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Fractional Separation of Hemicelluloses and Lignin in High Yield and Purity from Mild Ball-Milled Periploca sepium

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Abstract: A novel three-step procedure for separation of hemicelluloses and lignin with high yield and purity was proposed in this study, where wood is mildly milled and successively extracted to produce three hemicellulosic and lignin fractions representing the total hemicelluloses and lignin in wood. The sequential treatments of the mild ball-milled Periploca sepium with 80% aqueous dioxane containing 0.05 M HCl at 85°C for 4 h, DMSO at 85°C for 4 h, and 8% NaOH at 50°C for 3 h resulted in a total release of over 85% of the original hemicelluloses and 86% of the original lignin. In particular, approximately 36% of the original hemicelluloses and 50% of the original lignin were separated during the first mild acidolytic hydrolysis process after low intensity milling. The structure of the acidic dioxane-, DMSO-, and alkali-soluble hemicellulosic and lignin fractions were elucidated using wet chemical analysis, FT-IR, and solution-state ^{1}H, ^{13}C, and ^{31}P NMR techniques. Results showed that both the mild ball milling and the mild acidolysis under the conditions given did not affect the separated lignin macromolecular structure. On the other hand, the mild acidolytic hydrolysis condition did cause substantial hemicellulosic depolymerization exception for a significant cleavage the ether linkages between lignin and hemicelluloses. The

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acidic dioxane-soluble lignin fraction was structurally different from the DMSO- and alkali-soluble lignin preparations and may originate mainly from the primary wall, while the alkali-soluble lignin preparation was mainly released from the secondary wall of Periploca sepium. Furthermore, it was found that the acidic dioxane-soluble hemicelluloses mainly contained more branched and less acidic arabinoxylans, and the 8% NaOH-soluble hemicellulosic fraction H_3 was both less branched and less acidic in structure, whereas the DMSO-soluble hemicelluloses were more acidic but less branched and consisted mainly of 4-*O*-methylglucuronoarabinoxylan.

Keywords: Ball millings, hemicelluloses, mild acidolysis, native lignin, periploca sepiums, ^{1}H, ^{13}C, ^{31}P NMR

INTRODUCTION

Periploca sepium is one of the main shrubs and has been planted in the desert region of China since the 1960s to prevent wind erosion and control desertification (1). The shrubs not only have a great importance for reforestation of deserts and dry steppes, they also provide wood, fuel, fodder etc (2). Studies on the utilization of these shrubs have shown the potential of these lignocellulosic raw materials for a variety of applications such as papermaking and bio-ethanol production. These bio-based products from renewable agricultural and forestry residues are novel materials of the twenty-first century and would be of great importance to the materials world, not only as a solution to the growing environmental threat but also as a solution to the uncertainty of petroleum supply (3). In our program we are interested in the fractional separation of the cell wall components in high yield and purity and chemical modification of lignin, hemicelluloses, and cellulose isolated as novel materials for industrial utilization.

Cell walls have been demonstrated to play an essential role in the differentiation of tissues and organs. Composition and the macromolecular organization of particular cell wall components change in the course of development and the specialization of tissues (4,5). Among numerous cell wall components, cellulose, hemicelluloses, and lignin are the first, second, and third most abundant natural polymeric materials, respectively, in the plant kingdom (6). Over the last five decades, a major problem in native lignin structure determination has been in trying to separate as much of the lignin as possible while minimizing the extent of chemical modification (6). The first major advance toward separating lignin in a relatively unaltered state was made by Björkman (7,8), who developed a lignin separation procedure based on the extraction of extensive ball-milled wood with aqueous

dioxane. The yield of milled wood lignin (MWL) varies depending on the extent of milling, ranging from 25 to 50% and the structural alterations due to ball milling. In fact, increases in α-carbonyl groups via side-chain oxidation and phenolic hydroxyl content through cleavage of β-aryl ether linkages, as well as decreases in the degree of polymerization, have been reported as a result of the MWL separation procedure (6,9). In addition, based on the increase in α-aryl ether groups, it was suggested that some condensation reactions may occur during the ball-milling process (10).

Another improving method for the separation of lignin was the cellulolytic enzyme treatment of the ball-milled wood, introduced by Pew and Weyna (11). In this case, the ball-milled wood was treated with cellulolytic enzymes, and the lignin was obtained as an insoluble residue, which contained almost all of the lignin and as much as 12% of the polysaccharides. Thirteen years later, Chang et al. (12) treated milled wood with the enzymes having greater cellulolytic and hemicellulolytic activities. The insoluble residue obtained after the enzymatic hydrolysis was successively extracted with 96% and 50% aqueous dioxane. The combination of these two fractions offered higher yields of cellulolytic enzyme lignin (CEL) than MWL (12). However, the lignin fraction soluble in 50% aqueous dioxane contained twice as many polysaccharides as MWL. Recently, Wu and Argyropoulos (13) have developed a novel lignin separation procedure composed of an initial mild enzymatic hydrolysis of milled wood, followed by a mild acid hydrolysis stage. In this method, the initial cellulolytic action removes most of the polysaccharides while the mild acidolysis cleaves the remaining lignin-hemicellulosic linkages, liberating the enzymatic mild acidolysis lignin (EMAL) in high yield and purity. Nevertheless, no attempts have been made to investigate the effects of the milling and mild acidolysis conditions on the yield, purity, and structure of both lignin and hemicelluloses from Periploca sepium. To obtain both hemicelluloses and lignin in high yield and purity from Periploca sepium, in this study, the hemicellulosic and lignin preparations were isolated by sequential extractions of the mild ball-milled sample with dioxane-H_2O (80/20, v/v) containing 0.05 M HCl at 85°C for 4 h, dimethyl sulfoxide (DMSO) at 85°C for 4 h, and 8% NaOH at 50°C for 3 h, respectively. The lignin and hemicellulosic fractions separated were analysed to characterize their chemical composition, physicochemical properties, and linkages between units by using the degradation method such as acid hydrolysis and alkaline nitrobenzene oxidation, and non-degradation techniques such as ultraviolet (UV), Fourier transform infrared (FT-IR), and ^{1}H, ^{13}C, and ^{31}P magnetic resonance spectroscopy (^{1}H NMR, ^{13}C NMR and ^{31}P NMR) as well as gel permeation chromatography (GPC), and the results are comparatively reported.

EXPERIMENTAL METHODS

Materials

Periploca sepium, 5 years old, was harvested in October 2002, in the Yikezhao desert region of Inner Mongolia, China. The leaves were removed, and the stalks were chipped into small pieces. After drying at 60°C for 15 h in an oven, the chips were then ground to pass a l.0 mm size screen, and the powder was dewaxed with toluene-ethanol (2:1, v/v) in a Soxhlet for 6 h. The dewaxed sample was further dried in a cabinet oven with air circulation at 60°C for 15 h and stored in a desiccator under vacuum. The major composition (percent, w/w) was cellulose, 42.5% ± 1.6, hemicelluloses, 31.2% ± 1.1, and lignin, 20.1% ± 0.8 on a dry weight basis of the dewaxed Periploca sepium, determined in duplicate as the method for Populus euphratica described previously (14). All weights and calculations were made on an oven-dried basis. All chemicals, such as dioxane and DMSO, were purchased from Sigma Chemical Company (Beijing).

Fractional Separation

Rotary ball milling was performed in a 2 L porcelain jar in the presence of 68 porcelain balls (15 mm in diameter), which occupied 20% of the active jar volume. Ten grams of extractive-free powder was loaded into the jar, creating a porcelain ball to a sample weight ratio of 28.8. The milling process was conducted at room temperature for up to 3 days with a rotation speed of 70 rpm. The ball-milled sample was first submitted to a mild acid hydrolysis using aqueous dioxane (dioxane/water 80:20, v/v, containing 0.05 M HCl) under a nitrogen atmosphere at 85° for 4 h with a solid to liquid ratio of 1:20 (g/mL). The resulting suspension was filtered, and the residue was washed with 80% aqueous dioxane twice. The combined supernatant was neutralized with 1 M NaOH to pH 5.5. Then the supernatant was concentrated to a rotary evaporator under reduced pressure to about 50 mL, and then mixed with 3 volumes of 95% ethanol (2 h, 25°C) for separating dioxane-soluble hemicelluloses. The precipitated hemicellulosic fraction was filtered and purified by washing with 70% ethanol at room temperature and then dried in air. After evaporating ethanol and the remaining dioxane, the dioxane-soluble lignin fraction was obtained by re-precipitation at pH 1.5–2.0 adjusted with 6 M HCl from the corresponding supernatants. The separated acidic dioxane-soluble lignin fraction was purified by washing with acidified water (pH 2.0), freeze-dried overnight, and kept at 5°C until analysis.

The DMSO and 8% NaOH soluble hemicelluloses and lignin fractions were separated from the above residue by sequential treatment with DMSO at 85°C for 4 h and 8% NaOH (residue:extractant, 1:20, g/mL) at 50°C for 3 h, respectively. Note that the hemicelluloses and lignin separated during the first treatment with 80% aqueous dioxane containing 0.05 M HCl were labelled as acidic dioxane-soluble hemicellulosic fraction H_1 and acidic dioxane-soluble lignin fraction L_1, respectively. Similarly, the hemicelluloses and lignin separated during the sequential extraction of the 80% dioxane-treated residue with DMSO were named as DMSO-soluble hemicellulosic fraction H_2 and DMSO-soluble lignin fraction L_2, and the hemicelluloses and lignin dissolved during the treatment of the DMSO-extracted residue with 8% NaOH were coded as alkali-soluble hemicellulosic fraction H_3 and alkali-soluble lignin fraction L_3, respectively. A scheme for sequential separation of hemicelluloses and lignin from Periploca sepium is shown in Fig. 1. All of the treatments were performed in duplicate, and the yields of hemicelluloses and lignin are given on a dry weight basis related to the starting material (Table 1). The standard errors or deviations of the yield were observed to be lower than 5.6%.

Chemical Analysis

The neutral sugar composition of the three hemicellulosic fractions and the hemicellulosic moieties associated with the three lignin preparations was determined by hydrolysis with 6% H_2SO_4 for 2.5 h at 100°C. The liberated neutral sugars were analysed by gas chromatography (GC) of their alditol acetates (15). Total uronic acids were determined colormetrically by the method of Blumenkrantz and Asboe-Hansen (16). The error for analysis of the sugars and total uronic acids in duplicate was observed to be $<0.08\%$ and $<0.13\%$, respectively. The monomeric composition of the non-condensed monomeric units of the lignins in the three lignin fractions and in three hemicellulosic preparations was characterized by nitrobenzene oxidation and analysis of the resulting aromatic aldehydes and acids by high-performance liquid chromatography (HPLC) has been reported previously (17). All nitrobenzene oxidation results represent the mean of triplicate samples and each oxidation mixture was chromatographed twice. The standard errors or deviations were observed to be 6.5–13.9%. Methods for determining Klason lignin content in hemicellulosic fractions and the residue (cellulose), and measuring molecular weights of both hemicelluloses and lignin have been described in a previous paper (18). The analyses were run at least twice. The average relative error was 2.8–5.6% for molecular average weights and 3.5% for Klason lignin content.

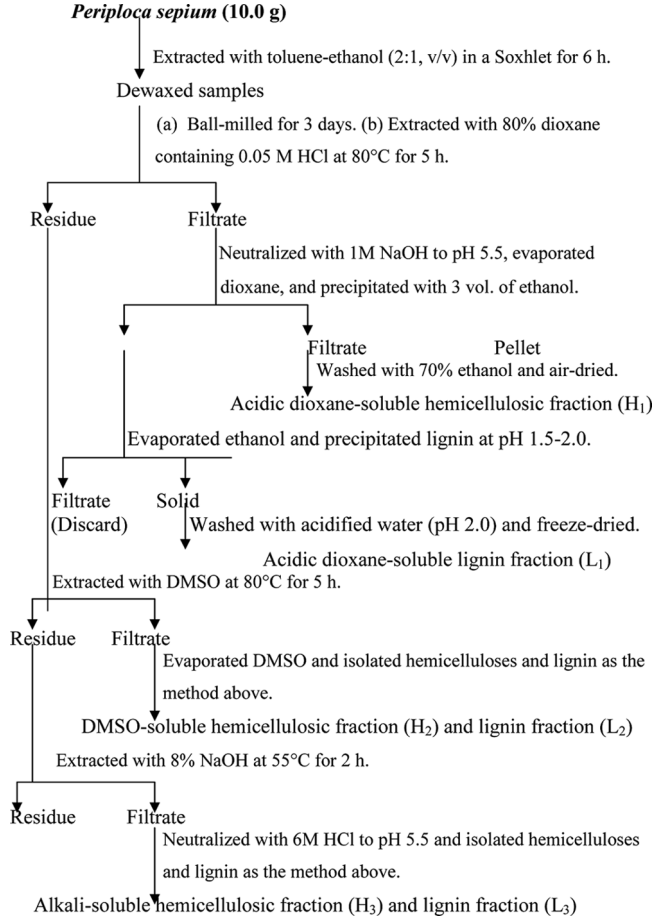

Figure 1. Scheme for separation of original lignin and hemicelluloses from Periploca sepium.

The FT-IR spectra of the hemicellulosic and lignin fractions were recorded from a KBr disc containing 1% finely ground samples on a Nicolet 510 FT-IR spectrophotometer in the range 4000–400 cm^{-1}. The solution-state ^1H and ^{13}C NMR spectra of hemicelluloses and lignin were obtained on a Bruker DRX-400 spectrometer operating in the FT mode at 74.5 MHz. The lignin sample (25 mg for ^1H, 250 mg for ^{13}C) was dissolved in 1 mL DMSO-d_6 (99.8% D), and the hemicellulosic sample (15 mg for ^1H, 100 mg for ^{13}C) was dissolved in 1 mL D_2O. The spectra were recorded at 25°C after 30 000 scans. A 60° pulse flipping angle, a

Table 1. The yield of hemicelluloses and lignin (% dry matter) obtained by sequential extractions of ball-milled Periploca sepium with dioxane-H_2O (80/20, v/v) containing 0.05 M HCl at 80°C for 5 h, DMSO at 80°C for 5 h, and 8% NaOH at 55°C for 2 h

Hemicellulosic and lignin fractions	Yield (% dry weight)
Acidic dioxane-soluble hemicelluloses (H_1)[a]	5.1
DMSO-soluble hemicelluloses (H_2)[b]	6.5
Alkali-soluble hemicelluloses (H_3)[c]	9.0
Hemicelluloses hydrolysed by acidic dioxane[d] (Oligosaccharides)	6.0
Total solubilized hemicelluloses	26.6
Acidic dioxane-soluble lignin (L_1)[a]	10.1
DMSO-soluble lignin (L_2)[b]	3.1
Alkali-soluble lignin (L_3)[c]	4.2
Total solubilized lignin	17.4
Residue	47.6

[a]Represent the hemicellulosic and lignin fractions separated during the extraction of ball-milled Periploca sepium with dioxane-H_2O (80/20, v/v) containing 0.05 M HCl at 80°C for 5 h.

[b]Represent the hemicellulosic and lignin fractions released during the sequential extraction with DMSO at 80°C for 5 h from the acidic dioxane-treated residue.

[c]Represent the hemicellulosic and lignin fractions separated by extraction with 8% NaOH at 55°C for 2 h from the DMSO-treated residue.

[d]Represents the hemicellulosic fraction of the degradation products (oligosaccharides) during the acidolysis which was still solubilized in the supernatant after precipitation of the hemicelluloses in 3 volumes of ethanol, calculated by total hemicelluloses (31.2%)–(hemicellulosic fraction released during the acidic dioxane extraction obtained by precipitation in ethanol + hemicelluloses solubilized during the sequential extraction with DMSO and 8% NaOH + hemicelluloses in the residue).

3.9 μs pulse width, a 0.85 s acquisition time, and 1.2 s relaxation delay time were used.

Quantitative ^{31}P-NMR spectra of the lignin fractions were recorded using published procedures (19). Approximately 40 mg dry lignin was placed into a 5 mm (O.D.) NMR tube and dissolved in 800 μL solvent mixture composed of pyridine-d_5 and deuterated chloroform (1.6:1,v/v). Cholesterol (100 μL, 40 mg mL^{-1}) and chromium(III) acetylacetonate (100 μL, 40 mg mL^{-1}) were added as an internal standard and relaxation reagent, respectively. Finally, 100 μL of phosphitylating reagent II (2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane) was

added, and the tube was closed and shaken for a few minutes to ensure proper mixing during the reaction. The spectra were acquired using a Bruker DRX-400 spectrometer equipped with a Quad probe dedicated to ^{31}P, ^{13}C, and ^{1}H acquisition. Approximately 1000 transients were acquired to ensure a high signal/noise ratio. The spectra were integrated and the precise amount of different functional groups was calculated as described by Argyropoulos (20). That is, the 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.

RESULTS AND DISCUSSION

Fractional Yield

To improve yields while minimizing damage on the lignin structure, the extent of mechanical action during milling must be reduced. Therefore, a period of ball milling time of 3 days, a considerably milder rotary ball-milling condition, was used in this study instead of traditional ball milling for 6 days. In addition, because the linkages between lignin and hemicelluloses are extensively cleaved during the mild acid hydrolysis, the lignin separated with the acidic dioxane-water solution is of a very high purity and less-modified structure. As the data shown in Table 1, treatment of the ball-milled Periploca sepium with 80% aqueous dioxane containing 0.05 M HCl at 85°C for 4 h separated 35.6% of the original hemicelluloses and 50.2% of the original lignin (w/w, based on the amount of Klason lignin of the starting wood and the isolated lignin). These data revealed that the concerted effect of ball milling action and mild acid hydrolysis offered significant yield improvements over the traditional procedure for separating lignin from wood without mild acid hydrolysis. However, it should be noted that the treatment with acidic dioxane under the condition given also cleaved some amounts of glycosidic linkages between the sugar units in macromolecule of hemicelluloses as shown by a yield of 6.0% oligosaccharides, which were soluble in the aqueous ethanol solution and resulted from the significant degradation of hemicelluloses in the acidic condition. Besides the oligosaccharides, minor amounts of lignin also became soluble during the process of precipitation of lignin at pH 1.5. However, no further attempts were made to recover such soluble lignin fractions.

When hemicelluloses and lignin are separated by alkali, any ester bonds present are simultaneously saponified. Therefore, to separate hemicelluloses and lignin in their native state, we isolated the acetylated

hemicelluloses and lignins with esterified *p*-coumaric acid using DMSO as a solvent before sequential application of alkaline solution. As expected, the sequential treatments of the acidic dioxane-treated Periploca sepium with DMSO at 85°C for 4 h and 8% NaOH at 50°C for 3 h resulted in a separation of 20.8 and 28.8% of the original hemicelluloses and 15.4 and 20.9% of the original lignin, respectively. Taking together, the sequential three-step treatment of the ball-milled Periploca sepium separated 85.3% of the original hemicelluloses and 86.6% of the original lignin. Apparently, the acidic dioxane-soluble lignin was the major fraction, comprising 58.0% of the total separated lignins. Similarly, the acidic dioxane hydrolysis also released and/or degraded 41.7% of the total dissolved hemicelluloses. This result indicated that the acidic dioxane treatment under the condition given significantly cleaved the ether linkages between lignin and hemicelluloses from the cell walls of ball-milled Periploca sepium.

Sugar Composition

Table 2 lists the content of neutral sugars and uronic acids in three hemicellulosic preparations and three lignin fractions as well as in the residue (cellulosic fraction). Evidently, xylose (33.56%) and arabinose (30.12%) are the major sugar components of the acidic dioxane-soluble hemicellulosic fraction H_1 compared to the dominance of xylose (48.07–48.61%) in the DMSO- and 8% NaOH-soluble hemicellulosic preparations H_2 and H_3. Interestingly, with the sequential separation stages from the 80%

Table 2. The content of neutral sugars and uronic acids (% dry sample, w/w) in hemicellulosic, lognin, and residue fractions

	Fractions[a]						
Sugars	H_1	H_2	H_3	L_1	L_2	L_3	Residue
Rhamnose	1.23	0.88	0.60	0.33	0.18	ND[b]	0.29
Arabinose	30.12	21.20	12.91	1.16	1.09	0.56	3.95
Xylose	33.56	48.07	48.61	1.32	1.21	0.78	7.01
Mannose	2.56	1.12	0.69	0.26	0.13	0.06	0.91
Galactose	1.68	0.70	0.77	ND[b]	0.10	0.05	1.10
Glucose	4.65	9.34	24.41	0.38	0.26	0.10	70.74
Uronic acids	4.00	13.63	5.21	ND	ND	ND	ND
Total	77.80	94.94	93.20	3.45	2.97	1.55	84.00

[a]Corresponding to the fractions in Table 1.
[b]ND, not detectable.

acidic dioxane, to DMSO, and to 8% NaOH, the content of xylose increased from 33.56% in H_1, to 48.07% in H_2, and to 48.61% in H_3. In contrast, the content of arabinose decreased from 30.12% in H_1, to 21.20% in H_2, and to 12.91% in H_3. Similar decreasing trend was also observed for rhamnose, mannose, and galactose. The data in Table 2 also showed that the content of uronic acids (probably glucuronic acid and/or 4-*O*-methyl-glucoronic acid) was much higher in H_2 fraction (13.63%) separated with DMSO compared to the hemicellulosic fractions H_1 (4.00%) and H_3 (5.21%). Because most of the xylose residues probably originate from the backbone of xylan, it can be concluded that the acidic dioxane-soluble hemicelluloses mainly contained more branched but less acidic arabinoxylans, and the 8% NaOH-soluble hemicellulosic fraction H_3 was both less branched and less acidic in structure. A much higher content of uronic acids in H_2 fraction implied that the DMSO-soluble hemicelluloses consisted mainly of 4-*O*-methylglucuronoarabinoxylan, which need to be further investigated, since based on the sugar composition alone it is difficult to draw conclusions on the structural patterns of the hemicelluloses. Furthermore, a large amount of glucose found in the hydrolysate of 8% NaOH-soluble hemicellulosic fraction H_3 probably arose from the degradation of cellulose under the alkaline condition used, since the glucose liberated was partially attributed to the cellulose fraction.

Analysis of the residue fraction showed that the three sequential treatments were not able to separate all hemicellulosic substances. About 52.4% of Periploca sepium was extracted leaving the residue, which consisted of about 70.7% cellulose and about 13.3% hemicelluloses. The hemicelluloses, which remain in the residue, revealed again that the hemicellulosic polymers are tightly bound to the cell wall component, cellulose.

During the ball-milling process, the amount of ester groups formed by polyoses and lignin decreased due to extensive mechanical treatments performed. Besides ester groups, other type of linkages such as ether bonds between lignin and hemicelluloses was also cleaved, assisting the separation of more pure lignin fractions (21). As the data shown in Table 2, the three lignin fractions contained only small amounts of associated hemicelluloses, ranging between 1.55 and 3.45%. The content of hemicelluloses in the 8% NaOH-soluble lignin fraction L_3, only 1.55% by weight, was remarkably lower than that of the acidic dioxane-soluble lignin fraction L_1 and DMSO-soluble lignin fraction L_2. This may be explained by the alkaline cleavage of linkages between lignin and hemicelluloses during the third stage treatment by alkali. Nevertheless, the hemicellulosic content of the acidic dioxane-soluble lignin fraction L_1 was still slightly higher when compared to that of alkali-soluble lignin fraction L_3, probably implying that the mild acidolysis performed did not cleave all

lignin-hemicellulose linkages under the condition given. Xylose (0.78–1.32%) and arabinose (0.56–1.16%) were found to be the major sugar components. Glucose, mannose, and rhamnose were identified in minor quantities, and uronic acids were absent in all the three lignin fractions. On the other hand, regardless of the sequential separation procedures applied, the occurrence of small amounts of associated hemicelluloses in the three lignin fractions verified that the hydroxyl groups of the sugar residues in the hemicelluloses may be tightly linked to the side chain of the lignin molecules, probably at the α position of the side chain of phenylpropane units by ether bonds.

Content of Phenolic Acids and Aldehydes

Table 3 lists the results concerning the analysis of phenolic acids and aldehydes from the three lignin fractions and the lignins associated in the hemicellulosic and residual preparations, obtained by alkaline nitrobenzene oxidation at 170°C for 2.5 h. Obviously, the predominant oxidation products were found to be vanillin and syringaldehyde, which together represented 80.6, 79.0, and 81.7% of the total phenolic compounds in L_1, L_2, and L_3 fractions, and resulted from the degradation of non-condensed guaiacyl (G) and syringyl (S) units, respectively (22). The presence of fewer *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid was indicative of non-condensed *p*-hydroxyphenyl (H) unit, indicating the incorporation of *p*-hydroxycinnamoyl alcohol in the lignin of Periploca sepium. In addition, the total yield from alkaline nitrobenzene oxidation of L_1, L_2, and L_3 fractions was practically identical, indicating that the acidic dioxane-, DMSO-, and alkali-soluble lignin fractions were representative, structurally, of the majority of lignin in Periploca sepium. However, it is worth noting that a slightly lowest yield of nitrobenzene oxidation of the lignin fraction L_1 indicated the most condensed lignin fraction separated during the treatment with acidic dioxane. Similar results have been reported by Ikeda and co-workers (6) in the studies on the effect of ball milling on lignin structure. The authors revealed that ball milling reduces the degree of polymerization, creating new free-phenolic hydroxyl groups through cleavage of β-aryl ether linkages and increasing α-carbonyl groups via side-chain oxidation. Furthermore, they also indicated that some condensation reactions may occur during the ball milling process, and ball milling primarily affects the side-chain structure of the C_9 phenyl-propane units in lignin.

It is well known that lignin in the primary wall (P), lignin in the secondary wall (S2), and lignin in the compound middle lamella (CML) have different chemical structures. (10,23,24) Lignin in the CML has lower

Table 3. The yield (% dry sample, w/w) of phenolic acids and aldehydes from alkaline nitrobenzene oxidation of lignin preparations, bound lignins in the hemicellulsic and residual fractions

	Fractions[a]						
Phenolic acids and aldehydes	H_1	H_2	H_3	L_1	L_2	L_3	Residue
p-Hydroxybenzoic acid	0.36	0.30	0.14	0.45	0.56	0.68	0.06
p-Hydroxybenzaldehyde	0.16	0.08	0.03	0.31	0.48	0.46	0.09
Vanillic acid	0.12	0.10	0.07	0.41	0.42	0.38	0.10
Vanillin	0.61	0.26	0.12	6.48	6.53	5.66	0.38
Syringic acid	0.10	0.05	0.03	0.28	0.41	0.42	0.07
Syringaldehyde	0.46	0.21	0.10	5.63	6.68	9.23	0.35
Acetovanillone	0.20	0.07	0.02	0.79	0.86	0.76	ND[b]
Acetosyringone	0.08	0.06	0.03	0.68	0.79	0.63	ND
p-Coumaric acid	0.03	0.06	ND	0.11	0.28	ND	ND
Ferulic acid	ND	0.09	ND	0.13	0.22	ND	ND
Total	2.12	1.28	0.54	15.27	17.23	18.22	1.05
Content of Klason lignin	15.92	5.13	3.21				3.61
Molar ratio (***g:s:h***)[c]				8.4:6.2:1	6.3:5.4:1	4.7:6.4:1	

[a]Corresponding to the fractions in Table 1.

[b]ND, not detectable.

[c]***g*** represents the sum of total moles of vanillin, vanillic acid, and acetovanillin; ***s*** represents the sum of total moles of syringaldehyde, syringic acid, and acetosyringone; and ***h*** represents the sum of total moles of *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid.

methoxyl content, and is richer in H units than lignin in the S2 (24). Because a large amount of guaiacyl lignin is formed in the early stage of xylem differentiation than in the later stages, lignin in the P enriches in G units (25). Based on an extensive study on the heterogeneity in the formation of lignin in different xylem of Ginkgo biloba, Fukushima and Terashima (26) reported that lignin in CML has a higher degree of condensation and contain much more H units than lignin in the S2. Scott et al. (27) using UV microscopy discovered that the majority of lignin in spruce (72% in early-wood and 81% in latewood) is in the S2. As shown in Table 3, the relative molar ratios of ***g*** (the relatively total moles of _vanillin, vanillic acid, and acetovanillin) to ***s*** (the relatively total moles of syringaldehyde, syringic acid, and acetosyringone) and to ***h*** (the relatively total moles of *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid) decreased from 8.4:6.2:1 in acidic dioxane-soluble lignin fraction L_1, to 6.3:5.4:1 in DMSO-soluble lignin fraction L_2, and to 4.7:6.4:1 in alkali-soluble lignin fraction L_3. Taking into consideration these earlier findings and our current results, it can be deduced that the lignin fraction L_1 likely originated mainly from the P because of its higher monomeric ratio of ***gls***. On the other hand, the alkali-soluble lignin fraction L_3 was separated mainly from the S2, which is rich in S units. Thus, three lignin fractions were isolated, representing the total lignin in Periploca sepium: L_2 is indicative of the lignins in both P and S2, L_3 is largely representative of lignin in the S2, whereas L_1 represents lignin in the P, and the separating procedure developed in this study achieved over 85% extractable lignin yield for a given ball milling period of 72 h.

It is commonly assumed that lignin is tightly linked to polysaccharides in the cell walls of plants by various linkage types, such as ether linkage of the hydroxyl group at the α-position of the lignin side chain with alcoholic hydroxyl of sugar residue (28). To verify the associated lignin in the three hemicellulosic fractions and in the residue of cellulose, all the hemicellulosic and cellulosic preparations were also oxidized by alkaline nitrobenzene, and the degraded phenolic monomers from the bound lignins are given in Table 3. Evidently, as compared to the lignin content in acidic dioxane-soluble hemicellulosic fraction H_1 (15.92%) and DMSO-soluble hemicellulosic fraction H_2 (5.13%), a much lower content of associated lignin in the alkali-soluble hemicellulosic fraction H_3 (3.21%) and the residue of cellulose (3.61%) demonstrated that the α-benzyl ether linkages between lignin and hemicelluloses were substantially cleaved during the final stage treatment with 8% NaOH under the condition given. On the other hand, a noticeable amount of bound lignin in the acidic dioxane-soluble hemicellulosic fraction H_1 implied that lignin in P has more linkages with hemicelluloses and is cleaved sufficiently only after ball milling for a longer period, such as 6 days, in a rotary ball mill.

Molar Mass

Table 4 gives the weight-average (M_w) and number-average (M_n) molecular weights and polydispersity (M_w/M_n) of the hemicellulosic and lignin fractions. Clearly, the molar mass of the hemicelluloses followed the order: alkali-soluble > DMSO-soluble > acidolysis hemicelluloses. The average molar mass (related to polysaccharides) of the DMSO- and alkali-soluble hemicellulosic fractions (H_2, M_w, 20520 g mol^{-1}; H_3, M_w, 22680 g mol^{-1}) was nearly twice as high as that of acidic dioxane-soluble hemicellulosic preparation (H_1, M_w, 13410 g mol^{-1}). The low molar mass of the hemicellulosic fraction H_1 obtained by acidolysis was probably due to the consequence of the cleavage of glycosidic ether linkages between sugar units in the macromolecular structure of hemicelluloses during the acidolysis step. Interestingly, although the acidic dioxane-soluble hemicelluloses contained noticeable amounts of lignin chains linked in hemicelluloses, these chains increased the apparent molar mass of the hemicelluloses when measured using GPC on the one hand. Furthermore, as the separation was sequentially processed, the accessibility to higher linear molecular weight hemicellulosic fragments, e.g. xylans, was also increased. On the other hand, the hemicelluloses themselves were also degraded by cleavage of the glycosidic bonds between the sugar units, and the partial cleavage of these lignin-hemicellulosic linkages during the acidolysis may decrease the apparent molar mass of the hemicelluloses.

The data of Table 4 show that for the case of three lignin samples, the apparent molecular weight average (M_w) were nearly constant, ranging between 4990 and 5360 g mol^{-1}. It is clear that the concerted effect of mild ball milling followed by mild acidolytic hydrolysis, DMSO extraction, and alkali treatment afforded the separation of significant amounts of lignin without any apparent damage on the lignin macromolecular structure. Overall, these data suggested that the combination of mild ball milling and mild acidolysis as well as alkali treatment allowed the

Table 4. Weight-average (M_w) and number-average (M_n) molecular weights and polydispersity (M_w/M_n) of the hemicellulosic and lignin fractions

	Fractions[a]					
	H_1	H_2	H_3	L_1	L_2	L_3
M_w	13410	20520	22680	4990	5120	5360
M_n	3240	5230	6260	1950	1920	2060
M_w/M_n	4.14	3.92	3.62	2.56	2.67	2.60

[a]Corresponding to the fractions in Table 1.

isolation of lignin fractions that were not accessible by neither of the alternative lignin separation procedures.

FT-IR Spectra

The analysis of FT-IR data showed that all the three hemicellulosic fractions clearly illustrated the typical signal pattern for hemicellulosic moiety, and had a specific maximum band at 1024 or 1048 cm^{-1} shown in Fig. 2. This band is dominated by the glycosidic bond stretching (C–O–C). The attribution of another main absorption is related to OH stretching at 3428 cm^{-1}. A small sharp band at 895 cm^{-1} is characteristic of β-glycosidic linkages between the sugar units (29). In the carbonyl stretching region, in addition to a strong signal due to the absorbed water (1628 or 1640 cm^{-1}), an intensive band at 1745 cm^{-1} in the spectra of acidic dioxane- and DMSO-soluble hemicellulosic fractions H_1 (spectrum not shown) and H_2, is originated from the acetyl and uronic ester groups of the hemicelluloses, while the absence of this signal in the spectrum of alkali-soluble hemicellulosic fraction H_3 stated that the alkaline treatment under the condition used completely cleaved this ester bond from the hemicelluloses. In addition, the occurrence of a noticeable signal at 1515 cm^{-1} in H_1, a small of this band in H_2 or a shoulder around

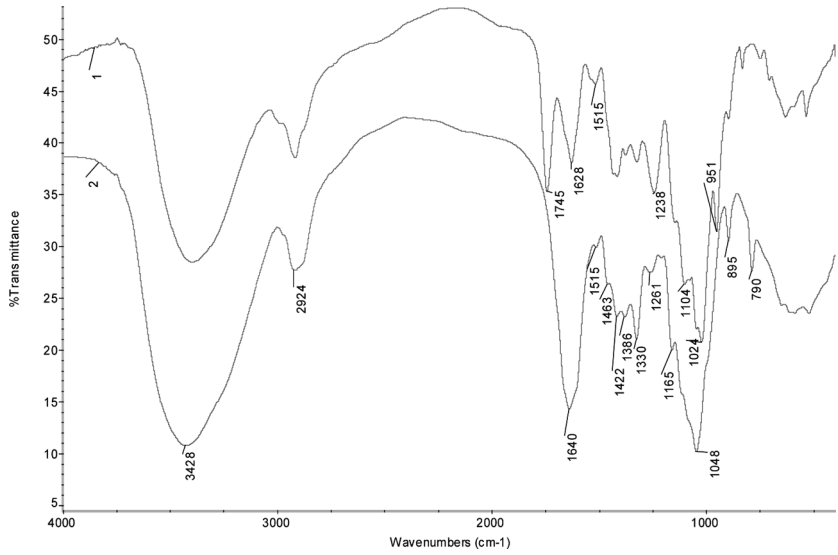

Figure 2. FT-IR spectra of hemicellulosic fractions H_2 (spectrum 1) and H_3 (spectrum 2).

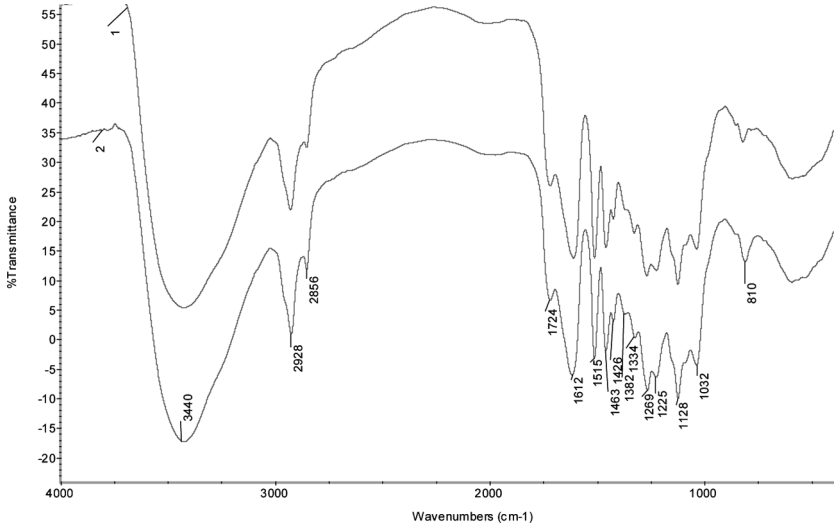

Figure 3. FT-IR spectra of lignin fractions L_1 (spectrum 1) and L_2 (spectrum 2).

$1515\,cm^{-1}$ in H_3 is undoubtedly due to the presence of small amounts of associated lignin in the hemicellulosic fractions, and corresponded to the values of Klason lignin bound to the hemicelluloses.

Figure 3 illustrates the FT-IR spectra of the acidic dioxane-soluble lignin fraction L_1 and DMSO-soluble lignin fraction L_2. As can be seen from the diagram, the relative intensities of the bands for aromatic skeleton vibrations, assigned at 1612, 1515, 1463, and $1426\,cm^{-1}$ are rather similar, indicating a similar structure of the lignin fractions. This typical spectra of lignin also revealed that the "core" of the lignin structure did not change significantly during the mild ball milling and acidolytic hydrolysis processes. A rather weak band at $1724\,cm^{-1}$, assigned to the unconjujated ketone and carboxyl group stretching, indicated that both the ball milling and acidolytic hydrolysis under the conditions given did not result in a significant oxidation of the lignin molecules. In Fig. 3, the bands at 1334 and $1128\,cm^{-1}$ are due to the syringyl units in lignin molecules, while the absorption at 1269, 1228, and $1032\,cm^{-1}$ belong to guaiacyl units in the lignin molecules.

^{1}H, ^{13}C, and ^{31}P NMR Spectra

The ^{1}H-NMR spectrum of the hemicellulosic fraction H_2, isolated with DMSO, is given in Fig. 4. Remarkably, the spectrum shows the typical

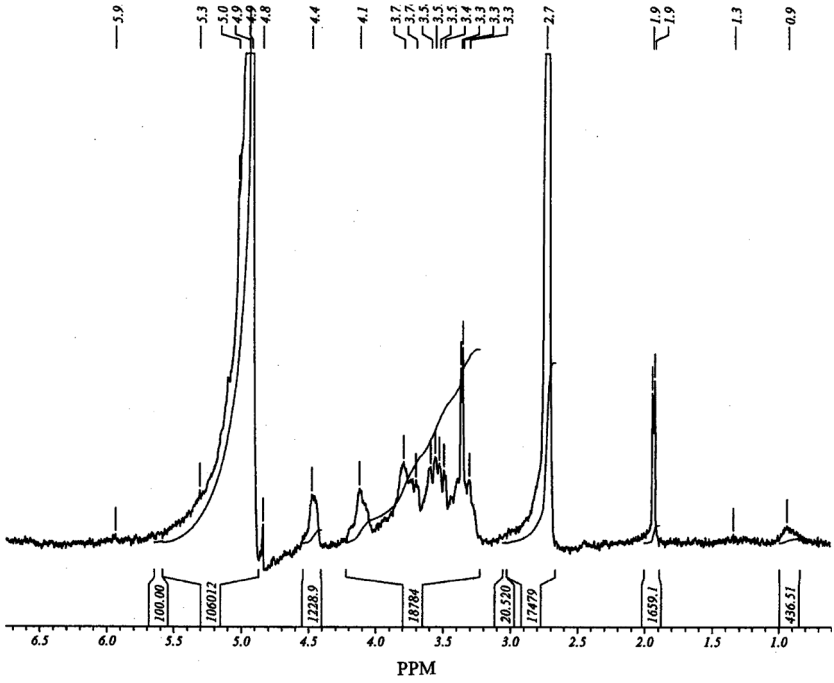

Figure 4. ^{1}H-NMR spectrum of hemicellulosic fraction H_2.

signal pattern expected for a hemicellulosic moiety. The chemical shifts of 2.7–4.4 ppm are attributed to the equatorial proton and other protons of anhydroxylose units of hemicelluloses. The methyl protons of few amounts of the acetyl group and 4-*O*-methyl-D-glucuronic acid produce peaks at 1.9 and 0.9 ppm, respectively. A shoulder at 5.3 ppm is originated from protons of terminal α-D-arabinofuranosyl residues. A weak signal at 4.8 ppm arises from protons of α-D-galactose. A strong signal at 4.9 ppm corresponds to the residual solvent (HDO) (30). Resonance signals originating from phenolic compounds (5.9 ppm) were visible in the spectrum, indicating the contaminated lignins (31).

In order to obtain deeper insight into the branched structure of the hemicelluloses, a ^{13}C NMR spectrum of the DMSO-soluble hemicellulosic fraction H_2 was recorded, and is shown in Fig. 5. The main 1,4-linked β-D-Xyl*p* units were obviously characterized by five strong signals at 102.4, 78.0, 75.9 or 75.0, 73.4 or 72.9, and 61.5 or 61.2 ppm, which are originated from C-1, C-4, C-3, C-2, and C-5 of the β-D-Xyl*p* units, respectively. The signals at 82.9, 78.1 (data not shown), and 57.3 ppm are attributed to C-4, C-2, and C-5 of α-L-Ara$_f$ residues, respectively.

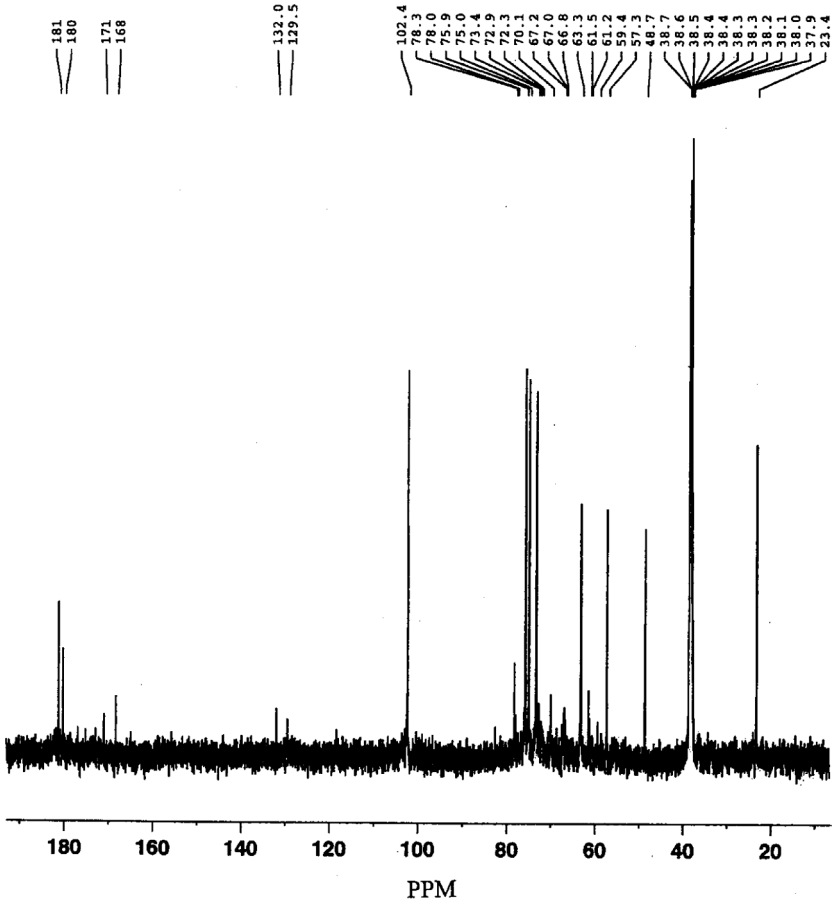

Figure 5. ^{13}C-NMR spectrum of hemicellulosic fraction H_2.

Small amounts of glucuronic acid (Glc*p*A) and 4-*O*-methyl-glucuronic acid were also present as identified from the spectrum with signals at 78.3 (C-4 in Glc*p*A), 67.0 or 66.8 (C-5 in Glc*p*A), and 59.4 ppm (4-*O*-methoxyl group of Glc*p*A). The carbonyl resonances from uronic acids, and acetyl and uronic ester groups may contribute to the signal at 171 ppm. An intense signal at 23.4 ppm is most likely due to CH_3 from acetyl groups in hemicelluloses. The presence of fragments of lignin is also verified by signals at 132.0 ppm (C-1, S non etherified; C-1, G non etherified) and 38.7–37.9 ppm (β- and α-methylene groups in *n*-propyl side chains), indicating the associated lignins. The signal at 168.0 (C-γ in etherified ferulic acid, FE) and 129.5 ppm (C-2/C-6 in esterified

p-coumaric acid, PC) are originated from etherified ferulic acid and esterified *p*-coumaric acid, respectively. It is therefore very likely that the major component of the hemicellulosic fraction H_2 was an acidic xylan and it was contaminated with small amounts of lignin and hydroxycinnamic acids. This finding is of significance in the understanding of the structure of the cell wall from Periploca sepiumin which 4-*O*-methylglucuronoarabinoxylan is a major component.

The ^{1}H NMR spectrum of lignin fraction L_1, isolated with 80% aqueous dioxane containing 0.05 M HCl from the mild ball-milled Periploca sepium is shown in Fig. 6. The signals at 6.9 and 6.7 ppm represent the aromatic protons in guaiacyl and syringyl units, respectively (32). The signals at 5.3 ppm is attributed to the H_α in condensed structures (β-5), and the signal at 4.8 ppm is originated from H_α and H_β of β-O-4 structures (33). Methoxyl protons ($-OCH_3$) give a strong signal at 3.4 ppm. An intense signal at 3.7 ppm arises from the proton of xylan residues. A sharp signal at 2.5 ppm is due to the protons in DMSO. The peaks between 2.1 and 1.9 ppm correspond to the methyl or methlene protons adjacent to double bonds or carbonyl groups. The signals between 1.4 and 0.8 ppm represent the protons in aliphatic groups of lignin side chains (34).

Figure 7 shows the ^{13}C NMR spectrum of the acidic dioxane-soluble lignin fraction L_1. Obviously, guaiacyl (G) residues were identified by

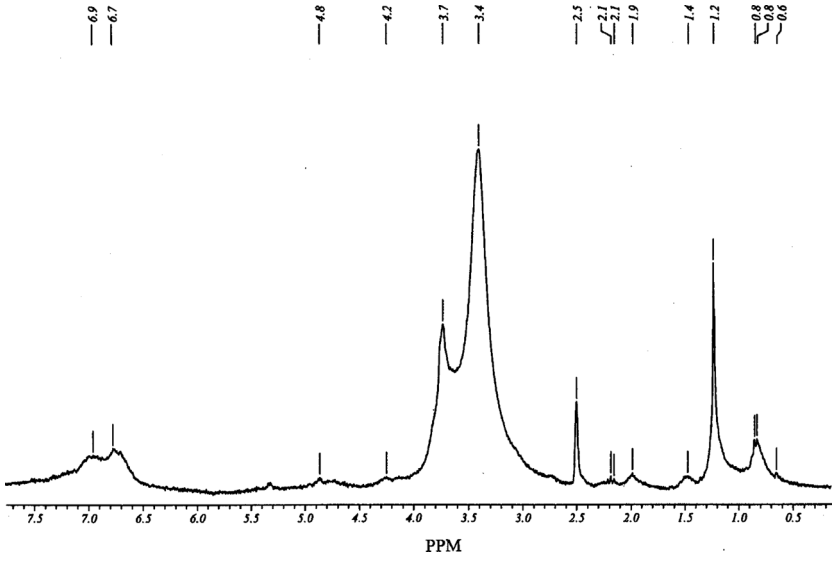

Figure 6. ^{1}H-NMR spectrum of acidic dioxane-soluble lignin fractions L_1.

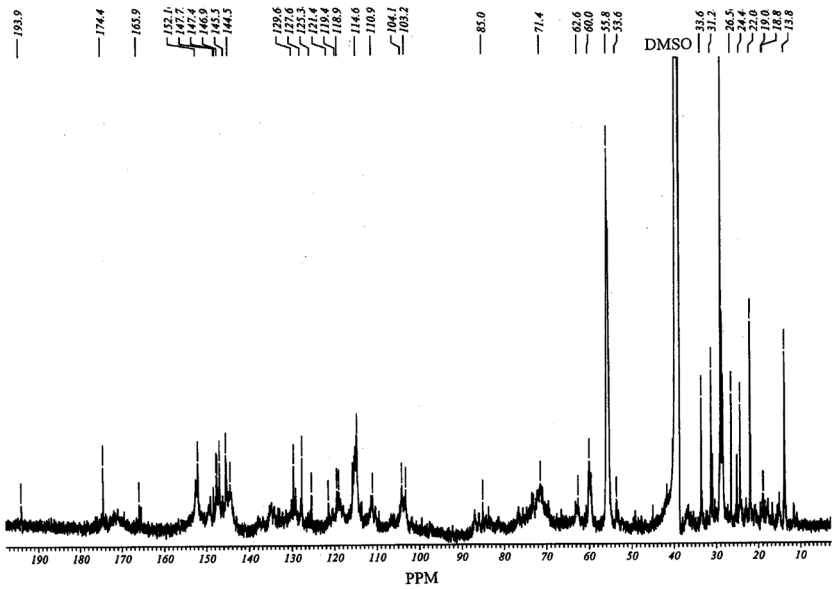

Figure 7. ^{13}C-NMR spectrum of acidic dioxane-soluble lignin fractions L_1.

signals at 147.7, 147.4, 146.9 (C-4, G etherified), 145.5 (C-4, G nonetherified), 135.0 (data not shown, C-1, G etherified), 119.4, 118.9 (C-6, G), 114.6 (C-5, G), and 110.9 ppm (C-2, G). The syringyl (S) residues were verified by signals at 152.1 (C-3/C-5, S), 147.7, 147.4, 146.9 (C-3/C-5, S nonetherified), and 135.0 ppm (data not shown, C-1, S etherified). The *p*-hydroxyphenyl (H) residues were observed at 127.6 ppm (C-2/C-6, H). These signals indicated that the lignin fraction could be justified as SGH lignin, corresponding to the results obtained by nitrobenzene oxidation. The signals at 165.9 (C-γ, PC ester), 144.5 (C-α, PC ester), and 129.6 ppm (C-2/C-6, PC ester) are attributed to the esterified *p*-coumaric acid. Etherified ferulic acid gives signals at 121.4 (C-6, FE ether) and 116.5 ppm (C-β, FE ether). These observations implied that *p*-coumaric acid is associated to lignin by ester bonds, while ferulic acid is linked to lignin by ether bonds (35).

In the region of carboxyl structure, the signal at 174.4 ppm is likely due to unconjugated aliphatic carboxyl groups, whereas the signal at 165.9 ppm is possibly conjugated carboxyl structures. In the carbonyl structure region, a signal at 193.9 ppm arises from cinnamaldehyde (Ar−CH=CH−CHO) or conjugated carbonyl group (α-C=O in β-*O*-4). The resonances of C-β, C-α, and C-γ in β-*O*-4 gives signals at 85.0, 71.4, and 60.0 ppm, confirming that the mild ball milling and

the acidolytic hydrolysis under the conditions given did not cleave the β-aryl ether structure to a significant extent. The common carbon-carbon linkages such as β-β (C-α in β-β units, 84.2 ppm, C-γ in β-β units, 72.1 ppm, data not shown in the spectrum) and β-5 (C-γ in β-5, 62.6 ppm overlapped with C-5 in xylose from the contaminated hemicelluloses) were also observed, suggesting that condensation reactions may also occur during the ball milling and acidolysis processes. A small signal at 53.6 ppm is indicative of C-β in phenylcoumaran (35). The signals representing the γ-methyl, α and β-methylene groups in n-propyl side chains occurs in the spectrum between 13.8 and 33.6 ppm. A very strong signal at 55.8 ppm is due to the OCH_3 in syringyl and guaiacyl units.

To obtain the different hydroxyl groups present in the lignin fractions, the lignin fraction L_3 was phosphitylated with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane in the presence of known amounts of cholesterol as internal standard and submitted to quantitative ^{31}P-NMR analysis (spectrum not shown). The signals at around 130.6 and 132.1 ppm can quite definitely be assigned to guaiacyl and syringyl hydroxyls, respectively, with a molar ratio of 0.89 (guaiacyl hydroxyls/syringyl hydroxyls) and a total content of phenolic hydroxyls of 0.33 mmol g^{-1} (36). A small signal at 127.3 ppm is attributed to the carboxylic hydroxyls in aliphatic acids with a value of 1.16 (mmol g^{-1}). Two sharp signals at 134.6 and 135.0 ppm represent the threo α-hydroxyls in arylglycerol-β-syringyl and guaiacyl units, and erythro α-hydroxyls in arylglycerol-β-guaiacyl units, respectively with a ratio of threo to erythro of 6.9, indicating that the threo formis the predominant form of the β-*O*-4 ether linkages. Similar results have been reported in MWL from transgenic poplars by Akim and co-corkers (19). The authors found that the erythro α-hydroxyl group content in β-*O*-4 bonds was rather low in MWL fraction. Further analysis found that the total content of β-*O*-4 ether bonds in the non condensed alkali-soluble lignin fraction L_3 was 0.33 mmol g^{-1}. With the study of effect of isolation method on the chemical structure of residual lignin from pulp, Jaaskelainen et al. (37) found that the content of β-aryl ether linkages in acidolysis lignin was lower than in two-step or enzymatic lignins. This result suggested 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. In the case of our experiment, a mild milling process (3 days) and a mild acidolysis (0.05 M HCl) were performed so as to keep these linkages constantly.

Guaiacyl and syringyl phenolic units give two shoulders at around 139.2 and 142.5 ppm. Three intensive signals at 147.0, 147.3, and 147.5 ppm are originated from the aliphatic hydroxyls with a value of

1.55 mmol g^{-1}. Based on the structural analysis of wheat straw lignin by quantitative ^{31}p spectroscopy, Grestini and Argyropoulos (38) reported that the phosphitylated lignin samples showed the presence of considerable amounts of *p*-phydroxyphenyl and guaiacyl units, while syringyl units were detected only low amounts. However, in the case of our experiment, the *p*-hydroxyphenyl phenolic units were identified to be in minor quantities. In fact, *p*-hydroxyphenyl units have been considered to be due mainly to the presence of esterified *p*-coumaric acid (39), which was significantly removed during the third stage treatment by 8% NaOH. Therefore, the *p*-hydroxyphenyl units were found to be eliminated after alkaline hydrolysis. In contrast to *p*-hydroxyphenyl units, the guaiacyl and syringyl units are not involved the ester bonds, and thus to remain unchanged after alkaline hydrolysis.

CONCULUSION

The current results showed that the combination of mild ball milling and sequential extractions with 80% aqueous dioxane containing 0.05 M HCl, DMSO, and 8% NaOH offers the possibility to separate lignin and hemicelluloses with high yield and purity. It appeared that guaiacyl-rich lignin structures in the primary wall are preferentially separated in the first acidic dioxane extraction step, and the sequential separation with DMSO and alkali resulted in dissolution of the lignin fractions-rich in syringyl units, resulting from the secondary walls of Periploca sepium. It was found that the acidic dioxane-soluble lignin was released mainly by cleaving lignin-hemicellulose linkages rather than via the degradation of ether bonds within lignin. The cleavage of the lignin-hemicellulose bonds afforded during the mild acidolysis step of the lignin protocol allows the separation of lignin fractions that are not accessed by any other separation procedures. However, it should be noted that the mild acidolytic hydrolysis with 80% aqueous dioxane containing 0.05 M HCl under the condition given did degrade the macromolecular structure of the hemicelluloses.

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