STUDIES ON THE HEMICELLULOSE FRACTION OF PLANT TISSUE

by

John Grant Armstrong

-0-

A Thesis

Presented to the Faculty of Graduate Studies and Research of McGill University, Montreal, in partial fulfilment of the requirements for the degree of

MASTER OF SCIENCE

September, 1941.

TABLE OF CONTENTS

HISTORICAL

An accurate and rapid method for the determination of hemicellulose both in woody and in succulent tissues is seriously needed at the present time. Such a method is desired by many workers in such widely different fields as plant physiology, microbiology, entomology, and animal nutrition. The methods now being used, although quite inadequate, will be briefly examined.

These methods may for convenience be considered as of three types (43):

(1) Estimation by hydrolysis and determination of the reducing sugars liberated.

(2) Estimation by a determination of the furfural yield with calculation therefrom of the apparent pentosan content.

(3) Isolation and weighing as hemicellulose.

In every method it is necessary to prepare the material for treatment and to remove interfering substances. In the different methods various treatments have been suggested in an attempt to insure the complete appearance of hemicellulose in the material finally determined, and to insure the absence of possible interfering substances.

Each of the three types of procedure will be considered in turn. Some of the more recently used methods in each case will be mentioned and some of the criticisms that have been brought against each type will be offered.

TYPE 1. Hydrolysis and Determination of Reducing Sugars.

Essentially this procedure involves the hydrolysis of a sugar and starch-free-residue with dilute mineral acid, followed by

a determination of the reducing sugars produced. The method of Williams and Olmsted (56) is a commonly used one. They have employed the Shaffer-Somogyi reagent to determine the total reducing sugars produced, then fermented a portion using the Somogyi washed yeast procedure and again determined the reducing sugars. Hemicellulose (or pentosan) is given as 0.88 x the non-fermentable reducing sugar.

This calculation is based on the assumption that all of this non-fermentable reducing material is xylose and arabinose and that these sugars are entirely non-fermentable. It is also of necessity based arbitrarily on a fixed ratio of xylose to arabinose, and because of a difference in reducing powers of these two sugars, will consequently involve some error should they be formed in a different ratio.

It has been shown by Malhotra (30) that results may be influenced by conditions of hydrolysis. Thus the concentration of the acid, the amount of the acid solution, and heating conditions may affect results. This makes any method of this type purely arbitrary.

Considerable differences are found among different workers in treatment of the material before hydrolysis. In some cases, (57) (22), (31) , and extraction has been made with diethyl ether to remove fats. In all cases the soluble sugars and starches are removed. Ethyl alcohol is commonly employed to remove sugars but the ratio of weight of solvent to weight of material varies with different workers. Starch has been removed by enzyme digestion using buffered takadiastase (58), pancreatic amylase digestion in buffered-bile salt solution (22), (56), (57), or saliva (30), (31).

It was observed by O'Dwyer (41) that a hemicellulose preparation on treatment with takadiastase was split up into two polysaccharide fractions. Thus it would seem that care should be taken to check the activity of enzyme preparations used in this work.

In one study in which hemicellulose was determined in three year old apple and pear twigs, and in six month old spinach and tomato plants by a method of this type (29), it was found that a 60-mesh or finer material was required to give uniform results. It was also shown that samples of 3.0 to 4.0 grams gave more satisfactory (higher) results than either smaller or larger ones. It was suggested that weighing errors are more serious with smaller samples, and that a large excess of reagents with small samples might cause undesirable destruction of materials while the same quantity of reagents is commonly used with the larger samples where it is possibly insufficient to completely react with the whole of the material.

In addition to these factors which have been shown possibly to influence results in methods of this type, there are certain fundamental objections:

(1) Certain hemicelluloses form comparatively resistent aldobionic acids which are decomposed only by treatments which cause degrading of some non-hemicellulosic materials.

(2) Uronic acids heated with mineral acids form degradation products of unknown composition and of indefinite reducing power.

(3) The final calculation results in an approximation to the true hemicellulosic material only because the reducing value must be expressed as apparent glucose (or xylose) although it is known to be due to a mixture of sugars.

TYPE 2. Determination of the Furfural Yield.

The methods following this type of procedure are based on the work of Tollens and co-workers (53), (32). The material is boiled with 12% HCl and the furfural produced is collected by allowing the vapors to distil over and pass through a water condenser.

Various methods have been used to determine the amount of furfural produced. The most commonly used method, and that adopted by the A.O.A.C., is to weigh the phloroglucide. Colorimetric methods have been proposed by Youngburg and Pucher (59), Hughes and Acree (24), and by Reeves and Munro (46). These methods have been proposed in attempts to obtain a more rapid estimation of furfural and greater accuracy in the estimation of small amounts of furfural. They have, however, not yet been proven of satisfactory reliability, and require careful attention in the preparation of calibration curves. The last of these (the Reeves and Munro method) does not require the distillation of the furfural from the residue of the substance which yields it, but rather involves its separation in a xylene layer. This method has not yet been shown to be applicable to different plant materials.

The most serious criticism of the methods of this type is, however, not in the estimation of the furfural produced from the sample, but rather in the way this furfural represents the hemicellulosic material in the plant. The pentoses present in hemicellulose are reported to yield only furfural when boiled with HCl (through splitting off three molecules of water), and the uronic acids to yield one molecule of CO_2 and one of furfural. Thus these parts of the hemicellulosic material may be estimated, although variable yields make even these estimates

somewhat inaccurate. There remains however, the hexosan part of hemicellulose which is not estimated, and the amount of this fraction seems to vary so widely that it would require a separate and detailed study of the composition of the hemicellulose to establish any measure of the extent of the error here introduced. This error will be generally greatest in the estimations of hemicellulose from non-woody tissues for these have commonly a higher hexose (galactose) content than woody tissues.

For the pentosan and polyuronide constituents of hemicellulose this type of analysis is however fairly satisfactory. The soluble sugars and then the pectic substances are removed. The total furfural yield on the residue is corrected for a small yield of furfural from the cellulosan in the Cross and Bevan cellulose fraction, and the result is taken to represent pentosans and uronic acids. The method is applicable (and is used as a routine test) on wood material where the hexose content of hemicellulose is low. Sometimes the uronic acid content is determined by measuring the CO_2 produced. Several methods (37), (15), also based on the work of Tollens and co-workers (53), (32), have been used for this.

It must be remembered however, that because of the variable yields mentioned above, even the pentosans are not fully accounted for in the furfural yield. Neither is the theoretical yield of furfural obtained from pentoses, nor do the individual pentose sugars give identical amounts. Kröber (25) constructed detailed tables for the furfural to be obtained from pentoses and pentosans, the latter figures being the mean of those given for araban and xylan. Thus even from

pentosans, unless the exact nature of the groups yielding the furfural is known their estimation by this method can be only an approximation.

A further error is suggested by work of Freudenberg and Harder (18) which showed that lignin, when subjected to the pentosan determination, liberates formaldehyde which forms a precipitate with phloroglucinol. Thus the lignin present in a sample might cause the apparent pentosan to have a higher value than is correct. It would seem that this error might be greatest in the estimation of hemicellulose in woody tissues because of the higher lignin content generally in these tissues.

Drying of samples has been shown by Campbell and Booth (13),(14), to cause a reduction in furfural yield in both hardwood (English oak) and softwood (Silver fir).

TYPE 3. Isolation and Weighing as Hemicellulose.

In this type of procedure an attempt is made to extract (with an alkaline reagent) the hemicellulose in a form that shall be as free as possible from any interfering substance, then precipitate and weigh this product. Corrections may be applied for impurities still present. Norman (38, p.64) says that the actual preparation and weighing is a somewhat tedious process, "but with certain modifications may be the most satisfactory means of obtaining a figure for hemicellulose content."

The discussion of this type of procedure falls into four sections.

(1) The Removal of Interfering Substances. Here will be considered some of the attempts that have been made to prepare the sample for the hemicellulose extraction.

(2) Extraction Methods. Many variations of method have arisen through attempts to develop an extracting technique which would avoid if possible the removal or degradation of non-hemicellulosic materials while still giving complete removal of hemicellulose.

(3) Holocellulose Preparation. By this means it is suggested that the lignin as an interfering substance may be removed while the hemicellulose is at the same time made more easily extractable.

(4) Some Comments on This Type of Procedure.

(1) The Removal of Interfering Substances. The main interfering substances that workers have attempted to remove before extraction of hemicellulose are: sugars, starch, pectic substances, proteins and fats. In that method which involves the preparation of holocellulose an attempt has been made to remove lignin completely. This is to be considered separately.

An extraction with water is commonly used. This will remove sugars, and, if hot, will be effective in the removal of starch, some pigments, and some proteins. Anderson and Krznarich (2) report that unless there is an extraction with hot water some starch will appear in the hemicellulose. There is generally also an extraction with some organic solvent which assists further with the removal of sugars, pigments, proteins and fats. Thus Phillips and Davis (44) reflux with a constant boiling mixture of ethyl alcohol and benzene for thirty hours, Anderson and co-workers (6) use diethyl ether and hot ethyl alcohol, and Krznarich (26) uses acetone and ethyl alcohol. Extractions with $(NH_4)_2\ C_2O_4$ are commonly used to remove pectic substances. Attempts have been made to remove a part of the lignin with dilute alcoholic NaOH, e.g., Phillips and Davis (44). Norman (38, p.57) cautions that this pretreatment should not be carried out unless it is previously

ascertained that no change in hemicellulosic materials is caused. He points out that, "if carried out at the boil this treatment seems to produce on many materials a degradative attack on the hemicelluloses, both polyuronide and cellulosan".

(2) Extraction Methods. The technique of extracting hemicellulose with alkaline reagents commonly employs NaOH but the concentration, time and temperature vary with different workers. Thus Anderson and Kinsman (1) extracted with 7% NaOH on a water bath for two hours; Burkhart (11) used 5% NaOH at room temperature in a shaking machine; Buston (12) used portions of 4% NaOH for several days at room temperature; MacMasters, Woodruff and Klaas (28) extracted with 2% $Na_2 CO_3$ on a steam bath for four hours, gave a short exposure to dilute hypochlorite and then extracted twice with 0.5% NaOH, first on a steam bath for thirty-five minutes, then at room temperature for eighteen hours; O'Dwyer (42) using mechanical stirring extracted twice with 0.2% NaOH for four hours and forty-eight hours respectively, then twice with 4% NaOH for forty-eight hours each; Preece (45) employed 4% NaOH first in the cold for two extractions of forty-eight hours each, then on a water bath for five extractions of six hours each, and finally refluxed for one extraction of one hour.

These many different techniques have been developed to minimize inaccuracies due to the fact that when the extracting procedure is sufficiently vigorous to give an approximately complete removal of hemicellulose a danger arises that cellulosan material will also be extracted and that preparations will be contaminated with lignin. It is

relevant to this problem that Reid et. al. (47) found another alkaline reagent - hot monoethanolamine - to be very satisfactory for the determination of cellulose "particularly for fibrous farm wastes". No other record was found of attempts to use this reagent for the extraction of hemicellulose.

It has been recognized that extraction procedures are made more difficult because of the fact that a portion of the hemicellulose is probably associated or combined with some other cell-wall constituent. Thus in 1931, Preece (45) made a distinction between what he called "free" and "combined" hemicellulose, the difference being exhibited in their ease of extraction. And in 1934 Harris, Sherrard, and Mitchell obtained evidence which suggested the existence of a hemicellulose - lignin complex.

Some workers, e.g., (50), (5), (28) have attempted to break this suggested bond by oxidizing the lignin. Commonly chlorine or hypochlorite have been used for this purpose, a portion of the hemicellulose being extracted before and a further portion extracted after this treatment. In one case (4) bromination has been reported more satisfactory than chlorination to free a hemicellulose preparation of lignin.

(3) Holocellulose Preparation. This method which attempts to remove lignin before the hemicellulose is extracted, differs considerably from the other methods of this type. The lignin-free product, called holocellulose, it is stated, "may be taken as representing virtually all polyuronide hemicelluloses together with natural cellulose..... and.....would seem to be an ideal starting

material for hemicellulose investigations".....Norman (38,pp.20, 58).

Ritter and co-workers (27), (48), (54), (55), (36), have been the leaders on this continent in the preparation of this material. In 1933 (48) and 1934 (27) procedures were developed to extract lignin from ethyl alcohol-benzene and hot water - extracted hardwood (maple) and softwood (spruce) by alternate extractions with Cl_2 and an alcoholic solution of pyridine. The hardwood extract could be bleached white with the complete removal of lignin by $CaOCl_2$. . There was, however, a loss of hemicellulose when this was done with softwood so it was omitted and the residue contained a trace of lignin.

In 1937 (54) and 1938 (55), alternate extractions with Cl_2 and 3% monoethanolamine in 95% ethyl alcohol greatly reduced the time required and also provided a sharp color end point for the extraction of lignin. This material has been used (36) as a source of hemicellulose. The latter was obtained in four fractions by successively extracting the holocellulose with boiling water, with 2% Na_2CO_3 in the cold, with 4% NaOH at room temperature, and with boiling 10% NaOH.

(4) Some Comments on This Type of Procedure. Certainly the methods now in use are too lengthy to be satisfactory for routine work. The use of other alkaline extracting agents as was suggested by the work of Reid, et. al. (47) might offer means of improving present methods.

Nevertheless it seems necessary to use mild extractions. Norman (38 p.56) states, "When extraction is performed with cold alkali the amount of cellulosan removed is probably small compared to the polyuronide. On the other hand, hot alkali undoubtedly produces an extensive removal of cellulosan accompanied by the less

resistant hexosan from the cellulose". Methods such as those of Phillips and Davis (44) have in this respect much value, but the times involved make them impractical for many purposes. Vigorous agitation during extractions would seem to offer a means of reducing the times required for these steps and possibly also to reduce the number of extractions needed. The use of a centrifuge would seem also to be valuable in some cases to reduce the time needed to separate extracts from residues.

Lignin presents a great problem, both in pretreatments for removal of possible contaminating materials, and in the freeing of hemicellulose to permit its complete extraction. The value of alcoholic NaOH as an agent for removal of lignin prior to the extraction of hemicellulose is not well established. Norman has been reported above cautioning against the use of a hot extraction, and he states (38, p.57) that "alternatively, if performed cold, this treatment is peculiarly ineffective as a delignifying agent, though a useful purpose may be served in the removal of a portion of the protein". Phillips and Davis (44) however, report the removal of lignin amounting to 8.2% of a ethyl alcohol-benzene, water, and $(NH_4)_2\ C_2O_4$ extracted sample of alfalfa hay or 5.0% of the ethanol-benzene extracted hay by three extractions, each consisting of covering the material with dilute alcoholic NaOH for 24 hours and stirring occasionally. The removal of lignin would seem more certain in the preparation of holocellulose. However to make this a part of a procedure for the estimation of hemicellulose, it would need to be shown definitely that no hemicellulosic materials are removed in this step. No record was found of this having been done.

Some means of removal lignin seems nevertheless to be necessary to permit complete extraction of hemicellulose. Norman (38, p.62) states, "Recognition of the existence of a hemicellulose-lignin complex will make preparatory work simpler in that the purpose of the initial treatment must be the rupture of this association which may be brought about not only by treatment with alkali, but by chlorination or oxidation of the lignin and probably by other means yet to be devised". Before adopting any procedure designed to remove lignin and so to free hemicellulose, it would seem necessary to ascertain the effect of this treatment on the non-lignin materials. Possible destruction of hemicellulosic materials or conversion of cellulose to a form that would be estimated with hemicellulose would have to be investigated. Although chlorination treatments have been used, their effects do not seem to have been fully studied.

O'Dwyer (40) has reported that the drying of wood materials may affect the hemicellulose extractable by 4% NaOH. This effect was so great as to give only 50% of fresh material yield if wood were dried in an oven at 100°C. Reference was made above to a decrease in furfural-yield from wood as a result of drying, (13), (14).

If the hemicellulose after extraction is to be estimated by precipitation and weighing some care should be taken to ensure completeness of precipitation. Angell and Norris (6), (7), showed that different hemicellulose preparations could have different optimal pH's for their precipitation. Norris and Preece (39) considered precipitation of hemicellulose to be complete after acidification and addition

of one volume of acetone. Phillips and Davis (44) obtain a further precipitate on the addition of one volume of 95% ethyl alcohol to this filtrate.

EXPERIMENTAL

The experiments which have been carried out were designed to give further information regarding certain possible means of improving the methods of estimating hemicellulose by its isolation and weighing. Specifically these experiments studied:

(1) The use of monoethanolamine as an extracting agent for hemicellulose.

(2) The extraction of hemicellulose by vigorous agitation with 5% NaOH following attempted removal of lignin
   (a) with alcoholic NaOH.
   (b) by preparation of holocellulose.

(3) The effect of alternate extraction with 4% NaOH and brief exposure to chlorine.

(1) The Use of Monoethanolamine as Extracting Agent.

Following the method of Reid et. al. (47) in the determination of cellulose in fibrous agricultural wastes, a sample of oat straw was extracted with monoethanolamine and a "crude cellulose" obtained as the residue.

This oat straw sample had been collected immediately after threshing and ground in a Wiley mill to pass 40 mesh. A small portion of the straw that was not reduced to 40 mesh size, but passed repeatedly through the mill, had been discarded. This amounted to 7.7% of the total weight of the fresh straw (180 grams). The whole of the material passing the 40 mesh sieve was saved and constituted the sample of oat straw used throughout this work.

The monoethanolamine had been redistilled and only that portion boiling 170° -173° C. was used in these experiments.

An all-glass reflux apparatus was used which consisted of a 250-ml. Erlenmyer flask fitted through a ground-glass joint to a water condenser.

Filtering was very slow with a 1-G-5 Jena fritted glass crucible but proceeded satisfactorily with the 1-G-2 filter.

From two samples of oat straw the crude cellulose obtained was 48.6% and 48.4% of the air-dry sample. The pentosan content of one of these as determined by the method of A.O.A.C. (35) was 24.3% of the crude cellulose sample. This represented 11.6% of the original air-dry sample of straw.

It must be noted here that the A.O.A.C. procedure recommends the use of a 300-ml. distillation flask. Such a flask was not readily available and these determinations, during the first part of this work, were carried out with the use of a 250-ml. flask.

The alpha-cellulose content of the second crude cellulose sample was found by the method of Bray (10) to be 73.8% which represents 35.8% of the original sample.

Reid et. al. give no values for analysis of oat straw by their method but report for rye straw as follows: crude cellulose 50.6%, pentosan in crude cellulose 34.0%, alpha-cellulose in crude cellulose 69.7%, and alpha-cellulose calculated on original material 35.3%. They conclude that the use of hot monoethanolamine as a reagent for the determination of cellulose in fibrous farm wastes was "found to be very satisfactory". Their crude cellulose fractions, corrected for ash, compared favorably with those of the Cross and Bevan, and Norman - Jenkins methods, and they report that "the precision" of their method was equal to that of the Cross and Bevan method.

The advantage they claim for their method over the other methods is in the saving of time and effort.

It is noticed that their reported values for rye straw are similar to the values here obtained for oat straw. The main difference lies in the lower value found for the pentosan content of the crude cellulose prepared from oat straw. It was considered however, that even this pentosan content was relatively high and indicated that a considerable portion of the hemicellulosic materials were not extracted by this reagent.

The efficiency of 4M aqueous monoethanolamine as an extracting agent was then studied. A portion of the ground oat straw sample described above, was pretreated as follows: dried in an oven at 105°C., extracted in a soxhlet with constant-boiling ethyl alcohol-benzene mixture for thirty hours, washed on a filter with alcohol, dried at 105°C., extracted with hot water (ten times the weight of the sample) by refluxing for three hours, then extracted twice with 0.5% $(NH_4)_2\ C_2O_4$ by refluxing for one and one-half hours each time, and finally washed with water on a filter and dried at 105°C. The loss in weight of the air-dry straw was followed by weighing the residue each of the three times it was dried in the oven at 105°C. The initial air-dry weight, and the three oven-dry weights are respectively: 70.0, 65.3, 60.3 and 55.5 grams.

Extractions with the 4M monoethanolamine were carried out on two-gram samples with fifty ml. of extracting solution in the same all-glass refluxing apparatus that had been used previously for extractions with the undiluted monoethanolamine. The flasks were here heated on wire gauze pads over flames instead of in an oil bath as in

the previous experiment. A few drops of caprylic alcohol were added to prevent foaming.

To determine the efficiency of removal of hemicellulose three samples were given successive half-hour extractions. The first sample received one of these extractions, the second received two, and the third three. After each extraction the mixture was filtered (Jena 1-G-2), washed with water and the residue then returned to the flask for the next extraction, or, if after the last extraction, the residue was saved for a pentosan determination. The pentosan contents of the residues (calculated to a percentage of the pretreated sample) were respectively 22.1%, 20.8%, and 20.6%.

An attempt was made to precipitate and weigh the hemicellulose removed in each of the six extractions. The precipitation was effected by acidifying with acetic acid and adding one volume of acetone (39), but filtration was found to be very difficult. Gooch crucibles soon became clogged and even with diatomaceous earth, filtration proceeded so slowly as to be impractical.

A third study of the action of monoethanolamine was made on a sample of straw following a pretreatment with a solution of urea (tried here because it has had some use as a depolymerizing agent). A ten-gram sample of the original 40-mesh oat straw was treated as follows: dried at 105°C., extracted in a soxhlet with a constant-boiling mixture of ethyl alcohol and benzene for thirty hours, washed on a filter with alcohol, dried at 105°C., extracted by refluxing with 4M urea solution, washed on a filter with water, dried at 105°C., then a portion extracted by refluxing with 4M monoethanolamine for one half hour period, washed with water and dried at 105°C.

The amounts of material extracted in each treatment were checked by weighing after each of the times the residue was dried in the oven. These weights calculated to the basis of 100 grams of oven-dry straw are respectively: 100, 94, 79, and 64 grams.

The pentosan content of the residue was 22.5% of the oven-dry weight of sample taken for the monoethanolamine extraction. This would indicate that the efficiency of extraction of hemicellulose is no greater than in the preceding experiment.

A change in technique of weighing the precipitated phloroglucides was made in this last pentosan determination and the changed technique was used in all subsequent determinations. The black precipitate quickly forms cracks if allowed to become dry in washing or filtering. This makes subsequent washing ineffective. To eliminate this danger and to improve the speed of filtering the Gooch crucibles were prepared with approximately two grams of a diatomaceous silica product "Dicalite" (McArthur Chemical Company, Ltd., Montreal) above the asbestos pad. In addition a weighed one-gram portion of the same material was added to the precipitate in the beaker and was washed with the precipitate into the crucible. This was found to make filtering more rapid and to entirely prevent any cracks forming in the precipitate when the crucible was sucked dry.

(2) (a) The Use of Vigorous Agitation with 5% NaOH Following Attempted Removal of Lignin with Alcoholic NaOH.

The method employed by Phillips and Davis (44) in the extraction of hemicellulose from alfalfa hay seemed sufficiently mild to ensure comparatively slight contamination of the product with materials of cellulosic origin, and claimed the removal of a large amount of lignin by a

treatment that was likewise mild. This latter feature seemed to indicate lessening the danger of contamination of the final product and to offer an opportunity for very complete extraction of the hemicellulosic materials. The method required, however, ten extractions each for twenty-four hours after the material had been ground and extracted for thirty hours with an ethyl alcohol-benzene mixture.

Experiments were carried out to determine the least time and number of extractions that, with vigorous agitation, would be required to reduce to a minimum each of the following:

(1) Pectic substances, by extracting, with 0.5% $(NH_4)_2\ C_2O_4$ solution at 85°C.

(2) Lignin, by extracting with the dilute alcoholic NaOH used by Phillips and Davis (44).

(3) Hemicellulose, by extracting with 5% NaOH.

The preliminary experiments were carried out on a sample of clover and later tests were made on a sample of turnip tissue.

The clover used was a sample of red clover containing about 1 - 2% timothy. It was cut in the early bloom stage and ground in a Wiley mill. The whole of the material passing a 40-mesh sieve was saved and used as the sample in the following work. Of the total sample taken for grinding (50.4 grams) a quantity (8.9 grams) was not reduced to 40-mesh size after passing through the mill three times. This portion was discarded.

The turnip sample was taken after three months storage in a root cellar. The whole turnips were washed, freed of smaller roots

and all green portions removed from the crown. Any turnips showing brown-heart when cut through were discarded. Healthy turnips thus prepared and selected were sliced and passed through a food chopper. A total sample of 1800 grams of turnip was thus finely chopped. The whole of this was immediately placed in hot ethyl alcohol, refluxed for forty-five minutes, and after cooling, filtered on a Büchner. The amount of alcohol used here was such that the final concentration (assuming turnips to contain 92% water) would be 70% by weight. The extracted turnip after filtering, washing with alcohol and drying at 105°C. weighed 90.7 grams, thus representing 5.04% of the original turnip sample. This dried material was ground to pass a 40-mesh sieve and was stored in a glass-stoppered bottle.

In all these experiments in which vigorous agitation was used to facilitate extraction, the stirrer employed to bring about this agitation was a "No. 22, Gilchrist model", manufactured by Hamilton Beach Mfg. Co., Racine, Wisc., U.S.A. It is the type of stirrer which is used at soda fountains in making ice-cream drinks and which was adopted by Bouyoucos for soil extractions. The metal solution-container provided by the manufacturer was replaced in this work by a tall-form 500-ml. pyrex beaker. To further avoid contact of solutions with metal, the stirring rod was covered with a tightly-fitting rubber tube.

For preliminary experiments a portion of the ground clover sample was extracted in a soxhlet with constant boiling ethyl alcohol-benzene mixture for thirty hours. It was then filtered, washed with alcohol and dried at 55°C. The clover lost in weight 32.2% of its air-dry weight.

To determine the extraction necessary to reduce to a minimum the content of pectic substances, a 0.5 gram sample of this pretreated clover was extracted with stirring for successive ten-minute periods with 150 ml. of 0.5% $(NH_4)_2\ C_2O_4$ at 85°C. Following each extraction period the mixture was filtered (Jena 1-G-2 and Pyrex glass filtering crucibles style 30 C), the pectic content of filtrate estimated by method of Fellers and Rice (16), and the residue returned to the beaker for a further extraction. In these tests four successive extractions were given. The amounts of pectic substances removed after the second extraction were very small. It was decided to use one extraction period of one-half an hour followed by one for ten minutes under these conditions in the next tests.

To determine the amount of extraction necessary to remove a maximum of lignin, the sample previously freed of pectic substances was now given successive ten minute extractions with the alcoholic NaOH solution at room temperature with similar agitation. After each extraction the filtrate was examined for lignin content and the residue returned to the beaker for the next extraction. To estimate the amount of lignin extracted, one-third of the filtrate, i.e. 50 ml., was treated as described by Phillips and Davis (44) to the point of precipitating the lignin by acidifying after removal of the alcohol at reduced pressure. This precipitated lignin was here separated from the filtrate in a 15 ml. centrifuge tube, and the amounts from different extracts compared as in the Fellers - Rice procedure for pectic substances. Again four successive extractions were made and it was found that after the second there was only very slight removal of lignin. It was decided on the next test to use one extraction

for one-half an hour followed by one for ten minutes.

Since only one-third of these lignin-containing filtrates had been used to estimate the lignin extracted, there remained approximately 100 ml. from each of the four extractions. These were combined, evaporated to dryness on a water-bath and the residue was distilled with 12% HCl. No furfural was found in the distillate. This was taken as an indication of the very slight effect this treatment has on the hemicellulose material.

There remained then to determine the extraction required under these conditions to remove as completely as possible the hemicellulose. This information was sought by extracting the residue from the last alcoholic NaOH treatment successively for ten-minute periods with 150 ml.portions of 5% NaOH. Following each extraction the mixture was filtered on a small Buchner funnel and the residue washed with water but the washings not added to the filtrate. The residue was returned to the beaker for the next extraction and the filtrate was analysed for hemicellulose content following the method of Phillips and Davis but adapted to the centrifuge tube technique previously employed. In this, two 5-ml. aliquots of the filtrate were placed in each of two 15-ml. centrifuge tubes, acidified with a small amount of glacial acetic acid, 3 ml. of acetone and 3 ml. of 95% ethyl alcohol added. The amounts of hemicellulose separating out were then compared. The duplicates checked closely and added to the reliability of the comparisons. Six successive extractions were made and each extract showed that a further, although smaller, amount of hemicellulose was being extracted each time.

After the sixth extraction, the residue was dried in an oven at 105°C. and then its pentosan content was determined. It was found

to contain 7.4% pentosan, or an amount representing 2.5% of the original weight of the sample. This pentosan content expressed as a percentage of the original sample is possibly low because of the great number of filtering and transfer operations each of which would involve some mechanical loss.

It should be noted here that the A.O.A.C. procedure being followed in these pentosan determinations requires that the precipitated phlorogluoide be dried four hours at the temperature of boiling water. In the earlier determinations this drying was carried out in a hot water oven. In this determination and in all following pentosan determinations drying was carried out in a gas-heated oven which was regulated to a temperature range of 94^{o} - 97^{o} C.

As a second test of this procedure two samples, one of clover and one of turnip tissue, were extracted in turn with $(NH_4)_2C_2O_4$, alcoholic NaOH and 5% NaOH. Since it was considered that ultimately, an attempt might be made to precipitate and weigh the hemicellulose extracted, it was thought advisable to choose for this experimental work a size of sample that should yield an amount of hemicellulose suitable for weighing. From the hemicellulose content of Alfalfa hay reported by Phillips and Davis (44) it was considered that a five-gram sample of ethyl alcohol-benzene extracted clover would probably give 0.3 - 0.5 grams of hemicellulose. From the analysis of rutabagas given by Holland and Jones (23) it was calculated that a four-gram sample of ethyl alcohol-extracted turnip would probably give a similar amount of hemicellulose. Accordingly, a five-gram sample of the ethyl alcohol-benzene extracted clover and a four-gram sample of the ethyl alcohol-extracted turnip were agitated twice with

$(NH_4)_2\ C_2O_4$ at 85°C. for periods of one-half an hour and ten minutes, then extracted twice with alcoholic NaOH at room temperature also for periods of one-half an hour and ten minutes, and finally extracted with 5% NaOH at room temperature for four periods of fifteen minutes each.

It was found that, because of swelling due to the starch content of the turnips, the volume of $(NH_4)_2\ C_2O_4$ and of the alcoholic NaOH solutions had to be increased to 250 ml. Otherwise 150 ml. of extracting solution was used in each case. It was considered necessary however to occasionally wash down with a fine stream of extracting solution (from a wash bottle) a ring of material that adhered to the flask at the surface of the solution.

It was also found that it was almost impossible to filter the turnip materials. To facilitate this separation these mixtures were centrifuged. The clover mixtures were found to be also more easily separated by this means so that centrifuging replaced entirely the filtering methods used in the first test. The extracting solutions were then kept to such a volume that they could each be washed completely into one 250-ml. centrifuge cup. Since washing was found to be important, each residue was shaken in the centrifuge cup with 100 cc of the solution with which it had just been extracted, and then separated again by centrifuging.

The efficiency of the removal of pectic substances and lignin were again checked as in the first test. It was found that in each case the first extractions removed large amounts and that washing of the residues after the first extractions was effective in removing further appreciable quantitites. The second extraction however removed relatively little of either pectic substances or lignin. It

was considered advisable nevertheless to continue the procedure of making the two extractions.

The completeness of removal of hemicellulose was measured by determining the pentosan content of the residues. The clover residue was dried at 105°C. and the pentosan remaining was found to be 9.8% of this oven dry weight. It was not possible to express this pentosan on the basis of the original sample because of a loss that had occurred in centrifuging at one point. The pentosan remaining in the turnip residue amounted to 4.9% of the original sample.

A study was made of the precipitation of hemicellulose from the combined clover extracts and from the combined turnip extracts. It was found that when acidified to a pH 4.5 - 5.0 the precipitation was for each solution as complete as could be obtained at either higher or lower pH. It was found also that one volume of acetone and one volume of alcohol added to the acidified extract gave as complete precipitation from each solution as could be obtained by further additions of alcohol or of acetone.

In an attempt to learn if more complete removal of hemicellulose were possible, a third experiment was carried out. Two four-gram samples of turnip were given the same extractions for pectic substances and for lignin as were given in the preceding experiment and were then extracted with 5% NaOH, one receiving a single four-hour extraction and the other receiving two two-hour extractions.

For a measure of the completeness of removal of hemicellulose the pentosan content of each residue was determined. It had been found in the last pentosan determinations that the turnip sample had a great tendency to foam in the distillation flask (250 ml.). This could not be

easily controlled by the use of caprylic alcohol because this material soon distilled off. For these determinations 500 ml. flasks were used and foaming was much less troublesome. In all future pentosan determinations the distillation flasks were of 500 ml.capacity. In these determinationa, the furfural yield indicated that after the single four-hour extraction the pentosan content of the residue was 2.6% of the sample.

The NaOH extracts were treated as had been found most satisfactory in the preceding experiment for the precipitation of the hemicellulose. The precipitates were separated and washed (with water) by centrifuging. They were then washed with water into platinum dishes. The water was evaporated and the precipitates dried in an oven at 95°C. The amounts of hemicellulose obtained were 5.5% and 9.9% of the original samples from the single four-hour and the two two-hour extractions respectively. It is believed that these results may be in error because of the washing with water. It is noticed however, that they are consistent with the values obtained for residual pentosan although the agreement is not exact.

Thus:

	Hemicellulose weighed	Pentosan remaining
The one four-hour extraction	4.0%	5.5%
The two two-hour extractions	2.6%	9.9%

As a further test of the possibility of removing hemicellulose using 5% NaOH with vigorous agitation, an extraction was carried out at a higher temperature. A four-gram sample of ethyl

alcohol extracted turnip was treated with $(NH_4)_2\ C_2O_4$ and with alcoholic NaOH as in the last experiment, then it was given two two-hour extractions with 5% NaOH at 60°C. The furfural yield of the residue indicated the presence of pentosans amounting to 1.3% of the original sample. The extracted hemicellulose was precipitated as before but was lost in an attempt to estimate its nitrogen-content by the method of Pregl (49).

(2) (b) The Use of Vigorous Agitation with 5% NaOH Following Attempted Removal of Lignin by Preparation of Holocellulose.

In a first experiment holocellulose was prepared from a two-gram sample of alcohol-extracted turnip by the method of Van Beckum and Ritter (55) and this was given two two-hour extractions with 5% NaOH. The pentosan remaining after these extractions amounted to 3.8% of the original sample.

Some difficulty was encountered in preparing holocellulose from this material. When wet with water the swelling of starch caused the formation of a gel-like mass so that washing was very slow on the filter. To facilitate filtering first sand was added to the sample on a glass crucible (Pyrex style 30 C). This sand was "Ottawa sand" which had been washed with acid and with alkali. A further attempt was made to aid filtering by transferring the sample to a small Büchner funnel where it was filtered through a piece of muslin placed over a piece of filter paper. The use of this muslin however causes a loss of some material when the holocellulose is to be transferred to the beaker for extraction with NaOH. The time required still seemed excessive, for to complete once the chlorination and extraction treatments required one hour.

During this extraction, if the washings were allowed to accumulate in the suction flask a white flocculent precipitate formed. It was feared that a part of this might be some hemicellulose removed by the action of monoethanolamine and precipitated from the alcoholic acid solution in the flask. It is shown below that a removal of hemicellulose actually did occur.

For the complete removal of lignin as indicated by the residue remaining white following chlorination and addition of hot solvent required five extraction treatments.

A second experiment involving this holocellulose-preparing technique was carried out by first removing pectic substances (two extractions with agitation at 85°C. as above) and the preparation of holocellulose from this pectin-free material. The hemicellulose was then extracted from this holocellulose by two two-hour extractions as used previously. The pentosan content of this residue was 1.2%. The hemicellulose was precipitated as described above and the lignin content estimated by the 72% H_2SO_4 method of Manning (33) to be 3.5%.

A test was made here of the completeness of the furfural formation during the distillation of 360 ml. by the method being used. The distillation of the residue from this experiment was continued to collect four additional 30-ml. portions. The precipitate which formed on the addition of phloroglucin to this distillate was found to be entirely due to methyl-furfural and to ω-hydroxy methyl-furfural as indicated by its complete solubility in 95% ethyl alcohol at 60° C. (51).

A third experiment including this technique was made by

removing a portion of the hemicellulose before a single chlorination and extraction treatment of the holocellulose preparation, and then removing further hemicellulose after this treatment. This was carried out on a sample of the alcohol-extracted turnip prepared after the manner of the last section for the NaOH extraction, viz., by giving two extractions with $(NH_4)_2 \quad C_2O_4$ and two with the alcoholic NaOH. This residue was then extracted once for two hours with 5% NaOH, once treated with chlorination and extraction for the lignin chloride, and finally extracted a second time for two hours with 5% NaOH. The residual pentosan was then 0.9% of the alcohol-extracted sample.

To form a better estimate of the efficiency of the hemicellulose extraction, the pentosan content was determined on some of the materials used in this work after the pretreatments had been given. All pentosan data express the amounts found as %'s of the sample of ethyl alcohol-extracted turnip.

Material	Percentage Pentosan
Ethyl alcohol-extracted turnip (average of two determinations: 13.9% and 14.3%)	14.2
Pectin-free, alcohol-extracted turnip (prepared as for second experiment with holocellulose technique)	4.8
Holocellulose prepared from pectin-free alcohol-extracted turnip	4.1

It is noticed that a decrease in pentosan-yielding material is shown to accompany the preparation of holocellulose.

An estimation of the amount of methyl-furfural-phloroducide and ω hydroxymethyl-furfural-phloroglucide included in the weighed phloroglucides was made at this point. The precipitate obtained from the determination of the pentosan content of pectin-free alcohol-extracted turnip was treated after the method of Schorger (51). It was found that these phloroglucides made up on 1.36% of the total weight of the precipitated phloroglucides. This amount is similar to that found by Schorger (52).

3. The Effect of Alternate Extraction with 4% NaOH and Exposure to Chlorine.

A study was made of the effect of chlorination between successive extractions with 4% NaOH on both straw and turnip tissues. The straw used was the 40-mesh sample described above, after a thirty-hour extraction with a constant boiling ethyl alcohol-benzene mixture. The turnip samples were taken from the ethyl alcohol-extracted turnip described above. The effects of the extraction and chlorination procedure were carefully studied by determining the pentosan content of the tissues following each step.

The tables below show the percentage of the sample that appeared as furfural-yielding material (calculated to pentosan) following each step. Letters are used to describe the material of which the pentosan content is recorded. Thus "O" indicates the original sample, "E" refers to a residue after one extraction with 4% NaOH for one half-hour with agitation, "EC" refers to a residue after an extraction with NaOH and one chlorination, "ECE", "ECEC", and "ECECE" similarly refer to samples after further extractions and chlorination.

The symbol "S" is used in connection with the turnip samples to indicate the residue after the removal of starch from the original sample by the method Hassid et. al. (21).

The residues from the NaOH extractions were in each case washed in the centrifuge with three 20-ml. portions of 4% NaOH before chlorination or before the HCl distillation. The chlorinations were carried out by gently bubbling Cl_2 gas through the residues from the NaOH extractions after the latter had been acidified with N acetic acid and water added to make the volume approximately 50 ml. Following this chlorination the residues were separated by centrifuging, then washed once with 20-ml. portions of H_2O, and then washed three times with 20-ml. portions of 95 % ethyl alcohol.

The following tables show the results of each single determination that was made. Three complete runs on straw samples and two on turnip samples have been studied. A few additional tests have been made on certain treatments. In the last column of each of the tables are given data concerning the total weight of hemicellulose that was removed in the three extractions of the "ECECE" samples. The data report (as a % of the original sample) the weights of hemicellulose obtained on precipitating from these three extracts combined in the manner described earlier.

STRAW:

O	E	EC	ECE	ECEC	ECECE	HEM.
22.7	10.7	1.6	0.9	3.8	0.4	25.4
23.8	9.0	9.7	1.0	0.3	1.1	29.2
26.9	10.0	9.2	1.5	1.8	2.1	18.3
26.9	10.1				0.9	

TURNIP:

O	S	SE	SEC	SECE	SECEC	SECECE	HEM.
13.9	7.3	4.0	8.3	2.5	7.2	1.4	6.6
14.3	5.2	4.1	5.4	1.9	4.5	1.8	8.0
			3.4		0.8		

The data for the two complete runs on turnip tissue are shown graphically on page 34 .

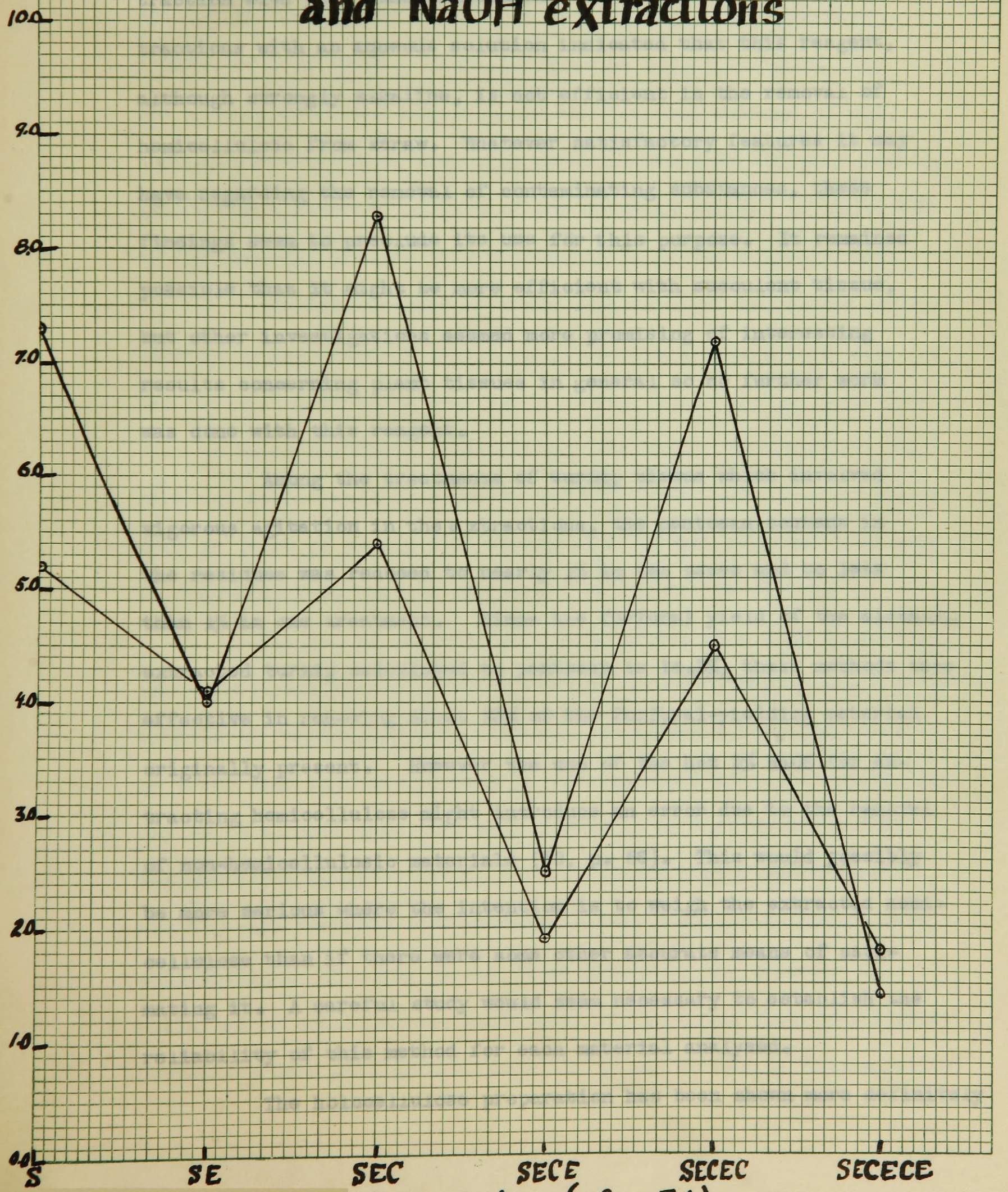
An attempt was made through this study to estimate the lignin content of the different extracts by the use of the Folin and Denis phenol reagent (17), after the method of Mehta (34). As well as it could be determined the lignin content of all the extracts was very low, and as was observed by Bailey (8), (9), it was found that this lignin was not preferentially soluble in butyl alcohol over aqueous NaOH even after heating in a pressure cooker at 158°C, for four hours. However this colorimetric estimation of lignin was found unsatisfactory for accurate work because of the formation of a white precipitate which obscured the blue color.

In this study it was observed that the filtrates from the first chlorinations, and to a lesser extent from the second chlorination, formed a very light white precipitate when the alcohol washings were added. In the determination of the pentosan-content of these residues immediately following chlorination a cloudy distillate was obtained in all cases. When the phlorogluoin reagent was added to these cloudy distillates the color at first turned reddish instead of greenish as is common. In some cases (especially in distillates from residues after the second chlorination) the color was at first a distinct orange, which darkened to a deep red and then to black. This reddish color seemed to be due to the precipitate which was forming for when filtered after standing overnight the filtrates had the characteristic greenish color, but these precipitates were brownish instead of black.

Attempts to identify this reddish or brownish material as a compound of phlorogluoin and methyl furfural or ω hydroxymethyl fur-

fural (51) have been inconclusive. There seems also the possibility that it was due to formaldehyde formed from lignin or from some product of lignin in the pentosan distillation. The precipitate formed by formaldehyde with phloroglucin is reddish-orange in color. This possibility has not yet been fully investigated.

Pentosan (by furfural yield) from turnip after successive chlorinations and NaOH extractions



Material (c.f. p.31)

DISCUSSION

The high pentosan content of the residues after extraction with pure monoethanolamine and after successive extractions with an aqueous solution indicated that this reagent, although strongly alkaline, is not efficient in the removal of hemicellulose from straw. Whatever satisfactory features it may have regarding the removal of contaminating substances, these findings seem to preclude its use for this purpose. It remained possible that it might be more efficient with succulent tissue, but other investigations seemed more promising of interesting results concerning plant tissues in general so no further work was done with this reagent.

Among the treatments of turnip tissue which involved vigorous agitation in the extractions, the pentosan content in the residues was reduced to nearly 1% in two cases and to less than 1% in one treatment. Since the furfural yield of the alcohol-extracted turnip calculated to pentosan is 14.2%, these methods were effective in removing about 93% of the furfural-yielding material originally present. However the use of the hot 5% NaOH for extracting hemicellulose might introduce an error due to the removal of non-hemicellulosic materials (38, p. 56). This would possibly be more serious where the intention is to weigh the extracted hemicellulose than if there were some other accurate means of estimating it. A careful study would seem necessary to establish the reliability of this method for each material analysed.

The holocellulose preparation has been shown more definitely

to introduce error. Here the danger is that some of the hemicellulosic materials may be removed in the chlorination and following alcoholic monoethanolamine extraction. This was indicated in the work with turnip tissue (p. 29). The pentosan content of pectin-free, alcohol-extracted turnip was 4.8% (of the alcohol-extracted sample), and the pentosan content of the holocellulose prepared from this material was 4.1% (on same basis), indicating a decrease of 0.7% of the original sample, or about 14% of the pentosan present before preparation of the holocellulose.

The inaccuracies of these methods might be reduced sufficiently to make them of considerable practical interest if they had the virtue of being relatively rapid. This does not seem however to be an immediate possibility. The number of transfers and washings cannot well be reduced, and high starch-containing tissues such as those of turnip will remain slow to wash when wet with water, or a special step must be introduced to remove the starch.

Studies on chlorinations alternating with NaOH extractions point to a possible satisfactory method through studying still other methods of removing lignin. There is indicated a possible source of error when chlorine is used in that non-hemicellulosic materials may be so altered that they will appear as of that nature. This was particularly shown in the case of turnip tissue where there was a marked increase in furfural-yielding material following each chlorination. This may be due to an attack by the chlorine on cellulose or other hexosan materials. It is known that oxycellulose contains uronic acid groups (19). Or it may be due to the formation

of formaldehyde from lignin as suggested by the work of Freudenberg and Harder (18).

Some indication is given of possible successful analysis through precipitating and weighing the extracted hemicellulose. The weights of hemicellulose obtained seem related (though not exactly) to the amount extracted as shown by decrease in furfural yield. Further study of this is needed. Especially should the nitrogen-content and lignin-content of these precipitates be known.

SUMMARY

Experiments have been carried out which were designed to assist in the development of a method for hemicellulose estimation in plant tissues.

Monoethanolamine has been shown to be an inefficient extracting agent for hemicellulose, both when it is employed undiluted and when in 4 M aqueous solution.

Extraction of hemicellulose by vigorous agitation with 5% NaOH was studied following attempted removal of lignin in each of two ways: (1) with alcoholic NaOH, (2) by the preparation of holocellulose. These experiments indicated that the residual hemicellulosic materials (as measured by furfural yield on distillation with 12% HCl) could be reduced to a low level. However, the most efficient procedure (involving the preparation of holocellulose) is lengthy and complicated, and in addition evidence has been obtained that some hemicellulosic material is lost in this removal of lignin.

A study was made of the use of chlorine in removing lignin by alternating the dilute NaOH extractions with mild chlorinations. In both straw and turnip tissue, especially the latter, the data suggested that chlorination tended to produce an increase in the amount of furfural-yielding materials. As has been mentioned this may be due to the formation of formaldehyde during the HCl distillation or it may be a result of an attack by the oxidizing agent on cellulose or other hexosan material. This would suggest that attempts to remove hemicellulose by alternating chlorinations and extracts with NaOH may result in the removal of some material properly belonging

to the cellulose fraction. This possibility does not seem to have been recognized before. Further, this effect of chlorination makes it difficult or impossible to use the furfural yield as a measure of the completeness of hemicellulose removal from certain tissues at any rate. It also introduces difficulties into the determination of the constitution of hemicellulose preparations isolated by such a procedure. It is suggested in this connection that a search should be made for an oxidizing or other agent to remove lignin since elimination of the latter seems to be an essential requirement for the complete extraction of hemicellulose from plant tissues.

ACKNOWLEDGMENTS

The investigations here reported were carried out while the author was employed as demonstrator in chemistry at Macdonald College. Grateful acknowledgment is given for this financial assistance.

The author also wishes to express his sincere thanks to Dr. W.A.DeLong, under whose instruction this work was carried out, for many helpful suggestions and personal interest in this work.

BIBLIOGRAPHY

1. Anderson, E. and S. Kinsman. - Studies on the hemicelluloses. II The composition of the hemicellulose from cottonseed hulls. J. Biol. Chem. 94: 39-47, 1931 - 32.

2. Anderson, E. and P.W.Krznarich. - Hemicellulose from oat hulls. J. Biol. Chem. 111: 549-552, 1935.

3. Anderson, E., L.W.Seigle, P.W.Krznarich, L. Richards, and W.W.Martiny. The isolation of pectic substances from wood. II J. Biol. Chem. 121: 165-174, 1937.

4. Anderson, E., J. Hechtman and M. Seeley. - Hemicelluloses from cottonseed hulls. J. Biol. Chem. 126: 175-179, 1938.

5. Anderson, E., M. Seeley, W.T.Stewart, J.C.Redd and D. Westerbeke. The origin and composition of the hemicelluloses obtained from hardwoods. J. Biol. Chem. 136: 189-198, 1940

6. Angell, S., and F.W.Norris. - Studies on hemicelluloses. V. Additional notes on the hemicelluloses of maize cobs. Biochem. J. 30: 2155-2158, 1936.

7. Angell, S., and F.W.Norris. - Studies on hemicelluloses. VI. The hemicelluloses of the flower of the hop (Humulus lupulus). Biochem. J. 30: 2159-2165, 1936.

8. Bailey, A.J. - The chemistry of lignin. II. The butanolysis of wood. Paper Trade J. 110: No. 2, 29-32, 1940.

9. Bailey, A.J. - The alcoholysis of wood. Pacific Pulp & Paper Ind. 14: No. 3,24-27, 1940.

10. Bray, M.W. - Chemical analysis of pulps and pulpwoods. Methods used at the Forest Products Laboratory. Paper Trade J. 87: No. 25, 59-68. 1928.

11. Burkhart, B.A. - Hemicellulose constituents in alfalfa roots. Plant Physiol. 11: 421-428, 1936.

12. Buston, H.W. - The polyuronide constituents of forage grasses. Biochem. J. 28: 1028-1037, 1934.

13. Campbell, W.G. and J. Booth - The chemical aspect of the drying of timber. Biochem. J. 24: 641-648, 1930.

14. Campbell, W.G. and J. Booth - The chemical aspect of the drying of timber. 2. The drying of a softwood. Biochem. J. 25: 756-762, 1931.

15. Dickson, A.D., H. Otterson and H.P.Link - A method for the determination of uronic acids. J. Am. Chem. Soc. 52: 775-779, 1930.

16. Fellers, C.R. and C.C.Rice - Rapid centrifugal method for pectic acid determination. Ind. Eng. Chem., Anal. Ed. 4: 268-271, 1932.

17. Folin, D., and W. Denis. - On phosphotungatic-phosphomolybdic compounds as color reagents. J. Biol. Chem. 12: 239 - 243, 1912.

18. Freudenberg, K. and M. Harder- Lignin. II. Formaldehyde as a cleavage product of lignin. Ber. 60B: 581-585, 1927. C.A. 21: 1818, 1927.

19. Godman, G.L., W.N.Haworth and S. Peat. Polysaccharides. Part XXXVII. oxycellulose. J. Chem. Soc. 1939: 1908-1914.

20. Harris, E.E., E.C.Sherrard and R.L.Mitchell Some chemical reactions of maple and spruce lignin. J. Am. Chem. Soc. 56: 889-893, 1934.

21. Hassid, W.Z., R.M.McCready, and R.S.Rosenfils. - Determination of starch in plants. Ind. Eng. Chem., Anal. Ed. 12: 142-144, 1940

22. Heller, V.G., and R. Wall. - Indigestible carbohydrates of feeds. J. Nutrition 19: 141-149, 1940.

23. Holland, E.B. and C.P.Jones. - The relation of"dark center" to the composition of rutabagas. J. Agr. Research. 48: 377-378, 1934

24. Hughes, E.E., and S.F.Acree. - Quantitative formation of furfural from xylose. J. Research Natl. Bur. Standards 21: 327-336, (Research Paper No. 1132). 1938.

25. Krober, E. - The hydrochloric-acid-phloroglucin method in the determination of pentosans. J. Landw. 48: No. 4, 357, 384, 1901. Expt. Sta. Record; 13: 320-321, 1901-1902.

26. Krzmarich, P.W. - The hemicelluloses from oat hulls. II Cereal Chem. 17: 457-459, 1940.

27. Kurth, E.F., and G.J.Ritter. - Spruce holocellulose and the composition of its easily hydrolysable fraction. J. Am. Chem. Soc. 56: 2720-2723, 1934.

28. MacMasters, M.M., S. Woodruff, and H. Klaas. - Studies on soybean carbohydrates. Ind. Eng. Chem., Anal. Ed. 13: 471-474, 1941.

29. Malhotra, R.C. - Effect of degree of pulverization and weight of samples on quantitative analyses with particular reference to plant tissues. Ind. Eng. Chem., Anal. Ed. 2: 398-401, 1930.

30. Malhotra, R.C. - Notes on the determination of hemicelluloses. Ind. Eng. Chem., Anal. Ed. 3: 161-163, 1931.

31. Malhotra, R.C. - The distribution of some reserve substances in hard winter wheat plant at successive growth stages and their possible utilization. J. Agr. Sci. 22: 485-496, 1932.

32. Mann, Kruger, and B. Tollens. - The determination of pentoses and pentosans by distillation with hydrochloric acid. Ztschr. angew. Chem. 1896: No. 2, p 33 - Expt. Sta. Record. 7: 830, 1895-6.

33. Manning, K.R. - M.Sc. Thesis, Agricultural Chemistry, McGill University, 1940.

34. Mehta, M.M. - Biochemical and histological studies on lignification. Part I. The nature of Lignin: its physiological significance and its estimation in timbers. Biochem. J. 19: 958-978, 1925.

35. ---------- - Methods of Analysis, A.O.A.C., 4th Edition, 1935, p 344-345.

36. Mitchell, R.L. and G.J.Ritter. - Composition of hemicellulose isolated from maple wood. J. Am. Chem. Soc. 62: 1958-1959, 1940.

37. Nanji, D.R., F.J.Paton, and A.R.Ling. - Decarboxylation of polysaccharide acids: its application to the establishment of the constitution of pectins and to their determination. J. Soc. Chem. Ind. 44: 253-258 T, 1925.

38. Norman, A.G. - The Biochemistry of Cellulose, The Polyuronides, Lignin, etc. London: H. Milford, Oxford Univ. Press.

39. Norris, F.W., and I.A.Preece. - Studies on hemicelluloses. I. The Hemicelluloses of wheat bran. Biochem. J. 24: 59-66, 1930.

40. O'Dwyer, M.H. - The hemicelluloses of the wood of English oak. I. The effect of drying of wood on the yield and composition of hemicellulose A. Biochem. J. 25: 2017-2022, 1931.

41. O'Dwyer, M.H. - The hemicelluloses of the wood of English oak. III. The fractionation of hemicellulose A. Biochem. J. 31: 254-257, 1937.

42. O'Dwyer, M.H. - The hemicelluloses of the wood of English oak. IV. The structure of hemicellulose A. Biochem. J. 33: 713-717, 1939.

43. Phillips, M. - The hemicellulose constituents of the nitrogen-free extract. J. Assoc. Official Agr. Chem. 23: 119-126,1940

44. Phillips, M. and B.L.Davis. Hemicelluloses of alfalfa hay. J. Agr. Research 60: 775-780, 1940.

45. Preece, I.A. - Studies on hemicelluloses. IV The proximate analysis of boxwood, and the nature of its furfuraldehyde-yielding constituents. Biochem. J. 25: 1304-1318, 1931.

46. Reeves, R.E., and J. Munro - Quantitative determination of pentoses. Ind. Eng. Chem., Anal. Ed. 12: 551-553, 1940.

47. Reid, J.D., G.H.Nelson, and S.I.Aronovsky. - Determination of cellulose in fibrous agricultural wastes. A rapid method using monoethanolamine. Ind. Eng. Chem., Anal. Ed. 12: 255-259, 1940.

48. Ritter, G.J., and E.F.Kurth - Holocellulose, total carbohydrate fraction of extractive-free maple wood. Ind. Eng. Chem. 25: 1250-1253, 1933.

49. Roth, H. - Pregl's Quantitative Organic Microanalysis. 3rd English ed. translated from the 4th revised and enlarged German ed. by E. Beryl Daw. Philadelphia: Blakiston's Son & Co.

50. Sands, L., and P. Nutter. - The hemicelluloses extracted from mesquite wood after chlorination. J. Biol. Chem. 110: 17-22, 1935.

51. Schorger, A.W.
- The chemistry of wood. I Methods and results of analysis of some American species.
Ind. Eng. Chem. 9: 556-561, 1917.

52. Schorger, A.W.
- The chemistry of wood. II Discussion of methods and results.
Ind. Eng. Chem. 9:561-566, 1917.

53. Tollens, B.
- On the pentoses and pentosans and their estimation by furfurol distillation.
Ztschr. Ver. Rubenz Ind., May, 1894, 426-437.
Expt. Sta. Record 6: 111, 1894-5.

54. Van Beckum, W.G. and G.J.Ritter
- Chemical composition of wood.
Paper Trade J. 104: No. 19, 49-50, 1937.

55. Van Beckum, W.G and G.J.Ritter
- Rapid methods for the determination of holocellulose and Cross and Bevan cellulose in wood.
Paper Trade J. 105: No. 18, 127-130, 1937.

56. Williams,R.D. and W.H.Olmsted
- A biochemical method for determining indigestive residue (crude fibre) in faeces: lignin, cellulose, and non-water-soluble hemicelluloses.
J. Biol. Chem. 108: 653-666, 1935.

57. Williams, R.D. L. Wicks, H.R.Bierman, and W.H.Olmsted.
- Carbohydrate values of fruits and vegetables.
J. Nutrition. 19: 593-604, 1940.

58. Winkler, A.J. and W.O.Williams.
- Carbohydrate metabolism of vitis vinifero: hemicellulose.
Plant Physiol. 13: 381-390, 1938.

59. Youngburg, G.E. and G.W.Pucher
- Studies on pentose metabolism. I. A colorimetric method for the estimation of furfural.
J. Biol. Chem. 61: 741-746, 1924.