Some Characteristics of Forage Plant Lignin

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Introduction

The nutritive value of a forage crop is considered as composed of a complex of parameters, (1) intake of feed, (2) digestibility of feed, and (3) efficiency of utilization of digested feed. Of these parameters the digestibility of feed is regarded as primary, because digestibility has a great influence on the other two parameters[6]. Although the best measure of forage digestibility is achieved by animal feeding trials, the trials are not applicable to small samples for plant breeding or agronomic experiments.

As a forage crop becomes maturer, its lignin content increases and its digestibility decreases; a close negative relationship is found between lignin content and digestibility. Thus lignin counts among the most important chemical components related to forage digestibility and can play a role as an indicator for evaluating the digestibility or nutritive value of forage.

The 72% sulphuric acid method, by which lignin in plant materials is isolated as Klason lignin, is most frequently applied to the determination of lignin. Proteins in plant materials condense with lignin components during digestion with 72% sulphuric acid to give a positive error. Herbaceous plants are generally characterized by much higher protein content than woody plants, resulting in a greater positive error for herbaceous plants than that for woody plants. Therefore, proteins should be removed prior to digestion. The acid detergent (2% w/v cetyl trimethylammonium bromide in 1 N sulphuric acid)[10] is usually used for removal of proteins in forage plant samples. It still remains whether this pretreatment is adequate procedure or not.

Investigating the relationships between *in vivo* digestibility and lignin content to obtain regression equations for predicting digestibility, we observed that the relationships differ markedly between grasses and legumes. We revealed that the discrepancy mainly results from a differential effect of the acid detergent on the determination of lignin.

Relationship between digestibility and lignin content

A total of 26 forage samples, 8 of Italian ryegrass, 8 of orchardgrass, 6 of perennial ryegrass, and 4 of alfalfa all in the form of artificially dried hay, were used to investigate the relationships between *in vivo* dry matter digestibility (DMD) and 14 chemical components; crude protein, crude fiber, ether extracts, neutral detergent fiber, lignin, silica, etc.[2]. Of the chemical components determined, lignin content was most highly correlated with DMD for individual forage species: $r=-0.98$ ($P<0.001$) in Italian ryegrass, $r=-0.97$ ($P<0.001$) in orchardgrass, $r=-0.97$ ($P<0.01$) in perennial ryegrass, and $r=-0.98$ ($P<0.05$) in alfalfa. According to the regression analyses, no significant differences in the regression of lignin content on DMD were present among three grass species but the regression for grasses significantly differed from that for alfalfa. The regression equations for grasses and alfalfa are as follows:

Grasses: DMD(%)=84.5−5.29×Lignin (% of dry matter)
Residual standard deviation: 1.31

Alfalfa: DMD(%)=119.0−6.20×Lignin (% of dry matter)
Residual standard deviation: 1.21

As shown in Fig. 1, alfalfa samples had higher lignin contents than grass samples when compared at the same DMD; the average difference amounted

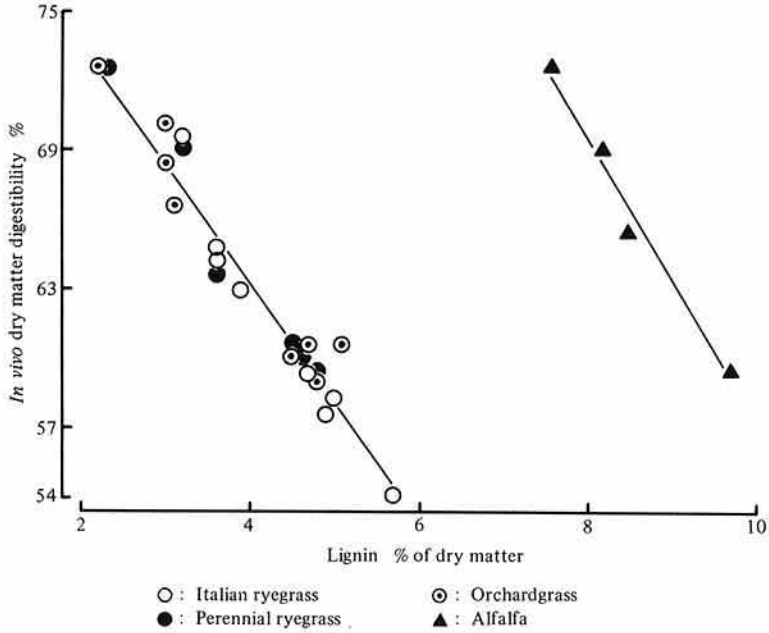

Fig. 1. Relationship between *in vivo* dry matter digestibility and lignin content in grass and alfalfa hays

to about 5%. Though the lignin preparations isolated from alfalfa samples by the 72% sulphuric acid method were contaminated with nitrogeneuos compounds including heat damaged protein[12] to a larger extent than those from grass samples, the correction of positive error caused by protein-lignin complex formation diminished only by an average of 1% the difference in lignin content between grass and alfalfa samples. Some workers[1,11] presumed that the lower lignin content of grasses reflects a more marked inhibitory effect of grass lignin on digestion of cellulose and hemicellulose by ruminant animals.

Here lignin was determined by successive treatment with the acid detergent–72% sulphuric acid. The acid detergent treatment (boiling for 1 hr under reflux) is known to remove plant proteins and to leave a fibrous residue which is chiefly composed of cellulosic carbohydrate and lignin. On the other hand, the treatment has been also reported to reduce the lignin yield[5,8]. If the acid detergent has a differential effect on the lignin yield from grasses and legumes, the difference in lignin content as shown in Fig. 1 may be explained by the different lignin loss during the acid detergent treatment. Then, the effect of acid detergent on the lignin yield was examined in two grasses and two legumes[3].

Effect of acid detergent on lignin yield

Low-protein residues (extractive free material) were prepared with pronase[4] from freeze-dried stem samples of four forage plants: Italian ryegrass and orchardgrass harvested at heading stage, and alfalfa and red clover at bud appearing stage. The lignin content of extractive free material was determined by the 72% sulphuric acid method with or without pretreatment with the acid detergent.

The original lignin in plant materials is quantitatively recovered in the form of an insoluble residue (Klason lignin) by treatment with 72% sulphuric acid followed by boiling with 3% sulphuric acid. However, in several plant species containing herbaceous plants, a small amount of the original lignin is lost as acid-soluble lignin in the filtrate. In this experiment, acid-soluble lignin was spectrophotometrically determined[9] and the total lignin content was calculated as the sum of Klason and acid-

Table 1. Effect of acid detergent on yield of 72% sulphuric acid lignin

	Untreated			Treated with acid detergent				
	Klason lignin	Acid-soluble lignin	Total lignin (A)	Residue	Klason lignin	Acid-soluble lignin	Total lignin (B)	Lignin* loss(%)
	% of extractive free material (DM)							
Forage plant								
Italian ryegrass	15.4(0.48)	1.8	17.2	65.8	7.8(0.58)	0.5	8.3	52
Orchardgrass	12.1(0.63)	2.3	14.4	60.7	5.3(0.69)	0.3	5.6	61
Alfalfa	17.5(0.92)	1.5	19.0	76.2	15.6(0.80)	0.6	16.2	15
Red clover	11.6(1.30)	1.7	13.3	71.3	9.9(0.92)	0.4	10.3	23
Woody plant								
Japanese red pine	28.1	—**	28.1	81.1	26.4	—**	26.4	5
Locust tree	20.4	3.1	23.5	78.1	15.8	1.9	17.7	25
Madake	24.8	1.4	26.2	71.8	19.7	0.7	20.4	22

*Calculated as: (A−B)×100/A. **Not determined.
Figures in parentheses show the nitrogen content of Klason lignin.

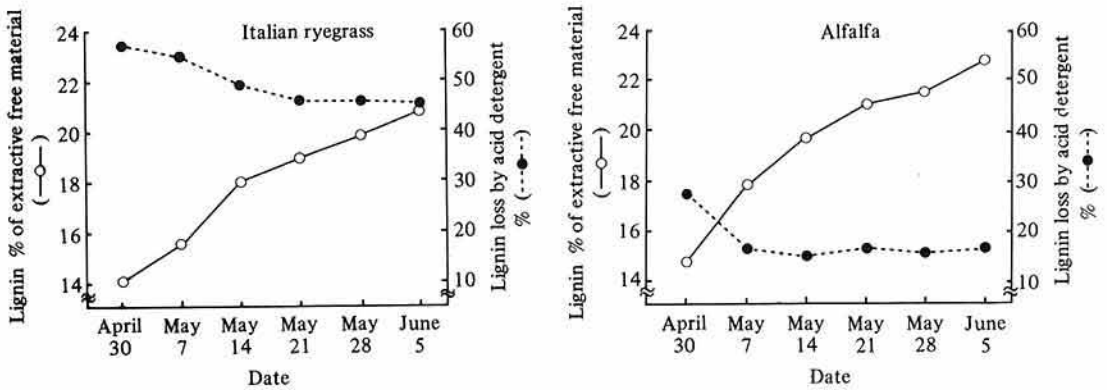

Fig. 2. Changes in lignin content and lignin loss by acid detergent with advancing growth

soluble lignin contents.

The acid detergent treatment caused the loss of Klason and acid-soluble lignin (Table 1). The extent of this loss depended on plant species; in Italian ryegrass and orchardgrass, the loss amounted to more than 50%, while in alfalfa and red clover it was less than 23%, and in Japanese red pine (coniferous tree) only 5%. The lignin loss was much more in Italian ryegrass than in alfalfa at each harvesting date and this loss decreased with advancing growth stage regardless of plant species (Fig. 2).

Fig. 3 shows the elution profiles of the acid detergent soluble fraction of extractive free material on Sephadex G-25. Two peaks (P1 and P2) of carbohydrate appeared in the profiles of both grasses and legumes. The major peak (P2) coincided with the peak of an authentic standard of glucose or xylose. The P2-sugar might be released by acidic hydrolysis from hemicellulosic carbohydrate. There were three peaks (F1, F2, and F3) of absorbance at 280 nm in the elution profiles. The legumes, however, lacked the F3 peak. With both grasses and legumes the F1 peak of absorbance overlapped the P1 peak of carbohydrate.

When the fraction F1 eluted at the void volume was rechromatographed on Sephacryl S300 with 0.5 M phosphate buffer (pH 6.8), it was eluted with Kav of 0.5 as a single peak. The fraction F1 reacted positively with phlorogrucinol-hydrochloric acid reagent, and produced *p*-hydroxybenzaldehyde, vanillin, and syringaldehyde by alkaline-nitrobenzene oxidation. Therefore, the fraction F1 seems to be homogeneous in molecular size and to contain lignin.

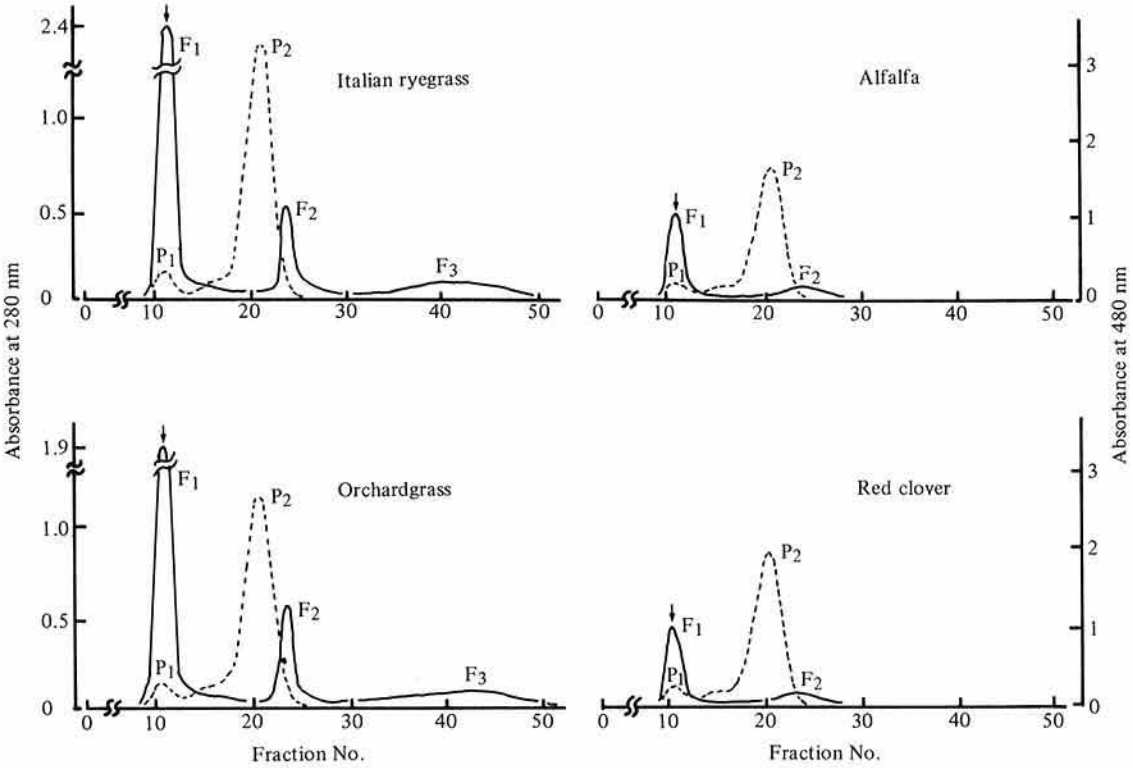

Fig. 3. Elution profiles of acid detergent solubles on Sephadex G-25
The column was eluted with water, eluting F1, F2, and F3 lignin-like fractions (solid line; absorbance at 280 nm) in that order. At the same time P1 and P2 carbohydrate fractions (dotted line; absorbance at 480 nm) were eluted. Arrows represent the void volume.

Discussion

The amount of lignin in forage plants is usually measured by using acid detergent-treated residue which is called as acid detergent fiber. Present results indicate that some portion of lignin dissolves in the acid detergent solution, resulting in an underestimation of lignin content. Porter and Singleton[5] pointed out that the acid detergent has a strong peptising effect and resultant colloidal lignin can not be recovered by salting out or changing of pH.

Van Soest[11] determined lignin by the successive procedure with the acid detergent–72% sulphuric acid in that order, and found that grasses have a lower lignin content than legumes at the same digestibility when plotted the lignin contents against digestibility. We obserbed that the difference in lignin content between grasses and alfalfa is an average of 5% at the same digestibility (Fig. 1). Such differences have to be caused considerably by the different loss of lignin during the treatment procedure with the acid detergent, since such loss is much more in grasses than in legumes.

Although the acid detergent method is not applicable to the estimation of the true lignin in forage plants, this method is sufficiently useful for the determination of the lignin fraction as a parameter for predicting the digestibility of forage. Using the regression equation of lignin content determined by the acid detergent method on *in vivo* digestibility, its low residual standard deviations indicate high accuracy in predicting the digestibility of forage.

Alkaline-nitrobenzene oxidation has been applied to the characterization of lignin. Alkaline-nitrobenzene oxidation of lignin or lignin containing

Table 2. Yields of phenolic aldehydes produced by alkaline-nitrobenzene oxidation

	Total yield (% of lignin)	Loss of yield by acid detergent treatment (%)	Molar ratio	
			p-H/V	Sy/V
	..... Extractive free material			
Forage plant				
Italian ryegrass	21.3	—	0.17	0.64
Orchardgrass	18.7	—	0.20	0.56
Alfalfa	21.8	—	0.07	0.54
Red clover	21.6	—	0.04	0.78
Woody plant				
Locust tree	27.8	—	0.04	2.00
Madake	26.4	—	0.47	1.05
	..... Acid detergent-treated residue			
Forage plant				
Italian ryegrass	19.1	57	0.19	0.73
Orchardgrass	17.2	64	0.21	0.67
Alfalfa	19.7	23	0.06	0.57
Red clover	19.5	31	0.03	0.75

p-H: *p*-hydroxybenzaldehyde, V: vanillin, Sy: syringaldehyde.

materials yield *p*-hydroxybenzaldehyde, vanillin, and syringaldehyde as major phenolic aldehydes. The total yield and composition of these compounds provide information on structural units of lignin. The results in Table 2 show that there are no large differences in the total amount and composition of phenolic aldehydes among four forage plants, with the exception of the higher ratio of *p*-hydroxybenzaldehyde to vanillin in grasses. Although the extent of lignin loss may not be related to the chemical structure of lignin shown by alkaline-nitrobenzene oxidation, some differences in lignin structure ought to have caused the lower lignin yield in grasses.

At the present time it appears that the difference in grass and legume lignin is one of the prime areas in need of clarification. This is important for not only a clear understanding of the inhibitory effect of lignin on digestion of forages by ruminant animals, but also a biochemical analysing of lignin accumulation in forage plants. We are investigating the chemical and physical properties of milled wood lignin, which is, in many respects, almost identical with the original lignin[7] isolated from Italian ryegrass and alfalfa.

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