# Evaluation of Lignin Preparations from Lignocellulosics by HPLC/Electrochemical Detection of Phenolics

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Lignins prepared by five extraction procedures ($H_2SO_4$, HCl, HF, NaOH, and dioxane/water) from wheat straw, corn stalks, alfalfa stems, and red oak leaves were compared on the basis of their phenolic composition. Analyses were performed by HPLC/electrochemical detection after alkaline nitrobenzene oxidation. Quali-quantitative results are reported, along with typical HPLC profiles. Statistical analysis of the quantitative results shows significant differences among the lignin preparations, the materials, and the interaction between preparations and materials.

## INTRODUCTION

Agroindustrial lignocellulosic residues represent a large and renewable source of energy. EEC surplus of straw residues is about 24 million tons (EEC, 1987). Considerations of environment protection and farm managing suggest restrictions in current practices of disposal, such as straw burning and ploughing, and release of pulp industry wastes into natural waters.

While the use of lignocellulosics as a fuel depends on oil price, industrial (polymer, fiber, paper, chemical) and livestock (ruminant feed) uses require various pretreatments. These pretreatments affect structure and interaction of the three main constituents of lignocellulosic biomass, namely hemicellulose, cellulose, and lignin. For instance, steam, acid, alkali, and fungal processings (Bender et al., 1970; Ben-Ghedalia et al., 1988; Silva and Ørskov, 1988; Zadrazil, 1984) have been used to make polysaccharides more digestible by rumen enzymes, thus upgrading lignocellulosics as an animal feed.

Analysis of lignin components can be valuable to monitor these processes (Janshekar and Fiechter, 1983) and to predict their effects, such as straw digestibility (Reeves, 1987).

Oxidative degradation of lignin into monomeric units (Chang and Allan, 1971) and their determination by HPLC with UV (Hartley and Buchan, 1979) and electrochemical (ElCh) detection (Chiavari et al., 1988; Galletti et al., 1990) are well established. ElCh detection has lower detection limits than UV detection and is selective toward electrochemically active compounds, such as phenolics. A recent paper reports the exploitation of such selectivity to simplify the workup of the nitrobenzene oxidation procedure for lignin analysis in wheat straw by eliminating the need of cumbersome and nonreproducible solvent extraction and the comparison between ElCh and UV detection (Galletti et al., 1989).

Because different methods of lignin preparation and/or straw treatment may lead to different quali-quantitative composition of lignin itself (Janshekar and Fiechter, 1983; Lai and Sarkanen, 1971), it seemed interesting to study the application of such a simplified procedure to determine the phenolic composition of lignins prepared according to five well-accepted procedures from four lignocellulosic byproducts.

Wheat straw, corn stalks, alfalfa stems, and red oak leaves were treated with HCl (Lai and Sarkanen, 1971), HF (Papadopoulos and Defaye, 1986), $H_2SO_4$ (Goering and Van Soest, 1970), dilute NaOH (Lai and Sarkanen, 1971), and dioxane/water (Himmelsbach and Barton, 1980) to obtain lignins. NaOH and HF lignins are of particular interest, since the former is commonly used for feedstuff treatments, whereas the latter is a well-known reagent for polysaccharide hydrolysis and has been used recently for the characterization of lignin in wood.

## MATERIALS AND METHODS

**Sample Preparation.** Wheat straw, corn stalks, alfalfa stems, and red oak leaves were collected at the Agricultural Research Center in Beltsville, MD, in November 1987. Samples were dried at 60 °C and homogenized in a UDY cyclone mill to 40 mesh. Fifteen grams was mechanically shaken with 200 mL of benzene/ethanol (1:1) for 24 h and filtered. The following lignin extraction procedures were performed on the residues of the described extraction. Extractions were not replicated. In a previous work, standard deviations of lignin analysis by $H_2SO_4$ extraction ranged from 3.2 to 7.6% of the lignin results (Piccaglia and Galletti, 1987).

**HCl Lignin.** Samples (2 g) were extracted with 37% HCl (30 mL) for 3 h at room temperature. The slurry was subjected to several cycles of centrifugation and washing with water and acetone until neutral pH was reached, and then the samples were dried.

**HF Lignin.** A modification of the Papadopoulos and Defaye (1986) procedure was used, treating the samples (0.5 g) with 48% HF (10 mL) for 3 h at room temperature in closed Teflon jars. The slurry was then treated as above.

**$H_2SO_4$ Lignin.** Samples (2 g) were extracted with 72% $H_2SO_4$ (30 mL) for 3 h at room temperature and then subjected to the same procedure as above.

**NaOH Lignin.** Samples (2 g) were extracted with 0.1 M NaOH (30 mL) for 3 h at room temperature, and the above centrifugation and washing procedure was followed.

**Dioxane/Water Lignin.** Samples (5 g) were extracted with dioxane/water (9:1) (100 mL, freshly distilled dioxane) for 6 days at room temperature with mechanical shaking. The slurry was filtered, and the filtrate was concentrated to about 5 mL in a rotary evaporator under vacuum at 50 °C and dispersed through syringe into a 1% $Na_2SO_4$ solution (75 mL). The cooled suspension was filtered, and the precipitate was dried over NaOH.

**Oxidative Hydrolysis.** Lignins prepared as described (100 mg) were added to 2 M NaOH (5 mL) and nitrobenzene (100 μL) and heated in a thick glass tube closed with a screw cap at 160 °C for 2 h under magnetic stirring. After cooling, the mixture was diluted to 25 mL and filtered. The filtrate (2 mL) was acidified with 1 M HCl (2 mL), diluted to 25 mL, filtered through a 0.22-μm cartridge filter and injected (20 μL) into a HPLC. Experiments were duplicated.

**HPLC Conditions.** A Waters chromatographic system consisting of a 590 pump and a U6K injector was connected with an ESA Coulochem detector (Model 5100 A) equipped with an analytical cell (Model 5011) set at +0.80 V. Chromatographic

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**Table I. Yield (Percent of Defatted Material) of Lignin from Different Extraction Procedures**

| | lignin | | | | |
|---|---|---|---|---|---|
| lignocellulosic | $H_2SO_4$ | HCl | HF | NaOH | diox/$H_2O$ |
| wheat straw | 22.5 | 56.5 | 80.8 | 91.5 | 0.82 |
| corn stalks | 18.0 | 40.0 | 54.2 | 65.5 | 1.28 |
| alfalfa stems | 22.5 | 53.5 | 89.0 | 74.5 | 1.52 |
| red oak leaves | 37.5 | 51.2 | 72.0 | 70.5 | 0.80 |

traces were displayed on a Perkin-Elmer 561 recorder. A reversed-phase column (120 × 4.6 mm), Viosfer C6, 5 μm (Violet, Rome), was employed under isocratic conditions with methanol/0.1% perchloric acid in water (15:85 v/v), 1 mL/min. Peaks were identified by comparison of their retention times and electrochemical behaviors (hydrodynamic voltammograms) with those of pure compounds. Quantification was performed by calibration with external standards.

**Statistical Analysis.** A 4 × 5 factorial analysis of variance was used for each phenolic: four materials (wheat, corn, alfalfa, and red oak leaves) and five lignin preparations ($H_2SO_4$, HCl, HF, NaOH, and dioxane/$H_2O$).

## RESULTS AND DISCUSSION

A preliminary ethanol/benzene extraction of all of the samples was carried out to work on extractive-free substrates. It was already pointed out (Lai and Sarkanen, 1971) that this is a common procedure, although extractives may contain so-called "low molecular weight" lignin. This soluble fraction of lignin was not examined, since the focus of this work was the insoluble lignin and the products of its chemical degradation.

Lignin yields (percent of defatted materials) (Table I) reflect the differential efficiency of the extraction media. The lowest yield of lignin was obtained with the dioxane/water system, which also showed a unique pattern of extraction from the four byproducts. Similar patterns of lignin yields were found for the NaOH, HCl, HF, and $H_2SO_4$ extractions of the lignocellulosics, although the absolute yields were quite different. It is well-known (Lai and Sarkanen, 1971) that conditions (time, temperature, media) and techniques of sample grinding strongly affect absolute lignin yields. However, our scope was a relative yield comparison by means of ordinary techniques and reasonable experimental times, rather than an attempt to maximize the yields by, for example, increasing the extraction time.

Under these circumstances, the very low dioxane/water lignin yields in spite of a 6-day extraction are consistent with a higher degree of selectivity of this method when compared to the others. The extraction with dioxane/water is therefore a interesting method to obtain "pure" lignins but is too cumbersome to be of practical application in screening tests.

As stated by Lai and Sarkanen (1971), "alkali lignins, in their crude form, may contain as impurities carbohydrates, silica and proteins". This may be an explanation of the relatively high residues after NaOH extraction.

Small recoveries after $H_2SO_4$ treatment, in comparison with other acidic treatments, may be explained with a complete hydrolysis of polysaccharides (Janshekar and Fiechter, 1983) and a partial removal of lignin itself, as already reported for some types of wood (Lai and Sarkanen, 1971). Furthermore, $H_2SO_4$ lignin is considered to have a high degree of condensation, hence a reduced yield due to loss of water. Condensation reactions should be less important when HCl is used, thus explaining the higher yields of HCl lignins. The relatively high residues after treatment with HF solution can be due to incomplete removal of carbohydrates bound to lignin (Papadopoulos and Defaye, 1986).

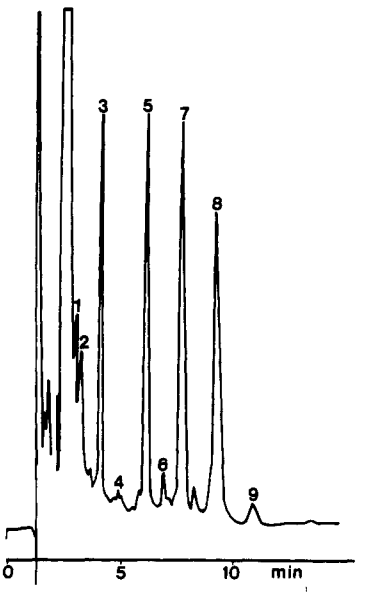


**Figure 1.** HPLC/ElCh profile of NaOH lignin of corn stalk. (1) *p*-Hydroxyphenylacetic acid; (2) *o*-hydroxyphenylacetic acid; (3) *p*-hydroxybenzaldehyde; (4) vanillic acid; (5) vanillin; (6) syringic acid; (7) *p*-coumaric acid; (8) syringaldehyde; (9) ferulic acid.

Figure 1 shows the HPLC/ElCh profile of NaOH lignin from corn, after direct injection of nitrobenzene hydrolysate.

The phenolic composition of the five lignin preparations from wheat straw, corn stalks, alfalfa stems, and red oak leaves is reported in Tables II–V. Total phenolic yields of $H_2SO_4$, HCl, HF, and NaOH lignin preparations ranged from 4.6 to 15.8% of crude lignin for the four lignocellulosic byproducts, whereas dioxane/water lignin yielded phenolics in a wider range (5.0–33.9% of crude lignin). The relatively low yields of phenolics in all of the lignins are consistent with poorly selective lignin preparations. In this respect, dioxane/water extraction can be regarded as the most selective lignin preparation. The lignocellulosics were clearly divided into two groups when total phenolic yields (particularly that from dioxane/water lignin) are considered: lignin preparations from the monocot materials (wheat, corn) yielded higher amounts of phenolics than the dicot materials (alfalfa, oak). A possible explanation of this observation is that lignin preparations from the tested dicots are impure of non-lignin constituents to a larger extent than the preparations from the monocots, assuming that the efficiency of nitrobenzene conversion of lignin into phenolics is comparable for the various lignins.

Differences in the lignin composition between the monocots and dicots are pointed out by the different contents of synapilic compounds (syringaldehyde and syringic acid) and ferulic acid, which were more abundant in wheat and corn than in alfalfa and oak.

Statistical analysis showed significant differences ($P < 0.01$) in the content of each phenolic among (A) the four materials (wheat, corn, alfalfa, and red oak leaves) and (B) the five lignin preparations. Furthermore, interactions between the materials and the methods of preparation (C) were found significantly different. In other words, statistical analysis results proved that (A) different materials yielded different amounts of phenolics when subjected to the same lignin preparation; (B) different lignin preparations yielded different amounts of phenolics from the same material; and finally (C) the relative

**Table II. Phenolic Composition in Wheat Straw as Percentage of Lignin Preparations (±SD)**

| compd | $H_2SO_4$ | HCl | HF | NaOH | diox/$H_2O$ |
|---|---|---|---|---|---|
| *p*-hydroxyphenylacetic acid | | | 0.065 (0.005) | 0.074 (0.008) | |
| *o*-hydroxyphenylacetic acid | 0.166 (0.007) | 0.211 (0.002) | | | 0.602 (0.042) |
| *p*-hydroxybenzoic acid | 0.048 (0.003) | 0.038 (0.003) | | | 0.183 (0.010) |
| *p*-hydroxybenzaldehyde | 0.166 (0.029) | 0.233 (0.006) | 0.136 (0.016) | 0.148 (0.002) | 1.004 (0.065) |
| vanillic acid | 0.536 (0.006) | 0.275 (0.006) | 0.208 (0.006) | 0.275 (0.003) | 0.468 (0.045) |
| vanillin | 1.695 (0.124) | 3.284 (0.059) | 1.625 (0.219) | 2.495 (0.054) | 7.402 (0.139) |
| syringic acid | 2.441 (0.033) | 1.674 (0.207) | 1.581 (0.211) | 0.950 (0.013) | 3.317 (0.118) |
| *p*-coumaric acid | 0.246 (0.001) | 1.075 (0.135) | 0.974 (0.088) | 0.994 (0.181) | 0.524 (0.037) |
| syringaldehyde | 4.020 (0.091) | 5.351 (0.368) | 3.497 (0.476) | 3.619 (0.091) | 7.087 (0.020) |
| ferulic acid | 0.372 (0.003) | 0.791 (0.072) | 0.991 (0.015) | 1.316 (0.143) | 0.149 (0.001) |
| sum | 12.690 (0.134) | 12.932 (0.579) | 9.077 (0.918) | 9.871 (0.113) | 20.736 (0.477) |

**Table III. Phenolic Composition in Corn Stalks as Percentage of Lignin Preparation (±SD)**

| compd | $H_2SO_4$ | HCl | HF | NaOH | diox/$H_2O$ |
|---|---|---|---|---|---|
| *p*-hydroxyphenylacetic acid | 0.169 (0.016) | 0.099 (0.003) | 0.076 (0.008) | 0.084 (0.008) | 0.515 (0.049) |
| *o*-hydroxyphenylacetic acid | | 0.276 (0.021) | | 0.149 (0.016) | 0.536 (0.049) |
| *p*-hydroxybenzoic acid | 0.203 (0.025) | 0.048 (0.001) | 0.048 (0.004) | | 0.187 (0.012) |
| *p*-hydroxybenzaldehyde | 0.548 (0.033) | 0.555 (0.004) | 0.680 (0.088) | 0.262 (0.002) | 3.416 (0.119) |
| vanillic acid | 0.368 (0.018) | 0.276 (0.001) | 0.388 (0.039) | 0.218 (0.001) | 0.393 (0.039) |
| vanillin | 1.266 (0.126) | 2.049 (0.023) | 1.898 (0.172) | 1.788 (0.442) | 6.799 (0.284) |
| syringic acid | 2.022 (0.023) | 0.730 (0.040) | 1.021 (0.018) | 0.634 (0.058) | 2.150 (0.071) |
| *p*-coumaric acid | 2.527 (0.318) | 4.420 (0.857) | 5.080 (0.882) | 5.142 (1.13) | 6.480 (0.169) |
| syringaldehyde | 6.875 (0.171) | 4.912 (0.198) | 5.827 (0.487) | 3.728 (0.753) | 13.154 (0.066) |
| ferulic acid | 0.635 (0.055) | 0.587 (0.023) | 0.759 (0.001) | 1.394 (0.289) | 0.254 (0.008) |
| sum | 14.613 (0.195) | 13.952 (0.899) | 15.777 (0.075) | 13.399 (2.53) | 33.884 (4.29) |

**Table IV. Phenolic Composition in Alfalfa Stems as Percentage of Lignin Preparation (±SD)**

| compd | $H_2SO_4$ | HCl | HF | NaOH | diox/$H_2O$ |
|---|---|---|---|---|---|
| *p*-hydroxyphenylacetic acid | 0.275 (0.021) | 0.161 (0.020) | 0.144 (0.023) | 0.149 (0.013) | |
| *o*-hydroxyphenylacetic acid | | | | | 0.463 (0.039) |
| *p*-hydroxybenzoic acid | 0.064 (0.008) | 0.109 (0.009) | 0.035 (0.002) | | 0.351 (0.027) |
| *p*-hydroxybenzaldehyde | 0.098 (0.001) | 0.068 (0.011) | 0.092 (0.005) | 0.025 (0.001) | 0.329 (0.030) |
| vanillic acid | 0.618 (0.007) | 0.413 (0.086) | 0.443 (0.045) | 0.332 (0.005) | 0.285 (0.022) |
| vanillin | 1.477 (0.108) | 3.068 (0.721) | 2.423 (0.221) | 2.560 (0.907) | 1.038 (0.088) |
| syringic acid | 0.437 (0.029) | 0.226 (0.040) | 0.239 (0.057) | 0.201 (0.003) | 1.009 (0.129) |
| *p*-coumaric acid | | 0.077 (0.014) | 0.182 (0.015) | 0.069 (0.001) | 0.369 (0.044) |
| syringaldehyde | 1.515 (0.045) | 2.419 (0.466) | 1.957 (0.192) | 1.393 (0.312) | 1.195 (0.078) |
| ferulic acid | 0.114 (0.014) | | | | |
| sum | 4.598 (0.132) | 6.541 (1.52) | 5.515 (0.354) | 4.729 (0.684) | 5.039 (0.457) |

**Table V. Phenolic Composition in Red Oak Leaves as Percentage of Lignin Preparation (±SD)**

| compd | $H_2SO_4$ | HCl | HF | NaOH | diox/$H_2O$ |
|---|---|---|---|---|---|
| *p*-hydroxyphenylacetic acid | | 0.039 (0.003) | 0.104 (0.008) | 0.117 (0.011) | |
| *o*-hydroxyphenylacetic acid | 0.344 (0.023) | 0.254 (0.023) | | | 0.521 (0.041) |
| *p*-hydroxybenzoic acid | 0.263 (0.024) | 0.141 (0.013) | 0.190 (0.015) | 0.121 (0.013) | 0.464 (0.037) |
| *p*-hydroxybenzaldehyde | 0.272 (0.011) | 0.275 (0.022) | 0.177 (0.003) | 0.053 (0.005) | 0.572 (0.040) |
| vanillic acid | 0.595 (0.064) | 0.269 (0.008) | 0.441 (0.007) | 0.310 (0.004) | 0.275 (0.021) |
| vanillin | 1.299 (0.180) | 1.853 (0.127) | 1.961 (0.001) | 2.507 (0.226) | 1.239 (0.086) |
| syringic acid | 0.499 (0.032) | 0.309 (0.008) | 0.523 (0.065) | 0.298 (0.018) | 0.634 (0.030) |
| *p*-coumaric acid | 0.231 (0.001) | 0.450 (0.025) | 0.538 (0.028) | 0.468 (0.057) | 0.598 (0.046) |
| syringaldehyde | 2.038 (0.299) | 2.965 (0.165) | 3.215 (0.319) | 3.181 (0.051) | 1.468 (0.046) |
| ferulic acid | 0.151 (0.001) | 0.193 (0.049) | 0.333 (0.047) | 2.230 (0.049) | |
| sum | 5.692 (0.178) | 6.748 (0.705) | 7.482 (0.664) | 9.285 (0.218) | 5.771 (0.346) |

selectivity of the lignin preparations is affected by the various lignocellulosic materials.

In conclusion, HPLC/ElCh analysis of nitrobenzene hydrolysates allowed a rapid determination of phenolic constituents of different lignin preparations from lignocellulosic byproducts. The lack of better correlations among the various procedures of lignin preparations shows that they cannot be determining the same chemical plant constituents. While it is possible to determine some of the coextracted non-lignin components (such as carbohydrates and proteins), it is arduous to predict how the interaction among these components in the cell-wall polymeric matrix can affect the selectivity of the various methods. Knowing the changes in phenolic composition of different lignins might be of some utility in understanding the chemical basis of the various lignin assays, and in this regard ElCh detection provides a useful tool for the rapid screening of several samples by HPLC.

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