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Phytochemistry 64 (2003) 1023–1031
PHYTOCHEMISTRY
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Changes in lignin content of leaf litters during mulching

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Received 26 December 2002; received in revised form 24 March 2003

Abstract

Alkaline nitrobenzene oxidation, ozonation and methoxyl content determinations were applied to decomposing leaf litter of *Ginkgo biloba* L., *Cinnamomum camphora* sieb., *Zelkova serrata* Makino and *Firmiana simplex* W. F. Wight, respectively, during mulching to investigate the properties and estimate changes in lignin composition and content . Since the Klason lignin residue originated from components highly resistant to degradation by acid, the methoxyl content of Klason residue was used to estimate the lignin content of leaf litter. Quantitative analysis of presumed lignin-derived fragments, by use of alkaline nitrobenzene oxidation and ozonation methods, suggested that the estimated lignin content approximates that of the real lignin content of leaves, which is greatly overestimated by the Klason procedure. The estimated lignin contents ranged from 3.9 to 10.0% while the Klason lignan residue varied from 37.1 to 46.7% in un-mulched leaf litter. The absolute amounts of the measured lignin somewhat decreased during mulching, while the structure of lignin remaining in leaf litters after mulching was considered not to be very different from its original structure.

Keywords: *Ginkgo biloba*; Ginkgoaceae; *Cinnamomum camphora*; Laaraceae; *Zelkova serrata*; Ulmaceae; *Firmiana simplex*; Sterculiaceae; Klason residue; Lignin; Alkaline nitrobenzene oxidation; Ozonation; Methoxyl content; Leaf litter decomposition

1. Introduction

Lignin is a decay-resistant biopolymer usually regarded as a rate-regulating factor in leaf litter decomposition (Berg and Staaf, 1980). Hence, lignin content may be used to predict the decomposition rate and weight loss of leaf litter (Meentemeyer, 1978; McClaugherty and Berg, 1987; Salamanca et al., 1998). Surprisingly, however, there is no adequate analytical method to measure lignin content, especially in the field of leaf litter decomposition (Theander and Westerlund, 1993). While the Klason procedure and acid detergent fiber (ADF) methods (Van Soest, 1963) are the most widely used methods in quantitative determination of leaf litter lignin (Table 1), these methods cannot discriminate between true lignin and lignin-like materials (Salamanca et al., 1998; Johnsson et al., 1986). That is, Klason lignin and ADF lignin may include both lignin and other non-hydrolyzable products (Salamanca et al., 1998). The aim of this paper therefore is to investigate the properties of lignin in decaying leaf litter and to estimate the lignin content of leaf litter during mulching, using alkaline nitrobenzene oxidation, ozonation (Matsumoto et al., 1986; Akiyama et al., 2000) and methoxyl content determination, all of which have been applied here to decomposing leaf litter.

2. Results and discussion

2.1. Total mass loss and yield of Klason residue during mulching

The term "Klason residue" instead of "Klason lignin" was used because the residue obtained by the Klason

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doi:10.1016/S0031-9422(03)00423-0

Table 1
Methods for lignin content determination of tree leaf litter

Tree species	Method	Comment of authors on the method	Authors
Acer saccharum *Populus grandidentata* *Quercus alba* *Pinus strobus* *Tsuga canadensis*	Klason procedure	Acid insoluble fraction	McClaugherty et al. (1985)
Quercus serrata	ADF	Lignin and non-hydrolyzable products	Salamanca et al. (1998)
Ceanothus megacarpus *Salvia mellifera*	Klason procedure	Lignin and other resistant compounds	Schlesinger and Hasey (1981)
Pinus silvestris *Betula pubescens*	Klason procedure	Lignin and humification products	Berg and Wessén (1984)
Carya glabra *Quercus alba*	ADF	Protein being complexed by polyphenols and/or lignin-like compounds	Suberkropp et al. (1976)
Picea abies *Pinus sylvestris*	Klason procedure	Lignin and humic substances	Johansson et al. (1986)

procedure seemed to contain significant amounts of non-hydrolyzable products other than lignin.

Mass loss of leaf litter ranged from 30.5 to 52.7% after 1 year of mulching (Fig. 1). A rapid mass loss was observed in *Ginkgo biloba* leaf litter followed by those of *Cinnamomum camphora* and *Firmiana simplex*. Mass loss in *Zelkova serrata* leaf litter was the least, with 69.5% mass remaining after 1 year of mulching. Previous studies indicated that decomposition of litter is mainly governed by the rate of lignin decomposition (Berg, 1986). Our present results also showed that Klason residue of leaf litter originated from components resistant to mulching. An absolute increase in the Klason residue of leaf litter at the early stage of mulching was observed (Fig. 2). When other methods (Table 1) are applied to lignin determination, an initial absolute increase in non-hydrolyzable products of leaf litter has also been noted by several researchers (Salamanca et al., 1998; Schlesinger and Hasey, 1981; Johansson et al., 1986). This phenomena has been attributed to increased contamination from non-hydrolyzable materials other than lignin.

2.2. Estimation of lignin content as assumed lignin

The most widely used method for quantitative determination of leaf litter lignin has been the Klason procedure, which affords an insoluble residue from hydrolysis with sulfuric acid which is measured gravimetrically (Table 1). When applied to leaf litter, values between 40 and 50% Klason residue were obtained (Fig. 3). Leaf litter, which is not extensively lignified, is not likely to contain 40–50% lignin. Hence, the Klason residue of the leaves could not have originated solely from lignin. In order to confirm this point, alkaline nitrobenzene oxidation, ozonation and methoxyl content determinations were performed on leaf litter of *G. biloba*, *C. camphora*, *Z. serrata* and *F. simplex*, and the results were compared with those of the Klason residue(s).

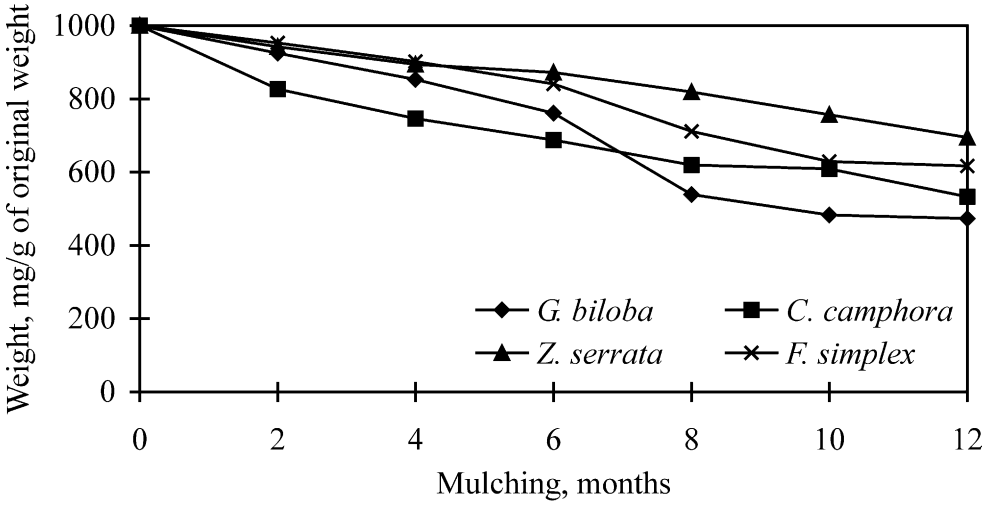

Fig. 1. Mass of leaf litter remaining after mulching.

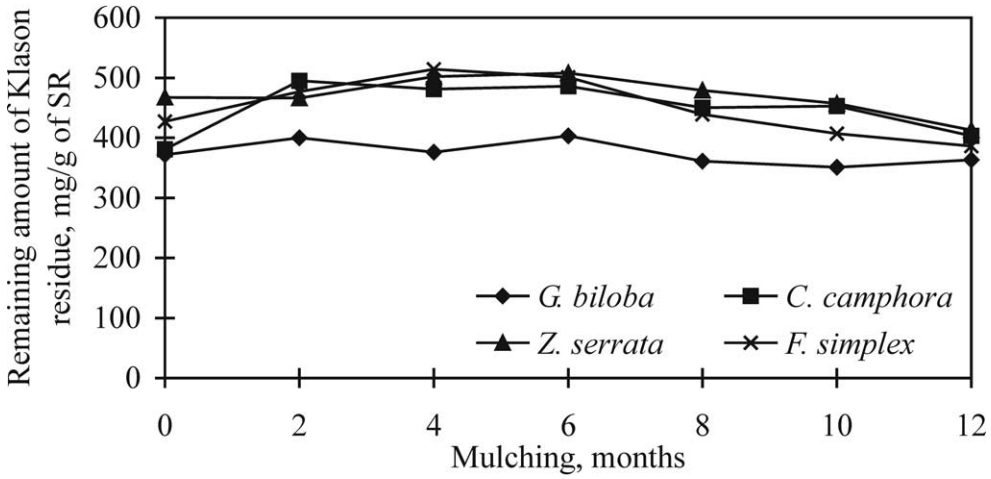

Fig. 2. Changes in absolute amount of Klason residues of leaf litter during mulching; SR = solid residue.

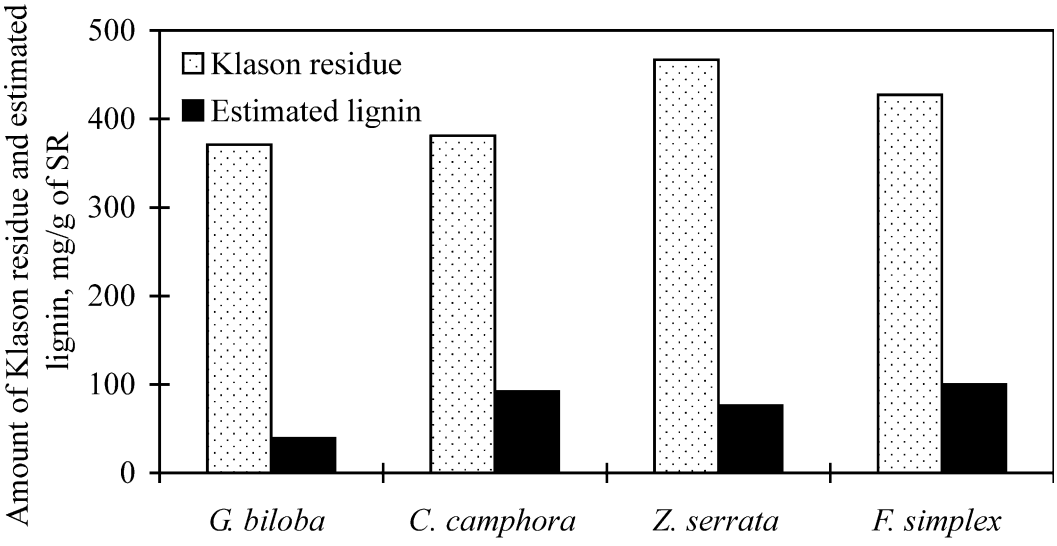

Fig. 3. Initial amount of Klason residues and estimated lignin contents; SR = solid residue.

The methoxyl group is a major functional group of lignin, which could serve as a useful means to provide an approximate measure of the lignin content. The precursor of gymnosperm lignin is coniferyl alcohol, whereas that of angiosperm is both coniferyl and sinapyl alcohol. Coniferyl alcohol carries one methoxyl group on its aromatic ring, whereas sinapyl alcohol has two. The contribution of *p*-hydroxycinnamyl alcohol, which has no methoxyl group, as the lignin precursor is usually very small in wood lignin. If it is assumed that Klason residues originate from lignin with similar characteristics to wood lignin, whose lignin units have more than one methoxyl group in the aromatic ring on average, then the methoxyl content of Klason residue must be more than 5000 mmol/kg. For example, the methoxyl content of Klason residue from birch wood meal approximates 5772.9 mmol/kg (unpublished data). However, when the methoxyl content in Klason residue of leaf litter was analyzed it was much lower than the value of birch wood meal (Table 2), ranging from 519.3 to 1217.0 mmmol/kg,

The low methoxyl content of the Klason residue suggested the following possibilities about leaf-tissue lignin.

1. The structural characteristics of leaf litter lignin are similar to those of wood lignin and the real content of lignin in leaf litter is much lower than the yield of Klason residue.
2. The main precursor of leaf lignin is *p*-hydroxycinnamyl alcohol and, thus, the lignin contents in leaf litter are much higher than those suggested from the methoxyl content in Klason residue.

If possibility 1 was the case, the real content of lignin can be approximately estimated on the basis of the methoxyl content of the Klason residue. When the methoxyl content of the Klason residue was converted to predict the lignin content with the solid residue,

Table 2
Methoxyl content and estimated lignin contents

	G. biloba	*C. camphora*	*Z. serrata*	*F. simplex*
MeO (mmol/kg)[a]	519.3	1217.0	806.4	1174.2
Klason residue (% SR[b])	37.1	38.1	46.7	42.7
Estimated lignin contents[c] (% SR)	3.9	9.2	7.6	10.0

[a] Methoxyl content of Klason residue.
[b] SR = solid residue.
[c] Calculated based on MeO according to the formula in Section 4.2.5.

according to the formula shown in Section 4, it ranged from only 3.9 to 10.0% among the various leaf litter, whereas the Klason residues varied from 37.1 to 46.7%. Thus, the predicted estimated lignin content of leaf litter was much lower than that of the Klason residue (Fig. 3).

The second possibility can be further classified into the following two possibilities.

(a) The main precursor of leaf lignin is *p*-hydroxycinnamyl alcohol but the linkages connecting precursors are wood lignin type.
(b) The main precursor of leaf lignin is *p*-hydroxycinnamyl alcohol and the linkages connecting precursors are quite different from wood lignin type.

If either possibility (a) or (b) is the case, the low methoxyl content in the Klason residue does not necessarily mean low lignin content in leaf litter. However, possibility (a) can be examined by analysis of lignin structure. For this purpose, alkaline nitrobenzene oxidation and ozonation methods were applied to the analysis of leaf litter lignin.

Among alkaline nitrobenzene oxidation products (Table 3), vanillin was the most abundant in *G. biloba* leaf litter, together with small amounts of *p*-hydroxybenzaldehyde, syringaldehyde and their oxidation products. Leaf litter of *C. camphora*, *Z. serrata* and *F. simplex* gave rise to vanillin and syringaldehyde as the major products together with small amounts of *p*-hydroxybenzaldehyde and other minor oxidation products. Although *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid were detected, the yields were very low. The total yields of alkaline nitrobenzene oxidation products from solid residue of original leaf litter ranged from 73.1 (*G. biloba*) to 162.3 mmol/kg (*F. simplex*) (Table 3) and those values were much lower than that of birch wood meal (577.1 mmol/kg). The low yield of *p*-hydroxybenzaldehyde seems to exclude possibility (a). However if the *p*-hydroxyphenyl unit in leaf lignin is mainly present as a condensed structure, possibility (a) cannot be excluded completely because in the latter case it could not contribute to formation of *p*-hydroxybenzaldehyde.

Several condensed type structures are known to be present in lignin, such as β-5 (phenylcoumaran), β-1 (diarylpropane), β-β (pinoresinol) and 5-5 (biphenyl). Among these condensed type structures, the relative importance of β-1 and β-5 structures can also be evaluated by the ozonation method. It has been generally accepted that the arylglycerol-β-aryl ether structure is the most important structural unit in wood lignin (Matsumoto et al., 1986), which accounts for about 50% of wood lignin, whereas β-1 and β-5 accounts for about 10–15% of the linkages in wood lignin. Among several ozonation products (Table 4), erythronic acid or threonic acid arises from the typical non-condensed structures of the arylglycerol-β-aryl ether structure (Matsumoto et al., 1986) and β-hydroxymethylmalic acid originates from typical condensed structures such as the β-1 or β-5 structure of lignin (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 evaluate the importance of β-5 and β-1 types of condensed structures in leaf litter lignin. These ratios were found to be 0.12, 0.13 and 0.06 for *G. biloba*, *C. camphora* and *F. simplex* leaf litter and this was similar to that of wood lignin (0.10) (unpublished data), indicating that β-5 or β-1

Table 3
Yield of alkaline nitrobenzene oxidation products of solid residue in original leaf litter

	SR[a] (mmol/kg)			
	G. biloba	*C. camphora*	*Z. serrata*	*F. simplex*
p-Hydroxybenzaldehyde	9.8	4.7	4.1	5.8
Vanillin	36.2	37.6	54.6	50.8
Syringaldehyde	4.4	23.4	32.9	72.6
p-Hydroxybenzoic acid	3.6	5.1	2.9	14.1
Vanillic acid	5.9	3.7	8.3	6.3
Syringic acid	13.1	8.5	21.7	12.7
Total yield	73.1	83.0	124.5	162.3
S/*V* ratio	0.12	0.62	0.6	1.43

[a] SR = solid residue.

Table 4
Yield of ozonation products of solid residue in original leaf litter

	SR[a] (mmol/kg)			
	G. biloba	*C. camphora*	*Z. serrata*	*F. simplex*
Erythronic acid	17.8	64.8	49.2	43.1
Threonic acid	18.9	43.7	38.7	47.7
Total yield	36.7	108.5	87.9	90.8
E/*T* ratio	0.9	1.5	1.3	0.9
Area ratio[b]	0.11	0.13	–[c]	0.06

[a] SR = solid residue.
[b] Area ratio of β-hydroxymethylmalic acid to total yield of erythronic and threonic acids.
[c] Not detected.

types of condensed structure are not predominant structures in leaf litter. This suggests that lignin structure in leaf tissue is not special from the view point of condensed/non-condensed ratio.

Since the structural characteristics of leaf litter lignin, as clarified by alkaline nitrobenzene oxidation and ozonation methods, excluded possibility (a), only possibility (b) remained to be examined. But the structure suggested by possibility (b) is that leaf lignin is mainly composed of the *p*-hydroxyphenyl unit connected together by 5-5 (biphenyl) linkages. Such lignin, if present, should be defined as polyphenols other than lignin. Hence, based on the arguments outlined in this section, the assumed lignin content estimated from the methoxyl content in Klason residue gives the most reasonable approximation of the real lignin content in leaf litters.

Table 5
Ratio of methoxyl groups found in Klason residue, to methoxyl groups found in solid residue

Mulching (months)	*G. biloba*	*C. camphora*	*Z. serrata*	*F. simplex*
0	0.48	0.77	0.62	0.73
4	0.56	0.73	0.69	1.07
8	0.86	0.81	0.70	0.85
12	0.89	0.79	0.71	0.86

2.3. Changes in content and structural characteristics of leaf litter lignin during mulching

Methoxyl groups in the Klason residue accounted for 48, 77, 62 and 73% of the total methoxyl groups in the solid residue in original leaf litters for *G. biloba*, *C. camphora*, *Z. serrata* and *F. simples*, respectively (Table 5). Presumably, the rest of the methoxyl groups not found in Klason residues came from other cell wall components, such as lignans, stilbenes and carbohydrates,

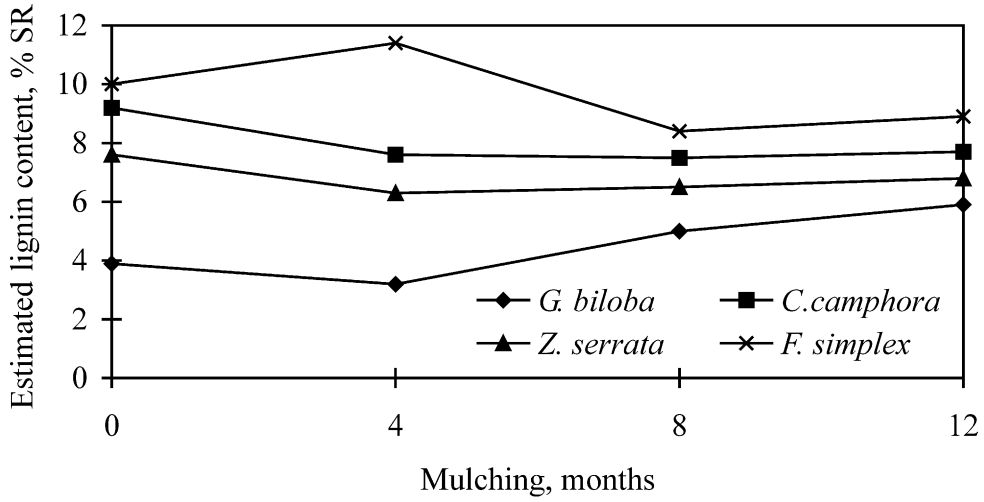

Fig. 4. Changes in absolute amount of estimated lignin contents during mulching; SR = solid residue.

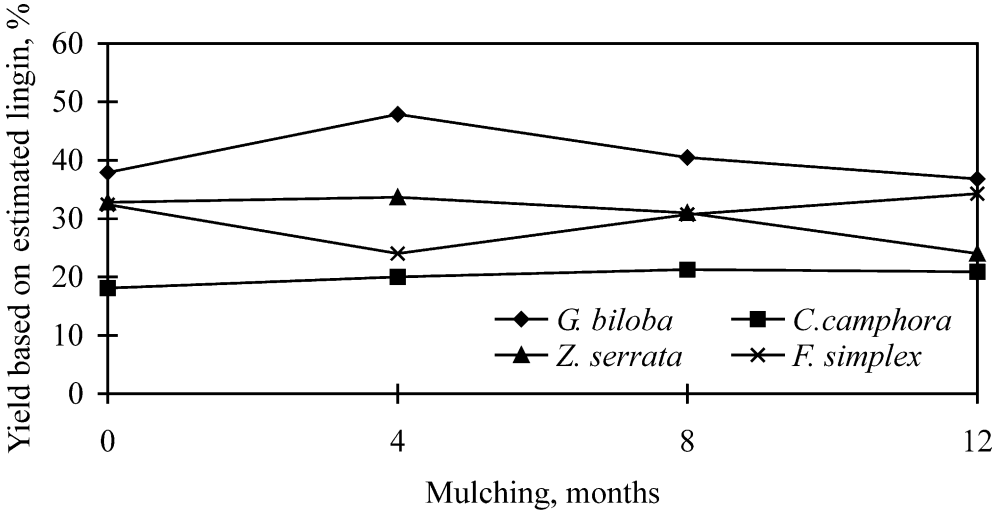

Fig. 5. Changes in yields of alkaline nitrobenzene oxidation products based on estimated lignin contents during mulching.

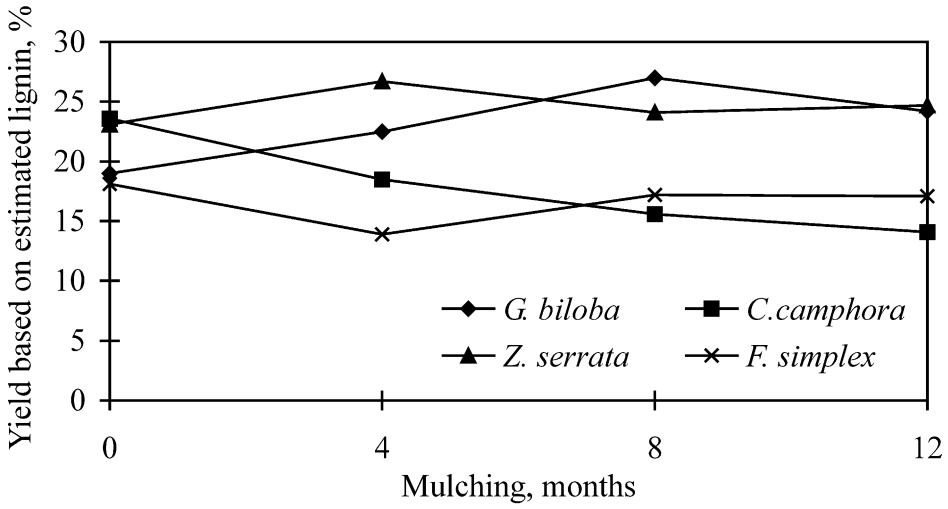

Fig. 6. Changes in yields of ozonation products based on estimated lignin contents during mulching.

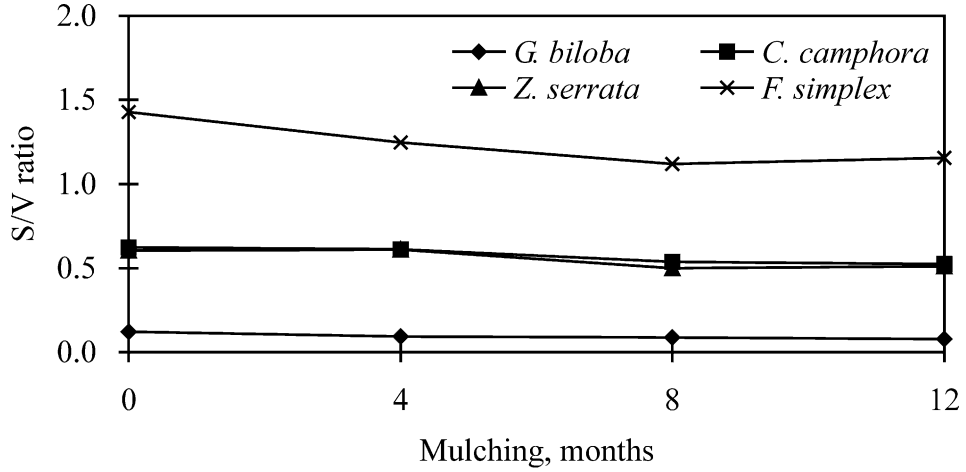

Fig. 7. Changes in S/V ratio during mulching.

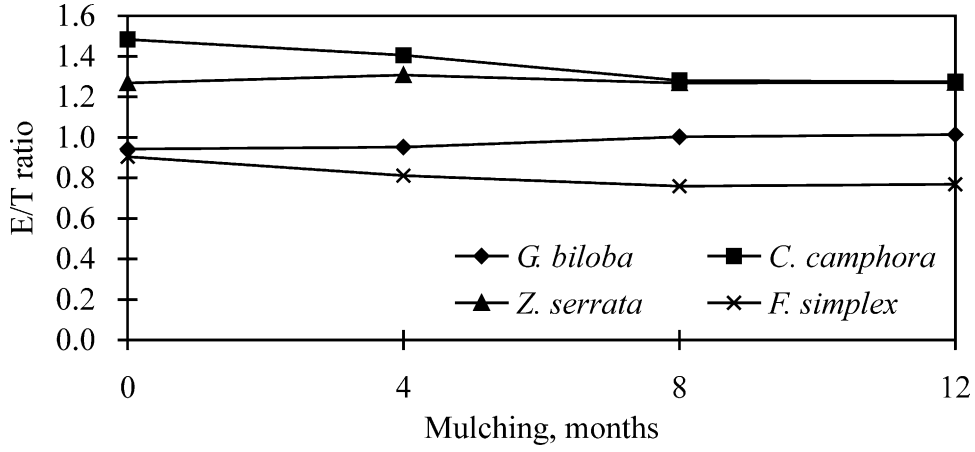

Fig. 8. Changes in E/T ratio during mulching.

which would also generate methyl iodide quantitatively during HI treatment. The ratio of methoxyl groups found in the Klason residue to those found in whole solid residue increased during mulching (Table 5). These results revealed that cell wall components other than lignin participated in the formation of methyl iodide to a great extent during an earlier stage of decomposition. The loss of methoxyl groups at the early stage of decomposition may be caused by rapid decreases in cell wall polysaccharide and extracts as was reported in our previous study (Jin et al., 2002).

Absolute amount of estimated lignin contents of *C. camphora*, *Z. serrata* and *F. simplex* decreased to some extent while for *G. biloba* the value increased slightly during mulching (Fig. 4). Yields of both alkaline nitrobenzene oxidation and ozonation products based on the assumed lignin content were almost constant throughout the mulching period (Figs. 5 and 6). In addition, molar ratios of syringaldehyde to vanillin (S/V ratio) (Fig. 7) and erythronic acid to threonic acid (E/T) (Fig. 8) were relatively constant during mulching.

It is interesting to note that absolute amounts obtained from the methoxyl group estimation of Klason residue did not decrease significantly during the time period of mulching. This result suggests that oxidation of lignin aromatic rings, either chemically or biochemically did not occur during the mulching conducted in this experiment.

3. Conclusions

Four conclusions were obtained from this work:

1. The Klason residue is representative of undegradable material of the solid residue, and not necessarily lignin.
2. The accurate content of lignin can be estimated from the methoxyl content of the Klason residue.
3. The estimated lignin content is much lower than the Klason residue, and its content in the solid residue remains almost constant throughout mulching.
4. The yields of alkaline nitrobenzene oxidation and ozonation products, based on estimated lignin levels, was almost constant throughout mulching, suggesting that the structure of lignin remaining in leaves after mulching is not very different from the original structure.

4. Experimental

4.1. Leaf litter collection and mulching experiment

Fallen leaves of *G. biloba*, *C. camphora*, *Z. serrata* and *F. simplex* were collected from the campus of the University of Tokyo. Leaves of *G. biloba*, *F. simplex*, *Z. serrata* were collected in early winter of 1999. *C. amphora* is an evergreen tree species and the leaves were collected in April 2000. The mulching experiment was conducted at the experimental field of the University of Tokyo (latitude 35°41′, longitude 139°46′) from December of 1999 for *G. biloba*, *Z. serrata* and *F. simplex* and April of 2000 for *C. camphora*. The litter bag technique was used to quantify decomposition rates. Litter bags (20×20 cm^2) made of nylon net with a mesh size of 0.1 mm were prepared. The samples were collected at 2-month intervals up to 1 year. The samples were cleaned of foreign materials and dried at room temperature then weighed. The moisture content of each sample was calculated after it was dried overnight at 105 °C.

4.2. Chemical analyses

Air-dried samples were milled in a Wiley mill to pass a 420 μm sieve, and extracted with boiling ethanol H_2O (4:1) (three times for 1 h) followed by water extraction overnight at room temperature. Extract-free residues (solid residue) were separated from inorganic solids (sands and stone particles) by a sedimentation procedure using CCl_4–CH_2Br_2 (1:1, s. g. ca 2.0) (Iiyama et al., 1995).

4.2.1. Klason residue

Klason residues of extractive-free samples (solid residue) were determined gravimetrically by the TAPPI Standard T 222om-88 used for determination of Klason lignin content.

4.2.2. Ozonation

Ozonation analysis was carried out according to the scheme presented by Akiyama et al. (2000). The ozonation method gives erythronic acid from the *erythro* type and threonic acid from the *threo* type of the typical non-condensed structure of arylglycerol-β-aryl ether structure and gives β-hydroxymethylmalic acid from the typical condensed structure such as β-1 or β-5 structure of lignin (Fig. 9).

On the basis of the yields of erythronic and threonic acids, the ratio of *erythro* to *threo* type (E/T ratio) can be measured. In addition, the ratio of the yield of β-hydroxymethylmalic acid to the total yield of erythronic and threonic acids was used to evaluate the importance of condensed structures in leaf litter lignin.

4.2.3. Alkaline nitrobenzene oxidation

The aromatic feature of leaf litter lignin was examined using the alkaline nitrobenzene oxidation as reported by Iiyama and Lam (1990). Upon alkaline nitrobenzene oxidation, normal softwoods (Gymnospermae) and their lignins give rise to vanillin as the major product, where hardwoods (Angiospermae) and their lignins

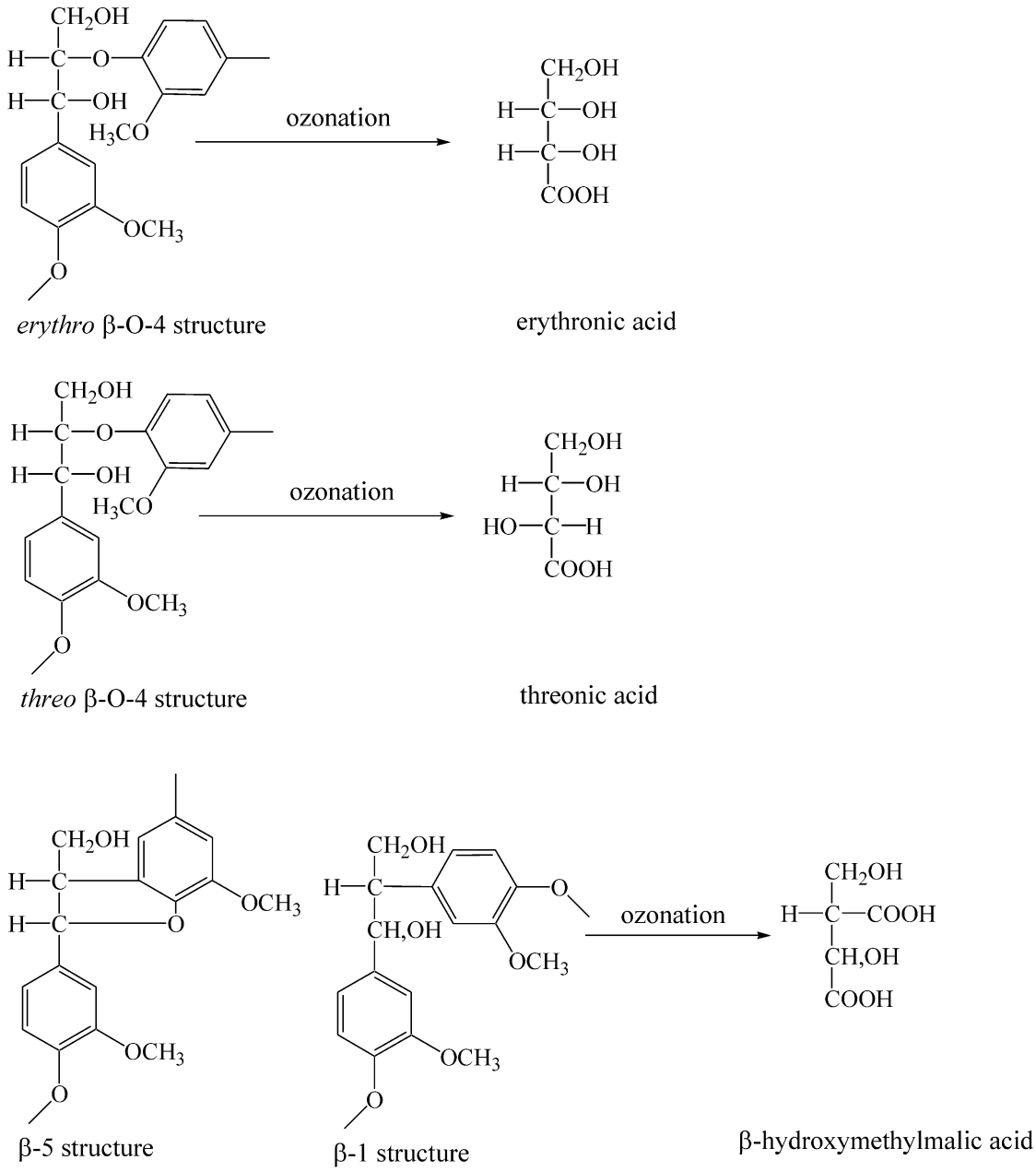

Fig. 9. Ozonation products.

mainly give vanillin and syringaldehyde. In addition, small amounts of *p*-hydroxybenzaldehyde and other minor oxidation products such as *p*-hydroxybenzoic acid, vanillic acid, syringic acid are obtained (Chen, 1992). The syringaldehyde/vanillin (*S*/*V*) ratio was used to investigate the structure of leaf litter lignin.

4.2.4. Methoxyl content determination

Samples (20 mg) were individually soaked in 5 ml of concentrated HI (57%). The mixture was kept in a heating block for 30 min at 130 °C and then cooled with ice. Iodoethane (60 mg in 20 ml CCl_4; 1 ml) was added as an internal standard, followed by 5 ml of CCl_4. After mixing well, an aliquot (1 ml) was taken from the organic layer and dried (anhyd Na_2SO_4). The products were analyzed using a CP-Sil 13 CB capillary column chromatography on a Shimadzu GC-14B system equipped with a flame-ionization detector. The injector temperature was 200 °C, the detector temperature was 230 °C, and column temperature was 40 °C for 5 min, then programmed at 10 °C min^{-1} to 180 °C. The injection quantity was 1 μl (Goto et al., 2000).

The reaction mechanism of the methoxyl group determination method is shown in Fig. 10.

4.2.5. Calculation of assumed lignin content

Methoxyl contents of Klason residues were used to calculate the estimated lignin content by the following

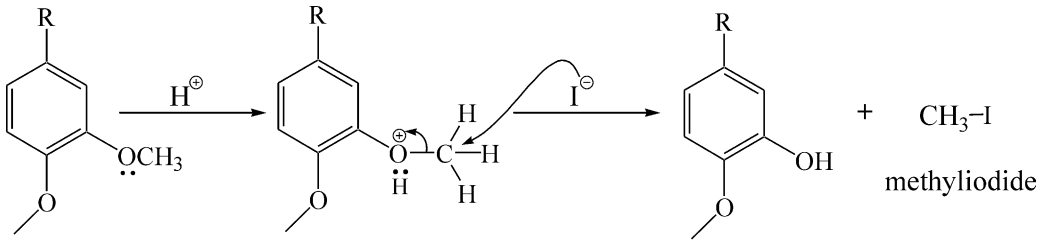

Fig. 10. Reaction mechanism of methoxyl content determination method.

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).

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