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Use of Lignin Extracted from Different Plant Sources as Standards in the Spectrophotometric Acetyl Bromide Lignin Method

Romualdo S. Fukushima*,† and Monty S. Kerley‡

†Departamento de Nutrição e Produção Animal, Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo, Av. Duque de Caxias Norte, 225, 13630-000, Pirassununga, SP, Brazil

‡Division of Animal Sciences, College of Agriculture, Food, and Natural Resources, University of Missouri, 920 E. Campus Drive, Columbia, Missouri 65211, United States

ABSTRACT: A nongravimetric acetyl bromide lignin (ABL) method was evaluated to quantify lignin concentration in a variety of plant materials. The traditional approach to lignin quantification required extraction of lignin with acidic dioxane and its isolation from each plant sample to construct a standard curve via spectrophotometric analysis. Lignin concentration was then measured in pre-extracted plant cell walls. However, this presented a methodological complexity because extraction and isolation procedures are lengthy and tedious, particularly if there are many samples involved. This work was targeted to simplify lignin quantification. Our hypothesis was that any lignin, regardless of its botanical origin, could be used to construct a standard curve for the purpose of determining lignin concentration in a variety of plants. To test our hypothesis, lignins were isolated from a range of diverse plants and, along with three commercial lignins, standard curves were built and compared among them. Slopes and intercepts derived from these standard curves were close enough to allow utilization of a mean extinction coefficient in the regression equation to estimate lignin concentration in any plant, independent of its botanical origin. Lignin quantification by use of a common regression equation obviates the steps of lignin extraction, isolation, and standard curve construction, which substantially expedites the ABL method. Acetyl bromide lignin method is a fast, convenient analytical procedure that may routinely be used to quantify lignin.

KEYWORDS: Dioxane, extinction coefficient, grasses, legumes, standard, woods

INTRODUCTION

Lignin is a complex phenylpropanoid polymer present in most vegetable cell walls[1] and has important physiological roles.[2] It has detrimental effects upon digestibility of forage cell wall carbohydrates by herbivores.[3] Lignin concentration is inversely proportional to efficient conversion of plant carbohydrates into human food such as meat and milk.[4] Lignin inhibits enzymatic hydrolysis of cell wall biomass for bioenergy production[5] and negatively influences paper and cellulose production.[6]

Thus, need for an accurate and precise analytical method for lignin quantification in plants is evident. Acetyl bromide lignin (ABL) was introduced for lignin determination in wood by Johnson et al.,[7] and modifications have been made to tailor this procedure for various forage substrates. For example, Morrison[8,9] adapted ABL for nonwoody samples because it was found that high protein content in grasses and legumes did not interfere with lignin quantification. However, as with any spectrophotometric method, there is a challenge in developing an appropriate calibration standard.[10,11] Several materials have been tested as potential standard: indulin,[12] beech kraft lignin,[13] native lignin,[14] and lignin extracted by acetyl bromide reagent.[15] Lignin concentration was estimated by using the monomers guaiacol and ferulic acid as standards.[16,17] Based on infrared spectra and nitrobenzene oxidation data, a specific absorption coefficient of 20.0 g^{-1} L cm^{-1} was proposed for lignin content estimation.[18] Unfortunately the wide array of lignin sources has led to questioning of spectrophotometric method feasibility.[10] This is furthermore complicated by the heterogeneity of lignin composition and diverse chemical bonding among phenolic moieties.[19] Variation of lignin structure among different lignin samples could lead to variation in extinction coefficients, resulting in error in calculation of lignin content.

More recently ABL was modified with acidic dioxane addition to extract lignin from forage samples.[10,11] Standard curves were developed which yielded lignin values different from those measured by Klason lignin, acid detergent lignin, or permanganate lignin procedures. Correlations with in vitro dry matter or cell wall digestibility were highest with ABL data.[11] Although high correlation with in vitro digestibility is not necessarily an indication of an ideal lignin concentration method, we view the relationship of digestibility to ABL mass as indicative that ABL more consistently quantified the lignin component of plant cell wall, assuming that lignin is responsible in large part for susceptibility of structural polysaccharide hydrolysis within plant cell wall structures. However, the traditional approach required extraction of lignin with dioxane, its isolation, and a standard curve built for each plant analyzed. This represents a methodological complexity because these steps are lengthy and laborious, which are magnified if there are many samples involved.

A possible solution to simplify the ABL procedure would be capability to use only one lignin extract to derive a single standard curve and to calculate lignin concentration in plant samples ranging from trees to forages. However, this would require that all lignins absorbed UV light (280 nm) equally irrespective of botanical origin. An exploratory study[10] reported that regression

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equations obtained from a few plant samples were similar to each other. Another study indicated extinction coefficient (EC) values ranging from 22.96 to 23.60 g^{-1} L cm^{-1} for *Arabidopsis thaliana* which was independent of accession, environmental growth conditions, and insensitive to lignin structure.[20] This demonstrated that although lignin has a heterogeneous composition, its absorption spectrum is like that of a pure compound most likely due to the spectroscopic similarities of monolignols that compose the lignin polymer.[10]

Objectives of this research were to verify if lignin extracted from a variety of plant species had similar absorption characteristics, allowing only one lignin extract to be used as a standard to measure lignin concentration across plant species.

MATERIALS AND METHODS

Sample Materials and Lignin Extraction. Fourteen plants were employed in this study: pine (*Pinus elliotti*), aspen (*Populus nigra*), pau-brasil (*Caesalpinia echinata*), two species of bamboo (*Chusquea oxylepis* and *Bambusa vulgaris*), corn stover (*Zea mays*), sugar cane bagasse (*Saccharum hibridas*), Napier grass (*Pennisetum purpureum*), annual ryegrass (*Lolium multiflorum*), tall fescue (*Festuca arundinacea*), Caucasian bluestem (*Bothriochloa caucasia*), alfalfa (*Medicago sativa*), red clover (*Trifolium pratense*), and lespedeza (*Lespedeza bicolor*). Also, three commercial lignins (Aldrich Chemical Co., Inc., Milwaukee, WI) were compared: hydrolytic, organosolv (propionate), and organosolv (2-acetoxyethyl ether).

Wood samples were taken from mature tree trunks as wood chips, and before grinding in an Udy mill (0.5 mm), they were ground (5.0 mm screen) through a hammer mill. Bamboos (internodes) were harvested from several year-old clumps and also ground in two steps as described above. Napier was harvested at past full bloom stage and corn stover came from mature plants, post corn harvest. Sugar cane bagasse came ground from a sugar/alcohol plant. Annual ryegrass, tall fescue, and Caucasian bluestem were harvested at late boot stage with flowering heads fully emerged. Alfalfa, red clover and lespedeza were harvested at full bloom stage. For this assay, mature plants were chosen because we wanted to extract and isolate lignin to build the standard curves, and for this purpose, a reasonable quantity of lignin is necessary. Yield of lignin from young plants (particularly legumes) is very low which would seriously affect standard curve construction.

Forage samples were dried (55 °C) in a forced air oven for 72 h and then ground (0.5 mm) in a cyclone mill. Cell wall (CW) used for lignin extraction was obtained as described previously[10] with the exception that filter bags (Ankom Technology Corp., Macedon, NY) were used instead of glass thimbles (ten filter bags placed in the Soxhlet extraction tube instead of an individual glass thimble). Also, chloroform:methanol (2:1) mixture replaced pure chloroform. Concentration of CW was used to calculate lignin values in the samples.

Lignins were extracted from CW employing acidified dioxane and purified by precipitation in distilled water and anhydrous diethyl ether[10] with following modifications. Ten grams of CW was placed in 250-mL round-bottom flask and 200 mL of 0.2 N HCl in dioxane (190 mL of dioxane + 10 mL of 2 N HCl) added. This mixture was refluxed under N_2 for 1 h. After cooling, the solution was vacuum filtered through a 13400 μm glass fiber filter (Sartorius AG, Goettingen, Germany). The filtrate was collected in 500 mL Erlenmeyer flask containing 15 g of sodium bicarbonate. Approximately 10 mL of 92% dioxane was used to wash residual CW. The Erlenmeyer was placed on a rotary shaker for a minimum of 3 h until the solution had reached a neutral pH (using pH strips) and then filtered through glass fiber filter. The filtered solution was concentrated (15−20 mL total volume) under vacuum on a rotary evaporator (water temperature 45−50 °C) and again filtered. A few milliliters of 92% dioxane was used to wash flask and filter. The filtrate was added dropwise into two centrifuge bottles containing 200 mL each of rapidly stirring water. Then 3.0 g of anhydrous sodium sulfate was added (while stirring) to flocculate the lignin. Lignin was pelleted by centrifugation (3000*g*, 15 min) and dried (55 °C) in a forced air oven. Lignin extract was dissolved with 4−5 mL of 92% dioxane, filtered through 0.8 μm nylon filter (Whatman International Ltd., Maidstone, England), and added dropwise to 200 mL of rapidly stirring anhydrous diethyl ether. The precipitate was centrifuged (3000*g*, 10 min) and solubilization in dioxane and ether repeated twice. Immediately upon discharging the last ether wash, the pelleted lignin was washed with 80 mL of water and dispersed into small particles using a spatula. The lignin extract was centrifuged (6000*g*, 15 min), water wash was discarded, and lignin was recovered after drying (55 °C for 24 h).

Five grams of each commercial lignin was dissolved in 30 mL of 92% dioxane and filtered through a 0.8 μm nylon filter. The filtrate was added dropwise into rapidly stirred water and lignin extracted as described above.

Lignin Quantification. Before building standard curves, it was necessary to correct for nonlignin compounds such as protein, which was determined as nitrogen (N × 6.25) using a nitrogen analyzer (Leco Corp., St. Joseph, MI), neutral sugars were determined by phenol−sulfuric acid method using glucose as standard,[21] and uronic acids were determined colorimetrically using galacturonic acid as standard.[22] Moisture and ash were also determined and where necessary discounted from total lignin weight. Nine standard solutions were prepared: three replicates each of 3.0, 6.0, and 9.0 mg of lignin were weighed in culture tubes and dissolved in 10.0 mL of 95% acetic acid. From each tube, a 1.0 mL aliquot was pipetted into glass culture tubes (with Teflon lined caps). A blank tube contained 1.0 mL of 95% acetic acid. One milliliter of 50% (v/v) acetyl bromide in acetic acid was added, and lignin samples were dissolved by immersion in a 50 °C water bath for 2 h with occasional mixing. Upon cooling, 5.0 mL of acetic acid and 2.0 mL of 0.3 N NaOH were added. After mixing, 1.0 mL of 0.5 M hydroxylamine hydrochloride was added and absorption read in a spectrophotometer (Beckman Instruments, Inc., Fullerton, CA) adjusted to 280 nm wavelength. A blank was included to correct for reagent background absorbance. All operations were performed in a ventilated hood. Based upon readings and respective lignin concentrations, regression curves were calculated to give the extinction coefficient (EC) for each plant.

Acetyl bromide lignin concentration in plant samples was determined as follows: approximately 100 mg of CW was weighed into a 50-mL screw capped glass tube (Teflon lined cap) and 10 mL of 25% acetyl bromide/acetic acid added. The mixture in the tube was allowed to digest in a 50 °C water bath for 2 h with occasional mixing. A blank tube was included. After cooling and centrifuging (3000*g*, 15 min), 0.5 mL was added to a tube containing 6.5 mL of acetic acid and 2.0 mL of 0.3 M NaOH. Contents were mixed and 1.0 mL of 0.5 M hydroxylamine hydrochloride solution pipetted. Absorption was measured at 280 nm wavelength and optical density inserted in the following ABL equation:

$$X = \frac{(Y - 0.0009)}{23.077}$$

where X is concentration of lignin (mg/mL), Y is the optical density reading of unknown sample, 0.0009 is the mean intercept value, and 23.077 is the mean extinction coefficient obtained from this work. The resulting X value is multiplied by CW content in the plant (on a DM basis) and accounted for the actual weight of CW utilized after all dilutions. This will give the lignin concentration in the plant (g/kg DM).

Although apparently there seems to be some discrepancy between ABL treatments of isolated lignins (for standard curve building) and intact cell wall samples (for lignin concentration determination), solution concentrations and volumes were calculated so that these two types of samples were treated exactly the same.

Table 1. Cell Wall Content and Composition of Extracted Lignins[a]

lignin type	cell wall (g/kg DM)	water (g/kg lignin)	sugars (g/kg lignin)	uronosyls (g/kg lignin)	protein (g/kg lignin)	ash (g/kg lignin)
Aldrich hydrolytic	–	16.6	10.6	1.2	46.9	3.9
Aldrich propionate	–	0	4.2	0.5	13.8	6.6
Aldrich ether	–	4.1	4.4	2.3	21.1	0
pine	971.5	1.9	21.4	0.6	5.6	0
pau-brasil	972.5	0	35.3	9.8	13.7	0
aspen	796.3	9.1	47.2	13.6	16.9	0
bamboo 2	911.3	4.7	46.1	11.2	18.1	0
bamboo 4	952.9	20.9	43.9	10.2	13.1	5.6
alfalfa	669.1	0	35.6	10.6	51.9	0
red clover	646.1	0	39.3	7.9	43.7	4.7
lespedeza	665.1	0	45.9	9.1	40.6	0
napier	741.9	0	48.3	10.5	22.5	2.6
caucasian bluestem	700.1	0	28.8	2.7	34.3	8.1
annual ryegrass	676.1	3.8	24.6	3.5	33.2	0
tall fescue	685.7	19.6	39.1	5.8	26.9	4.2
corn stover	858.1	28.1	34.2	10.8	17.5	9.1
sugar cane bagasse	879.6	16.1	50.2	13.2	16.8	4.6

[a] Mean of two observations.

Laboratory analyses were done in duplicate or triplicate. Estimates of lignin concentration were compared by *F* tests. A simple linear regression was performed to measure main effects between lignin methods using PROC GLM from SAS.[23]

RESULTS AND DISCUSSION

Lignin composition. Neutral sugars content among plant species ranged from 21.4 to 50.2 g/kg of lignin (Table 1). These values were similar to previous studies[10,11] but slightly higher than reported for accessions of *Arabidopsis*.[20] As expected, sugar cane bagasse exhibited the highest value. Concentration of uronic acids varied from 0.6 to 13.6 g/kg of lignin with pine showing the lowest content of uronosyls. Other studies[10,11] have also showed pine with low uronosyl values. Commercial lignins used had lower concentrations of carbohydrates than reported previously,[24] due probably to different extraction procedures. Carbohydrates are common contaminants in isolated lignins,[18] usually constituting between 50 to 150 g/kg.[8]

Extracted lignins contained nitrogen that was presumed to be protein (13.3 to 51.9 g/kg) (Table 1). Chang et al.[20] reported protein content varying from 26.2 to 53.4 g/kg of lignin for several accessions of *Arabidopsis*. Cell wall contains protein, generically called extensins, which have structural roles within the wall matrix and have been suggested to be cross-linked to lignin.[25] However, cell wall of woods is virtually absent of protein,[1] reflected in the low protein content of isolated lignins from woods. Pine lignin had the lowest protein content, agreeing with previous research.[11] Mature grasses (bamboos, corn stover, and sugar cane bagasse) exhibited lower protein values than other grasses harvested at younger growth stages. As expected, lignin from legumes had high concentrations of protein, following high concentration of this nutrient in the whole plant. These findings were supported by lower nitrogen values in acid detergent and Klason lignin residues of grasses compared to alfalfa.[26] Commercial lignins had elevated contents of protein, particularly hydrolytic lignin.

Not all lignin extracts contained ash and when present its concentration was less than 10 g/kg. Similar data was reported with three commercial and one synthetic lignin.[24] Ash, water, and other nonlignin components were considered when calculating standard curves. Isolated lignins were on the average 923.8 g/kg pure.

Table 2. Slopes and Intercepts of Standard Curves and Concentrations of Lignin

sample	slope	intercept	lignin[a] (g/kg DM)	lignin[b] (g/kg DM)	lignin[c] (g/kg DM)
Aldrich hydrolytic	23.826 A[d]	−0.018	–	–	–
Aldrich propionate	23.027 A,B,C	0.0267	–	–	–
Aldrich ether	23.318 A,B,C	−0.0039	–	–	–
pine	23.833 A	0.0079	231.7 b	225.7 c	267.6 a
pau-brasil	22.606 A,B,C	−0.001	192.9 c	197.1 b	222.9 a
aspen	22.861 A,B,C	0.0004	105.7 b	106.8 b	122.0 a
bamboo 2	22.711 A,B,C	−0.0264	181.4 b	180.3 b	209.8 a
bamboo 4	23.733 A,B	−0.037	222.1 b	210.5 c	256.6 a
alfalfa	22.667 A,B,C	−0.033	55.4 b	54.6 c	64.0 a
red clover	23.444 A,B,C	0.0131	52.2 b	52.1 b	60.3 a
lespedeza	22.994 A,B,C	−0.0063	50.1 b	49.9 b	57.8 a
napier	22.956 A,B,C	0.0531	126.1 b	126.5 b	145.5 a
caucasian bluestem	23.683 A,B,C	−0.0149	76.1 b	74.3 c	87.9 a
annual ryegrass	22.483 B,C	0.0269	83.3 c	87.2 b	96.3 a
tall fescue	23.217 A,B,C	−0.0181	95.9 b	96.4 b	110.7 a
corn stover	22.444 B,C	0.0277	156.4 c	164.8 b	180.5 a
sugar cane bagasse	22.509 B,C	−0.0122	144.2 c	146.2 b	166.6 a
mean	23.0772	−0.0009			
SEM	0.118	0.006			

[a] Lignin concentration calculated by using this work's mean extinction coefficient. [b] By using the plant extinction coefficient. [c] By using the Iiyama and Wallis extinction coefficient. [d] Different capital letters in the column and different small letters in the rows indicate differences ($p < 0.05$).

Standard Curves. Each plant (or commercial lignin) had its own standard curve. Most standard curves had similar slopes (Table 2). Although statistical differences were detected between the extreme slopes, the difference between the lowest slope (corn stover $-22.444\ g^{-1}\ L\ cm^{-1}$) and the highest slope (pine $-23.833\ g^{-1}\ L\ cm^{-1}$) was less than 6% which would alter little in

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lignin concentration and would probably have little impact in terms of biological effect. An average extinction coefficient of 23.077 g^{-1} L cm^{-1} was then calculated.

In this work the mean EC was somewhat higher than previous works[10,11] which averaged 17.901 and 17.084 g^{-1} L cm^{-1}, respectively. One possible explanation could be predominance of a given monomer over others (syringyl, guaiacyl, or *p*-hydroxyphenyl nuclei) that could interfere on the resulting EC. However, this seems not to be the case because we used a range of plant species which probably would not show such predominance. Also, Iiyama and Wallis[27] reported that wavelength (280 nm) and ECs of both softwood (guaiacyl type lignin) and hardwood (mix of guaiacyl and syringyl nuclei) lignins were similar after acetyl bromide treatment, indicating that monomer type would not interfere with ultraviolet maximum or EC. Other reasons could be humidity and ash contents in the extracted lignins which were not determined in those previous reports.[10,11] However, this work coefficient was consistent with other papers: from 22.6 to 24.0 g^{-1} L cm^{-1} for various softwoods and 22.4 to 24.5 g^{-1} L cm^{-1} for hardwoods.[7] Chang et al.[20] reported a mean EC value of 23.35 g^{-1} L cm^{-1} for *Arabidopsis thaliana* which was independent of the *Arabidopsis* accession and environmental growth conditions and insensitive to lignin structure (syringyl to guaiacyl ratio). The work of Iiyama and Wallis[18] suggested a slightly lower EC of 20.0 g^{-1} L cm^{-1}, on the basis of infrared spectra and nitrobenzene oxidation data for herbaceous plant samples, containing diverse syringyl to guaiacyl ratios. This confirmed that EC values using the ABL method are insensitive to differences in lignin composition which would support the hypothesis that a single EC could be utilized to quantify lignin concentration in plant materials.[10,20] Intercepts showed no statistical differences among standard curves ($p > 0.05$).

Cell Wall and Lignin Quantification. A fibrous preparation such as crude cell wall which is obtained after treatment with water and organic solvents is used in the ABL method. This is followed to avoid interference of other phenolic compounds such as tannins, flavonoids, etc.[8,9] Woods (except aspen) and bamboos tended to have higher CW content, as expected (Table 1). Napier, corn stover, and sugar cane bagasse samples were from mature plants which also had high CW contents. Plants harvested at earlier growth stages had proportionally less CW. As the plant matures CW concentration increases because of higher stem proportion in total plant biomass and CW thickening.[28] Our research used CW instead of neutral detergent fiber (NDF) to capture pectin and other cell wall nonstarch polysaccharides (β-glucans, galactans, and gums) dissolved by NDF solution.[29] It seems that CW preparation better reflects the total plant cell wall matrix.[30]

A precipitate formed after centrifuging digested CW with acetyl bromide solution. Morrison[8,9] concluded that the precipitate consisted almost entirely of proteins, which would not interfere with optical density for lignin determination. Nitrogen analyses of sediments from samples unrelated to this work confirmed Morrison's observation, with crude protein ranging from 515.4 to 693.0 g/kg of precipitate (data not published).

To calculate ABL concentration, the mean EC (23.077 g^{-1} L cm^{-1}) was utilized. Woods (except aspen) and bamboos had the highest lignin content. Legumes had the lowest lignin content (Table 2). Overall, plants exhibited a typical maturity/lignin concentration relationship. To better assess if a mean EC can be utilized in substitution for individual standard curves, lignin concentration was estimated for each plant utilizing the mean EC and its own standard curve. For comparative purposes, the EC of 20.0 g^{-1} L cm^{-1} [18] was also utilized. Although the EC obtained by Iiyama and Wallis[18] gave the highest lignin concentrations ($p < 0.05$) (Table 2), on the average the distance between concentrations was less than 13%, probably because the methods utilized by these latter authors for obtaining EC was different than ours. The mean 23.077 g^{-1} L cm^{-1} extinction coefficient came from regression analysis of standard curves derived from 17 isolated lignins. The EC determined by Iiyama and Wallis[18] was obtained on the basis of infrared spectra and nitrobenzene oxidation data for milled sample lignins. When comparing lignin concentrations obtained by employing the mean and the individual EC, a number of samples showed no statistical differences, and in those where significance was detected, results did not follow a uniform tendency and the difference was smaller than 6%. Statistical significance was easily detected in the ABL method (for instance, alfalfa showed 55.4 versus 54.6 g/kg DM) because of low variance characteristic of this method. Obviously, for practical purposes such difference will have little, if any, impact over the lignin effect on cell wall digestion.

Utilization of a standard curve for individual plant species to calculate lignin concentration requires that steps of lignin extraction and isolation, including the determination of contaminants, as well the construction of calibration curve for each plant be followed. On the other hand, by employing the mean EC, there is no need to follow these steps. Introduction of mean EC in calculation of total lignin concentration simplified the ABL method, making it a convenient and easy procedure. It may be a good option for routine laboratory analyses.

AUTHOR INFORMATION

Corresponding Author

*Tel: +55 (19) 3565-4231; fax: +55 (19) 3565-4295; e-mail: rsfukush@usp.br.

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