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Extraction and Isolation of Lignin for Utilization as a Standard to Determine Lignin Concentration Using the Acetyl Bromide Spectrophotometric Method

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Lignin extracted with acidic dioxane was investigated as a possible standard for quantitatively determining lignin content in plant samples using the spectrophotometric method employing acetyl bromide. Acidic dioxane lignins were analyzed for carbohydrate, total protein, nitrobenzene oxidation products, and UV spectral characteristics. Total carbohydrate content of isolated lignins ranged from 2.21 to 5.70%, while protein ranged from 0.95 to 6.06% depending upon the plant source of the original cell wall sample. Nitrobenzene analysis indicated differences in the amount of guaiacyl and syringyl units making up the lignins, but this did not alter the UV spectrum of lignin solubilized in acetyl bromide. Regression equations developed for the acetyl bromide method using the isolated lignins for all the plant samples were similar to each other. Lignin values obtained by the acetyl bromide method were similar to the lignin values obtained as acid insoluble residues following a Klason lignin procedure.

Keywords: *Acetyl bromide lignin assay; forages; dioxane; lignin; spectrophotometric method*

INTRODUCTION

Cell walls of forages provide an essential source of potential energy to ruminants. However, utilization of this energy source is far from complete leaving a large portion (as high as 60−70% for some warm season grasses) to be excreted by the animal. It is generally believed that lignin and cross-linking to cell wall polysaccharides are the major impediments to fiber digestion (*1*). Lignin is a complex phenolic polymer, composed of phenylpropanoid units and is an integral component of forage cell walls that cannot be digested by mammalian enzymes (*2*). There is usually a strong negative correlation between herbage age and dry matter digestibility for most forages; as forages mature, cell wall concentration increases as a function of both thickening of walls and a decrease in leaf-to-stem ratio (*3*). Lignin concentration also increases with maturity and is generally higher in legume tissues than those of grasses, although in grasses it is usually more inhibitory to digestion than that in legumes (*1*).

Lignin content is often correlated with fiber digestibility (*4*, *5*); however, any digestibility marker must be chemically determined with acceptable precision and accuracy (*6*). On the basis of previous work (*7*), a rapid method was developed for measuring lignin in forages (*8*, *9*). This method, referred to as the acetyl bromide soluble lignin (ABSL) method, is based on solubilization of lignin into a solution of 25% acetyl bromide in glacial acetic acid. Lignin concentration is read at 280 nm; however, as with any spectrophotometric method, a

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reliable standard is needed to develop calibration curves (*10*). For pure compounds development of extinction coefficients becomes a convenient method of quantifying the compound of interest. Lignin itself is not a pure compound, varying in composition (monolignols) and bonding patterns but may behave like a pure compound due to the spectral similarities of the monolignols that compose the macromolecule. Several materials have been used as standards, such as lignin derivatives such as Indulin (*11*), beech kraft lignin (*12*), native lignin (*13*), and more recently, an acetyl bromide solubilized lignin (*14*). Estimates of lignin values were obtained by using optical absorbances of guaiacol and ferulic acid as standards (*15*, *16*). The acetyl bromide method was modified to include the use of perchloric acid to produce a more complete digestion of woody and herbaceous samples ensuring a complete solubilization of lignin (*17*). Using isolated lignin samples they developed a specific absorption coefficient of 20.0 L g^{-1} cm^{-1}. Later work has shown that the inclusion of perchloric acid must be handled carefully to prevent excessive degradation of xylans that interfere with the lignin absorption at 280 nm (*18*).

Objectives of this research included: (i) developing a convenient method of obtaining lignin that has minimal nonlignin contamination; (ii) comparing the lignins isolated from different plant materials to determine if all extracted lignins (irrespective of structure, composition and origin) respond in a similar fashion in the ABSL method; and (iii) using isolated lignins to develop standard curves for lignin quantification based on the spectrophotometric analyses of plant samples using the ABSL method. The information gained from this study allows us to compare our results to previous studies and to determine whether a single extinction coefficient for lignin is suitable for the acetyl bromide lignin method.

MATERIALS AND METHODS

Plant Material. Most plant materials for this study were grown in the greenhouse under supplemental light (high-pressure sodium lamps, 500 W). The lighting consisted of a 14/10 h day/night regimen, and the temperature was maintained at 26/18 °C (day/night). All plants were watered biweekly with a soluble fertilizer (Petes Soluble fertilizer, 20−20−20, N−P−K). Red clover used in these experiments was field grown and harvested from the University of Wisconsin Experimental Farm, Arlington, WI. Pine samples were a gift from the USDA-Forest Products Laboratory (S. Ralph and L. Landucci). For the acetyl bromide extraction experiments the following plant/plant tissues were used: corn (*Zea mays* L.) stalk; alfalfa (*Medicago sativa* L.) leaf and stem tissues; bromegrass (*Bromus inermis* L.) leaf and stem; and loblolly pine (*Pinus taeda* L.). Corn stalks were harvested when seed development was at the early dent stage, alfalfa was harvested at early flowering (10−20% bloom stage), bromegrass was harvested at late boot stage, and the pine was a mature wood sample. For the acidic-dioxane extraction experiments, the following plant stem samples were investigated; corn, alfalfa, bromegrass, red clover (*Trifolium pratense* L.), and loblolly pine. Maturity stages and specific tissues of forage samples were as follows: corn rind tissue at 10 days past anthesis; alfalfa stems 10−20% bloom stage; red clover stems at late bud stage; bromegrass stems samples were of three maturity stages, I − young plant, late boot stage with flowers just beginning to emerge; II − flowering heads fully emerged and inflorescences beginning to open; and III − mature, past pollen shed of the full bloom stage. Harvested forages were dried in a 60 °C forced air oven for 72 h and the stems were milled to pass a 0.5 mm sieve using a Wiley Mill (Arthur H. Thomas Co., Philadephia, PA). To prepare the cell walls (CW), ground samples were sequentially extracted with water, ethanol, chloroform and acetone in a Soxhlet apparatus. Each solvent extraction was continued until no additional color leached from the walls; typically extraction times were 4−8 h for each solvent.

Lignin Extraction. Acetyl bromide lignin (AcBrL), as its name indicates, was extracted with 25% acetyl bromide in glacial acetic acid (*14*). Acidic-dioxane lignin (DL) was isolated using dioxane acidified with HCl (*19*) incorporating some minor modifications. Five grams of dry cell wall material was placed in a 250-mL round-bottom flask and 100 mL of acidic dioxane (90 mL of dioxane + 10 mL of 2 N HCl solution) was added; the flask was connected to a reflux condenser and N_2 gas was blown onto the liquid surface for a 20−30 s. The solution was then refluxed under N_2 for 30 min. After cooling, the solution was filtered through a glass fiber filter (GF/C, 47 mm, Whatman Inc., USA) and collected in an Erlenmeyer flask, 20 mL of 96% dioxane was used to wash the residue collected on the filter and the wash was combined with the original filtrate. Sodium bicarbonate (4.0 g) was added to the Erlenmeyer flask and the sample placed on a rotatory shaker for several minutes until neutralization of the solution (measured with a pH strip). The solution was filtered through a 0.45 μm nylon membrane (Schleicher & Schuell, Keene, NH) before concentrating to 10−15 mL under reduced pressure on a rotary evaporator (water temperature 40 °C). The solution was added dropwise to a 250-mL centrifuge bottle containing ~200 mL of rapidly stirring distilled water. Any insoluble residue remaining in the flask was washed with 2.0 mL of 96% dioxane solution and added dropwise to the water. Generally, a fine precipitate would immediately form. For some samples that did not readily form a precipitate, the addition of 2.0 g of anhydrous sodium sulfate (while stirring) helped flocculate the lignin. After stirring, the precipitate was pelleted by centrifugation (9000*g*, 20 min) and the supernatant was removed. The pellet was partially dried by placing the centrifuge bottle in a 60 °C forced air oven for 15 min. Lignin residues were dissolved in 4−5 mL of dioxane (100%), filtered through a 0.45 μm nylon membrane, and added dropwise to 200 mL of rapidly stirring anhydrous diethyl ether in a 250-mL centrifuge bottle. The resulting precipitate was pelleted by centrifuging (9000*g*, 15 min, 0 °C) and the entire solubilization in dioxane and ether wash step was repeated to remove hydrophobic nonlignin contaminants. After removing the diethyl ether, 60 mL of petroleum ether was added while stirring (with a spatula) to thoroughly wash the lignin residue. This solvent was removed after allowing the residue to settle. The lignin residue (referred to dioxane lignin − DL) was freeze-dried (Virtus Co., Gardiner, NY) for 48 h and stored in a freezer over desiccant.

Standard Curves and ABSL Method. Briefly, 10 mg of isolated lignin (after corrections for carbohydrate and protein contaminants) was dissolved in 5.0 mL of dioxane and aliquots of 0.2, 0.3, 0.4, 0.5 and 0.6 mL were pipetted into culture tubes (12.5 × 1.6 cm), frozen in liquid N_2, and placed on a freeze-drier overnight. To each tube 0.5 mL of 25% acetyl bromide in glacial acetic acid (HAcBr) was added (cautionary note on acetyl bromide: corrosive; causes burns; irritating to eyes and respiratory system; reacts violently with water; lachrymator). A blank was included to correct for reagent background absorbance. Tubes were tightly capped (Teflon lined caps) and put in a 50 °C water bath for 30 min. After cooling the samples, all tubes received 2.5 mL of acetic acid (HAc), 1.5 mL of 0.3 M NaOH, and 0.5 mL of 0.5 M hydroxylamine hydrochloride solution. Tubes were shaken and HAc was added to give a final volume of 10.0 mL. Solutions were read at 280 nm in a Beckman, DU-50 model spectrophotometer and scanned from 250 to 350 nm. Standard curves were developed from duplicate sample series.

For quantitative evaluation of lignin in forages and wood, a basic acetyl bromide protocol was conducted (*17*); however, cautionary notes were followed (*18*). The procedure consisted of digesting 100 mg of the CW preparation with 4.0 mL of AcBrHAc reagent at 50 °C for 2 h, with occasional mixing. After cooling the sample, the volume was made to 16.0 mL with HAc and centrifuged (3000*g*, 15 min), and 0.5 mL of this

solution was added to a tube containing 2.5 mL of HAc and 1.5 mL of 0.3 M NaOH. After shaking the sample, 0.5 mL of 0.5 M hydroxylamine hydrochloride solution was added and the volume was made up to 10 mL with HAc. Optical density at 280 nm was measured and the concentration determined from the respective standard curve using a regression equation. A blank was included to correct for reagent background absorbance.

Chemical Analyses. Klason lignin (KL) was determined as the residue remaining after two-step sulfuric acid hydrolysis of cell wall polysaccharides (*20*). This method allowed the determination of neutral sugars, total uronosyls, and acid insoluble residues in original cell wall, acetyl bromide isolated lignin, and DL samples. Neutral sugars were quantified using an HPLC procedure (*21*) (DX-500 Carbohydrate System using a Carbopac PA10 4 × 250 mm column; Dionex Corp, Sunnyvale, CA). Total uronosyls were determined using the phenyl phenol method (*22*). Total N content of isolated lignin samples was determined by the Carlo Erba NA 1500 nitrogen analyzer (Fision Instruments, Saddle Brook, NJ) (*23*). Crude protein was estimated as N × 6.25.

Nitrobenzene oxidation was used to compare monolignol composition among isolated DL samples. The procedure basically followed the original reaction (*24*) with some modifications (*25*−*27*). Briefly, approximately 10 mg of DL was weighed into a stainless reaction vessel and 4 mL of 2 N NaOH and 0.25 mL of nitrobenzene was added to each reaction vessel. Vessels were tightly closed and kept in a fluidized dry bath (170 °C) for 2.5 h. The content of each vessel was quantitatively transferred to a 25-mL culture tube with the aid of distilled water (3× with 5 mL). Isovanillin was added as an internal standard (50 μL of 10 mg/mL ethanol solution). The basic nitrobenzene reaction mixture was extracted twice with chloroform ($CHCl_3$, 5 mL), and the $CHCL_3$ extract was properly discarded. The remaining aqueous fraction was acidified with 10 N HCl to pH <2.5 and slowly passed through a solid phase extraction column (3 mL, Envir-18 Supelco) which had been previously conditioned with the following sequence of solvents: 2 mL each of dH_2O, methanol (MeOH), MeOH/$CHCl_3$ (1:1), $CHCl_3$, MeOH/$CHCl_3$ (1:1), MeOH, dH_2O, and a 2 N NaOH solution (pH adjusted to <2.5 with 10 N HCl). After loading the sample, the column was washed with dH_2O (pH adjusted to <2.5 with TFA); all wash solutions were properly discarded. Components retained on the columns (aldehydes, ketones, and acids) were eluted from the column using methanol (2 mL 2×) and tetrahydrofuran (2 mL 2×) and collected in a clean 15 mL conical tube. The volume of the methanol + THF extract was reduced to approximately 1/3 under a stream of dried-filtered air. A sub-sample (1.0 mL) was placed in an 8-mL reaction vial, evaporated to dryness, dissolved in 10 μL of THF and 40 μL of silylating agent *N*,*O*-bis (trimethylsilyl) trifluoroacetimide (BSTFA), and heated at 60 °C for 20 min. One microliter was injected (split mode 50:1) into a GC-FID (HP 6890 Agilent) and separated on a capillary column (DB-1 25 m × 0.2 mm − J&W Scientific) using a temperature program that consisted of an initial temperature of 160 °C (held for 5 min), ramp at 4 °C/min to 200 °C, ramp at 10 °C/min to 240 °C (held for 5 min), followed by a ramp of 15 °C/min to 300 °C (held for 5 min). Phenolic standards (100 μg of each): *p*-hydroxybenzaldehyde; vanillin; isovanillin; acetovanillone; syringaldehyde; 3,5-dimethoxy-4-hydroxy-acetophenone; vanillic acid; syringic acid; *p*-coumaric acid and ferulic acid were subjected to the same nitrobenzene procedure to determine response/recovery factors for each.

Statistical Analysis. Experimental design employed was a completely randomized design with duplicate analysis for each assay. Statistical evaluation was performed by analysis of variance for the yield of DL, total carbohydrate, and protein content in the DL samples, on nitrobenzene oxidation products yield and on ABSL and Klason results. When the analysis of variance indicated difference ($P < 0.05$), the individual treatments were compared by means of the General Linear Model Procedure (*28*). Intercepts and slopes of regression equations derived from the standard curves of isolated lignins were compared using the REG Procedure (*28*).

Table 1. Cell Wall (CW) Content (g kg^{-1} DM) and Acetyl Bromide Lignin (AcBrL) Yields (g kg^{-1} CW) and Total Uronosyls, Neutral Sugars, and Protein Content in the AcBrL (g kg^{-1} Lignin)[a]

sample name	CW	AcBrL	total uronosyls	neutral sugars	total sugars	protein
corn stalk	392.3^{d}	76.0^{d}	5.2^{e}	212.4^{b}	217.6^{b}	15.6^{d}
alfalfa leaf Y	195.6^{f}	39.1^{e}	11.7^{b}	357.5^{a}	369.2^{a}	26.9^{c}
alfalfa stem Y	484.6^{c}	187.0^{b}	18.8^{a}	158.6^{c}	177.4^{c}	33.6^{b}
bromegrass leaf Y	362.3^{e}	81.3^{c,d}	7.3^{c}	352.3^{a}	359.6^{a}	18.0^{d}
bromegrass stem Y	494.4^{b}	112.5^{c}	6.0^{d}	97.7^{d}	103.7^{d}	36.5^{a}
pine	950.9^{a}	312.7^{a}	3.7^{f}	10.7^{e}	14.4^{e}	2.3^{e}
mean	480.0	134.8	8.8	198.2	207.0	22.2
S.E.	70.11	27.77	1.55	38.24	38.67	3.52

[a] Data are means of two observations. Y − young; S.E. − standard error. Means, within a column, followed by different small letters are different ($P < 0.05$).

RESULTS AND DISCUSSION

Acetyl Bromide Lignin Extraction Trial. Relatively low yields are generally obtained when lignin is isolated from plant sources. Less than 1% of alfalfa lignin was extracted using Brauns' native lignin procedure (*13*). Several other investigators have used different organic solvents to extract lignin from woods, and yields were from 2.0 to 81.5% of lignin as compared with Klason lignin content (*29*, *30*). Earlier work (*14*, *31*), using a range of different forage species, utilized the acetyl bromide reagent to solubilize lignin from cell wall samples. This was a convenient procedure for obtaining high yields of lignin with recoveries ranging from 2.2 to 33.5% of the cell wall. However, samples often contained significant amounts of other wall materials in addition to the lignin. We have utilized this procedure to produce lignin samples for comparison to the lignin obtained using acidic-dioxane extractions. Yield of acetyl bromide lignin (AcBrL) ranged from 39.1 to 312.7 g kg^{-1} CW (Table 1), and as one might expect leaves yielded lower amounts of AcBrL than stems. The large range in recoverable AcBrL not only varied with different plant species, tissue, and maturity stage but also showed considerable variation in the amounts of wall components in addition to the lignin.

Higher concentrations of total sugars were detected in the AcBrL isolates from leaf tissue as compared to stems ($P < 0.05$). As a general trend, it appears that the less total AcBrL extracted from the cell wall, the higher the nonlignin components. This may be a reflection of the type of cell wall and its chemical composition. Total sugar concentration in the AcBrL varied from 14.4 to 369.2 g kg^{-1} lignin. It is evident that total sugar components in this type of lignin were high. High contamination of AcBrL with carbohydrate was reported somewhere else, varying from 11.2 to 29.7% (*31*).

Neutral sugar composition of the extracted carbohydrate indicated a large portion appeared to be coming from cellulose as the major sugar in each sample was glucose (Table 2). Earlier work has shown that acetyl bromide reagent can readily degrade xylans but not other types of cell wall polysaccharides (*18*). Perhaps the high level of glucose in the AcBrL is due to acetylation of the cellulose, which would render it more soluble in the acetyl bromide reagent. Upon acid hydrolysis, the glucose would be recovered whereas xylose would not due to its degradation. NMR analysis of acetyl bromide isolated lignin samples produced complex spectra indicating significant amounts of acetylated

Table 2. Content of Individual Neutral Sugars in the Acetyl Bromide Lignin (g kg^{-1} Lignin)[a]

sample name	Fuc	Ara	Rha	Gal	Glc	Xyl	Man
corn stalk	0	6.0	0	0.5	198.1	7.8	0
alfalfa leaf Y	0	0.4	1.3	2.0	348.5	3.2	2.0
alfalfa stem Y	0	0	0.4	0.4	156.5	1.3	0
bromegrass leaf Y	0	7.5	0.2	0.5	340.8	3.3	0
bromegrass stem Y	0	7.7	0	0	84.6	5.4	0
pine	0	0	0	0	9.4	0	1.3
mean	0	3.6	0.3	0.6	189.7	3.5	0.6
S.E.	0	1.06	0.15	0.20	37.56	0.77	0.38

[a] Data are means of two observations. Fuc − fucose; Ara − arabinose; Rha − rhamnose; Gal − galactose; Glc − glucose; Xyl − xylose; Man − mannose; Y − young; S. E. − standard error.

carbohydrates as well as carbohydrate degradation products (data not shown). Protein content of AcBrL was low (Table 1) and similar to values reported previously (*32*), which ranged from 1.1 to 10.0% in AcBrL extracted from crude cell wall, neutral detergent fiber, or acid detergent fiber.

Although acetyl bromide reagent is a convenient method for isolating lignin from cell wall matrixes, the presence of high levels of nonlignin components, particularly carbohydrates, hampers its use as a routine method for extracting lignin. Also, it has been shown that the lignin is acetylated during the acetyl bromide extraction (*17*, *33*). Acetylation reactions of the lignin molecules will alter the UV absorbance spectra of lignin in the 250 nm and lower regions, but the major absorbance maximum for lignin around 275−280 nm is not affected. This does present a problem when considering using AcBrL as a standard because of the increased weight that must be accounted for due to the added acetyl groups. Since the amount of acetylation would vary with the composition and linkage pattern within individual lignins it would be difficult to accurately correct for this additional weight. For these reasons, a new method was sought that would allow us to isolate a lignin with minimal nonlignin components and was easy to use on a routine basis if necessary. The acidic dioxane method seemed a likely candidate for such a procedure (*19*).

DL Extraction Trial. Utilization of acidic dioxane to isolate lignin was primarily targeted to reduce the level of contamination, particularly carbohydrates. We focused on the stem fractions of forages primarily because as forages mature there is generally a decrease in leaf-to-stem ratio and an increase in cell wall content including increasing levels of lignin (*3*). The plant materials for this study are shown in Table 3 as well as general cell wall characteristics used for acidic-dioxane lignin (DL) extraction and composition of the recovered DL. Neutral sugar composition of individual wall samples is given in Table 4. The concentration of uronosyls was higher in the legume CW as compared to the CW of grasses or pine ($P < 0.05$) (Table 3). This is in agreement with the general observation of elevated content of pectin in legumes (*2*) and the specific determination that pectic carbohydrates form a significant proportion of alfalfa stem cell walls (*34*). The actual amounts of pectic polysaccharides in the alfalfa and red clover cell walls is lower than normal based on the pectic neutral sugars (Table 4; Ara, Gal, Rha) and total uronosyls (*34*). This is most likely due to the extraction method used to prepare the cell walls. Continued extraction with water for 8 h using the Soxhlet apparatus will remove part of the total pectic carbohydrates in pectic rich walls such as those found in red clover and alfalfa. Pine tended to contain similar amounts of uronosyls as grasses. However, uronosyl content of pine DL was the lowest among all plants ($P < 0.05$). Legumes exhibited a lower proportion of uronosyls in their DL extracts as compared to the grass samples despite the higher content within the original cell walls (Table 3). This is most likely due to differences in the polysaccharides that the uronosyls are associated with. In grasses, the major portion of uronosyls are glucuronic or 4-*O*-methyl-glucuronic acids that are attached to xylans, whereas in the legumes it is galacturonic acid that forms the main backbone of the pectic fraction (*35*, *36*). When one compares the neutral sugar composition of the DL extracts (Table 5) the major sugar is xylose, indicative of xylans being extracted with these lignin fractions. It has been shown that xylans are frequent components of lignin complexes isolated from plant walls (*37*−*41*). Evidence that glucuronoxylans can

Table 4. Content of the Individual Neutral Sugars in the Cell Wall (g kg^{-1} CW)[a]

sample name	Fuc	Ara	Rha	Gal	Glc	Xyl	Man
corn stalk	0.1	22.5	0.5	6.9	504.5	243.2	2.0
alfalfa Y	1.5	25.3	5.5	19.4	459.0	129.7	19.9
alfalfa M	1.0	12.5	4.9	16.0	490.0	125.9	19.7
bromegrass Y	0.2	27.9	1.0	7.1	449.7	243.4	2.1
bromegrass M1	0.2	32.9	1.3	8.9	431.3	245.0	1.0
bromegrass M2	0.2	30.6	1.3	8.3	451.8	261.5	1.2
red clover	2.1	37.3	6.1	25.5	430.1	112.3	19.7
pine	0.3	12.0	0.6	23.4	449.8	69.8	111.7
mean	0.7	25.1	2.7	14.4	458.3	178.8	22.2
S.E.	0.2	2.6	0.5	1.8	6.5	18.6	9.0

[a] Data are means of two observations. Fuc − fucose; Ara − arabinose; Rha − rhamnose; Gal − galactose; Glc − glucose; Xyl − xylose; Man − mannose; Y − young; M − mature (1 and 2 refer to two different maturity stages); S.E. − standard error.

Table 3. Cell Wall (CW) Content (g kg^{-1} DM), Concentration of Neutral Sugars and Uronosyls in the CW, and Dioxane Lignin (DL) Yield (g kg^{-1} CW); Protein, Neutral Sugars, and Uronosyls Content in the DL (g kg^{-1} Lignin)[a]

sample name	CW	uronosyls (CW)	neutral sugars (CW)	DL	protein (DL)	uronosyls (DL)	neutral sugars (DL)	total sugars (DL)
corn stalk	500.6^{f}	23.0^{g}	779.8^{a}	59.1^{c}	22.5^{d}	16.9^{a,b}	38.3^{a,b}	55.2^{a}
alfalfa Y	728.8^{c}	88.0^{b}	660.3^{d,e}	31.2^{d}	52.5^{b}	12.1^{d}	12.4^{d}	24.5^{b}
alfalfa M	807.3^{b}	73.3^{c}	670.0^{d}	55.7^{c}	33.4^{c}	13.0^{c,d}	11.4^{d}	24.4^{b}
bromegrass Y	658.1^{d}	33.3^{e,f}	731.4^{b,c}	63.0^{c}	26.4^{d}	16.7^{a,b}	40.3^{a}	57.0^{a}
bromegrass M1	645.8^{d}	33.6^{e}	720.7^{c}	79.7^{b}	34.2^{c}	14.5^{b,c,d}	34.1^{a,b}	48.1^{a}
bromegrass M2	706.1^{c}	32.8^{e,f}	754.9^{a,b}	96.1^{a}	37.2^{c}	15.3^{b,c}	36.6^{a,b}	51.9^{a}
red clover	597.3^{e}	122.4^{a}	633.0^{e}	17.8^{d}	60.6^{a}	15.5^{b,c}	13.4^{d}	28.9^{b}
pine	950.9^{a}	29.9^{f}	667.6^{d}	62.8^{c}	9.5^{e}	3.7^{e}	18.4^{c,d}	22.1^{b}
mean	699.4	54.5	702.2	58.2	34.5	13.5	25.7	39.2
S.E.	33.0	8.7	12.8	6.0	4.0	1.1	3.2	3.8

[a] Data are means of two observations. Y − young; M − mature (1 and 2 refer to two different maturity stages); S.E. − standard error. Means, within a column, followed by different small letters are different ($P < 0.05$).

Table 5. Content of the Individual Neutral Sugars in the Dioxane Lignin (g kg^{-1} Lignin)[a]

sample name	Fuc	Ara	Rha	Gal	Glc	Xyl	Man
corn stalk	0	7.6	0.4	0.7	3.9	25.7	0
alfalfa Y	0	1.2	0.2	0.9	1.4	8.7	0
alfalfa M	0	0.6	0.2	0.9	1.4	8.3	0
bromegrass Y	0	12.2	0	0.5	3.2	24.4	0
bromegrass M1	0	11.5	0	0.2	2.7	19.7	0
bromegrass M2	0	11.9	0	0.5	2.8	21.4	0
red clover	0	1.4	0	0.5	2.4	9.1	0
pine	0	1.1	0	3.3	3.6	4.9	5.5
mean	0	5.9	0.1	0.9	2.7	15.3	0.7
S.E.	0	1.3	0.1	0.3	0.3	2.1	0.5

[a] Data are means of two observations. Fuc − fucose; Ara − arabinose; Rha − rhamnose; Gal − galactose; Glc − glucose; Xyl − xylose; Man − mannose; Y − young; M − mature (1 and 2 refer to two different maturity stages); S.E. − standard error.

become ester linked to lignins through the glucuronosyl residues has been presented (*38*), accounting for the coextraction of xylans with a lignin fraction using the acidic dioxane procedure.

A comparison of the AcBrL with DL (Tables 2 and 3) indicates that although the DL yields are lower the level of nonlignin components was also lower providing a cleaner lignin preparation. Neutral sugar concentration of cell walls is shown in Table 3. Legumes exhibited lower levels of these sugars in relation to the walls of grasses ($P < 0.05$). Carbohydrates are a common contaminant in most isolated lignins (*17*), usually constituting between 5 and 15% of the total soluble lignin complex (*8*, *42*).

Isolated lignins from forages typically contain nitrogen which is indigestible to animals, whereas wood lignins are virtually N free (*2*). The DL samples contained N, presumed to be protein, that ranged from 22.5 to 60.6 g kg^{-1} lignin. Grass DLs generally had a lower N concentration than legumes (with the exception of mature alfalfa). As one might expect, mature pine had the lowest N content (Table 3). Acid insoluble lignin from alfalfa had a higher concentration of N than lignins from grasses (*20*). The recalcitrant N may reflect cell-wall proteins, particularly those that have structural roles (*43*) and can be cross-linked into the matrix and are no longer extractable (*20*).

DL Oxidation Products from Nitrobenzene Reaction. Nitrobenzene analysis, a reaction that oxidizes releasable lignin subunits to their aldehydes (*24*, *25*), was used to compare the composition of lignin extracts. Although aldehydes are the prominent reaction products, other phenolics are also recoverable from the nitrobenzene reaction on DL samples (Table 6). As with many analytical tools for assessing lignin structure, nitrobenzene analysis does not result in the complete disassociation of lignin molecules into its monolignol subunits. Because of possible effects of heterogeneity of lignin structures upon the yields of nitrobenzene reaction products no attempt was made to apply correction factors to account for total lignin composition (*27*, *44*). The values listed in the table are the molar amounts recovered from each DL sample.

With the exception of pine (total guaiacyl type lignin), all other plants contained lignins composed of both guaiacyl and syringyl units resulting in a mixture of vanillin and syringaldehyde as the most abundant oxidative products. Yields of nitrobenzene products were similar among all the plants. The higher yield from corn is due to the high amounts of *p*-coumaric acid (*p*CA) attached to corn lignin that are recovered as *p*-hydroxybenzaldehyde and *p*CA. Corn stalks contain significant amounts of regiospecifically esterified (all esterified to the C-9 primary alcohol of monolignols) on its lignin (*45*). *p*-Coumaric acid can be a prominent component of grass walls but is not found at any substantial level in either legumes or woods (*46*). Ferulic acid and *p*CA can be oxidized to vanillin and *p*-hydroxybenzaldehyde respectively (*47*) and be counted as part of the lignin. Detection of hydroxycinnamic acids among nitrobenzene reaction products in grasses has been reported (*26*, *27*), and recovery factors have been proposed to allow their quantification. Recovery of oxidation products, particularly of those hydroxycinnamic acids, was determined to be a function of both temperature and time (*25*). We adopted the procedure of Iiyama to minimize oxidation of hydroxycinnamates found in the walls.

Table 6. Nitrobenzene Oxidation Products from Dioxane Lignin (mmol g^{-1} Lignin)[a]

sample name	*p*HBA	Van	Syr	VanA	SyrA	*p*CA	FA	total
corn stalk	0.14	0.35	0.61	0.02	0.12	0.60	0.12	1.96
alfalfa Y	0.01	0.85	0.45	0.10	0.09	0	0.01	1.23
alfalfa M	0.02	0.83	0.47	0.11	0.09	0	0.01	1.27
bromegrass Y	0.03	0.62	0.54	0.02	0.06	0.22	0.04	1.53
bromegrass M1	0.05	0.69	0.58	0.02	0.05	0.20	0.05	1.64
bromegrass M2	0.06	0.59	0.60	0.03	0.07	0.25	0.06	1.66
red clover	0.01	0.67	0.61	0.09	0.14	0	0.02	1.39
pine	0.07	1.03	0	0.23	0	0	0	1.33
mean	0.05	0.70	0.48	0.08	0.08	0.16	0.04	1.58
S.E.	0.01	0.05	0.05	0.02	0.01	0.05	0.01	0.05

[a] Values are means of two observations. *p*HBA − *p*-hydroxybenzaldehyde; Van − vanillin; Syr − syringaldehyde; VanA − vanillic acid; SyrA − syringic acid; *p*CA − *p*-coumaric acid; FA − ferulic acid; Y − young; M − mature (1 and 2 refer to two different maturity stages); S.E. − standard error.

Spectra and Regression Equation of Isolated DL. Acetyl bromide soluble lignin spectral characteristics of DL extracts were similar to those of the original cell walls (Figure 1A and B). One would expect lignin to give similar spectra irrespective of the original source from which it is extracted since the major components that make up lignin macromolecules are the same (i.e., coniferyl and sinapyl alcohols). The spectra are useful for evaluating the acetyl bromide reactions to determine if any side reactions have occurred. For example, excessive degradation of carbohydrates or lignin perhaps due to too much water in the samples tends to broaden the spectral maximum at 280 nm and increase the absorbance below the 275−280 nm region. Acetyl bromide solubilized lignin should produce an absorbance maximum at 280 nm. If the lignin is unusually rich in syringyl units, the maximum can be shifted slightly to 275 nm. The shoulders at 300 nm, evident in the grass samples, are due to hydroxycinnamates attached to the lignin.

Generally slopes of regression lines for the different DL standard curves were similar. One exception was corn stalk DL standard that had a higher slope ($P <$ 0.05, 0.01, or 0.001) than all other samples (Table 7). The deviation of the corn sample may be due to the unusually high levels of hydroxycinnamates, especially *p*CA attached to the lignin. This can be seen in the nitrobenzene products (Table 6) where *p*CA is the second largest component recovered. Such high levels of *p*CA could alter the acetyl bromide lignin spectrum. Although *p*CA has an absorption maximum 300−310 nm, it also has a strong shoulder at 280 nm that would add to the lignin absorption maximum at 280 nm. Earlier work with milled-wood-enzyme lignins isolated

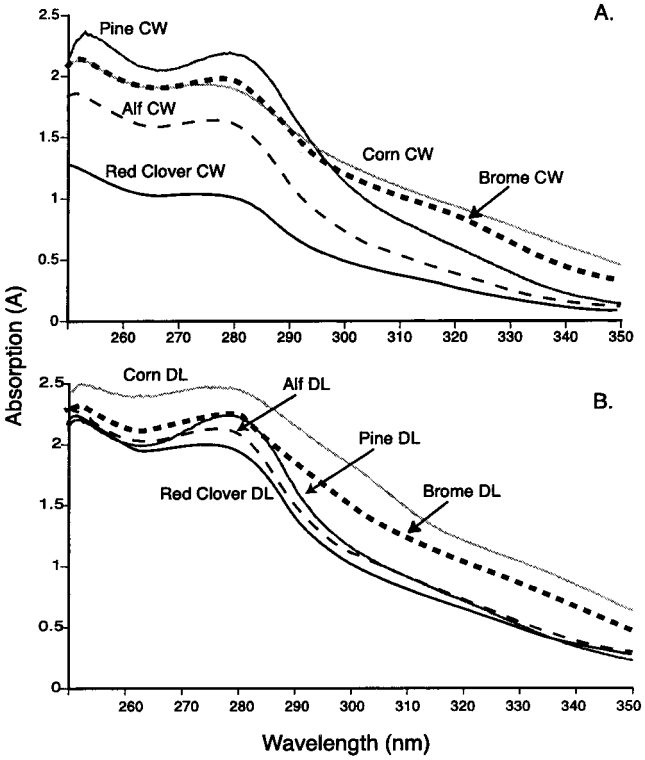

Figure 1. Spectral characteristics of acetyl bromide soluble lignins. (A) Acetyl bromide soluble lignin spectra obtained from isolated cell wall samples. (B) Spectra obtained from dioxane-HCl extracted lignins used to produce standard curves of acetyl bromide soluble lignins.

Table 7. Regression Equations Obtained from Standard Curves of DL Preparations, Absorbance Readings of Cell Walls, and Lignin Concentration (g kg^{-1} DM)[a]

sample name	regression equation	ABSL	KL	ABSL/KL
corn stalk	X = (Y′ + 0.1244)/20.48	81.3^{g}	76.7^{e}	1.06
alfalfa Y	X = (Y′ + 0.0702)/17.20	99.7^{f,B}	123.0^{c,d,A}	0.81
alfalfa M	X = (Y′ + 0.0502)/17.15	127.9^{d}	130.4^{c}	0.98
bromegrass Y	X = (Y′ + 0.0709)/18.63	112.7^{e}	102.2^{d}	1.10
bromegrass M1	X = (Y′ + 0.0662)/17.89	115.7^{e}	100.4^{d}	1.15
bromegrass M2	X = (Y′ + 0.0981)/17.98	132.2^{c}	109.8^{c,d}	1.20
red clover	X = (Y′ + 0.0154)/16.13	63.1^{h,B}	71.2^{e,A}	0.89
pine	X = (Y′ + 0.0955)/17.747	305.3^{a,A}	256.0^{a,B}	1.19
mean		142.2	121.2	
S.E.		26.2	14.2	

[a] Values are means of two observations. Y − young; M − mature (1 and 2 refer to two different maturity stages); X − Concentration of ABSL (mg/mL); Y′ − absorbance readings; ABSL − acetyl bromide soluble lignin; KL − Klason lignin; S.E. − standard error. Means, within a column, followed by different small letters are different ($P < 0.05$). Means, within a row, followed by different capital letters are different ($P < 0.05$).

from corn revealed that approximately 20% of this lignin fraction was made up of *p*CA (*45*). Bromegrass in this study had *p*CA levels that were one-third those of corn. It would appear that for most forage plants a single regression equation could be used to estimate total lignin content within their cell walls. The one exception would be corn with high levels of hydroxycinnamates attached to lignin, which would possibly be better served with its own regression curve. Using the corn regression slope for measuring lignin content of corn samples may be valuable in that it would take into account the added absorption form hydroxycinnamates and one would not have to worry about pretreatment of wall samples to remove these compounds when using the ABSL procedure (*46*).

Lignin Quantification. A comparison of the acetyl bromide soluble lignin (ABSL) method with a modified Klason method (acid insoluble lignin) showed generally good agreement among the plant samples analyzed in this work (Table 7). Alfalfa young and red clover ABSL values were lower than those obtained as an acid insoluble residue ($P < 0.05$). The pine sample had a higher lignin value with the ABSL procedure ($P < 0.05$), and remaining samples showed no differences (Table 7). The ratio between ABSL and KL ranged from 0.81 to 1.20. Legumes typically had ratios below 1.0, and this may be attributed to the hypothesis that acid insoluble lignins, especially for legume samples, contain protein contamination. On the other hand, the ABSL method may account for any acid soluble lignin that otherwise would be lost during the strong acid digestion (*48*) in Klason type lignin procedures.

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