Quantitative Examination of Pre-Extraction Treatment on the Determination of Lignin Content in Leaves

Morikazu Toda,* Takuya Akiyama, Tomoya Yokoyama, and Yuji Matsumoto

It has been suggested that the Klason method overestimates the lignin content of non-wood tissues of plants. To evaluate the effect of pre-extraction treatments on lignin determination in leaves, nine kinds of pre-extraction treatment were applied to ginkgo leaves and zelkova leaves. The apparent lignin contents (lignin determination by the Klason method) of ginkgo and zelkova leaves without pre-extraction treatments were 30.7% and 42.6%, respectively. After the various pre-extraction treatments, the apparent lignin contents were still high. On the other hand, the yield of nitrobenzene oxidation products (NPs) per apparent lignin content was maintained at a very low level before and after pre-extraction treatments (maximum value was 6% for ginkgo leaf and 11% for zelkova leaf after extraction treatment) compared with the value from wood (25 to 60%). These results suggested that the Klason method overestimates the lignin content of leaves even after the pre-extraction treatments examined in this study. In addition, a considerable part of the sample from which NPs or neutral sugars originate was lost during these pre-extraction stages. These results implied that some parts of the cell wall components were also removed by these pre-extraction treatments.

Keywords: Lignin; Leaf; Extractions; Klason lignin; Biomass

Contact information: Department of Biomaterial Sciences, Graduate School of Agricultural and Life Sciences, University of Tokyo, Yayoi 1-1-1, Bunkyo, Tokyo, Japan;
** Corresponding author: 2073375825@mail.ecc.u-tokyo.ac.jp*

INTRODUCTION

Studies on the utilization of plant biomass have been carried out widely because biomass is a renewable resource. To isolate and utilize polysaccharides from biomass, degradation and removal of lignin is necessary in most cases. Consequently, it is important to have an accurate understanding of the quantity, structure, and reactivity of lignin. The components of secondary xylem of wood are rather simple because it is mostly composed of cell walls. However, non-woody tissues, such as leaves and bark, contain substantial amounts of non-cell wall components. The study of lignin in non-secondary xylem has not been well developed compared with that of secondary xylem, so there is no definitive method to accurately determine the quantity of lignin in non-secondary xylem. The Klason method is commonly used for the determination of lignin in secondary xylem, where the lignin content in wood is usually 20 to 30%. When the Klason method is utilized with leaves, lignin determination values are sometimes much higher than those of secondary xylem (Jin *et al*. 2003; Chung *et al*. 2008). Furthermore, it is known that the lignin basis yields of nitrobenzene oxidation products (NPs) are very low compared with secondary xylem samples (Jin *et al*. 2003).

These results strongly suggested that the Klason method overestimates the lignin content of leaves. If the Klason method overestimates the lignin content, such an error

can be important. The Klason determination value is the basis for almost all the other indirect lignin determination methods such as near IR, IR, and UV methods, including the acetyl bromide method and others. In these indirect methods, measured values are translated into lignin determination values by the use of a calibration line or curve that expresses the relationship between measured value and actual lignin determination value obtained by a direct method such as Klason method. Even in the case of the Kappa number method, which is one of the few direct determination method, correlation between values obtained by the Klason method is important.

For the analysis of cell wall component including lignin, it is common practice to remove non-cell wall components by a pre-extraction treatment before analysis. For a sample obtained from secondary xylem, ethanol-benzene extraction is usually employed to remove a variety of extractable components. However, for wood samples such as bark and for some tropical trees, it is difficult to remove all extractable components by ethanol-benzene treatment. For such samples, sodium hydroxide solution treatment or methanol treatment is sometimes conducted after ethanol-benzene treatment. There is no established extraction method to determine the lignin content of non-secondary xylem. For lipid extraction, chloroform-methanol is also used sometimes in addition to ethanol-benzene. There are some cases in which hydrophilic organic solvents, such as acetone or 80% aqueous ethanol, are used for the removal of lipids as well as water-soluble components. For pre-extraction treatment of wood and leaves, which have many different kinds of water-soluble contents, hot or cold aqueous organic solvents or above-mentioned 80% aqueous ethanol is used to remove water-soluble contents. It has been suggested that these pre-extraction treatments can remove alkaloids, tannins, and free polysaccharides. Proteins are usually extracted with aqueous sodium chloride or diluted aqueous sodium hydroxide. Alkaline solutions, however, carry a risk of degrading polysaccharides and lignin. For the determination of lignin in forage samples, neutral detergent treatment followed by an acid detergent method is often applied. However, Kondo *et al*. reported that acid detergent could dissolve lignin in some species (Van Soest and Robertson 1980; Kobayashi 1987; Kondo *et al.* 1987).

Nitrobenzene oxidation is widely used to analyze the aromatic structure of lignin (Chen 1992). By nitrobenzene oxidation of softwood lignin, *p*-hydroxybenzaldehyde and vanillin are obtained. In addition to these two compounds, syringaldehyde is also obtained from hardwood lignin. The lignin basis yield of nitrobenzene oxidation products (NPs) varies depending on the lignin structure. Usually, lignin basis yield of NPs from softwood lignin is 26 to 39% and that from hardwood lignin is 32 to 59% (Akiyama *et al.* 2005). On the other hand, Jin *et al.* (2003) reported that the yield of NPs from wood leaves is extremely lower (2% for gingko) than wood lignin's value (Jin *et al*. 2003). Based on this fact, as well as the low methoxyl group content in leaf lignin, they concluded that the amount of lignin in leaves is overestimated by the Klason method.

The necessary conditions for pre-extraction treatment before lignin determination are that cell wall components, especially lignin, must be left intact, and non-cell wall components that cross-react together with lignin, such as protein and lipids, must be removed. If these conditions are achieved by a pre-extraction method, lignin values of leaves determined by the Klason method should be lower and the lignin-basis yield of NPs may be close to the level of wood lignin. In this study, nine different pre-extraction methods were examined with ginkgo and zelkova leaves to elucidate the effects of pre-extraction treatments on lignin determination. It is thought that leaves contain various non-lignin components that are measured as lignin by the Klason method. This study

focused on components containing nitrogen, such as proteins. To follow the behavior of these components during pre-extraction treatment, the nitrogen content was analyzed.

EXPERIMENTAL

Materials

Ginkgo (*Ginkgo biloba*) leaves and zelkova (*Zelkova serrata*) leaves were collected from the tree in the field in August 2008. The leaves were about four months old. They were washed with water, freeze-dried (-45 °C, over night), and then milled using a Wiley mill so that the material passed through a 1-mm mesh. Beech (*Fagus japonica*) wood was milled in the same manner and extracted using ethanol-benzene (1:2).

Pre-Extraction Treatment of Leaves

Each leaf sample (3 g) was treated with 200 mL of extraction solvent under the following condition.

Ethanol-benzene (1:2)

Prepared samples were subjected to soxhlet extraction for 8 h then dried by vacuum drying in an oven at 40 °C.

80% aqueous ethanol

Prepared samples were subjected to extraction under reflux three times for 1 h each. After extraction, the samples were washed with methanol then dried by vacuum drying in an oven at 40 °C.

Hot water

Prepared samples were subjected to extraction under reflux three times for 1 h each. After boiling, the samples were filtered and dried at 105 °C in an oven.

50% aqueous acetone

Prepared samples were soaked in 50% acetone at room temperature for three days. The samples were then filtered and dried in a vacuum drying oven at 40 °C.

Dichloromethane

Prepared samples were soaked in dichloromethane at room temperature for three days. The samples were filtered and dried in a vacuum drying oven at 40 °C.

KOH-water (10 g/L)

Prepared samples were subjected to extraction under reflux for 1 h. After boiling, the samples were filtered and dried at 100 °C in an oven.

KOH-methanol (10 g/L)

Prepared samples were subjected to extraction under reflux for 1 h three times. After extraction, the samples were washed with methanol then dried by vacuum drying in an oven at 40 °C.

Chloroform-methanol (1:1)

Extract from prepared leaves was subjected to Soxhlet extraction for 8 h then dried in a vacuum drying oven at 40 °C.

Neutral detergent

Prepared samples were subjected to extraction under reflux for 1 h. After boiling, the samples were filtered and dried at 100 °C in an oven. The composition of the aqueous neutral detergent was 30.0 g/L sodium lauryl sulfate, 18.61 g/L disodium edetate dehydrate, 6.81 g/L sodium tetraborate decahydrate, 4.56 g/L anhydrous disodium phosphate, and 10.0 mL/L triethylene glycol.

Analysis

Klason method for apparent lignin content

Samples (0.5 g) were soaked in 72% sulfuric acid for 3 h. After that, 187.5 mL of water was added to dilute the acid to 3%; then, the sample was heated for 30 min at 121 °C in an autoclave. After cooling overnight, Klason residue was filtered using a glass filter and dried at 105 °C in an oven. Acid-soluble material was measured by UV spectroscopy at 205 nm using a value of 20 for gram absorptivity. The total of Klason residue and acid-soluble materials are expressed as apparent lignin content in this study.

Nitrogen content

Nitrogen content was measured by elementary analysis using a 2400 Series II (PerkinElmer, USA).

Nitrobenzene oxidation

Dried samples (20 mg) were reacted in a 10-mL autoclave with 4 mL of 2 mol/L aqueous sodium hydroxide and 0.25 mL of nitrobenzene for 2 h at 170 °C. After the reaction, the sample was cooled and internal standard (ethylvanillin) was added. The reactant was removed from the autoclave and the autoclave was washed with 5 mL of 0.1 mol/L sodium hydroxide three times. Washings were combined with the reactant. The sample was washed with 15 mL of dichloromethane three times, and the pH was adjusted to a pH of 1 by addition of 4 mol/L hydrochloric acid. The acidified sample was extracted with 20 mL of dichloromethane twice then with 15 mL of diethyl ether once. The combined organic layer was washed with deionized water, dehydrated with sodium sulfate, and then evaporated.

A total of 0.1 mL of N,O-bis(trimethylsilyl)acetamide was continuously added by titration to the dried sample for 10 min at 100 °C. After the reaction, the sample was analyzed using a gas chromatogram (GC2014, Shimadzu, Tokyo, Japan). The GC peak area of the aimed compound relative to that of the ethylvanillin (internal standard) was translated into the weight by the use of a calibration line that expresses the relationships between the peak area ratio and weight ratio of the aimed compound and ethylvanillin.

Neutral sugar analysis

Samples (0.5 g) were soaked in 72% sulfuric acid for 4 h at room temperature. Afterwards, 140 mL of water was added to dilute the acid to 4% and the sample was heated for 60 min at 120 °C in an autoclave. After cooling overnight, the solution was filtered using glass filter and diluted to 500 mL. Internal standard (inositol) was added to

10 mL of the diluted sample and then neutralized with barium hydroxide. After the removal of barium sulfate by centrifugation, the supernatant was reduced by the addition of sodium borohydride. Acetic acid was added to degrade excess sodium borohydride, and the supernatant was removed in an evaporator. The addition and evaporation of methanol was repeated several times to remove borate, and the dried sample was further dried at 105 °C in an oven. Acetic anhydride (3 mL) was added and reacted for 3 h at 120 °C. After the reaction, the sample was analyzed using a gas chromatogram (GC-14B, Shimadzu, Tokyo, Japan).

RESULTS AND DISCUSSION

Tables 1 and 2 show the results of analyses of leaf samples of gingko and zelkova with and without pre-extraction treatments, respectively. In the tables, analytical results are expressed not only as the extracted sample-basis yield but also as non-extracted sample-basis yield, and by the use of the latter yield, retention during pre-extraction treatment was also calculated (retention = 100 × non-extracted sample-basis yield of extracted sample/yield obtained for non-extracted sample).

In the case of the wood sample, alcohol-benzene extraction did not cause significant weight loss because only a small amount of extractable material was removed and the cell wall components remained after treatment. However, in the case of leaf samples, weight loss caused by pre-extraction treatment was sometimes very large, as shown as extraction yield in Tables 1 and 2. This result was not surprising, however, because leaves contain large amounts of non-cell wall materials such as vacuole components, storage polysaccharides, and various kinds of extractable components. As a whole, the weight retention (extraction yield) was smaller when extraction used aqueous solvents than with non-aqueous solvents. The weight retention after pre-extraction treatment was lower for ginkgo leaves than for zelkova leaves. Minimum retention was observed with neutral detergent extraction for both ginkgo and zelkova leaves at 25% and 57%, respectively.

Extracted sample-basis yields of Klason residue and apparent lignin content (lignin determination value by the Klason method) of extracted ginkgo leaves tend to increase with aqueous solvent extraction, while the values were similar or smaller with non-aqueous solvent extraction. On the other hand, for zelkova leaves, the extracted sample-basis yield of Klason residue and apparent lignin content did not change significantly with either aqueous or non-aqueous solvent extraction except for neutral detergent extraction. Even though there were such differences depending on the extraction solvents and species, the extracted sample-basis yield of Klason residue was still high after pre-extraction treatment, ranging from 18.1% to 40.2% for gingko leaves and from 30.6% to 40.3% for zelkova leaves. The lignin content obtained by the Klason method still seemed to be overestimated. This suggests that substances that behave similar to lignin during Klason treatment and participate in the formation of Klason residue are, as a whole, not easily removed by pre-extraction treatments.

Jin *et al.* (2003) showed that ginkgo leaves produce only small amounts of NPs in spite of their extremely high apparent lignin content. Based on this result, they proposed that the lignin content of gingko leaves was overestimated by the Klason method because of the presence of compounds that behave together with lignin during Klason treatment. If a pre-extraction treatment with a solvent can remove such compounds efficiently and

selectively, the ratio of the extracted sample-basis yield of NPs to the apparent lignin content will be higher than that of a non-extracted sample and will approach the level of wood. In Fig. 1, the yield of NPs is plotted against apparent lignin content. All the values from leaves were found to be lower than those of 21 wood species (data from Akiyama *et al*. (2005)), indicating that NP yield on the basis of apparent lignin content was extremely low even after various pre-extraction treatments. Acid-soluble lignin will be overestimated if non-lignin contents give absorbance at 205 nm.

Table 1. Results of Analysis on Extracted and Non-Extracted Ginkgo Leaf

		Extraction yield (%)	Neutral sugar (%)	NP (%)	Klason method (%)	Klason residue (%)	Nitrogen contents (%)	NP / Klason residue
Before extraction		-	24.6	1.36	30.7	25.1	2.25	0.054
Dichloro-methane	After extraction	90.9	23.0	1.56	25.9	20.0	1.97	0.078
		-	20.8[a]	1.41[a]	23.5[a]	18.15[a]	1.79[a]	-
	Retention	-	84.7	104.1	76.7	72.4	79.6	-
Ethanol-benzene	After extraction	90.7	19.3	1.03	23.7	18.1	2.04	0.057
		-	17.5[a]	0.93[a]	21.5[a]	16.4[a]	1.85[a]	-
	Retention	-	70.9	68.5	70.0	65.6	82.2	-
Chloroform-methanol	After extraction	68.76	29.0	0.83	28.7	26.7	2.65	0.031
		-	19.9[a]	0.57[a]	19.8[a]	18.4[a]	1.82[a]	-
	Retention	-	80.8	41.8	64.3	73.3	80.9	-
KOH-methanol	After extraction	56.39	35.3	0.92	25.6	23.2	2.95	0.040
		-	19.9[a]	0.52[a]	14.4[a]	13.1[a]	1.66[a]	-
	Retention	-	80.7	38.3	47.0	52.3	73.8	-
Hot water	After extraction	58.49	23.4	0.91	41.5	37.6	1.67	0.024
		-	13.7[a]	0.53[a]	24.3[a]	22.0[a]	0.98[a]	-
	Retention	-	55.5	39.1	79.1	87.8	43.6	-
50% aqueous acetone	After extraction	55.24	26.9	0.94	35.1	30.9	1.74	0.031
		-	14.9[a]	0.52a	19.3[a]	17.0[a]	0.96[a]	-
	Retention	-	60.3	38.4	63.0	68.0	42.7	-
KOH aqueous solution	After extraction	40.56	45.7	2.03	39.1	38.4	0.43	0.053
		-	18.5[a]	0.82[a]	15.9[a]	15.6[a]	0.17[a]	-
	Retention	-	75.2	60.6	51.7	62.2	7.6	-
80% aqueous ethanol	After extraction	38.54	24.0	1.01	28.9	24.3	1.87	0.042
		-	9.3[a]	0.39[a]	11.1[a]	9.4[a]	0.72[a]	-
	Retention	-	37.5	28.6	36.3	37.4	32.0	-
Neutral detergent	After extraction	25.08	55.7	1.90	41.3	40.2	1.28	0.047
		-	14.0[a]	0.48[a]	10.4[a]	10.1[a]	0.32[a]	-
	Retention	-	56.6	35.2	33.7	40.2	14.2	-
Beech wood			57.5	11.67	26.4	24.0	0.1	0.486

a: Calculated value based on original sample

Retention: 100 × non-extracted sample-basis yield of extracted sample / yield obtained for non-extracted sample

Table 2. Results of Analysis on Extracted and Non-Extracted Zelkova Leaf

		Extraction yield (%)	Neutral sugar (%)	NP (%)	Klason method (%)	Klason residue (%)	Nitrogen contents (%)	NP / Klason residue
Before extraction		-	27.9	2.54	42.6	39.7	1.68	0.064
Dichloro-methane	After extraction	95.7	24.2	2.47	42.1	38.7	1.67	0.064
		-	23.2[a]	2.37[a]	40.3[a]	37.1[a]	1.60[a]	-
	Retention	-	83.3	93.1	94.7	93.5	95.2	-
Ethanol-benzene	After extraction	96.8	27.8	2.6	42.1	39.5	1.7	0.067
		-	26.9[a]	2.5[a]	41.2[a]	38.2[a]	1.6[a]	-
	Retention	-	96.6	100.1	96.7	96.4	97.6	-
Chloroform-methanol	After extraction	98.29	31.1	2.46	40.5	37.3	1.65	0.066
		-	30.5[a]	2.41[a]	39.8[a]	36.7[a]	1.62[a]	-
	Retention	-	109.6	95.0	93.4	92.4	96.4	-
KOH-methanol	After extraction	88.67	30.5	2.91	37.6	35.0	1.34	0.083
		-	27.1[a]	2.58[a]	33.4[a]	31.0[a]	1.19[a]	-
	Retention	-	97.2	101.6	78.3	78.2	70.8	-
Hot water	After extraction	71.95	34.9	2.47	41.5	39.8	1.60	0.062
		-	25.1[a]	1.78[a]	29.9[a]	28.7[a]	1.15[a]	-
	Retention	-	90.1	70.7	70.2	72.2	68.5	-
50% aqueous acetone	After extraction	82.99	24.7	2.04	42.1	39.9	1.52	0.051
		-	20.5[a]	1.69[a]	34.9[a]	33.2[a]	1.26[a]	-
	Retention	-	73.7	66.5	81.9	83.6	75.0	-
KOH aqueous solution	After extraction	83.30	28.0	2.48	37.8	35.9	1.35	0.069
		-	23.3[a]	2.06[a]	31.5[a]	29.9[a]	1.12[a]	-
	Retention	-	83.6	81.2	74.0	75.3	66.7	-
80% aqueous ethanol	After extraction	85.91	29.9	2.54	42.6	40.3	1.64	0.063
		-	25.7[a]	2.18[a]	36.6[a]	34.6[a]	1.41[a]	-
	Retention	-	92.1	85.7	86.0	87.3	83.9	-
Neutral detergent	After extraction	57.80	47.6	3.46	31.2	30.6	1.37	0.113
		-	27.5[a]	2.00[a]	18.0[a]	17.7[a]	0.79[a]	-
	Retention	-	98.8	78.7	42.3	44.6	47.0	-
Beech wood			57.5	11.67	26.4	24.0	0.08	0.486

a: Calculated value based on original sample

Retention: 100 × non-extracted sample-basis yield of extracted sample / yield obtained for non-extracted sample

Because of this reason, NP yield is plotted against Klason residue in Fig. 2, and even here the ratios of NPs to Klason residue in leaf samples were 0.02 to 0.11, which are extremely low compared with those of wood samples (0.26 to 0.68). This result indicated that the amount of lignin measured by the Klason method includes components that will not produce NPs, even after pre-extraction treatment. Furthermore, sometimes the retention of whole sample weight was lower than the retention of Klason residue, especially for aqueous solvent extraction of gingko leaves. Therefore, it was considered that the pre-extraction treatments carried out in this study could not selectively remove non-lignin substances measured as lignin by the Klason method.

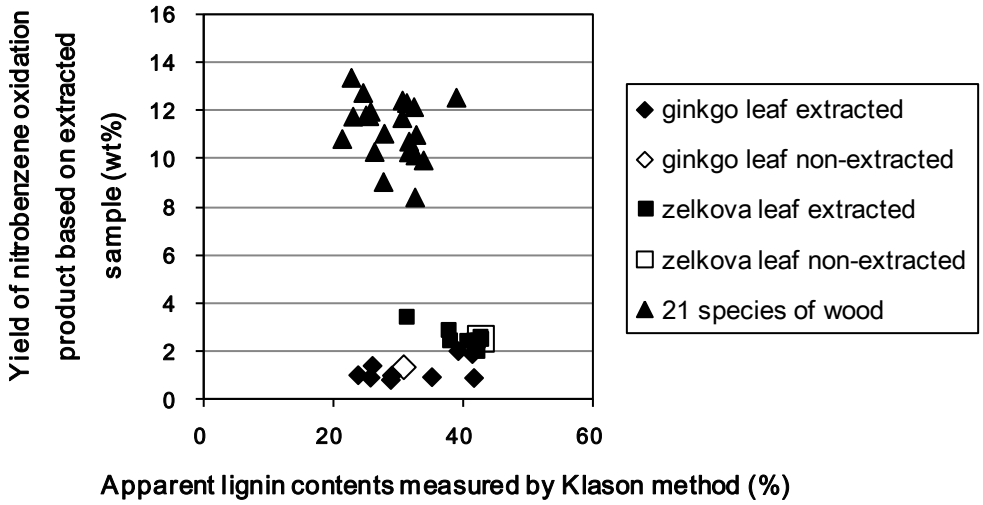

Fig. 1. Correlation between yield of nitrobenzene oxidation products and apparent lignin content measured by the Klason method (total of Klason residue and acid-soluble material)

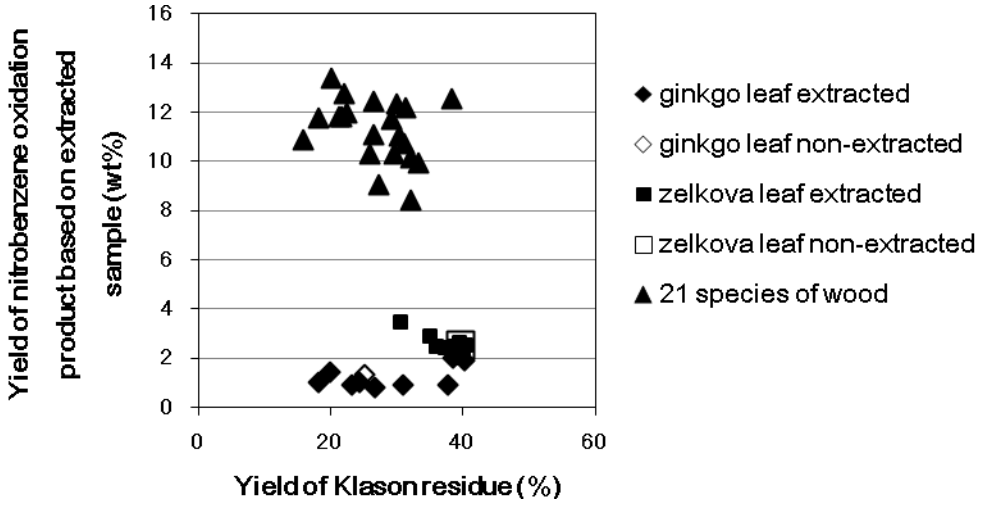

Fig. 2. Correlation between yield of nitrobenzene oxidation products and Klason residue

Contrary to such resistance of non-lignin substances against pre-extraction treatment, some data were obtained that suggest that some cell wall components (lignin, cellulose, and hemicellulose) could be removed during pre-extraction treatment. If parts of the lignin and polysaccharide cell wall components are removed during the pre-extraction process, then yields of specific degradation products obtained from such cell wall components in extracted samples will be reduced when the yields are expressed on the basis of the non-extracted sample. From this point of view, yield and retention of NPs and that of neutral sugars were examined (Tables 1 and 2). Retention of neutral sugars during the pre-extraction treatment of ginkgo leaves was not high (38 to 85%), but that of zelkova leaves was 74 to 110%. The retention of NPs during the pre-extraction treatment of ginkgo leaves was small (28.6 to 104.1%), except for dichloromethane-extraction, and that of zelkova leaves was not high either (66.5 to 101.6%), although pre-extraction

treatment of zelkova leaves resulted in a higher retention of NPs than gingko leaves as a whole. If wood samples were subjected to the same analysis, the retention of both NPs and neutral sugars will be very close to 100%. The result that the retention of both neutral sugars and NPs during pre-extraction treatment was low implied the possibility that part of the cell wall components were removed by the pre-extraction treatments used in this study.

Of course, the presence of storage polysaccharides in leaves will result in the low retention of neutral sugars during pre-extraction treatment. In particular, glucose can be obtained from both cell wall polysaccharides (cellulose and some hemicelluloses) and storage polysaccharides such as starch. For gingko leaves, the retention of glucose was lower than other hemicellulosic neutral sugars except for the KOH-water extracted sample. It is unlikely that cellulose is more easily removed than hemicellulose by pre-extraction treatment. Therefore, this result suggested that part of the glucose removed from gingko leaves during pre-extraction treatment will be derived from storage carbohydrates such as starch or sucrose. For zelkova leaves, retention of both glucose and other hemicellulosic neutral sugars was high. Samples extracted with aqueous solvents showed lower retention of rhamnose and arabinose, which suggested that aqueous solvents removed some of the pectic polysaccharides from leaf samples.

The nitrogen content of leaf samples was still high even after pre-extraction treatments. When gingko leaves were extracted with aqueous solvent, the retention of nitrogen was lower than the weight retention of the whole sample but did not approach zero, except for KOH-water extraction. When these samples were extracted with non-aqueous solvents, the retention of nitrogen was high and sometimes higher than the weight retention of the whole sample. In case of zelkova leaves, the retention of nitrogen was high for both aqueous and non-aqueous solvent extractions. If the origin of nitrogen is protein, then these results suggest that protein cannot be removed selectively and efficiently and may, therefore, participate in the formation of Klason residue during Klason treatment even after pre-extraction treatments.

CONCLUSIONS

1. Pre-extraction treatments of leaf samples using a variety of solvents could not effectively and selectively remove non-cell wall components, and some cell wall components were suggested to be removed by these pre-extraction treatments.
2. Even when the same extraction solvent was used, its effects on cell wall analysis of gingko and zelkova leaves were different, which indicated that the effects of pre-extraction treatments differed depending on the species.
3. The potential overestimation of lignin content in leaves by the Klason method cannot be prevented by adopting pre-extraction treatments of leaves.

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