ABSTRACT

THE USE OF ULTRAVIOLET SPECTROMETRIC PROCEDURES IN FORAGE EVALUATION AND IN DETERMINATION OF DIFFERENCES IN LIGNIN STRUCTURE

by James L. McCampbell

Twenty-one forage lignins (13 grass and 8 legume) and their respective fecal lignins were analyzed by ultraviolet spectrometric procedures and quantitative color reactions to determine if the lignin structure changed during the digestion process. Twenty forage lignins (6 grass and 14 legumes) were analyzed by these same procedures to determine if there were differences in lignin structures among the forages. The values obtained from the ultraviolet spectrometric procedures and color reactions for the twenty forage lignins were used to develop prediction equations estimating digestibility and digestible dry matter intake/cwt. in sheep.

The lignin molecule did undergo a change during the digestion process and the change was greater in grasses than in legumes. This change was either: a reduction in the amount of phenolic hydroxyl and methoxyl groups attached to the benzene ring, but with a proportionally greater reduction in the phenolic hydroxyl content; or alternatively the phenolic hydroxyl groups were replaced on the benzene

ring by methoxyl groups. This is a minor change considering all of the possible changes that could have taken place.

Lignins from legumes and lignins from grasses differed in the chemical make up of the lignin molecules. The aromatic compounds in the lignin from grass molecule had a greater proportion of methoxyl groups and/or less phenolic hydroxyl groups than did the aromatic compounds in the lignin from legumes.

The methoxyl content increased with maturity of the alfalfa hay, and lignin in forages with high in vitro dry matter digestibility contained less methoxyl groups than did forage lignin with low in vitro dry matter digestibility.

Alfalfa lignin had a greater phenolic hydroxyl content than did birdsfoot trefoil lignin and siberian reed canary grass contained more methoxyl groups than did the reed canary grass lignin.

The prediction equations using data for combined grasses and legumes were developed to estimate digestible dry matter and digestible dry matter intake/cwt. These prediction equations had low squared multiple correlation coefficients (.52 and .80 respectively) and complicated mathematics which would limit their usefulness. The four prediction equations for grasses and legumes when taken separately have squared multiple correlation coefficients (.93 and .96 for legumes and .90 and .99 for grasses) sufficiently great to be of practical

importance. The prediction equation for digestible dry matter intake/cwt. in grasses was $\hat{y} = 3.64 - .74X_3 - 6.03X_9$. The independent variable phenol (X_3) was a quantitative color reaction for phenol-like compounds. The independent variable 243 mμ (X_9) was the absorbtivity at 243 mμ on the difference spectrum.

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INTRODUCTION

Proper evaluation of forage is of academic importance and there is still a practical need for quick and accurate evaluation of forage samples. Attempts have been made to use lignin content of the forage as an indication of forage quality but the results have been variable and of limited usefulness in estimating the nutritive value of a forage. Current lignin analytical procedures do not take into account differences in the structure of lignin molecules. Differences in the basic units of the lignin molecules might influence dry matter digestibility of the plant to a greater extent than does the total amount of lignin.

If the basic units are as or more important than the total lignin content, more knowledge should be obtained about the structures of the lignin units, the type of compounds present, the type of groups attached to these compounds, and chemical linkages among the compounds. Ultraviolet spectrometry methods which are specific measures of certain lignin characteristics were developed by wood chemists to provide information about the lignin structure of wood. These lignin ultraviolet characteristics were analyzed in this thesis on

forage and fecal lignin to determine if the lignin structure changed during digestion of the forage. The same lignin ultraviolet characteristics were used to determine if lignin from grasses differed from that of legumes, if stage of maturity or treatments had an effect on the lignin structure, if the structure of lignin from first and second cutting hay differed, or if species of grasses or legumes differed in lignin structure. Prediction equations were also developed using these lignin ultraviolet characteristics to predict forage digestibility and intake by sheep.

REVIEW OF LITERATURE

Extensive reviews on chemistry of wood lignin can be found in the literature (Brauns, 1952), (Brauns and Brauns, 1960), (Hachihama and Jyodai, 1946), (Harborne, 1964), (Schubert, 1965), (Gould, 1966) and (Pearl, 1967). Since the chemistry of wood lignin is adequately reviewed in the above books and articles, the literature review in this thesis will discuss lignin as it applies to forage plants, such as legumes and grasses, and only refer to wood lignins where pertinent.

Chemical Properties of Lignin

Definition. The term lignin has different meanings to different people in various disciplines. The botanists, agronomists, and animal nutritionists consider lignin as a metabolite of the growing plant or a structural element contributing strength to the mature plant and as an undigestible feed ingredient. The microbiologists and soil chemists consider lignin a residue of decay, while the enzymologists and plant biochemists think of it as an end product of the enzymatic dehydrogenation of specific phenylpropane monomers.

Organic chemists consider lignin as a complex polymer that challenges their fundamental interest in chemical structure.

Brauns (1952) defined lignin as that encrusting material of the plant which: (1) is built up mainly, if not entirely, of phenylpropane building stones; (2) carries the major part of the methoxyl content of the wood; (3) is unhydrolyzable with acids, readily oxidized, and soluble in hot alkali and bisulfate, and (4) readily condenses with phenols and thio compounds. Brauns and Brauns (1960) updated this definition to include the production of aromatic aldehydes upon the oxidation of lignin with alkaline nitrobenzene and the production of "Hibbert monomers" (vanillin, ethoxypropiovanillone, and vanilloyl methyl ketone) upon subjection to ethanolysis.

The structure or structures of lignin in their native state have not been determined to date principally because of inability to isolate lignin in its native state unchanged chemically or physically.

Lignin Complex. There is a bond between lignin and cell wall carbohydrates but little is known about this linkage. Siegel (1956) has shown that when cellulose, in the form of filter paper, is added to a system of eugenol, peroxidase, and hydrogen peroxide there is deposited on the paper a lignin-like polymer similar to that observed in the presence of tissue sections. The author suggests that ether linkages may be essential for lignification. Bolker (1963)

used high-resolution differential infrared spectroscopy to study the existence of a bond between lignin and cell wall carbohydrates. He concluded that an acetal or hemiacetal bond exists between the carbonyl groups of lignin and the hydroxyl groups of some portion of the holocellulose and on cleavage liberated a ketone group in the lignin. Freudenberg proposed a model structure of lignin, as reported by Pearl (1964), that contained a disaccharide attached to a carbonyl group on one of the monomeric units.

Chemical Composition. The actual structure of lignin is not known, but research workers have determined either theoretically or by actual experiments some important aspects of the lignin structure. Pearl (1967) hypothesized that coniferous lignin was a polymerized product derived from a building unit R-C-C-C, in which R is the 4-hydroxy-3-methoxyphenyl group. Most recently lignin chemists have referred to the 4-hydroxy-3-methoxyphenyl groups as the guaiacyl group and the phenylpropane structure as a C_6-C_3 unit. Alkaline oxidation with nitrobenzene (Creighton et al., 1941) and with cupric oxide (Pearl, 1942), giving vanillin in yields above 25%, has been accepted as proof of the guaiacyl moiety existing in lignin. Harris et al. (1938) subjected isolated lignin to high pressure hydrogenation and obtained a combined yield of over 50% of 4-n-propylcyclohexanol, 4-n-propylcyclohexane-1,2-diol and 3-(4-hydroxycyclohexyl)-1-propanol. These high yields of hydrogenation products with

cyclohexylpropane structures proved that lignin was essentially aromatic and demonstrated that for the most part it was a condensation polymer of guaiacylpropane monomers.

Kefford (1958) concluded that linkages between the building units involved the phenolic hydroxyl group and the three carbon side chain but there is variation in the actual linkages formed. A model of a lignin molecule is shown in Figure 1, this model demonstrates the type of compounds that can be present in the lignin molecule and the type of bonds that can be formed between the basic units. Three aromatic compounds are represented in the model: compound 1 is p-hydroxycinnamic acid; compound 2 is coniferyl alcohol; and compound 3 is sinapyl alcohol. Other compounds may be present in lignin. Two types of bonds can be formed according to this model and these bonds are ether and carbon-carbon bonds. The ether bonds can be formed between side chains or between a phenolic hydroxyl group and a side chain. A carbon-carbon bond can be formed between any two carbon atoms.

Lignin has been isolated by many different procedures and the isolated lignin takes its name from the isolation procedure. Examples of this naming procedure are: ethanol lignin extracted by means of ethyl alcohol in the presence of small amounts of mineral acid; and sulfuric acid lignin extracted by concentrated sulfuric acid from the plant material.

In studies of lignin isolated from the stalk and leaves of normal maize and brown-midrib mutants Gee et al. (1968)

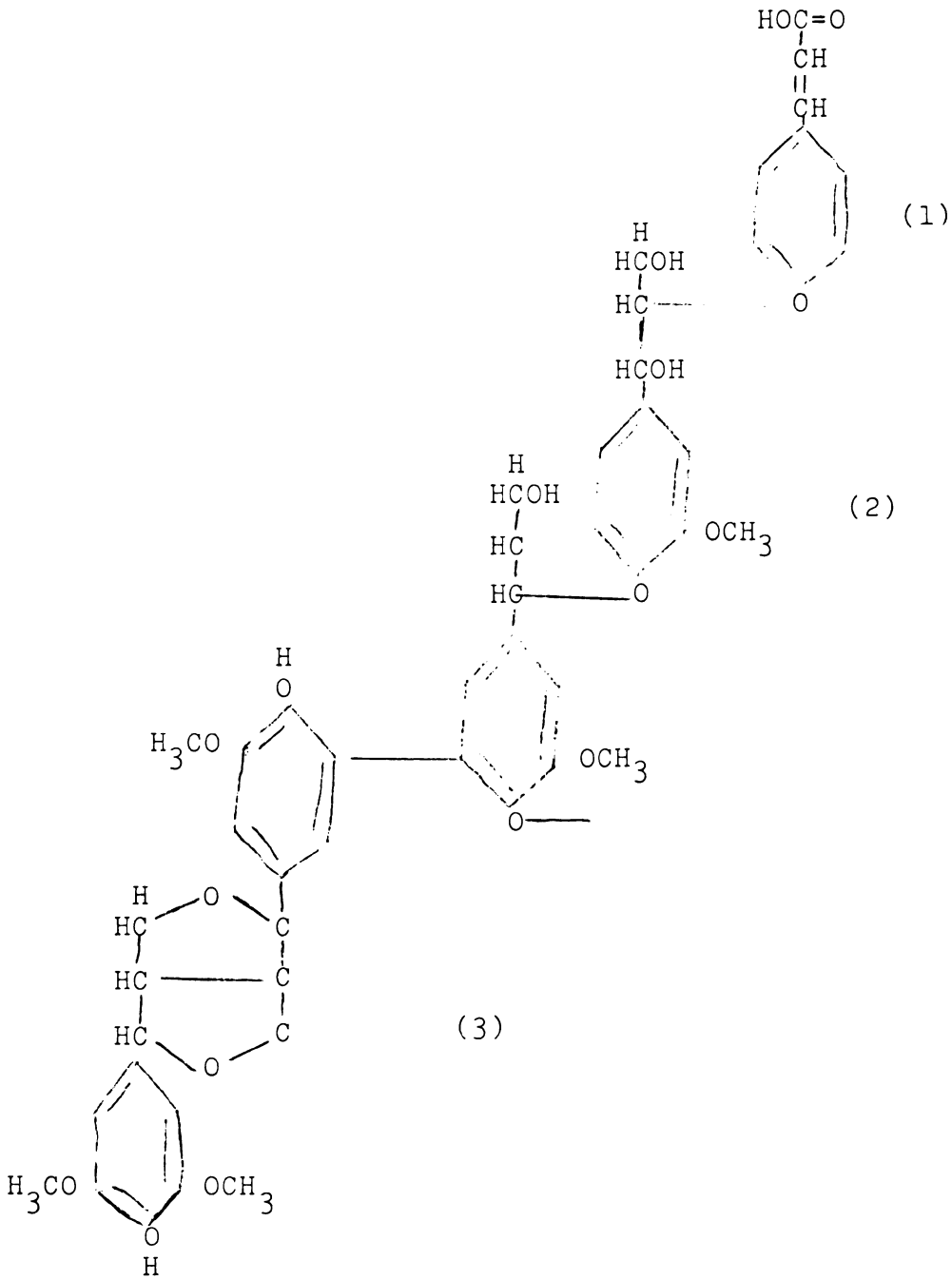


Figure 1. A model structure of lignin showing three different phenylpropane units [(1) p-hydroxycinnamic acid, (2) coniferyl alcohol, and (3) sinapyl alcohol] and the type of bonds that can be formed between these units.

found that the elemental analysis of normal dimethylformamide (DMF) lignin was C, 62.23%; H, 5.51%; N, 0.77%; O, 29.61%; and DMF lignin from brown-midrib mutants contained C, 60.72%; H, 5.43%; N, 1.24%; O, 30.10%. The phenolic acids in DMF lignins were isolated by thin layer chromatography after liberation by alkaline hydrolysis and extraction from lignin with anhydrous ether. Ferulic and p-coumaric acids were the predominant products liberated. There was no difference in the amount of erulic acid liberated from normal or mutant lignin, but the amount of p-coumaric acid liberated from the mutant lignin was approximately 50% less than that from normal lignin. Alkaline nitrobenzene oxidation of the lignins resulted in lower yields of syringaldehyde, vanillin, and p-hydroxybenzaldehyde in the brown-midrib mutant lignin when compared to normal maize lignin. The authors have suggested that when the mutant gene is present the lignin core has fewer sites at which p-hydroxycinnamic acid can be esterified thus changing the lignin formed by the mutant plants. This research indicates that mutant plant might be found that have higher digestibility due to a change in the lignin structure. Kato et al. (1967) found that the elemental analysis of tobacco milled wood lignin from the stalk was C, 59.28%; H, 6.16%; O, 34.56% and from the midrib C, 62.01%; H, 5.86%; O, 32.13%. The yields of p-hydroxybenzaldehyde, vanillin, and syringaldehyde from alkaline nitrobenzene oxidation of the stalk was 2.19%, 15.7%, and 5.88% respectively, and from the

midrib 3.74%, 10.63% and 0.0% respectively. The change in the alkaline nitrobenzene oxidation products as well as the elemental analysis indicates that the lignin composition is different in different locations of the plant.

Nitrogen Content of Lignin. Wood lignin is practically free from nitrogen. The very small quantities of nitrogen (0.2-0.3%) found in wood lignin are considered by Brauns (1952) to be accidntal impurities caused by protein materials hydrolyzable only with difficulty. Bondi and Meyer (1948) found that the nitrogen content of lignin prepared from green plants is due neither to accidental contamination with protein nor to the presence of lignin-protein complexes. They found that grass lignin contains about 1.5% nitrogen and legume lignin contains about 3.0% nitrogen.

In studies comparing lignin from the stalk and leaves of normal maize with that of brown-midrib mutants of maize Gee et al. (1968) found that the brown-midrib mutants contained almost twice as much nitrogen as did normal maize, 3.22 and 1.70, respectively. Ely et al. (1953) found that the nitrogen content of lignin isolated from orchardgrass hay decreased with maturity of the plant. The immature plant lignin contained 4.25% nitrogen and the most mature plant lignin contained 2.41%. Lignin isolated from corresponding feces had an average nitrogen content of 5.19 and 2.19 percent, respectively.

Czerkawski (1967) characterized the nitrogenous impurities in the purified grass lignin. He hydrolyzed the lignin three

times with 6 N HCl for 16 hours of 105° and the free α-amino groups were estimated by the ninhydrin method with alanine as a standard. The nitrogen content left in the residue was determined by the Kjeldahl method. More than half of the resistant nitrogen could be removed as α-amino nitrogen presumably in the form of amino acids and the rest of the nitrogen remained in the residue. This leads one to conclude that part of the nitrogen in lignin is protein and part is nitrogen attached in some way to the lignin molecule. This nitrogen could possibly be amine groups that were not cleaved from the amino acids L-tyrosine or L-phenylalanine when these monomers were used in the formation of lignin. Brown (1961) proposed a lignification pathway that included the synthesis of these two amino acids and then the formation of the monomeric units of lignin from them. Since the structure of these amino acids have the basic C_6-C_3 unit it is possible that the nitrogen in grasses and legumes is part of the structure of lignin.

Molecular Weight. Some of the physical methods that have been used for the determination of the molecular weight of lignin are diffusion, depression of freezing point by β-naphthol, osmotic pressure methods and the ultracentrifuge. The values vary between 250 and 11,000 (Brauns, 1949). The molecular weight can be determined by the introduction of cleavage of known groups, such as methoxyl, methanol, or formaldehyde by quantitative reactions that measure the

amount of groups cleaved or introduced. The molecular weight when determined this way is approximately 800.

Brown et al. (1967) used selective decay of wood by fungi to determine the molecular size of lignin in different parts of the plant. They concluded that lignin was a polymer of finite rather than infinite size and that it differed at least in molecular size according to its location within wood cell walls. The larger molecular weight lignins were located close to the center of the cell.

Methoxyl and Hydroxyl Groups in Lignin. The methoxyl content of different wood lignins vary from 14 to 20% and is higher in hard wood lignins than in soft wood lignins. According to Phillips et al. (1939) and Bondi and Meyer (1943) the methoxyl content of lignin isolated from green plants is lower than that of spruce wood lignin, and increases with the age of the plant. Bondi and Meyer (1948) found that lignin from grass contains 9-10% methoxyl groups while lignin from legumes contains approximately one half of this amount. They also found that the methoxyl content of the lignin isolated from feces differs very little from that of the corresponding plant lignin therefore there is no noticeable rupture of ether linkages during digestion in the animal. Ely et al. (1953) found similar results when working with orchardgrass lignin.

Data concerning the phenolic-hydroxyl content of lignins is small in volume. Gee et al. (1968) found that the phenolic-hydroxyl content of dimethylformamide lignin was 10.41×10^{-4}

moles per gram in normal maize and 6.23×10^{-4} moles per gram in the mutant. Brauns and Bruans (1960) reported that the total hydroxyl content of milled wood lignin prepared in air was 10-11% and of native spruce lignin was 10.2%. The phenolic hydroxyl content was 4.6% for milled wood lignin and 3.4% for native spruce lignin.

Physical Properties of Lignin

Color and Solubility. The color of various lignins extracted by 0.5 N NaOH is light to dark brown. The lignins are readily soluble in dilute NaOH, ethanol, and in acetone. Up to 70% of the lignin is soluble in 90% acetic acid. Phenol and β-naphtol dissolve up to 15% of their weight of lignin when heated about 20°C above their melting points. Lignins are not soluble in other common solvents. Brauns (1949) concluded that the color of lignin and lignin derivatives depend upon the mode of preparation. He also stated that there was evidence that protolignin was almost as white as cellulose and the color of wood and other lignified materials were caused by extraneous coloring matter rather than by lignin.

Color Reactions of Lignin. Certain color reactions become important in the study of lignin because they are specific for definite chemical structures or groups in the lignin molecule Gierer (1954) found that quinonemonochloroimide reacts with lignin in a weak alkaline solution to yield a blue "dyestuff". He investigated this reaction using 80 model compounds

chemically related to lignins and found that the reaction was specific for the free p-hydroxybenzylalcohol group in the lignin preparation. A positive Wiesner color reaction (Adler et al., 1948) indicates the presence of a coniferylaldehyde group in the lignin, possibly as an end group, by the production of a red purple color. A positive Cross-Bevan color reaction (Migita et al., 1955) indicates the presence of a syringyl group at the terminal end of the lignin molecule by the production of a red-violet color. The Maule color reaction (Spearin and Dresler, 1954) was developed to differentiate between hardwood and softwood lignins. A dark-purple color indicates the presence of hardwood-like lignin and a brown color indicates the presence of softwood-like lignin.

Ultraviolet Characteristics. The use of ultraviolet spectra for characterization of lignin preparations was developed in the past decade and it has been an invaluable aid to the lignin chemist. Patterson and Hibbert (1943) found that the ultraviolet absorption spectra of ethanol lignins indicate that lignin is aromatic in nature and that a carbonyl group or an ethylenic double bond is present in conjugation with the aromatic nucleus. Aulin-Erdtman (1949) reported that the pH value of the solvent influenced the ultraviolet absorption spectrum of the lignin preparations. The minimum shifted toward higher absorbances and longer wavelengths in alkaline solutions than in neutral solutions. Aulin-Erdtman (1949)

also reported that the shift in wavelengths from the peak on the neutral spectrum to the peak on the alkaline spectrum was roughly proportional to the content of phenolic hydroxyls present. In the case of lignin preparations studied by Aulin-Erdtman (1949) the position of the maximum was practically unchanged in all cases; therefore, they probably contained very little phenolic hydroxyls. Figure 2 shows an example of a difference and direct spectra. Wexler (1964) employed ultraviolet spectrometry for interpreting the characterization of lignosulfonic acids. He found the direct spectrograms useful in establishing the gross features of the material, whereas the difference spectrogram was of value in determining the aromatic hydroxyl content besides being a characteristic physico-chemical property of the material. The difference spectrogram (spectrum) is the difference in the absorbance between the neutral and alkaline spectrograms. Goldschmid (1954) found two maximums in the difference spectra; one at 250 mμ and the other at 300 mμ. These maximums are characteristic for the absorption of the phenolate ion of simple substituted aromatic hydroxyl compounds. The 250 mμ maximum is common to carbonyl and phenolic hydroxyl groups while the 300 mμ maximum of the difference curve is characteristic for phenolic hydroxyl groups only. He also found that the precise structure of the side chain had very small effects on the ultraviolet absorption characteristics of model compounds.

Figure 2. Difference and direct absorption spectra of a grass lignin preparation. These spectra illustrate the type of data obtained from forage lignins.

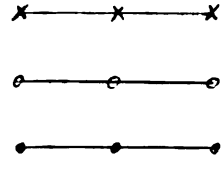
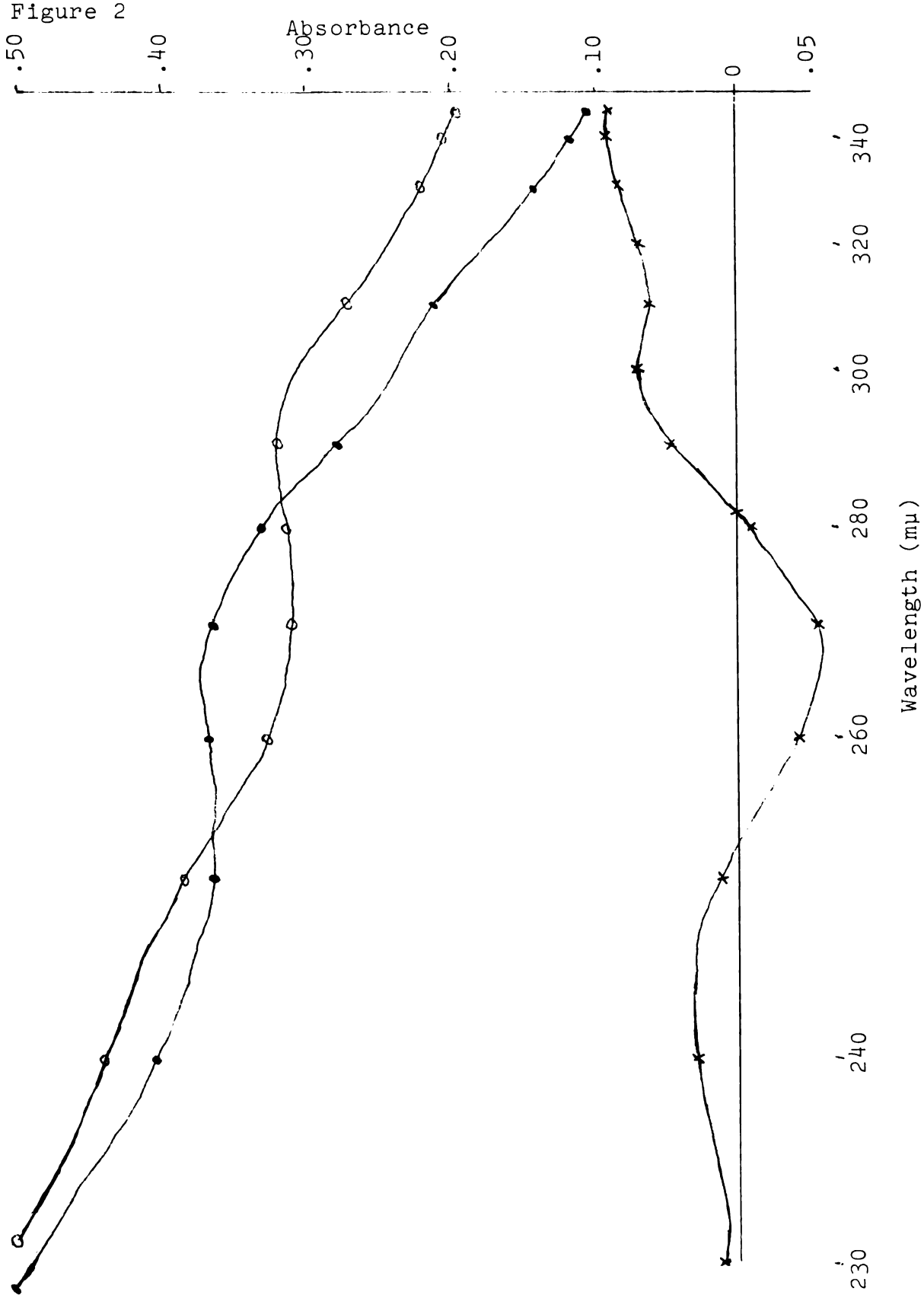


Figure 2


Absorbance
.50
.40
.30
.20
.10
0
.05
340
320
300
280
260
240
230
Wavelength (mμ)

To date most of the ultraviolet spectrometry work has been done with wood lignin, but Stafford (1960) used the ultraviolet absorption spectra to see if there was a difference between young green timothy shoots and mature timothy hay. She concluded that mature hay had greater absorbances in the difference curve than did young green shoots at the same wavelength. The peaks of the difference curve were at 250, 300, and 350 mμ with an area in the region of 270 to 280 mμ where the optical density of the alkaline spectrum which makes the difference spectrum go below zero absorbance. In further research Stafford (1962) found that lignin isolated from the sclerenchyma cell of the leaf blade differed from the lignin of the rest of the plant. The lignin from the other parts of the plant had a greater absorbance in the difference spectrum at 350 mμ than at 250 or 300 mμ but the lignin from the blade had a greater absorbance at 250 mμ than at 350 mμ. Therefore she concluded that lignin isolated from different parts of the plant differed in structure. Gee et al. (1968) used ultraviolet spectroscopy to determine the amount of phenolic hydroxyl present in lignin isolated from maize. These authors calculated the percent phenolic hydroxyl in maize lignin by taking the absorptivity at 300 mμ on the difference spectra times 0.414.

Allinson (1966) studied lignin extracted from three varieties of alfalfa. Each variety had two sample clones,

one with high in vitro digestibility and the other with low in vitro digestibility. Optical density peaks occurred on the difference spectrum at 250, 300, and 350 mμ and with an area in the region of 270 to 285 mμ where the optical density of the neutral spectrum was greater than the optical density of the alkaline spectrum which makes the difference spectrum go below zero. The observed spectra were different not only in spectral conformation but also quantitatively. These differences were present especially between clones grouped on the basis of nutritive value. This work suggests that ultraviolet spectrometry values could be used as a method of estimating the relative nutritive value of forages or as one or several parameters in a regression equation related to nutritive value.

Infrared Characteristics. During the last twenty years infrared absorption spectra have been developed to assist in characterization of lignin. Data on infrared absorption curves can be used to determine whether lignins from different species or isolation procedures differ in gross characteristics or structure, such as fewer hydroxyl or carboxyl groups. Sell et al. (1961) studied the infrared spectra of lignins isolated from alfalfa stems, timothy stems, and aspen wood by the potassium hydroxide pellet method. They concluded that the alfalfa stem lignin and timothy stem lignin showed some differences, but were more alike than when compared to aspen wood lignin. Infrared

characteristics were not investigated in this thesis project.

Lignin Digestibility and Its Value as a Predictor of Forage Quality. As a forage plant matures the composition changes. Protein and the highly digestible carbohydrate fractions decrease in amount while the lignin and cellulose fractions increase. Phillips et al. (1939) found this to be the case in the oat plant. Miller et al. (1967) analyzed baled hay that was allowed to dry to four different moisture contents ranging from 26 to 58%. There was a greater percent ash, cell wall constituents, cellulose, acid detergent fiber, and lignin in the hays baled at the higher moisture content than those baled at the lower moisture content. The increase in cell wall constituents and fiber which decreased the nutritive value was probably due to the extent of the temperature rise which was caused by the amount of moisture in the hay at the time of baling. The higher the moisture content the higher the temperature rose resulting in greater oxidation of nutrients which lowered the nutritive value in the final product. Bechtel et al. (1945) observed an increased percentage of lignin, ash, and crude fiber in brown and black hays because other organic constituents had been lost in the process leaving indestructible lignin as a greater percent of the material remaining. Pigden and Heinrich (1957) studied the lignin content of the leaves and stems of six clonal lines of wheatgrass and notes that there were significant differences in lignin content between

clones for both leaf and stem. They also found a highly significant difference in lignin content between years and in percent leaf between lines. This suggests that the amount of lignin formed in the plant is not constant from year to year and may be influenced by the environment.

Besides variation due to the amount of lignin formed by the plant, there is a possibility of forming artifact lignin during the laboratory analysis procedure of the sample. Van Soest (1964) found that when wet forage samples were heated above 50°C during drying the apparent lignin content increased by as much as 300%. He also reported that negative estimates of digestibilities can occur due to the formation of artifact lignin in processing the feed and feces. This occurs when more artifact lignin is formed in heating feces than feed. In the same study, Van Soest found that the lignin content of forages increases as the digestibility of the dry matter decreased. The correlation coefficient between lignin and digestibility was -.82 for grasses, -.74 for alfalfa, and -.40 when all were combined. In later research Van Soest (1965) found that heat-drying of forages at temperatures above 50°C showed analytically significant increases in yields of lignin and fiber. The increase in acid-detergent fiber was largely accounted for by the production of artifact lignin via the non-enzymatic browning reaction.

Stallcup *et al.* (1955) found that the lignin content

of hays influenced the digestibility of forages and decreased the passage of nutrients through the rumen. Digestibilities of hays were negatively related to their lignin content and there were more ingesta remaining in the rumen 12 hours after feeding when sheep were fed hays high in lignin as compared to hays low in lignin.

The digestibility of cell-wall carbohydrates is affected by the extent of lignification and this mode of action is by physical barrier or by some other means. Dehority et al. (1962) used four stages of maturity of timothy, three of alfalfa, and two of orchardgrass as substrates to study the rate and extent of hemicellulose fermentation decreased as the plant matured but when forage particle size was reduced by ball-milling, to remove any physical barrier by lignin, the extent of hemicellulose fermentation was increased. Additionally, the rate and extent of pectin digestion was estimated with the three stages of maturity of alfalfa. Both the rate and extent of pectin digestion decreased as the alfalfa matured and the effect of maturity was lessened considerably after reduction of particle size by ball-milling. These data indicate that both hemicellulose and pectin digestion are influenced by maturity of the forage and suggest that this is the result of lignin forming a physical barrier between the plant hemicellulose or pectin. This then results in a decrease in the ability of the forage to be degraded by rumen bacteria. Goering and Van Soest (1968) reported that

when sodium chlorite was ensiled with forages the estimated true digestibility increases as the level of sodium chlorite increased up to 3-5%. They found that true digestibility of orchardgrass increased from 86% with 0% $NaClO_2$ and that the lignin content decreased from 5.9% to 2.6%, respectively, after being ensiled for 15 days. This shows that the sodium chlorite is chemically removing the physical or chemical barrier that protects the cell-wall constituents from attack.

Results concerning the digestibility of lignin are varied but most research workers agree that lignin from grasses and legumes is undigestible. Bondi and Meyer (1948) found that the solubility, the apparent molecular weight, the methoxyl content, and the nitrogen content of the lignin remained unchanged by digestion. On the other hand the disappearance of the aldehyde fraction from the degradation products of fecal lignin showed that a change in side chains had occurred and that the nonphenolic hydroxide groups had disappeared. Crampton and Maynard (1938) found that 97.8% and 99.3% of the dietary lignin was recovered in the feces of rabbits and steers, respectively. Ellis et al. (1946) concluded that lignin from sudangrass hay was not digested by the cow, sheep, or rabbit. The percent recovery of lignin in the feces was 102%, 100%, and 97% for the cows, sheep, and rabbits, respectively.

Forbes and Garrigus (1948) found that the average recovery of lignin was 102±7 percent on bluegrass pasture that

was cut each day in a series of digestion trials with steers. With sheep, the average recovery of lignin was 105.4 percent on orchardgrass and ladino clover. From this study dry matter digestibility and total digestible nutrient content of the various forages were found to vary inversely with the lignin content of the forage. Kane et al. (1950) concluded that there was no loss of lignin during the process of digestion when they were able to recover 98.8% of the lignin in alfalfa hay fed to three cows.

Ely et al. (1953) found that the apparent digestion coefficients of lignin in dairy cow rations containing orchardgrass hay cut at different stages of maturity ranged from 3.6 to 16.0 percent. Davis et al. (1947) found that when four yearling and two year-old Hampshire ewes were fed dehydrated pea and lima bean vines, the digestion coefficients for lignin were 16.2% and 10.6%, respectively. Hale et al. (1947) reported that the digestion of lignin in the rumen was about 3.1% but the fecal digestion of lignin in the hay was about 30%, indicating that most of the digestion of lignin occurred after the forage left the rumen.

The wide variation in the recorded estimates of lignin digestibility is not surprising especially since a variety of determination or isolation procedures and techniques were used to obtain these estimates.

Tomlin et al. (1965) used orchardgrass, bromegrass, timothy, reed canary grass, alfalfa, red clover, and

birdsfoot trefoil at three stages of maturity to study the relationship of lignification to in vitro cellulose digestibility in a 12 hour incubation system. Lignin content was found to be negatively correlated with in vitro cellulose digestibility for grasses and legumes, and the regression equations for the two groups were significantly different. Lignification was linearly and negatively related to cellulose digestibility as the grasses matured, but this relationship did not exist for alfalfa. Patton (1943) studied the lignin and cellulose content of nine Montana grasses. In general, the increase in lignin and cellulose content during growth appeared to have been similar in the various species with a coefficient of correlation between lignin and cellulose greater than +0.9 in the 123 samples analyzed. Patton and Gieseker (1942) determined lignin and cellulose in five species of grasses, at five different stages of maturity, and in two localities in Montana. Lignin increased with advancing season, from about 5% in May to 18% in September, with considerable species differences. They found that the lignin content closely paralleled the cellulose content, both increasing to maturity. They believed lignin to be of definite value in predicting the feeding value of forage plants.

Van Soest (1967) concluded from his research that the amount of lignification did not affect the digestibility of the cellular contents. The digestibility of the structural carbohydrates of the cell wall declined with the maturity of

the forage and these components tended to form an increasing proportion of the dry matter of the plant with age. Digestibility of the cell wall carbohydrates were highly negatively correlated with lignification. This was also reported by Van Soest and Marcus (1964). Van Soest (1965) found that the lignin was highly correlated with cell wall constituents, +0.73; acid-detergent fiber, +0.83; and cellulose, +0.71. These correlations held true within species but not between species. Colburn et al. (1968) carried out digestion trials on 17 forages (16 orchardgrass, 1 alfalfa) with growing Jersey steers. The digestible dry matter of first cutting orchardgrass hay declined at the rate of 0.4 percent for each day's delay in harvest from May 11 to June 3. The prediction of percent digestible dry matter by chemical composition was expressed by the equation $\hat{y} = 59.2 - 2.75X_1 - 0.24X_2 + 0.66X_3 + 0.50X_4$, where X_1, X_2, X_3, X_4 represent percent lignin, hemicellulose, crude protein, and cellulose respectively. The coefficient of variation was 3% and the coefficient of correlation was 0.80.

Lignin has been used as an indigestible marker to determine the coefficient of digestibility of other feed constituents. Ellis et al. (1946) developed the lignin ratio method for determing the digestibility of other feed constituents. The formula is shown below.

$$y = 100 - 100 \frac{x}{z} \cdot \frac{n \text{ in feces}}{n \text{ in feed}}$$

y = percent digestibility of a specific nutrient, n

n = percent of a specific nutrient in either feed or in feces

x = percent lignin in feed

z = percent lignin in feces

Advantages of this method are that the digestibility of feeds may be determined without measuring total feed intake and total fecal output. This technique also permits studies of the digestibility of pasture forages under grazing conditions. The differences between the digestion coefficients when measured by this method and by the conventional method were not significantly different.

One disadvantage of the lignin ratio method is the difficulty of obtaining a representative sample of pasture forage. A second disadvantage is the daily variation in lignin content of the feces. Because of the two reasons stated above it may be hard to get repeatability using the lignin ratio method for pasture studies.

MATERIALS AND METHODS

The experiment was designed to answer three major questions. 1) Has the ultraviolet characteristics of lignin changed during digestion? 2) Are the ultraviolet characteristics and specific color reactions of lignin different in legumes and grasses, between species of grasses or species of legumes, or between different stages of maturity? 3) Are these lignin characteristics related to sheep intake or digestibility and can prediction equations be developed to predict the digestibility and the digestible dry matter intake of a population of forages?

Several sheep digestion trials have been run on forages at Michigan State University over the last six years. Representative samples of the dried forages used in these trials and their feces were collected and stored in a refrigerated room for future use. There were different species of grasses and different species of legumes in these samples which provided a good sample of the population. Among these forages were samples of the same forage but with different harvesting treatments or additives and other of the same forage but harvested at different stages of maturity.

A representative sample of a difference spectrum and direct spectra is shown in Figure 2 and will help to explain how the lignin ultraviolet characteristics were obtained. Any of the numerous values from the spectra could have been used in this study. Those selected are defined and described in the top twelve lines of Table 1. Most of the selected observations have been related to the content of specific functional groups of wood lignin preparations. Their significance will become clearer when discussed in the results and discussion section.

To provide information concerning the first question the lignin from 21 forage (13 grass and 8 legume) samples and their respective feces samples (combined from 3 or 4 sheep) were analyzed by ultraviolet spectrometry. The difference spectrum and direct spectra were obtained for each forage and fecal sample. Data on nine of the fourteen lignin ultraviolet characteristics listed on Table 1 were used in the analysis of the forage and fecal samples. The nine values or characteristics used were 243 mμ, 265 mμ, 300 mμ, and 337 mμ, % POH, Ratio O.D., Δ mμ, and mμ shift.

To provide information concerning the second and third questions twenty forage (6 grass and 14 legume) samples were analyzed by ultraviolet spectrometry and values for the fourteen lignin ultraviolet characteristics listed in Table 1 were used. These fourteen characteristics were analyzed to determine if differences in lignin structures occur between

Table 1. The lignin ultraviolet characteristics and their designations.

Designation	Definition of Characteristic
243 mμ	absorbtivity[a] at 243 mμ on the difference spectrum[b]
265 mμ	absorbtivity at 265 mμ on the difference spectrum
270 mμ	absorbtivity at 270 mμ on the difference spectrum
300 mμ	absorbtivity at 300 mμ on the difference spectrum
337 mμ	absorbtivity at 337 mμ on the difference spectrum
% POH	the % phenolic hydroxyl groups in lignin based on the percent increase in absorbtivity of the neutral peak to the alkaline peak on the direct spectra[d] X 0.21
Ratio O.D.	the ratio of optical density of the minor peak to the maximum peak on the difference spectrum
Δ mμ	the distance in mμ between the minor peak and the maximum peak on the difference spectrum
mμ shift	the distance in mμ between the neutral peak and the alkaline peak of the direct spectra
300 POH	the % phenolic hydroxyl groups in lignin, based on the absorbtivity at 300 mμ on the difference spectrum X 0.414
250 POH	the % phenolic hydroxyl groups in lignin based on the absorbtivity at 250 mμ on the difference spectrum X 0.192
250 C POH	the % phenolic hydroxyl groups in lignin based on the absorbtivity at 250 mμ corrected for base line[a] X 0.192
Guaiacol	mg of guaiacol like compounds/gm lignin
Phenol	mg of phenolic acids/gm lignin

[a] $\text{absorbtivity} = \frac{\text{absorbance}}{(\text{concentration})\ (\text{cell length})}$

(b) (c) (d) See Figure 2.

grasses and legumes, between treatments of the same forage, between stages of maturity of the same forage, or between species of grasses or legumes. Values for these same lignin characteristics were also related to animal intake and digestibility as single predictors or in multiple prediction equations to determine their value in estimating the nutritive value of forages. A list of twenty forages and their descriptions is presented in Table 2.

Chemical Analysis

Lignin Extraction

The lignin was extracted from the forage in two steps as shown by Figure 3. The forage was first extracted with a detergent-acid mixture to obtain a lignino-cellulose (acid detergent fiber) preparation free from proteins, fats, pigments, pectins, sugars, starch and hemicellulose. The acid detergent fiber procedure (Van Soest, 1963) dissolves all plant components except for lignin and cellulose thereby leaving a lignino-cellulose (acid detergent fiber) residue. The lignin was extracted from the acid detergent fiber by dilute sodium hydroxide when the mixture was heated. Lignin is soluble in hot alkaline solutions but cellulose is not. The lignin extraction procedure used was a modification of the procedure described by Stafford (1960). A slightly different extraction procedure was used for the first part of the study than used for the second part. Details for both

Table 2. Forage descriptions and their code number.

Sample No.[a]	Description
6401-01	Alfalfa hay first cutting
6401-02	Alfalfa hay second cutting
6502-01	Alfalfa hay early cut
6502-02	Alfalfa hay late cut
6701-21	Alfalfa silage control
6701-22	Alfalfa silage formic acid treated
6701-01	Alfalfa hay (control to 6701 silage study)
6201-11	Birdsfoot trefoil first cutting
6201-12	Birdsfoot trefoil second cutting
6702-OV	Alfalfa hay var. Vernal
6702-OD	Alfalfa hay var. DuPuits
6703-02	Alfalfa hay breeder line
6703-03	Alfalfa hay breeder line
6704-01	Alfalfa hay breeder line
6401-51	Bromegrass first cutting
6401-52	Bromegrass second cutting
6401-41	Reed canary grass first cutting
6401-42	Reed canary grass second cutting
6401-31	Siberian reed canary grass first cutting
6401-32	Siberian reed canary grass second cutting

[a] Year harvested is indicated by the first two digits of the number, sequential sheep trial by fourth digit, forage species by fifth digit, and cutting number or other information by last digit.

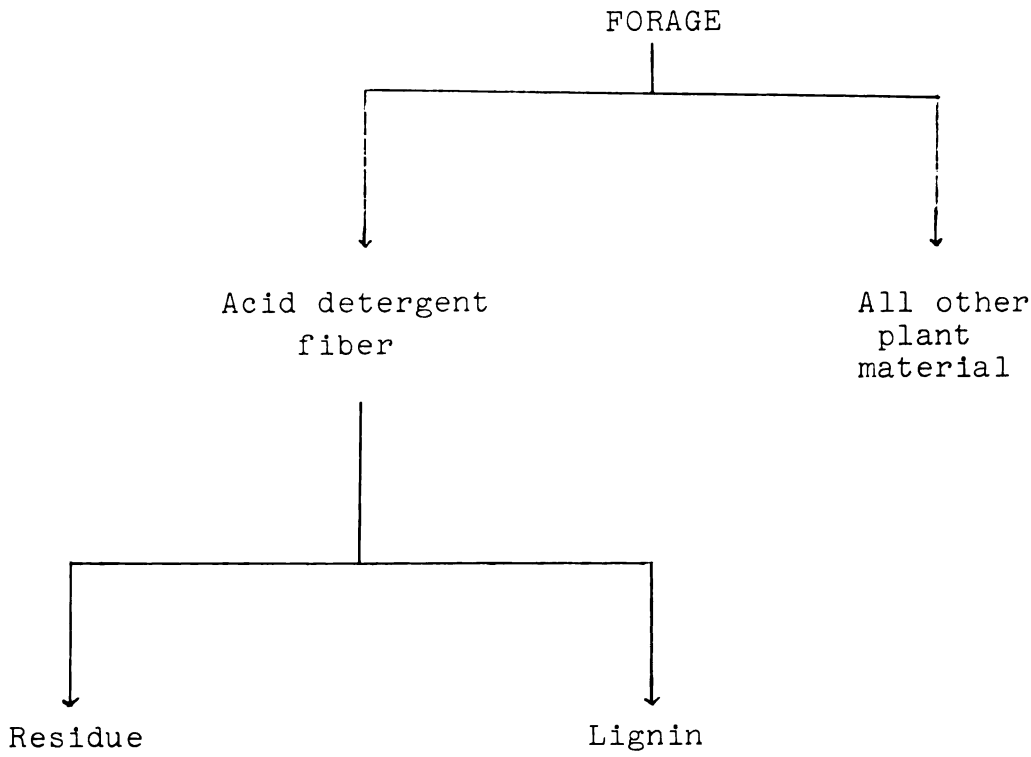


Figure 3. Schematic diagram of the lignin extraction procedure.

these procedures are given.

1. Forage vs Feces

A 0.10 gm sample of the acid detergent fiber was added to 10 ml of 0.5 N NaOH in a stoppered test tube and the mixture set in an oven for 16 hours at 70°C. At the end of this extraction time the test tube was centrifuged at 2000 rpm for 10 minutes and the supernatant was then transferred to a beaker or similar type vessel. The residue was washed twice using 5 ml of distilled water each time, centrifuged at 2000 rpm and the supernatants transferred to the same beaker. The supernatant and washes were combined for analysis.

The residue was re-extracted using 10 ml of 0.5 N NaOH for 24 hours at 70°C. At the end of this period the extraction mixture was treated as before. The first and second extracts were analyzed separately in the laboratory then the values combined in all analysis of results.

2. Forage Analysis

The lignin extraction was the same as described above except that the initial extraction time was 48 hours instead of 16. It was thought that with the extra length of the initial extraction there would be no need to run a second extraction. The second extract did contain lignin but in very small quantities.

Ultraviolet Spectrometry Analysis

The procedure used was a modification of the procedure described by Stafford (1960). Each sample of extracted lignin was neutralized to about pH 8.5 to 9.0 then the total volume was brought up to 50 ml with distilled water. The ultraviolet spectrometry analysis was made on aliquots of the sample. One 1 ml aliquot was added to a test tube containing 10 ml of 0.05 M phosphate buffer at pH 7.0. The other 1 ml aliquot was added to a test tube containing 10 ml of 0.05 N NaOH at a pH of about 12.0.

The Beckman model DK-2A ratio recording spectrophotometer was used to determine the ultraviolet absorption spectra on both the neutral and basic solutions. The range of wavelengths scanned was 220 to 340 mμ. The neutral solution was placed in the reference cell and the basic solution was placed in the sample cell to determine the difference spectrum. Distilled water was placed in the reference cell and either the neutral or basic sample was placed in the sample cell to obtain the direct spectra of the forage. The difference spectrum measures the difference between the neutral and basic direct spectra and the direct spectra measure the absorbance of the neutral or basic solution over the wavelengths scanned. Twelve of the fourteen lignin ultraviolet characteristics come from the difference and direct spectra (10 from the difference spectrum and 2 from the direct spectra). The lignin preparations had to be analyzed within about 5 hours after the pH was

standardized to 8.5-9.0 because the added acid tended to change the lignin ultraviolet properties of the lignin preparations. This time factor limits the number of samples that could be analyzed in one day.

Guaiacol Analysis

The lignin preparations were analyzed to determine the amount of free guaiacol like compounds present in the preparations. The procedure used was similar to the one described by Stafford (1960). A 4 ml aliquot of the extracted lignin solution was added to a test tube that contained 3 ml of 0.5 M tris (hydroxymethyl) amino-methane buffer at pH 9.0 and 0.5 ml of a freshly prepared alcoholic solution containing 25 µg of 2,6-dichloroquinonechlorimide. The compounds were mixed and allowed to stand at room temperature for one hour. Absorbance readings were made at the end of the hour at 610 mµ with a Beckman DU spectrophotometer, using guaiacol as a standard. The guaiacol was purchased from Matheson, Coleman, and Bell. The absorbance readings were converted to mg of guaiacol-like compounds per gm of lignin in the original forage.

Stafford (1960) developed a formula for the conversion of mg of guaiacol to mg of lignin (mg guaiacol x 32 = mg lignin) in the forage. This formula was used to estimate the amount of lignin in the forage and these results were compared to total lignin found by the method of Van Soest (1963).

Phenol Analysis

The extracted lignin solutions were analyzed to determine the amount of free phenol-like compounds present in the preparations. The procedure used was similar to the one described by Swaim and Hillis (1959). A 0.5 ml aliquot of the lignin preparation was added to a test tube containing 10.5 ml distilled water. Next 1.25 ml of Folin-Denis reagent was added and then, after about one minute, 1.25 ml of saturated $Na_2 CO_3$ was added. The compounds were mixed and allowed to stand for one half hour at room temperature. If the test tube became cloudy or a precipitate formed the solution was filtered or centrifuged before the absorbance readings were taken. After one-half hour absorbance readings were made at 760 mμ with a Beckman DU spectrophotometer, using phenol as the standard. The phenol analysis was determined at the same time as the guaiacol analysis and absorbances read 0.5 hour apart.

The Folin-Denis reagent was made by adding 25 mg sodium tungstate, 5 gm phosphomolybdic acid, and 12.5 ml of phosphoric acid to 187.5 ml distilled water. This mixture was refluxed for two hours, cooled overnight and diluted to a volume of 250 ml with distilled water. The solution was then stored in a cool dark place until used.

The saturated sodium carbonate solution was made by adding 35 mg of anhydrous sodium carbonate to 100 ml distilled water. All of the sodium carbonate went into solution when

the solution was heated to 80°C. The solution was then allowed to cool overnight. This super-saturated solution was seeded with crystals of sodium carbonate and the supernatant saturated solution was removed as needed.

Statistical Analysis

Standard procedures for analysis of variance, orthogonal contrasts, correlation coefficients, and multiple linear regressions as outlined by Steel and Torrie (1960) were employed.

RESULTS AND DISCUSSION

Change in Lignin Structure During Digestion

Differences were found in the lignin ultraviolet characteristics between forages and their respective feces which indicates that the lignin molecule had undergone changes during the digestion process. These changes will be discussed in some detail, first for grasses and legumes separately and then for the combined grasses and legumes.

Grasses. The lignin extracted from grasses differed significantly from the lignin extracted from the corresponding feces in five of the nine lignin characteristics studied (Table 3). The absorbtivities of the lignin at 265 and 270 mμ were significantly less in the forage lignin than in the fecal lignin. Wexler (1964) indicated that the difference spectra of phenolic substances may reveal negative absorption in regions where the neutral form absorbs more strongly than the ionized or phenolate form. Both the forage and fecal lignin had negative absorption in the range of 280 to 260 mμ which indicated that the difference spectra were similar in shape, but not in the amount of absorbtivity (Figure 4). The difference in absorbtivities between the forage and fecal

Table 3. The effect of digestion process on changes in lignin U.V. characteristics of the legumes and grasses combined and separate (mean ± SE).

Characteristic[d]	Combined[a] Forage	Feces	SE	Legumes[b] Forage	Feces	SE	Grasses[c] Forage	Feces	SE
1. 243	1.57	2.66	±0.46	0.85	1.21	±0.45	2.01	3.56	±0.62
2. 265	-2.97[e]	-0.82	±0.36	-1.37	-0.29	±0.53	-3.94[e]	-1.14	±0.37
3. 270	-2.83[e]	-1.14	±0.38	-1.54[f]	-0.21	±0.47	-3.78[e]	-1.71	±0.32
4. 300	4.58[e]	2.96	±0.32	2.88	2.05	±0.22	5.63[e]	3.52	±0.41
5. 337	6.58	4.59	±0.72	2.62	1.92	±0.32	8.99[e]	6.25	±0.67
6. mμ shift	23.2[e]	13.71	±1.05	26.0[e]	15.0	±2.18	21.54[e]	12.92	±0.92
7. % POH	1.68	1.37	±0.34	2.56	2.56	±0.42	1.18	0.68	±0.13
8. Ratio O.D.	0.49	0.52	±0.05	0.65	0.53	±0.10	0.39	0.52	±0.05
9. Δ mμ	66.05	73.57	±5.67	56.88	74.12	±8.03	71.69	73.23	±7.74

(a) N = 21 forages analyzed

(b) N = 8 legumes analyzed

(c) N = 13 grasses analyzed

(d) Characteristic designation same as for Table 1.

(e) Forage differs from feces at $P < .01$

(f) Forage differs from feces at $P < .05$

Figure 4. The difference spectra of a sample forage lignin preparation and a sample feces lignin preparation.

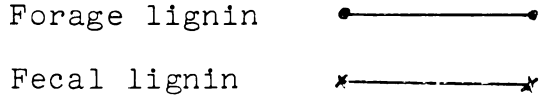
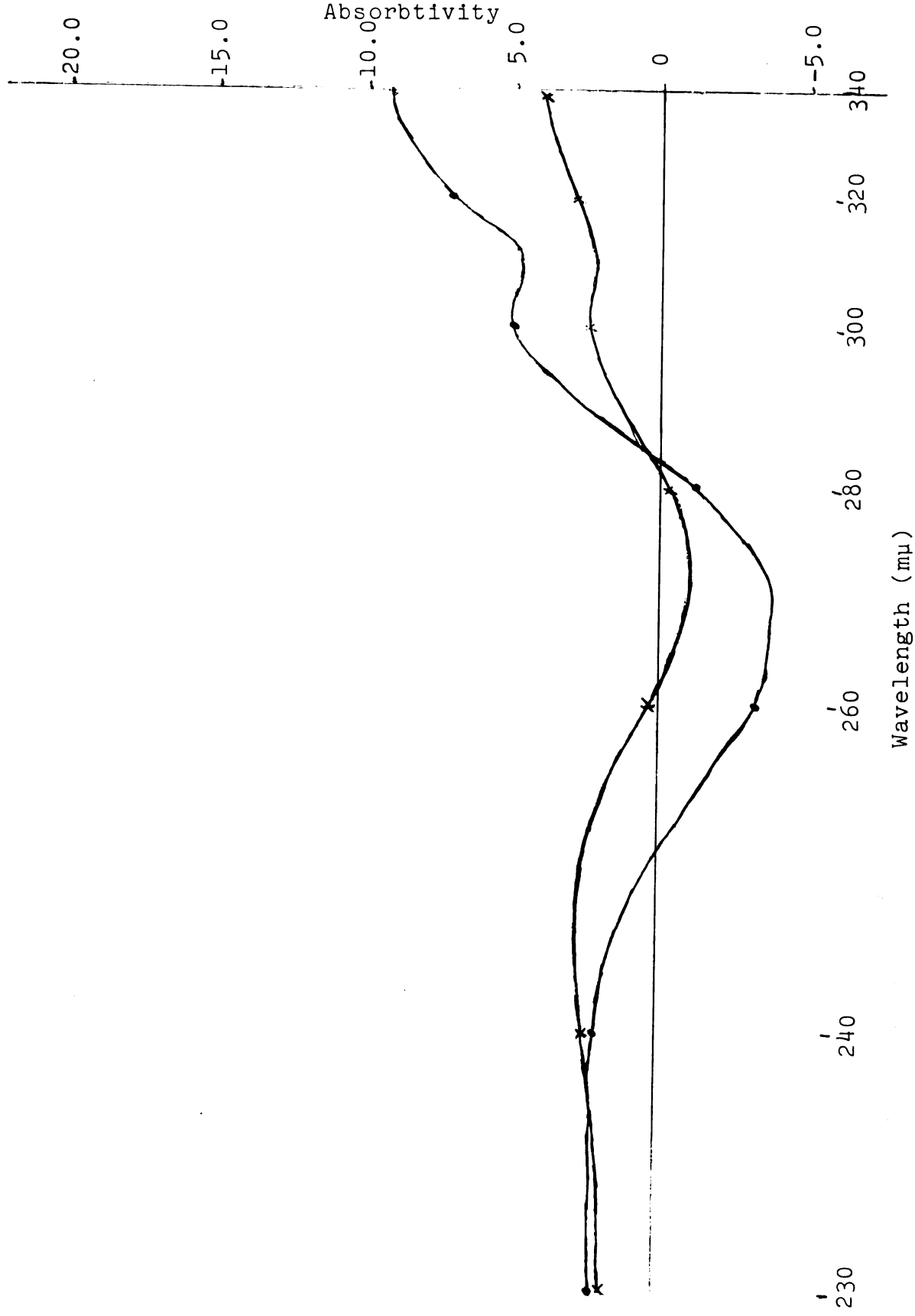


Figure 4


Absorbtivity
20.0
15.0
10.0
5.0
0
-5.0
340
320
300
280
260
240
230
Wavelength (mμ)

lignins in this region must be due to the fact that the difference between the neutral spectrum and alkaline spectrum of the forage lignin is greater than that of the fecal lignin (Figure 5). These differences indicated that the forage lignin contained more ionizable groups on the benzene ring than did the fecal lignin.

The absorbtivity of the lignin at 300 mμ was significantly greater in the forage lignin than in the fecal lignin. Goldschmid (1954) found that the 300 mμ maximum of the difference spectra is a characteristic of phenolic hydroxyl groups. This suggests that the observed differences between the forage and the fecal lignins were due to differences in amount of phenolic hydroxyl groups present in the lignins. However, the direct spectra data showed that there was no significant difference between the forage lignin and the fecal lignin in amount of phenolic hydroxyl groups.

The absorbtivity of the extracted lignin at 337 mμ was significantly greater in the forage lignin than in the fecal lignin. Wexler (1964) found that the interference of vanillin, syringealdehyde and similar conjugated phenol compounds can be detected by the appearance of a peak at about 340 mμ on the difference spectrum. The difference spectrum of the forage in Figure 4 showed a peak at about 340 mμ which suggests that in the forage lignins there were more conjugated phenol compounds present than in the fecal lignins.

Figure 5. The direct spectra of a sample forage lignin preparation and a sample feces lignin preparation.

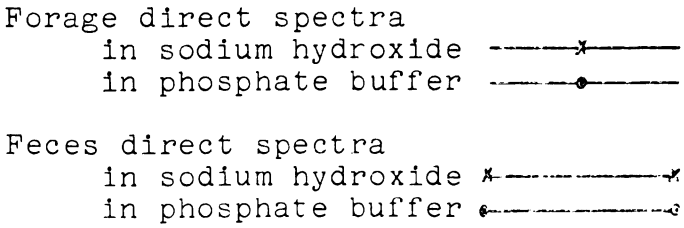
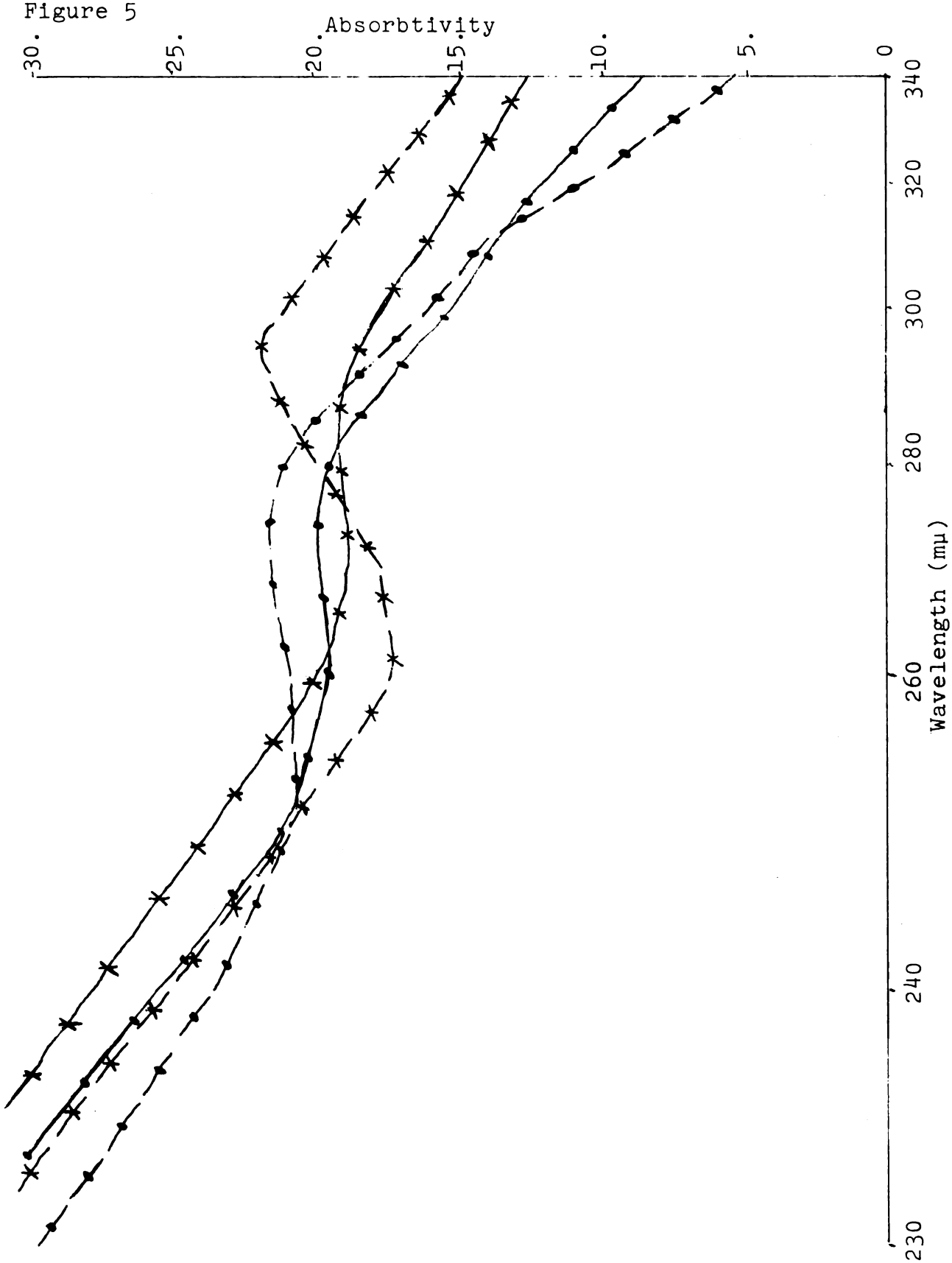


Figure 5


Absorbtivity
30.
25.
20.
15.
10.
5.
0
340
320
300
280
260
240
230
Wavelength (mμ)

There was a significant decrease in the mμ shift (defined in Table 1) from the forage lignin to the fecal lignin. Wexler (1964) found that methoxyl groups tended to stabilize the absorption at 280 mμ and the mμ shift decreased as the proportion of hydroxyl to methoxyl and other ether groups decreased. It would appear that either hydroxyl groups were lost or methoxyl groups were formed during digestion but since the % POH was not different this would indicate that methoxyl groups were formed during the digestion process.

There were no significant differences between forage lignin and fecal lignin in the absorbtivity at 243 mμ. There are no differentially ionizable groups on the lignin molecule that absorb at the 243 mμ wavelength. If a difference had been present, it would have been due to something other than ionizable groups on the benzene ring.

The ratio OD and Δ mμ characteristics were not signifcantly different between forage and fecal lignins indicating similarity in the difference spectra of lignin from these two sources.

Legumes. The forage lignins significantly differed from their respective fecal lignins in two of the nine lignin characteristics studied (Table 3). The absorbtivity on the difference spectrum at 270 mμ was less in the forage lignins than in the fecal lignins which was similar to that found for grasses, only the difference was not as great in legumes as in grasses. The standard error of the mean was higher in

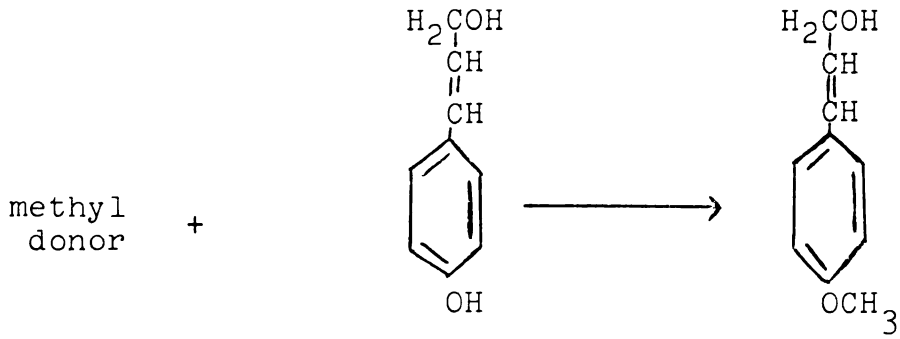
the legumes and the variation was greater between samples, causing the difference to be less than that observed in the grasses. There was a significant decrease in the mμ shift for the fecal lignin as compared to the forage lignin which indicates the loss of hydroxyl groups or the formation of methoxyl groups in the lignin molecule. Since there was no difference between the forage and feces in the absorbtivity on the difference spectrum at 300 mμ or in the phenolic hydroxyl content it can be assumed that there was a formation of methoxyl groups on the lignin molecule without the loss of a significant amount of phenolic hydroxyl groups. The absence of any other differences indicates that the general form of the spectra, both direct and difference, were similar in shape and size for both lignins. Therefore the lignin molecule does not undergo as much change during digestion in legumes as in grasses.

Combined Legumes and Grasses. When data for legumes and grasses were combined the forage lignin significantly differed from the fecal lignin in four of the nine lignin characteristics (Table 3). Differences in these four characteristics were also significant in grasses and two of these four were significant in the legumes. The combined analysis showed no difference on the difference spectrum at 337 mμ. The absorbtivity of the forage lignin was significantly less at 265 and 270 mμ than was the fecal lignin because the difference between the neutral spectrum and

alkaline spectrum of the forage lignin was greater than that of the fecal lignin.

Decreased values for the absorbtivity at 300 mμ on the difference spectrum, mμ shift, and phenolic hydroxyl content were obtained from fecal lignin when compared to forage lignin. The absorbtivity of the forage lignin at 300 mμ was significantly greater than that of fecal lignin which indicated that the amount of phenolic hydroxyl groups decreased during digestion. The decreased mμ shift indicated that the ratio of phenolic hydroxyl groups to methoxyl groups was less in the fecal lignin than in the forage lignin.

These data indicate that there was a change in the lignin molecule during the digestion process and that the change was greater in grass lignins than in legume lignins. The change might be a reduction in the amount of phenolic hydroxyl and methoxyl groups attached to the benzene ring but with a proportionately greater reduction in the amount of the phenolic hydroxyl groups than in the amount of methoxyl groups. Another possible explanation for this change is that a phenolic hydroxyl group is replaced on the ring by a methoxyl group as shown below (Brewster and McEwen, 1961). There are pH conditions in the gastro-intestinal tract that would allow this to take place if a methyl donor was present.



Differences Between Grasses and Legumes in the Lignin Ultraviolet Characteristics

There were significant differences between the grasses and legumes in twelve of the fourteen lignin ultraviolet characteristics studied (Table 4). The grass lignins had significantly greater absorbtivities at 243, 300, and 337 mμ and significantly greater negative absorbtivities at 265 and 270 mμ. The peak at about 340 mμ in the difference spectrum of grass lignins indicated the presences of vanillin and syringealdehyde or similar conjugated phenols in the lignin preparations but no such prominent peak was noted in the difference spectrum of legumes lignins. The conjugated phenols in the lignin preparations tend to increase the absorbtivities at 243 and 300 mμ but probably not enough to account for all of the differences noted between the legume and grass lignins. The grass lignin preparations have three absorbtivity peaks on the difference spectrum, a 240, 300, and 340 mμ; whereas the legume lignin preparations have only two

Table 4. Differences in lignin ultraviolet characteristics between legumes and grasses (mean ± SE).

Characteristics[a]	Legume	Grass	SE
243 mμ	-1.55[b]	8.95	± 0.94
265 mμ	-8.05[b]	-15.57	± 0.48
270 mμ	-6.55[b]	-15.62	± 0.51
300 mμ	8.04[b]	15.29	± 0.90
337 mμ	5.63[b]	25.41	± 0.44
Guaiacol	6.50[b]	14.33	± 0.59
Phenol	213.8[b]	487.3	±35.2
Ratio O.D.	0.62	0.35	± 0.10
mμ shift	27.3[b]	23.2	± 1.4
Δ mμ	46.3[b]	99.2	± 1.4
300 POH	0.59	0.62	± 0.10
250 POH	-0.08[b]	0.08	± 0.06
250 C POH	0.12[b]	0.25	± 0.02
% POH	7.27[b]	3.49	± 1.24

(a) Characteristic designations same as for Table 1.

(b) Legumes differ from grasses at $P < .01$.

absorbtivity peaks on the difference spectrum, at 300 and possibly at 340 mμ. Lignins from legumes had a negative absorbtivity over a wider range of wavelengths (280-235 mμ) than did the grasses (280-250 mμ). The grass lignins had a greater negative absortivity in the region of 280 to 260 mμ than did the legume lignins (-6.25 vs -3.25). The difference spectra of grass lignin differed from that of the legume lignin in both the absolute values for absorbtivity at a given wavelength and the shape of the respective spectrograms (Figure 6).

The grass lignin preparations contained more free guaiacol-like compounds than did the legume lignin preparations (14.33 vs 6.5 mg/gm lignin). The grass lignin preparations also had more free phenol-like compounds present than did the legume lignin preparations (487 vs 214 mg/gm lignin). These differences are similar to those observed between the difference spectra of the forage lignins. This indicates that the legume lignin is either more tightly bound and resistant to chemical breakdown or has different monomeric units than does the grass lignin.

The distance in mμ between the maximum peak and minor peak on the difference spectrum was greater for the grass lignins than for the legume lignins. The maximum peak in the grass lignin was found at approximately 340 mμ whereas the maximum peak in the legume lignin was at approximately 300 mμ. The minor peak of the grass lignin was distinct and

Figure 6. The difference spectra of lignin from a grass and a legume.

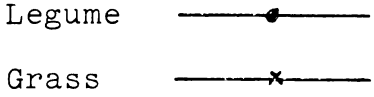
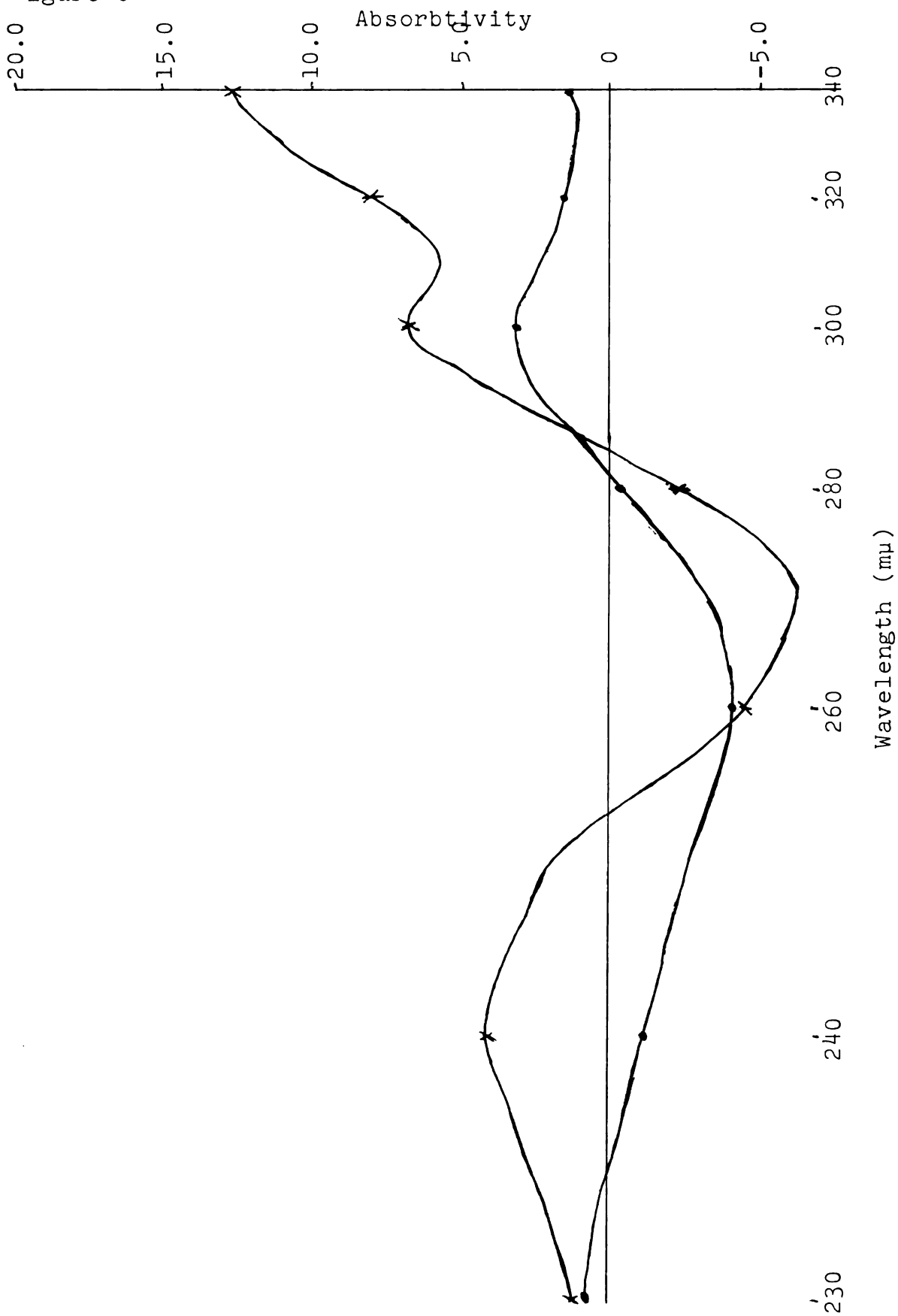


Figure 6


Absorbtivity
20.0
15.0
10.0
5.0
0
-5.0
340
320
300
280
260
240
230
Wavelength (mμ)

occurred at approximately 240 mμ while the minor peak in the legume lignin was indistinct and found at approximately 340 mμ. The difference in the number of peaks and the position of the maximum peak shows that there were different compounds ionizing in the grass than in the legume samples. This indicates a difference in basic structure.

The direct spectra of the grass and legume lignins had similar differences to those found on the difference spectra. Also, the absolute absorbtivity of the grass lignin was greater than the absolute absorbtivity of the legume lignin throughout the wavelengths scanned (Figure 7). This shows that the lignin molecule of grasses contained a greater proportion of aromatic compounds which had a greater absolute absorbtivity than the aromatic compounds that made up the legume lignin molecule. The shift in mμ also indicated that the basic structures are different in that the ratio of phenolic hydroxyl groups to methoxyl groups is greater in the legume lignins than in the grass lignins. There are four methods for estimating the phenolic hydroxyl content of the lignin. These were developed using ligno-sulfonate preparations to determine the conversion factors to multiply times a specific ultraviolet measurement. These methods showed varying results in the data on forages. The 300 POH method showed no difference in the percent phenolic hydroxyl groups between the grass and legume lignins. The 250 POH and 250 C POH methods showed that the grass lignins contain

Figure 7. The direct spectra of lignin from a grass and a legume.

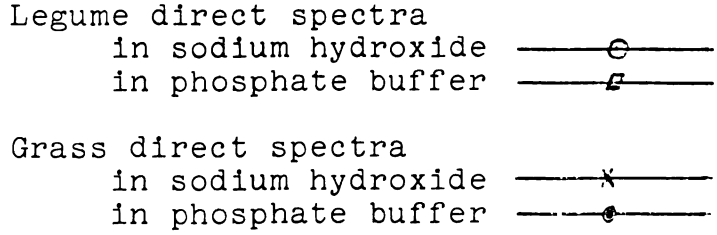
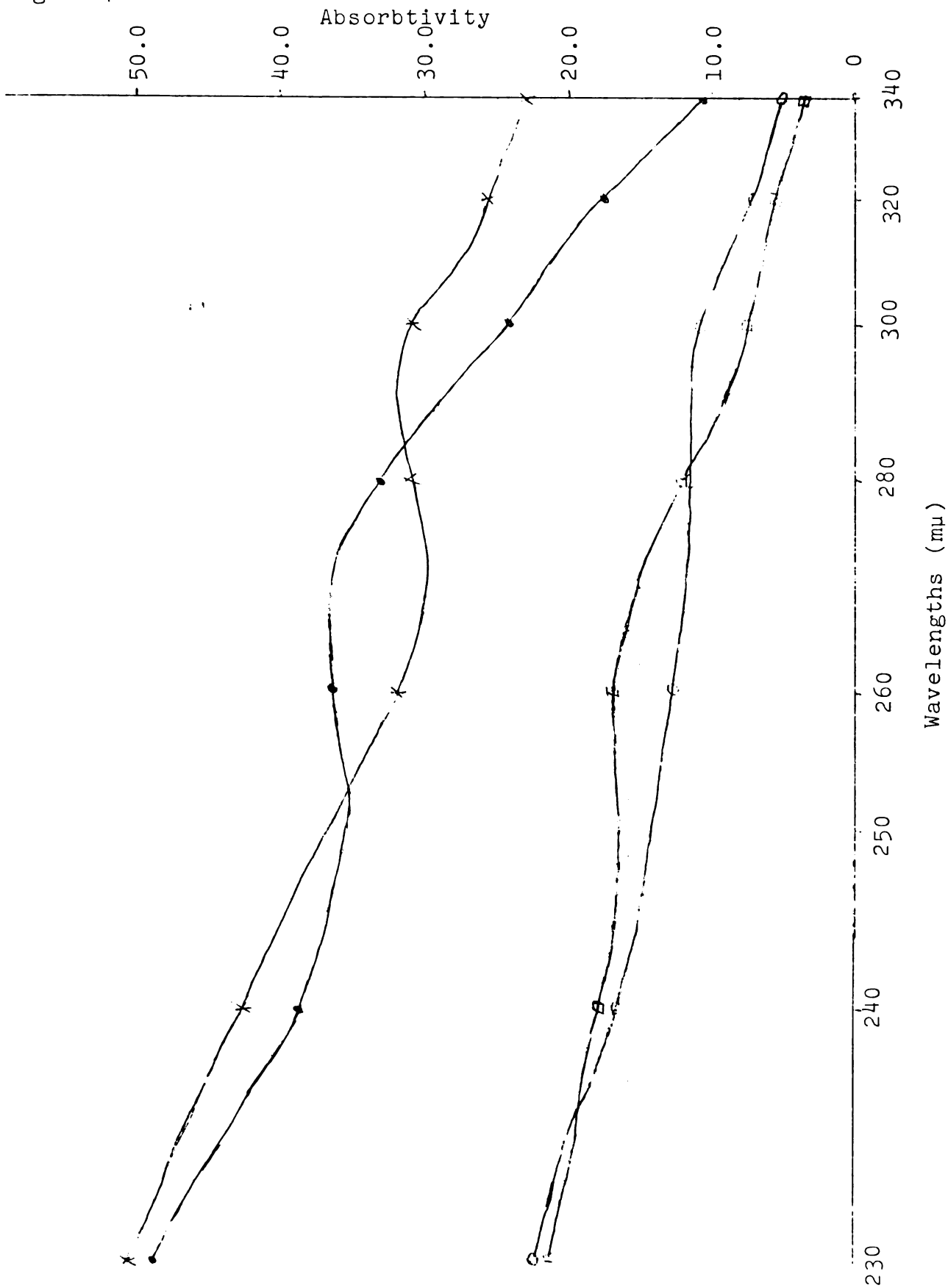


Figure 7


Absorbtivity
50.0
40.0
30.0
20.0
10.0
0
340
320
300
280
260
250
240
230
Wavelengths (mμ)

a significantly greater percent phenolic hydroxyl groups than did legume lignins. The % POH method showed that the legume lignins contain a significantly greater percent phenolic hydroxyl groups than did grass lignins. Wexler (1964) concluded that the phenolic hydroxyl values obtained from the difference spectrum were more accurate than the value obtained from the direct spectra when using none pure compounds. Therefore there are more phenolic hydroxyl groups in lignin from grasses than in lignin from legumes and also more methoxyl groups because the ratio of phenolic hydroxyl groups to methoxyl groups is less in the lignin from grasses.

There was a difference between legume lignin preparations and grass lignin preparations in the chemical make up of the lignin molecule. The grass lignin molecule had a greater proportion of aromatic compounds with a greater specific absorbtivity than the aromatic compounds which make up the legume lignin molecule. These aromatic compounds seem to have more methoxyl groups and/or less phenolic hydroxyl groups attached to the benzene ring. The percentage of this aromatic compound present in the lignin sample could be a factor related to the digestibility of the forage.

Alfalfa Hay Early Cut (6502-01) vs Late Cut (6502-02)

Three of the fourteen lignin characteristics were significantly different between the early cut alfalfa hay and the late cut hay (Table 5). The late cut alfalfa hay had a significantly greater negative absorbtivity than did the early cut hay at 265 mμ while the 250 POH and 250 C POH lignin values showed that the early cut alfalfa hay had more phenolic hydroxyl groups than did the late cut hay. The negative absorbtivity on the difference spectra at 265 mμ is due in part to the absorption by the non-ionized phenolic hydroxyl group at this wavelength compared to no absorption by the ionized phenolic hydroxyl group. Greater values for this difference (or the greater the negative absorbtivity) indicate increased amounts of phenolic hydroxyl groups present. This is different than the observed difference at the 250 POH and 250 C POH lignin characteristics but similar to the trends observed in the 300 POH and % POH lignin characteristics. If there had been a difference in the amount of phenolic hydroxyl groups present then there would have to have been a difference in the methoxyl content since there were no observed differences between the ratios of phenolic hydroxyl group to methoxyl groups. Therefore as the alfalfa plant matures there is an increase in the phenolic hydroxyl content and methoxyl content. Either or both might affect the digestibility of the hay.

Table 5. Differences in lignin ultraviolet characteristics for 20 samples of forages (mean ± standard error).

Forage Sample[b]	Lignin Ultraviolet Characteristics[a] 243 mμ	265 mμ	270 mμ	300 mμ	337 mμ
6401-01	3.02	- 3.44^{f}	- 3.81^{f}	9.27	7.10
6401-02	7.63	- 0.20^{e}	- 0.76^{e}	11.08	8.09
6502-01	3.37	- 3.51^{G}	- 3.84	6.81	5.24
6502-02	- 2.04	- 7.59^{H}	- 6.32	7.73	4.64
6701-21	- 8.06	-13.47^{Kl}	-10.90^{k}	12.25	6.96^{k}
6701-22	- 5.75	-10.16^{Kj}	- 9.01^{k}	9.70	8.13^{k}
6701-01	- 5.66	- 8.40^{I}	- 6.50^{i}	7.69	5.04^{i}
6702-OV	- 6.55	-12.34^{P}	-10.15	9.90	5.78
6702-OD	- 2.92	- 8.99^{o}	- 7.58	8.26	6.35
6703-02	- 4.14	- 8.76	- 7.18	6.98	3.35
6703-03	- 4.11	- 9.27	- 7.92	6.22	3.81
6704-01	- 4.83	- 9.24	- 8.04	6.19	3.59
6201-11	2.31	- 5.96	- 5.47	5.58	4.91^{N}
6201-12	5.99	- 3.27	- 4.17	4.83	5.88^{M}
6401-51	9.88	-19.13^{R}	-18.52^{R}	19.38^{Q}	28.39^{Q}
6401-52	4.37	-10.88^{Q}	-10.09^{Q}	9.53^{R}	12.71^{R}
6401-41	5.72	-17.79	-17.17	15.82	24.97
6401-42	8.05	-14.96	-14.96	14.04	24.16
6401-31	15.45	-17.30^{T}	-18.17^{t}	19.78^{S}	36.46^{S}
6401-32	10.24	-13.34^{S}	-14.74^{s}	13.19^{T}	25.76^{T}
Std. error	±0.94	±0.48	±0.51	±0.90	±0.44

(a) Characteristic designations are same as for Table 1.

(b) Forage sample descriptions are in Table 2.

Table 5 (continued).

Forage Sample[b]	Lignin Ultraviolet Characteristics[a] Guaiacol	Phenol	Ratio OD	mμ shift	Δ mμ
6401-01	9.11	283.2	0.43	29.5	47.0
6401-02	8.03	294.7	0.78	29.0	47.0
6502-01	7.51	204.4	0.52	29.0	47.0
6502-02	6.07	171.1	0.62	30.5	45.5
6701-21	6.00	270.3	0.62	26.0	46.0^{J}
6701-22	6.93	273.1	0.86	27.5	40.5^{L}
6701-01	4.61	184.1	0.74	26.5	46.5
6702-OV	8.91	226.9	0.61	28.5	46.0
6702-OD	8.49	216.0	0.83	28.0	45.5
6703-02	5.08	170.7	0.48	27.5	47.5
6703-03	6.35	167.4	0.50	24.5^{P}	44.0
6704-01	5.64	160.4	0.46	30.5^{O}	45.0
6201-11	3.43	175.0	0.42^{n}	23.0	58.5^{M}
6201-12	4.86	196.1	0.76^{m}	22.5	42.0^{N}
6401-51	19.78^{Q}	515.6^{q}	0.34	23.5	99.5^{R}
6401-52	9.50^{R}	302.1^{r}	0.35	23.5	103.5^{Q}
6401-41	11.34	493.4	0.25	26.0	99.5
6401-52	14.75	509.9	0.33	24.0	97.0
6401-31	19.18^{S}	693.4^{s}	0.42	23.0	98.5
6401-32	11.42^{T}	412.8^{t}	0.41	19.0	97.0
Std. error	±0.59	±35.2	±0.10	±1.4	±1.4

(a) Characteristic designations are same as for Table 1.

(b) Forage sample descriptions are in Table 2.

Table 5 (continued).

Forage Sample[b]	Lignin Ultraviolet Characteristics[a] 300 POH	250 POH	250 C POH	% POH
6401-01	0.63[F]	0.10[F]	0.20[F]	5.27[e]
6401-02	1.07[E]	0.43[E]	0.43[E]	4.49[f]
6502-01	0.58	0.12[G]	0.23[G]	6.66
6502-02	0.71	-0.19[H]	0.08[H]	7.79
6701-21	0.66	-0.27	0.04	5.30
6701-22	0.57	-0.19	0.09	4.92
6701-01	0.52	-0.22	0.05	5.63
6702-OV	0.52	-0.22	0.03	10.66
6702-OD	0.48	-0.13	0.04	9.31
6703-02	0.59	-0.24	0.03	10.34
6703-03	0.48	-0.23	0.03	10.63
6704-10	0.53	-0.29	0.03	11.26
6201-11	0.52	-0.02[n]	0.14[N]	4.97
6201-12	0.44	0.18[m]	0.24[M]	4.50
6401-51	0.65	0.07	0.25[Q]	3.28
6401-52	0.52	0.04	0.17[R]	3.81
6401-41	0.60	-0.03	0.20[P]	3.11
6401-42	0.68	0.05	0.28[O]	4.08
6401-31	0.65	0.18	0.32	3.32
6401-32	0.60	0.13	0.29	3.35
Std. error	±0.10	±0.06	±0.02	±1.24

(a) Characteristic designations are same as for Table 1.

(b) Forage sample descriptions are in Table 2.

Table 5 (continued).

E >F at P <.01

e >f at P <.05

G >H at P <.01

I >K at P <.01

i >k at P <.05

J >L at P <.01

j >l at P <.05

M >N at P <.01

m >n at P <.05

o >p at P <.05

O >P at P <.01

Q >R at P <.01

q >r at P <.05

S >T at P <.01

s >t at P <.05

Alfalfa Hay First Cutting (6401-01) vs Second Cutting (6401-02)

Six of the fourteen lignin characteristics were significantly different between the first cutting of alfalfa hay and the second cutting hay (Table 5). All six of these values, 265 and 270 mμ, 300 POH, 250 POH, 250 C POH, and % POH, are affected by the amount of phenolic hydroxyl groups present in the lignin. The absorbtivities at 265 and 270 mμ and the % POH indicate that the first cutting hays have more phenolic hydroxyl groups present than the second cutting hays, but the 300 POH, 250 POH and 250 C POH shows that the second cutting hays contain a greater percentage of phenolic hydroxyl groups than the first cutting hays. Since the ratio of phenolic hydroxyl groups to methoxyl groups is not significantly different then there is probably little or no difference in the structure of the lignin molecule between first and second cutting alfalfa hay.

Birdsfoot Trefoil Hay First Cutting (6201-11) vs Second Cutting (6201-12)

Five of the lignin characteristics were significantly different between the first cutting of birdsfoot trefoil hay and the second cutting hay (Table 5). The absorbtivity at 337 mμ was significantly greater in the second cutting than in the first cutting trefoil hay which indicates that the second cutting lignin preparations contained more conjugated

phenol compounds, such as vanillin, than did the first cutting lignin preparations. The greater amount of conjugated phenol compounds in the second cutting hays caused the ratio O.D. to be significantly different and it also significantly reduced the mμ difference between the maximum peak and minor peak on the difference spectra. The 250 POH and 250 C POH values showed the second cutting hay contained significantly more phenolic hydroxyl group than did the first cutting but the 300 POH and % POH values showed no difference between the cuttings. Therefore there was a greater amount of phenolic hydroxyl groups in the second cutting hay, which might have been due to the presence of more conjugated phenol compounds present in the second cutting hay.

Alfalfa Hay vs Birdsfoot Trefoil Hay

Ten of the fourteen lignin characteristics were significantly different between alfalfa hay and trefoil hay (Table 6). The absorbtivities at 265, 270, 300 and 337 mμ were significantly greater in the alfalfa lignin samples than in the trefoil lignin samples. The alfalfa hay had a greater ratio of phenolic hydroxyl groups to methoxyl groups than did the trefoil hay and the trefoil hay had a greater mμ difference between the maximum and minor peaks than did the alfalfa hay. Three of the methods for determining amount of phenolic hydroxyl groups present showed that the alfalfa hay contained more phenolic hydroxyl groups than did the trefoil

Table 6. Differences in lignin ultraviolet characteristics between alfalfa and birdsfoot trefoil (mean ± SE).

Characteristic[a]	Alfalfa	Birdsfoot Trefoil	SE
265 mμ	-1.82[b]	-4.61	±0.48
270 mμ	-2.28[b]	-4.82	±0.51
300 mμ	10.17[b]	5.20	±0.90
337 mμ	7.59[c]	5.39	±0.44
Guaiacol	8.57[c]	4.14	±0.59
mμ shift	29.2 [c]	22.7	±1.4
Δ mμ	47.0 [b]	50.2	±1.4
300 POH	0.85[c]	0.48	±0.10
250 POH	0.26[c]	0.10	±0.06
250 C POH	0.31[c]	0.19	±0.02

(a) Characteristic designations same as for Table 1.

(b) Alfalfa differs from birdsfoot trefoil at $P < .05$

(c) Alfalfa differs from birdsfoot trefoil at $P < .01$

hay and the other method showed no difference between the species.

Apparently alfalfa lignins have a greater proportion of aromatic compounds that contain more phenolic hydroxyl groups than do the aromatic compounds that make up trefoil lignins. This would explain the difference in the absorbtivities at specific wavelengths on the difference curve, the difference in the amount of guaiacol-like compounds in the lignin preparation, the difference in the mμ shift (or ratio of phenolic hydroxyl groups to methoxyl groups), the difference in the mμ distance between the maximum and minor peaks on the difference spectrum and the greater phenolic hydroxyl content.

Alfalfa Silage (6701-21 & 22) vs Alfalfa Hay (6701-01)

The only difference between the silages and hay in the lignin characteristic studied were the absorbtivities at 265, 270 and 337 mμ (Table 5). The silages contained significantly more conjugated phenol compounds than did the hay. The silages had significantly greater negative absorbtivities at 265 and 270 mμ than did the hay. Therefore there is very little difference in the lignin molecules of alfalfa silage and alfalfa hay.

Alfalfa Silage (6701-21) vs Alfalfa Silage Treated with Formic Acid (6701-22)

The alfalfa silage had a greater negative absorbtivity at 265 mμ than did the formic acid treated silage and a greater mμ difference between the maximum peak and minor peak on the difference spectrum than did the treated silage (Table 5). This shows that the differences in the lignins in these two silages were very small.

Alfalfa Variety Vernal (6702-OV) vs Variety DuPuits (6702-OD)

The only difference in the lignin characteristics between the two varieties of alfalfa was that Vernal had a greater negative absorbtivity at 265 mμ than did DuPuits (Table 5). Therefore, there is no difference in the lignin structures between the two varieties of alfalfa. The forages were harvested on the same day but the variety DuPuits was more mature than the variety Vernal.

Alfalfa Breeder Line (6704-01) vs Breeder Line (6703-03)

The ratio of phenolic hydroxyl groups to methoxyl groups was the only characteristic that showed a significant difference between the breeder lines (Table 5). Breeder line 6704-01 had a greater ratio than did breeder line 6703-03

while the phenolic hydroxyl content was similar. Therefore, the difference must be due to the fact that the breeder line 6704-01 had fewer methoxyl groups than the other line. This greater methoxyl content might be contributing to the low digestible dry matter content of the 6703-03 line.

Bromegrass Hay First Cutting (6401-51) vs Second Cutting (6401-52)

First cutting bromegrass hay had significantly greater negative absorbtivities at 265 and 270 mμ and had significantly greater absorbtivities at 300 and 337 mμ than did the second cutting hay (Table 5). The first cutting hay also had more free guaiacol-like and phenol-like compounds in the lignin preparation than did the second cutting hay. The second cutting hay had a greater difference in mμ between the maximum and minor peaks than did the first cutting hay. These observations indicate that there is a small difference in structure of the compounds analyzed, such as position of the groups on the benzene ring, even though there was no difference in the ratios of phenolic hydroxyl groups to methoxyl groups.

Reed Canary Grass Hay First Cutting (6401-41) vs Second Cutting (6401-42)

There were significantly more phenolic hydroxyl groups present in the second cutting hay than in the first cutting hay by the 250 C POH method. There is a difference, but just what this difference is cannot be identified at present since the methods for determining phenolic hydroxyl content were based on wood lignin and give variable values for forage lignin.

Siberian Reed Canary Grass Hay First Cutting (6401-31) vs Second Cutting (6401-32)

The first cutting hay had significantly greater negative absorbtivities at 265 and 270 mμ and had significantly greater absorbtivities at 300 and 337 mμ than did the second cutting hay (Table 5). The first cutting hay also had more free guaiacol-like and phenol-like compounds in the lignin preparations than did the second cutting hay. These observations indicate that there were differences in the structure of the lignins between the two different cuttings.

Reed Canary Grass Hay vs Siberian Reed Canary Grass Hay

Siberian reed canary grass hay had significantly greater absorbtivities at 243 and 337 mμ than did reed canary grass hay

which indicates that there are more conjugated phenol compounds, such as vanillin, present in Siberian reed canary grass lignin preparation (Table 7). Reed canary grass hay had a greater ratio of phenolic hydroxyl group to methoxyl groups than did Siberian reed canary grass hay. Siberian reed canary grass hay contains more methoxyl groups than does reed canary grass hay which might be a factor since reed canary grass is more digestible than Siberian reed canary grass hay.

Bromegrass Hay vs Reed Canary Grass and Siberian Reed Canary Grass Hays

Reed canary grass hays had a significantly greater negative absorbtivity at 270 mμ and had a significantly greater absorbtivity at 337 mμ than did bromegrass hay which indicates that there were more conjugated phenol compounds present in the reed canary grass lignin preparations (Table 8). There was a significantly greater phenolic hydroxyl content in the reed canary grass hays than in the bromegrass hay with the 250 C POH method, and the reed canary grass hays had the most conjugated phenol compounds present.

Lignin Determination

The correlation between Stafford's method (1960) for lignin content and the 72% sulfuric acid method was not significantly different from zero (Table 9). Stafford's

Table 7. Differences in lignin ultraviolet characteristics between reed canary grass and Siberian reed canary grass (mean ± SE).

Characteristics[a]	Reed Canary Grass	Siberian Reed Canary Grass	SE
243	6.88[b]	12.84	±0.94
337	24.56[b]	31.11	±0.44
mμ shift	25.0 [b]	21.0	±1.4
250 POH	0.01[c]	0.15	±0.06
250 C POH	0.24[d]	0.30	±0.02

(a) Characteristic designations same as for Table 1.
(b) Reed canary grass differs from Siberian reed canary grass at P <.01.
(c) Reed canary grass differs from Siberian reed canary grass at P <.05.

Table 8. Differences in lignin ultraviolet characteristics between the reed canary grasses (common and Siberian) and bromegrass (mane ± SE).

Characteristics[a]	Reed Canary Grasses	Bromegrass	SE
270	16.26[b]	14.30	0.51
337	27.84[c]	20.55	0.44
250 C POH	0.28[c]	0.21	0.02

(a) Characteristic designations same as for Table 1.
(b) Reed canary grasses differ from bromegrass at P <.05.
(c) Reed canary grasses differ from bromegrass at P <.01.

Table 9. Comparison of two methods for the determination of lignin content in forages.

Forage	Lignin by Stafford's Method	Lignin by 72% Sulfuric Acid Method
	%	%
6401-01	9.20	6.31
6401-02	13.91	9.54
6502-01	10.15	8.03
6502-02	8.87	10.81
6701-21	6.16	6.33
6701-22	7.54	6.78
6701-01	7.42	8.96
6201-11	4.85	8.73
6201-12	6.96	8.50
6702-OV	7.13	5.00
6702-OD	8.40	6.11
6703-02	6.57	8.08
6703-03	8.02	7.89
6704-01	6.26	6.93
6401-51	9.02	2.85
6401-52	6.26	3.86
6401-41	5.62	3.12
6401-42	6.48	2.88
6401-31	8.94	2.74
6401-32	7.99	4.14

$r = 0.25$; $P < .15$

suggested method is based on the mg of guaiacol-like compounds present in lignin. This indicates that the amount of free guaiacol-like compounds in the lignin preparation is not related to the amount of lignin in that preparation and should not be used to estimate the amount of lignin present in the forage.

The Relationship of the Extracted Material to the Lignin Content in the Forage

The correlation of the amount of extracted material and the mg of the lignin in the original ADF residue was determined on legumes and grasses separately (Table 10). Neither correlation significantly differed from zero which means that a varying amount of cellulose was extracted. Some cellulose may have been so tightly bound to the lignin that both were extracted simultaneously. The extracted cellulose does not affect the spectrograms obtained because carbohydrates in neutral or alkaline solutions do not absorb in the ultraviolet region scanned (Aulin-Erdtman, 1949).

Relationship Between the Lignin Characteristics Animal Data and Lignin Content

Combined Legumes and Grasses. Correlation coefficients were determined between the values per unit of acid detergent fiber for the fourteen lignin characteristics and three

Table 10. Comparison of the amount of extracted material to the mg of lignin in the forage.

Forage	mg extracted material 0.1 gm ADF by weight loss	mg lignin 0.1 gm ADF by analysis
	Legume	
6401-01	31.8	18.9
6401-02	29.4	24.9
6502-01	32.6	24.2
6502-02	27.4	22.0
6701-21	32.7	15.5
6701-22	33.6	17.2
6701-01	38.4	22.6
6201-11	46.1	26.9
6201-12	34.0	27.5
6702-OV	33.7	15.6
6702-OD	34.7	17.8
6703-02	34.3	25.3
6703-03	34.7	23.6
6704-01	32.0	24.2
	Grass	
6401-51	27.5	8.1
6401-52	26.2	12.6
6401-41	28.8	9.6
6401-42	20.4	8.7
6401-31	28.2	8.1
6401-32	32.4	12.9

Legumes $r = 0.25$; $P < .20$

Grasses $r = 0.42$; $P < .22$

measures of animal response as well as lignin content (Table 11). Most of the correlations were not significantly different from zero and those that were significantly different from zero were of low magnitude and not useful as single predictors of animal performance or lignin content.

Legumes. Correlation coefficients were determined for the legume forages between the lignin characteristics (expressed as per unit of acid detergent fiber and per gm lignin) and animal data and lignin content (Table 12). Most of the correlations were not significantly different from zero and those that did differ from zero were not of such magnitude to be important biologically as single predictors of animal preformance or lignin content.

Grasses. Correlation coefficients for the grass hays were determined between the lignin characteristics (expressed as per unit of acid detergent fiber and per gm lignin) animal data and lignin content (Table 13). Several of the correlation coefficients were not significantly different from zero or were of such low magnitude as to be unimportant biologically as single predictors of animal performance or lignin content. The correlation coefficient between the phenol content and digestible dry matter/cwt was -.98 and the correlation coefficient between the absorbance at 337 mμ on the difference spectra and digestible dry matter/cwt was -.96. These two correlations with digestible dry matter/cwt were of sufficient significance that they could be used as single

Table 11. Correlations between the lignin characteristics of the legumes and grasses combined, animal data and % lignin.

Characteristics[a]	Digestible Dry Matter	Max D.M./cwt	Digestible D.M./cwt	% Lignin
Guaiacol[b]	-.21	.33	.11	.04
Phenol[b]	-.31	.19	.12	.11
243[b]	-.15	.48	.37	-.23
265[b]	-.07	.54	.51	.10
270[b]	-.04	.49	.48	.26
300[b]	-.43	-.05	-.20	.42
337[b]	-.24	.03	-.11	-.59
300 POH	-.39	.15	-.05	.10
250 POH	-.18	.44	.35	-.16
250 C POH	-.24	.35	.22	-.29
% POH	.03	-.20	-.20	.41
Ratio OD	-.01	-.42	-.26	.60
mμ shift	.21	.13	.13	.47
Δ mμ	-.01	.14	.04	-.82

Any $r > 0.44$ gives $P < .05$

(a) Designation same as in Table 1.

(b) These are per 0.1 gm A.D.F.

Table 12. Correlations between the lignin characteristics of legumes, animal data and % lignin.

Characteristics[a]	Digestible Dry Matter		Max DM/cwt		Digestible DM/cwt		% Lignin	
	(b)		(b)		(b)		(b)	
Guaiacol	-.20	.19	.49	.14	.27	.09	.05	-.51
Phenol	-.25	.16	.41	.01	.43	.18	.33	-.34
243	-.12	-.17	.63	.70	.62	.65	.38	.44
265	-.11	-.26	.60	.71	.57	.61	.26	.51
270	-.17	-.33	.54	.68	.47	.55	.27	.56
300	-.48	.03	.08	-.28	-.08	-.25	.30	-.34
337	-.21	.19	.26	-.06	.33	.13	.33	-.23
300 POH		-.48		.21		-.02		.38
250 POH		-.16		.57		.55		.32
250 C POH		-.25		.49		.48		.38
% POH		.03		-.21		-.34		-.31
Ratio OD		.08		-.49		-.36		.03
mμ shift		.03		.22		.02		-.08
Δ mμ		-.02		.25		.28		.20

Any r >.53 gives P <.05.

(a) Designations same as in Table 1.

(b) These are per 0.1 gm A.D.F.

Table 13. Correlations between the lignin characteristics of grasses, animal data and % lignin.

Characteristics[a]	Digestible Dry Matter		Max DM/cwt		Digestible DM/cwt		% Lignin	
	(b)		(b)		(b)		(b)	
Guaiacol	-.23	-.24	-.50	-.44	-.50	-.28	-.22	-.78
Phenol	-.53	-.50	-.95	-.77	-.98	-.58	-.07	-.80
243	-.76	-.25	-.83	-.89	-.91	-.80	.14	-.40
265	.49	.34	.02	.27	.36	.22	-.37	.79
270	.70	-.03	.35	.51	.66	.48	-.41	.71
300	-.48	.34	-.57	-.53	-.79	-.45	.03	.77
337	-.68	.22	-.80	-.83	-.96	-.76	.11	-.61
300 POH		.14		-.48		-.29		-.76
250 POH		-.51		-.78		-.74		-.01
250 C POH		-.47		-.90		-.83		-.29
% POH		-.02		.27		.49		.07
Ratio OD		-.63		-.59		-.60		.27
mμ shift		-.91		.37		.56		-.63
Δ mμ		-.46		.66		.64		.26

Any r >0.81 gives P <.05 and any r >0.92 gives P <.01.

(a) Designations same as in Table 1.

(b) These are per 0.1 gm A.D.F.

predictors of digestible dry matter/cwt.

Prediction Equations for Digestible Dry Matter and Digestible Dry Matter Intake/Cwt

Multiple linear regression analysis was used to develop prediction equations for digestible dry matter and digestible dry matter intake/cwt. Six different equations were developed; two for combined legumes and grasses, four for legumes (two per unit A.D.F. and two per gm lignin), and two for grasses. The importance of these equations is discussed below.

Combined Legumes and Grasses. The digestible dry matter prediction equation that was developed using data for the combined legumes and grasses had no biological importance due to a low multiple correlation coefficient (R^2) of .51 at $P < .28$ (Table 14). This equation included eight independent variables (lignin ultraviolet characteristics) and with this many variables the mathematics would be too long and involved to use as a practical predictor. The standard partial regression coefficients indicate that the variables 250 C POH, mμ shift, and 243 mμ were more important than the other five variables in the equation.

A prediction equation for digestible dry matter/cwt was developed using nine of the independent variables which had a R^2 of .80 at $P < .01$ (Table 14). The standard partial regression coefficients were of about equal size indicating that they were of equal importance in the equation. This

Table 14. Prediction equations on combined legume and grass forages for digestible dry matter (DDM) and digestible dry matter intake per hundred pounds of live body weight (DDM/cwt).

DDM

$$\hat{y} = 46.11 - 220.30X_1 - 64.26X_4 + 1.57X_6 - 103.19X_7 + 109.46X_8 - 143.07X_9 - 265.97X_{11} - 1.14X_{12}$$

$R^2 = .51$

Standard partial regression coefficients.

$b^*_1 = -.17$	$b^*_8 = .20$
$b^*_4 = -.73$	$b^*_9 = -.68$
$b^*_6 = .46$	$b^*_{11} = -.15$
$b^*_7 = -.13$	$b^*_{12} = -.26$

Independent variables (see Table 1 for details).

X_1 = 300 mμ	X_5 = 337 mμ	X_{10} = 265 mμ
X_2 = 300 POH	X_6 = mμ shift	X_{11} = 270 mμ
X_3 = Phenol	X_7 = Guaiacol	X_{12} = % POH
X_4 = 250 C POH	X_8 = 250 POH	X_{13} = Δ mμ
	X_9 = 243 mμ	X_{14} = Ratio OD

DDM intake/cwt

$$\hat{y} = 4.32 + 49.57X_{10} - 56.06X_{11} - 12.83X_9 + 6.57X_8 - 1.03X_{14} + .05X_6 - .50X_3 - 18.40X_7 - 7.06X_5$$

$R^2 = .80$

Standard partial regression coefficients.

$b^*_{10} = .46$	$b^*_8 = .22$	$b^*_3 = -.22$
$b^*_{11} = -.52$	$b^*_{14} = -.31$	$b^*_7 = -.40$
$b^*_9 = -.27$	$b^*_6 = .28$	$b^*_5 = -.18$

R^2 value is not of sufficient size for precise prediction of digestible dry matter intake/cwt. The low predictability and the complicated mathematics of using several variables in an equation would limit the usefulness and application of these relationships although the variables could easily be determined.

Legumes per unit A.D.F. The independent variables used were good predictors of digestible dry matter and digestible dry matter intake/cwt for legumes. Ten independent variables were used in the prediction equation for digestible dry matter and gave an R^2 of .93 at P <.15 (Table 15). The standard partial regression coefficients indicate that the variables phenol, 250 C POH, 265 mμ, mμ shift were more important than the other six variables in the equation. The independent variables are easily obtained from the direct and difference spectra and from chemical analysis for phenol. The only difficulty would be using calculations involving ten variables.

A prediction equation for digestible dry matter intake/cwt was determined using seven independent variables which gave an R^2 of .96 at P <.001 (Table 15). The prediction equation with a multiple correlation coefficient of this magnitude would be a good predictor of digestible dry matter intake/cwt even if the calculations would be difficult. The standard partial regression coefficients were of the same magnitude and of equal importance in the equation. The

Table 15. Prediction equations for DDM and DDM intake/cwt on legume forages.

DDM

$$\hat{y} = -18.07 + 2078.09X_1 - 136.37X_2 - 30.80X_3 - 260.14X_4 + 682.37X_5 - 2304.43X_{11} + 243.69X_9 + 1963.81X_{10} - 15.89X_{14} + 2.08X_6$$

$$R^2 = .93$$

Standard partial regression coefficients.

$b^*_1 = .64$	$b^*_{11} = -.93$
$b^*_2 = -.48$	$b^*_9 = .21$
$b^*_3 = -.80$	$b^*_{10} = .92$
$b^*_4 = -.92$	$b^*_{14} = -.51$
$b^*_5 = .50$	$b^*_6 = .84$

DDM intake/cwt

$$\hat{y} = 5.05 + 49.74X_{10} - 4.48X_8 + 1.98X_3 + .22X_{12} - 22.66X_5 - 42.25X_7 - 17.39X_1$$

$$R^2 = .96$$

Standard partial regression coefficient.

$b^*_{10} = .93$	$b^*_5 = -.88$
$b^*_8 = -.85$	$b^*_7 = -.92$
$b^*_3 = .93$	$b^*_1 = -.91$
$b^*_{12} = .89$	

difference in probability levels between the two R^2's is due to the amount of degrees of freedom available for error.

Per gm Lignin. Nine independent variables were used in the prediction equation for digestible dry matter and gave an R^2 of .98 at P <.01 and the standard partial regression coefficients indicate that the variables are of equal importance in the equation (Table 16). The prediction equation for digestible dry matter intake/cwt was determined using six variables which gave an R^2 of .76 at P <.03 (Table 16). The standard partial regression coefficients show that the variables 265 and 270 mμ were more important than the other four variables in the equation.

Grass per unit A.D.F. The prediction equation for digestible dry matter had an R^2 of .90 at P <.15 with only three independent variables (Table 17). The standard partial regression coefficients showed that the variable mμ shift was more important than the other two in the equation. Such a prediction equation is of biological importance due to its high multiple correlation coefficient and ease with which calculations can be made using only three variables.

The prediction equation for digestible dry matter intake/cwt had an R^2 of .99 at P <.005 with two independent variables (Table 17). The standard partial regression coefficients were of the same magnitude and of equal importance in developing the equation. This prediction equation is of practical and biological importance due to its very high multiple

Table 16. Prediction equations for DDM and DDM intake/cwt on legume forages.

DDM

$$\hat{y} = 40.22 - 8.32X_{11} + 5.12X_{10} - 222.87X_4 - 4.17X_7 - 1.91X_9 + 0.85X_3 + 181.17X_8 - 4.70X_{14} + 2.31X_6$$

$$R^2 = .98$$

Standard partial regression coefficients.

$b^*_{11} = -.97$	$b^*_7 = -.98$	$b^*_8 = .98$
$b^*_{10} = .94$	$b^*_9 = -.88$	$b^*_{14} = -.72$
$b^*_4 = -.98$	$b^*_3 = .89$	$b^*_6 = .98$

DDM intake/cwt

$$\hat{y} = 0.18 + 0.71X_{10} - 0.74X_{11} - 3.23X_4 + 0.83X_{13} + 0.13X_1 - 0.86X_5$$

$$R^2 = .76$$

Standard partial regression coefficients.

$b^*_{10} = .78$	$b^*_4 = -.50$	$b^*_1 = .53$
$b^*_{11} = -.76$	$b^*_{13} = .60$	$b^*_5 = -.24$

Table 17. Prediction equations for DDM and DDM intake/cwt on grass forages.

DDM

$$\hat{y} = 15.60 + 1.83X_6 + 26.09X_{14} - 2.74X_3$$

$$R^2 = .90$$

Standard partial regression coefficients

$b^*_6 = 1.83$ $b^*_3 = -.27$

$b^*_{14} = .26$

DDM intake/cwt

$$\hat{y} = 3.64 - .74X_3 - 6.03X_9$$

$$R^2 = .99$$

Standard partial regression coefficients

$b^*_3 = -.73$ $b^*_9 = -.60$

correlation coefficient and ease of which calculations can be made with only two variables.

The independent variable phenol content of ADF was used in all of the prediction equations. Therefore it was relatively important in all the equations, judged by the magnitude of the standard partial regression coefficients, and strongly suggests its importance as a determinant of digestibility.

Any prediction equation is useful only if the independent variables are easy to obtain in the laboratory and the R^2 or predictability is sufficiently high to be of practical importance. The prediction equations for grasses and legumes are useful to get an estimation of the digestible dry matter and digestible dry matter intake/cwt. The prediction equations should not be used when the predictor values fall outside of the ranges used to develop the equations.

SUMMARY

The lignin molecule underwent a change during the digestion process and this change was greater in grass lignins than in legume lignins. The change might have been a reduction in the amount of phenolic hydroxyl and methoxyl groups attached to the benzene ring, but with a proportionally greater reduction in the phenolic hydroxyl content. Another possible explanation for this change is that a phenolic hydroxyl group was replaced on the benzene ring by a methoxyl group.

There was a difference between legume lignin preparations and grass lignin preparations and this difference was in the chemical make up of the lignin molecules. The grass lignin molecule had a greater proportion of aromatic compounds with a greater specific absorbtivity than the aromatic compounds which make up the legume lignin molecule. These aromatic compounds have more methoxyl groups and/or less phenolic hydroxyl groups attached to the benzene ring and can account for the difference in specific absorbtivity.

The phenolic hydroxyl and methoxyl content tended to increase with maturity of the alfalfa hay. This is

consistent with the data reported by Phillips et al. (1939).

An alfalfa breeder line with high in vitro and in vivo dry matter digestibility had a lower methoxyl content than did an alfalfa breeder line with low in vitro and in vivo dry matter digestibility. This indicates that forage having a lignin molecule that is high in methoxyl content will have low dry matter digestibility.

Alfalfa hay lignin contains a greater proportion of aromatic compounds that contain more phenolic hydroxyl groups than do the aromatic compounds that make up trefoil lignin.

The lignin from Siberian reed canary grass hay contained more methoxyl groups than did the reed canary grass hay lignin. This might be related to the observation that this sample of reed canary grass hay was more digestible than the Siberian reed canary grass hay.

Other comparisons were made among the forages studied but there were no differences found in the structure of lignin in these comparisons.

Lignin content was determined by a procedure that was based on the amount of guaiacol-like compounds present in lignin. These lignin values were compared to values obtained from a standard laboratory method and found that the content of guaiacol-like compounds cannot be used to determine the amount of lignin present in the forage.

Prediction equations for estimating sheep digestibility and digestible dry matter intake/cwt were developed for

grasses and legumes separately and combined. The prediction equations for the combined legumes and grasses had low multiple correlation coefficients and complicated mathematics that would limit the usefulness of these equations. The prediction equations for grasses and legumes when taken separately have squared multiple correlation coefficients high enough to be of practical importance in estimating the digestible dry matter and digestible dry matter intake/cwt in sheep (.90 and .99 respectively for grasses and .93 and .96 respectively for legumes). The prediction equation for digestible dry matter in grasses was $\hat{y} = 15.60 + 1.83X_6 + 26.09X_{14} - 2.74X_3$. The independent variables used were mμ shift, ratio O.D., and phenol respectively. The mμ shift variable (X_6) was a measurement of the distance in mμ between the peak on the neutral spectrum and the peak on the alkaline spectrum. The phenol independent variable (X_3) was a quantitative color reaction for phenol-like compounds. The ratio O.D. (X_{14}) was the ratio of the optical density of the maximum peak to the optical density of the minor peak on the difference spectrum. This is an example of the type of prediction equations that are presented in the text.

BIBLIOGRAPHY

Adler, E., K. J. Bjorkquist and S. Haggroth. 1948. Uber die Ursache der Farbreaktionen des Holzes. Acta Chem. Scand. 2:93.

Allison, D. W. 1966. Factors Affecting the Nutritive Value of Forages. Ph.D. Thesis, Department of Crop Science, Michigan State University.

Aulin-Erdtman, G. 1949. Ultraviolet Spectroscopy of Lignin and Lignin Derivatives. TAPPI 32:160.

Bechtel, H. E., A. O. Shaw, and F. W. Atkeson. 1945. Brown Alfalfa Hay. Its Chemical Composition and Nutritive Value in Dairy Cattle Rations. J. Dairy Sci. 28:35.

Bolker, H. I. 1963. A Lignin Carbohydrate Bond as Revealed by Infra-red Spectroscopy. Nature 197:489.

Bolker, H. I. and N. G. Somerville. 1962. Ultraviolet Spectroscopic Studies of Lignins in the Solid State. I. Isolated Lignin Preparations. TAPPI 45:826.

Bondi, A. H. and H. Meyer. 1943. On the Chemical Nature and Digestibility of Roughage Carbohydrates. J. Agricultural Sci. 33:123.

Brauns, F. E. 1949. The Physical Properties of Lignin. TAPPI 32:157.

Brauns, F. E. 1952. The Chemistry of Lignin. Academic Press, New York, New York.

Brauns, F. E. and D. A. Brauns. The Chemistry of Lignin. Supplemental Volume. Academic Press, New York, New York.

Brauns, F. E. and H. Seiler. 1952. Reactions with Homogenized Spruce Wood. TAPPI 35:67.

Brewster, R. Q. and W. E. McEwen. 1961. Chemical Properties of Phenol. Organic Chemistry. Prentice-Hall, Inc., Englewood Cliffs, N. J., p. 602.

Brown, Stewart A. 1961. Chemistry of Lignin. Science 134:305.

Brown, W., S. I. Falkehag, and E. B. Cowling. 1967. Molecular Size Distribution of Lignin Wood. Nature 214:410.

Colburn, M. W., J. L. Evans, and C. H. Ramage. 1968. Apparent and True Digestibility of Forage Nutrients by Ruminant Animals. J. Dairy Sci. 51:1450.

Crampton, E. W. and L. H. Maynard. 1938. The Relation of Cellulose and Lignin to the Nutritive Value of Animal Feeds. J. Nutrition 15:383.

Creighton, R. H. J., J. L. McCarthy and H. Hilbert. 1941. Aromatic Aldehydes from Spruce and Maple Woods. J. Am. Chem. Soc. 63:312.

Czerkawski, J. W. 1967. The Determination of Lignin. British Journal of Nutrition 21:325.

Davis, R. E., C. O. Miller and I. L. Lindahl. 1947. Apparent Digestibility by Sheep of Lignin in Pea and Lima Bean Vines. J. of Agric. Res. 47:285.

Dehority, B. A., R. R. Johnson and H. R. Conrad. 1962. Digestibility of Forage Hemicellulose and Pectin by Rumen Bacteria In Vitro and the Effect of Lignification Thereon. J. Dairy Sci. 45:508.

Ellis, G. H., G. Matrone and L. A. Maynard. 1946. A 72% Sulfuric Acid Method for the Determination of Lignin and Its Use in Animal Nutrition Studies. J. Animal Sci. 5:285.

Ely, R. E., E. A. Kane, W. C. Jacobson and L. A. Moore. 1953. Studies on the Composition of Lignin Isolated from Orchardgrass Hay Cut at Four Stages of Maturity and from Corresponding Feces. J. Dairy Sci. 36:346.

Forbes, R. M. and W. P. Garrigus. 1948. Application of a Lignin Ratio Technique to the Determination of the Nutritive Intake of Grazing Animals. J. Animal Sci. 7:373.

Gee, M. S., O. E. Nelson, and J. Kuc. 1968. Abnormal Lignins Produced by the Brown-Midrib Mutants of Maize. II. Comparative Studies on Normal and Brown-Midrib -1 Dimethylformamide Lignins. Archives of Biochemistry and Biophysics 123:403.

Gierer, J. 1954. Die Reaktion van Chinonmonochlorimid mit Lignin. Acta Chemica Scandinavica 8:1319.

Goering, H. K. and P. J. Van Soest. 1968. In Vitro Digestibility of Lignified Materials Ensiled with Sodium Chlorite. J. Dairy Sci. 51:974.

Goldschmid, O. 1954. Determination of Phenolic Hydroxyl Content of Lignin Preparations by Ultraviolet Spectrophotometry. Analytical Chemistry. Vol. 26, pp. 1421.

Gould, R. F. 1966. Lignin Structure and Reactions. American Chemical Society, Washington, D.C.

Hachihama, Y. and S. Jyodai. 1946. The Chemistry of Lignin. Nippon Hyoronsha, Tokyo, Japan.

Hale, E. B., C. W. Duncan and C. F. Huffman. 1947. Rumen Digestion Studies. II. Studies in the Chemistry of Rumen Digestion. J. Nutrition 34:747.

Harborne, J. B. 1964. Biochemistry of Phenolic Compounds. Academic Press, New York, New York.

Harris, E. E., J. D'Ianni, and H. Adkins. 1938. Reaction of Hardwood Lignin with Hydrogen. J. Am. Chem. Soc. 60:1467.

Kane, E. A., W. C. Jacobson, and L. A. Moore. 1950. A Comparison of Techniques Used in Digestibility Studies with Dairy Cattle. J. Nutrition 41:583.

Kato, K., J. Nakano, and N. Migita. 1967. Studies on Tobacco Lignin. Part I. Some Characteristics of Tobacco Milled Wood Lignin. Agricultural and Biological Chemistry 31:1010.

Kefford, N. P. 1958. Lignification of Plants in Relation to Ruminant Nutrition. Journal of the Australian Institute of Agricultural Science 24:297.

Kuc, J. and O. E. Nelson. 1964. The Abnormal Lignins Produced by the Brown-Midrib Mutants of Maize. Archives of Biochemistry and Biophysics 105:103.

Migita, N., J. Nakano, T. Okada, J. Ohashi, and M. Noguchi. 1955. Lignin XXIII. Cross-Bevan Color Reaction of Hardwood Lignin. J. Japan Forestry Soc. 37:26.

Miller, L. G., D. C. Clanton, L. F. Nelson, and O. E. Hoehne. 1967. Nutritive Value of Hay Baled at Various Moisture Contents. J. Animal Sci. 26:1369.

Patterson, R. F. and H. Hibbert. 1943. Studies on Lignin and Related Compounds LXXIII. The Ultraviolet Absorption Spectra of Ethanol Lignins. J. Am. Chem. Soc. 65:1869.

Patton, A. R. 1943. Seasonal Changes in the Lignin and Cellulose Content of Some Montana Grasses. J. Animal Sci. 2:59.

Patton, A. R. and L. Gieseker. 1942. Seasonal Changes in the Lignin and Cellulose Content of Some Montana Grasses. J. Animal Sci. 1:22.

Pearl, I. A. 1942. Vanillin from Lignin Materials. J. Am. Chem. Soc. 64:1429.

Pearl, I. A. 1964. Century-Old Puzzle. Chemistry and Engineering News, p. 81.

Pearl, I. A. 1967. The Chemistry of Lignin. Marcel Dekker, Inc., New York.

Phillips, M., M. J. Goss, B. L. Davis, and H. Stevens. 1939. Composition of the Various Parts of the Oat Plant at Successive Stages of Growth with Special Reference to the Formation of Lignin. Journal of Agricultural Research 59:319.

Pigden, W. J. and D. H. Heinrich. 1957. The Lignin Content of Six Clonal Line of Intermediate Wheat Grass. Canadian Journal of Plant Science 37:128.

Schubert, W. J. 1965. Lignin Biochemistry. Academic Press, New York.

Sell, H. M., C. W. Duncan, and L. D. Brown. 1961. Factors Affecting the Utilization of Feed by Ruminants. Annual Report of Cooperative Regional Project NC-25.

Siegel, S. M. 1956. The Biosynthesis of Lignin: Evidence for the Participation of Celluloses as Sites for Oxidative Polymerization of Eugenol. J. Am. Chem. Soc. 78:1753.

Spearin, W. E. and R. C. Dresler. 1954. Stain Technique for Differentiating Hardwood and Softwood Mechanical Pulps. TAPPI 37:39.

Stafford, H. A. 1960. Differences between Lignin-Like Polymers Formed by Peroxidation of Eugenol and Ferulic Acid in Leaf Sections of Phleum. Plant Physiology 35:108.

Stafford, H. A. 1962. Histochemical and Biochemical Differences Between Lignin-Like Materials in Phleum pratense L. Plant Physiology 37:643.

Stallcup, O. T., J. L. Cason, and B. J. Walker. 1955. Influence of the Lignin Content of Hay on the Passage of Nutrients Through the Rumen. Arkansas Experiment Station Bulletin #572, pp. 3-16.

Swain, T. and W. E. Hillis. 1959. The Phenolic Constituents of Prunus Domestica. I. The Quantitative Analysis of Phenolic Constituents. J. of the Science of Food and Agriculture 10:63.

Thompson, S. O. 1967. Ph.D. Thesis, University of Wisconsin, Madison, 1965, as reviewed by I. A. Pearl in The Chemistry of Lignin. Marcel Dekker, Inc., New York.

Tomlin, D. C., R. R. Johnson, and B. A. Dehority. 1965. Relationship of Lignification to In Vitro Cellulose Digestibility of Grasses and Legumes. J. Animal Sci. 24:161.

Van Soest, P. J. 1963. Use of Detergents in the Analysis of Fibrous Feeds. II. A Rapid Method for the Determination of Fiber and Lignin. J. of The Association of Official Agricultural Chemists 46:829.

Van Soest, P. J. 1964. Symposium on Nutrition and Forage and Pastures: New Chemical Procedures for Evaluating Forages. J. Animal Sci. 23:838.

Van Soest, P. J. 1965. Symposium on Factors Influencing the Voluntary Intake of Herbage by Ruminants: Voluntary Intake in Relation to Chemical Composition and Digestibility. J. Animal Sci. 24:834.

Van Soest, P. J. 1965. Use of Detergents in Analysis of Fibrous Feeds. III. Study of Effects of Heating and Drying on Yields of Fiber and Lignin in Forages. J. of the Association of Official Agricultural Chemists 48:785.

Van Soest, P. J. 1967. Development of a Comprehensive System of Feed Analysis and Its Application to Forages. J. Animal Sci. 26:119.

Van Soest, P. J. and W. C. Marcus. 1964. A Method for the Determination of Cell Wall Constituents in Forages Using Detergent and the Relationship Between this Fraction and Voluntary Intake and Digestibility. Paper 81 presented at the annual meeting of the American Dairy Science Association, Tucson, Arizona.

Van Soest, P. J. and R. H. Wine. 1965. Development and Use of Chemical Methods for Determining the Nutritive Value of Forages. NE-24 Annual Report.

Wexler, A. S. 1964. Characterization of Lingosulfonates by U.V. Spectrometry. (Direct and Diff. Spectrograms). Analytical Chemistry 36:213.

Appendix Table I. Lignin concentration, % digestible dry matter, maximum and digestible dry matter intake/cwt for the forages studied.

Legumes	% Lignin	DDM %	Max. intake cwt lbs.	DDM/cwt lbs.
6401-01	6.31	63.15	4.18	2.44
6401-02	9.54	54.68	3.16	1.66
6502-01	8.03	62.28	3.69	1.86
6502-02	10.81	55.70	3.49	1.45
6701-21	6.33	62.54	2.10	1.30
6701-22	6.78	63.70	2.38	1.51
6701-01	8.96	57.91	2.41	1.40
6201-11	8.73	61.71	3.20	1.98
6201-12	8.50	62.58	3.34	2.09
6702-OV	5.00	63.41	2.86	1.68
6702-OD	6.11	66.33	2.15	1.42
6703-02	8.08	61.16	3.12	1.66
6703-03	7.89	53.78	2.58	1.15
6704-01	6.93	63.39	2.78	1.76
Grasses				
6401-51	2.85	63.86	3.36	1.85
6401-52	3.86	61.65	3.61	2.08
6401-41	3.12	63.17	3.23	1.71
6401-42	2.88	61.02	3.08	1.84
6401-31	2.74	60.95	2.35	1.22
6401-32	4.14	53.62	2.82	1.27