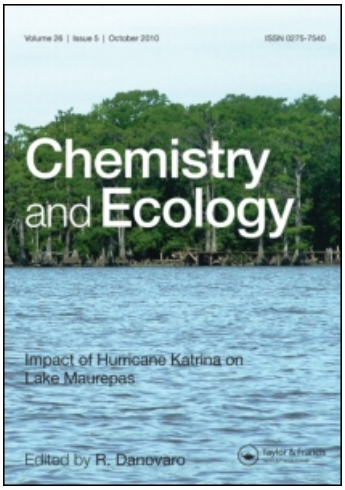

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Comparative studies of grass compost lignin and the lignin component of compost humic substances

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Comparative studies of grass compost lignin and the lignin component of compost humic substances

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The aim of this research was to compare the structural features of lignin and alkali-soluble humic substances isolated from grass compost at different stages of composting, and to estimate the applicability of basic analytical techniques used in lignin chemistry for characterising the lignin structures transformed during composting. Elemental, functional analyses, alkaline nitrobenzene oxidation, analytical ozonation and FT-IR techniques were applied. Compost Björkman lignins were used as reference samples. FT-IR of the compost Björkman lignins was typical for guaiacyl–syringyl lignins. The humic substances contained methoxyl groups and gave the same products as Björkman lignins by nitrobenzene oxidation and ozonation. The lignin component of humic substances, calculated on the basis of chemical markers, was lower than that for the Klason procedure and decreased in following order: methoxyl group > nitrobenzene oxidation products > ozonation products. Methoxyl groups are recommended for lignin content evaluation in compost because of their higher stability in the compost environment among the chemical markers under study. Our results showed that lignin macromolecules were already sufficiently modified at the early stage of composting to be dissolved in alkali. Modified lignin constituted a significant part of grass compost humic substances.

Keywords: grass compost; lignin; humic substances

1. Introduction

Grass-dominated ecosystems comprise approximately one third of the Earth's vegetative cover [1], and the use of grass biomass could play an important role in soil fertility management. Based on analyses of 300 plant species, Palm et al. [2] suggested that different uses of plant biomass depend on the N and lignin content. It was suggested that composting should be used in the case of a sufficient N content and high lignin content, which makes provision for grass resistance to microbial degradation [2].

Composting is one of the most promising treatment methods for solid organic waste and may be considered as a biomimetic process in the formation of humic substances (HS) in soil at the early stages. Many studies have focused on lignin degradation by white-rot fungi [3], but little attention has been paid to the main lignin degraders within a compost environment, i.e. thermophilic microfungi and actinomycetes [4]. Lignin is not totally mineralised during composting. The

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ultimate goal in composting is to produce humus-like products, and lignin fragments become the building blocks for compost HS, which makes the evaluation of lignin degradation in compost difficult. There is a constant search for chemical methods that may be related to the decomposition state of the compost and the chemical changes that occur during the transformation of organic matter while being composted. According to Tuomela et al. [5], 12–14% of the radiolabelled lignin ^{14}C-DHP was bound to humic and fulvic acids and 30–39% to humin after 45-day composting experiments. With increasing lignin content, the biodegradation of polysaccharides decreased. A linear correlation between lignin content and biodegradation has been found in pulp and paper products [6].

Many studies clearly demonstrate that lignin biodegradation is monomer specific in the soil environment. The turnover kinetics of lignin-derived guaiacyl moieties is slower than that of syringyl and *p*-hydroxyphenyl moieties [7]. The chemical composition of grass lignin differs from that of hardwood and softwood lignins and undergoes biodegradation more easily [3]. Grass lignin is composed of all three phenylpropanoid units: guaiacyl, syringyl and *p*-hydroxyphenyl, which are interconnected through ether and C−C bonds and contain ester- and ether-linked hydroxycinnamic acids: *p*-coumaric and ferulic acid [8].

Stevenson wrote that the biochemistry of HS formation is one of the least understood aspects of humus chemistry and one of the most intriguing [9]. Lignin plays an important role in terrestrial HS formation; however, the mechanisms of this process have not been completely elucidated [9,10]. Therefore, the objectives of this study were to characterise grass compost lignin and the structural features of the lignin components of the HS fraction by the analytical tools used in lignin chemistry, with the purpose of revealing the applicability of these methods and gaining new knowledge about the changes in lignin during composting and its transformation to HS.

2. Materials and methods

2.1. *Materials*

Samples of grass compost were kindly provided by the Itako compost yard of Nihon Doro Kodan (the Japan Highway Public Corporation, Ibaraki Prefecture, Japan). Grasses were collected from slopes beside highways. The major raw materials of the compost were common grasses growing in Japan such as *Phalaris* spp., *Cynodon* spp. and *Eleusine* spp. Composting was carried out in open compost piles, where the composting conditions such as aeration and moisture were not controlled. Samples were taken at different stages of composting, namely, a 2-week-old compost at the thermophilic stage (compost I) and a 2-month-old final compost (compost II). Grass compost samples were stored at −20 °C. Before analysis, samples were freeze-dried and ground using a Wiley mill to pass a 420 μm sieve. The ground samples were successively extracted by boiling in 80% ethanol for 1 h (three times) then kept overnight with water (30 °C, with shaking). Extract-free compost was used for the isolation of HS (Figure S1, available online only).

2.1.1. *Isolation of alkali-soluble humic substances*

Soluble HS were extracted and purified following procedures recommended by the International Humic Substances Society (IHSS) using 0.1 M NaOH from the extract-free compost [11]. HS from composts I and II were designated as HS I and II, respectively.

2.1.2. *Isolation of Björkman lignin*

Following the isolation of HS, the compost was milled without solvent for 96 h using a vibratory ball mill, cooled (< 25 °C) by water flow. Björkman lignin was extracted with dioxane/water

(9:1) and purified following the procedure described by Björkman [12]. Björkman lignin from composts I and II was designated as lignin I and II, respectively.

2.2. *Analytical methods*

Elemental analyses were performed with a CE instrument Flash EA1113 (Thermo Quest, Italy). Ash content was determined by the combustion of samples at 700 °C for 3 h.

Neutral sugar composition was determined using an alditol acetate procedure [13] in the composts, lignins and HS after treatment with 72% sulphuric acid for 1 h at room temperature; the acid was diluted with water to give 4% sulphuric acid and heated at 121 °C for 1 h. Alditol acetates were quantified by gas chromatography (Shimadzu GC18A, column TC17) using myo-inositol as an internal standard. The total amount of sugars was expressed as a sum of the obtained rhamnose, arabinose, xylose, mannose, galactose and glucose.

The lignin content of the extract-free compost was determined using an acetyl bromide method and Klason procedure supplemented with determination of acid-soluble lignin. Klason lignin was determined gravimetrically. Acid-soluble lignin was determined spectrophotometrically using the absorption coefficient $\varepsilon = 110\,\mathrm{L} \cdot \mathrm{g}^{-1} \cdot \mathrm{cm}^{-1}$ at 205 nm [14]. Determination of lignin using the acetyl bromide method was carried out using with an absorption coefficient $\varepsilon = 20\,\mathrm{L} \cdot \mathrm{g}^{-1} \cdot \mathrm{cm}^{-1}$ at 280 nm [15]. UV spectra were recorded using a Hitachi Spectrophotometer U3010 ($b = 1.000$ cm).

Determination of the methoxyl group content in composts, lignins and HS was carried out using the classical Zeisel–Viebock–Schwappach method with 57% hydroiodic acid (HI). The methyl iodide formed was determined by gas chromatography (Shimadzu GC14, column CP7506) using ethyl iodide as an internal standard [16].

p-Coumaric and ferulic acids in the lignins and HS were determined by gas chromatography (Shimadzu GC17, column NB1) as trimethylsilyl derivatives after their release by alkaline hydrolyses with 4 M NaOH at 170 °C for 2 h [17].

The aromatic constituents of lignin were examined using an alkaline nitrobenzene oxidation procedure [18]. Lignins and HS were oxidised with nitrobenzene in 2 M NaOH at 170 °C under an N_2 atmosphere for 2 h. The lignin-derived phenolic acids and aldehydes were quantified by gas chromatography (Shimadzu GC17, column NB1) as trimethylsilyl derivatives using ethylvanillin as an internal standard.

Ozonation was carried out using a method described by Akiyama et al. [19]. Lignins and HS were suspended in a mixture of solvents (acetic acid/water/methanol 16:3:1), and oxygen gas containing $\sim$ 3% ozone was bubbled into the mixture at 0 °C for 2 h. The tetronic acids formed were quantified by gas chromatography (Shimadzu GC17, column NB1) as trimethylsilyl derivatives using erithrol as an internal standard.

Table 1. Chemical composition of grass mixture and grass composts (% dry matter).

	Grass mixture	Compost I	Compost II
C	39.3	37.6	28.5
N	1.1	2.0	1.7
C/N ratio	36	19	17
Ash	16.0	27.7	38.0
Total sugars (%)[a]	58.2	24.7	12.4
Klason lignin	13.3	24.0	45.6
Methoxyl group	1.04	2.02	2.21

Note: [a]Sum of rhamnose, arabinose, xylose, mannose, galactose and glucose.

The potentiometric titration of lignins and HS was carried out using a Hiranuma Comtite Auto Titrator COM 550. All measurements were carried out under an N_2 atmosphere.

Analytical pyrolysis of the lignins was performed using a micro-furnace pyrolyser (PYR4A, Shimadzu) at 500 °C. The products of pyrolysis were identified using GCMS (Shimadzu GC17/QP5000 system, column NB1).

Fourier transform infrared (FT-IR) spectra of lignins and HS were recorded in KBr pellets by a Jasco FT/IR615 (resolution: 4 cm^{-1}, number of scans: 64).

3. Results and discussion

3.1. *Chemical composition of grass compost*

The results of elemental analysis, and the amounts of polysaccharide, lignin and methoxyl groups in the parent grass mixture and grass composts following 2 weeks and 2 months of composting are presented in Table 1. The C/N ratio is often used to estimate compost maturity. At the beginning of composting, the C/N ratio of the grass mixture was 36, and it decreased rapidly to 19 during the first 2 weeks of composting and then continued to decrease slightly to 17. The polysaccharide content decreased rapidly at the early stages of composting. This indicates that polysaccharides serve as a source of easily degradable carbon. The relative amount of ash increased with the mineralisation of organic compounds.

Determination of the lignin content was carried out using two independent methods. An acetyl bromide UV spectroscopic method has been recommended for lignin determination in herbaceous plants [14]. However, absorption of the solution obtained after digestion of the compost sample was very high and therefore use of the specific absorption coefficient recommended for lignin determination [15] was completely inapplicable for calculating the lignin content in grass compost samples.

Determination of lignin using the Klason procedure is a common method for analysis of lignocellulosic materials; however, in compost analysis, this gravimetrical method is not selective enough. Sulphuric acid promotes the formation of so-called 'false lignin', which is impossible to separate from true lignin. The amount of lignin measured using the Klason procedure increased rapidly to almost twice its initial value during composting, while the methoxyl groups content increased only slightly. The amount of methoxyl groups recalculated on Klason lignin in the starting grass mixture and compost I was $\sim$ 8%, but decreased to 4.8% after 2 months of composting (compost II). This coincides with data obtained by Jin et al. [20]. These results could be explained first by the presence of non-lignin admixtures in the residues obtained by the Klason procedure and, to some extent, by lignin demethoxylation, which was noted in earlier studies on lignin changes during composting [21]. However, on the basis of our data, it was not possible to establish the real level of demethoxylation.

HS and Björkman lignins were isolated from the compost to investigate lignin transformation during composting. The yields of alkaline extracts were 24.3 and 21.8% for composts I and II, respectively. Björkman lignin was isolated from the composts after alkaline extraction. The yield of Björkman lignins was 1.7 and 2.3% for composts I and II, respectively.

3.2. *FT-IR spectra of compost lignins and humic substances*

The FT-IR spectra of both Björkman lignins were very similar, and showed typical absorption maxima for lignins with a dominating maximum at 1126 cm^{-1} of aromatic C−H in the plane deformation of the syringyl group plus C=O stretch (Figure 1). According to the classification by Faix [14], these spectra belong to the group of guaiacyl–syringyl lignins, which is characterised by

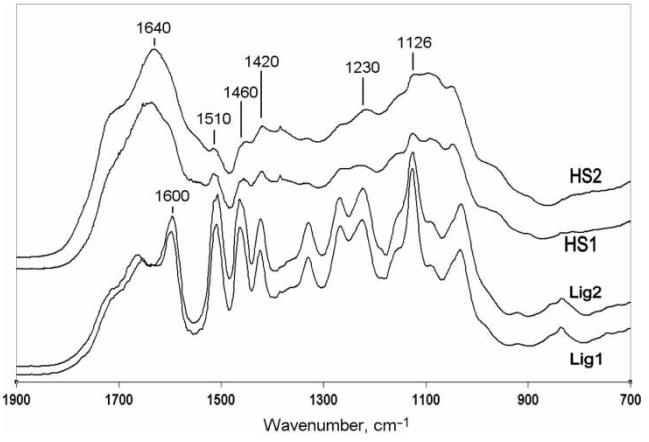

Figure 1. FT-IR spectra of grass Björkman lignin and humic substances.

a medium amount of syringyl units and a low amount of *p*-hydroxyphenyl units. The lignins were characterised by an elevated absorption in the C=O stretch range between 1800 and 1600 cm^{-1}.

HS showed a smooth FT-IR profile corresponding to the transformed macromolecular mixture (Figure 1). Only a few group frequencies and bands are assigned unequivocally, and many bands can be interpreted in various ways. Nevertheless, the spectra showed a fairly well-defined band pattern with diagnostic peaks centred at 1510 (aromatic skeletal vibrations), 1460 (aliphatic C−H deformations; asym. in $-CH_3$ and $-CH_2-$), 1420 (aromatic skeletal vibrations combined with C−H in plane deform.), 1230 (C−OH stretching of aromatic groups and C−O−C stretching of aryl ethers) and 1126 cm^{-1}, which could be interpreted by the presence of the preserved lignin component structure in humic substances. This indicates the integration of lignin into HS.

3.3. *Elemental composition and functional groups of compost lignins and humic substances*

The results of elemental analysis, and the amounts of polysaccharide, functional groups and hydroxycinnamic acids in lignins and HS are presented in Table 2. The elemental composition of lignin I and II was very close. Lignins also contained a small amount of nitrogen compounds and sugar admixture. Absorption coefficients (ε) for lignins I and II were 17.5 and 17.8 $L \cdot g^{-1} \cdot cm^{-1}$ at 280 nm in methylcellusolve. The methoxyl group content of Björkman lignin was higher than

Table 2. Elemental and functional composition of compost Björkman lignins and humic substances (dry matter).

	Lignin I	Lignin II	HS I	HS II
C (%)	60.6	60.6	43.0	41.4
N (%)	0.95	0.88	3.1	2.4
MeO ($mmol \cdot g^{-1}$)	4.81	5.25	1.29	0.87
$OH_{(phen)}$ ($mmol \cdot g^{-1}$)	1.51	1.50	1.29	1.18
COOH ($mmol \cdot g^{-1}$)	0.83	0.81	2.20	2.69
p-Coumaric acid ($mmol \cdot g^{-1}$)	0.15	0.06	0	0
Ferulic acid ($mmol \cdot g^{-1}$)	0.10	0.04	0	0
Total hydroxycinnamic acid ($mmol \cdot g^{-1}$)	0.25	0.10	0	0
Total sugars (%)	3.3	1.5	7.3	7.9
Ash (%)	n.d.	n.d.	10.5	12.1

Note: HS, humic substance.

that in compost Klason lignin; this may arise from the non-lignin admixture of compost Klason lignin. Methoxyl group content for both Björkman lignins was $\sim 5\,\text{mmol} \cdot \text{g}^{-1}$ or ~ 1 methoxyl group per monolignol unit based on the assumption that an equivalent molecular mass of one unit of lignin was 200 Da. This was higher than the methoxyl group content of the parent grass mixture recalculated for lignin. The data showed that compost Björkman lignin contained less *p*-hydroxyphenyl than parent grass lignin. No clear differences in the functional groups between Björkman lignin I and lignin II were observed from chemical analysis. The substitutions of OH groups were calculated as 0.47 OH · unit^{-1} for lignin I, and 0.46 OH · unit^{-1} for lignin II. Grass lignin also contained etherified and esterified *p*-coumaric and ferulic acids. The total amount of hydroxycinnamic acids in lignin decreased during composting. Nevertheless, the total acidity of lignin, judged by the COOH group content, did not change.

Analytical pyrolysis of Björkman lignins methylated with CH_2N_2 confirmed that lignin contains new acidic groups. Methyl ester of vanillic acid was identified between typical lignin $C-C_6$, C_2-C_6 and C_3-C_6 pyrolysis products (Figure 2). This suggested the cleavage of $C\alpha-C\beta$ bonds in the side chains of lignin during composting.

HS samples differed not only from compost Björkman lignins, but also between themselves. Both HS samples had a lower carbon content and higher nitrogen content than Björkman lignins. The amount of methoxyl groups in HS was lower than in the lignins and decreased during composting. These results made it possible to suggest the demethoxylation of lignin during its integration into HS. The total acidity of HS was significantly higher than for the lignins and increased during composting. Hydroxycinnamic acids were not detected in HS after alkaline hydrolysis.

3.4. *Nitrobenzene oxidation of compost lignins and humic substances*

Alkaline nitrobenzene oxidation is a method for the qualitative and quantitative determination of the minimal amount of uncondensed lignin-building blocks. The yields of nitrobenzene oxidation products for Björkman lignins and HS are summarised in Table 3. Björkman lignins gave a high yield of phenolic aldehydes and acids by nitrobenzene oxidation of 27.3 and 28.1% for lignins I and II, respectively; the amount of uncondensed units in compost lignins would be expected to be at least one third. The same products were obtained after nitrobenzene oxidation of HS, but with a lower yield. The syringyl/guaiacyl ratio for all lignins and HS was almost the same. The yield

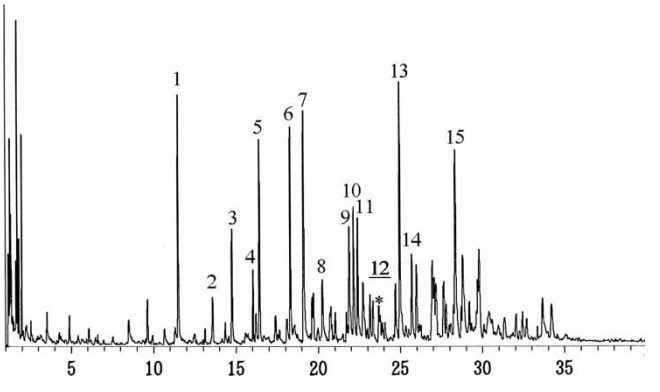

Figure 2. Analytical pyrogram of compost Bjorkman lignin I methylated with CH_2N_2.
Notes: 1, 2-methoxyphenol; 2, 4-vynilanisole; 3, 4-methylguaiacol; 4, 4-methylveratrol; 5, 1-methoxy-4(1-propenyl) benzene; 6, 4-vynilguaiacol; 7, syringol; 8, vanillin; 9, 4-methylsyringol; 10, isoeugenol; 11, 3,4-dimethoxybenzaldehyde; 12, vanillic acid methyl ester; 13, 4-vynilsyringol; 14, 3,4,5-trimethoxybenzaldehyde; 15, 4-propenylsyringon.

Table 3. Alkaline nitrobenzene oxidation of compost Björkman lignins and humic substances (mmol · g^{-1} dry matter).

	Lignin I	Lignin II	HS I	HS II
p-Hydroxybenzaldehyde	0.032	0.015	0.017	0.006
Vanillin	0.592	0.657	0.098	0.060
Syringaldehyde	0.758	0.757	0.100	0.060
p-Hydroxybenzoic acid	0.006	0.003	0.007	0.004
Vanillic acid	0.063	0.076	0.020	0.014
Syringic acid	0.148	0.144	0.049	0.029
Total yield	1.60	1.65	0.29	0.16
Acid/aldehyde ratio	0.16	0.16	0.35	0.41
Syringyl/guaiacyl ratio	1.4	1.2	1.3	1.4
Hydroxyphenyl/guaiacyl ratio	0.06	0.02	0.20	0.13

Note: HS, humic substance.

of *p*-hydroxybenzaldehyde and the corresponding acid after nitrobenzene oxidation was low for lignin and HS, and decreased during composting.

The molar ratio of aromatic acids/aldehydes in nitrobenzene oxidation products for both lignins was 0.16. The aromatic acids/aldehydes ratio in nitrobenzene oxidation products for HS was more than twice that in compost lignin after 2 weeks (0.35) of composting and increased up to 0.41 during composting. These results indicate that the lignin component of HS is more oxidised.

The yield for the lignin-derived phenolic aldehydes and acids obtained from nitrobenzene oxidation is comparable with that obtained by alkaline CuO oxidation [14]. Alkaline CuO oxidation has been used extensively in the study of soil lignins [9]. The yield for lignin-derived phenolic aldehydes and acids from soil lignin is low, but the phenolic acid to aldehyde ratio changes in a broad range from 0.2–0.3 for arable soil to 1.3–2.1 for forest soil [7]. In our case, the phenolic acid to aldehyde ratio for the compost HS was close to the typical values for soil lignin.

Nitrobenzene oxidation results indicate that the compost Björkman lignin is not significantly condensed and oxidised, in contrast to the lignin component of HS.

3.5. *Ozonation of compost lignins and humic substances*

The ozonation procedure is a comparatively rare method for characterising the lignin component of HS, although it allows valuable quantitative and qualitative information about lignin aliphatic chains to be obtained. Selective degradation of the aromatic nuclei of lignin by ozone gives low molecular mass compounds that retain the stereo-selective structures of the original stereo side chain structures of lignin, and makes it possible to estimate the aryl glycerol, aryl ether or β-O-4 structure content of lignin [14]. The results of analytical ozonation are presented in Table 4. The total yield of tetronic acids from HS was very low in comparison with that obtained for lignins, and decreased twice during composting. The ratio of the different erythro/threo forms for the compost lignins and HS suggests that the lignin component of HS is degraded stereo-selectively; the erythro form in the HS fraction is degraded faster than the threo form.

3.6. *Comparison of different analytical procedures for lignin component determination in humic substances*

The lignin components of HS were calculated on the basis of data from different analytical procedures: Klason residue content, methoxyl group content, total yield of alkaline nitrobenzene oxidation products and total yield of erythronic and threonic acids obtained by analytical

Table 4. Yield of ozonation products of compost Björkman lignins and humic substances (mmol · g^{-1} dry matter).

	Lignin I	Lignin II	HS I	HS II
Erythronic acid	0.44	043	0.049	0.022
Threonic acid	0.26	0.25	0.035	0.018
Total tetronic acids	0.70	0.68	0.084	0.039
Erythro/Threo ratio	1.7	1.7	1.4	1.3

Note: HS, humic substance.

Table 5. Estimation of the lignin component content in humic substances calculated on the basis of the results of different analytical methods (% dry matter).

Basis for calculation	HS I	HS II
Yield of Klason residue	49.7	41.9
MeO group content	27	17
Yield of NBO products	18	10
Yield of ozonation products	12	6

Note: HS, humic substance; NBO, nitrobenzene oxidation.

ozonation, and their comparison with data obtained for compost Björkman lignins (Table 5). This allowed us to draw a conclusion about the transformation of lignin during composting.

The lignin content of HS estimated using chemical markers was lower than that determined using the Klason procedure. The highest value for the lignin content of HS I, namely 27%, was calculated on the basis of the methoxyl group content, whereas the values calculated from the results of nitrobenzene oxidation products and ozonation products were lower. HS II contained 17, 10 and 6% lignin, calculated on the basis of the methoxyl group, nitrobenzene oxidation and ozonation, respectively.

These results show that lignin macromolecules have been sufficiently biologically modified at the earlier stage of composting to be dissolved in alkali. The solubility of lignin may be connected to the increase in the hydrophilic carboxylic group content, as confirmed by functional analysis. The alkali-soluble lignin component constituted a significant part of the grass compost HS. This coincides with the observed numerous positive effects of lignin on humic acid formation, e.g. during co-composting lignin with yard and kitchen biowastes [22].

These results are in agreement with the viewpoint that HS are a supramolecular association of low molecular mass organic molecules, including recognisable biomolecules such as lignin-derived structures [23].

4. Conclusions

Our results have shown that part of the lignin macromolecules had been significantly biologically modified at the earlier stage of composting and underwent a deep chemical transformation. A significant part of lignin, probably including demethoxylated lignin fragments, was integrated into alkali-soluble HS during composting. The application of analytical pyrolysis, nitrobenzene oxidation and ozonation allowed us to estimate the cleavage of $C\alpha-C\beta$ bonds in lignin side chains, the oxidation of lignin and the increase in the amount of acidic groups. The acetyl bromide and Klason procedures have a significant drawback for lignin determination in compost samples. Of the chemical markers under study, methoxyl groups could be recommended for evaluating the lignin content in compost because of their higher stability in a compost environment in comparison

with uncondensed lignin aromatic units and lignin aliphatic chains, which may also be used as a basis for similar calculations. Our results have shown that the lignin component of soluble HS was oxidised and characterised by a higher condensed structure and a lower amount of aryl glycerol aryl ether or β-O-4 linkages than compost Björkman lignin at the corresponding stage of composting.

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