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Comparison of the Acetyl Bromide Spectrophotometric Method with Other Analytical Lignin Methods for Determining Lignin Concentration in Forage Samples

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Present analytical methods to quantify lignin in herbaceous plants are not totally satisfactory. A spectrophotometric method, acetyl bromide soluble lignin (ABSL), has been employed to determine lignin concentration in a range of plant materials. In this work, lignin extracted with acidic dioxane was used to develop standard curves and to calculate the derived linear regression equation (slope equals absorptivity value or extinction coefficient) for determining the lignin concentration of respective cell wall samples. This procedure yielded lignin values that were different from those obtained with Klason lignin, acid detergent acid insoluble lignin, or permanganate lignin procedures. Correlations with in vitro dry matter or cell wall digestibility of samples were highest with data from the spectrophotometric technique. The ABSL method employing as standard lignin extracted with acidic dioxane has the potential to be employed as an analytical method to determine lignin concentration in a range of forage materials. It may be useful in developing a quick and easy method to predict in vitro digestibility on the basis of the total lignin content of a sample.

KEYWORDS: Acetyl bromide soluble lignin; acid detergent lignin; dioxane; in vitro digestibility; Klason lignin; permanganate lignin

INTRODUCTION

Lignin is a complex phenolic polymer found in plant cell walls. Lignification of forage cell walls is considered to be a primary factor limiting cell wall degradability by rumen microbes (*1*). To assess its exact role and the mechanisms involved in the inhibition of cell wall structural carbohydrate digestion, knowledge of lignin content in the plant is of primary importance. There are several analytical procedures for determining the lignin content of cell walls; however, these methods often give quite different estimates of lignin content (*2*). The most commonly employed procedures fall into two categories: analytical methods that remove cell wall constituents except lignin and methods that oxidize the lignin polymer out of the cell wall matrix.

In the first category, the result is an acid insoluble residue (lignin) left after a sulfuric acid solubilization/hydrolysis of cell wall polysaccharides, such as the Klason lignin (KL) (*3*) and the acid detergent lignin (ADL) (*4*). However, results may be underestimated by a lignin fraction that is potentially soluble in the acid detergent solution (*5*, *6*) or in the 72% sulfuric acid reagent (*7*). Loss of soluble lignin using the ADL technique was up to 50% of the lignin present in tropical grasses (*8*). The loss is smaller in the KL method, one of the reasons being the type of pre-extracted material utilized, such as neutral detergent fiber (*9*) or starch-free cell walls (*7*). However, results may also be biased by proteins bound to lignin (*10*) or by a contaminant, particularly cutin, present in the acid insoluble residues (*11*, *12*).

In the second category, lignin can be determined by difference after reaction with an oxidizing reagent such as potassium permanganate (*11*). This procedure has the advantage of employing reagents that are not as corrosive as the 72% sulfuric acid and do not require standardization. However, this method also has setbacks; permanganate oxidizes phenolic and unsaturated substances (e.g., tannins, pigments, or proteins) that are not completely removed during the cell wall preparation steps (acid detergent washing) and that appear as lignin (*11*). Noncellulosic polysaccharides constituted most of the dry matter removed from the neutral detergent fiber by the $KMnO_4$ solution (*13*), suggesting it is critical that only cellulose remain before treatment.

The current popular methods for lignin determination have weaknesses and strengths with no dominant method being chosen to use for forage analyses (*14*). Alternatively, a spectrophotometric procedure to quantify lignin in woods (*15*) or in forages (*16*, *17*) has been proposed, which is based upon

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solubilization of lignin into a solution of 25% acetyl bromide in glacial acetic acid and lignin concentration calculated from the absorption at 280 nm. The acetyl bromide soluble lignin (ABSL) method, however, as with any spectrophotometric method, requires a reliable standard to develop calibration curves (*18*). Previously, we reported the utilization of a lignin extracted with acidic dioxane solution as a reference standard to the ABSL method (*19*). It is a relatively straightforward procedure that produces a lignin extract that has minimal carbohydrate and protein contamination.

Objectives of this work included (1) applying the spectrophotometric method (ABSL) employing lignin extracted with acidic dioxane to a broader set of samples, (2) comparing these results with data obtained through other analytical procedures, (3) correlating the results from analytical lignin methods with in vitro measurements of dry matter and cell wall digestibility.

MATERIALS AND METHODS

Plant Material. Alfalfa (*Medicago sativa* L.), bromegrass (*Bromus inermis* L.), and corn (*Zea mays* L. B73XMo17) used in this study were grown in the greenhouse under supplemental light consisting of a 14/10 h day/night regimen; 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, 2.5 g/pot). Red clover (*Trifolium pratense* L.) was field grown and harvested from the University of Wisconsin Experimental Farm, Arlington, WI. Aspen (*Populus* sp.) and loblolly pine (*Pinus taeda*) samples were obtained from the USDA Forest Products Laboratory (S. Ralph and L. Landucci). Oat (*Avena sativa* L.) and wheat (*Triticum aestivum* L.) straws were from a commercial source. One sample of bromegrass and another of setaria (*Setaria verticillata* L.) were harvested from uncultivated areas.

Maturity stages and specific tissues of samples were harvested as follows: corn stalk rind tissue at 10 days past anthesis, alfalfa stems, 10−20% bloom stage, early development stage (alfalfa Y); alfalfa stems, full bloom, cut at 5 cm from the soil up to 30 cm height (alfalfa FB lower 30); alfalfa stems, full bloom, the upper segment from the previous cut (alfalfa FB upper 30); alfalfa stems, past seed development and shedding (alfalfa PSD); red clover stems, harvested at full bloom stage; bromegrass stems, harvested at three maturity stages [young plant, late boot stage with flowers just beginning to emerge (bromegrass Y); flowering heads, fully emerged (bromegrass M1); flowering heads, mature, past pollen shed of the full bloom stage (bromegrass M2); wild field grown grass, harvested at past seed shed stage (bromegrass M3)]; setaria, wild grown and very mature (tiller growth from setaria was separately analyzed). Both stem and leaf tissues of straw were analyzed. Aspen and pine were mature wood samples.

All materials (except the wood samples, which came pre-extracted) were dried in a 60 °C forced-air oven for 72 h and milled to pass a 0.5 mm sieve using a Wiley mill (Arthur H. Thomas Co., Philadephia, PA). Cell walls were prepared by sequential extraction of ground samples with water, ethanol, chloroform, and acetone in a Soxhlet apparatus.

Chemical Analyses. Lignin used as reference standard was isolated from each plant through extraction of cell walls with HCl acidified dioxane [dioxane lignin (DL)] (*19*) except that two additional filtering steps (with 0.45 μm nylon membrane) were included to avoid mineral contamination from added sodium bicarbonate. Briefly, 5 g 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) added. The suspension was refluxed under N_2 for 30 min, cooled to room temperature, and filtered through a glass fiber filter (GF/C, 47 mm, Whatman Inc.). Insoluble cell wall residue, collected on the filter, was washed with 20 mL of 96% dioxane and the wash combined with the original dioxane extract. Sodium bicarbonate (4.0 g) was added, and the flask was placed on a rotary shaker for several minutes until neutralization of the solution. The solution was filtered through a 0.45-μm nylon membrane (Schleicher & Schuell, Keene, NH) before concentration to 10−15 mL under reduced pressure on a rotary evaporator (water temperature of 40 °C). It was again filtered through a 0.45-μm nylon membrane. The solution was added dropwise to a 250-mL centrifuge bottle containing ~200 mL of rapidly stirring distilled water. Generally a fine precipitate would form. For some samples that did not 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 removed. The pellet was partially dried by placing the centrifuge bottle in a 60 °C forced-air oven for 15 min. The lignin residue was dissolved in 4−5 mL of dioxane, filtered through a 0.45-μm nylon membrane, and added dropwise to 200 mL of rapidly stirring anhydrous diethyl ether. The resulting precipitate was pelleted by centrifuging (9000*g*, 15 min, 5 °C). The entire solubilization in dioxane and ether wash step to remove hydrophobic non-lignin contaminants was repeated twice. After removal of the diethyl ether, 60 mL of petroleum ether was added to wash the lignin residue. This solvent was removed after the residue had settled. The lignin residue was freeze-dried (Virtus Co., Gardiner, NY) for 48 h and stored in a freezer over desiccant.

Extinction coefficients were calculated from standard curves as follows: 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, frozen in liquid N_2, and placed on a freeze-dryer overnight. To each tube was added 0.5 mL of 25% acetyl bromide in glacial acetic acid (HAcBr). A blank was included to correct for reagent background absorbance. Tubes were tightly capped (PTFE-lined caps) and put in a 50 °C water bath for 30 min. After cooling, 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 in a spectrophotometer at 280 nm.

The procedure for determining ABSL concentrations consisted of digesting 100 mg of the cell wall (CW) preparation with 4.0 mL of AcBrHAc reagent at 50 °C for 2 h, with occasional mixing. After cooling, the volume was made up 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, 0.5 mL of 0.5 M hydroxylamine hydrochloride solution was added and the volume made up to 10 mL with HAc. Optical density at 280 nm was measured and concentration determined from the respective extinction coefficient.

Klason lignin (KL) was determined as the insoluble residue remaining after a two-step sulfuric acid hydrolysis of cell wall polysaccharides (*3*). ADL and permanganate lignin (PerL) were performed on acid detergent fiber residues (*20*). The KL method has the flexibility to allow the determination of neutral sugars and total uronosyls as well as acid insoluble residues in the cell wall 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 using the Carlo Erba NA 1500 nitrogen analyzer (*23*) (Fision Instruments, Saddle Brook, NJ); crude protein was estimated as N × 6.25. Nitrobenzene oxidation was used to compare monolignol composition among isolated DL samples (*24*).

Duplicate material samples (including the wood and straw samples) were subjected to in vitro rumen determinations of dry matter and cell wall digestibility (*20*). Rumen fluid was collected from two lactating Holstein cows fed a total mixed ration that contained corn silage, corn grain, soybean meal, vitamins, and minerals. Fluid from both animals was obtained through rumen fistulas and then combined into one inoculum. Mineral solution containing cysteine as a reducing agent and resazurin as a redox indicator was reduced at a faster rate by illuminating the mineral solution with two 500-W quartz-halogen lamps (*25*). In vitro dry matter digestibility (IVDMD) and in vitro cell wall digestibility (IVCWD) were determined after a 48-h fermentation followed by a 48-h digestion with acid pepsin. Both sets of fermentations were run at the same time using the same rumen fluid inoculum.

Statistical Analysis. The experimental design employed was a completely randomized design with duplicate analysis for the lignin

Table 1. Cell Wall (CW) Content, Dioxane Lignin (DL) Yield, and Protein, Neutral Sugars, and Uronosyls Content in the DL[a]

sample	CW (g kg^{-1} of DM)	DL (g kg^{-1} of CW)	protein (DL) (g kg^{-1} of lignin)	neutral sugars (DL) (g kg^{-1} of lignin)	uronosyls (DL) (g kg^{-1} of lignin)
bromegrass Y	658.1	88.0	19.6	31.6	17.8
bromegrass M1	664.8	79.7	17.9	32.1	17.8
bromegrass M2	696.5	123.0	22.7	32.9	18.6
bromegrass M3, W	715.5	104.0	19.4	26.6	18.5
setaria M3, W	691.9	128.8	20.9	26.6	15.4
setaria M3, W (tiller)	712.7	103.7	23.3	28.9	13.4
oat straw (stem)	847.7	114.2	7.2	29.4	11.3
oat straw (leaf)	776.9	57.8	18.2	24.2	5.9
wheat straw (stem)	890.5	142.2	10.3	46.9	12.2
wheat straw (leaf)	841.5	78.0	12.6	30.2	6.7
corn stalk PA	491.3	172.1	11.6	29.5	18.9
alfalfa Y	745.3	33.3	24.7	17.2	14.2
alfalfa FB, lower 30	795.4	57.5	32.9	14.5	14.3
alfalfa FB, upper 30	660.0	29.5	31.7	21.0	17.2
alfalfa PSD	722.5	52.8	24.5	21.7	15.8
red clover FB	677.5	43.1	25.6	19.9	16.9
aspen wood	942.2	166.2	3.9	13.6	4.1
pine wood	960.3	171.4	4.0	15.1	14.4
mean	749.5	97.0	18.4	25.7	14.1
SE	27.25	11.05	2.03	1.38	0.74

[a] Data are means of two observations. Y, young; M, mature (1–3 refer to three different maturity stages); W, wild; PA, past anthesis; FB, full bloom; PSD, past seed development; SE, standard error.

assays. For the in vitro experiment, a randomized block design was employed where rumen fluid sampling corresponded to 2 weeks, each week designated as a block. Analyses of variance were conducted on lignin data (ABSL, ADL, PerL, and Klason results), on nitrobenzene oxidation products from DL and on the extinction coefficients derived from the standard curves. Analysis of variance was performed using the ANOVA protocol of the SAS system (*26*). Tukey's test was used to separate individual means at a significance level of $P < 0.05$. Correlations were made between lignin data from each analytical procedure and in vitro digestibility values.

RESULTS AND DISCUSSION

Cell Wall and Dioxane Lignin. This work focused on stem fractions, because as forages mature there is generally a decrease in leaf-to-stem ratio and an increase in cell wall content of the stem fraction, including increasing levels of lignin (*27*). It is known that a neutral detergent solution dissolves pectic substances (*28*) from cell walls, resulting in material that is not a true reflection of the total wall polysaccharides. To avoid this problem, cell wall isolates were obtained by sequential extraction with water, ethanol, chloroform, and acetone using the Soxhlet apparatus. This procedure usually yields higher numbers than neutral detergent fiber; however, continued extraction with water for several hours may remove part of the total pectic carbohydrates in pectic-rich walls such as legumes (*19*). This experiment showed that cell wall (CW) contents of samples were close to data reported previously, including the CW concentration of corn stalk (rind tissue), which was somewhat low (*19*). As expected, the straws contained a substantial amount of CW, and mature wood samples were composed almost exclusively of CW (**Table 1**). Mature bromegrass exhibited a higher content of CW than younger samples. Increasing plant maturity usually leads to higher CW content because of the increased proportion of stem in the total biomass, CW thickening, and lower contents of protein and other cell solubles (*28*). Higher accumulation of CW in legumes was due to maturity (*1*); however, in this study an older sample of alfalfa showed a lower CW concentration that could be attributed to a different variety, part of the plant being studied, or growth conditions. As expected, the CW content of alfalfa lower 30 cm was higher than that of the upper internodes, reflecting developmental accumulation of structural carbohydrates and lignin in the lower part of the plant (*29*).

Table 2. Neutral Sugar Composition of Dioxane Lignins[a]

	g kg^{-1} of lignin						
sample	Fuc	Ara	Rha	Gal	Glc	Xyl	Man
bromegrass Y	0	10.3	0	0.4	1.9	18.9	0
bromegrass M1	0	11.1	0	0.3	1.5	19.0	0
bromegrass M2	0	10.9	0	0.4	1.9	19.7	0
bromegrass M3, W	0	9.8	0	0.2	1.5	15.0	0
setaria M3, W	0	11.0	0	0.4	0.6	14.6	0
setaria M3, W (tiller)	0	12.8	0	0.5	1.8	13.8	0
oat straw (stem)	0	10.8	0	0.4	1.8	16.3	0
oat straw (leaf)	0	12.5	0	0.4	1.8	8.9	0.4
wheat straw (stem)	0	11.4	0	0.4	3.4	31.7	0
wheat straw (leaf)	0	13.3	0	0.8	2.6	13.5	0
corn stalk PA	0	9.5	0	0.4	2.0	17.6	0
alfalfa Y	0	1.7	0	1.0	1.0	12.9	0
alfalfa FB, lower 30	0	1.1	0.5	0.9	1.6	10.4	0
alfalfa FB, upper 30	0.4	3.3	0.4	1.3	3.1	12.4	0
alfalfa PSD	0.4	2.3	0.6	1.5	4.2	12.3	0.2
red clover FB	0	1.4	0.6	1.4	2.0	14.4	0
aspen wood	0	1.0	0	1.8	2.3	3.3	5.1
pine wood	0	0.4	0.2	0.2	0.9	13.3	0.2
mean	0	7.5	0.1	0.7	2.0	14.9	0.3
SE	0.02	0.81	0.04	0.08	0.15	0.96	0.20

[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 (1–3 refer to three different maturity stages); W, wild; PA, past anthesis; FB, full bloom; PSD, past seed development; SE, standard error.

Yield of acidic-dioxane lignin (DL) is also shown in **Table 1**. Wood samples gave higher yields of lignin compared to the other plant samples, possibly due to their higher lignin concentrations in the cell walls. Apparently, the lignin composition had little impact upon the extractability of the lignin because pine lignin is a guaiacyl-type lignin and aspen lignin is approximately a 70:30 combination of syringyl and guaiacyl units (**Table 3**), and both woods yielded approximately the same amount of DL.

Corn rind tissue resulted in the highest lignin extraction, similar to the woods; in fact, grasses (except the young plants

Table 3. Nitrobenzene Oxidation Products from Dioxane Lignins[a]

	mmol g^{-1} of lignin						
sample	*p*HBA	Van	Syr	VanA	SyrA	*p*CA	FA
bromegrass Y	0.14abc	0.49bcde	0.62ef	0.05bcde	0.08de	0.29cd	0.17abcd
bromegrass M1	0.13abc	0.49bcde	0.67def	0.05cde	0.08e	0.35c	0.18abcd
bromegrass M2	0.15abc	0.52bcde	0.70cde	0.06bcde	0.09de	0.26cd	0.22abcd
bromegrass M3, W	0.15abc	0.53bcde	0.68def	0.07bcd	0.10bcde	0.20cde	0.19abcd
setaria M3, W	0.21ab	0.40def	0.84b	0.04de	0.12bcd	0.72ab	0.28ab
setaria M3, W (tiller)	0.20ab	0.40ef	0.76bcd	0.04de	0.10bcde	0.59b	0.26ab
oat straw (stem)	0.14abc	0.52bcde	0.69cde	0.05bcde	0.16a	0.34c	0.29a
oat straw (leaf)	0.10bc	0.49bcde	0.45gh	0.06bcde	0.14ab	0.13def	0.18abcd
wheat straw (stem)	0.09bc	0.44cdef	0.67def	0.05cde	0.16a	0.12def	0.21abcd
wheat straw (leaf)	0.07bc	0.52bcde	0.43h	0.06bcde	0.16a	0.13def	0.20abcd
corn stalk PA	0.26a	0.30f	0.82bc	0.03e	0.13abc	0.88a	0.23abc
alfalfa Y	0.02c	0.63b	0.55fgh	0.09b	0.09de	0.01f	0.09cd
alfalfa FB, lower 30	0.01c	0.60bc	0.62ef	0.07bcd	0.08de	0.01f	0.12abcd
alfalfa FB, upper 30	0.02c	0.58bcd	0.55fgh	0.07bcd	0.08e	0.03ef	0.12abcd
alfalfa PSD	0.02c	0.63b	0.57efg	0.08bc	0.08e	0.01f	0.08cd
red clover FB	0.01c	0.61bc	0.76bcd	0.07bcd	0.10cde	0.01f	0.11bcd
aspen wood	0.02c	0.38ef	1.14a	0.06bcde	0.17a	0.01f	0.15abcd
pine wood	0.04c	0.86a	0.06i	0.14a	0.01f	0.02f	0.05d
mean	0.10	0.52	0.64	0.07	0.11	0.23	0.17
SE	0.013	0.021	0.036	0.004	0.007	0.044	0.012

[a] Data 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 (1–3 refer to three different maturity stages); W, wild; PA, past anthesis; FB, full bloom; PSD, past seed development; SE, standard error. Means, within a column, followed by different letters are different ($P < 0.05$).

and leaf tissues) exhibited high DL recoveries. All legumes gave low DL yields. Grasses typically have a high content of *p*-coumarates (*p*CA) in their walls (**Table 3**). Earlier work with milled-wood-enzyme lignins isolated from corn revealed that ~20% of this lignin fraction was made up of *p*CA (*30*). Recent work revealed that 30.6% of the nitrobenzene oxidation products from corn lignin was *p*CA (*19*). Studies have shown that *p*CA is associated with lignin (*31*, *32*). It is unknown at this time if the significant amounts of *pCA* help render corn lignin or other grass lignins more soluble in the acidic dioxane solution. The *p*-hydroxycinnamic acids have not been found in legumes or, if present, they are in quantities much lower than in grasses (*33*, *34*). Absence of these acids may render legume lignin less soluble in acidic dioxane solution. On the other hand, woods that were good sources of DL have no *p*CA. Legume lignin apparently has a more condensed structure and thus is less reactive than grass lignin (*35*). Condensed lignin polymers could be less soluble in acidic dioxane solution, thus yielding less.

All DL samples contained small amounts of nitrogen that were assumed to be from protein and used to estimate protein contamination (6.25 × N) (**Table 1**). However, it is not clear whether the nitrogen present in the DL residues represents true protein or nonprotein nitrogen. Cell walls contain proteins that have structural roles within the wall matrix and have been suggested to be cross-linked to lignin (*36*). Legume DL contained more protein than grasses; grasses are known to contain less wall protein (*37*). Lower nitrogen values were generally found in lignin residues of grasses compared to alfalfa (*7*). DL samples contained less protein (ranging from 3.9 to 32.9 g kg^{-1} of lignin) than reported in a previous work [9.5–60.6 g kg^{-1} of lignin (*19*)]. DL from aspen and pine exhibited the lowest protein values; lignins from wood contain virtually no protein (*10*).

DL from legumes and woods contained less neutral sugar than grasses (**Table 1**). The neutral sugar composition of DL (**Table 2**) indicates that carbohydrate contamination was probably from xylans due to the predominance of xylose and arabinose. Several studies have shown that xylans are frequently the major polysaccharide found in isolated lignin complexes (*38*). There is evidence that glucuronoxylans can become ester linked to lignins through the glucuronosyl residues (*39*), which may account for the coextraction of xylans with lignin when using the acidic dioxane procedure. The uronosyl content in the DL failed to show any trend except that leaf tissue from both straws and aspen had smaller uronosyl concentrations.

Dioxane Lignin Nitrobenzene Oxidation. The main alkaline nitrobenzene oxidation products from DL samples were vanillin and syringaldehyde. Grasses showed higher concentrations of *p*-hydroxybenzaldehyde (*p*HBA) than legumes and woods (**Table 3**). The origin of *p*HBA is from *p*-hydroxycinamyl alcohol residues (*40*), and in grasses *p*CA that is predominantly esterified to lignin can be oxidized to *p*HBA (*41*). Ferulic and *p*-coumaric acids can be detected among nitrobenzene oxidation products. The recovery efficiency is dependent upon an interaction between time and temperature (*24*) with hydrolysis of the ester linkage under the alkaline conditions of the reaction followed by a partial oxidation to *p*-hydroxybenzaldehyde and vanillin (*41*, *42*) from the respective molecules of *p*-coumaric and ferulic acid. Significant amounts of *p*CA were recovered from the grass DLs as a product of nitrobenzene oxidation. This is in agreement with observations that grasses contain high levels of *p*CA, whereas legumes and woods do not contain this molecule (*19*).

Leaf tissue from wheat and oat straws had lower contents of syringaldehyde than the respective stems (**Table 3**); this agrees with nitrobenzene oxidation data from lignin fractions of maize and wheat (*43*). Internodes are rich in secondary walls that contain higher amounts of syringyl units contributing to the mechanical strength of the plant (*44*). On the other hand, the leaves, low in supporting tissues, have a network that is proportionally more abundant in guaiacyl monomer units (*42*).

Lignin Analytical Methods. To calculate the acetyl bromide soluble lignin (ABSL) values, extinction coefficients (EC) derived from the standard curves were employed. This work showed an average EC of 17.08 g^{-1} L cm^{-1} (**Table 4**), slightly lower than the 17.90 g^{-1} L cm^{-1} from a previous paper (*19*). On the basis of infrared spectra and nitrobenzene oxidation data

Table 4. Extinction Coefficients from the Dioxane Lignin Standard Curves

sample	extinction coeff (g^{-1} L cm^{-1})
oat straw (leaf)	20.097a
wheat straw (leaf)	19.808ab
setaria M3, W	18.955abc
oat straw (stem)	18.910abc
setaria M3, W (tiller)	18.433bcd
aspen wood	17.898cde
corn stalk PA	17.747cde
wheat straw (stem)	17.542cdef
bromegrass Y	17.443cdef
bromegrass M3, W	17.377cdef
bromegrass M1	17.107defg
bromegrass M2	16.727efgh
alfalfa FB, upper 30	15.988fghi
alfalfa Y	15.693ghij
alfalfa PSD	15.297hijk
red clover FB	14.478ijk
alfalfa FB, lower 30	14.232jk
pine wood	13.785k
mean	17.084

[a] Data are means of six observations. Y, young; M, mature (1–3 refer to three different maturity stages); W, wild; PA, past anthesis; FB, full bloom; PSD, past seed development. Means followed by different letters are different ($P < 0.05$).

for milled sample lignins, a value of 20.0 g^{-1} L cm^{-1} was proposed for the specific absorption coefficient (*43*).

In most cases, ABSL concentrations were higher than other analytical procedures, except Klason lignin (KL) and permanganate lignin (PerL) measured in legumes (**Table 5**). Previous data also reported the spectrophotometric procedure yielding higher lignin values than either ADL or PerL (*2*). Acid detergent lignin (ADL) yielded the lowest values except for leaf tissue from both straws, where the PerL method gave lower results. KL concentration was, in general, the second highest; however, PerL for legumes tended to be higher than KL (**Table 5**). In agreement with results of previous research (*46*), KL concentrations were higher than corresponding ADL measurements for all samples in the present study.

All methods reflected maturity trends. The upper portion of alfalfa stems showed a lower concentration of lignin than the lower stems internodes; this was expected because the bottommost internodes were physiologically older than the upper part. The observation that alfalfa PSD yielded slightly lower lignin concentrations for all methods than alfalfa lower 30 cm FB reflects possibly that alfalfa PSD was a mix of total stem.

For all methods, stems from the straws exhibited higher lignin contents than leaves, as expected, except the ADL method. Both in vitro dry matter and cell wall digestibility data agreed as leaves were more digested than the stems. As mentioned earlier, the leaves have a lignin matrix that is proportionally more abundant in guaiacyl monomer units than the stems (syringyl/guaiacyl ratios were 0.81 and 0.74 for DL of leaves from oat and wheat straw, respectively; these ratios were 1.00 and 1.18 for the respective stems). Syringyl/guaiacyl ratios of 0.60 and 0.53 were found for KL and ADL residues, respectively (*7*), lower than the original lignin, which suggests syringyl-rich lignins are preferentially lost during sulfuric acid hydrolysis. The significant shift in S/G ratio when KL was compared to ADL may be an indication that the syringyl-rich proportions of the lignin are more susceptible to acid detergent solubilization. It is known that acid detergent solution solubilizes lignin (*6*−*8*), especially in grasses. As a result, guaiacyl-rich tissues would be more preserved from the acid solubilization/hydrolysis of carbohydrates, and it seems to be the case concerning the straw leaves.

Aspen ADL content was surprisingly low (**Table 5**). Nitrobenzene oxidation of aspen DL showed it was predominantly a syringyl-type lignin (syringyl/guaiacyl = 2.19). The second highest syringyl/guaiacyl ratio was from corn stalk (1.70), which happened to exhibit the lowest ADL content. However, young mature bromegrass also showed low ADL concentrations but its syringyl/guaiacyl ratios were all around unity. Pine, on the other hand, with a guaiacyl-type lignin, had an ADL value similar to those of KL and PerL.

PerL and KL determined in legume samples were generally higher than ABSL and ADL. Potassium permanganate can be applied to cell wall preparations and the loss in weight recorded as lignin (*11*); however, there is the risk of removing carbohydrates, in particular, noncellulosic carbohydrate contamination of ADF (*10*). Legume cell walls were particularly rich in uronosyls (**Table 1**), which could overestimate lignin values upon permanganate oxidation. On the other hand, KL of legumes could suffer from protein contamination (*47*); cetyl trimethylammonium bromide is included in the acid detergent solution specifically for protein removal in the ADL method (*20*). The KL method does not contain a protein-removal step except if a cell wall proteolytic enzyme treatment is added. It was found that with the exception of alfalfa leaves, the N content of KL residues was always greater than the N content of ADL residues (*7*).

For other samples, the higher lignin values obtained with the ABSL method could reflect phenolic components that contribute to the optical density and are easily removed by the H_2SO_4 treatment (*48*). These phenolic compounds could be represented in part by the ester-linked hydroxycinnamic acids (*49*). The solution after a 2 M NaOH treatment (24 h at room temperature) of DL from some grasses revealed typical peaks of mixed *p*-coumaric and ferulic acids between 250 and 350 nm. These peaks were not present when the DL came from legume or pine (*50*).

These hydroxycinnamic acids could explain why extinction coefficients for grasses were higher than for legumes and pine. However, aspen was similar to the grasses (**Table 4**), and it has little *p*CA and ferulic acid. Corn DL exhibited higher optical density readings than other lignins that contributed toward higher 250−350 nm scanning and regression slope (*19*). However, unlike the latter finding, corn did not have the highest extinction coefficient despite still showing the highest *p*CA plus ferulic acid concentration. Removal of these phenolics prior to building a standard curve may be an option to verify improvement in lignin determination in grasses if it is accepted that ester-linked hydroxycinnamic acids are not an integral part of the lignin molecule (*49*). Also, non-lignin components—other phenolics such as tannins—if not removed by a preparatory cell wall step may be dissolved by the acetyl bromide solution and could provide interfering absorbance (*10*, *16*). Proteins would not be soluble in the acetyl bromide/acetic acid final solution and should not contribute to the absorption at 280 nm (*17*).

The accuracy of lignin determination assumes great importance when it comes to equations estimating energy value of forages. Less reliable predictions of indigestible NDF were obtained when ADL was employed as compared to PerL (*14*). These authors reported that ADL values were ~76% of those determined by the PerL method. The work reported here revealed that ADL was ~71% of PerL. Lignin values determined

Table 5. Lignin Concentrations Obtained through Four Analytical Procedures plus in Vitro Dry Matter Digestibility (IVDMD) and in Vitro Cell Wall Digestibility (IVCWD)[a]

	g kg^{-1} of DM					
sample	ADL	PerL	KL	ABSL	IVDMD	IVCWD
bromegrass Y	28.5 ± 1.05d	56.0 ± 0.5c	102.2 ± 4.1b	123.3 ± 0.15a	527	365
bromegrass M1	30.4 ± 0.1d	64.1 ± 1.2c	100.4 ± 9.75b	127.5 ± 0.3a	451	298
bromegrass M2	36.5 ± 0.9d	69.8 ± 4.55c	109.8 ± 11.0b	144.6 ± 9.1a	401	253
bromegrass M3, W	45.6 ± 1.2c	67.0 ± 0.75b	130.1 ± 0.1a	139.0 ± 3.65a	366	140
setaria M3, W	72.5 ± 1.7b	80.9 ± 6.75b	135.3 ± 0.95a	136.0 ± 2.3a	337	160
setaria M3, W (tiller)	61.6 ± 2.2b	65.8 ± 6.65b	126.0 ± 0.79a	130.9 ± 3.35a	377	171
oat straw (stem)	83.3 ± 0.45d	111.4 ± 3.85c	171.1 ± 1.15b	186.3 ± 4.2a	124	93
oat straw (leaf)	106.9 ± 2.0c	71.3 ± 5.7d	138.0 ± 1.35a	123.5 ± 0.2b	254	331
wheat straw (stem)	89.1 ± 7.65d	122.0 ± 9.1c	184.2 ± 1.5b	213.0 ± 10.6a	130	62
wheat straw (leaf)	103.4 ± 7.45b	74.3 ± 8.2c	141.5 ± 0.05a	149.9 ± 2.4a	234	336
corn stalk PA	24.8 ± 0.15d	45.2 ± 9.3c	76.7 ± 5.5b	91.9 ± 2.05a	508	289
alfalfa Y	83.6 ± 1.4c	134.6 ± 4.75a	123.0 ± 2.2ad	116.5 ± 3.05bd	438	321
alfalfa FB, lower 30	92.5 ± 2.1c	157.5 ± 11.85a	144.8 ± 0.3ab	134.7 ± 1.1bd	418	241
alfalfa FB, upper 30	59.3 ± 0.2c	95.3 ± 7.7b	111.4 ± 0.4a	71.3 ± 3.45c	616	395
alfalfa PSD	90.6 ± 1.0d	153.7 ± 7.45a	138.8 ± 0.7b	117.2 ± 1.65c	443	254
red clover FB	41.7 ± 0.9d	115.5 ± 5.75a	71.2 ± 1.05c	90.4 ± 0.8b	531	410
aspen wood	69.5 ± 0.2c	190.5 ± 8.25a	158.6 ± 5.9b	181.5 ± 0.1a	75	10
pine wood	245.5 ± 3.65b	255.3 ± 8.55b	249.7 ± 6.3b	401.2 ± 9.95a	9	11
mean	75.9	107.3	134.1	148.8	347	235

[a] Data are means ± SE of two observations. ADL, acid detergent lignin; PerL, permanganate lignin; KL, Klason lignin; ABSL, acetyl bromide soluble lignin; Y, young; M, mature (1–3 refer to three different maturity stages); W, wild; PA, past anthesis; FB, full bloom; PSD, past seed development. Means, within a row for analytical method, followed by different letters are different ($P < 0.05$).

Table 6. Correlation between Analytical Method and in Vitro Digestibility Data[a]

	IVDMD			IVCWD		
method	group 1	group 2	group 3	group 1	group 2	group 3
acid detergent lignin	–0.66****	–0.60***	–0.22^{NS}	–0.49**	–0.21^{NS}	–0.30^{NS}
permanganate lignin	–0.54***	–0.09^{NS}	–0.21^{NS}	–0.50**	–0.12^{NS}	–0.19^{NS}
Klason lignin	–0.84****	–0.81****	–0.38^{NS}	–0.78****	–0.69****	–0.63*
acetyl bromide soluble lignin	–0.79****	–0.89****	–0.89****	–0.74****	–0.79****	–0.85****

[a] IVDMD, in vitro dry matter digestibility; IVCWD, in vitro cell wall digestibility; NS, not significant; group 1, containing all plants; group 2, all plants but wood species; group 3, forages usually fed to ruminants. * = $P \leq 0.1$; ** = $P \leq 0.01$; *** = $P \leq 0.001$; **** = $P \leq 0.0001$.

from sulfuric acid are usually lower than the values determined by using the permanganate method (*11*).

In Vitro Digestibility and Correlation with Lignin Content. With the exception of leaf tissue from both straws, in vitro dry matter digestibility (IVDMD) was higher than in vitro cell wall digestibility (IVCWD) (**Table 5**). As expected, there was a maturity trend with regard to both harvesting date and plant height (alfalfa lower versus upper internodes). The decline in DM and CW digestibility as forage matures is usually credited to an increasing concentration of lignin (*10*, *51*). Wood samples were virtually indigestible, which probably can be attributed to their high lignin content.

Analytical lignin methods yield different estimates of lignin concentration and therefore may not provide the same accuracy in predicting forage digestibility. The results from the analytical methods of this study were correlated with in vitro measurements of dry matter and cell wall digestibility. Plants were separated into three groups: group 1, containing all plants; group 2, all plants but wood species; and group 3, forages usually fed to ruminants (bromegrass Y, M1, and M2; corn stalk; alfalfa Y, lower and upper 30 cm; and red clover). Across all plants, all four methods were negatively correlated with both IVDMD and IVCWD (**Table 6**). The literature contains several examples of data that show negative correlation between lignin concentration (obtained through a variety of methods) and either DM or NDF digestibility (*46*, *52*).

ABSL was the analytical procedure that consistently yielded significantly high correlation values for both dry matter and cell wall digestibility ($P \leq 0.0001$). Correlation coefficients between dry matter digestibility and the ABSL method were considerably higher than the coefficients obtained with ADL and PerL (*2*).

Grouping according to forage classes showed varied relationships between digestibility and lignin method (*46*). In this work it was observed that ADL, PerL, and KL were not correlated with IVDMD in the forages only, group 3. However, there was a strong correlation for all lignin methods in group 1 containing all plant samples. In other words, the correlation of lignin to digestibility decreased for ADL and PerL (to a lesser degree for KL) when highly lignified plants were removed. For forages usually fed to ruminants, only the ABSL method showed a strong relationship with both IVDMD and IVCWD. When grass and legume samples were combined into a single data file, there was no correlation between digestibility and ADL, although forage species taken individually showed negative correlations for digestibility with ADL (*53*).

The fact that grasses and legumes had different slopes for the correlation of ADL with digestibility (*52*, *53*) and that separate equations for the prediction of indigestible neutral detergent fiber were necessary for the two lignin procedures (ADL and PerL) commonly used (*14*) strongly suggests that some analytical problem may exist in either method or both.

The observation that ADL and KL were similarly related to digestibility of both grasses and legumes raises an interesting question because the ADL method may result in the loss of a substantial portion of the total forage lignin (*46*). The difference between ADL and KL values was of too great a magnitude to be accounted for simply as residual protein condensed in the residues (*7*).

This work ran in vitro digestibility with isolated cell wall because the deleterious lignin effects are upon the cell wall and not on cell solubles (*47*). Dry matter digestion was less affected by lignin content than was cell wall digestion (*51*). However, for most samples, it was detected that negative correlations were numerically lower for lignin concentration and IVCWD than with IVDMD (**Table 6**). The apparent relationship between forage lignin content (percent of DM) and indigestible NDF results as a consequence of maturity because NDF increases while crude protein and cell solubles decline (*14*).

Conclusions. Using acidic dioxane lignin extracted from a range of plant materials provides suitable standards for developing absorptivity coefficients to use with the spectrophotometric acetyl bromide assay for lignin. Although the ABSL method gives relatively high numbers for lignin concentration, compared to other methods, it is better correlated with both IVDMD and IVCWD, indicating that this method could be a good predictor of digestibility. Acid detergent solubilizes some of the lignin, particularly in grasses, resulting in lower ADL values. The Klason method may overestimate lignin content, especially in legumes, due to the presence of protein in the CW, and PerL gives high lignin values for legumes that may be due to oxidation of noncellulosic carbohydrates contaminating ADF. We see only one inconvenience in this method, which is related to the reference standard. In this work and in a previous one, the standard was extracted from the individual plant being assayed and, for each one, a standard curve developed. Although it is a relatively straightforward procedure that produces a lignin extract, when several different plant samples (species) are being investigated, this can be quite time-consuming, although building a standard curve is required only once for each species. A research project that is presently being conducted is investigating the hypothesis of a "universal standard"; in other words, could a lignin extracted from, say, pine be employed as standard to determine lignin concentration in any wood, grass, or legume? On the basis of the findings of this research, it is concluded that the ABSL method is a fast and convenient method for determining the total lignin concentration of a cell wall sample. It has some advantages over ADL, PerL, and KL and may prove to be useful in developing a quick and easy method to predict in vitro digestibility. Further studies on this analytical procedure are necessary to clearly establish its usefulness in predicting digestibility.

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