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Structural difference between leaf blade and petiole of original and mulched leaf litter of *Ginkgo biloba*

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Introduction

The processes of leaf litter decomposition, as opposed to woody litter, are poorly understood, in most cases, leaf litter decomposition is more complex (HAMMEL, 1997). The initial chemical composition of plant litters affects on its decomposition rate as the chemical changes during decomposition (MCCLAUGHERTY and BERG, 1987). Chemical composition of plant litters can be divided into three broad groups, namely soluble substances, polysaccharides (cellulose and hemicellulose), and acid insoluble aromatic compounds including lignin and polyphenolic compounds other than lignin. Lignin is a decay resistant biopolymer usually regarded as a rate regulating factor in leaf litter decomposition (BERG and STAAF, 1980). Therefore, lignin content has been used to predict the decomposition rate and weight loss of leaf litters (MEENTEMEYER, 1978; MCCLAUGHERTY and BERG, 1987; SALAMANCA *et al.*, 1998). However, there is no adequate analytical method to measure lignin content of leaf litters (THEANDER and WESTERLUND, 1993). The most widely used method for quantitative determination of leaf litter lignin has been the Klason method, which affords an insoluble residue from hydrolysis with sulfuric acid. Klason method is the standard method for lignin determination of wood samples, while the method usually gives an overestimated value for the lignin of herbaceous plants (IIYAMA and WALLIS, 1990). IIYAMA and WALLIS (1990) developed acetyl bromide method for determination lignin content of non-wood samples.

The aim of this study is to investigate lignin structural difference of leaf blade and petiole of original and mulched leaf litter of *G. biloba* and to estimate lignin content of leaf blade and petiole, not only by Klason method or acetyl bromide procedure, but also by a combination of alkaline nitrobenzene oxidation, ozonation, methoxyl content determination and analytical pyrolysis.

Materials and Methods

Sample preparation

Fallen leaves of *G. biloba* were collected from Yayoi campus of the University of Tokyo. Leaves were separated into mesophyll, vein and petiole, and grounded finely with a vibratory ball

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mill (Retsch type MM200) for 15 min at a vibration rate for 30 s^{-1}, and were then subjected to pyrolysis.

Mulching experiment was conducted at the experimental field in Yayoi campus (35º41´N, 139º46´E) of the University of Tokyo from early winter in 1999. Litter bag (20×20 cm) made of nylon net with a mesh size of 0.1 mm was used. Original and 16 months mulched leaves were separated into the leaf blade and petiole, respectively. Sample was air dried and ground in a Wiley mill to pass a 420 µm sieve. The ground sample was extracted three times with boiling 80% aqueous ethanol (v/v) for 1 h followed by water extraction overnight at room temperature with shaking. Extract free sample was dried in a vacuum oven over P_2O_5 overnight at 40ºC. The extract free samples were further ground using a Vibratory Ball Mill MM200 (Retsch, Germany) for 15 min at a vibration rate of 30 s^{-1}, and then subjected to neutral sugar analysis, Klason lignin and acetyl bromide lignin determination, alkaline nitrobenzene oxidation, ozonation and methoxyl content determination.

Analysis of chemical composition

The contents of carbon and nitrogen of the extract-free finely ground samples were determined with a Perkin Elmer elemental analyzer (PE 240 CHN).

The neutral sugars were obtained by hydrolysis samples with 3% sulfuric acid (w/w) for 1 h at 121ºC after treatment with 72% sulfuric acid (w/w) for 1 h at room temperature. The neutral sugars in the hydrolysate were analyzed as their alditol acetates (BLAKENEY *et al.*, 1983) using *myo*-inositol as an internal standard by Shimadzu GC-1700 Gas Chromatograph (TC 17 capillary column: 30 m×0.25 mm id., column temperature 210ºC. Carrier gas: He).

Lignin content was determined according to Klason method (Tappi Standard T 22 om-88) with minor modifications. The sample was treated with 72% H_2SO_4 for 3 h at room temperature. The reaction mixture was diluted with water to 3% concentration of sulfuric acid, and then heated in an autoclave at 121ºC for 30 min. Insoluble fraction (Klason residue) was separated by filtration using a glass filter. Klason residue was measured gravimetrically after drying at 105ºC overnight. Acid soluble lignin was determined by absorption at 205 nm (SCHÖNING and JOHANSSON, 1965). Klason lignin content was determined as the total amount of Klason residue and acid soluble lignin.

A spectrophotometric method for analyzing lignin after dissolution with acetyl bromide was also performed (IIYAMA and WALLIS, 1990). The UV absorption spectrum was measured against a blank solution which was run in conjunction with the sample. Lignin content of sample was determined by measuring the absorbance at 280 nm using the specific absorption coefficient (SAC) of 20 g^{-1}. L. cm^{-1} for lignin. The lignin content of sample was calculated as follows:

Lignin content (%) = (absorbance/sample) / 20

Methoxyl content determination

Methoxyl content determination was carried out as described by GOTO *et al.* (2005). The methoxyl group is a major functional group of lignin, which could serve as a useful means to

provide an approximate measure of lignin content. The precursor of softwood lignin is coniferyl alcohol, whereas that of hardwood is both coniferyl and sinapyl alcohol. Coniferyl alcohol carries one methoxyl group on its aromatic ring, whereas sinapyl alcohol has two. Methoxyl contents of Klason residues were used to calculate the estimated lignin content as the following formula based on the assumption that one lignin C_6-C_3 unit (equivalent 200) carries one methoxyl group.

Assumed lignin content (%) = Methoxyl/1000 × (200/1000) × KR

where, KR is yield of Klason residue (%) and methoxyl is the methoxyl content in the Klason residue (mmol/kg).

Alkaline nitrobenzene oxidation

Aromatic feature of lignin was examined by alkaline nitrobenzene oxidation (IIYAMA and LAM, 1990). The reaction products were trimethylsilylated with N,O-bis(trimethylsilyl)acetamide (BSA). Ethyl vanillin was used as an internal standard. The products were analyzed by a Shimadzu GC-17A Gas chromatograph using NB1 capillary column (25 m × 0.25 mm id) equipped with a frame ionization detector (FID). Both injector and detector temperature were 280°C. The column temperature was kept at 150°C for 10 min, and then programmed at 5 °C min^{-1} to 250°C.

Ozonation

Ozonation analysis was carried out according to the scheme presented by AKIYAMA *et al.* (2000). The ozonation products were analyzed by a Shimadzu GC-17A Gas chromatograph using NB1 capillary column (25 m × 0.25 mm id) equipped with FID. Both injector and detector temperature were 280°C. The column temperature was kept at 120°C for 5 min, and then programmed at 4°C min^{-1} to 170°C followed by 10°C min^{-1} to 280°C. Erythritol was used as internal standard.

Pyrolysis-GC/MS

Sample (50-100 µg) was pyrolyzed for 4 sec at 500°C using a microfurnace pyrolyzer (PYR-4A: Shimadzu). The pyrolyzer was interfaced (interface temperature 270°C) with a GC/MS system consisting of a Shimadzu GC-17A Gas Chromatograph coupled to a Shimadzu QP-5000 Mass Spectrometer. NB-1 (GL Science, 30m × 0.25mm, film thickness: 0.4 µm) capillary column was used for chromatograph. Column temperature was kept at 50°C for 1 min, then programmed at 5°C min^{-1} to 270°C.

Results and Discussion

Neutral sugar composition

Total yield of neutral sugars in petiole were higher than those in leaf blade for both original and mulched samples (Table 1). While even for the petiole, total yield of neutral sugars was much lower comparing with wood meal, which usually gives 65% of neutral sugars (SUZUKI *et al.*,

1998). Of the neutral sugar composition, glucose was the major component, and the value of petiole was about two times higher than that of leaf blade. Most of glucose would be from cellulose. Yields of mannose and xylose were similar to each other both in leaf blade and petiole suggesting mannose and xylose would be from *O*-acetyl-galactoglucomannan and arabino-4-*O*-methylglucuronoxylan, respectively, which are the major hemicellulose in gymnosperms. Rhamnose, arabinose and galactose would be originated from pectic substances and the relative content of those monosaccharides in petiole was higher than those of leaf blade.

Cell wall polysaccharides (cellulose and hemicellulose) are main source of carbon and energy, and are decomposed during the early stage of decomposition (BERG, 1986). After 1 year, about 45.4% and 28.1% of cellulose were decomposed, and hemicelluloses were degraded 46.8% and 29.0% for leaf blade and petiole, respectively (Table 1). These results were in agreement with earlier observations (BERG *et al.*, 1982).

Lignin content

Klason lignin and nitrogen content of leaf blade were higher than those of petiole, and increased both for leaf blade and petiole after 16 months mulching (Table 2). The increase of Klason lignin would be due to more rapid losses of soluble substances and neutral sugar

Table 1. Neutral sugar composition of leaf blade and petiole (% of extract free samples)

	G_0 leaf blade	G_0 petiole	G_{16} leaf blade	G_{16} petiole
Rhamnose	1.9	1.6	0.7	0.9
Arabinose	3.3	4.8	1.2	2.0
Xylose	1.8	4.4	1.0	4.1
Mannose	1.8	3.0	1.4	3.1
Glucose	11.9	22.4	6.5	16.1
Galactose	2.1	3.1	1.5	1.9
Total	22.8	39.4	12.4	27.7

G_0: original; G_{16}: 16 months mulched sample

Table 2. Lignin content of leaf blade and petiole (% of extract free samples)

%	G_0 leaf blade	G_0 petiole	G_{16} leaf blade	G_{16} petiole
Klason residue	32.8	21.4	52.9	45.3
Acid soluble lignin	3.1	1.9	3.3	2.0
Total	35.9	23.3	56.2	47.3
Protein (KR)	8.1	7.5	13.8	11.3
Corrected with protein	27.8	15.8	42.4	36.0
AcBr	13.5	17.6	22.5	20.4

G_0: original; G_{16}: 16 months mulched sample
Protein (KR): Nitrogen content of Klason residue × 6.25
AcBr: Lignin content obtained by acetyl bromide procedure

components. The high value of Klason lignin in leaf blade and petiole would be due to proteins and/or polyphenolic compounds co-precipitated with lignin during H_2SO_4 treatment (NORMAN and JENKINS, 1934; IIYAMA and WALLIS, 1988). Klason method for the determination of lignin has mainly been developed for wood materials which are very low in nitrogen constituents. Therefore, when the method is applied to non-woody samples, it is essential to correct Klason lignin content by subtracting protein content (NORMAN and JENKINS, 1934). Klason lignin contents of leaf blade and petiole corrected by subtracting protein still gave very high value (Table 2).

An improved acetyl bromide procedure (IIYAMA and WALLIS, 1990) was performed to estimate lignin content of leaf blade and petiole. Lignin intermonomer linkages such as α- and β-ether and glycosidic linkages of polysaccharides are cleaved quickly during acetyl bromide treatment and dissolved in acetic acid, while protein coagulates with perchloric acid ($HClO_4$) and precipitates as insoluble matter in reaction mixture (IIYAMA and WALLIS, 1990). Lignin content is determined spectrophotometrically, therefore even if sample contains protein, lignin content do not affect by protein. Lignin content of petiole (17.6%) obtained using the improved acetyl bromide method was higher than that of leaf blade (13.5%) in original sample, while Klason lignin was high in leaf blade.

Lignin contents of petiole determined with an improved acetyl bromide procedure gave similar values with Klason lignin content corrected by subtracting protein. However Klason lignin content corrected by protein in leaf blade was higher than the value obtained by acetyl bromide procedure, suggesting unknown materials other than protein such as polyphenols still contaminate in Klason lignin of leaf blade.

Methoxyl content

Methoxyl is a major functional group of lignin, which could serves as the useful means to provide an approximate measure of the lignin content. Yield of methoxyl content in Klason residue and solid residue were shown in Table 3. Methoxyl content in Klason residue of leaf blade was lower comparing with petiole, suggesting real lignin contained in leaf blade was low despite the higher Klason lignin content. Assumed lignin content was estimated based on methoxyl content of Klason residue. Assumed lignin content was lower comparing with lignin content

Table 3. Methoxyl content (mmol/kg) and assumed lignin contents (%)

	G_0 leaf blade	G_0 petiole	G_{16} leaf blade	G_{16} petiole
Methoxyl (SR)	331.4	731.4	299.3	776.8
Methoxyl (KR)	357.9	1413.7	508.3	1450.4
Assumed lignin %	2.4	6.1	5.4	13.1

SR: solid residue, namely extract free sample
KR: Klason residue
Assumed lignin %: Calculated based on methoxyl content of Klason residue according to the formula as described in Materials and Methods section

obtained by Klason lignin method and acetyl bromide procedure indicating that Klason lignin and acetyl bromide lignin were contaminated by unknown materials other than lignin. Assumed lignin content based on methoxyl content was high in petiole (leaf blade, 2.4%; petiole, 6.1%), while Klason residue (leaf blade, 33%; petiole, 21%) was low.

Alkaline nitrobenzene oxidation

Upon alkaline nitrobenzene oxidation, normal softwoods and their lignins give rise to vanillin as major product, while hardwoods and their lignins mainly give vanillin and syringaldehyde. In addition small amount of *p*-hydroxybenzoic acid, vanillic acid, syringic acid are obtained (CHEN, 1992). Both leaf blade and petiole presented typical softwood lignin characteristics. Vanillin was the predominant product together with insignificant amounts of syringic acid and *p*-hydroxybenzaldehyde were detected from original samples (Table 4). *p*-Hydroxybenzaldehyde was not detected from leaf and petiole after 16 months mulching. A part of *p*-hydroxyphenyl nuclei of lignin may take part in condensation reaction and another portion may involve in oxidative cleavage between C_α and C_β to produce *p*-hydroxybenzoic acid during mulching.

Syringic acid is produced from syringaldehyde by cannizzaro reaction with yield of 5-10% of syringaldehyde during alkaline nitrobenzene oxidation (IIYAMA and LAM, 1990). However, syringaldehyde was not detected both in original leaf blade and petiole, suggesting syringic acid was not production of cannizzaro reaction from syringaldehyde, namely the origin of syringic acid is not syringyl lignin. The most interesting result of alkaline nitrobenzene oxidation was the detection of syringaldehyde in both mulched leaf blade and petiole. Generally softwood lignin is composed of only guaiacyl lignin, but not syringyl lignin. Syringaldehyde was not detected in both original leaf blade and petiole, suggesting syringaldehyde detected from mulched leaf blade and petiole would be contaminant of the Klason residue other than lignin.

Table 4. Yield of alkaline nitrobenzene oxidation products of leaf blade and petiole

(mmol/kg)	G_0 leaf blade	G_0 petiole	G_{16} leaf blade	G_{16} petiole
p-Hydroxybenzaldehyde	8.8	7.7	0.0	0.0
Vanillin	26.0	97.0	52.5	189.9
Syringaldehyde	0.0	0.0	6.5	13.2
p-hydroxybenzoic acid	0.0	0.0	11.1	5.9
Vanillic acid	0.0	0.0	11.5	22.3
Syringic acid	11.3	7.4	14.1	10.0
Total	46.1	112.1	95.7	241.3
Acid/adehyde ratio	0.32	0.07	0.62	0.19
Yield calculated based on				
Klason residue	2.8	10.5	3.6	10.7
AcBr lignin	6.8	12.7	8.5	23.7
Assumed lignin	38.4	36.8	35.4	36.8

Acid/aldehyde ratio increased from 0.32 to 0.62 in leaf blade and 0.07 to 0.19 in petiole, respectively (Table 4). Those acids would arise from oxidation of lignin side chain during decomposition and the increase of acid to aldehyde ratio was in agreement with earlier results (IIYAMA *et al.*, 1994).

Generally, softwood produce vanillin as major product in the yield of 24-28% based on Klason lignin (CREIGHTON *et al.*, 1944; Chen, 1992). Total yields of alkaline nitrobenzene oxidation products based on Klason residue and acetyl bromide lignin were lower comparing with wood meal, while the yield based on methoxyl content coincided with the value of wood samples (Table 4).

Ozonation

It is well known that ozone attacks lignin aromatic nuclei selectively and releases side chain portion. Arylglycerol-β-aryl ether (β-O-4) intermonomer linkage, which is the most dominant linkage in wood lignin, gives erythronic and threonic acid as ozonation products depending on its stereo structures (MATSUMOTO *et al.*, 1986). Total yield of ozonation products of petiole was higher than that of leaf blade, suggesting that β-O-4 intermonomer linkage of lignin in petiole would be higher comparing with leaf blade. The molar ratio of erythronic acid to threonic acids (E/T ratio) of leaf blade and petiole was 0.9 and 1.1, respectively, presenting softwood lignin characteristics. The *erythro*-form is the predominant stereoisomeric form of β-O-4 intermonomer linkage in hardwood lignin, while softwood lignin contains approximately equal amounts of *erythro*- and *threo*-forms of this linkage (AKIYAMA *et al.*, 2005).

Several condensed type structures (β-1, β-5, β-β, 5-5) are known to be present in lignin. β-1 and β-5 structures, which accounts for about 10-15% of linkages in wood lignin, can also be evaluated by the ozonation method (HABU *et al.*, 1988). Erythronic acid or threonic acid arises from the typical non-condensed structures of the arylglycerol-β-aryl ether structure and β-hydroxymethylmalic acid originates from typical condensed structures of β-1 and β-5 structure of lignin (MATSUMOTO *et al.*, 1986; HABU *et al.*, 1988). Therefore the ratio between the yield of β-hydroxymethylmalic acid to the total yield of erythronic acid and threonic acid can be used to

Table 5. Yield of ozonation products of leaf blade and petiole

(mmol/kg)	G_0 leaf blade	G_0 petiole	G_{16} leaf blade	G_{16} petiole
Erythronic acid	16.2	53.3	24.2	99.3
Threonic acid	17.8	48.8	28.1	97.1
Total yield	34.0	102.1	52.3	196.4
E/T	0.9	1.1	0.9	1.0
Yield calculated based on				
Klason residue	2.1	9.5	2.0	8.7
AcBr lignin	5.0	11.6	4.6	19.3
Assumed lignin	28.3	33.5	19.4	30.0

evaluate the importance of β-1 and β-5 types of condensed structures. The ratio was 0.12 and 0.07 (unpublished data) for leaf blade and petiole, respectively, indicating that β-1 or β-5 types of condensed structure are not predominant structures in leaf blade and petiole.

AKIYAMA *et al.*, (2005) reported that total yield of erythronic and threonic acids from wood meals ranged from 22 to 37 (100×mol/200g Klason lignin). Total yields of ozonation products based on Klason residue and acetyl bromide lignin were significantly low, whereas the value calculated based on assumed lignin was 28.3 and 33.5 (100×mol/200g assumed lignin) (Table 5), suggesting the assumed lignin content estimated from the methoxyl content in Klason residue gives the most reasonable approximation of the real lignin content.

Pyrogram

The total ion chromatography (pyrogram) of analytical pyrolysis products showed significant differences among mesophyll, vein and petiole (Figs 1, 2 and 3). High intensity of 4-vinylguaicol, which is particularly characterized by guaiacyl units of lignin was detected in petiole and vein, while the peak was absent from mesophyll. The pyrogram of vein showed high intensities of

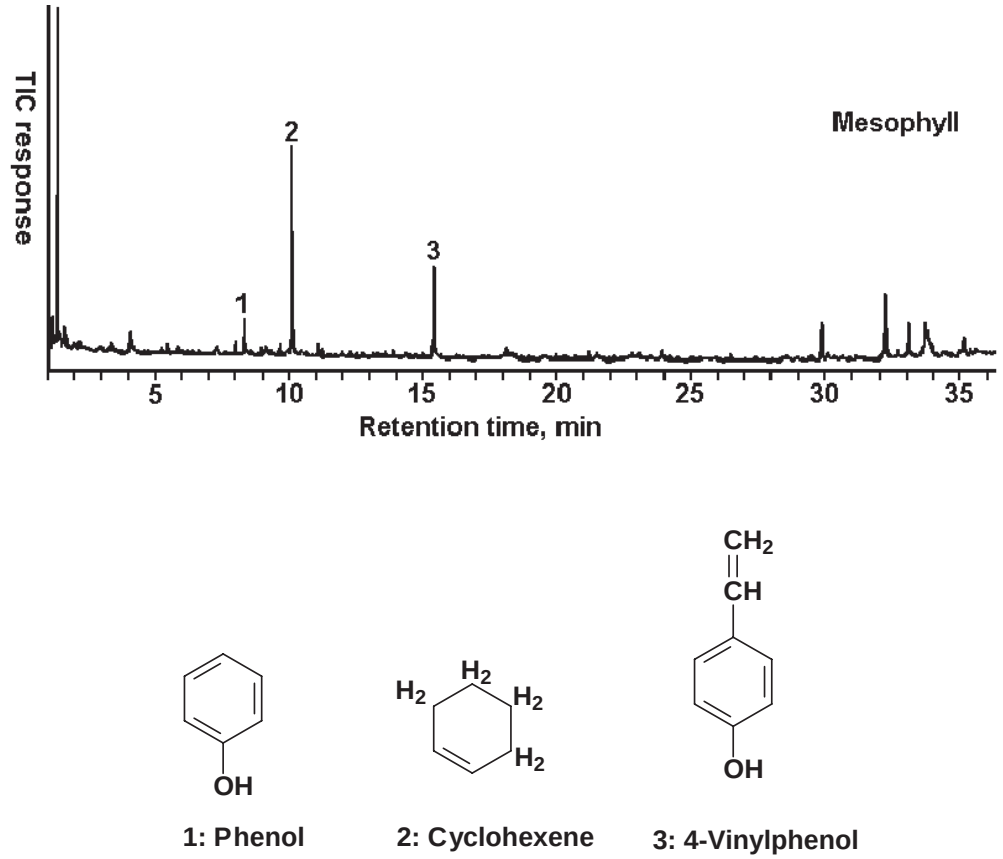

Fig. 1. Analytical pyrolysis of mesophyll fragment

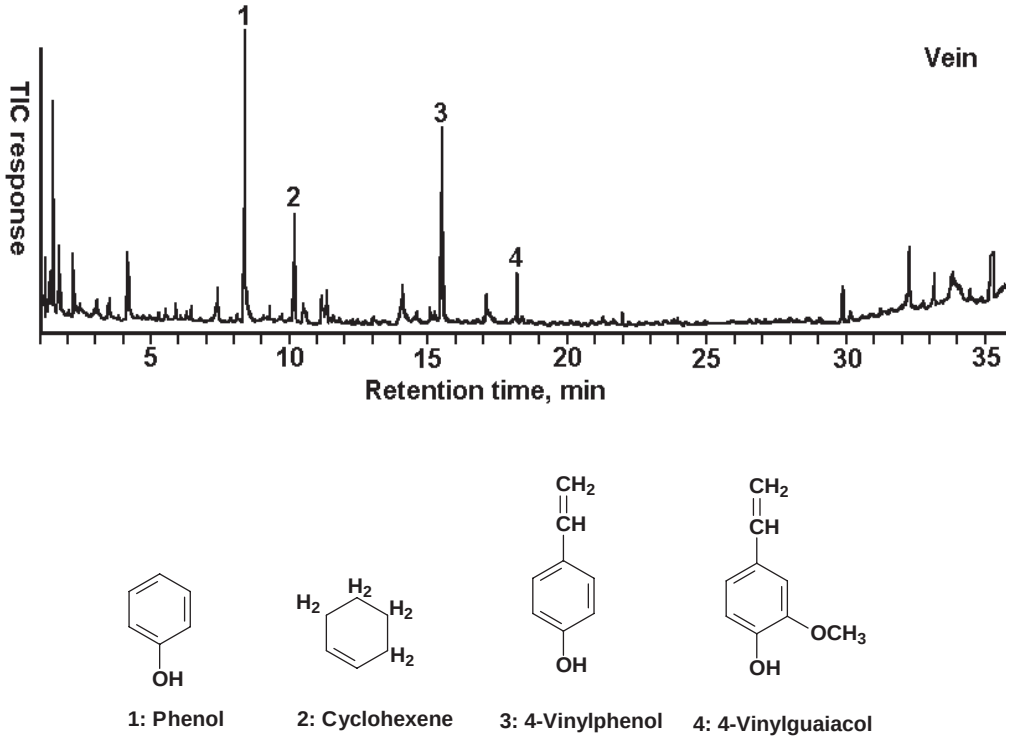

Fig. 2. Analytical pyrolysis of vein section

phenol and 4-vinyphenol, while very low intensity of 4-vinylguaicol. The pyrogram of mesophyll showed high intensity of cyclohexene together with low intensity of 4-vinyl phenol. These results were in agreement with the results of methoxyl contents of Klason residue, alkaline nitrobenzene oxidation and ozonation in leaf blade and petiole.

Conclusions

1. Total yield of neutral sugar in petiole were higher than those in leaf blade both original and mulched samples. Lignin content obtained by acetyl bromide procedure of petiole gave similar value with Klason lignin content corrected by subtracting protein content. Assumed lignin content based on methoxyl content of Klason residue was high in petiole (leaf blade, 2.4%; petiole, 6.1%), while Klason residue (leaf blade, 33%; petiole, 21%) was low.
2. Total yields of alkaline nitrobenzene oxidation and ozonation products based on Klason residue and acetyl bromide lignin were lower comparing with wood meal, while the value based on assumed lignin content coincided with wood meal. These results revealed that Klason method and acetyl bromide procedure gave an overestimated value for the lignin content of leaves.
3. The accurate content of lignin can be estimated from the methoxyl content of the Klason residue.

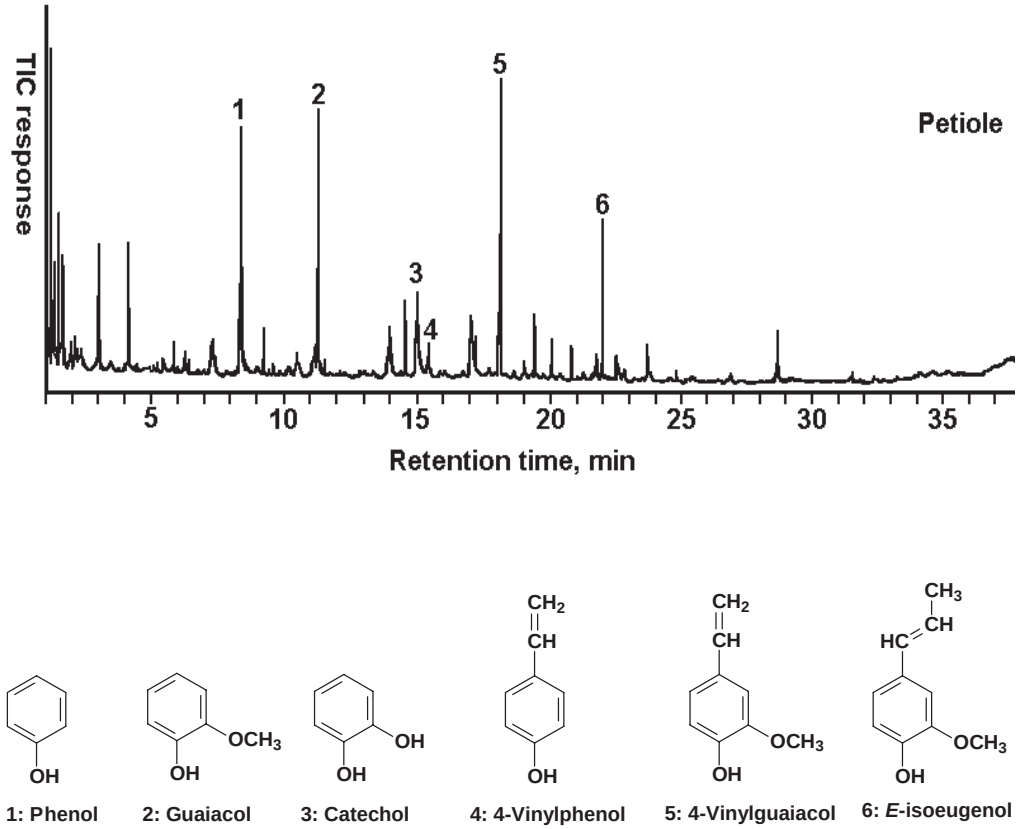

Fig. 3. Analytical pyrolysis of petiole section

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Summary

Lignin structural differences in leaf blade and petiole tissue from original and mulched leaf litter of *G. biloba* were analyzed. The total yield of neutral sugar in petiole was higher compared to that of leaf blade in both original and mulched samples. Lignin content obtained by an acetyl bromide procedure in petiole gave similar values to Klason lignin content corrected by subtracting the protein content. Total yields of alkaline nitrobenzene oxidation and ozonation products based on Klason residue and acetyl bromide lignin were lower compared to wood

samples, while the yield based on assumed lignin content coincided with the value from wood samples. These results revealed that the Klason method and acetyl bromide procedure overestimated the value for the lignin content of leaves. The accurate content of lignin can be estimated from the methoxyl content of the Klason residue.

Key words: Leaf blade, Petiole, Mulching, Lignin structural characteristics, Lignin content

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マルチング前後のイチョウ落葉の葉片および葉柄細胞壁の化学構造

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要　　旨

落葉のマルチング過程における化学成分の動態を詳しく見るためには，葉の組織ごとの化学成分の違いを考慮する必要がある。そこで，イチョウ落葉とそのマルチング処理物を，葉片と葉柄に分けて成分分析を行い，マルチングによる変化を調べた。細胞壁多糖の中性糖総量は葉片に比べ葉柄のほうが多かった。リグニンについて，Klason法に加えてアセチルブロミド法によって直接測定するとともに，メトキシル基量をもとに推定値を求めた。葉柄では Klason 残渣量をタンパク質について補正するとアセチルブロミド法の値とほぼ一致し，高い Klason 残渣量はタンパク質の混入によることが明らかとなった。しかし，葉片については Klason 残渣にタンパク質以外の成分が大量に混入していた。Klason残渣およびアセチルブロミドリグニンをベースとした葉片のニトロベンゼン酸化生成物量およびオゾン酸化によるリグニン由来の生成物も極めて少なかった。メトキシル基量をもとに計算したリグニン量推定値をベースとしたそれらの収率は葉片および葉柄ともに木粉のそれと一致する値が得られた。Klason 法およびアセチルブロミド法は葉，特に木質化してない葉片のリグニン含有量を過大評価することが明らかになった。

キーワード： 葉片・葉柄・マルチング・リグニン化学構造・リグニン含有量