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Quantitative Analysis of Detailed Lignin Monomer Composition by Pyrolysis-Gas Chromatography Combined with Preliminary Acetylation of the Samples

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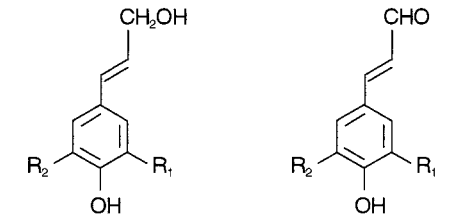
Detailed quantitative analysis of lignin monomer composition comprising *p*-coumaryl, coniferyl, and sinapyl alcohol and *p*-coumaraldehyde, coniferaldehyde, and sinapaldehyde in plant has not been studied from every point mainly because of artifact formation during the lignin isolation procedure, partial loss of the lignin components inherent in the chemical degradative methods, and difficulty in the explanation of the complex spectra generally observed for the lignin components. Here we propose a new method to quantify lignin monomer composition in detail by pyrolysis-gas chromatography (Py-GC) using acetylated lignin samples. The lignin acetylation procedure would contribute to prevent secondary formation of cinnamaldehydes from the corresponding alcohol forms during pyrolysis, which are otherwise unavoidable in conventional Py-GC process to some extent. On the basis of the characteristic peaks on the pyrograms of the acetylated sample, lignin monomer compositions in various dehydrogenative polymers (DHP) as lignin model compounds were determined, taking even minor components such as cinnamaldehydes into consideration. The observed compositions by Py-GC were in good agreement with the supplied lignin monomer contents on DHP synthesis. The new Py-GC method combined with sample preacetylation allowed us an accurate quantitative analysis of detailed lignin monomer composition using a microgram order of extractive-free plant samples.

Lignin, the second most abundant naturally occurring organic material, is a three-dimensional cross-linked polymer existing as the major polyphenol in plants.[1] It functions to waterproof and strengthen cell walls.[2] Since a massive amount of lignin is removed as a byproduct in pulp and papers industrially, it has significant economical and ecological importance.[3] It is generally considered that monomers for lignin consist of mainly cinnamyl alcohols, namely, p-coumaryl alcohol (**1**), coniferyl alcohol (**2**), and sinapyl alcohol (**3**) and a small percentage of cinnamaldehydes, namely, *p*-coumaraldehyde (**4**), coniferaldehyde (**5**), and sinapaldehyde (**6**), which are to be dehydrogenatively polymerized to form lignin.[4,5]



1 R_1, R_2 = H
2 R_1 = OCH_3, R_2 = H
3 R_1, R_2 = OCH_3

Cinnamyl alcohols

4 R_1, R_2 = H
5 R_1 = OCH_3, R_2 = H
6 R_1, R_2 = OCH_3

Cinnamaldehydes

The main monomer component (cinnamyl alcohol) has been shown to have variation between species, trees, and morphological origins,[6−8] and even within a tree,[9] and affects the digestibility, that is, the efficiency of pulp production.[10] In contrast to the main monomer, the compositional variation of the minor monomer, cinnamaldehydes, has been investigated although it will contribute considerably to digestibility and energy yield.[11]

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Table 1. Molar Ratio of Lignin Monomers in Feed of Dehydrogenative Polymers (DHPs) as Synthetic Model Lignins

DHP	*p*-coumaryl alcohol	coniferyl alcohol	sinapyl alcohol	*p*-coumar-aldehyde	conifer-aldehyde	sinap-aldehyde
1	1	1	1			
2					1	1
3	1	3	5	7	9	11
4	9	11	1	3	5	7
5	11	1	3	5	7	9
6	1	1	1	1	1	1

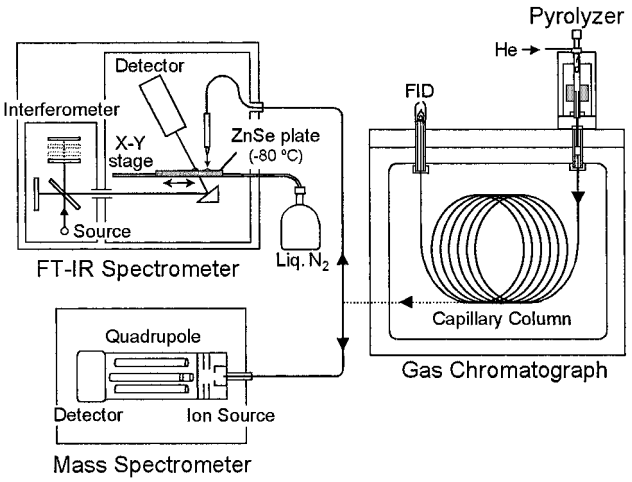

Figure 1. Schematic diagram of Py-GC system combined with MS and FT-IR.

The cinnamaldehydes in lignin have been determined by various methods.[12−18] First, the carbonyl group determination was reported using the reaction between lignin and hydroxylamine hydrochloride[12] and by ultraviolet spectroscopy.[13] However, these methods require lignin isolation, which always causes an increase in carbonyl groups artificially formed during the isolation procedure.[12] Furthermore, this kind of method cannot distinguish each cinnamaldehyde. Second are degradative methods such as thioacidolysis[14] and nitrobenzene oxidation,[15] which have not always been achieved quantitatively because the relatively labile cinnamaldehyde components might be partly lost during the degradation procedure.[5,15] Third, spectroscopic methods such as Fourier transform infrared (FT-IR)[16] and nuclear magnetic resonance (NMR) have been also employed.[17,18] However, FT-IR has been adopted only qualitatively because of the difficulty in discrimination of individual specific peaks on the observed complicated spectra while NMR requires the isolation of lignin, which often causes structural changes as mentioned above.

On the other hand, recently, pyrolysis-gas chromatography (Py-GC) has often been used for structural analysis of lignin, which allows us microgram-scale analysis without isolation of analyte from the samples. For example, Py-GC has been applied to the compositional analysis of lignin mainly focused on lignin main structural species.[19−21] So far, however, quantitative analysis between cinnamyl alcohols and cinnamaldehydes has not been reported by using Py-GC mainly because of possible formation of cinnamaldehydes from the corresponding alcohols during pyrolysis. In fact, the amounts of cinnamaldehydes estimated by conventional Py-GC always showed significantly higher values[19−21] compared to the previously assessed ones.[22]

In this work, the lignin samples are acetylated at their OH groups before pyrolysis to prevent the secondary formation of cinnamaldehydes during pyrolysis. By using the acetylated lignin samples, a new method using Py-GC is proposed for detailed determination of lignin monomer composition including minor cinnamaldehydes. Furthermore, this technique was also applied for compositional analysis of lignin components in wood samples.

EXPERIMENTAL SECTION

Materials. Six kinds of synthetic lignin, dehydrogenative polymer (DHP) were synthesized using various ratios of lignin monomer in buffer solution in the presence of peroxidase and hydrogen peroxide by the conventional mixing method.[23] The reaction was continued with further addition of peroxidase and hydrogen peroxide until lignin monomers and dimers disappeared in the thin-layer chromatograms of the reaction solution. After the reaction was completed, the products were centrifuged and the isolated precipitates were washed with water and vacuum-dried. Table 1 summarizes the molar ratio of lignin monomers in feed for the DHP samples. Holocellulose sample, "a model for lignin free wood", was prepared by a small-scale method.[24] As plant samples, clones of *Eucalyptus camaldulensis* trees ages 40, 2, and 1 year by cutting were utilized. The samples were taken from the trunk for age 40 and from the branch for ages 1 and 2. After debarking, the samples were milled to a 40-mesh pass, then extracted by the Soxhlet apparatus with a sequence of ethanol/toluene (1:2, v/v) for 6 h, ethanol for 4 h, and distilled water for 2 h, and finally vacuum-dried to make extractive-free samples.[24] All the samples obtained were cryomilled into fine powders by a Spex freezer mill 6,700 (Metuchen, NJ) and subjected to Py-GC analysis before and after acetylation.

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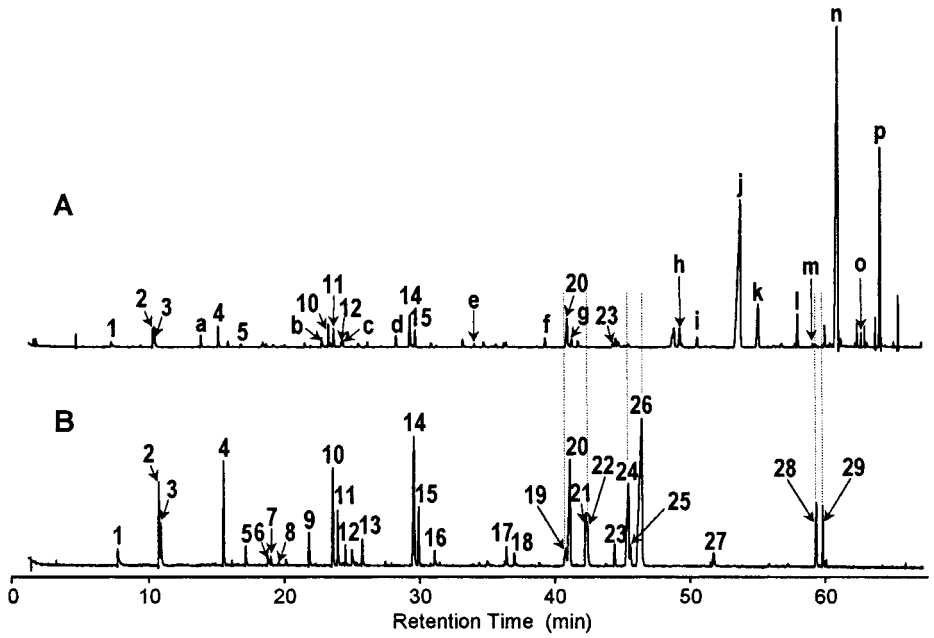

Figure 2. Typical pyrograms of DHPs before (B) and after (A) acetylation made from only cinnamyl alcohols (DHP1) detected by TIC of MS. The peak assignments are given in Table 2.

Acetylation. Acetylation of the samples was performed by the conventional method.[25] About 10 mg of samples was reacted with 75 μL each of dry pyridine and acetic anhydride at 30 °C. After 48 h for DHP and 120 h for wood samples and holocellulose, the reaction mixture was poured into ice-cooled 1% hydrochloric acid solution with stirring. The centrifuged precipitate was washed with distilled water and vacuum-dried. The reaction length for DHP was decided by observing the disappearance of the OH stretching peak at 3200−3250 cm^{-1} in its infrared (IR) spectrum[26] and, for wood samples and holocellulose, by observing the disappearance of cinnamyl alcohols peaks in their pyrograms.

IR. An Hitachi infrared spectrophotometer 260-50 (Tokyo, Japan) was used at 8-cm^{-1} resolution in the range 250−4000 cm^{-1}. About 1 mg of samples was dissolved in 80 μL of acetone; spectra of the solutions absorbed on the 3M IR card type 62 (St. Paul, MN) were collected after air-drying.

Py-GC. A schematic diagram of the Py-GC system, which was basically the same as that in our previous work,[21] combined with a mass spectrometer (MS) or a FT-IR is shown in Figure 1. Here, a vertical microfurnace pyrolyzer (Frontier-Lab PY2010D, Koriyama, Japan) was adopted to minimize the undesired changes in lignin structure caused by the interface heating before final pyrolysis.[21] The pyrolyzer was directly attached to a GC (Shimadzu GC14B, Kyoto, Japan) with a flame ionization detector (FID). The samples were pyrolyzed at 450 °C under a flow of helium carrier gas. Various amounts of the DHP samples ranging between 5 and 100 μg were subjected to Py-GC measurements for calibration. For the wood samples, ~100 μg each was pyrolyzed. A metal capillary column (Frontier-Lab Ultra-Alloy PY1, 30 m × 0.25 mm i.d., coated with 0.25 μm of poly(dimethylsiloxane) through chemical cross-linking, Koriyama, Japan) was used. The 50 mL min^{-1} helium carrier gas flow rate at the pyrolyzer was reduced to 1.0 mL min^{-1} at the capillary column by means of a splitter (split ratio 1:50). The column temperature was first held at 40 °C for 1 min, then programmed from 40 to 80 °C at 5 °C min^{-1}, to 150 °C at 2.5 °C min^{-1}, kept for 2.5 min and to 156 °C at 2.5 °C min^{-1}, kept for 2.5 min, reprogrammed to 180 °C at 4 °C min^{-1} and to 310 °C at 10 °C min^{-1}, and finally held for 10 min.

The identification of the peaks on the pyrograms was carried out by using both a GC/MS (Shimadzu QP2000A, Kyoto, Japan) with an electron impact ionization source (70 eV) and a GC (Hewlett-Packard 6890, Avondale, PA)-FT-IR (Bourne Scientific InfraRed Chromatograph, Acton, MA), to both of which the pyrolyzer was directly attached under the same measuring conditions for the Py-GC-FID described above. In the Py-GC/FT-IR system used, pyrolysates transferred through a heated capillary transfer line into the evacuated interface chamber are deposited on a ZnSe plate cooled at −80 °C by liquid N_2 placed on the X−Y stage. They were fixed as a strictly sharp solid line, which enables highly sensitive on-line FT-IR measurement of each component keeping inherent GC separation.[27] Furthermore, to enhance the sensitivity for minor components, relatively larger amounts (~400 μg) of samples were used under the smaller split ratio of 1:25 for Py-GC/FT-IR.

RESULTS AND DISCUSSION

Pyrograms of DHPs before and after Acetylation. Pyrograms of DHPs made from only cinnamyl alcohols (DHP1; Table 1) before and after acetylation were shown in Figure 2A and B, respectively. Table 2 summarizes peak assignment by MS for formulas and by FT-IR for cis and trans isomers based on the absorbance at 966 cm^{-1} specific to the trans isomer[33] together

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Table 2. Peak Assignment in the Pyrograms of DHPs

peak no.[a]	name	original monomer unit[b]	*m*/*z*	effective carbon no.[c]
1	phenol	H-Alc/Ald	94	5.25
2	methylphenol	H-Alc/Ald	108	6.25
3	guaiacol	G-Alc/Ald	124	5.45
4	methylguaiacol	G-Alc/Ald	138	6.45
5	vinylphenol	H-Alc/Ald	120	7.15
6	methoxycatechol	G-Alc/Ald	140	4.70
7	*cis*-propenylphenol	H-Alc/Ald	134	8.15
8	methylcatechol	G-Alc/Ald	124	5.50
9	vinylguaiacol	G-Alc/Ald	150	7.35
10	syringol	S-Alc/Ald	154	5.65
11	*trans*-propenylphenol	H-Alc/Ald	134	8.15
12	*cis*-isoeugenol	G-Alc/Ald	164	8.35
13	vanillin	G-Alc/Ald	152	5.65
14	methylsyringol	S-Alc/Ald	168	6.65
15	*trans*-isoeugenol	G-Alc/Ald	164	8.35
16	acetoguaiacone	G-Alc/Ald	166	6.65
17	vinylsyringol	S-Alc/Ald	180	7.55
18	(oxyallyl)guaiacol	G-Alc/Ald	178	7.55
19	*trans-p*-coumaryl alcohol	H-Alc	150	7.55
20	syringaldehyde	S-Alc/Ald	182	5.85
21	*cis*-coniferyl alcohol	G-Alc	180	7.75
22	*trans-p*-coumaraldehyde	H-Ald	148	7.35
23	*trans*-propenylsyringol	S-Alc/Ald	194	8.55
24	*trans*-coniferaldehyde	G-Ald	178	7.55
25	acetosyringone	S-Alc/Ald	196	6.85
26	*trans*-coniferyl alcohol	G-Alc	180	7.75
27	propiosyringone	S-Alc/Ald	210	7.85
28	*trans*-sinapaldehyde	S-Ald	208	7.75
29	*trans*-sinapyl alcohol	S-Alc	210	7.95
a	acetoxytoluene	H-Alc/Ald	150	7.75
b	acetoxybenzaldehyde	H-Alc/Ald	164	6.95
c	acetoxybenzene	H-Alc/Ald	136	6.75
d	*trans*-acetoxypropenylbenzene	H-Alc/Ald	176	9.65
e	*cis*-acetoxymethoxypropenylbenzene	G-Alc/Ald	206	9.85
f	*trans*-acetoxymethoxypropenylbenzene	G-Alc/Ald	206	9.85
g	*trans*-propenylphenol acetate	H-Alc	192	8.90
h	*cis*-acetoxypropenyl benzene acetate	H-Alc	234	10.40
i	*cis*-propenylguaiacol acetate	G-Alc	222	9.10
j	*trans*-acetoxypropenylbenzene acetate	H-Alc	234	10.40
k	*trans*-propenylguaiacol acetate	G-Alc	222	9.10
l	*cis*-acetoxymethoxypropenylbenzene acetate	G-Alc	264	10.60
m	*cis*-acetoxydimethoxypropenylbenzene acetate	S-Alc	294	10.80
n	*trans*-acetoxymethoxypropenylbenzene acetate	G-Alc	264	10.60
o	*trans*-propenylsyringol acetate	S-Alc	252	9.30
p	*trans*-acetoxydimethoxypropenylbenzene acetate	S-Alc	294	10.80
q	acetoxydimethoxybenzene	S-Ald	196	10.65
r	*trans*-acetoxyphenylpropenal[d]	H-Ald	190	8.85
s	*trans*-acetoxymethoxyphenylpropenal	G-Ald	220	9.05
t	*trans*-acetoxydimethoxyphenylpropenal	S-Ald	250	9.25

[a] Peaks 1−29 mainly observed on the pyrograms of DHP samples before acatylation. Peaks a−t for acetylated compounds were only observed on the pyrograms of the acetylated samples. The peak notations correspond to those in Figure 2. [b] H-Alc, p-coumaryl alcohol; H-Ald: p-coumaraldehyde; G-, coniferyl-; S-, sinapyl-. [c] Molar sensitivity corrections for FID. [d] This compound was observed in the pyrograms of DHPs 3−6 after acetylation. (The pyrograms did not show.)

with that for the other DHP samples. The peaks denoted a−t were assigned to the acetylated products observed for the acetylated samples. Some of the pyrolysis products were already identified in our previous report.[21] A pyrogram before acetylation (Figure 2B) gave many peaks with considerable intensities; contrary to this, that after acetylation (Figure 2A) became significantly simpler although various minor peaks were still observed. This fact suggests that secondary decomposition of lignin could be suppressed through acetylation. Furthermore, as was explained in the introduction section, relatively larger amounts of three cinnamaldehydes (peaks 22, 24, and 28) were observed on pyrogram B before acetylation mainly due to the secondary reaction. On the other hand, fairly different characteristic products were observed on pyrogram A after acetylation. One specific series consists of the products on which both phenolic and alcoholic OH groups in side chains are acetylated (peaks h, j, l, m, n, and p). Another consists of those only alcoholic OH in side chains

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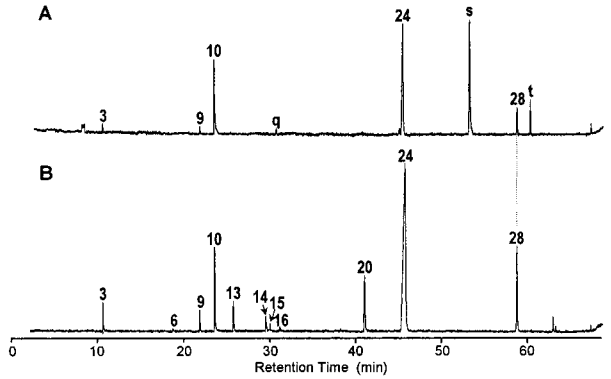

Figure 3. Typical pyrograms of DHPs before (B) and after (A) acetylation made from only cinnamaldehydes (DHP2) detected by TIC. The peak assignments are given in Table 2.

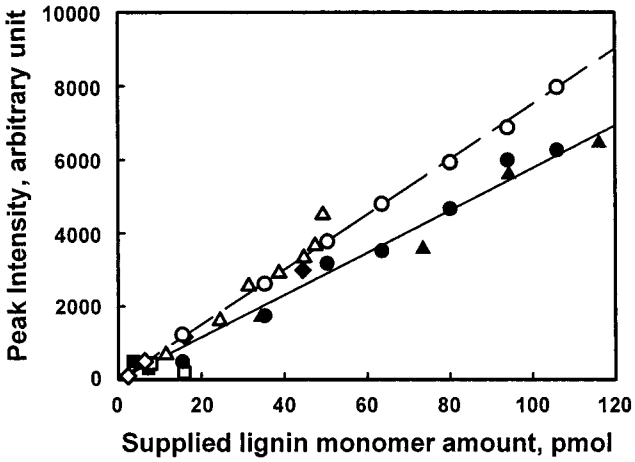

Figure 4. Relationship between supplied lignin monomer amount (coniferyl alcohol and coniferylaldehyde) and peak intensity in Py-GC measurement. Blank and closed symbols are for coniferyl alcohol and coniferaldehyde, respectively: △, ▲, DHP **3**; □, ■, DHP **4**; ◇, ◆, DHP **5**; ○, ●, DHP **6** in Table 1.

are acetylated (peaks g, i, k, and o). On pyrogram A, however, the original cinnamyl alcohols with free alcoholic OH were not observed at all. This fact demonstrated that acetylation was almost completely accomplished at alcoholic OH groups. Moreover, additional peaks assigned to neither cinnamaldehydes nor the corresponding acetylated ones were observed. These data suggest that acetylated cinnamyl alcohols did not yield any corresponding cinnamaldehydes during pyrolysis.

Parts A and B of Figure 3 show pyrograms of DHPs made from only cinnamaldehydes (DHP2; Table 1) before and after acetylation, respectively. On the pyrogram before acetylation (Figure 3B), original cinnamaldehydes (peaks 24 and 28) were observed as the major peaks. On the other hand, after acetylation (Figure 3A) peaks of acetylated cinnamaldehydes only at phenolic OH (peaks s and t) were observed along with original cinnamaldehydes (peaks 24 and 28). These data suggest that aldehyde groups were almost completely retained in the side chain during acetylation and pyrolysis. Thus, the above-mentioned results clearly support that acetylation had almost no interfering effect on the analysis of cinnamaldehydes present in the original lignin structures by Py-GC. Consequently, preacetylation of lignin samples should contribute to suppress the secondary thermal reactions to form cinnamaldehydes from cinnamyl alcohols during

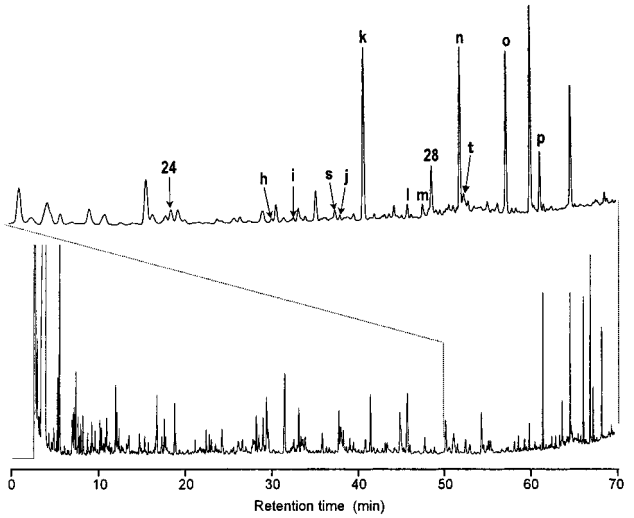

Figure 5. Typical pyrogram of acetylated extractive free sample of *E. camaldulensis* tree, age 40 years, detected by FID. The peak assignments are given in Table 2.

pyrolysis. Probable mechanisms for lignin pyrolysis before and after acetylation are proposed in Scheme 1 based on the results mentioned above "direct pyrolysis before acetylation" significantly causes the conversion of cinnamyl alcohol (**A**) into cinnamaldehyde (**B**), while "pyrolysis after acetylation" gives acetylated cinnamyl alcohols ((**C**) and (**D**)) derived from the cinnamyl alcohol units and acetylated (**E**) and original cinnamaldehyde (**B**) derived from the cinnamaldehyde units without accompanying any secondary reactions.

Relationship between Lignin Monomer Amount in the Sample and the Corresponding Peak Intensities in the Pyrograms. For the quantitative analysis, relationship between lignin monomer amounts in the samples, and the sum of FID intensities of their corresponding peaks observed in the pyrograms was examined for 4 kinds of acetylated DHPs prepared with various lignin monomers (3−6 in Table 1). Figure 4 shows the typical relationships for coniferyl alcohol and coniferaldehyde. Here, the intensities of the corresponding peaks, peaks i, k, and l for coniferyl alcohol and peaks 24 and s for coniferaldehyde, were corrected by molar sensitivities for FID using the effective carbon number (ECN) concept[29] whereas the lignin monomer amount was obtained from the lignin monomer ratio in feed multiplied by the sample weight for Py-GC measurement of a given DHP sanple.

The observed correlation coefficients both for three alcohols and for three aldehydes, were highly significant over 0.95. This result suggests that these relationships can be used to calibrate the corresponding components contained in the lignin samples. Although the ratio of linkage types between monomers was reported to be different by supplied amount of lignin monomers in DHP preparation,[30−32] the proposed method in this study could be valid for the evaluation of a given component regardless of its linkage type in lignin. This is unique when one considers the fact that the results obtained by the other degradative methods are general dependent on the linkage type.[14,15]

Application of a Novel Method for Compositional Analysis of Lignin Monomer Component in Wood Samples. Using the above-mentioned relationships obtained with DHPs for calibration,

Scheme 1. Proposed Mechanism for Lignin Pyrolysis before and after Acetylation

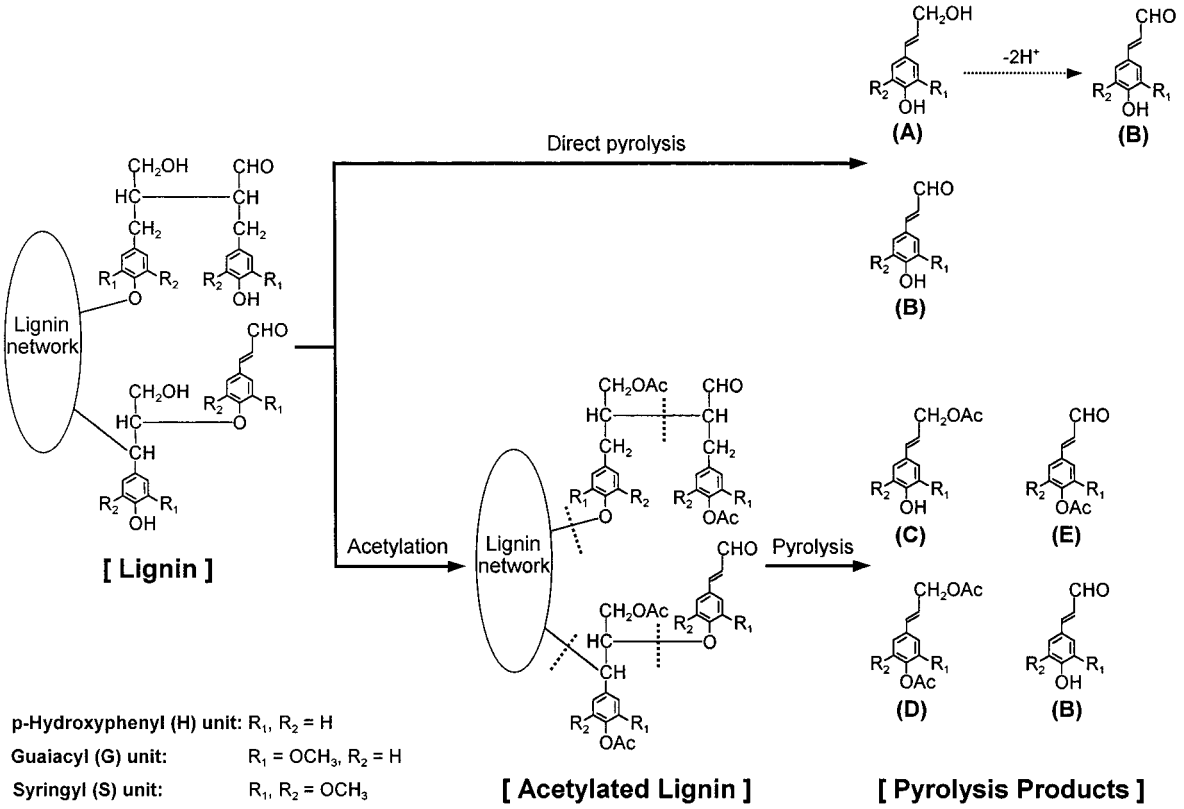

Figure 6. Lignin monomer composition obtained for samples of different ages in *E. camaldulensis* clones.

lignin monomer composition was examined with acetylated wood samples. Figure 5 shows a pyrogram of an acetylated extractive-free sample of *E. camaldulensis* tree, age 40 years, detected by FID. After elution of many peaks mostly derived from chemical constituents other than lignin up to a retention time of 50 min, original (peaks 24 and 28) and acetylated cinnamaldehydes (peaks s and t) and acetylated cinnamyl alcohols (peaks h−p) were clearly observed. Here, acetylation of lignin components in the wood sample was also considered to be carried out almost quantitatively because the original cinnamyl alcohols were not detected in the pyrogram and the observed aldehydes exclusively originated from the aldehyde structure in lignin. Consequently, this method can be applied to discriminate subtle differences in wood samples such as the age difference of clones.

Then, in order to investigate the age effect on the differences in lignin composition, two cloned samples of ages 1 and 2 years from their mother tree, age 40 years, were examined by the proposed method. Figure 6 summarizes the result of the estimated lignin monomer composition. The total cinnamaldehyde composition in the lignin samples was ∼12% for all the wood samples. Judging from the fact that the proposed method was independent of the lignin linkage type, the estimated amount of cinnamaldehydes could be fairly accurate. On the other hand, lignin monomer composition varied slightly depending on the age; for example, although each lignin monomer composition hardly varied during aging from 1 to 2 years old, coniferyl alcohol increased from about 16 to 20% and sinapyl alcohol decreased from about 70 to 66% by aging from 1 and 2 years old to 40 years old. However, the cinnamaldehydes hardly varied with age.

CONCLUSIONS

The new analytical method of lignin monomer composition in plants was successfully established by using Py-GC combined with preacetylation of lignin samples before pyrolysis. Preacetylation of the samples prohibits the secondary formation of cinnamaldehydes from cinnamyl alcohols during pyrolysis, which allows quantitative analysis of lignin monomer composition including minor components of cinnamaldehydes using a microgram order

of extractive-free plant samples. Therefore, this new method is expected to be useful for the quantitative characterization of plant samples such as the following: (1) many kinds of plant and plant-derived materials containing lignin, (2) genetically engineered plants downregulating the enzyme activity of cinnamyl alcohol dehydrogenase (CAD), which catalyzes the conversion of cinnamaldehydes to cinnamyl alcohols, containing more cinnamaldehydes than normal plants by qualitative analysis,[5,33] and (3) natural mutants deficient in CAD, which contain more cinnamaldehydes than normal plants.[34,35]

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