method described on TAPPI Useful Method UM-250 and counted up. The lignin content in the SELs was measured by the acethyl bromide spectrophotometric (Johnson et al. 1961) as modified by van Zyl (1978).

Acetylated lignin

To obtain a soluble sample for GPC and NMR spectroscopy, lignin was acetylated by the method of Chen and Robert (1988).

Analyses

^{13}C NMR spectra were obtained with a Brucker MSL-400 spectrometer, operating at 100.6 MHz for ^{13}C nuclear frequency, and using a standard decoupling pulse sequence. The spectra were obtained at ambient temperature with 75 mg samples dissolved in deuterated dimethylsulfoxide (DMSO-d_6). Internal reference was tetramethylsilane (TMS).

IR spectra were recorded with KBr pellets with a JASCO IR A-202 spectrophotometer.

GPC analyses were obtained with a Yanagimoto HPLC L-400W (Shodex GPC column, KF-804 and KF-802.5) and a ultraviolet (UV)-detector (Shodex M-315), operating at 280 nm. Tetrahydrofuran (THF) was used as an eluent, the flow rate was 0.8 ml/min, and the concentration of injection samples was 1%. Monodisperse polystyrene was used as a standard for determining the molecular weight of the lignin.

Results and discussion

Effect of pretreatment on the extent of enzymatic degradation

Differently pretreated TD samples were digested with enzyme, the digested solutions were centrifuged, and the precipitates were washed with water and freeze dried. The amounts remaining were calculated from the weight loss. After 12 days' digestion, the residue was 82.0% for the Wiley milled sample, and 40.2% for the sample that had also been ball-milled, indicating that some of carbohydrate material remained undegraded in the residue. For the sample swelled with DMAc-LiCl, the residue was only 28.5%, similar to Klason lignin content (28.8%), after enzymatic digestion for only one day. These results show that after swelling the carbohydrates had adequate contact with enzyme and that their degradation was fast and complete.

Effect of swelling solvent on lignin loss

In order to establish if the swelling solvent would dissolve any lignin, the following test was carried out. SUKP was swelled with DMAc-LiCl by the same method as in the SEL preparation, and then regenerated washed with water. The Klason lignin content in regenerated SUKP was 3.6%, while the original lignin content was 3.9%, indicating that only 5% of lignin has been lost. In comparison with the loss in the purification step, this loss can be deemed to be negligible.

Water solubility of the SEL-C from pulps

Unlike wood, the SEL-C from the pulps SUKP and BUKP contained some water soluble components. Enzymatically digested SUKP was resolved into an insoluble part (S3), a water soluble portion that could be regenerated at a low pH (S2), and a water soluble part (S1) as in Fig. 2. The amount of lignin in each fraction was determined. The results are shown in Table 1. About 90% of the lignin in SUKP was present in fractions S2 and S3. The water-soluble fraction S2 contained 72% of the lignin in the pulp. Most of the SEL-C could be precipitated at pH 3.0, and most of the loss of lignin could thus be avoided by washing at pH 3.0. Acetic acid was selected for adjusting the pH, because it could be easily removed and had no serious effect on the lignin.

Yields and lignin purity of the SELs

Figure 3 shows the yields of SELs and MWLs. The MWLs were isolated from the same kind materials for the SELs by the standard MWL method. It was found that greater part of the crude MWL was lost in the purification process, especially for the MWL or SUKP.

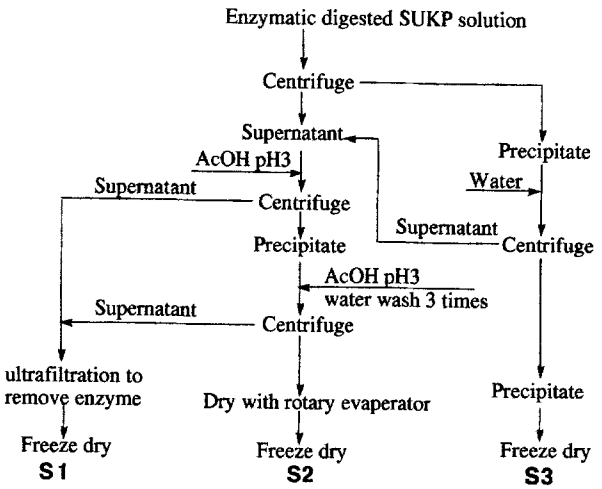

Fig. 2. Fractionation of SELN-C on the basis of water solubility

Table 1. Fractions of SELS-C grouped on the basis of water soubility and their lignin contents

Fraction	Weight of lignin in SUKP	Lignin content of the fraction	Lignin as percent of total lignin in SUKPs
Water soluble (S1)	0.28	—	—
Water soluble and regenerated at pH3 (S2)	3.78	74.5	72.2
Water insoluble (S3)	1.9	57.0	27.7

All values are in per cent

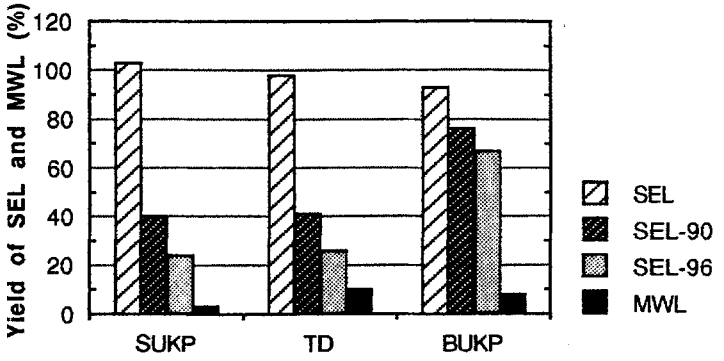

Fig. 3. Yield of SELs and comparison with MWLs (Yield was based on total lignin in the material)

Therefore, the yields of purified MWL were very low, 9.9% for TD and 7.8% for BUKP, and only 3.1% for SUKP. This is because the lignin is chemically linked to hemicelluloses. Evidently, the lignin-carbohydrate complex (LCC) could not be extracted by the lignin solvent (Chang et al. 1975). In a pulp with a low lignin content such as SUKP, most of the residual lignin was chemically linked to hemicelluloses, and the yield was very low (Isogai et al. 1987). The enzymes used in the SEL method, usually includes some hemicellulases that can degrade some of the hemicelluloses and free some of the lignin from the LCC. The yields of SELs are therefore much higher than those of the MWL. The yields of SELS-96 was 24% (almost 8 times of MWLS), and 66.8% for SELBK-96 (about 9 times that of MWLB). The purity of the SELs is shown in Table 2.

SEL-C had purity values from 68.6 to 80.2%, SEL-90 from 86.5 to 92.4% and SEL-96 from 86.5 to 96.2%. For all materials, SEL-90 were obtained in higher yield than SEL-96, but they were less pure. It is suggested that SEL-C be extracted with the 96% dioxane for NMR spectroscopy.

The relationships between lignin yield and purity are shown in Fig. 4. Lignin yield decreased as the purity increased.

IR spectra

IR spectra of the SELs and MWLs are shown in Fig. 5. The assignment of these bands is in accordance with those of Hergert (1971). For each material, the main characteristics

Table 2. Lignin purity of the SELs

Material	Purity (%)		
	SEL-C	SEL-90	SEL-96
SUKP	68.6	86.5	94.5
TD	74.2	88.8	96.2
BUKP	80.2	87.4	93.6

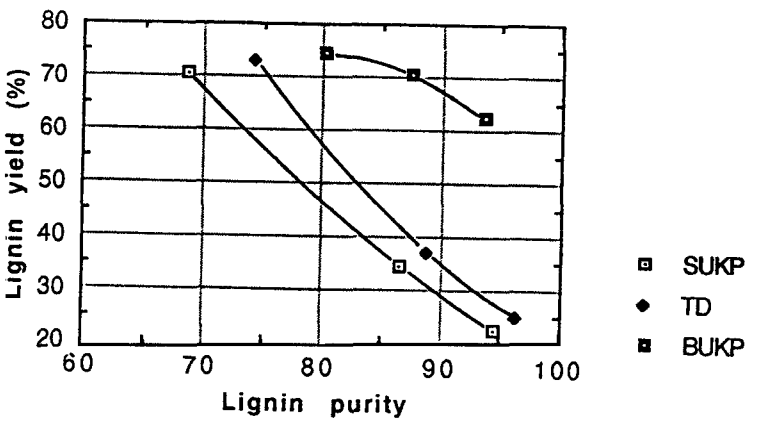

Fig. 4. The relationship between lignin yield and purity

$$\text{*Lignin yield} = \frac{(\text{yield of SEL}) \times \text{purity}}{\text{total lignin in the material}} \times 100\%$$

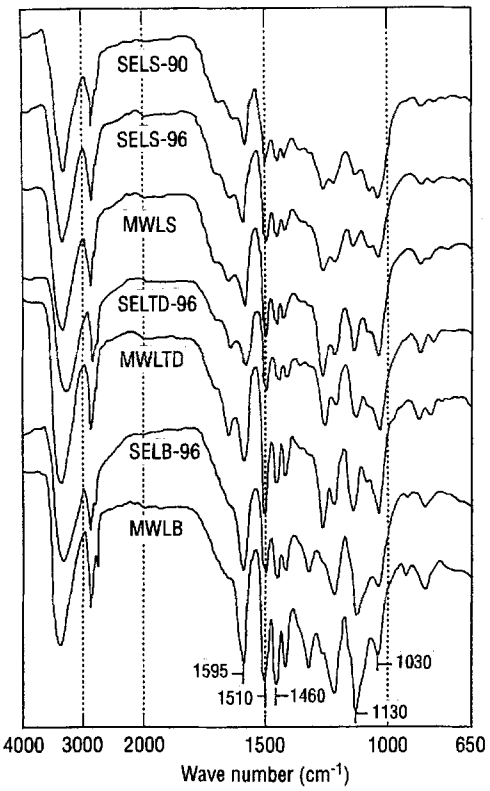

Fig. 5. IR spectra of the SELs and MWLs

of absorbance for the SELs were very similar to those of the MWLs, indicating that the SELs were chemically similar to the MWLs. The band at 1595 cm^{-1} and the next three bands from 1400 to 1510 cm^{-1} are aromatic skeletal bands. Absorbances of these bands for all dioxane extracted SELs are lower than for MWL, suggesting a lower aromaticity. These bands were also lower in SELS-90· than in SELS-96 for the same reason. The strength ratios of the bands at 1510 and 1460 cm^{-1}, as well as at 1130 and 1030 cm^{-1}, have been used as an indication of distinguishing between softwood and hardwood. For the softwood, the strength of 1510 cm^{-1} is stronger than that of 1460 cm^{-1}, and the strength of 1130 cm^{-1} is weaker than that of 1030 cm^{-1}, while the opposite applies to the hardwood (Kawamura, Higuchi 1964). This applied to both the MWLs and the SELs.

Elemental analyses

The elemental analyses of the SELs and MWLs are shown in Table 3. The element compositions of MWLs and the SELs were very similar for the same material. The SELs had a high carbon content, indicating a high aromaticity. The carbon contents of MWL seem to be a little higher than those of the SELs for all materials, suggesting that the SELs contained slightly more carbohydrates than the MWLs.

SELS-90 and SELBK-96 contained 0.32% and 0.47% of nitrogen, while the MWLs did not. This was probably due to adsorption of enzyme. After extraction with the 96% dioxane, SELBK-96 still contained some nitrogen. Because such a low nitrogen content had no obvious effect on the IR, GPC and NMR spectra, no further attempt was made to remove it.

Table 3. Elemental analyses of the SELs and MWLs

Sample	C%	H%	N%	S%
MWLS	60.85	5.36	0	0.88
SELS-90	60.76	5.43	0.32	0.86
SELS-96	60.63	5.56	0	0.85
MWLTD	60.84	6.10	0	0
SELTD-96	59.19	5.95	0	0
MWLBK	61.72	5.91	0	1.06
SELBK-96	60.05	5.80	0.47	1.05

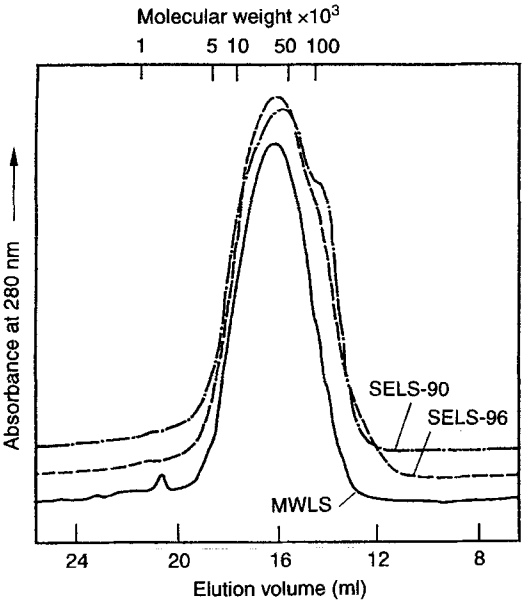

Fig. 6. GPC of the lignins isolated from SUKP

GPC charts

The GPC charts are shown in Figs. 6, 7 and 8. The SELs and MWLs showed similar molecular weight distributions and maximum peaks at almost the same molecular weights. However, in Fig. 6, SELS-90 showed a small additional shoulder at a molecular weight of 112,000, which almost disappeared in SELS-96, suggesting that the fraction in the shoulder was water soluble and difficult to dissolve in dioxane, and that it therefore could be a trace of LCC. For preparation of NMR samples, 96% dioxane should be used for purification. All SELs had a slightly wider molecular-weight distributions than did the MWLs, indicating that more of the lignin became extractable with dioxane after enzyme treatment. Small peaks appeared in the region of low-molecular weight in the MWLs, while there were hardly in the SELs. These fractions were perhaps dissolved and washed out in the SEL preparation procedure.

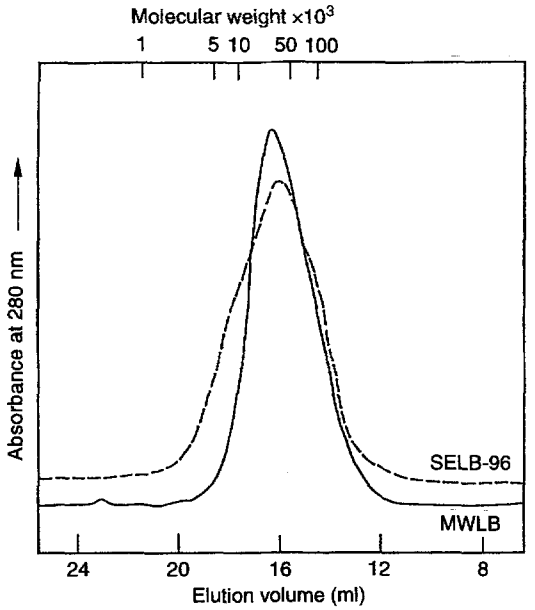

Fig. 7. GPC of the lignins isolated from BUKP

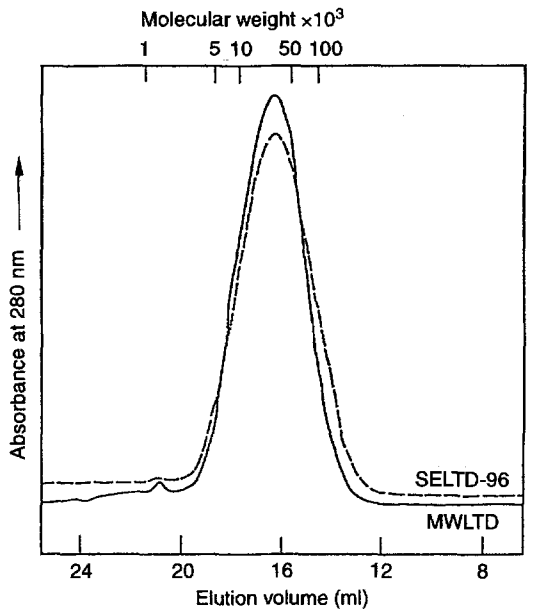

Fig. 8. GPC of the lignins isolated from TD

^{13}C NMR spectra

The ^{13}C NMR spectra of the isolated lignins are shown in Fig. 9. Proposed assignments of their signals are listed in Table 4.

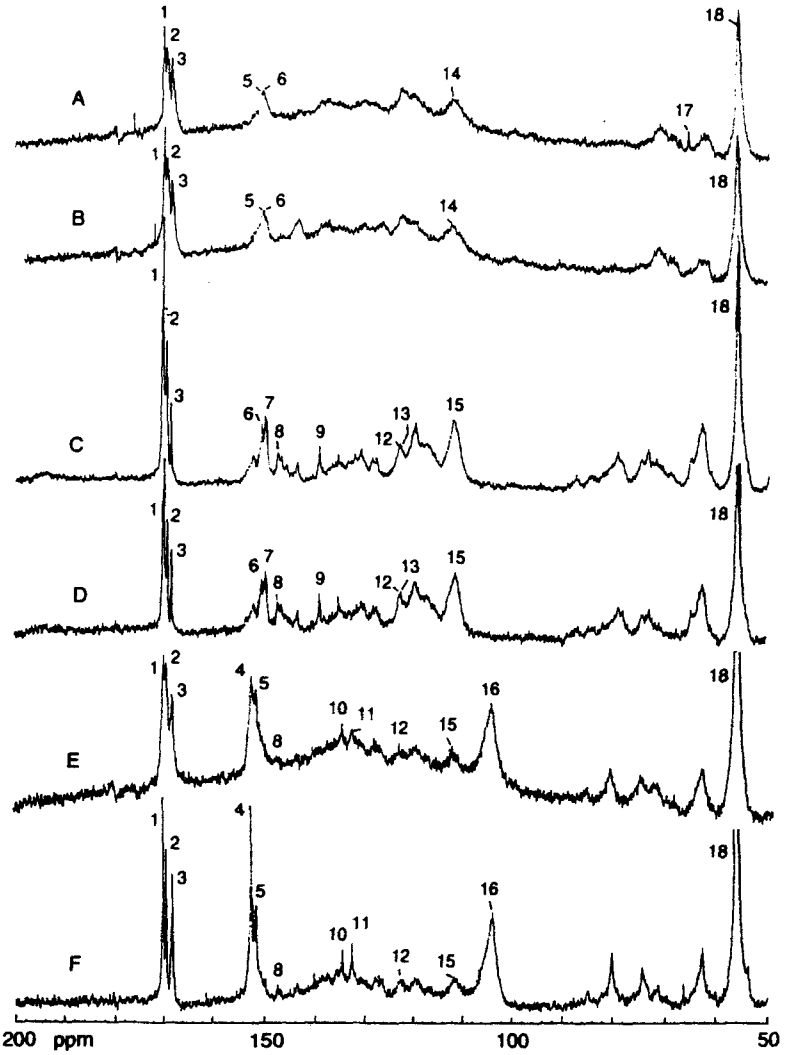

Fig. 9. ^{13}C NMR spectra of the SELs and MWLs

Signals 1, 2, 3 were assigned to the carbonyl carbons in primary, secondary and phenolic acetyl groups. Signals 4, 10 and 16 were assigned to syringyl, signals of 5, 6, 7, 8, 9, 10, 11, 12, 14 and 15 to guaiacyl, and 18 belongs to methoxyl.

The spectra of SELS-90 (A) and SELS-96 (B) were similar. The signals in the region from 100 to 60 ppm were week, indicating that the carbohydrate content was low in both A and B. Signal 17 was assigned to the non-reducing end unit of xylan. It appeared in A but not in B, suggesting that A contained a xylan that had not dissolved in 96% dioxane. The spectra of SELTD-96 (C) and MWLTD (D), as well as SELBK-96 (E) and MWLB (F) were all similar, showing that there were no obvious structure differences between SEL-96 and MWL.

Table 4. Assignment of signals in ^{13}C NMR spectra of the lignin

No.	Chemical shift (ppm)	Intensity						Assignment
		SUKP		TD		BK		
		A	B	C	D	E	F	
1	171.4-170.4a	6.49	6.75	13.1	10.9	7.59	10.2	Primary aliphatic acetyl CO
2	169.7-169.6a	5.40	5.20	6.94	5.63	7.17	7.54	Secondary aliphatic acetyl CO
3	168.4a	4.13	4.92	3.89	4.15	5.66	6.33	CO in aromatic acetyl
4	152.1a					6.68	9.85	C-3,5 in S, in B ring of 4-O-5
5	151.6-151.5a	2.85	2.10			5.97	4.84	Ga3, G4 and –OAc
6	150.6-150.4	3.35	2.82	2.96	2.76			C-3 Ge
7	149.9a			3.33	3.16			C-3 (α-OR)
8	147.5a			1.55	1.59	2.77	0.93	Ga3 (with α-acetyl);
9	138.9-138.7a			1.90	2.02			C-1,4 G–CH_2–O-; G–CH=CH–
10	134.2a					4.39	2.68	C-1 in Ge; C-4 β-β
11	132.4a					4.08	3.02	C-1 Ge
12	122.6-122.5a			2.06	2.18	3.39	1.21	C-α G–CH=CH; C-5 Ga
13	122.4-122.3a			2.04	2.20			Ha 3/5
14	112.2	2.91	2.28					C-2 in G
15	111.8-111.6a			3.31	3.23	3.29	1.32	G-2 (with α-acetyl); C-2 G–CH(OAc)–; C-2 in cinnamaldehyde
16	104.6-103.5					5.36	4.49	C-2/C-6 in S;
17	66.2	1.24						C-5, xyl non reducing end unit
18	55.6-55.8a	7.24	6.72	12.8	11.9	16.1	22.0	MeO

A =SELN-90; B =SELN-96; C =SELTD-96; D =MWLTD; E =SELBK-96; F =MWLBK; a =Acetylated; S =syringyl; G =Guaiscyl; Ge =Guaiacyl units, etherified; H =p-hydroxyphenyl units

The signals of syringly (4, 16) were strong in the lignin from BUKP, while very week in those from SUKP and TD. Obviously, the lignin in SUKP and TD consisted mainly of guaiacyl while that of BUKP contained both syringyl and guaiacyl unit. The structure of G–CH=CH– (signals 9 and 12) was only found in the TD wood.

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