Lignin concentration in oat (*Avena byzantina* L.) aerial part as measured by four analytical methods

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Abstract

The current analytical methods to quantify lignin in forages are not satisfactory. A spectrophotometric method, the acetyl bromide lignin (ABL), has been employed to determine lignin concentration in forages; however, it suffers from the lack of an ideal standard with which the optical density readings of samples are compared to. A lignin, extracted from the plant with a solution of acidic dioxane, was employed to build a calibration curve for this method. This procedure was then compared with other methods (acid detergent lignin - ADL, Klason lignin – KL and potassium permanganate lignin – PerL) to determine lignin content on different fractions (stem, leaf and whole plant) of eight oat cultivars (*Avena byzantina* L.). There was no agreement among the four methods. In general, ABL and KL methods yielded the highest values while ADL method yielded the lowest, particularly for the young plants. Lignin concentration was higher in the stem fraction as compared to leaf. It was detected influence of maturity stage in the investigated samples. It is concluded that the ABL method employing as standard lignin extracted with acidic dioxane has potential to be employed as a method to determine lignin concentration.

Key-words:
Acetyl bromide.
Cell wall.
Dioxane.
Lignin analyses.

Introduction

Lignin is a complex phenolic polymer found in the plant cell wall. Lignification of forages is considered the primary mechanism by which rumen cell wall degradability is inhibited[1]. Ruminants are capable of utilizing the fibrous fraction through the symbiotic interaction with microorganisms living in the rumen. Lignin content may be used as a predictor of forage nutritive value through mathematical calculations[2,3]. However, the main obstacle resides in relying on a method that determines lignin precisely and accurately; methods currently in use usually yield different lignin concentrations for the same samples[4,5,6].

Among some analytical procedures for quantifying lignin, there are the methods that oxidize the carbohydrates out of the cell wall matrix and the result is an insoluble lignin residue which is left after a sulfuric acid hydrolysis of the cell wall polysaccharides such as the acid detergent lignin method - ADL of Van Soest[7] and the Klason lignin – KL[8]. Lignin content can also be determined by difference after reaction with an oxidizing reagent such as the potassium permanganate lignin (PerL) procedure of Van Soest and Wine[9]; this procedure has the advantage of employing reagents that are not as corrosive as the sulfuric acid.

However, these methods are not completely satisfactory. The ADL procedure usually yields low concentrations. These lower ADL values may result from underestimation of lignin because it is potentially soluble in the acid detergent solution and in the 72% sulfuric acid reagent[10, 11]. On the other hand, the KL procedure, developed for use with

woody species, is not suitable for several forages, especially for those with high levels of protein[3]. These higher values could be a reflex of nitrogen content which was significantly higher (2-6 times) in the KL residues[6]. The detergent cetyl trimethylammonium bromide is included in the acid detergent solution intended for protein removal in the ADL method[7], whereas the KL method does not contain a protein-removal step.

The potassium permanganate method also has problems; permanganate oxidizes other phenolic substances (e.g., tannins, pigments, or proteins) that are not completely removed during the acid detergent preparatory step and appear as lignin[9]. Some cell wall carbohydrates such as hemicellulose and pectin may also be removed. Barton and Akin[12] showed that hemicellulose constituted most of dry matter removed from the plant NDF by the permanganate solution. Cell walls which are rich in uronosyls could overestimate lignin values upon permanganate oxidation of the pectic substances[5].

Alternatively, it has been reported a spectrophotometric procedure which is based upon absorbance reading at 280 nm after lignin is dissolved in a solution of 25% acetyl bromide in acetic acid (acetyl bromide lignin - ABL)[13] . Morrison[14] correlated absorbance values with sulfuric acid lignin data from the same samples and lignin content was calculated. However, due to the disadvantages of sulfuric acid lignin such comparisons would also carry out the same setbacks to the spectrophotometric results.

Concentration of a given substance determined by a spectrophotometric method must rely on a calibration curve; however, for this application it is necessary a reliable standard[15]. For pure compounds development of extinction coefficients becomes a convenient method of quantifying the compound of interest. Fukushima and Dehority[4] extracted and isolated lignin from forages with acetyl bromide reagent to be used as a standard for the spectrophotometric method; nevertheless, due to its high contamination with carbohydrate and acetate, another extraction procedure was proposed which extracts lignin with an acidic solution of dioxane[16].

Among those analytical methods, neither procedure is dominant; thus, the choice of a lignin method to use is at the discretion of the scientist performing the forage analyses[2]. The present research was undertaken to determine the lignin content in oat aerial part through four different methods and verify the usefulness of employing the lignin extracted with dioxane as a reference standard for the ABL methodology.

Materials and Methods

Plant material and cell wall preparation

Eight cultivars of oats (*Avena byzantina* L.) namely, "Centro de Treinamento Contrijuí (CTC) 1, CTC 2, CTC 3; Universidade Federal do Rio Grande do Sul (UFRGS) 7, UFRGS 10, UFRGS 18; Universidade de Passo Fundo (UPF) 14 and UPF 15", were harvested at three maturity stages (45-, 55- and 65-day old) from an experimental forage plot located at "Faculdade de Zootecnia e Engenharia de Alimentos da Universidade de São Paulo", Pirassununga Campus, SP, Brazil. Plants were cut at 10 cm from the soil. Harvested forages were dried in a 65°C forced air oven for 72 h, and separated into: 1/3 as they were (whole plant) and the remaining 2/3, manually sorted into leaf and stem. Samples were ground to pass a 1-mm pore size screen in a Wiley mill. Crude cell walls (CW) were obtained as outlined by Iiyama and Wallis[17], except that an initial step was added which consisted of a 45-min extraction of the samples in hot water (70°C). Each solvent extraction was continued until no additional color leached from the walls; extraction times were around 2-3 h for each solvent.

Chemical analyses

All laboratory analyses were run in duplicates. Acid detergent fiber (ADF), neutral detergent fiber (NDF) and ADL

were performed as outlined by Van Soest, Robertson and Lewis[18]. Permanganate lignin was determined as outlined by Van Soest and Wine[9]. Klason lignin was obtained as the unhydrolyzed residue remaining after a two-stage sulfuric acid hydrolysis of cell wall polysaccharides, as described by Hatfield et al.[6].

Extraction of lignin with acidic dioxane and the construction of standard curve for the ABL determination followed the procedure outlined by Fukushima and Hatfield[16].

For the quantitative evaluation of lignin in the aerial part of oats, a basic acetyl bromide protocol was conducted[17]; however, cautionary notes were followed[19]. The procedure consisted of digesting 100 mg of the CW preparation with 4.0 mL of a 25% acetyl bromide in acetic acid reagent at 50ºC for 2 h, with occasional mixing. After cooling, the volume was made up to 16.0 mL with acetic acid (HAc) and centrifuged (3000g, 15 min). Half milliliter 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 standard curve using a regression equation. A blank was included to correct for reagent background absorbance.

Because the leaf tissue yielded very low amounts of lignin, DL extraction was performed only in the stem fraction and the resulting regression equation employed to calculate lignin concentration in the corresponding leaf and whole plant fractions. Although it might be expected that lignin monomer composition may differ between different tissues[4,20], differences in phenolic composition would not alter the intensity of ultra-violet absorption and change the 280 nm absorption peak (Hatfield, R. D. – personal communication).

Carbohydrate content in dioxane lignin samples was measured by anthrone technique[21], and protein by micro-Kjeldahl[22] method.

Statistical analyses

The main focus of this research was concentrated on lignin analytical methods; thus, statistical analyses were reported within cultivars, comparing the methods. Estimates of lignin concentration determined by the methods (duplicate analyses) were analyzed as an 84 factorial design. When analysis of variance indicated difference ($p<0.05$), slicing interactions were compared. Means were compared by the Tukey test at a probability level of 5%, through the GLM procedure[23].

Results and Discussion

Studies on lignin concentration in vegetable products usually show substantial variation. Part of this explanation relies on the different methods available to determine lignin concentration in vegetable products[5]. Buxton and Russel[1] registered differences for core and noncore lignins among C_3 species. Jung and Vogel[24] also found that variation for all measures of lignification seemed to be as great within species as between grass species. Thus, the intention of narrowing down number of species to just one, but increasing diversity in terms of cultivars seemed appropriate in this study.

As expected, generally, lignin content obtained through all four methodologies increased as plants matured (Tables 1 to 3). For most cultivars, stem fraction had higher lignin content, as Jung and Vogel[24] had already reported. Some exceptions were detected on the usual increase in lignin content; it should follow the order: leaf => whole plant => stem. It is well known that laboratory conditions (time, temperature, media) and techniques of sample grinding strongly affect absolute lignin yields, regardless of the adopted methodology[25].

In general, ADL was the method showing the lowest lignin values, followed by the PerL procedure ($P\leq0,05$). Acetyl bromide method yielded for most samples,

Table 1 - Lignin concentration determined through four methodologies in three vegetable fractions from eight cultivars of oats, at 45-day harvesting age, expressed on a dry matter basis (%)[1]

Cultivar	Plant fraction / lignin methodology											
	Stem				Leaf				Whole plant			
	ADL	PerL	KL	ABSL	ADL	PerL	KL	ABSL	ADL	PerL	KL	ABSL
CTC 1	6.6^{d}	9.2^{c}	12.2^{b}	15.5^{a}	3.7^{b}	3.9^{b}	10.1^{a}	11.0^{a}	6.5^{c}	4.6^{c}	10.0^{b}	12.6^{a}
CTC 2	5.0^{c}	5.4^{c}	12.0^{b}	16.7^{a}	3.0^{c}	4.7^{c}	10.4^{b}	13.5^{a}	3.3^{c}	4.5^{c}	10.2^{b}	14.4^{a}
CTC 3	7.4^{b}	2.9^{c}	12.0^{a}	12.7^{a}	4.5^{b}	5.8^{b}	10.1^{a}	9.1^{a}	5.0^{b}	6.3^{b}	9.2^{a}	8.8^{a}
UFRGS 7	7.0^{b}	2.3^{c}	12.6^{a}	13.2^{a}	4.5^{b}	2.9^{b}	11.3^{a}	9.1^{a}	7.7^{b}	7.8^{b}	11.7^{a}	12.3^{a}
UFRGS 10	7.2^{c}	10.2^{b}	13.8^{a}	11.0^{b}	4.8^{c}	4.9^{c}	11.8^{a}	6.9^{b}	6.0^{c}	9.0^{b}	12.6^{a}	9.2^{b}
UFRGS 18	5.3^{d}	8.1^{c}	12.3^{b}	14.0^{a}	3.2^{b}	4.8^{b}	10.7^{a}	10.9^{a}	3.7^{b}	5.1^{b}	10.8^{a}	11.3^{a}
UPF 14	6.0^{c}	6.2^{c}	11.4^{b}	17.6^{a}	3.8^{c}	5.3^{c}	9.4^{b}	14.0^{a}	4.6^{c}	4.8^{c}	9.8^{b}	14.0^{a}
UPF 15	5.0^{c}	9.9^{b}	13.0^{a}	10.9^{b}	3.5^{c}	5.8^{bc}	12.1^{a}	7.7^{b}	4.2^{c}	3.0^{c}	11.9^{a}	8.8^{b}
Average	6.2	6.8	12.4	14.0	3.9	4.8	10.7	10.3	5.1	5.6	10.8	11.4
S.E.M.	0.22	0.69	0.18	0.63	0.14	0.28	0.23	0.63	0.27	0.48	0.29	0.57

[1] Values are means of three observations. ADL: acid detergent lignin; PerL: permanganate lignin; KL: Klason lignin; ABSL: acetyl bromide soluble lignin; CTC: Centro de Treinamento Contrijuí; UFRGS: Universidade Federal do Rio Grande do Sul; UPF: Universidade de Passo Fundo; S.E.M.: standard error of the mean.Means within a row followed by different letters, for each vegetable fraction, are different (P≤0.05) by Tukey's test.

Table 2 - Lignin concentration determined through four methodologies in three vegetable fractions from eight cultivars of oats, at 55-day harvesting age, expressed on a dry matter basis (%)[1]

Cultivar	Plant fraction / lignin methodology											
	Stem				Leaf				Whole plant			
	ADL	PerL	KL	ABSL	ADL	PerL	KL	ABSL	ADL	PerL	KL	ABSL
CTC 1	8.4^{b}	10.3^{a}	12.9^{a}	11.1^{a}	4.8^{d}	6.9^{c}	12.5^{a}	9.1^{b}	6.7^{b}	7.8^{b}	13.1^{a}	8.7^{b}
CTC 2	7.3^{d}	10.0^{c}	13.6^{b}	19.1^{a}	5.8^{c}	5.9^{c}	13.3^{b}	15.0^{a}	5.6^{d}	8.8^{c}	13.6^{b}	17.6^{a}
CTC 3	7.0^{c}	9.3^{c}	13.1^{b}	16.3^{a}	4.3^{b}	6.3^{b}	12.4^{a}	13.2^{a}	5.2^{c}	8.2^{b}	13.9^{a}	14.7^{a}
UFRGS 7	6.8^{c}	7.8^{b}	11.9^{a}	9.4^{ab}	5.9^{c}	7.6^{b}	12.4^{a}	8.6^{b}	6.6^{c}	7.9^{b}	13.4^{a}	9.5^{b}
UFRGS 10	7.6^{c}	11.0^{ab}	13.9^{a}	13.7^{a}	5.5^{c}	10.2^{b}	15.1^{a}	15.0^{a}	7.6^{c}	8.9^{b}	14.1^{a}	14.5^{a}
UFRGS 18	6.8^{c}	8.7^{c}	12.8^{b}	14.3^{a}	5.9^{c}	10.5^{b}	17.0^{a}	17.9^{a}	5.9^{c}	8.9^{b}	13.4^{a}	14.4^{a}
UPF 14	7.2^{c}	9.6^{b}	14.8^{a}	16.0^{a}	4.5^{c}	7.2^{b}	13.0^{a}	14.4^{a}	6.2^{c}	7.7^{b}	13.6^{a}	15.3^{a}
UPF 15	6.5^{c}	9.7^{b}	14.8^{a}	16.2^{a}	6.5^{d}	9.0^{c}	15.9^{a}	13.4^{b}	6.3^{c}	8.2^{b}	12.8^{a}	11.0^{a}
Average	7.2	9.5	13.5	14.5	5.4	8.0	14.0	13.3	6.3	8.3	13.5	13.2
S.E.M.	0.14	0.28	0.25	0.77	0.18	0.43	0.44	0.77	0.17	0.17	0.14	0.76

[1] Values are means of three observations. ADL: acid detergent lignin; PerL: permanganate lignin; KL: Klason lignin; ABSL: acetyl bromide soluble lignin; CTC: Centro de Treinamento Contrijuí; UFRGS: Universidade Federal do Rio Grande do Sul; UPF: Universidade de Passo Fundo; S.E.M.: standard error of the mean. Means within a row followed by different letters, for each vegetable fraction, are different (P≤0.05) by Tukey's test.

the highest lignin values. In some instances, ABL was similar to KL method (P>0,05) (Tables 1 to 3). Similar results were reported earlier by Fukushima and Hatfield[5].

Jung, Mertens and Payne[26] found less variability for KL than ADL and concluded that KL procedure preserves the lignin structure better, and was a more accurate measure than ADL. KL values were consistently higher than ADL for all forage samples. For grass species, the KL residues were about 2 to 3.5 times greater than ADL residues[6]. This loss of soluble lignin for the ADL was up to 50% of the lignin present in tropical grasses[27]. Younger grasses seem to suffer more of this phenomenon; perhaps the lignin molecule is still in the process of formation and more susceptible to hydrolytic attack by the chemical reagents used for isolation, particularly in the ADL procedure.

Table 3 - Lignin concentration determined through four methodologies in three vegetable fractions from eight cultivars of oats, at 65-day harvesting age, expressed on a dry matter basis (%)[1]

Cultivar	Plant fraction / lignin methodology											
	Stem				Leaf				Whole plant			
	ADL	PerL	KL	ABSL	ADL	PerL	KL	ABSL	ADL	PerL	KL	ABSL
CTC 1	10.6^{c}	12.2^{b}	15.1^{b}	18.8^{a}	4.4^{c}	7.5^{b}	15.3^{a}	16.3^{a}	8.7^{b}	9.6^{b}	15.0^{a}	16.29^{a}
CTC 2	8.5^{b}	9.1^{b}	13.0^{a}	14.8^{a}	7.9^{b}	9.8^{b}	14.5^{a}	14.4^{a}	6.8^{d}	9.9^{c}	12.3^{b}	14.29^{a}
CTC 3	8.5^{c}	10.8^{b}	12.0^{b}	14.9^{a}	4.7^{c}	8.5^{b}	12.9^{a}	15.4^{a}	6.4^{c}	9.1^{b}	13.2^{a}	14.06^{a}
UFRGS 7	8.2^{c}	11.5^{b}	14.1^{ab}	14.5^{a}	7.8^{c}	12.7^{b}	17.3^{a}	15.8^{a}	8.6^{c}	12.6^{b}	14.6^{a}	14.76^{a}
UFRGS 10	9.3^{b}	11.0^{b}	13.4^{b}	19.0^{a}	9.1^{c}	10.8^{c}	15.5^{b}	20.9^{a}	8.6^{c}	7.8^{c}	15.1^{b}	19.76^{a}
UFRGS 18	8.8^{b}	9.5^{b}	15.1^{a}	17.6^{a}	10.0^{b}	10.0^{b}	18.4^{a}	20.6^{a}	8.2^{c}	10.4^{c}	15.0^{b}	17.67^{a}
UPF 14	15.8^{b}	11.8^{c}	14.1^{b}	22.2^{a}	6.8^{c}	10.4^{b}	13.1^{b}	21.8^{a}	9.1^{c}	11.0^{c}	13.1^{b}	23.37^{a}
UPF 15	7.3^{c}	10.1^{b}	13.2^{ab}	15.0^{a}	5.8^{c}	10.4^{b}	17.4^{a}	17.2^{a}	7.5^{b}	9.8^{b}	15.9^{a}	16.78^{a}
Average	9.6	10.8	13.8	17.1	7.1	10.0	15.5	17.8	8.0	10.0	14.3	17.12
S.E.M.	0.55	0.28	0.27	0.69	0.39	0.38	0.54	0.71	0.21	0.36	0.31	0.78

[1] Values are means of three observations. ADL: acid detergent lignin; PerL: permanganate lignin; KL: Klason lignin; ABSL: acetyl bromide soluble lignin; CTC: Centro de Treinamento Contrijuí; UFRGS: Universidade Federal do Rio Grande do Sul; UPF: Universidade de Passo Fundo; S.E.M.: standard error of the mean.Means within a row followed by different letters, for each vegetable fraction, are different (P≤0.05) by Tukey's test.

However, this loss was smaller in KL method. This could be because the KL method does not contain a protein-removal step, which could explain the reason KL values were close to ABL, finding also reported by Fukushima and Hatfield[16]. The acid detergent solution employed in the ADL method is known to remove some of the lignin fraction, particularly in grasses[6,10].

Potassium permanganate lignin values were slightly higher than ADL, but lower than either KL or ABL (P≤0,05). Fukushima and Hatfield[5] also found this same trend. Accuracy on lignin determination also assumes great importance when it comes to equations estimating energy value of forages. Lower predictions of indigestible NDF were obtained when ADL was employed as compared to PerL[2]. These authors reported that ADL values were approximately 76% of those determined by the PerL method. Lignin values determined from sulfuric acid are usually lower than the values determined by the permanganate method[9].

For all cuts, the ratio between PerL/ ADL was higher in the leaf sections as compared to stems. This could be interpreted that some loss of leaf ADL was taking place or that PerL content in the leaf tissue was being overestimated. At this point, there are no reasons to explain why PerL would be overestimating lignin content in the leaf. On the other hand, some loss of leaf lignin on the ADL method could be because the leaves have a lignin matrix that is proportionally more abundant in guaiacyl monomer units than the stems. Guaiacyl-type lignin may be less resistant to acid hydrolysis than syringyl rich lignin present in the stems, which is a supporting tissue. These observations strongly suggest that analytical problems may be taking place in the ADL method.

On the other hand, the ABL method exhibited the highest values for most samples. The higher lignin values obtained with ABL could be to the phenolic compounds that contribute to the optical density, some of which are easily removed by the H_2SO_4 treatment[28]. For instance, ABL quantifies the ester linked hydroxycinnamic acids, typically found in grasses. Basic solutions, for instance sodium hydroxide solutions, remove the hydroxycinnamic acids. The solution after alkaly treatment of lignin grasses revealed typical peaks of p-coumaric and ferulic acids between 250 and 350 nm. These peaks were not present when the lignin came from

legume or pine[29], which is coherent because these materials do not have ester linked hydroxycinnamic acids to the lignin moiety. Corn lignin exhibited higher optical density readings than other lignins, which could be attributed to the presence of high concentration of p-coumaric acid[16]. Also nonlignin components – other phenolics such as tyrosine and tannins – if not removed by a preparatory cell wall step, may be dissolved by the acetyl bromide solution and could produce interfering absorbance[14]. Proteins should not be a problem because it is not soluble in the acetyl bromide/acetic acid solution then, should not contribute to the absorption at 280 nm.

Another artifact that may inflate ABL are some carbohydrate degradation products, particularly those coming from xylans present in the cell wall[19] which is aggravated by the presence of perchloric acid as catalyst. Because of this observation, these authors recommended that water bath temperature initially set at 70ºC[17] should be reduced to 50ºC and incubation time increased from 30 min to 2 hours; they also recommended to avoid use of perchloric acid.

Conclusions

1.There was no agreement among the four methods. In general, the ABL and KL methods yielded the highest values while the ADL yielded the lowest, particularly for the young plants.

2.It was not possible to assess which analytical method would be better than others; probably other ancillary techniques are necessary to draw a more concrete figure. However, the ABL method employing lignin extracted with acidic dioxane as standard has potential to be employed as a method to determine lignin concentrations. It is relatively easy to handle and less caustic than the sulfuric acid lignin method.

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Concentração de lignina na parte aérea da aveia (*Avena byzantina* L.) medida por quatro métodos analíticos

Resumo

Os métodos analíticos para quantificar a concentração de lignina atualmente em uso não se tem mostrado satisfatórios. Um método espectrofotométrico, a lignina brometo de acetila (LBA) tem sido empregado para determinar o teor de lignina em plantas forrageiras; entretanto, padece da inexistência de um padrão de referência ideal, com o qual as leituras de densidade óptica das amostras são comparadas. Uma lignina, extraída da planta com solução ácida de dioxano, foi empregada para a construção de uma curva de calibração para o método em questão. Este procedimento foi comparado com outros métodos (lignina detergente ácido - LDA, lignina Klason – LK e lignina permanganato de potássio - LPer) na estimativa do teor de lignina em diferentes frações vegetais (caule, folha e parte aérea) de oito cultivares de aveia (*Avena byzantina* L.). Não houve concordância de valores entre os quatro métodos analíticos. Num âmbito geral, LBA e LK forneceram as maiores estimativas enquanto a LDA resultou nos menores valores, particularmente nas amostras de plantas mais jovens. A concentração de lignina foi mais elevada na fração caule do que na folha. Foi detectado efeito da maturidade nas amostras analisadas. Conclui-se que o método LBA usando como padrão de

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referência a lignina extraída com dioxano ácido tem potencial para ser empregado nas determinações dos teores de lignina.

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