# A COMPARISON OF ANALYTICAL METHODS FOR THE PARTITION OF INDIGESTIBLE RESIDUE OF FORAGE GRASSES

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TABLE OF CONTENTS

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# INTRODUCTION

For the past forty years animal feeding stuffs have been divided by proximate analysis into six fractions: Moisture, ether extract, protein, ash, crude fiber, and nitrogen-free extract, as a common basis for their nutritional classification. The three fractions, protein, fat, and fiber, have been particularly used in feed control measurements. Most of the coefficients of digestibility data reported in the literature have been calculated from values obtained with this system of analysis. The significance of the specific values obtained, however, has frequently been overestimated because of the failure of these analytical procedures to define accurately each of the nutritional factors present in the feed. This system of analysis also involves certain assumptions as to the digestibility and uniform composition of each fraction which are not always substantiated by more rigorous investigation. This pertains especially to crude fiber values.

The Association of Official Agricultural Chemists procedure for the determination of crude fiber is a slight modification of the method of Hennenburg and Stohman, proposed in 1864, and now commonly known as the Weende method. It consists essentially of a weak acid digestion followed by a weak alkali digestion, which leaves an insoluble residue commonly referred to as "crude fiber". Many workers have proposed modifications of the original procedure which have been used advantageously (3,7,28,32,47). Other methods with different principles from the original have been reported. Simon and Lohrisch (60) used strong potassium hydroxide and hydrogen peroxide; Konig (25), glycerol and sulfuric acid; Steigler (63), 10 percent hydrochloric acid at 100°,

and a stream of air; Fellenberg (9), a micro-chemical method, using nitric and acetic acids with subsequent oxidation with potassium dichromate; Scharrer and Kürschner (55), acetic acid, nitric acid, and trichloroacetic acid. Most chemical methods for the determination of indigestible residue depend upon the insolubility of the constituents. The product is, therefore, defined by the process itself.

It has been reported by Williams and Olmsted (67), working with feces, that a variable percentage of the constituents commonly considered indigestible (lignin, cellulose, and hemicellulose) were not included in the crude fiber when determined by the Weende method. Norman (33) found that crude fiber from plants consists almost exclusively of cellulose and lignin, and that the cellulose and lignin appearing in the crude fiber fraction varies from 40 to 84 percent of the total cellulose and from 4-67 percent of the total lignin. The variations depended principally upon the type of plant tissue used for the determination. In 1936, Horwitt, Cowgill, and Mendel (23) reported that the crude fiber as determined by the Weende method amounted to approximately 30 percent of the crude fiber as determined by a biochemical enzymatic treatment. Schultz (57), in 1892, and Heuser (21), in 1921, also showed the compositional variability of crude fiber as determined by the Hennenburg hydrolysis.

There is much controversial literature regarding the fate of lignin during digestive processes. Woodman and Stewart (68) concluded that ligno-cellulose is completely indigestible in the stomach of the ruminant. Rogozinski and Starzewska (52) reported that the amount of lignin excreted in the feces of the cow was comparable to the amount ingested. Crampton and Maynard (6) similarly recovered from 93 to

100.3 percent of the dietary lignin in the feces of the steer. However, other experimenters have reported results not so conclusive as to the indigestibility of lignin. Heller and Wall (20) found the coefficients of digestibility of lignin dependent upon the nutrients of the diet. Phillips (43) showed that lignin is demethoxylated in the animal body. Hale and others (5,16,42) have found lignin digestibility to be confusing. Much of the conflicting evidence is probably due to arbitrary methods for the analysis of lignin. In view of these generally inclusive results, and in the absence of any clear-cut evidence as to any nutritive significance of lignin, it seems best to include all of it with the indigestible residue.

The percentage utilization of cellulose and hemicellulose has also been difficult to study. There are no enzymes produced by the digestive tract that are able to hydrolyze cellulose and pentosans. However, these substances are attacked by bacteria in the first three compartments of the stomach of ruminants, in the caecum and colon of the horse, and to a lesser extent in the large intestine of other animals. These bacteria produce organic acids (chiefly acetic and butyric) from cellulose and hemicellulose. It is quite possible that simple sugars, such as glucose, galactose, fructose, mannose, and xylose are also produced. These acids and glucose, galactose, and fructose would be nutritionally valuable to the animal, but since their production depends upon the bacterial flora present, upon species and individual animal differences, and also upon the nature of the association of cellulose and hemicellulose with other substances in the plant, it is not practical to attempt to estimate by in-vitro experimentation the utilization of these cell wall materials. There is no evidence in the

literature that pentoses are ever utilized in the animal body. It is believed rather that upon entering the blood stream they are soon excreted in the urine (30,59,69).

The digestibility of lignin, cellulose, and hemicellulose depends to a great extent upon their association with each other and with the readily digestible nutrients. Hummel, Shephard, and Macy (24) found that the distribution of unavailable carbohydrates in a diet could be altered without change in the total fiber intake or the caloric value of the daily diet. Heller and Wall (20) demonstrated changes in the degree of utilization of fiber with variability of the more nutritious factors of the diet. Pryanischnikov and Tomme (46), and Woodman and Stewart (35), concluded that the relative digestibility of pentosans and cellulose is decreased by the presence of lignin in the ingested material. These data indicate that lignin may be physically or chemically associated with the other constituents of the indigestible residue and thus have an inhibiting effect upon the bacterial action on the associated carbohydrates.

Crampton and Maynard (18) reported data calculated from coefficients of digestibility which showed that the relative digestibility of crude fiber in one-fourth of the feeds they analyzed (dry roughages, green roughages, silages and concentrates) was greater than that of the nitrogen-free extract. This anomaly is very probably due to the inherent inaccuracy of the Weende method, in that only part of the lignin and cellulose and little of the hemicellulose are isolated as crude fiber, and the remainder included with the nitrogen-free extract as determined by difference. It seems evident that the digestibility of the dietary carbohydrates does not strictly follow their partition

into crude fiber and nitrogen-free extract.

In summary, the reports in the literature indicate that crude fiber as determined by the usual chemical methods includes variable and unpredictable fractions of the total lignin, cellulose, and hemicellulose. In the digestive tract of animals equally indeterminant portions of the lignin, cellulose, and hemicellulose are made soluble. The type of bacterial flora present in ruminants is an important factor. The degree of solution is further dependent upon the complex chemical and physical associations of the lignin, cellulose, hemicellulose, and other factors of the feed. Whatever is dissolved may or may not be of nutritive value.

It is concluded that for a method to be satisfactorily used in calculating digestibility data it must isolate an indigestible residue containing all of the lignin, cellulose, and hemicellulose of a sample.

It may now be realized that while the value obtained by the purely empirical methods for crude fiber may indicate the amount of bulk or roughage in the material, it should not be considered to have any definite or regular relationship to any particular plant constituent or to its degree of digestibility.

The trend in recent years has been to isolate by some biological means a fraction of the plant material containing the cellulose, lignin, and hemicellulose, which is in turn broken down into these constituents.

Remy (48), in 1931, described a biological reagent consisting of a mixture of pepsin, malt diastase, and pancreatin, which separated starch, protein, and fat from the indigestible constituents. After comparison of his results with the crude fiber values obtained by the

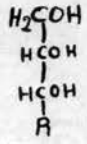
Weende method, he concluded that the Weende digestion caused a great loss of indigestible materials. The use of enzymes, he reported, gave a much closer approximation of the true indigestible portion of feed. In 1935 Williams and Olmsted (67) substantiated this idea and introduced a new enzyme preparation, pancreatin in neutral solution, which proved to have a less hydrolytic effect on the hemicelluloses and yet removed as much of the starch, protein, and fat as did Remy's digestion. Horwitt, Cowgill, and Mendel (23) found that pepsin, clarase, and trypsin would remove starch and protein more efficiently from vegetable materials than would the other enzyme preparations. On forage grasses their method gave a much lower indigestible residue than did the Williams and Olmsted method, although approximately the same values on samples of feces. In 1939, Davis and Miller (8) reported their modification of the Horwitt, Cowgill, and Mendel method to be the most satisfactory of the enzymatic procedures for isolating the indigestible residue of forage grasses with a minimum of starch, fat, and protein remnants.

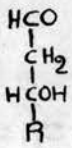
The enzymatic digestion of feed materials has many distinct advantages over the older and more empirical chemical technics. While it does not duplicate the actual mammalian digestive processes, it gives a close approximation of those materials not easily broken down by the normal enzymatic action of the animal digestive tract. This type of method isolates a residue which bears a definite relationship to the indigestible fraction of a specific plant material, and also one which may be compared with the indigestible fraction of other plant materials. Enzymatic treatment proves more efficient than chemical treatment in separating incrusting material (proteins, starches,

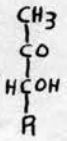
and fats) from the indigestible residue, and yet does not attack important indigestible constituents. While enzymatic treatment demands a longer period of time for manipulation, it seems particularly advisable, in view of the more satisfactory experimental results and theoretical considerations, to employ this method in research studies of indigestible residue.

The second problem involves the separation of the indigestible residue into its various chemical entities, cellulose, lignin, and hemicellulose. The true structure and properties of cellulose, lignin, and hemicellulose are only postulated and, therefore, strictly chemical methods cannot be used in their estimation. The methods for the determination of these membrane substances are dependent upon their differences in chemical and/or physical properties.

Hibbert (22) and Freudenberg (10) have reported that lignin may be regarded as a product resulting from the etherification and condensation of the following and similar units:







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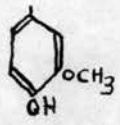
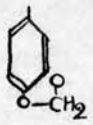
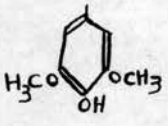
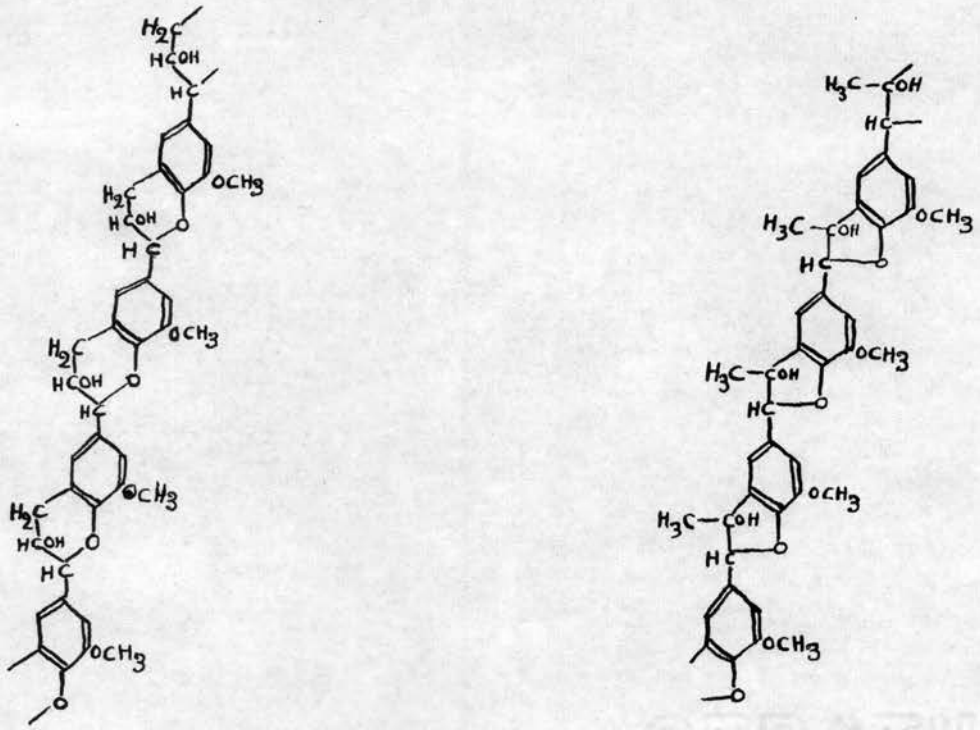






The condensation products which result have no free phenolic hydroxyl, carbonyl, and carboxylic groups (15).

The general structure of the lignin molecule is postulated by Freudenberg to be (10):



LIGNIN

The length of the lignin chain is as yet undetermined. A satisfactory molecular weight estimation has not yet been possible, since lignin is either completely or partially altered by all procedures which dissolve it. Each method for the partition of lignin from the indigestible residue employs one of four types of chemical reactions characteristic of the lignin molecule. The first type of reaction concerns the functional groups, which are: The aliphatic hydroxyl, methoxyl, and methylenedioxy groups, the C-methyl groups, and their derivatives. Methods based on these reactions are not very satisfactory, owing to the variability of the amounts of these functional groups in lignin derived from different sources or from the same source at different times. The second type of reaction involves replacement of hydrogen; i.e., substitution by chlorine, bromine, iodine, nitric acid, and mercuric acetate. This probably occurs on the aromatic ring and/or through reaction with alpha-phenylhydroxyl groups. The third type of reaction consists of degradation without rupture of the carbon to carbon linkages, and is produced by hydrolysis with alkalies, aminolysis, sulfite reaction, alcoholysis, etc. All of these reactions take place at the alpha-phenyl-hydroxyl groups and the open or cyclic ether groups. The last type of reaction involves degradation of the carbon framework of the lignin molecule, resulting from drastic reaction with potash, oxidation, hydrogenation, or thermal degradation. These reactions may attack any portion of the molecule, but usually the ether linkages are first broken followed by the oxidation of the side chain with the formation of the corresponding aromatic acid.

It will be noticed that any method employing one of these reactions would probably isolate hemicellulose and/or cellulose by render-

ing lignin extractable by some suitable solvent. This is the basis for the determination of cellulose by the following methods: The Cross and Bevan (7) chlorination procedure; the chlorine dioxide method of Schmidt (56); the Norman and Jenkins (39) method using sodium hypochlorite solution; the Van Beckum and Ritter (64) direct chlorination procedure for the isolation of holocellulose; and the Kürschner and Hoffer (27) method, which uses nitric acid and alcohol as reagents for removing lignin.

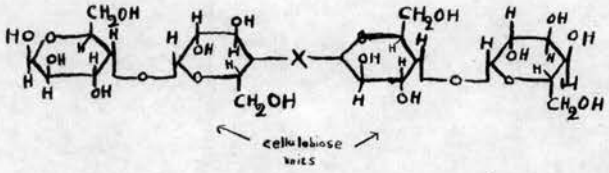
In 1938 Crampton and Maynard (21) proposed a lignin method for feeds based on a procedure of Ross and Potter (53) for wood, which was developed from the reaction of formaldehyde and lignin to yield a rapidly flocculating lignin precipitate. This method has been rejected by all lignin chemists as being theoretically unsound, because the compound formed is a substituted and resinous material containing formaldehyde, and thus yields unreasonably high lignin values. This reaction of lignin, supposedly a phenol-aldehyde resin formation, takes place in concentrated acid solutions. As will be shown later, the reaction is also characteristic of furfural, formed from pentoses, and causes high results when pentoses are present in reaction media intended to isolate lignin.

The lignin molecule is more resistant to hydrolysis or degradation by mineral acids, is more easily substituted or oxidized, and is more reactive with alkalies than is cellulose. The analytical separations of these two materials are based upon one or more of these differences in their chemical behavior.

Cellulose was long considered to be the typical compound of the whole polysaccharide group. Structurally, it is considered to consist of long chains of glucopyranose molecules linked together in the 1,4

position. The essential structural difference between starch and cellulose is that the glucoside linkages are alpha in starch and beta in cellulose. Freudenberg and Blomqvist (12) have also pointed out that starch and glycogen may have open or branched chains, while cellulose has open chains without any detectable branching.

While the nature of the chemical bonds between the glucose units of cellulose is fairly well understood, the number of glucose molecules in the cellulose chain is uncertain. Its postulated chain structure is:




Cellulose

The value of X has been estimated by Haworth (19) to be between 100 and 200, and by Staudinger and Huseman (62) to be 1000-2000 glucose molecules. This indicates the variation of opinion on the molecular size of cellulose.

Measurements on the kinetics of cellulose hydrolysis correlated with optical rotation (44) have shown that in pure cellulose the beta linkage (cellobiose linkage) predominates, and that no other type of linkage can occur in greater proportion than one in fifty or one in a hundred. These cellobiose linkages are split almost quantitatively by acid hydrolysis to yield glucose. Thus cellulose may be calculated

from the amount of glucose produced. Various methods for the determination of lignin have been developed based on the differential solubility of lignin and cellulose in strong acid. The method of the Association of Official Agricultural Chemists (2) makes use of fuming hydrochloric acid for isolating lignin. The Williams and Olmsted (67) procedure depends upon the conversion of cellulose and hemicellulose to glucose and pentoses by concentrated acid treatment. The mixture is subsequently diluted and refluxed to separate the insoluble lignin from the soluble pentoses and glucose. The strong sulfuric acid hydrolysis of cellulose and hemicelluloses has also been used by Norman and Jenkins (40) and by the Forest Products Laboratory (51) as a basis for the determination of lignin.

The resistance of cellulose to hydrolysis, oxidation, substitution, and solution permits its isolation by vigorous chemical reactions which attack and remove other materials associated with it. Such reactions are applied in methods for the isolation of cellulose as an insoluble residue from treatments which remove associated substances.

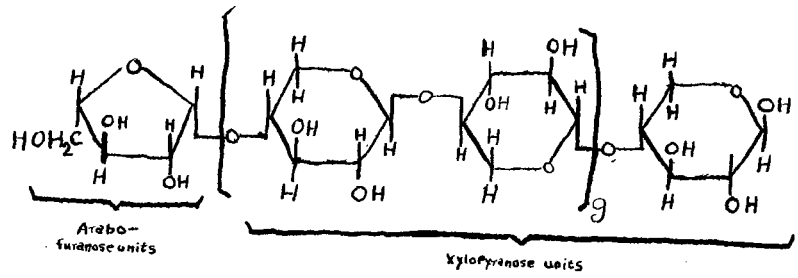
The chemistry of hemicelluloses is rather chaotic. Hemicelluloses are rather generally defined as those cell wall polysaccharides extractable by dilute alkalies, either hot or cold, and hydrolyzable by hot dilute acids to their constituent sugars or uronic acids. The group includes short chain hexosans and pentosans (cellulosans) associated and oriented with the cellulosic aggregate (50), polysaccharides related to starch and inulin, uronic acids, and incrusting amorphous polysaccharides which may conceivably be in part linked to lignin (54), and which seem invariably to contain uronic units (polyuronides). That is, the term hemicelluloses includes everything of a carbohydrate

nature in plants except starch, cellulose, lignin, and soluble sugars. Hydrolysis of hemicelluloses may therefore yield relatively pure monosaccharides or a variable mixture of monosaccharides and uronic acids. The monosaccharides may consist of mannose, glucose, galactose, arabinose and xylose. Also galacturonic and glucuronic acids are yielded, as well as traces of some other materials (1).

Of the various types of hemicelluloses the pentosans have received the most consideration, owing primarily to their greater abundance and to the ease of their determination by the furfural method. The hemicelluloses may comprise the entire cell wall, or they may simply form incrustants on the cellulose framework of the wall. In most plants they rank next to cellulose in quantitative importance.

The simple pentose sugars formed by the hydrolysis of hemicelluloses are reducing, but are not fermentable by Saccharomyces cerevisiae (baker's yeast). A method based on this fact has been devised by Williams and Olmsted (67) for the quantitative differentiation of pentoses from the glucose formed by the concurrent hydrolysis of cellulose, and the cellulose and hemicelluloses of a sample may be calculated as a result of a single hydrolytic procedure.

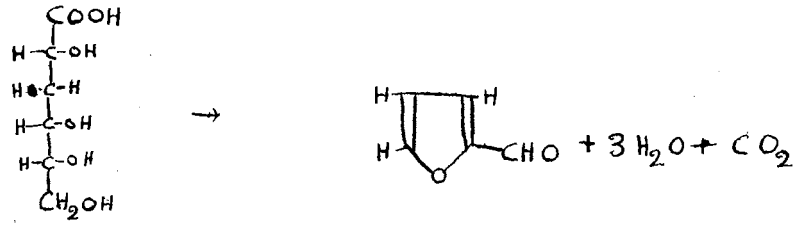
The study of the structure of hemicelluloses has been confined mainly to the hemicelluloses of wood. The structure of the hemicellulose xylan, is stated by Haworth (19) to be as follows:




Xylan

It is highly probable that the molecule consists of eighteen or nineteen xylopyranose units connected by 1,4 linkages and terminating at one end with an arabofuranose group. Similarly, it was shown by Anderson, Hechtman, and Seeley (1) that in cottonseed hulls sixteen xylose units are present for every galacturonic acid group. Weihe and Phillips (66) identified xylose, arabinose, and hexuronic acid among the products of hydrolysis of wheat straw. They were present in a ratio of 23-0.9-1. Phillips and Davis (45) found the hemicellulose of alfalfa hay to consist of 77.3 percent xylose, a trace of l-arabinose, and 12.13 percent of uronic anhydride, values probably representative of the hemicelluloses of forage crops.

The reaction of uronic anhydrides with various concentrations of acids has been studied by Link, Otterson, and Dickson (29), who demonstrated that when glucuronic acid (anhydride or lactone) is heated with 12 percent hydrochloric acid under conditions similar to those for determining pentoses, it is decomposed according to the following equation:



The yield of furfural is less than the theoretical, while that of carbon dioxide is practically quantitative. They also state that in order to insure complete decarboxylation of uronic acids it is necessary to heat with 12 percent hydrochloric acid at 130-140° C. for a period of 4 to 5 hours. Their procedure is rather time-consuming, and since the percentage of uronic acids in hemicellulose is relatively small, the specific analysis for polyruonides is not usually made.

The discussion of the constituents of indigestible residue has been primarily concerned with the chemical properties of the individual constituents. Actually, cellulose, hemicellulose, and lignin are closely interrelated, both chemically and physically, in the natural cell wall.

It has been shown by Norman (35) that a chemical bond probably exists between polyuronide hemicelluloses and lignin. Harris and his co-workers (18) report that in efforts to methylate the lignin of maple wood no methoxyl groups were added over the normal amount found in the lignin. However, upon mild hydrolysis of the wood before methylation, an increase in methoxyl was observed. They therefore concluded that a chemical bond between hemicellulose and lignin existed. The evidence points toward a glucoside linkage, involving the secondary carbonyl groups. A complete separation of lignin and hemicellulose involves an acid hydrolysis, which, however, also alters the lignin. The highly

polymerized lignin occurring in older tissues tends to be sugar-free; considerable sugar is associated with lower lignin polymers predominating in young tissues. There is a regular gradation between the two classes, and many species differ. The hydrolytic procedures used to remove sugars further condense the lower lignin polymers into larger ones, and aids in quantitative analysis.

There is no evidence of a chemical linkage between lignin and cellulose (10,34); nevertheless, it is impossible to extract the cellulose from lignified tissues by use of a specific solvent, inasmuch as a swelling of the cellulose chain occurs in copper, viscose, or acetate reagent which prevents its diffusion into solution.

The cellulose of most plant materials and woods differs from cotton cellulose in many respects, due principally to the presence of other polysaccharides intimately associated with and tenaciously retained by the true cellulose (alpha-cellulose) of the common plant and wood fibers. Norman (34) has suggested the name "cellulosans" for this group, implying thereby that hexosans or pentosans are found with the cellulose and held in some way by it. The forage grasses have a high content of cellulosans with as much as 30 percent xylose existing in the crude cellulose fraction.

Cellulose preparations containing cellulosans undergo an irreversible change on oven-drying, resulting in a fraction soluble in hot water. The main constituent affected by this treatment is xylose; however, heat drying also renders cellulose more susceptible to extracting and hydrolyzing agents.

The cellulosans are probably formed concurrently with the cellulose and are oriented in the bundles of molecules comprising the

colloidal structure of the fibers. They would be retained by secondary valence bonds, just as the cellulose chains are themselves stabilized in this manner. The molecular size, or rather the length of the chain of sugar units, is considerably less in the case of cellulosans than with cellulose itself. As a result of the shorter chain length in cellulosans the adhesive forces due to secondary valence bonds would be weaker than those of cellulose. Partial removal of cellulosans from some cellulose fractions during their isolation is probably due to differences in the solubilities of these two materials. The cellulosans undoubtedly form a normal integral part of the cellulosic structure of the plant cell wall and fibers, and any analytical methods for isolating natural celluloses should include this group in as little changed a condition as possible. Van Beckum and Ritter (64) have substantiated Norman's findings, and have shown that Cross and Bevan cellulose, as well as hydrolyzed holocellulose (carbohydrate fraction of extractive-free wood), contains a considerable quantity of unextractable furfural-yielding substances.

The general physical association of lignin, cellulose, and hemicellulose with each other in the cell wall is evidently very close, because of the vigorous chemical reactions necessary to segregate each one from the other.

Four chemical methods for the separation and estimation of lignin, cellulose, and hemicellulose appear superior in accuracy and rapidity of manipulation to many others also developed in recent years, and are briefly described below.

The Williams and Olmsted (67) procedure for indigestible residue of feces involves digestion with strong sulfuric acid followed by dilute

acid hydrolysis to isolate lignin. Fermentable and non-fermentable sugars are determined on the filtrate and calculated to cellulose and hemicellulose by suitable factors.

The combination of Norman and Jenkins (39,41) and Norman (36,37) methods isolates lignin, cellulose, and hemicellulose separately by established individual methods. Lignin is determined, after suitable pretreatment, as the residue from strong acid hydrolysis (37). Pentoses are determined by furfural yield (36). Cellulose is determined by sodium hypochlorite treatment (41). Uronic acids are determined by direct measurement of the carbon dioxide evolved upon treatment with strong acid (39). This method has many advantages but was not used in this study because of one serious disadvantage, i.e., the length of time required for manipulation.

The method of Davis and Miller (8) for the partition of the less easily hydrolyzed carbohydrate complex of forages involves a weak acid pretreatment of the indigestible residue to remove hemicellulose, followed by a short treatment with strong sulfuric acid, and a subsequent weak acid hydrolysis to remove cellulose and to isolate lignin. Cellulose is determined by the Kürschner and Hanak (26) acetic-nitric acid method, and hemicellulose is obtained by difference.

The newly developed rapid method of Van Beckum and Ritter (64) isolates the holocellulose of wood by alternate chlorination and extraction of the lignin with 3 percent monoethanolamine in 95 percent ethyl alcohol. "Chlorolignin" is extracted quantitatively, leaving as a residue holocellulose, i.e., crude cellulose plus hemicellulose. The hemicellulose is calculated from the weight loss of the holocellulose upon weak acid hydrolysis.

The methods of Williams and Olmsted and Van Beckum and Ritter are arbitrary means of partitioning the lignin, cellulose, and hemicellulose of materials other than forages. Satisfactory adaptations and modifications of these methods seem necessary before they may be satisfactorily employed in partitioning the indigestible residue of grasses.

A procedure was therefore developed in this laboratory involving modifications and adaptations of established methods to the partition of the indigestible residue derived from the type of sample analyzed in this laboratory. The procedure of Williams and Olmsted is the basis of this modified method. The procedures of Williams and Olmsted, Davis and Miller, Van Beckum and Ritter, and this modification were compared relative to their efficiency in partitioning indigestible residue.

## MATERIALS AND METHODS

Samples of the following dry-land forage grasses were analyzed.

| Scientific Name | Common Name |
|---|---|
| Sporobolus cryptandrus | Sand dropseed |
| Andropogen hallii | Sand bluestem |
| Andropogen scoparius | Little bluestem |
| Eragrostis trichodes | Sand lovegrass |
| Panicum vergatum | Switch grass |
| Paspalum stramineum | Sand paspalum |
| Redfieldia flexuosa | Blowout grass |
| Triplasis purpurea | Purple sandgrass |
| Cracca virginianna | Sand legume |
| Calamovilfa gigantia | Giant reedgrass |
| Buchloe dactyloides | Buffalo grass |
| Bonteloua hirsuta | Hairy grama |

| | |
|---|---|
| Eragostis curvula | Weeping lovegrass |
| Rhus trilobata | Skunk brush |

The samples were air-dried, ground through a 0.5 mm. screen in a large Wiley mill, and reground through a 60 mesh screen in a medium Wiley mill. They were stored in glass bottles until analyzed.

It was necessary first to select a method which would isolate an indigestible residue free from contaminants to use as an identical substrate for each of the subsequently applied methods of chemical fractionation. The Davis and Miller enzymatic digestion procedure (8) was selected as being the most satisfactory method for preparing indigestible residue. The adaptation used is as follows: A 3 gram air-dried sample is weighed into a fat-determination tube and extracted in a continuous extraction apparatus with anhydrous diethyl ether for 16 hours. After drying at 100° C. for 15 minutes it is transferred to a 250 ml. Erlenmeyer flask. It is then treated with 150 ml. of .1 N hydrochloric acid containing .3 g. of pepsin, and the mixture is incubated at 40° C. for 48 hours. During this and subsequent incubations the mixture is shaken occasionally. The incubate is then made just acid to methyl red (pH 4.5) by successive additions of 2 N sodium hydroxide and .1 N hydrochloric acid, and 10 ml. of a 3 percent aqueous solution of clarase are added. The addition of a few drops of toluene aids materially in preventing mold growth. After again being incubated for 48 hours at 40° C. the mixture is filtered and the residue washed with water and returned to the original flask. A solution of 0.3 g. of trypsin in 150 ml. of water is added, a few drops of 1 N sodium hydroxide are added (on account of buffer action this raises the pH to only about 7.8) and the mixture is incubated for 96 hours at 40° C. The

incubate is then filtered, and the residue washed with hot water, hot 95 percent alcohol, and hot benzene. It is then placed in a weighed 50 ml. beaker, dried at 80° C. for 3 hours, and allowed to remain in a desiccator over night. This residue is then weighed and the percent indigestible residue of the original sample calculated. The dry material is transferred to a stoppered flask where it is stored until analyzed. All filtrations are made through a 200-thread silk bolting cloth.

In a brief study of substances contaminating the Davis and Miller indigestible residue, nitrogen was determined by the methods of analysis of the Association of Official Agricultural Chemists (2) and ash by ignition in alundum crucibles. While traces of other foreign materials probably remain in the indigestible residue as determined, their significance is relatively small in comparison with that of protein and ash, and no specific analysis was made to determine errors introduced by their presence.

The indigestible residue was first fractionated by the following modified Williams and Olmsted procedure: A 0.5 g. sample of the indigestible residue in a 100 ml. tall-form beaker is thoroughly mixed with 20 ml. of cold 72 percent (by weight) sulfuric acid and placed in a refrigerator at 6-10° C. for 16-24 hours. It is stirred at hourly intervals during the first 5 hours. The mixture is then transferred to a tall-form 1 liter beaker, diluted to 480 ml. (4 percent sulfuric acid) with distilled water, and gently refluxed for 3 hours, a flask of cold water set on the beaker serving as condenser. The flocculent lignin is filtered into an alundum crucible, washed with hot water, and dried at 105° C. for 4 hours. The crucible is then weighed,

ignited, and reweighed, and the loss in weight on ignition reported as lignin. The filtrate is neutralized to phenol red (pH 7.8) with 50 percent sodium hydroxide (about 40 ml. required) and transferred to a 1000 ml. volumetric flask where it is made to volume with distilled water. The total reducing value of the sugars is determined on a 5 ml. aliquot by the Shaffer-Somogyi copper reduction method (58). A 10 ml. aliquot is yeast fermented by the Somogyi procedure (61) for 20 minutes at 25° C. and then centrifuged at 3500 to 4000 r.p.m. for 15 minutes to clear the solution of all yeast cells. Non-fermentable reducing sugars are subsequently determined on a 5 ml. aliquot of this yeast-free solution.

In order to convert the titers of the fermentable and non-fermentable reducing sugars to the percent of the specific sugars from which they were derived, it was necessary to prepare two curves based on the titration values of known amounts of fermentable and non-fermentable reducing sugars.

To prepare the curve for reading fermentable reducing sugar values, the titers of a series of known amounts of glucose were plotted against the amounts used. Aliquots of a 1:1 xylose-arabinose standard solution were yeast fermented and the titration values subsequently determined for plotting against concentration to obtain the non-fermentable reducing sugar curve. In preparing both of these curves the exact procedure used on the unknown solutions was followed and sodium sulfate was added to make the salt concentration equivalent to that of the sample, so that each curve included a complete blank on the analysis.

In calculating, the titers of the unfermented samples minus the titers of the fermented samples represents glucose and is used in read-

ing the glucose value from the prepared glucose curve. This value is converted by calculation to percent glucose, and the percent glucose is multiplied by 0.9 to secure the equivalent percent of cellulose. The factor 0.9 is derived from the ratio of the molecular weight of glucose to the molecular weight of the glucose unit in cellulose. The titration value of the non-fermentable reducing sugars is considered to represent pentoses derived from hemicelluloses, and the equivalent sugar values are read from the prepared xylose-arabinose curve, converted to percent, and the percent of pentoses multiplied by 0.88 to convert to hemicelluloses on the basis of the ratio of the molecular weight of the pentose molecule to the molecular weight of the pentose unit in hemicelluloses.

The Davis and Miller method for the determination of lignin was used without modification, as follows: A 0.5 g. sample of indigestible residue is placed in a 100 ml. beaker, 25 ml. of 5 percent (by weight) sulfuric acid added, and the mixture heated at boiling point for 1 hour to remove hemicelluloses. The digest is filtered and the residue returned to the original beaker and dried at 80° C. for 1 hour. The beaker containing this residue is cooled in an ice bath and 20 ml. of cold 72 percent (by weight) sulfuric acid are added. The mixture is stirred at 0° constantly for 15 minutes, removed from the bath, and allowed to stand for 45 to 60 minutes at room temperature to complete the reaction. The reaction mixture is then diluted to 480 ml. in a tall-form 1 liter beaker and refluxed for 2 hours, with a flask of cold water placed on the top of the beaker as a condenser. For remainder of the procedure refer to the previous description of the Williams and Olmsted method.

Davis and Miller suggested that cellulose be determined by the Kürschner and Hanak procedure, as follows: A 0.5 g. sample of indigestible residue is placed in a 250 ml. Kjeldahl flask and 15 ml. of 80 percent (by weight) acetic acid and 1.5 ml. of concentrated nitric acid are added. The mixture is boiled gently on a hot plate for 20 minutes with a glass thimble in the mouth of the flask. The digest is then transferred to a 50 ml. centrifuge tube by means of a stream of 95 percent ethyl alcohol, and centrifuged for 10 minutes at 2500 to 3000 r.p.m. The supernatant liquid is decanted and the residue resuspended in alcohol and again centrifuged out. The residue is then rinsed into an alundum crucible with a stream of alcohol, and washed with hot benzene, hot alcohol, and ether. The crucible containing the crude cellulose is dried at 105° C. for 2 hours, weighed, ignited at a low temperature, and reweighed. The loss in weight upon ignition is reported as cellulose.

Davis and Miller calculated hemicelluloses by subtracting the sum of the lignin and cellulose percentages from the percentage of the indigestible residue. Since the indigestible residue of forage grasses is contaminated by an appreciable percentage of ash, the ash content of the indigestible residue was determined and correction made for it when percentages of hemicelluloses were calculated according to this system.

The analytical technique of the Van Beckum and Ritter procedure (64) for the isolation of holocellulose was modified for better adaptation to forage grasses, as follows: A 0.5 g. sample of indigestible residue is weighed accurately into a tared, sintered-glass filtering crucible, moistened with cold distilled water (about 10° C.), and the

excess moisture removed by applying suction. By means of moderate suction, chlorine is drawn through the sample from a funnel inverted over the crucible, which is still supported in the suction flask. After 3 minutes chlorination, the residue is stirred thoroughly and then rechlorinated for 2 minutes. Alcohol is added to remove excess chlorine and hydrogen chloride, and after 1 minute is removed by suction. The suction is released, and sufficient hot 3 percent solution of monoethanolamine in 95 percent alcohol ("chlorolignin" solvent) to cover the material completely is added. After 2 minutes this also is removed by suction. The solvent treatment is repeated and the residue then washed with 95 percent ethanol and twice with cold water to remove monoethanolamine. The chlorination and extraction is repeated until the residue is white following chlorination and is no longer colored by the addition of the hot "chlorolignin" solvent. The monoethanolamine is finally removed by washing twice with alcohol, twice with cold water, and again with alcohol until the residue is neutral to litmus. The remaining residue, holocellulose, is dried to constant weight at 105° C. The percentage of lignin in the indigestible residue is calculated from the loss in weight during the chlorination and extraction procedure.

The holocellulose is transferred to a 400 ml. beaker containing 200 ml. of hot 3 percent (by weight) sulfuric acid, and the mixture digested just below boiling for 1 hour. The residue, which is crude cellulose, is filtered into the original crucible and dried to constant weight at 105° C. The percentage of hemicelluloses is calculated from the loss in weight of the holocellulose. The crude cellulose is then transferred to a tared crucible, ignited, and the percentage of cellu-

lose is calculated from the loss in weight upon ignition.

The method finally developed in this laboratory by modifying and combining these methods is as follows:

The indigestible residue is determined by the Davis and Miller procedure previously described.

Lignin is determined by the previously described method of Davis and Miller, with the added details that the hydrolysate from the preliminary 5 percent acid treatment is reserved for the determination of the pentoses of the hemicellulose fraction, and the filtrate from the subsequent strong acid hydrolysis is reserved for the determination of glucose derived from cellulose.

Hemicelluloses are determined from the amount of pentoses in the 5 percent acid hydrolysate as follows:

The filtrate of the 5 percent sulfuric acid treatment is transferred to a 500 ml. volumetric flask and neutralized to litmus paper with 50 percent sodium hydroxide. Then 16 g. of anhydrous sodium sulfate are added to make the salt concentration comparable to that obtained by neutralization of the strong acid digestion mixture, for which salt concentration the standard sugar curves are prepared. The solution is made to volume, and a 5 ml. aliquot pipetted into a 200 x 26 mm. test tube. Then 5 ml. of Shaffer-Somogyi sugar reagent (58) are added, and the test tube is covered and heated in a boiling water bath for 15 minutes. The tube is removed from the bath, cooled rapidly in cold water, and 5 ml. of 1 N sulfuric acid are added to liberate iodine. The tube is shaken and the excess iodine is titrated with .05 N sodium thiosulfate, using soluble starch as the indicator. The difference between this titer and the titer of a blank containing 0.2055 g. of

sodium sulfate in 5 ml. of water which is run concurrently with the sample represents the total reducing sugars of the 5 percent sulfuric acid hydrolysate. A 10-15 ml. portion of the same hydrolysate is placed in a centrifuge tube containing 3-5 g. of washed baker's yeast, thoroughly stirred, fermented at 30° C. for 30 minutes, and then centrifuged at 3500 to 4000 r.p.m. A 5 ml. aliquot of the supernatant liquid is immediately analyzed for reducing sugars as just described. The difference between this titer and the titer of a blank (containing 0.2055 g. of sodium sulfate per 5 ml.) that was subjected to yeast fermentation and analyzed parallel with the sample analysis represents the non-fermentable sugars of the 5 percent acid hydrolysate. The titration values are converted to the respective sugar values by use of standard curves prepared as described for the Williams and Olmsted method. The difference between the percentages of total reducing sugars and those of non-fermentable reducing sugars represents glucose derived from the short chain hexosans present in the cellulosan fraction and is converted to percentage of cellulose and included with the cellulose fraction.

Cellulose is determined from the amount of glucose in the filtrate from the lignin determination. The filtrate is placed in a 1 liter volumetric flask, neutralized to litmus paper with 50 percent sodium hydroxide, and total reducing sugars and non-fermentable reducing sugars are determined exactly as described for hemicelluloses. The difference between the titration values of the total reducing sugars and the non-fermentable reducing sugars represents glucose. By reading the sugar equivalent on the glucose standard curve and calculating as in the Williams and Olmsted procedure, a value for pure cellulose is

secured. The non-fermentable reducing sugar value of this fraction is discarded, since a satisfactory estimation of pentoses in strong acid digestion mixtures is not practical. The percentage of glucose in the 5 percent sulfuric acid filtrate plus that of the pentoses of the cellulosans, differentiated by the following procedure, are converted to percentage of cellulosans and added to the pure cellulose value to represent crude cellulose.

Total pentoses are estimated by conversion to furfural and its quantitative estimation by the method of Halsworth (17) as follows: A 0.5 g. sample of indigestible residue is transferred to a 250 ml. distilling flask containing 22 g. of sodium chloride, and 100 ml. of 13.15 percent (by weight) hydrochloric acid are added. The flask is closed with a one-hole rubber stopper fitted with a thistle tube which is adjusted to extend well below the surface of the mixture. The flask is attached to a Liebig condenser and the flask half immersed in a wax bath previously heated to 168-170° C. A 500 ml. volumetric flask is used as a receiver for the distillate. Distillation should progress so that 25 ml. of distillate are collected every 10 minutes. At 10 minute intervals 25 ml. of 13.15 percent hydrochloric acid are added through the thistle tube to keep the volume of the mixture constant within limits. After 300 ml. of distillate are collected the receiving flasks are removed and the distillate made to volume. A 100 ml. aliquot is pipetted into a glass stoppered 250 ml. Erlenmeyer flask and 25 ml. of bromine reagent (0.1 N potassium bromate containing about 5 percent potassium bromide) are added. The flask is kept in a dark room at 20° C. for 1 hour. An excess of potassium iodide is added and the iodine liberated is titrated with standard .1 N sodium thiosulfate,

starch being used as the indicator. A blank correction is determined on a solution made up to contain 4 percent hydrochloric acid and 25 ml. of the potassium bromate-potassium bromide reagent run concurrently with the sample. The difference between the titration values of the blank and the sample represents the ml. of .1 N bromine required to react with the furfural of the sample, and is multiplied by the factor 0.002402 to convert to mg. of furfural (14,17). This value multiplied by the experimental factor 1.835 (17) yields mg. of pentoses equivalent to the furfural.

The difference between the percentage of pentoses calculated from the amount of non-fermentable reducing sugars of the 5 percent acid hydrolysate and the percentage of total pentoses determined from the furfural represents pentoses derived from the cellulosans of the crude cellulose. Multiplication of the value of these pentoses of the cellulosans by the factor .88 converts them to the equivalent values of the cellulosans. These pentose values of the cellulosans plus the glucose value of the cellulosans derived from the determination of the hemicelluloses represent total cellulosans and are added to the percentage of the pure cellulose fraction indicated by the fermentable sugars to obtain a percentage representing crude cellulose.

## RESULTS AND DISCUSSION

A study was made to detect possible protein adhering to the Davis and Miller indigestible residue. It seemed that the presence of protein would be manifested by two effects; first, by the incompleteness of recovery of the indigestible residue as measured by the summation of the fractions determined, and second, by high lignin percentages, as was indicated by Norman and Jenkins (40).

In the partition of the indigestible residue, errors may result from failure to account for the presence and distribution of proteinaceous material. This is true particularly of the methods of Davis and Miller, and Van Beckum and Ritter, which determine one or more of the important constituents of the indigestible residue by difference. While the presence of protein would also prohibit the complete recovery of the indigestible residue when each constituent is determined separately, as is done in the Williams and Olmsted method or the procedure developed in this laboratory, this error in recovery is to be expected, and it is not as significant as the error resulting from calculating the amount of a constituent by difference.

This study of the protein remnants not removed by the enzymatic digestion of Davis and Miller was made to determine the extent and position of errors introduced by such remnants. The proportion of the total nitrogen which was removed by the enzymatic digestion of Davis and Miller was determined, and the values are reported in table 1. The variation in the amount of nitrogen extracted (48-76 percent) is probably due to the nature of the different grass samples. Whether the unremoved protein is held by some form of chemical bond to the membrane carbohydrates or whether it is mechanically incorporated as a part of the colloidal structure of these carbohydrates is a matter of speculation. It seems reasonable to suspect that the protein has a definite and functional part in the colloidal structure of the plant cell membrane. If it is assumed that the nitrogen of indigestible residue exists as protein, the errors introduced by its presence may be appreciable, especially if recovery of all the indigestible residue is desired. It was thought that the determination of the protein of the indigest-

ible residue and addition of this value to the amounts of other constituents of the residue might correct this error. However, even if the total amount of the protein present in the indigestible residue were known, the mode of distribution of this protein among the fractions determined would not be known, and the accurate correction for the protein content of each would be impossible. Owing to the unavoidable appearance of protein in the determined fractions, the addition of the total protein present to these constituents in calculating recovered indigestible residue would produce an error. For this reason it was thought advisable to disregard the protein fraction when calculating the recovery of indigestible residue after its partition.

TABLE I. The Removal of Nitrogen by the Davis and Miller Enzymatic Digestion*

| Kind of grass | N present | | In i. r.** | |
|---|---|---|---|---|
| | Treated | Untreated | N % | Protein % |
| Sand bluestem | 10.15 | 3.05 | .58 | 3.6 |
| Little bluestem | 11.70 | 3.82 | .61 | 3.8 |
| Switch grass | 17.60 | 8.06 | 1.42 | 8.8 |
| Hairy grama | 15.45 | 3.76 | .68 | 4.2 |
| Weeping lovegrass | 16.35 | 3.35 | .56 | 3.3 |

*Unless otherwise indicated the results recorded are expressed in mg./g. original sample.
**Abbreviation of the term indigestible residue.

Norman and Jenkins (40) observed that protein added to the strong sulfuric acid digestion mixture caused lignin values much higher than were obtained when protein was absent. The effect of protein was thought to be due to the linkage of protein fission products with lignin. The size of these nitrogen-containing incrustants is probably rather small. It does not seem reasonable that a peptide linkage

could withstand the vigorous hydrolytic action of the strong sulfuric acid for any considerable length of time. By adding a definite amount of protein to a lignin-sulfuric acid mixture, Norman and Jenkins showed that the amount of nitrogen added to the lignin relative to its increase in weight would not permit the addition of a very large nitrogen-containing molecule.

A study of the nitrogen adhering to the Davis and Miller lignin and its relation to the distribution of the nitrogen within the indigestible residue is recorded in table 2. It will be observed that from 50 to 67 percent of the nitrogen of the indigestible residue is retained as a contaminant of the lignin fraction. Since the size of the nitrogen-containing fission product of the protein is apparently very small, the error introduced by it in increasing the lignin value is probably not of great consequence. There is no stoichiometric relation between the amounts of nitrogenous component and of lignin, as shown in columns 2 and 4 of table 2.

TABLE 2. The Nitrogen Contaminations of Davis and Miller Lignin and Their Relation to the Distribution of the Nitrogen of Indigestible Residue

| Kind of grass | N | | Lignin N % | Total N of i. r. in lignin % |
|---|---|---|---|---|
| | I. R. | Lignin | | |
| Sand paspalum | 4.2 | 2.1 | 1.6 | 50.0 |
| Blue grama | 5.7 | 2.2 | 1.5 | 66.7 |
| Switch grass | 5.3 | 3.3 | 1.8 | 62.5 |

While the presence and the effect of ash in the indigestible residue is not as difficult to study as is that of protein, it nevertheless may cause considerable error if ignored entirely. Table 3 shows

the relative amounts of ash removed by the extracting processes to which samples are subjected in the preparation of indigestible residue. The amount of ash present is sufficient to require its determination and the application of corrections for its presence in those fractions isolated to represent a definite indigestible constituent.

TABLE 3. The Removal of Ash from Grasses by Solution During the Davis and Miller Enzymatic Digestion Procedure

| Kind of grass | Ash | | | |
|---|---|---|---|---|
| | Untreated | Treated | Removed | I. R. |
| | | | % | % |
| Purple sandgrass | 52.8 | 23.0 | 55.5 | 3.7 |
| Sand lovegrass | 58.1 | 12.2 | 79.0 | 2.0 |
| Blowout grass | 48.0 | 27.6 | 42.5 | 3.6 |
| Sand legume | 68.8 | 19.2 | 72.2 | 3.0 |
| Switch grass | 46.8 | 17.5 | 62.6 | 2.6 |

In these experiments no lignin was isolated that did not contain some ash. This causes no particular concern, however, as lignin is determined by difference upon ignition. Ash is a very definite contaminant in the case of both Van Beckum and Ritter cellulose and Kürschner and Hanak cellulose, as shown in table 4. The cellulose of the latter method is determined as weight loss upon ignition, and the presence of ash would therefore not affect its yield. However, the Van Beckum and Ritter cellulose is weighed directly, and its apparent value would therefore be increased by the presence of ash. As shown in table 4, the process of Van Beckum and Ritter removes very little of the ash contaminating the indigestible residue. It therefore seems desirable to determine the amount of ash in this cellulose and correct for its presence in calculating cellulose results. This has been the procedure followed in this experiment.

Values for hemicelluloses obtained by the Davis and Miller procedure are calculated by difference as total indigestible residue minus the sum of the lignin and cellulose values directly determined. The error in hemicelluloses, therefore, includes, in addition to all experimental errors, the errors due to the presence of protein and ash in the indigestible residue, unless proper corrections are applied. Corrections for ash contaminations were made in this research for all values obtained by this method.

TABLE 4. The Ash Contaminations of the Kürschner and Hanak Cellulose and Van Beckum and Ritter Cellulose and Their Relation to that Present in Indigestible Residue

| Kind of grass | Ash | | |
|---|---|---|---|
| | I. R. | Van Beckum and Ritter cellulose | Kürschner and Hanak cellulose |
| Purple sandgrass | 23.0 | 22.5 | 13.5 |
| Hairy grama | 48.2 | 43.0 | 35.6 |
| Giant reedgrass | 27.5 | 22.8 | 18.7 |
| Switch grass | 17.5 | 15.8 | 16.5 |

The Williams and Olmsted procedure previously used in this laboratory (20) was the first method studied for the partition of the indigestible residue into lignin, cellulose, and hemicellulose. In the original method the residue of an enzymatic digest was treated with 60 percent sulfuric acid to dissolve hemicelluloses and cellulose and isolate lignin. The work of Waksman and Tenney (65) and of Norman (38) showing the failure of this concentration of sulfuric acid to dissolve natural cellulose quantitatively was substantiated in this laboratory. When 60 percent sulfuric acid was employed the lignin flocs were very gelatinous, and extremely high values, even exceeding at times the original weight of the sample taken for analysis, were obtained.

The hemicelluloses, cellulose, and lignin of several samples were determined by the Williams and Olmsted procedure using two different concentrations of acid, and the results are compared in table 5. The cellulose results obtained with 72 percent sulfuric acid are higher, and the lignin values lower than when 68 percent sulfuric acid is used. This indicates that 72 percent sulfuric is a more effective cellulose solvent than the 68 percent acid. For this reason 72 percent sulfuric acid was used in subsequent analysis.

TABLE 5. The Effect of the Concentration of Sulfuric Acid on Partition of Indigestible Residue by Williams and Olmsted Procedure

| Kind of grass | Lignin | | Cellulose | | Hemicellulose | |
|---|---|---|---|---|---|---|
| | 68% | 72% | 68% | 72% | 68% | 72% |
| | Acid digestion | | Acid digestion | | Acid digestion | |
| Little bluestem | 293.9 | 254.6 | 211.2 | 246.0 | 126.4 | 126.4 |
| Sand paspalum | 211.6 | 190.7 | 215.0 | 234.0 | 121.2 | 119.4 |
| Hairy grama | 251.0 | 191.5 | 204.0 | 231.0 | 91.2 | 94.7 |
| Blowout grass | 267.3 | 204.0 | 168.0 | 180.0 | 129.7 | 129.7 |

In table 6 the results of the complete partition of the indigestible residue of several samples are presented. The recoveries are determined by the summation of these fractions; in most of the samples they are quite low.

Norman (38) stated that it is impossible to recover all the natural cellulose of forage grasses by hydrolysis and subsequent determination of glucose, but that it may be possible to demonstrate complete recovery upon materials yielding pure glucose. As shown in table 7, 96 percent of maltose and filter paper can be thus recovered. This indicates that pure substances yielding reducing sugars stable to mineral acid hydrolysis may be determined by such a procedure.

TABLE 6. The Amount of Indigestible Residue Recovered from the Williams and Olmsted Partition Employing the Use of 72 Percent Sulfuric Acid

| Kind of grass | Lignin | Cellulose | Hemicellulose | Recovered | I. R. |
|---|---|---|---|---|---|
| Little bluestem | 254.6 | 246.0 | 126.4 | 627.0 | 650.3 |
| Sand paspalum | 190.7 | 234.0 | 119.4 | 545.9 | 672.6 |
| Blowout grass | 204.0 | 180.0 | 129.7 | 511.2 | 622.7 |
| Blue grama | 129.2 | 200.6 | 63.4 | 392.2 | 523.2 |

TABLE 7. The Recovery of Glucose from Strong Acid Hydrolysis

| Kind of material | Weight sample mg. | Glucose recovered mg. | Percent recovery |
|---|---|---|---|
| Maltose | 300.0 | 300.0 | 96.0 |
| Filter paper | 300.0 | 320.0 | 96.0 |
| Glucose | 200.0 | 200.0 | 100.0 |

Pentose sugars are unstable in strong mineral acid. A xylose-arabinose mixture treated for one hour with cold 72 percent sulfuric acid is only 80 percent recoverable, as shown in table 8. This table also shows that the recovery of the xylose-arabinose mixture after treatment with boiling 5 percent sulfuric acid is 98 percent of the theoretical. Norman believed that the effect of strong acid hydrolysis would be even more marked upon the pentosans of the cell walls of plants. It is shown in column 5 of table 9 that the percent of pentoses recovered when the cell wall materials are subjected to the hydrolytic action of strong acid is in most cases even less than that obtained when pure pentoses are similarly treated. Thus the Williams and Olmsted analysis accounts for only part of the pentoses of the hemicelluloses contained

in the indigestible residue. Also, pentoses derived from cellulosans surviving the strong acid treatment would not be included as part of the cellulose fraction, but would be included with apparent hemicelluloses. However, the glucose derived from pure cellulose may reasonably be expected to be accurately estimated.

TABLE 8. The Effect of Sulfuric Acid Concentration on the Recovery of Pentose Sugars

| Sugars used | Weight sample | Weight recovered from 72% sulfuric acid cold | 5% sulfuric acid boiling | distilled water boiling |
|---|---|---|---|---|
| 1:1 ratio xylose-arabinose | 200.0 | 160.0 | 196.0 | 200.0 |

TABLE 9. Comparative Results of Lignin Determinations by the Williams and Olmsted and the Davis and Miller Methods and the Effect of the Former on Pentose Yield

| Kind of grass | Lignin | | Total pentoses | |
|---|---|---|---|---|
| | Williams and Olmsted | Davis and Miller | Olmsted and Williams lignin filtrate | I. R. |
| Little bluestem | 254.6 | 220.5 | 126.4 | 231.0 |
| Sand paspalum | 190.7 | 144.8 | 121.2 | 147.2 |
| Hairy grama | 191.5 | 155.0 | 94.7 | 162.5 |
| Blowout grass | 204.0 | 167.0 | 129.7 | 204.0 |
| Buffalo grass | 174.8 | 140.0 | 125.0 | 191.3 |
| Sand dropseed | 204.0 | 163.4 | 148.5 | 179.7 |

Norman and Jenkins (40) observed that the apparent lignin value is greatly increased by the presence of pentoses in the strong acid digestion mixtures used in isolating lignin. This is probably owing to the synthesis of furfural from pentoses, followed by the formation of an insoluble furfural-lignin complex which is weighed as apparent lignin.

The Williams and Olmsted lignin values presented in table 9 are all considerably higher than are the respective values of Davis and Miller. The 5 percent sulfuric acid pretreatment of Davis and Miller removes all or nearly all of the pentoses comprising the true hemicellulose fraction. Thus the difference between these two lignin values represents the differences due to the presence of pentoses in the digestion mixture. It may also be noted that the increase in apparent lignin is not correlated with the decrease in apparent hemicelluloses. An accurate estimation of either lignin or hemicellulose without a separation of the hemicelluloses from the indigestible residue previous to strong acid hydrolysis does not seem possible.

Hemicelluloses are defined as those polysaccharides convertible into simple sugars by heating with dilute acids at atmospheric pressure. The use of 5 percent sulfuric acid was adopted by Norman as a pretreatment in the determination of lignin by 72 percent sulfuric acid hydrolysis. Davis and Miller have made use of 5 percent acid hydrolysis to remove hemicelluloses from the indigestible residues of forage grasses, previous to the determination of lignin. It is shown by table 10 that from 65 to 75 percent of the total pentoses of the indigestible residue are removed by 5 percent sulfuric acid treatment. Norman (34) has shown that an extremely vigorous reaction with acid or alkali is necessary for the removal of the remaining pentoses. It was concluded that boiling 5 percent sulfuric acid extracted most of the pentoses due to hemicelluloses and, therefore, this hydrolytic treatment was incorporated in the Williams and Olmsted procedure to facilitate in partitioning the indigestible residue of forage grasses.

TABLE 10. Comparative Results of Pentose Determinations by the Reducing Sugars and Furfural Yield after Separation of Pentoses by the Davis 5 Percent Sulfuric Acid Treatment

| Kind of grass | Reducing sugars | | Furfural yield | |
|---|---|---|---|---|
| | Filtrate | Residue | Filtrate | Residue |
| Sand bluestem | 215.5 | 83.5 | 226.7 | 82.7 |
| Sand dropseed | 207.0 | 96.0 | 209.0 | 95.0 |

The pentoses that are not removed from the indigestible residue by the 5 percent sulfuric treatment apparently have no appreciable affect on the lignin results. The reason may be their low concentration. After strong sulfuric acid hydrolysis of the 5 percent acid pretreated residue, most of the remaining pentoses can be accounted for by the reducing sugar technique, as is shown in table 10. This would indicate that the pentoses of the cellulosan fraction are reasonably stable in strong sulfuric acid; thus neither furfural nor the consequent lignin-furfural complex would be formed. By careful technique it is possible to determine the amount of non-fermentable reducing sugars in this strong acid hydrolysate. Addition of the value thus received to the cellulose value yields results that are both more satisfactory analytically and more conformable to the theory presented in the introduction. Alternatively the percent of pentoses due to cellulosans may be calculated from the difference between the total pentoses of the indigestible residue and the pentoses of the hemicelluloses that are extracted by 5 percent sulfuric acid. As illustrated in table 10, furfural determination (17) was found to be an accurate and easily manipulated method of estimating pentoses. Almost identical results were obtained for the hemicelluloses partitioned with 5 percent sulfuric acid, and

for the cellulosan pentoses of the subsequent strong acid hydrolysis by both the furfural and the reducing sugar methods. In view of the somewhat uncertain stability of the pentoses of the cellulosans in strong acid solution, and the errors inherent in determining sugars at such low concentrations it appeared that the pentoses of cellulosans could be more reliably estimated by an indirect method employing the furfural determination. By subtracting the percent pentoses of the hemicelluloses determined by the reducing sugar technique applied to 5 percent sulfuric acid extract from the total percent of pentoses determined on the indigestible residue by the furfural procedure, a value representing the pentoses of the cellulosans was secured which was comparable to that determined by the direct reduction method. Accordingly, the Williams and Olmsted method was further modified to include a determination of total pentoses by furfural yield, and the error in both hemicelluloses and celluloses due to the neglect of the cellulosan fraction by the original procedure is corrected. Table 11 shows the results of the modified method as applied to partitioning of the indigestible residues derived from forage grasses. The sum of the percentage of the fractions partitioned is practically identical with the percentage of the indigestible residue.

It seemed desirable to check the values for certain of the fractions determined by the modified method by comparing with values determined by direct and independent methods based on different principles. The determination of cellulose by the Kürschner and Hanak procedure was used in this research to check the cellulose values of the modified method. It is based on the principle that lignin is nitrated by boiling dilute nitric acid and the resulting "nitrolignin" is sol-

uble in concentrated acetic acid. Also, hemicelluloses are hydrolyzed and extracted by the acid treatment. Reid and his co-workers (49) have shown that lignin-free cellulose is not isolated by the Kürschner and Hoffer procedure. Their results indicate that by analysis of this cellulose for lignin by the 72 percent sulfuric acid method and correction for the lignin contamination, low results for cellulose were obtained as compared to those from other procedures. The reason for this was not determined. Likewise, lignin-free cellulose was not obtained by the Kürschner and Hanak procedure in this laboratory. Also, low cellulose values were obtained by correcting for lignin contamination. It appeared that a critical analysis of the Kürschner and Hanak cellulose should be made in order to discover the sources of these errors.

TABLE 11. The Partition and Recovery of Indigestible Residue by the Modified Procedure

| Kind of grass | I. R. | Lignin | Cellulose | Hemicellulose | Ash | Recovered |
|---|---|---|---|---|---|---|
| Sand dropseed | 43.37 | 12.78 | 21.70 | 6.70 | 1.66 | 42.84 |
| Switch grass | 44.13 | 8.06 | 22.56 | 7.89 | 1.75 | 40.26 |
| Hairy grama | 48.29 | 9.30 | 25.50 | 5.70 | 4.80 | 45.33 |
| Blowout grass | 42.11 | 9.55 | 23.21 | 5.64 | 2.76 | 41.26 |
| Sand paspalum | 44.22 | 11.20 | 23.89 | 4.26 | 3.63 | 42.98 |
| Sand bluestem | 51.22 | 18.00 | 22.42 | 9.45 | 1.06 | 50.93 |

The value of the pure cellulose fraction retained by the Kürschner and Hanak cellulose was calculated from the amount of glucose yielded by strong acid hydrolysis. The results of this analysis are compared with the Williams and Olmsted cellulose in columns 4 and 5 of table 12. The consistently lower value for Kürschner and Hanak pure cellulose may indicate that some of the hexosans are extracted from the cellulosans by the acid mixture used in this procedure. A further analysis of the Kürschner and Hanak cellulose was made to discover the effect of this acid mixture on the pentose fraction of the cellulosans. The values for the pentoses of the cellulosans determined by the modified procedure, are compared to those of the pentoses from the Kürschner and Hanak cellulose determined by the furfural yield, as shown in columns 1 and 2 of table 13. This comparison shows that a considerable amount of the pentoses of the cellulosans is removed by the acid mixture of the nitrating medium. The lignin contamination of the Kürschner and Hanak cellulose is appreciable, as shown in column 2 of table 12. The analysis indicated in this paragraph shows that the Kürschner and Hanak cellulose contains only part of the cellulosans and is contaminated by small amounts of lignin, and thus this procedure does not isolate a fraction of the indigestible residue truly representative of cellulose. However, the Kürschner and Hanak procedure does give a cellulose value comparable to the modified cellulose value providing that no correction for the presence of lignin in the former cellulose is made. While this procedure is not theoretically sound, it seemed advisable to adopt it, since by so doing hemicelluloses could be calculated by subtracting the sum of lignin and cellulose from the indigestible residue. This makes possible complete partition

of the indigestible residue.

TABLE 12. The Composition of Kürschner and Hanak Cellulose and the Comparison of Its Pure Cellulose Value to that Determined by the Williams and Olmsted Procedure

| Kind of grass | Kürschner and Hanak cellulose | | | | Williams and Olmsted cellulose |
|---|---|---|---|---|---|
| | Total | Lignin | Pentoses | Pure cellulose | |
| Sand lovegrass | 359.3 | 59.3 | 52.8 | 243.0 | 267.5 |
| Switch grass | 230.0 | 26.0 | | 176.4 | 193.4 |
| Blowout grass | 358.0 | 40.3 | 53.0 | 256.5 | 266.5 |
| Buffalo grass | 306.3 | 65.5 | | 205.2 | 216.0 |

TABLE 13. Comparison of the Amount of Pentoses in the Cellulose as Isolated by the Kürschner and Hanak and by the Van Beckum and Ritter Procedures to that of the Modified Method

| Kind of grass | Pentoses of cellulose | | |
|---|---|---|---|
| | Kürschner and Hanak | Van Beckum and Ritter | Modified |
| Sand lovegrass | 52.8 | | 102.6 |
| Blowout grass | 53.0 | | 100.0 |
| Switch grass | | 65.0 | 68.7 |
| Purple sandgrass | | 58.5 | 56.8 |
| Sand bluestem | | 67.5 | 73.0 |

The method reported by Van Beckum and Ritter (64) for the partition of lignin, cellulose, and hemicelluloses of wood offers possibilities for partitioning indigestible residue. This procedure is based on the principle that in a moist medium chlorine will substitute into the lignin molecule, and render it extractable by an alcohol solution of monoethanolamine. The chlorination and extraction process leaves a residue containing the entire carbohydrate fraction of wood. Subsequent hydrolysis of this residue with weak acid extracts the pentoses derived

from the hemicelluloses thus isolating crude cellulose. Since the determination of lignin and cellulose by this procedure is based on entirely different principles than the modified method, it was thought that a comparison of the fractions determined by this method with those determined by the modified procedure would prove valuable in establishing the experimental accuracy of the modified procedure. A partition of indigestible residue was accordingly made by the Van Beckum and Ritter method.

It was found that the cellulose value, corrected for ash, was higher than the value secured by the modified procedure. Therefore, an analysis was made of the Van Beckum and Ritter cellulose to determine the percent of contaminants, of pure cellulose, and of pentoses derived from the cellulosans to discover which of these fractions caused the high result. The crude cellulose fraction was subjected to the usual hydrolysis with strong sulfuric acid followed by filtration to isolate lignin. Lignin values, which are reported in column 2 of table 14, were found to be of the same order as those of the Kürschner and Hanak cellulose. This may indicate that there exists a linkage between lignin and the polysaccharides which is not broken by the substitution reactions and subsequent extractions of these two methods. Norman (38) claims that the failure to break carbohydrate-lignin linkages is a common weakness of most lignin-substitution and -extraction methods when they are applied to forage grasses. The weight of pure cellulose present in the crude cellulose is calculated from the glucose yield determined on the acid hydrolysate. A comparison of the amount of pure cellulose in the Van Beckum and Ritter crude cellulose with that of the Williams and Olmsted cellulose shows them

to be equal, as shown in columns 4 and 5 of table 14. This indicates that glucose derived from the cellulosan fraction of the crude cellulose is probably not removed by the chlorination and extraction employed by the Van Beckum and Ritter procedure. Since the removal of glucose derived from cellulosans is not probable, the removal of the pentoses from the same source would not be expected. The weight of pentoses derived from the Van Beckum and Ritter cellulose as determined by the furfural yield is equal to the value for pentoses derived from cellulosans as determined by the modified method. (See columns 3 and 4 of table 13). It may be concluded from the above analysis of crude cellulose that the Van Beckum and Ritter procedure isolates a cellulose comparable to that from the modified procedure except that it is contaminated lignin.

TABLE 14. The Composition of Van Beckum and Ritter Cellulose and the Comparison of Its Pure Cellulose Value to that Determined by the Williams and Olmsted Procedure

| Kind of grass | Van Beckum and Ritter cellulose | | | | Olmsted cellulose |
|---|---|---|---|---|---|
| | Total | Lignin | Pentoses | Pure cellulose | |
| Blue grama | 381.5 | 43.5 | 59.0 | 288.0 | 290.0 |
| Sand bluestem | 366.0 | 53.0 | 67.5 | 234.0 | 235.0 |
| Purple sandgrass | 311.0 | 46.6 | 58.5 | 206.5 | 214.8 |
| Sand dropseed | 370.0 | 71.6 | 33.0 | 242.2 | 244.6 |

The lignin results determined by the Van Beckum and Ritter procedure are usually lower than determined by the modified method. This would be expected, since part of the lignin is retained by the holocellulose fraction. However, the lignin values corrected for the lignin retained by the holocellulose are greater than those of the modified procedure. This anomaly is very likely due to the presence of

protein in the indigestible residue, which would probably also be removed by the chlorination and extraction process. With this situation existing, apparent lignin values may even be high, although a considerable portion of the lignin is actually retained as a contaminant of the cellulose fraction. For this reason the determination of lignin by the Van Beckum and Ritter procedure is not dependable when applied to the indibestible residue of forage grasses. Modifications in this procedure to eliminate the lignin error in the cellulose value and to remove a greater amount of the protein from the indigestible residue would be desirable.

The weight of the hemicelluloses as determined by the Van Beckum and Ritter procedure is comparable to that determined by the modified method. This would be expected, since the Van Beckum and Ritter chlorination and extraction should have little effect on the hemicelluloses or those polysaccharides associated with them as has been indicated in the introduction. Therefore, the holocellulose fraction should be identical, in respect to polysaccharides, with the indigestible residue. The hydrolysis with weak acid, employed by these two methods, should yield equal percentages of hemicelluloses.

Table 15 records the results of a complete analysis of the indigestible residue by the Van Beckum and Ritter, Davis and Miller, and modified procedures. A comparison of these results shows that for some grasses (purple sandgrass, sand bluestem, and switch grass) the methods of Van Beckum and Ritter and Davis and Miller yield in all fractions the results obtained by the modified procedure. In other grasses (giant reedgrass, sand dropseed, and hairy grama) variations in results by the different methods occur in some fractions of the

partitioned residue. This would be expected because of the failure of these methods to produce identical fractions, and because of the differences in the nature of the various indigestible residues analyzed.

TABLE 15. Comparison of the Results of the Partition of the Indigestible Residue of Forage Grasses by the Modified, the Van Beckum and Ritter and the Davis and Miller Procedures

| Kind of grass | Indigestible residue | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Total | Lignin | | | Cellulose | | | Hemicelluloses | | |
| | | Modified | Van Beckum and Ritter | Davis and Miller | Modified | Van Beckum and Ritter | Davis and Miller | Modified | Van Beckum and Ritter | Davis and Miller |
| Switch grass | 671.9 | 221.2 | 183.6 | 221.2 | 312.0 | 324.3 | 312.0 | 130.5 | 128.8 | 122.2 |
| Hairy grama | 681.4 | 226.5 | 170.0 | 226.5 | 294.0 | 353.0 | 296.0 | 105.4 | 117.8 | 100.7 |
| Giant reedgrass | 713.5 | 249.8 | 247.6 | 244.8 | 386.0 | 384.0 | 424.0 | 87.7 | 66.8 | 39.7 |
| Purple sandgrass | 651.5 | 210.2 | 190.0 | 210.2 | 317.0 | 324.3 | 301.5 | 129.7 | 129.7 | 123.2 |
| Sand dropseed | 577.8 | 139.3 | 117.4 | 139.3 | 328.4 | 344.0 | 318.0 | 67.4 | 75.0 | 106.4 |
| Sand bluestem | 652.1 | 208.8 | 211.5 | 208.8 | 326.5 | 346.0 | 322.0 | 104.3 | 107.0 | 106.7 |
| Sand bluestem (Month younger) | 646.7 | 181.8 | 211.5 | 181.8 | 335.0 | 336.0 | 326.0 | 135.6 | 151.5 | 129.9 |

## RESULTS AND CONCLUSIONS

The Davis and Miller procedure was chosen for preparing indigestible residue. The effects of ash and protein contaminations were studied, and corrections were made for errors resulting from the presence of ash.

It was found that the Williams and Olmsted procedure did not properly allocate the pentoses of the cellulosans of the crude cellulose fraction, that pentoses were condensed in the 72 percent sulfuric acid solution to furfural, and that a furfural-lignin complex was formed which caused an increase in apparent lignin values.

A modified method was developed which made corrections for the errors of the Williams and Olmsted procedure. The results were in substantial agreement with those obtained by the Davis and Miller and Van Beckum and Ritter procedures for partitioning cellulose.

It is concluded that the modified procedure is theoretically sound and experimentally dependable for the partitioning of the indigestible residue of forage grasses.

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