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# Fractional Separation and Structural Characterization of Chlorophyll and Lignin from Perennial Ryegrass (*L. perenne*) and Cocksfoot Grass (*D. glomerata*)

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# Fractional Separation and Structural Characterization of Chlorophyll and Lignin from Perennial Ryegrass (*L. perenne*) and Cocksfoot Grass (*D. glomerata*)

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**Abstract:** One chlorophyll rich fraction and two lignin preparations were separated from perennial grass and cocksfoot grass by sequential three-stage treatments with 80% ethanol containing 0.2% NaOH, 2.5% $H_2O_2-0.2\%$ EDTA containing 1.5% NaOH, and 2.5% $H_2O_2-0.2\%$ TAED containing 1.0% NaOH at 75°C for 3 h, respectively, which released 4.6 and 3.6% chlorophyll rich fraction, 2.3 and 5.8%, and 0.9 and 1.0% lignin preparations, except for releasing 8.0 and 10.4%, 79.1 and 77.0%, and 12.9 and 12.5% of the original hemicelluloses, respectively. The lignin fractions obtained from the two different grasses had very similar molecular weights and structural composition. The NMR spectra of the lignin preparations revealed the presence of *p*-hydroxyphenyl, guaiacyl, and syringyl structures, and the lignin in chlorophyll rich fraction contained more guaiacyl and syringyl units than *p*-hydroxyphenyl unit, whereas the reverse trend was found in the two lignin preparations. The lignin preparations are distinguished with straw and wood lignins by relatively higher contents of *p*-hydroxyphenyl unit and lower amounts of condensed units ($\beta$-5 and 5-5′) and resinol units ($\beta$-$\beta$). This difference in distribution of structural units indicated some structural heterogeneity between grass and straw/or wood lignin.

**Keywords:** Perennial ryegrass, cocksfoot grass, chlorophyll, lignin, separation



## INTRODUCTION

In recent years, there has been an increasing trend towards more efficient utilization of agricultural residues, such as cereal straw, sugarcane bagasse, and grass. These renewable, recyclable, sustainable, and biodegradable materials can unhook widespread dependence on fossil fuel and make a difference in the environmental today and tomorrow (1, 2). Among the biomass materials in grasses, perennial ryegrass (*L. perenne*) and cocksfoot grass (*D. glomerata*) are the main species of grasses in UK and Europe, and present some valuable advantages: simple cultivation and harvesting, good yield (>20 tons of dry matter per hectare), high calorific value (20 kJ/kg of dry matter), and well-described material (3). The juvenile leaves of the grasses contain 28–31% cellulose, 34–41% hemicelluloses, 4–7% lignin, 3–4% chlorophyll, together with small amounts of proteins, ash, and pectin, and their composition and structure of the polysaccharides have been investigated in our previous studies (3). One of the significant applications of grasses in our laboratory has been for the production of biofuel (ethanol) and chemicals such as xylitol, chlorophyll and lignin for industries by fractional separation of the grasses. The new awareness of the importance of utilizing grasses for value addition has also led to an environmentally friendly separation process. Although the economy of such processes is severely affected by the high cost of product separation, simultaneous separation and marketing of enzymes have made economics to recover somewhat. However, it remains a fact that in spite of these advances, the commercial exploitation of grass-based separation remains limited.

Chlorophyll is the green molecule in plant cells that carries out the bulk of energy fixation in the process of photosynthesis. Chlorophyll itself is actually not a single molecule but a family of related molecules, designated chlorophyll *a*, *b*, *c*, and *d.* Chlorophyll *a* is the molecule found in all plant cells and therefore its concentration is what is reported during chlorophyll analysis. On the other hand, chlorophylls *b* and *c* are common in fresh water, and chlorophyll *d* is found only in marine red algae (4). Interestingly, due to the green color of chlorophyll, it has many uses as dyes and pigments. It is used in coloring soaps, oils, waxes, and confectionary (5).

Lignin is a complex phenylpropanoid polymer that derives mainly from the oxidative condensation of three *p*-hydroxycinnamyl alcohol monomers differing in their degree of methoxylation, the so-called *p*-coumaryl, coniferyl, and sinapyl alcohols. These monolignols produce the *p*-hydroxyphenyl, guaiacyl, and syringyl units respectively when incorporated into the lignin polymer (6–9). In general, lignin is a component of vascular plants, that is predominant in woods (20–40%), and less predominant in grass (2–15%) (3). In particular, within grass cell walls, the relative abundance of lignin and the frequency of ferulate cross-links with arabinoxylans appear to be the most important factors limiting its separation and utilization (10). In addition, it has become evident that it is difficult to separate chlorophyll and lignin fractionally without sufficient structural information, since lignin is linked with other matrix components and greatly influences cell wall properties, including the enzymatic degradability of structural polysaccharides (11). The lignins isolated can be commercially used in a wide range of products. Some of these applications are materials for automotive brakes, wood panel products, phenolic resins, biodispersants, polyurethane foams, epoxy resins for printed circuit boards, and surfactants (12).

In this paper, we proposed an experimental approach to fractional separation of chlorophyll and lignin from perennial ryegrass and cocksfoot grass using an environmentally friendly method, in which the chlorophyll was first separated from the grasses by extraction with 80% ethanol. Lignin was then fractionated from the ethanol-treated residue by sequential treatments with alkaline peroxide. The lignins separated were characterized by Fourier transform infrared (FT-IR) and hydrogen-$H^1$ and carbon-13 nuclear magnetic resonance ($^{13}C$ NMR) spectroscopy, gel permeation chromatography (GPC), and thermogravimetric analysis (TGA).

## EXPERIMENTAL METHODS

### Materials

Perennial ryegrass (*L. perenne*) and cocksfoot grass (*D. glomerata*), age of 10 weeks, were harvested from the farm of Biochem Wales Ltd in mid-September 2005, and was approximately 15 cm in height. They are freed

from any weeds by hand and transported to the laboratory as quickly as possible. The grasses were dried in a forced draught oven at 60°C for 16 h and then ground by hand before use.

## Fractional Separation of Chlorophyll and Lignin

In this study, all the cell wall separation procedures and the analyses of the cell wall components were performed at least in duplicate, then averaged for each grass sample. Yields of chlorophyll and lignin are given on a dry weight basis related to the starting grass (Table 1). The scheme for the sequential processes of perennial ryegrass and cocksfoot grass, and the fractional separation of chlorophyll and lignin solubilized is shown in Fig. 1. Firstly, the ground perennial ryegrass and cocksfoot grass (30.0 g) were treated with 750 mL 80% aqueous ethanol containing 0.2% NaOH for 3 h at 75°C, respectively. The green slurry was filtered through a 45-$\mu$m nylon cloth, resulting in a green suspension and the insoluble residue fraction left in the cloth. The residue was thoroughly washed with 80% ethanol (three times), and then oven dried at 60°C for 16 h. The supernatant was combined and then neutralized with 6 M HCl to pH 5.5, then kept for 5 h at 22°C to precipitate the low molecular weight of hemicelluloses released. The hemicelluloses that formed



***Table 1.*** The yield of chlorophyll and lignin (% dry matter) solubilized in the sequential treatments of perennial ryegrass and cockffot grass with 80% ethanol containing 0.2% NaOH, 2.5% $H_2O_2$−0.2% EDTA containing 1.5% NaOH, and 2.5% $H_2O_2$−0.2% TAED containing 1.0% NaOH at 75°C for 3 h

| Yield (%) | Perennial grass | Cockffot grass |
|---|---|---|
| Solubilized chlorophyll rich fraction in 80% ethanol containing 0.2% NaOH[a] | 4.6 | 3.6 |
| Solubilized lignin in 2.5% $H_2O_2$–0.2% EDTA containing 1.5% NaOH[b] | 2.3 | 5.8 |
| Solubilized lignin in 2.5% $H_2O_2$–0.2% TAED containing 1.0% NaOH[c] | 0.9 | 1.0 |
| Total solubilized chlorophyll and lignin | 7.8 | 10.4 |

[a]Represent for the two chlorophyll rich fractions solubilized during the treatment of perennial ryegrass and cockffot grass with 80% ethanol containing 0.2% NaOH at 75°C for 3 h.

[b]Represent for the two lignin preparations solubilized during the treatment of 80% ethanol extracted perennial ryegrass and cockffot grass with 2.5% $H_2O_2$−0.2% EDTA containing 1.5% NaOH at 75°C for 3 h.

[c]Represent for the two lignin preparations solubilized during the treatment of alkaline peroxide extracted perennial ryegrass and cockffot grass with 2.5% $H_2O_2$−0.2% TAED containing 1.0% NaOH at 75°C for 3 h.

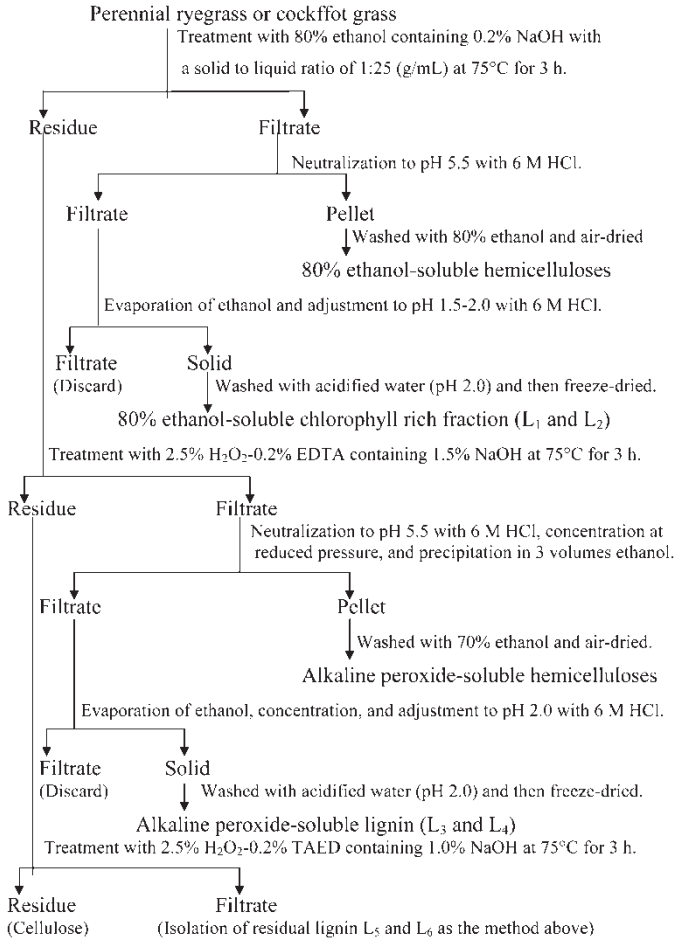


***Figure 1.*** Scheme for fractional separation of chlorophyll and lignin from perennial ryegrass and cockffot grass.

were recovered by filtration, washed with acidified 70% ethanol, and air-dried. The solubilized chlorophyll rich material was then obtained from the corresponding supernatants by precipitation at pH 1.5−2.0. It was washed with acidified water (pH 2.0), freeze-dried, and named as 80% ethanol-soluble chlorophyll rich material $L_1$ from perennial ryegrass and $L_2$ from cocksfoot grass, respectively.

The residue of the grasses was then sequentially extracted with 2.5% $H_2O_2$−0.2% EDTA (ethylene diamine tetraacetic acid) containing 1.5% NaOH at 75°C for 3 h, and 2.5% $H_2O_2$ − 0.2% TAED (tetraacetylethylenediamine) containing 1.0% NaOH at 75°C for 3 h with a solid to liquid ratio of

1:20 (g/mL), respectively. The solubilzed hemicelluloses were separated from the insoluble residue by filtration with a nylon cloth. The residue was subsequently washed with distilled water and 95% ethanol, and then oven dried at 60°C for 16 h. The filtrate was combined and concentrated with a rotary vacuum evaporator at 40°C. The concentrated supernatant was neutralized to pH 5.5 with 6 M HCl, and then mixed with 3 volumes of 95% ethanol for separating the hemicelluloses released. The solubilized lignins were obtained from the corresponding supernatants by re-precipitation at pH 1.5–2.0 after separation of the hemicellulosic fractions. The separated lignins were purified by washing with acidified water (pH 2.0) at room temperature and freeze-dried. Note that the lignins solubilized during the treatment of the 80% ethanol extracted perennial ryegrass residue with alkaline peroxide containing 0.2% EDTA and alkaline peroxide containing 0.2% TAED were labelled as lignin fractions $L_3$ and $L_5$, and the lignins released during the corresponding alkaline peroxide treatments of the 80% ethanol extracted cocksfoot grass residue were coded as the lignin fractions $L_4$ and $L_6$, respectively.



## Characterization of the Chlorophyll and Lignin

The monomeric composition of the non-condensed monomeric units of the lignins in the fractions was characterized by nitrobenzene oxidation and analysis of the resulting aromatic aldehydes and acids by high-performance liquid chromatography (HPLC) has been reported previously (13). The hemicellulosic moieties associated with chlorophyll rich and lignin fractions were hydrolysed with 6% $H_2SO_4$ for 2.5 h at 100°C. The liberated neutral sugars were analyzed by high performance anion exchange chromatography using a Dionex GP50 gradient pump, ED50 electrochemical detector, AS50 autosampler, and a Carbopac$^{TM}$ PA1 column. The content of uronic acids linked in the lignin fractions was determined colormetrically by the method of Blumenkrantz and Asboe-Hansen (14).

UV-vis spectra were recorded on a Hewlett-Packard 8425A Diode Array spectrophotometer. The FT-IR spectra of the chlorophyll rich and lignin preparations were recorded from a KBr disc containing 1% finely ground samples on a Nicolet 750 FT-IR spectrophotometer in the range 4000–400 $cm^{-1}$. The solution-state $^1H$- and $^{13}C$-NMR spectra were obtained on a Bruker MSL400 spectrometer operating in the FT mode at 74.5 MHz. The sample (25 mg for $^1H$, 250 mg for $^{13}C$) was dissolved in 1 mL DMSO-$d_6$ (99.8% D). The $^{13}C$-NMR spectrum was recorded at 25°C after 30 000 scans. A 60° pulse flipping angle, a 3.9 μs pulse width, a 0.85 s acquisition time, and 1.2 s relaxation delay time were used. The methods for determination of molecular weights and thermal analysis of the chlorophyll rich and lignin samples have been described in a previous paper (15).

## RESULTS AND DISCUSSION

### Yield of Chlorophyll and Lignin

To separate chlorophyll from the plants, homogenization by grinding of the filter enhances the rupture of the algal cells and increases the extraction efficiency of the solvent. Homogenization is an absolute necessity with an acetone solvent, but some have found that other extractants such as ethanol or methanol apparently do not need grinding to extract all of the chlorophyll (16). In addition, it was found that the solvents like ethanol or methanol are more efficient than acetone for extracting pigments from some green and blue-green algal cells. Methanol, however, is more toxic. In this study, the chlorophyll was separated from the ground perennial ryegrass and cocksfoot grass by treatment with 80% ethanol containing 0.2% NaOH at 75°C for 3 h, which yielded 4.6% and 3.6% of the chlorophyll rich materials, respectively.

As illustrated in Fig. 2, the UV spectra of the two chlorophyll rich fractions $L_1$ and $L_2$ gave an absorption maximum around 408 nm, implying that both perennial ryegrass and cocksfoot grass contained noticeable amounts of chlorophyll *a*, which absorbs strongly at a UV wavelength of 400–430 nm. A higher absorption coefficient at 408 nm in $L_1$ than in $L_2$ fraction implied that the $L_1$ fraction, isolated from perennial ryegrass, contained a higher amount of chlorophyll than in $L_2$ fraction extracted from cocksfoot grass. It should be noted that the treatment with 80% ethanol also dissolved small amounts of other non- chlorophyll materials such as lignin, protein, wax, free sugars, and low molecular weight of hemicelluloses, since in plants chlorophyll is associated with specific proteins and other materials (17). This was confirmed in our previous studies on the separation of polysaccharides from perennial ryegrass and cocksfoot grass. It was

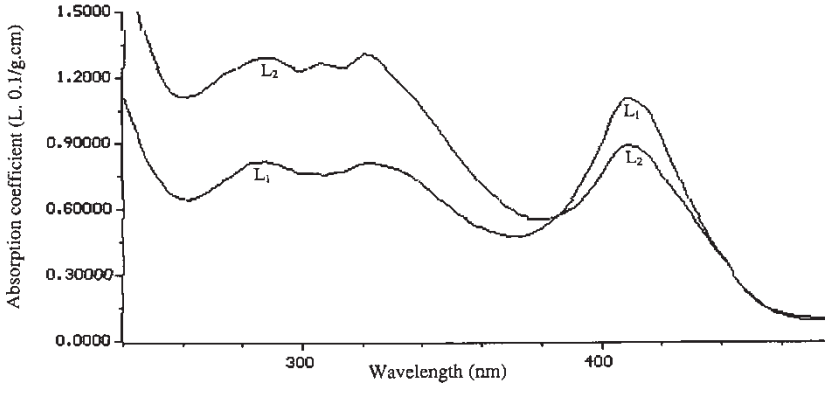


***Figure 2.*** UV spectra of the chlorophyll rich fractions $L_1$ and $L_2$.

found that the treatment with 80% ethanol under the condition given also released 8.0% and 10.4% of the original hemicelluloses from perennial ryegrass and cocksfoot grass, respectively (data not shown in Table 1) (18).

It is well known that hydrogen peroxide reacts with lignin under alkaline conditions and has been widely used for many years to bleach high-lignin wood pulps. The bleach effect of hydrogen peroxide has been attributed to its ability to react with various colored carbonyl-containing structures in lignin. This reaction has been explained through the reactions of the hydroperoxide anion ($HOO^-$), formed in alkaline media (19). On the other hand, hydrogen peroxide is unstable under alkaline conditions and readily decomposes into hydroxyl radicals ($HO^\bullet$) and superoxide anion radicals $O_2$. These radicals generated by the decomposition of hydrogen peroxide, in particular, in the presence of metal ions, are responsible for delignification and solubilization of hemicelluloses. In the present study, to remove interference from metal ions, 0.2% EDTA, a metal chelating agent, was added during the alkaline peroxide treatment. As the data shown in Table 1, treatment of the 80% ethanol-extracted perennial grass and cocksfoot grass residues with 2.5% $H_2O_2$ − 0.2% EDTA containing 1.5% NaOH at 75°C for 3 h released 2.3% and 5.8% lignin (percent dry starting matter) except for dissolving 79.1% and 77.0% of the original hemicelluloses, respectively. These results indicated that alkaline peroxide is a strong agent both for dissolving lignins and for solubilizing hemicelluloses from grass.

TAED is an effective bleach activator, and it has been commonly used in the thermomechanical pulp during the last decades (20). It was supposed that TAED would react with weakly alkaline aqueous solutions like hydrogen peroxide to form peracetic acid, a stronger oxidizing agent that is a highly effective bleaching agent at low temperatures (21). In this case, the bleaching efficiency of the activated peroxide greatly depends on the amounts of peracetic acid formed. In addition, since both hydroperoxide anion and peracetic acid react in different ways with certain organic molecules such as lignin, the combination of hydrogen peroxide and TAED should exhibit a synergistic effect on the bleaching of the hemicelluloses solubilized and the dissolving of the residual lignins from the grass residues. To enhance the release of the residual lignin and increase the whiteness of the hemicelluloses solubilized and the fibers (residue of the peroxide treatment), minor quantities (0.2%) of TAED were used in the third-stage treatment of the alkaline peroxide-treated grass residues. As the data given in Table 1, the third treatment of the 2.5% $H_2O_2$ − 0.2% EDTA extracted residues of perennial ryegrass and cocksfoot grass, with 2.5% $H_2O_2$ − 0.2% TAED containing 1.0% NaOH at 75°C for 3 h solubilized 0.9 and 1.0% lignin, and 5.3 and 4.2% hemicelluloses (percent dry starting grass), respectively. The yield of white crude cellulose was 28.4% from perennial ryegrass and 30.9% from cocksfoot grass, which are relatively free of the associated lignin. Taken together, the two sequential alkaline peroxide treatments of the 80% ethanol extracted perennial ryegrass and cocksfoot grass led to a

total dissolution of 3.2 and 6.8% lignin, and 37.8 and 30.0% hemicelluloses (percent dry starting grass), respectively.

## UV Spectra of Lignin

In the UV determination of lignin, the extinction maximum at about 280 nm is normally used and its absorbance is proportional to the lignin content of the sample (22). In this study, UV-vis absorption measurements of the lignin fractions were performed using a dioxane-water mixture, which solubilized the lignins but which is limited to wavelengths above 240 nm. Figure 3 illustrates the UV absorption spectra of the lignin fractions $L_3$, $L_4$, $L_5$, and $L_6$. The maximum absorption near 280 nm originates from non-conjugated phenolic groups (aromatic rings) in lignins. The presence of a second characteristic region of lignin absorption around 320 nm is assigned to the bound ferulic and *p*-coumaric acids (23). In comparison, a stronger absorption at 320 nm in $L_3$ and $L_4$ than that in the corresponding lignin fractions of $L_5$ and $L_6$ indicated that the lignin fractions isolated with 2.5% $H_2O_2$ − 0.2% EDTA containing 1.5% NaOH at 75°C for 3 h from the 80% ethanol-treated grasses contained higher amounts of hydroxycinnamic acids than those of the lignin fractions separated with 2.5% $H_2O_2$ − 0.2% TAED containing 1.0% NaOH at 75°C for 3 h from the corresponding alkaline peroxide treated residues. In addition, the lower absorption coefficient of the lignin fractions $L_3$ and $L_5$ extracted from the 80% ethanol-treated perennial ryegrass, was undoubtedly due to the relatively higher amounts of co-precipitated other non-lignin materials such as hemicelluloses, protein, and salts in these two lignin fractions. On the other hand, the higher absorption coefficient of the lignin preparations $L_4$ and $L_6$ isolated from the 80% ethanol-treated cocksfoot grass, implied a higher purity of the lignins. Furthermore, the much weaker absorption at 410 nm is

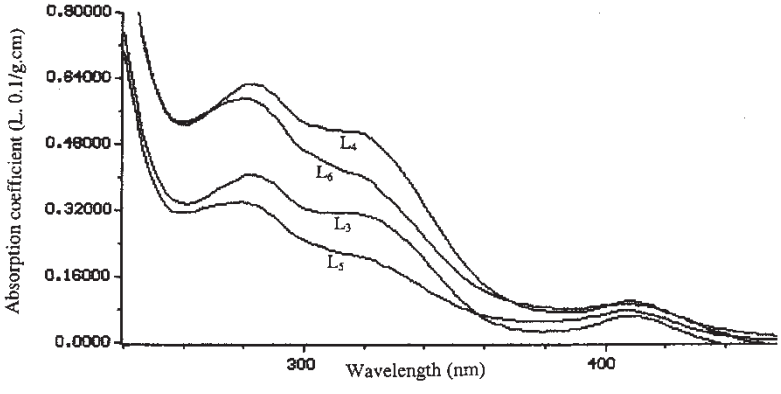


***Figure 3.*** UV spectra of the lignin preparations $L_3$, $L_4$, $L_5$, and $L_6$.

attributed to the co-extracted residual chlorophyll a in the lignin fractions, indicating that the lignin preparations were relatively free of chlorophyll.

## Content of Bound Hemicelluloses

The hemicelluloses in grasses are predominantly composed of unbranched mixed-linkage (1–3, 1–4)-$\beta$-D-glucan and glucuronoarabinoxylan that tightly bind to lignin macromolecules (24). Pectins, however, have been reported to be a rather scarce component of grass cell walls (25). In grasses, glucose and xylose are predominant components of $\beta$-D-glucan and/or cellulose and xylans, respectively. The origin of arabinose, galactose, and uronic acids is a little more complicated in that they are associated with pectin and hemicelluloses. Arabinose is found as a major substitution residue in xylans, particularly in primary cell walls (26). Table 2 lists the content of neutral sugars in the two chlorophyll rich fractions $L_1$ and $L_2$ and the four lignin fractions $L_3$, $L_4$, $L_5$, and $L_6$. Obviously, the chlorophyll rich fractions contained noticeable amounts of associated hemicelluloses, as indicated by their sugar content (12.9–13.0%), whereas the four lignin fractions contained small amounts of bound hemicelluloses, ranging

***Table 2.*** The content of neutral sugars and uronic acids (% dry weight, w/w) in the separated chlorophyll rich fractions and the lignin preparations

| | Chlorophyll rich fraction and lignin preparation[a] | | | | | |
|---|---|---|---|---|---|---|
| Sugars (%) | $L_1$[a] | $L_2$[a] | $L_3$[b] | $L_4$[b] | $L_5$[c] | $L_6$[c] |
| Arabinose | 0.4 | 0.3 | 0.6 | 0.4 | 0.5 | 0.3 |
| Rhamnose | 0.2 | 0.2 | 0.2 | 0.1 | 0.1 | 0.1 |
| Galactose | 2.8 | 3.2 | 0.5 | 0.4 | 0.6 | 0.5 |
| Glucose | 8.8 | 8.6 | 2.7 | 2.3 | 2.9 | 2.6 |
| Xylose | 0.3 | 0.3 | 0.5 | 0.4 | 0.8 | 0.5 |
| Mannose | 0.5 | 0.3 | 0.4 | 0.3 | 0.2 | 0.3 |
| Uronic acids | T[d] | T | T | T | T | T |
| Total | 13.0 | 12.9 | 4.9 | 3.9 | 5.1 | 4.3 |

[a] $L_1$ and $L_2$ represent for the 80% ethanol-soluble chlorophyll rich fractions solubilized during the treatment of perennial ryegrass and cockffot grass with 80% ethanol containing 0.2% NaOH at 75°C for 3 h.

[b] $L_3$ and $L_4$ represent for the lignin preparations solubilized during the sequential treatment of 80% ethanol extracted perennial ryegrass and cockffot grass with 2.5% $H_2O_2$ − 0.2% EDTA containing 1.5% NaOH at 75°C for 3 h.

[c] $L_5$ and $L_6$ represent for the lignin preparations solubilized during the sequential treatment of alkaline peroxide extracted perennial ryegrass and cockffot grass with 2.5% $H_2O_2$ − 0.2% TAED containing 1.0% NaOH at 75°C for 3 h.

[d] T, trace.

between 3.9 and 5.1%. On an increasing the NaOH concentration in the extractants from 0.2 to 1.5%, it resulted in a decrease in the content of associated hemicelluloses from 12.9–13.0% in $L_1$ and $L_2$ to 3.9–4.9% in $L_3$ and $L_4$. Similarly, as the NaOH concentration in 2.5% $H_2O_2$ extractant decreased to 1.0%, the content of neutral sugars rose to 4.3–5.1% in $L_5$ and $L_6$ fractions. This suggested that the post-treatments with alkaline peroxide substantially cleaved the linkages between lignin and hemicelluloses in the cell walls of grass in addition to saponification of hydroxycinnamic esters. Furthermore, as the data shown in Table 2, a relatively higher amount of glucose (2.3–8.8%) was indicative of noticeable amounts of $\beta$-D-glucan and/or cellulose bounded in the chlorophyll rich and lignin fractions. The presence of minor quantities of galactose (0.4–3.2%), xylose (0.3–0.8%), arabinose (0.3–0.6%), mannose (0.2–0.5%), rhamnose (0.1–0.2%), and traces of uronic acids indicated that the bound polysaccharides also arose from the hemicelluloses in the secondary cell walls of grass. In comparison with the lignin fractions isolated from cereal straws (13), a relatively higher content of the bound hemicelluloses in the lignin preparations separated from the grasses in this study was probably due to the juvenile grasses used. Generally, the individual sugars that make up polysaccharides in grass cell walls vary depending upon the grass species and the stage of development, and tissues of grasses harvested early in development have relatively higher molar fractions of arabinose, galactose, and uronic acids and lower fractions of cellulose, xylans, lignin, and bound phenolic monomers than tissues of plants harvested at later stages of development (27, 28).



## Composition of Phenolic Monomers

To elucidate the differences in the structures of the fractions, the two chlorophyll rich fractions and the four lignin preparations were submitted to alkaline nitrobenzene oxidation at 170°C for 2.5 h, and their phenolic monomers are given in Table 3. Obviously, the total yield of oxidation products was higher in the two chlorophyll rich fractions (17.59–18.54%) than in the four lignin preparations (9.76–11.63%), indicating that the lignins in the two chlorophyll rich fractions ($L_1$, $L_2$) had a lower degree of condensation than the four lignin preparations ($L_3$, $L_4$, $L_5$, $L_6$). These low yields were indicative of an accumulation of condensed structures in the four lignin fractions, since some carbon-carbon linkages, such as $\beta-\beta$ and $5-5'$ biphenylics, are not cleaved during the oxidation by nitrobenzene. This enrichment of condensed lignin in the four lignin fractions also resulted from either the formation of condensed structures during the sequential alkaline peroxide treatments or the selective dissolution of condensed structures of the lignin. *p*-Hydroxybenzaldehyde, vanillin, and syringaldehyde were found to be the predominant oxidation products in all the chlorophyll rich and lignin fractions, and resulted from the degradation of

***Table 3.*** The contents (% lignin sample, w/w) of phenolic acids and aldehydes from nitrobenzene oxidation of the chlorophyll rich fractions and the lignin preparations

| | Chlorophyll rich fraction and lignin preparation[a] | | | | | |
|---|---|---|---|---|---|---|
| Phenolic acids and aldehydes | $L_1$ | $L_2$ | $L_3$ | $L_4$ | $L_5$ | $L_6$ |
| *p*-Hydroxybenzoic acid | 0.86 | 0.68 | 0.74 | 0.75 | 0.63 | 0.61 |
| *p*-Hydroxybenzaldehyde | 3.89 | 3.23 | 3.65 | 3.13 | 2.78 | 2.96 |
| Vanillic acid | 0.46 | 0.45 | 0.34 | 0.42 | 0.33 | 0.32 |
| Vanillin | 4.89 | 4.18 | 3.19 | 3.12 | 2.51 | 3.01 |
| Syringic acid | 0.48 | 0.55 | 0.23 | 0.36 | 0.28 | 0.27 |
| Syringaldehyde | 5.93 | 6.21 | 2.09 | 3.10 | 2.68 | 2.65 |
| Acetovanillin | 0.56 | 0.69 | 0.21 | 0.23 | 0.16 | 0.15 |
| Acetosyringone | 0.63 | 0.96 | 0.19 | 0.18 | 0.18 | 0.16 |
| *p*-Coumaric acid | 0.46 | 0.35 | 0.21 | 0.19 | 0.11 | 0.13 |
| Ferulic acid | 0.38 | 0.29 | 0.15 | 0.15 | 0.10 | 0.15 |
| Total | 18.54 | 17.59 | 11.00 | 11.63 | 9.76 | 10.44 |
| Molar ratio (h:v:s)[b] | 1:1.24:1.48 | 1:1.36:1.97 | 1:0.85:0.57 | 1:0.97:0.95 | 1:0.88:0.91 | 1:0.97:0.88 |

[a]Corresponding to chlorophyll rich fractions and the lignin preparations in Table 2.

[b]h represents the relatively total moles of *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid, v represents the relatively total moles of vanillin, vanillic acid; and acetovanillin, and s represents the relatively total moles of syringaldehyde, syringic acid, and acetosyringone.

non-condensed *p*-coumaryl, guaiacyl, and syringyl units, respectively. The relative molar ratios of h (the relatively total moles of *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid), v (the relatively total moles of vanillin, vanillic acid, and acetovanillin), and s (the relatively total moles of syringaldehyde, syringic acid, and acetosyringone) were found to be l:l.24:1.48 in $L_1$, l:l.36:1.97 in $L_2$, 1:0.85:0.57 in $L_3$, l:0.97:0.95 in $L_4$, 1:0.88:0.91 in $L_5$, and 1:0.97:0.88 in $L_6$. The relatively higher monomeric ratios of s to v and to h in the two chlorophyll rich fractions $L_1$ and $L_2$ indicated that the lignins released during the 80% ethanol-0.2% NaOH treatment, resulted mainly from the secondary wall of grass since the secondary wall lignin contains higher amounts of non-condensed $\beta$-*O*-4 structures and many more syringyl units than the middle lamella lignin. In contrast, the relatively lower monomeric ratios of s to v and to h in the four lignin fractions $L_3$, $L_4$, $L_5$, and $L_6$ revealed that these lignins were substantially released from the middle lamella of the grass. It is clear that syringyl units in the lignin macromolecules of the grasses are more reactive to alkaline 80% ethanol than guaiacyl lignin units. In addition, although considerable amounts of *p*-coumaric and ferulic acids had been converted into *p*-hydroxybenzaldehyde or *p*-hydroxybenzoic acid and vanillin or vanillic acid, respectively, during the alkaline nitrobenzene oxidation at 170°C, the remaining occurrence of small amounts of *p*-coumaric acid (0.11–0.46%) and ferulic acid (0.10–0.38%) suggested that these two hydroxycinnamic acids are chemically linked to the lignin molecules in the cell walls of grass.

Based on the study of grass lignins at different developmental stages, Chen et al. (29) demonstrated that lignin content increased moderately during stem elongation of tall fescue, but a major increase occurred when plants changed from the elongation stage to the reproductive stage. Simultaneously, the content of syringyl (S) unit and the ratio of syringyl/guaiacyl (G) units in lignins increased with progressive maturity of stems. In addition, the incorporation of *p*-hydroxyphenyl (H), G, and S units in grass lignins is spatially and temporally regulated and it varies between primary and secondary cell walls and among tissues (30). Guaiacyl-rich lignins in middle lamella/primary walls are thought to be highly branched due to rapid polymerization and coupling by mainly condensed bonds. In contrast, syringyl-rich lignins may be deposited more gradually in secondary walls and would be more linear due to extensive coupling by $\beta$-*O*-4 bonds (31). These previous findings confirmed again that the lignins in the two chlorophyll rich fractions were mainly released from the secondary walls, whereas the four lignin fractions resulted substantially from the middle lamella and/or primary walls of the grasses. Thus, the lignin cores of both perennial ryegrass and cocksfoot grass were composed of H, G, and S units, and their ratios strongly depended on the grass maturity and the separating solvents used. The current results also explained why the various conclusions of grass lignin compositions were obtained during the last decades. For example, Harkin (32) stated that grass lignin contrasts from dicot lignin by having

large amounts of *p*-coumaryl alcohol in the core lignin. On the other hand, based on the study of lignin-cell wall matrix interactions, Grabber and coworkers (30) reported that lignin cores of grasses were composed predominantly of coniferyl and sinapyl alcohols, with only small amounts of *p*-coumaryl alcohol.

## Molecular Weight

Weight-average ($M_w$) and number-average ($M_n$) molecular weights and polydispersity ($M_w/M_n$) of the two chlorophyll rich fractions and the four lignin preparations separated from perennial ryegrass and cockffot grass are given in Table 4, and the molecular weights are based on polystyrene standards. It can be seen that the differences in average molecular weights between the two chlorophyll rich fractions and the four lignin preparations were not significant ($M_w$ 5080–6470 g/mol). However, the $M_w$ between the two chlorophyll rich fractions and the four lignin preparations appeared to be different. Due to the relatively higher contents of syringyl-rich lignins, which are lowly branched, in the two chlorophyll rich fractions, the molecular weights of the two chlorophyll rich fractions ($M_w$ 5080–5150 g/mol) were lower than was observed for the four lignin preparations ($M_w$ 5480–6470 g/mol). These values revealed that the grass lignin was not extensively degraded by alkaline peroxide under the separating conditions used.



## FT-IR Spectra

FTIR spectra of chlorophyll rich fraction $L_1$, and lignin fractions $L_3$ and $L_5$ separated sequentially from perennial ryegrass are illustrated in Fig. 4. Clearly, the intensity of the bands in the spectra b and c is rather similar, indicating a similar structure of the two lignin fractions. The aromatic skeletal

***Table 4.*** Weight-average ($M_w$) and number-average ($M_n$) molecular weights and polydispersity ($M_w/M_n$) of the two chlorophyll rich fractions and the four lignin preparations isolated from perennial ryegrass and cockffot grass

| | Chlorophyll rich fraction and lignin preparation[a] | | | | | |
|---|---|---|---|---|---|---|
| | $L_1$ | $L_2$ | $L_3$ | $L_4$ | $L_5$ | $L_6$ |
| $M_w$ | 5080 | 5150 | 5930 | 6470 | 5480 | 6020 |
| $M_n$ | 4030 | 3410 | 4220 | 4950 | 3790 | 4510 |
| $M_w/M_n$ | 1.26 | 1.51 | 1.41 | 1.31 | 1.45 | 1.33 |

[a]Corresponding to the two chlorophyll rich fractions and the four lignin preparations in Table 2.

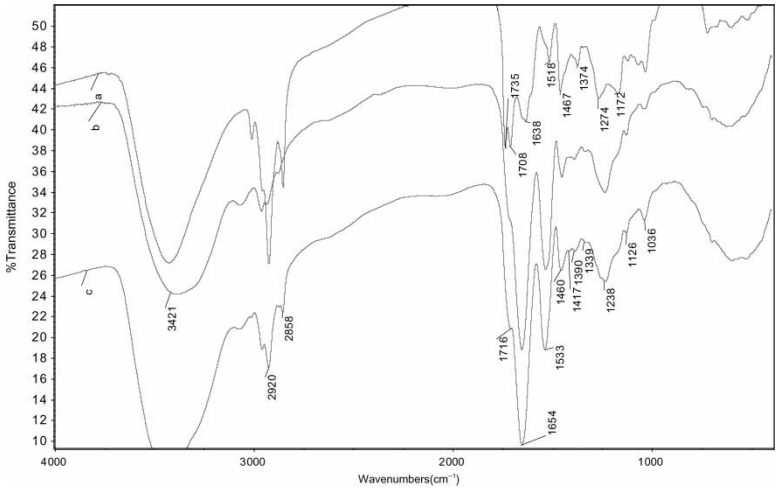


***Figure 4.*** FT-IR spectra of the chlorophyll rich fraction $L_1$ (spectrum a), and two lignin preparations $L_3$ (spectrum b) and $L_5$ (spectrum c).

vibrations in lignins exhibit absorption at 1654, which overlapped with the ring-conjugated $\alpha$, $\beta$ unsaturated bond, 1533, and 1417 $cm^{-1}$. The aromatic methyl group vibrations occur at 1460 $cm^{-1}$. The C═O stretch in unconjugated ketone and carbonyl groups was observed in the chlorophyll rich fraction $L_1$ at 1708 $cm^{-1}$ and 1716 $cm^{-1}$ in the lignin fractions $L_3$ and $L_5$. The band at 1238 $cm^{-1}$ is assigned to the syringyl and guaiacyl ring breathing with C-O stretching. Aromatic C-H in-plane deformation is clearly seen at 1126 (syringyl type) and 1036 $cm^{-1}$ (guaiacyl type). The band at 3421 $cm^{-1}$ is originated from the OH stretching in chlorophyll and lignin molecules. The CH stretching of methyl, methylene, or methine group in both chlorophyll and lignin molecules occurs at 2920 and 2858 $cm^{-1}$. An intensive peak at 1735 $cm^{-1}$ in the spectrum of chlorophyll rich fraction $L_1$ is characterized by the ester bonds in chlorophyll. The C═N and C-N stretching in chlorophyll exhibits the peaks at 1638 and 1172 $cm^{-1}$, respectively, in the chlorophyll rich fraction $L_1$. The bands at 1638 (overlapped with C═N stretching), 1518, and 1467 $cm^{-1}$ in the chlorophyll rich fraction $L_1$ are due to the aromatic skeleton vibrations in lignin molecules.

The spectra in Fig. 5 show bands characteristic of grass lignin at 1650, 1526, 1460, and 1421 $cm^{-1}$ (aromatic ring), 1650 and 1716 $cm^{-1}$ (conjugated and non-conjugated carbonyl groups), 1390 $cm^{-1}$ (aliphatic C-H stretching), 1332 $cm^{-1}$(S ring breathing with C-O stretching), 1227 $cm^{-1}$ (phenolic OH), 1133 $cm^{-1}$(S ring), and 1033 $cm^{-1}$ (methoxy groups and G ring). Bands at 1526 and 1460 $cm^{-1}$ were useful in demonstrating the presence of lignin in both one chlorophyll rich fraction and the two lignin preparations,

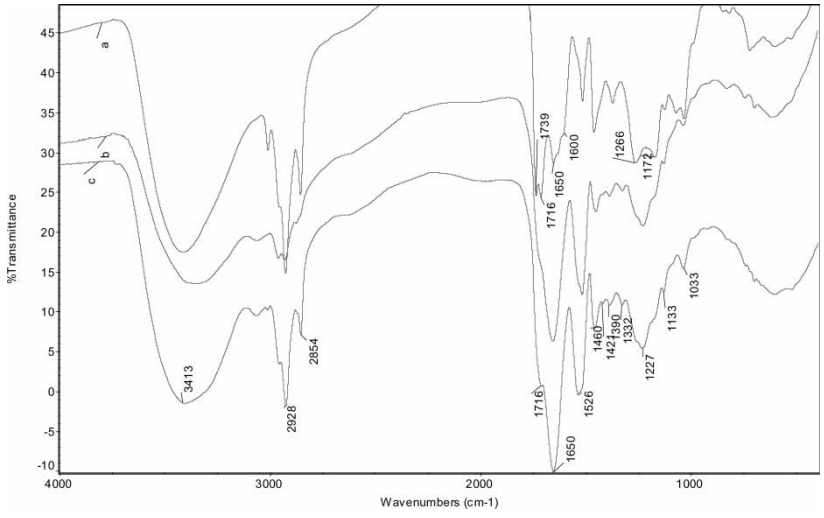


***Figure 5.*** FT-IR spectra of the chlorophyll rich fraction $L_2$ (spectrum a), and the two lignin preparations $L_4$ (spectrum b) and $L_6$ (spectrum c).

since they do not overlap with bands from other polymers. Similarly, peaks at 1650, 1600, and 1172 $cm^{-1}$ in the chlorophyll rich fraction $L_2$ are mainly attributed to the C═N, N-H, and C-N stretching in chlorophyll molecules, and indicative of the occurrence of chlorophyll in fraction $L_2$.

## $^{1}H$ and $^{13}C$ NMR Spectra

Figure 6 illustrates the $^{1}H$ NMR spectrum of lignin fraction $L_3$, isolated with 2.5% $H_2O_2$–0.2% EDTA containing 1.5% NaOH from the 80% ethanol treated perennial ryegrass. A strong signal at 7.2 ppm is assigned to the aromatic protons in positions 2 and 6 in structures containing a $C_\alpha$═O group, to aromatic protons in positions 2 and 6 of *p*-hydroxyphenyl units conjugated with a double bond, to the protons in $C_\alpha$═$C_\beta$ structure, and to aromatic protons in *p*-coumaric and ferulic acids, confirming the presence of *p*-hydroxyphenyl units, $C_\alpha$═O groups, and *p*-coumaric and ferulic acids in the lignin fraction (33). The integrals of signals centred at 6.6 and 6.8 ppm (data not shown) are originated from aromatic protons in syringylpropane and guaiacylpropane structures, respectively, indicating the presence of syringyl and guaiacyl units in the lignin (34). Two signals at 5.3 and 4.2 ppm are due to the $H_\alpha$ and $H_\beta$ in *β-O*-4 structures, respectively. The methoxyl protons (-$OCH_3$) give a strong signal at 3.4 ppm. A sharp and intense signal at 2.5 ppm relates to the protons in DMSO. Signals between 0.8 and 2.0 ppm are indicative of the protons in aliphatic groups at the lignin side chains.

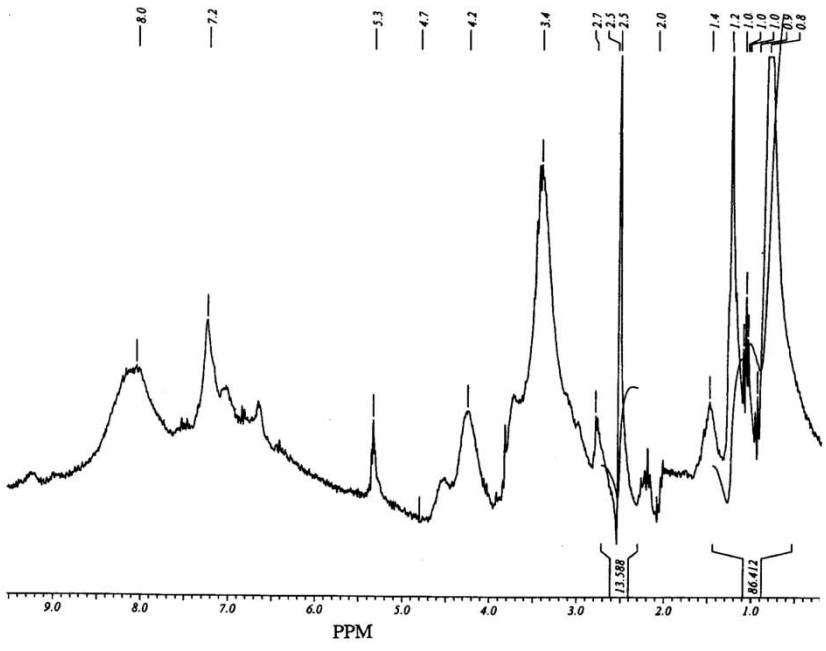


***Figure 6.*** $^{1}$H-NMR spectrum of the lignin preparation $L_3$.

The $^{13}$C-NMR spectrum of the lignin fraction $L_3$ (Fig. 7) showed the characteristic signals of the carbons in the different structures of this macromolecule: 171.9 ppm (C-$\gamma$ in esterified *p*-coumaric acid), 138 ppm (C-4 in etherified S unit), 131.5 ppm (C-1 in non-etherified S and G units), 129.3 ppm (C-2/C-6 in esterified *p*-coumaric acid), 128.0 ppm (C-2/C-6 in H unit), 117.1 ppm (C-$\beta$ in etherified ferulic acid), 115.0 ppm (C-5 in G unit), 111.2 ppm (C-2 in G unit), 104.5 ppm (C-2/C-6 in S unit), 60.2 ppm (C-$\gamma$ in $\beta$-*O*-4 linkages), and 56.0 ppm ($-OCH_3$ groups) (35). The signals representing the $\gamma$-methyl and $\alpha$- and $\beta$-methylene groups in *n*-propyl side chains occur in the regions of 11.1–15.4 and 18.6–33.7 ppm, respectively. A sharp signal at 174.1 ppm is presumed due to C-6 in methyl urinates, which are esterified to the side chains of lignins (36). These signals revealed that the lignin fraction contained *p*-hydroxyphenyl-, guaiacyl-, and syringyl structures, and was mainly composed of $\beta$-*O*-4 ether bonds, which could be justified as typical SGH grass lignin, corresponding to the results obtained by nitrobenzene oxidation. The results obtained also verified that *p*-coumaric acid is linked to lignin by ester bonds, whereas ferulic acid is linked to lignin by ether bonds.

It should be noted that there were no any signals for common carbon-carbon linkages such as $\beta$-$\beta$, $\beta$-5, and 5-5′ in the spectrum of the lignin fraction. This suggested that the grass lignin had much fewer condensed

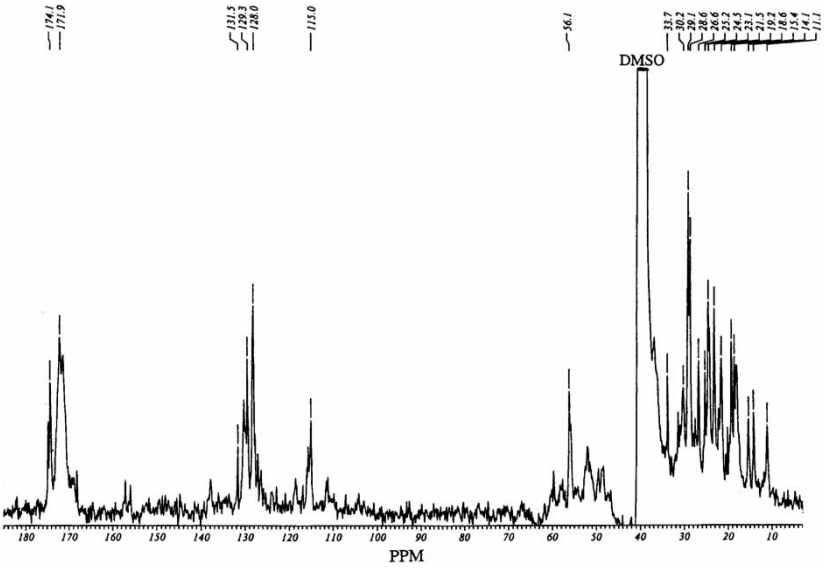


***Figure 7.*** $^{13}$C-NMR spectrum of the lignin preparation $L_3$.

units, which was consistent with the findings from the lignins of reed canary grass by Galkin et al. (34), but did not agree with the results obtained from the straw and wood lignins (13, 15). This structural difference raised the question of lignin heterogeneity in various plants or in morphologically different parts or maturities of the plant.

## Thermal Analysis

As illustrated in Fig. 8, the decomposition for the lignin fractions $L_4$ and $L_6$, separated by sequential treatments of the 80% ethanol-extracted cocksfoot grass with alkaline peroxide-0.2% EDTA and alkaline peroxide-0.2% TAED, started at about 180°C with a maximum of degradation at 320°C. Above 400°C, the main evolution is the deoxygenation of the char and the loss of aliphatic structures leading to the formation of a semi-coke by decarboxylation, decarbonylation, dehydrogenation, and loss of methane. It is also interesting to note that the char yield at 600°C is greater in the case of the lignin fraction $L_4$ (35% wt.%) than fraction $L_6$ (25% wt.%). Therefore, the differences observed in the thermal behavior of the two lignin fractions could be explained, at least, by two different processes, and the higher char yield of $L_4$ was probably due to the higher content of ash or salts in the lignin fraction $L_4$, isolated with 2.5% peroxide-0.2% EDTA containing 1.5% NaOH than in the lignin $L_6$, extracted with 2.5% peroxide-0.2% TAED containing 1.0% NaOH under the conditions used.

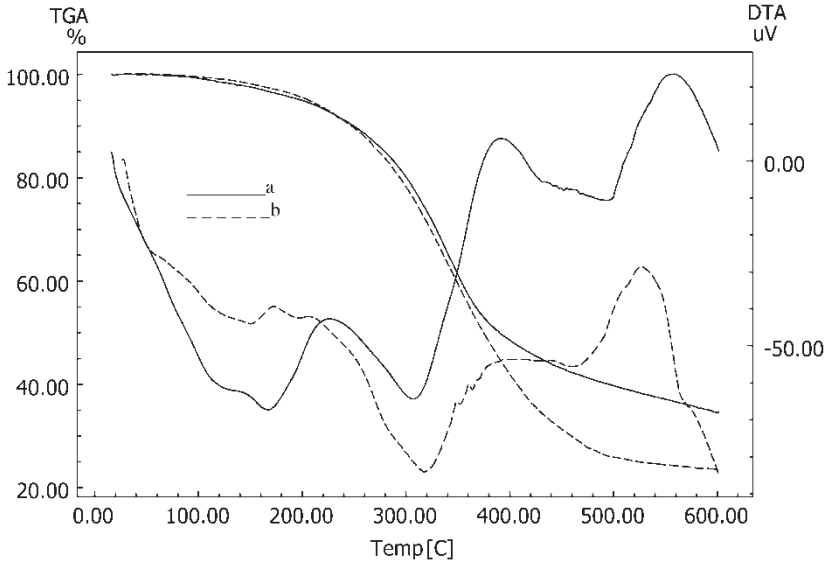


***Figure 8.*** Thermograms of the lignin preparations $L_4$ (a) and $L_6$ (b).

The differential thermal analysis (DTA) showed that the glass transition temperature of the lignin fractions $L_4$ and $L_6$ occurred at 158 and 170°C, respectively, and their thermograms showed an endothermic peak at 303°C for $L_4$ and 320°C for $L_6$. In addition, an intense exothermic transition at around 400°C was observed only for the lignin fraction $L_4$. In general, the fusion and/or decomposition process can be involved when DTA curves show an endotherm, whereas volatile substances release and/or decomposition can be involved as DTA curves show an exotherm.

## CONCLUSION

The results of our studies show that the sequential treatments of perennial ryegrass and cocksfoot grass with alkaline 80% ethanol and 2.5% alkaline peroxide under the conditions used successively separated the grass into chlorophyll rich, lignin, hemicelluloses, and cellulose fractions. The technique developed in this study may be considered an improvement upon other published methods. More importantly, while negating the risk of toxic chlorine dioxide gas inherent in the use of sodium chlorite for delignification, the quantity of the peroxide used is minimal (2.5%), which also represents a totally chlorine-free method for separating chlorophyll, lignin, hemicelluloses, and cellulose from other grass and biomass. Further work is required to confirm if these results can be gained from the more practical situation.

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