STUDIES ON THE CHEMICAL DETERMINATION OF PLANT LIGNIN.

by

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CLAIMS TO ORIGINAL RESEARCH

1. The method of isolation of plant lignin has been modified to permit the use of fresh material.

2. An extensive study of the removal of nitrogenous substances prior to determining the apparent lignin content of plant tissues has been carried out. It was found that extractions with hot, dilute mineral acid was necessary for the removal of interfering material from old tissue, but not from young plants.

3. The effect of reducing conditions on both the pretreatment extractions and the final isolation process has been investigated. It was found that under the conditions of these experiments these had no effect on the amount or composition of the lignin isolated.

4. The ultraviolet absorption spectra of lignin solutions from oat and timothy plants cut at different growth stages have been studied. The specific absorption appears to increase with the age of the plant from which the lignin is isolated.

5. High pressure hydrogenation of the extracted material from both young and old timothy has been carried out. The amount of fractionatable oil in the reaction products increases with the age of the plant.

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GENERAL INTRODUCTION.

The problem has its origin in the empirical nature of existing methods for the chemical determination of plant lignin. It is generally recognized that the apparent lignin content of plant tissues depends largely on the pretreatments employed, and on the method and conditions of final isolation of the lignin. By using various pretreatments and carefully regulating the conditions of the final determination of lignin, methods have been developed which give reproducible results on any given tissue.

However, the apparent lignin content of any particular tissue may vary greatly, depending on the method used for its determination.

Consequently, an investigation of more efficient methods for the removal of materials which interfere in the final isolation of lignin was indicated. In this connection, special attention has been paid to obtaining a lignin relatively free from nitrogen, and also, to the prevention of changes in the lignin itself during its isolation.

The most commonly used criteria of plant lignin purity have been its nitrogen and methoxyl contents. As an additional criterion of the purity of isolated lignin ultraviolet absorption spectra of lignin solutions have been studied. With the same end in view, high pressure hydro-

genation studies have been carried out on pre-extracted materials. These latter studies also throw some light on the chemical composition of lignin from succulent plant tissue as compared with that from mature wood.

Part A

The Removal of Substances which interfere in the Chemical Determination of Plant Lignin.

Historical Review.

1. Chemical Methods of Determination:

Plant lignin may be determined either directly or indirectly. The indirect methods involve the formation of some lignin derivative which can be quantitatively estimated. The indirect methods have proven generally unsatisfactory. because, in most cases, they are neither specific nor quantitative. Consequently, they are seldom referred to now.

Direct methods for the determination of lignins are of two main types. On the one hand are methods which involve the removal of all other plant constituents, leaving the lignin as a residue. On the other hand are methods involving extraction of the lignin. Included in this second group are methods involving solution of the lignin in alkali or organic solvents (various alcohols, phenols, etc.) The material is treated with the organic solvent in the presence of a suitable catalyst, usually hydrochloric acid. The lignin so dissolved is precipitated by pouring into an excess of water. In general, it has been found that these methods do not completely remove the lignin from plant tissue. Since these treatments are milder than those used in any of the other methods, the lignin isolated by

them is probably more like native lignin in the plant than that isolated by the use of more drastic reagents.

The most satisfactory methods for the estimation of plant lignin have proved to be those involving the removal of all other plant constitutents. In general, these methods involve removal of interfering substances by a series of pre-extractions and final removal of the cellulose by treatment with a strong acid under carefully controlled conditions. The residue remaining is weighed as lignin. Most common are the 72% sulphuric acid method of Ost and Wilkening (75) as modified by many later workers; the fuming hydrochloric acid method of Willstatter (99) which has also been extensively studied as to reaction conditions (81); the hydrochloric-phosphoric acid method of Urban (93); or the cuprammonium method of Freudenberg (32). Of these methods the first two give the most satisfactory results. The greater convenience of handling of the 72% sulphuric acid method has rendered its use more general.

2. Effect of Preliminary Drying of Tissue:

Most investigators have used wood in their study of lignin. One would expect the interference due to proteins, and possibly carbohydrates also, to be relatively greater in succulent than in woody tissues. This is because of the fact that proteins and carbohydrates constitute a considerably higher proportion of the dry matter of the former type of

tissue. It is also possible that the lignin of rapidly growing plant tissues is of a more reactive nature than that from mature tissue such as wood. In a study of the chemical aspect of the drying of timber, Campbell and Booth (12, 13)found that oven-drying resulted in an increase in the apparent lignin content of both soft and hard woods. At the same time, there was a corresponding decrease in furfuraldehyde - yielding material. They also claimed that there was definite evidence of hydrolysis in the oven-dried material, and that this reaction does not take place in air-dried or kiln-dried samples.

The dangers of high temperature drying of tissues from which the lignin is to be isolated have been recently pointed out by the author (58). Experiments were carried out on turnips, young rye, silage, rhubarb and beets. In every case it was found that high temperature drying resulted in a large increase in the apparent lignin content of the tissue. In some cases there was six times as much apparent lignin in the high temperature-dried as in the air-dried material.

Waksman and Iyer (94) report experiments in which protein was added to lignin preparations. They maintain that the formation of a ligno-protein took place and that the resistance of such a complex to microbial decomposition was rendered greater by drying and by an elevated temperature of formation. It seems probable that similar complexes may

be formed in succulent tissues on drying at an elevated temperature or, if such are previously formed, they may at least be rendered more resistant to subsequent decomposition. Waksman and Iyer (94) admit that the nature of the union of lignin with protein is unknown, but state that the complex in soil appears to be closely bound with other substances such as hemicellulose. High temperature drying may cause formation of an analogous complex system in succulent plant tissue.

3. Interference due to Tannins, Waxes, Resins, pigments, etc.

The interference due to these substances is, in general, small, but Cohen and Dadswell (22) report that in woods such as Eucalyptus gum-like or kino-like substances may cause a considerable error, Mahood (60) suggested a preliminary extraction with a minimum boiling mixture of ethanol-benzene (1:2) for four hours, but he gives no explanation as to the nature of the interference caused by the substances so removed. This treatment has been found to effectively remove the interference due to the presence of resins and waxes. Norman (69) compared the relative effects of ethanol-benzene and ether extractions and obtained a greater decrease in apparent lignin content with the former. Ritter and Barbour (85) showed that ethanol-benzene does not remove catechol tannins effectively, and therefore, they propose an

additional extraction with 95% ethanol to remove these. Shrikhande (87) points out that 95% ethanol also removes chlorophyll from tissues containing it.

The efficiency of ether as a fat extractant both with and without a preliminary hydrochloric acid digestion was studied by Holcomb (53) on chicken meat. He found that a preliminary hydrolysis allowed much more complete removal of fat by the subsequent ether extraction. In some cases as much as 25% of the fat remained in the tissue when no preliminary hydrolysis was used. In view of these results, it seems that the efficiency of the ethanol-benzene extraction might be increased if it followed rather than preceded the 1% hydrochloric acid extraction, as outlined by the A.O.A.C. Book of Methods (1).

4. Interference of Carbohydrates and Proteins.

The proportion of carbohydrate interference in the lignin determination on all succulent tissues is large. Paloheimo (76) pointed out that fructose and sucrose form insoluble reversion products when treated with concentrated mineral acids such as are used in the lignin determination. Norman and Jenkins (67) noted that cellulose preparations yielded about three percent apparent lignin by the sulphuric acid method, and they show that this figure could be lowered by treating the cellulose with hot dilute sulphuric acid which

they claimed removed the pentosan fraction. They are of the opinion that some sort of condensation product of the pentosan fraction was formed in the presence of 72% sulphuric acid. This product would then be weighed as lignin. They presented evidence to show that xylose was the main cause of this interference. Norman and Jenkins (67a) conclude that the pentose disturbance is caused by the slow production of furfuraldehyde and its condensation with lignin to form an insoluble phenol-furfuran resin. Hilpert and Littman (52) report the formation of insoluble substances with humin-like properties on treatment of sugars with 72% sulphuric acid for 48 hours at 20-22 deg.C. They show that xylose, fructose, and substances yielding these sugars on hydrolysis give by far the greatest amount of insoluble residue.

Modern theories of the structure of lignin make no provision for the inclusion in its molecule of nitrogen. The work on the structure of lignin has all been carried out on wood lignin, and the material isolated from this source contains no nitrogen. However, no one has ever succeeded in isolating nitrogen-free lignin from succulent plant tissues. The possibility that nitrogen is a fundamental component of native plant lignin, but not of wood lignin, remains open. However, most workers in the field, chief among whom is Norman (71), believe that the nitrogen in isolated lignin is of protein, or, at least, non-lignin

origin, and is condensed with the lignin in the pretreatment or isolation procedures. Norman and Jenkins (68) claim that there is a condensation reaction between protein and pentosans or their decomposition products. These workers believe that the reaction is concerned with the production of furfuraldehyde from the pentoses and its subsequent linkage with amino acids. They suggest, too, that the combining power of lignin with such nitrogenous substances is limited. Norman (70) states that the main disturbance is due to the condensation of large protein fragments with the lignin, and that interference due to simple amino acids is of a much smaller order.

Phillips (84) added various proteins to rye straw before determining its lignin content by the fuming hydrochloric acid method. Of the seven proteins which he used, five caused an increase in the weight of the apparent lignin obtained, although the actual increase varied with the particular protein used and was not directly proportional to the amount of protein added. It should be pointed out that the proteins used by Phillips (84) were globulins and albumins, whereas leaf proteins, according to Chibnall (18) are largely glutelin in nature. Phillips (84) found that the proteins which he added differed markedly from the nitrogenous materials of rye straw judging by their resistance to hydrolysis and he claims, on this basis, that the latter must be considered

as being quite unlike protein in nature. It appears, however, that Norman's (70) conclusion that proteins and lignin have only a limited combining power might explain the fact that the percentage of the original nitrogen present in the lignin deceased as the percentage of protein was increased.

The removal of nitrogenous material from plant tissue prior to the final lignin determination has been studied by several workers. Norman (70) studied dilute acid hydrolysis with this end in view. He found that successive short-time extractions with 5% sulphuric acid gave lignin yields from straw and oak wood which were lower than if one continuous hydrolysis were given. This may have been due either to the formation of some non-lignin insoluble material from the hydrolysis products when they were allowed to remain in the extractant, or to the condensation of some soluble product with the lignin, or to both. Goss and Phillips (39) employed extraction with 1% hydrochloric acid instead of 5% sulphuric acid for the removal of readily-hydrolyzable interfering materials.

The effect of dilute acid extraction on the lignin itself has been studied by several workers. Bamford and Campbell (2) critisize the method because in the case of some woods the apparent lignin content was higher with than without the acid pretreatment. Norman (70) claims that he has never found such a case, and he believes that some condensation product was formed in the experiments of Bamford

and Campbell (2). The latter workers also suggest that lower results are obtained where successive short treatments with acid are used instead of one long treatment. Norman (70) confirms this but claims such a treatment is only necessary with tissues where removal of the hydrolyzed products may be necessary.

Cohen and Harris (23) objected to the dilute acid treatment on the basis that the apparent lignin content of wood was greatly lowered by it. As the methoxyl content of the isolated lignin was the same before as after the removal of the hydrolyzable material they concluded that it was lignin and not carbohydrate which was being removed. Harris and Mitchell (43) confirmed the results of the above workers on spruce and maple woods. They claim that spruce wood which had been extracted by six three-hour treatments with 3% sulphuric acid lost 16.8% of its lignin. Maple wood lost 22.5% of its lignin when heated for four hours with 3% sulphuric acid. Treatment of wood with 2% hydrochloric acid for three hours at 90 deg. C was claimed to remove about 15% of its lignin. However, these workers have no conclusive evidence that the material removed by acid hydrolysis was not methoxyl-containing carbohydrates. It is generally assumed in the case of succulent plant tissues that the interference avoided by the use of the acid pretreatment is greater than any caused by solution of part of the lignin.

Hewson and Hibbert (49) recently studied the re-ethanolysis of isolated lignins. Their results show that the re-ethanolysis of ethanol lignins extracted under very mild conditions brings about depolymerization to low molecular weight units to a much greater extent than does similar treatment of ethanol lignins isolated by more drastic treatment. This supports the view that the action of ethanolic hydrochloric acid on wood lignin involves both polymerization and depolymerization changes. If isolated lignin can be so readily dissolved in ethanolic hydrochloric acid it would seem likely that aqueous hydrochloric acid might also remove some lignin from plant tissue when used as a pretreatment extractant.

In view of the necessity of removing nitrogenous materials from succulent plant tissue prior to determining its lignin content, a brief survey of methods for the extraction of leaf proteins seems pertinent. Chibnall (15,16) studied the efficiency of various organic extractants for removal of leaf proteins. He found that ether-saturated water was the most efficient extractant for these. In some cases he was able to remove almost 90% of the nitrogenous material from leaves by grinding with this solvent. Lugg (57), on the other hand, tested the relative efficiency of a large number of buffer and salt solutions for the removal of proteins from leaves. He found that mildly alkaline buffers were most effective for dispersing the protein-containing

portions of the cell. It should be stated that no one has yet devised a reagent capable of completely removing the proteins from green leaves. The known efficiency of hydrochloric acid in extracting protein from green leaves may be due, to some extent, to the formation of soluble protein-hydrochloric acid compounds such as that which Osborne (74) postulates for edestan, the insoluble denaturation product formed from the globulin edestin. He (73) found that the amount of denaturation of edestin caused by water at 50 deg.C was about seven times that which occurred in the same time at 20 deg. C. In the same way high-temperature drying or hot extraction of fresh plant material may render relatively easily soluble plant proteins insoluble.

5. Chemical Changes in Lignin During its Isolation.

In addition to the necessity for removal of materials which might interfere in the chemical determination of plant lignin, care must also be taken to arrange conditions so that the lignin itself will not be decomposed. For many years chemists have known that lignin is the most easily oxidized portion of the plant cell wall. Yet, owing to the drastic methods formerly employed in its oxidation, little was known concerning the break-down products of the lignin molecule. Hibbert et al (29) have recently shown that with increasing ozonization of lignin an increase in solubility in sodium bisulphite and a decrease in methoxyl is obtained.

Alkaline oxidation of lignin sulphonic acid was found by Tomlinson and Hibbert (91) to yield about seven percent of vanillin. It has also been shown that smaller amounts of acetovanillone and guaiacol (55) are obtained. With oak lignin sulphonic acid syringaldehyde (3), acetosyringone (56) and 1-3 dimethoxypyrogallol (3) are obtained, in addition to the other compounds mentioned. Workers in the same laboratory have recently succeeded in isolating extremely reactive substances such as vanilloyl methyl ketone from the ethanolysis products of wood (8, 9). More stable products such as vanillin have also been obtained. It is believed by these workers that the isolated products result from the break-down of the lignin molecule. Isolation of products believed to be lignin precursors in wood has also been accomplished by the same workers. West and Hibbert (98) recently showed that the action of acids and alkalies on 3 hydroxy - 1 - (4 hydroxy - 3 - methoxy phenyl) - 1 - propanone brings about a partial conversion into amorphous lignin-like products. They found that the product obtained by the action of concentrated sulphuric acid has the same empirical composition as a typical Klason lignin from spruce wood.

Hagglund (42) pointed out the possibility that changes in the essential nature of lignin might take place during the isolation process. He claimed that an oxygen bridge was split leaving free phenolic hydroxyl groups. He also mentions the possibility of condensation of other reactive plant

substances with the freed groups to give a high apparent lignin content. In view of the possibility of such changes as Hagglund (42) suggests, and the production from lignin through oxidation of reactive products such as Hibbert (51) has described, great care should be exercised to avoid oxidizing media in both the pretreatment process and the final isolation of plant lignin.

6. Relation of age of Plants to their Lignin Content.

Phillips et al (82, 83) have carried out extensive studies on the chemical composition of barley and oat plants at various stages of growth. They found that in the case of barley plants (82) the amount of lignin increased gradually and regularly with increasing age, as did also its methoxyl content. On the other hand, with the oat plants (83) there were definite periods in development when marked changes in the amount and nature of the apparent lignin occurred. The degree to which the changes were due, on the one hand, to changes in the lignin itself, and on the other, to changes in the materials known to interfere in the lignin determination is not known.

EXPERIMENTAL.

1. General Methods of Analysis.

Lignin Determination:

It was decided to determine the lignin content of the various tissues by the 72% sulphuric acid method, as this procedure has been most widely used by earlier workers. The actual conditions used for the determination were those recommended by Manning and DeLong (61). The lignin residues were filtered on asbestos in Gooch crucibles. The asbestos was extracted with hot hydrochloric acid, for several hours, filtered, washed free of the acid, dried, and finally ignited in the muffle furnace before being used.

To aid filtration, diatomaceous earth was added to the acid mixture and a layer was also placed over the asbestos in the crucible. The filter-aid was treated in the same way as the asbestos before being used. The residual lignin was thoroughly washed with hot water, and the Gooch crucibles were dried at 105 deg. C., weighed, ignited in the muffle furnace at a dull red heat for half an hour, and re-weighed. The loss in weight on ignition was taken as the weight of ash-free lignin in the original sample.

The determinations were carried out on air-dry material which had been extracted with ethanol-benzene (1:2), hot water, and 1% hydrochloric acid as prescribed in the official A.O.A.C. method (1). Material treated in this way is referred to as having received standard treatment.

Nitrogen Determination:

Nitrogen determinations on both the fresh and pre-extracted materials were carried out by the Kjeldahl method using mercury as a catalyst.

Lignin samples for nitrogen determinations were filtered in sintered glass crucibles. Naphthalene was used to aid filtering. This filtering technique was originally described by Mueller and Herrmann (65). The naphthalene was sublimed off on the water bath and the residue was dried at 105 deg. C., and weighed. The lignin thus obtained was ground to a fine powder and re-dried at the same temperature. Samples of the dry ground lignin were weighed into 100 cc. Kjeldahl flasks. Approximately 30 mgm. samples were used. Three cc. of the digestion mixture recommended by Campbell and Hanna (14) were added to each flask. Digestion was continued for about 10 minutes after clearing, which occurred in about 3 minutes. The micro-distillation was carried out in the standard all-glass apparatus. A 4% solution of boric acid was used in the receiver. This was titrated back to its original red color (methyl red) with 0.01N hydrochloric acid.

Methoxyl Determination:

Lignin for methoxyl was prepared in the same way as that for nitrogen determinations. Approximately 50 mgm. samples of lignin were weighed out in gelatin capsules as recommended by Samsel and McHard (86). The capsules used were size No. 3

manufactured by Eli Lilly and Company. The actual methoxyl determinations were carried out according to the Viebock and Schwappach modification of the Ziesel method as described by Clark (19) except that a suspension of red phosphorus as recommended by Samsel and McHard (86) was used in the scrubber.

The hydriodic acid used was prepared as described by Clark (20).

All the results recorded for lignin, nitrogen, and methoxyl are expressed on an oven-dry, ash-free basis. For several samples there was insufficient dried, extracted material to allow for the usual ash determination. In these cases all the lignin was isolated with naphthalene and oven-dry samples of the ground lignin were weighed into platinum crucibles and ashed in the muffle furnace. Ashing was accomplished by heating in the muffle furnace at a dull red heat for one hour.

(2)

Description of Research:

(a) Effect of Drying on Apparent Lignin Content of Succulent Plant Tissues:

Previous experiments by the author (58) have clearly illustrated the dangers involved in high temperature drying of material whose lignin content is to be determined. In view of this work, it was deemed advisable to determine whether air-drying appreciably affects the apparent lignin content of succulent plant tissues. Accordingly, a technique was developed which allows treatment of fresh material and does not involve drying until the bulk of the soluble and interfering substances have been removed.

The amount of interference caused by drying tissue would be expected to be relatively greater in young than in older tissues because of the high carbohydrate and nitrogen and low lignin content of the former. Because of this, it was decided to study the effect of drying on plants of different ages. Two experiments were carried out to study this effect. The first was carried out on oats grown in the green house; results are contained in Table I - Part A - The second experiment was carried out on oats grown in the field and the results of this are contained in Table I, part B.

Seed for the crop of greenhouse oats was sown on

December 14, 1942. The variety used was Banner 44. The seed was from the 1941 crop. Seed was sown in six-inch pots and in flats. Six plants were started in each pot and after growth was well started, these were thinned and only three plants were grown in each pot. Previous workers at Macdonald College had found this to be a satisfactory method of growing oats in the greenhouse. Artificial lighting was used from January 29th, till all the oats were finally harvested. The lights were turned on at 4. p.m., and remained on till about midnight. Owing to extremely dull weather during the latter half of December and the month of January, growth was slow, and the plants were inclined to be etiolated.

As the plants were about ten inches in height when they were pulled, it was felt that it would be wise to obtain material at a younger growth stage for comparison. Therefore, a second crop was sown on March 14th, 1943. Growth was much faster than with the previous crop and these plants were harvested on April 5th - 32 days after seeding. Samples from the original crop were harvested 59, 94, and 140 days after seeding.

Oats for the field experiment were sown on June 3, 1943. The variety used was Mabel. Samples were harvested 27,, 34, 41, 56, 60 and 75 days after seeding.

For all analyses the entire above ground portion of plant was used, except in the case of the 75 day sample from the field experiment. In this latter case the grain was removed before the material was analyzed.

The procedure finally adopted for fresh material is designated "A" in the tables which follow. In this procedure 75 gram samples of the fresh material were weighed out and then cut into 1/8 inch lengths. Twenty gram samples of similarly cut material were taken for moisture determinations. The cut (75 gram) sample was transferred to a Model "B" Waring Blendor,# and 350 cc. of water and 50 cc. of absolute ethyl ether were added. The sample was then extracted in the Blendor for half an hour. At the end of this time the sample was transferred to 250 ml. centrifuge bottles and centrifuged at about 2500 - 3000 r.p.m. for five minutes. The supernatant liquid was then drawn off through a sintered glass filter stick and the residue was transferred to the Waring Blendor again. The extraction was repeated twice. After the third extraction the residue was transferred to a round-bottom flask and was refluxed with 1% hydrochloric acid for three hours. The amount of acid solution used was approximately equal to 150 cc. for each gram of dry weight in the original sample. The extracted material was next filtered on a Buchner funnel, washed thoroughly with hot water, and finally air-dried at the water pump. The air-dry material was transferred to a Soxhlet extraction apparatus and was extracted for 30 hours with ethanol-benzene (1:2). On completion of this extraction the excess ethanol-benzene was driven off by gentle heating on the steam bath. The air-dry extracted material was ground by rubbing it between two large files. The samples were ground to pass a 40-mesh sieve. Final lignin determinations were carried out on the

#manufactured by Waring Corp., New York City.

air-dry ground material according to the method described by Manning and DeLong (61).

The results of this experiment are contained in Table I (Page 23).* In order to test whether the differences between treatment A and the standard method were entirely due to the drying of the samples in the latter case, some air-dry samples were analyzed by treatment "A". This treatment is referred to as "B" in Table I. In connection with these results it should be noted that those samples from the greenhouse experiment which received treatment "A" received only two extractions with ether-water in the Blendor.

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*Results in all tables are followed by the average deviation from the mean and the number of determinations in brackets.

TABLE I

COMPARISON OF TREATMENT "A" AND STANDARD METHOD.

Part "A" - Greenhouse Oats:

Age of Oat Plants	Treatment	% Lignin	% Methoxyl in Lignin	% Nitrogen in Lignin
32 days	A	2.62±.18(4)	3.80±.12(3)	3.70±.10(2)
	Standard	3.57±.13(3)	4.25±.15(3)	5.84±.05(3)
59 days	A	2.90±.35(6)	4.53±.08(2)	2.70±.03(2)
	Standard	3.68±.11(2)	7.18±.04(3)	4.29±.05(3)
66 days	A	2.89±.02(2)	8.56±.17(3)	2.92±.02(2)
80 days	A	3.22±.19(4)	11.87±.10(3)	1.82±.04(3)
94 days	A	4.82±.05(2)	13.01±.17(3)	1.52±.01(3)
	Standard	6.67±.04(3)	11.47±.15(3)	2.56±.08(3)
140 days	A	6.25±.01(3)	15.44±.24(3)	1.33±.01(2)
	Standard	6.05±.01(3)	13.37±.23(3)	2.66±.11(3)

Part "B" - Oats From Field Trial:

Age of Oat Plants	Treatment	% Lignin	% Methoxyl in Lignin	% Nitrogen in Lignin
27 days	A*	1.51±.16(3)	8.29±.30(3)	3.08±.01(2)
	B2	2.28±.14(3)	6.16±.16(3)	4.71±.11(3)
	Standard	2.97±.10(4)	4.71±.02(2)	6.98±.40(3)
34 days	B	1.91±.12(3)	9.13±.20(3)	2.61±.15(3)
	Standard	3.93±.04(3)	10.18±.55(3)	3.46±.04(3)
41 days	A	4.77±.15(5)	12.66±.13(3)	1.50±.02(3)
	B	5.51±.02(3)	13.42±.15(3)	2.04±.03(2)
	Standard	6.81±.10(3)	13.28±.14(3)	2.34±.15(3)
56 days	A	5.62±.13(6)	14.59±.13(3)	1.82±.15(3)
	B	6.14±.14(3)	14.43±.15(3)	1.36±.05(2)
	Standard	6.90±.05(3)	13.22±.59(2)	2.71±.03(2)
60 days	A	6.62±.16(6)	14.69±.03(2)	1.36±.04(3)
**75 days	A	10.88±.11(8)	15.34±.28(4)	0.48±.04(4)
	B	9.91±.18(3)	16.32±.37(3)	0.96±.04(2)
	Standard	10.61±.19(3)	14.88±.23(3)	1.22±.04(2)

A - Blendor Treatment on fresh Material
B - Blendor treatment on air-dry Material

*See Table 7 for variations of treatment given this sanple.

**Only straw was analyzed in this case,

It was thought desirable to discover whether the interference caused by high-temperature drying was removed if this was omitted until after the pre-extractions had been completed. Accordingly this effect was tested on young air-dry clover which had been harvested on May 28, 1942. The results of this experiment are contained in Table II. Both samples 1 and 2 received the standard treatment as described, except that sample 2 was dried at 105 deg. C., after the pretreatments, while sample 1 was air-dried.

Table I clearly illustrates the advantages of treatment "A" over the standard method. It will be noted that less lignin containing less nitrogen is obtained by the former procedure, except in the case of the mature straw. In addition, lignin isolated by the Blendor treatment contains more methoxyl in the case of the older samples, although this is not always true in case of the young material. This indicates that the interference due to methoxyl containing carbohydrates was relatively greater in the case of the young than of the older material.

The results on the 140 day old sample in Part "A" and the 75 day sample in part "B" are particularly interesting. It will be noted that for these two samples, treatment "A" gave more apparent lignin containing more methoxyl and less nitrogen than that obtained by the standard method. This is very difficult to explain unless condensation of nitrogenous

material with the lignin prevented similar condensation of methoxyl-containing material.

Comparison of treatment "B" with the standard procedure in Part B shows that approximately 50% of the apparent advantage of treatment "A" over the usual treatment is due to the alterations in the order and nature of the extractions, and not to the fact that the samples for the standard treatment were air-dry. It will be noted that the interfering material removed by treatment "B" as compared with the standard method has a high nitrogen content. The possibility of removal by treatment "B" of soluble lignin precursors which are condensed and thus rendered insoluble by the standard method should be kept in mind.

That the lignin content of tissues increases with increasing plant age is clearly illustrated by both parts of the above table. It will be noted that in the case of the greenhouse oats, the increase in the lignin was gradual and regular, and that even the ripe straw does not contain as much as the corresponding sample from the field trial. The methoxyl content of the lignin isolated from the greenhouse oats was very low and rose only very slowly while growth was poor. However, it increased very rapidly when the plants commenced to grow better.

The lignin content of oats grown in the field rises suddenly between the 34 and 41 day stages, then more slowly till after the 60 day stage when there is another marked rise.

This corresponds more closely to the results of Phillips (83) than do those for the greenhouse oats. On the other hand, the methoxyl content of the lignin from the young field oats was much higher than that from the young greenhouse oats. This may have been a result of the sturdier growth made in the field.

The differences between the results recorded in part "A" and those recorded in part "B" may have been due to varietal differences, but were much more probably due to the difference in growth conditions in the two cases.

TABLE II

Effect of oven-drying pre-extracted residue prior to lignin determination.

Sample No.	% Lignin	% Methoxyl	% Nitrogen
1	5.91±.06(3)	5.09±.09(3)	6.16±.06(3)
2	7.76±.21(3)	4.08±.20(3)	4.86±.16(3)

1. Air-dried after pretreatments
2. Oven-dried after pretreatments.

The results in Table II show clearly that for air-dry material all the interfering substances are not removed by the standard pretreatments. This indicates that part of the interference introduced by high temperature drying is caused by materials which are very difficultly extractable even from air-dry material. It will be noted that the residue in both cases contained approximately the same absolute amount of both nitrogen and methoxyl. The interference thus seems to be due to non-nitrogenous and non-methoxyl containing materials.

In view of the lower results obtained by treatment "B" than the standard method, it was thought that it would be wise to check whether reversal of the order of the 1% hydrochloric acid and ethanol-benzene extractions had caused the effect, or whether it was due to the removal of nitrogenous material with the ether-water mixture before heating. Accordingly the experiment recorded in Table III was carried out on young air-dry timothy harvested May 29, 1941. Sample 1 received the standard treatment. Sample 2 was first extracted for 3 hours with hot 1% hydrochloric acid. It was then air-dried and extracted for 30 hours in the Soxhlet extractor with ethanol-benzene (1:2). The possibility of more complete extraction of ethanol-benzene soluble materials due to the preliminary hydrolysis was also kept in mind.

TABLE III

Effect of Reversal of the 1% HCl and Ethanol-Benzene Extractions.

Sample No.	% Lignin	% Methoxyl in Lignin	% Nitrogen in Lignin
1.	5.56±.07(3)	9.59±.01(2)	2.94±.06(2)
2.	6.41±.06(3)	7.39±.15(3)	4.24±.06(2)

1. Standard pretreatments.

2. Three hours with 1% hydrochloric acid, followed by 30 hours with ethanol-benzene.

It appears from the results obtained that reversal of the extraction order has a detrimental effect, but it should be remembered that the usual hot water extraction was omitted in the case of sample 2. In addition, Table I, Part B, shows that reversal of the order of the 1% hydrochloric acid and ethanol-benzene extractions does not appear to have an adverse effect if the water soluble substances are first removed.

This study has shown that the use of fresh material results in a lower apparent lignin content than if air-dry material is used; that the Blendor treatment on air-dry tissue results in a lignin value lower than that obtained by the standard method, but higher than that obtained if fresh material is used; that all the material which interferes if tissue is dried at a high temperature is not removed by the usual pretreatments; and that reversal of the usual order of the hydrochloric acid and ethanol-benzene extractions does not have a detrimental effect on the lignin values obtained if water soluble materials are first

2. (b) Effect of Reducing Conditions on Apparent Lignin Content of Succulent Plant Tissues.

In view of the known ease of oxidation of lignin an investigation has been made of the effect of reducing conditions on the apparent lignin content of several tissues. Young air-dry timothy and air-dry clover, as well as fresh oat plants at various stages of growth were used in these experiments.

The reducing agent used in the first experiments was hydrogen sulphide. The results of experiments with this substance are recorded in Part "A" of Table IV.

Treatment C is simply the standard procedure modified by reversal of the usual order of the ethanol-benzene and 1% hydrochloric acid extractions and omission of the hot water extraction. Treatment A has been described in the previous section. The results here are for samples which received only two extractions with ether-water in the Waring Blendor.

In the case of samples for which the treatment is A + H_2 S or C + H_2 S all the extracting solutions were saturated with hydrogen sulphide.

In the case of the young timothy samples, hydrogen sulphide was passed into the mixture throughout the extraction with 1% hydrochloric acid. The residue was washed

with hot hydrogen sulphide saturated water. The material was then washed with 95% ethanol and transferred to Soxhlet thimbles. A stream of hydrogen sulphide was passed through the Soxhlets throughout the 30 hour extractions. The ethanol-benzene was then replaced by carbon tetrachloride and the extraction was continued 4-5 hours, hydrogen sulphide still being passed through the apparatus. The carbon tetrachloride extraction was designed to remove any precipitated sulfur from the sample. The samples were removed from the Soxhlets and dried in a vacuum desiccator in an atmosphere of nitrogen. Final lignin determinations were carried out by the usual method. Preliminary experiments on the use of a nitrogen atmosphere during the incubation period with 72% sulphuric acid indicated that this had no effect on the amount of lignin obtained, and therefore it was not used in these experiments. A stream of hydrogen sulphide was passed through the mixtures during the final hydrolysis with 3% sulphuric acid.

The oat samples received the same treatment as the timothy samples except that the ether-water was also saturated with hydrogen sulphide. The pre-extracted samples were air-dried instead of being dried in a vacuum desiccator in a nitrogen atmosphere, and the treatment of the 3% sulphuric acid mixture with hydrogen sulphide was omitted in the case of the oat sample.

The results in Part "B" of Table IV are of an experiment carried out on young air-dry clover harvested May 28, 1944. Sample 1 received the standard treatment.

Samples 2 - 3 and 4 also received the standard treatment, but modified as follows. In the case of sample 2 the 72% sulphuric acid used contained 10% by weight of hypophosphorus acid in order to introduce reducing conditions during the incubation period. Sample 3 was given a 3 hour extraction with hot 1% hydriodic acid instead of the usual 1% hydrochloric acid extraction. For sample 4 constant boiling hydriodic acid was used instead of 72% sulphuric acid for the final lignin determination.

(See Table IV next page)

TABLE IV.

Part "A" - H_2S Treatment.

Material	Age of Plants	Treatment	% Lignin	% Methoxyl in lignin	% Nitrogen in lignin
Young air-dry timothy	Harvested May 27/42.	C	6.41±.06(3)	7.39±.15(3)	4.24±.06(2)
		C + H_2S	5.52±06(3)	7.52±.19(2)	2.15±.02(2)
		C + H_2S	5.82±.08(3)	7.81±.38(3)	2.52±.08(3)
Young oat plants	32 days	A	2.62±.18(4)	3.80±.12(3)	3.70±.11(2)
		A + H_2S	2.24±.08(4)	5.07±.01(3)	4.47±.05(2)
	66 days	A	2.89±.02(2)	8.56±.17(3)	2.92±.02(2)
		A + H_2S	2.91±.03(2)	7.92±.12(3)	3.12±.03(2)
	80 days	A	3.22±.19(4)	11.87±.10(3)	1.82±.04(3)
		A + H_2S	3.47±.11(4)	12.70±.11(3)	1.49±.03(3)
	94 days	A	4.82±.05(2)	13.01±.17(3)	1.52±.01(3)
		A + H_2S	5.53±.17(2)	11.25±.11(3)	2.04±.04(3)
	140 days	A	6.25±.13(3)	15.44±.24(3)	1.33±.01(3)
		A + H_2S	6.85±..10(3)	14.48±.03(3)	1.12±.06(3)

A.- Blendor treatment on fresh material.

C.- Air-dry material - 3 hour extraction with 1% hydrochloric acid - 30 hours extraction with ethanol-benzene.

TABLE IV
(con'd)

Part "B" - Effect of Reducing Conditions at Various Stages in Standard Determination.

Sample No	% Lignin	% Methoxyl in lignin	% Nitrogen in lignin
1.	5.91±.06(3)	5.09±.09(30	6.16±.06(3)
2.	5.91±.15(30	4.87±.12(3)	6.77±.15(3)
3.	6.65±.15(4)	3.78±.26(2)	7.08±.41(4)
4.	18.8 ±.0.2(2)	1.53±.01(3)	2.65±.33(3)

1. Standard treatment

2. 72% sulphuric acid containing 10% hypophosphorous acid.

3. 1% hydrochloric acid treatment replaced by 1% hydriodic acid extraction.

4. 72% sulphuric acid replaced by constant boiling hydriodic acid.

The only case where the introduction of reducing conditions appears to have been of any value was in the case of the young air-dry timothy. In this case it apparently prevented the inclusion in the lignin residue of a large portion of the nitrogenous material included when the standard method is used. In the case of the fresh,cut oat plants, reducing conditions appear to have had very little effect.

In the experiment with young clover we see that the hypophosphorous acid had no significant effect in either the amount of lignin isolated or its composition.

Extraction with 1% hydriodic acid is not nearly as efficient a pretreatment for nitrogen removal as is 1% hydrochloric acid. Constant boiling hydriodic acid apparently fails to remove the cellulose under the conditions used.

From these experiments it appears that the introduction of reducing conditions during the lignin determination has no beneficial effect.

2. (c) Removal of Nitrogenous Material From Plants:

(1) Studies on acid pretreatment

The standard method for lignin determinations relies on extraction with hot 1% hydrochloric acid to remove the nitrogenous materials from plant tissues. Norman (79) tried successive extractions with 5% sulphuric acid and claimed that this aided the removal of nitrogenous material. In view of these findings it was decided to attempt a continuous extraction with hot 1% hydrochloric acid.

The study was carried out on young rye plants which had been killed by dropping them into boiling 95% ethanol. The material had been stored for two years, and was merely used to study the technique of the modified method described.

The tissue was cut into short lengths and ten gram samples were placed in the Waring Blendor with 300 cc. of distilled water and 30 cc. of absolute ethyl ether. This extraction in the Blendor was continued for 30 minutes after which time each sample was filtered with suction into a small Soxhlet thimble . This was accomplished by placing the thimble in a glass Gooch crucible holder. The thimbles were then transferred to an extraction apparatus arranged for continuous extraction with hot 1% hydrochloric acid. The extractor consisted of a can with a false floor in which holes the size of the thimbles were cut. In order to obtain a tight fit and, at the same time, to allow easy removal of the thimbles when they were wet and soft, the holes were lined with collars about 3/4" in width. A tube passing through the centre of the false floor was connected with a siphon from a large flask in which 1% hydrochloric acid was kept boiling under a reflux condenser. The rate of flow of the hot acid was controlled by a screw-clamp on a rubber connection in the siphon. The extractor was placed in a boiling water bath. The acid passed through the siphon into the bottom of the can and then rose up through the samples in the paper extraction thimbles. As the acid diffused out of the upper portion of the thimbles it was drawn off to a constant level. A continuous extraction with hot 1% hydrochloric acid was thus obtained.

After the three hour extraction the thimbles were removed from the extractor and placed in small Soxhlet extractors. Ninety-five percent ethanol was first used as the extractant in order to dry the samples. After 2 - 3 hours, ethanol-benzene was substituted for the alcohol alone. The extraction was continued for 30 hours. After removing the excess ethanol-benzene on the steam bath, lignin determinations were carried out in the usual way. It was found that the residue from 10 grams weighed approximately 1 gram. Therefore, after the extraction apparatus had been modified in some details the experiment was repeated starting with 5 gram samples of material. In this way approximately 1/2 gram residues were obtained after pre-extraction, and therefore it was unnecessary to weigh out the samples for the final lignin determination.

The results of this experiment are tabulated below. Sample 1 is the average of the results of the first trial and sample 2 the average of the second trial with the continuous extractor.

In order to obtain a comparison, a 50 gram sample of the rye was weighed out and treated by the standard method. Because of the nature of the material it was almost impossible to grind it either before or after the preliminary extractions. Sample 3 may therefore not be strictly comparable with 1 and 2, as the latter were very thoroughly sub-divided in the Waring Blendor treatment.

The continuous extraction with hot 1% hydrochloric acid was also tried with fresh oat plants harvested 59 days after seeding. The plants were about 10 inches in height. Ten gram samples were weighed out. Each of these was extracted for 2 half-hour periods with ether-water in the Waring Blendor. Four of the samples then received the same treatment as that given the rye described above. The remaining six samples were refluxed for 3 hours with 300 cc. of 1% hydrochloric acid. For the oat samples alundum thimbles of coarse porosity were used in the continuous extraction apparatus instead of paper thimbles. These were found to be much more satisfactory than the Soxhlet thimbles. After drying with 95% ethanol the ten samples were all placed in the multiple fat extraction apparatus and extracted with ethanol-benzene for 30 hours. Standard lignin determinations were carried out on the residues obtained without further weighing. The average of the samples which received the continuous hydrochloric acid extraction is recorded as sample 4 in Table V. The average of the six samples which were extracted in the usual manner is recorded as sample 5. Oat sample 6 was prepared according to the standard procedure.

TABLE V

Material	Sample	% Lignin	% Methoxyl in lignin	% Nitrogen in lignin
Young Rye	1	3.43±.44 (3)		
	2	3.59±.40 (3)		
	3	3.32		
Oat Plants harvested 59 days after seeding	4	2.90±.09 (4)	2.75±.01 (2)	1.98±.01 (2)
	5	2.90±.35 (6)	4.53±.08 (2)	2.70±.03 (3)
	6	3.68±.11 (2)	7.18±.04 (3)	4.29±.05 (3)

1. Continuous HCl extraction on 10 gram samples.
2. Continuous HCl extraction on 5 gram samples.
3. Standard method.
4. Continuous HCl extraction of small samples.
5. Ordinary HCl extraction of small samples
6. Standard method

The results on young rye indicate that the procedure described gave results very closely similar to those obtained by the standard method. The poor reproducibility of the results was probably due to inexperience in handling the method. oat samples 4 and 5 afford the only absolute comparison of the continuous extraction with the ordinary method. Apparently continuous extraction has little effect on the absolute amount of lignin isolated.

Apparently the continuous extraction removes more nitrogenous material than a single extraction. On the other

hand, the methoxyl content of the lignin isolated in this way is also lower than where one extraction was given. This is difficult to explain in view of the constancy of the lignin content. Harris and Mitchell (43) have shown, however, that treatment with mineral acids tends to lower the methoxyl content of lignin isolated. This would seem to be in line with the above finding.

The advisability of using dilute mineral acid for the pretreatment of materials on which the lignin content is to be determined has been questioned. This raised the problem of designing a method of pretreatment which did not involve such extraction. Accordingly the procedure designated as "D" in Table VI was devised. For lignin determination a 75 gram sample of finely cut fresh material was placed in the Waring Blendor along with 500 c.c. of 5% acetic acid solution. The material was extracted in the Blendor for half an hour and was then filtered on a Buchner filter and washed.. After air-drying the sample it was placed in the Blendor with 500 c.c. of ethanol-benzene and was extracted for a half hour. The ethanol-benzene was then removed with a sintered glass filter-stick and the extraction was repeated. The sample was then air-dried and ground to pass a 40-mesh sieve. Lignin determinations were carried out on the residue.

Treatment "E" was exactly the same as treatment "D" except that, in order to obtain more complete removal of nitrogenous materials, the fresh material received two half

hour extractions with ether-water before the acetic acid extraction in the Blendor.

TABLE VI

Part "A" - Effect of Acetic Acid Treatment.

Greenhouse Oats:

Age	Treatment	% Lignin	% Methoxyl	% Nitrogen
32 days	D	3.27±.54(7)	5.08±.01(3)	5.03±.01(2)
	A	2.62±.18(4)	3.08±.12(3)	3.70±.11(2)
	Standard	3.57±.13(3)	4.25±.12(3)	5.84±.05(3)
66 days	D	6.62±.12(2)	7.78±.20(3)	4.08±.06(3)
	A	2.89±.02(2)	8.56±.17(3)	2.92±.02(2)
140 days	D	11.61±.14(3)	11.84±.32(3)	2.20±.06(3)
	A	6.25±.13(3)	15.44±.24(3)	1.33±.01(3)
	Standard	6.05±.01(3)	13.37±.23(3)	2.66±.11(3)

Part "B" - Oats from Field:

Age	Treatment	% Lignin	% Methoxyl	% Nitrogen
27 days	D	3.82±.52(6)	5.94±.05(2)	5.58±.62(2)
	E	2.59±.09(4)	8.22±.18(3)	5.73±.26(3)
	A#	1.51±.16(3)	8.29±.30(3)	3.08±.01(2)
	Standard	2.97±.10(4)	4.71±.02(2)	6.98±.40(3)

A - 3 ether-water extns., in Blendor - 3 hrs. with hot 1% HCl plus 30 hrs. with alc-benz.
D. 1 extn. with 5% acetic acid plus 2 extns with alc-benz. in Blendor.
E. 2 extns with ether-water and treatment D.

#This sample received two extractions with ether-water and two extractions with 0.5N sodium benzoate instead of the usual three extractions with ether-water.

The results in Table VI indicate that the acetic acid treatment is just as efficient as the standard method for young material. It will be noted that for the 35-day old oats the lignin isolated by the acetic acid method contained less nitrogen and considerably more methoxyl than that obtained by the standard method. It would thus appear that hydrolysis with a mineral acid is unnecessary in the determination of the lignin content of young oat plants.

Part "B" of Table VI shows that treatment "E" is more efficient than the standard method, but still results in a higher lignin value than is obtained by modification "A". It is to be noted that the absolute amount of methoxyl isolated by treatments "D" and "E" was much higher than that obtained by either treatment "A" or the standard method. This may be due to the inclusion in the lignin of methoxyl-containing carbohydrate material, or to removal of a soluble fraction of lignin in the hot mineral acid extraction. It is also possible that the mineral acid extraction demethoxylates the lignin to some extent.

This procedure effects a great saving in time and labour over the standard method, but before it can be adopted it will have to be modified in such a way as to remove more of the interfering nitrogenous and carbohydrate material from older tissues.

2 - (c)

(2) Studies on the Relative Efficiency of Protein Extractants.

In view of the foregoing experiments, an investigation of the efficiency of removal of nitrogenous material from plant tissue by methods other than hydrolysis with mineral acid seemed to be indicated. Experiments were accordingly carried out on fresh oat plants at various growth stages and on young air-dry oats, as well as on air-dry clover which was harvested July 15, 1942. The results of these experiments are contained in Table VII (p. 46, 47).

Samples 1 - 4 inclusive were harvested 27 days after planting. Sample 1 consisted of 40 grams of fresh material, and it was extracted for two half-hour periods in the Waring Blendor with 300 cc. of ether-water. This sample was then divided into two parts, and its nitrogen content was determined.

Sample 2 was of the same size, and received the same treatment as sample 1, except that after the ether-water treatments it was extracted in the Blendor with 100 cc. of 0.5N sodium benzoate for one hour. The extract was then removed and the extraction was repeated. The nitrogen content of the extracted residue was then determined. The method of extraction with sodium benzoate was similar to that described by Morrow (64) for edestin. Samples 3 and 4 were treated in the same way as samples 1 and 2 respectively, but they then

received the 1% hydrochloric acid and ethanol-benzene extractions, as described for treatment "A".

Samples 5 to 10 inclusive were of oats harvested 34 days after seeding. Each consisted of 40 grams of the fresh material. The volume of extractant used in every case (samples 5 to 10) was 300 cc. Sample 5 received three extractions with ether saturated water. Sample 6 was treated in the same way as sample 5, but after air-drying, it received two additional extractions with ethanol-benzene. Samples 7 and 8 were prepared in the same way as samples 5 and 6, except that they were extracted with distilled water instead of ether-water. Samples 9 and 10 also received the same treatment as samples 5 and 6, but in this case the ether-water was replaced by a phosphate buffer solution. The phosphate buffer was similar to that used by Lugg (57). It was prepared according to the method of Clark (21). The p^H of the buffer was 7.3 and it was prepared by dissolving 6 grams of sodium dihydrogen phosphate in 1 litre of water and 6.28 grams of disodium phosphate in 4 litres of water. One litre of the first solution was then mixed with 3750 cc. of the second solution to give a buffer solution of p^H 7.3 containing 1.5 grams of phosphorus per litre.

Samples from oats harvested 41 days after seeding were treated in the following manner. Each of the samples consisted of two parts: 40 grams of fresh material which was extracted with 300 cc. of the extractant in question and 75

grams of similar material for which 500 cc. of each extractant was used. After the preliminary extractions had been carried out, the smaller portions were used for nitrogen determinations, while the larger portions were used for lignin determinations.

Sample 9 received exactly the same treatment as that given sample 6. Sample 12 was also treated in the same way, except that, in this case, two extractions with 0.5% ammonium oxalate were inserted between the ether-water and ethanol-benzene extractions. The oxalate extractions were used in an attempt to obtain a more complete removal of pectic and hemicellulose substances from the tissue in question. The concentration of ammonium oxalate used was that recommended by Norris and Schryver (72). Sample 13 received treatment "A" as described previously (p.21).

In order to check the efficiency of the Blendor treatment for air-dried, ground samples, experiments similar to those just described were carried out on such material. Young oat plants harvested 27 days after seeding, and clover harvested July 15, 1942, were used in this study. The results are contained in part "B" of Table VII. For samples 1, 2 and 3, ten gram samples of the ground air-dry material were weighed out. The volume of the extractant used was 500 cc. in every case. Sample 1 received three extractions with ether-water in the Waring Blendor. Sample 2 received similar treatment but, in addition, it was extracted twice with

ethanol-benzene. Sample 3 was treated in the same way as sample 1, but was given an additional three hour extraction with hot 1% hydrochloric acid. Nitrogen determinations were carried out on the original material and on each of the extracted samples.

Four 2 gram samples of the air-dry clover were weighed out for analysis. Each of these was then given three extractions with 200 cc. of the phosphate buffer described. Two of the samples were then used for Kjeldahl nitrogen determinations. The remaining two samples were extracted twice with ethanol-benzene before their nitrogen content was determined. The results obtained on the first two samples are reported as sample 4 in Table VII, Part B, while the second pair of samples are reported as sample 5:

TABLE VII

Part "A" - Removal of Nitrogenous Material From Fresh Oat Plants:

Age	Sample No.	% N.in orig. Material	% of orig.N. in extr'd Material	% Lignin	% N. in Lignin	% orig. N. in Lignin
	1	4.41±.02(3)	16.6			
27	2	"	5.44			
days	3.	"		1.57±.10(3)	3.61±.05(2)	1.29
	4.	"		1.51±.16(3)	3.08±.01(2)	1.05
	5.	2.17±.01(2)	11.21			
	6.	"	7.93			
34	7.	"	18.01			
days	8.	"	7.93			
	9.	"	12.36			
	10.		7.65			
41	11.	1.81-.01(2)	31.0	8.28±.25(5)	1.96±.49(3)	8.97
days	12.	"	27.0	7.35±.16(6)	3.33±.08(2)	13.51
	13.	"	4.37	4.77±.15(5)	1.50±.02(3)	3.96

1. Two ether-water extractions in Blendor.
2. Two ether-water and two sod.benzoate extractions in Blendor.
3. Two ether-water extractions and 3 hours with hot 1% HCl and 30 hours with eth.-bz.
4. Two ether-water and two sod. benz. extractions and 3 hours with hot 1% HCl and 30 hours with eth.-bz.
5. Three extractions in Blendor with ether-water.
6. Three extractions with ether-water and two extractions with eth.-bz. in Blendor.
7. Three extractions in Blendor with distilled water.
8. Three extractions with dist'd-water and two extractions with eth.-bz. in Blendor.
9. Three extractions with sod. phosphate buffer in Blendor.
10. Three extractions with phosphate buffer and two extractions with eth.-bz. in Blendor.
11. Three extractions with ether-water and two extractions with eth.-bz. in Blendor.
12. Three extractions with ether-water and two with amm. oxalate and two with eth.-bz. in Blendor.
13. Three extractions with ether-water and 3 hours with 1% HCl and 30 hours with eth.-bz.

TABLE VII

Part "B" - Removal of Nitrogen from Air-dry Material:

Material	Sample No.	% N. in orig. Material	% orig. N. in Residue
Air-dry Oat Plants	1.	3.87	41.1
Age - 27 days	2.	"	25.6
	3.	"	6.2
Air-dry Clover. harvested July 15, 1942	4.	1.85	56.2
	5.	"	38.5

1. Three extractions with ether-water in Blendor
2. Three extractions with ether-water and two extractions with alc-bz. in Blendor.
3. Three extractions with ether-water and 3 hours with hot 1% HCl.
4. Three extractions in Blendor with phosphate buffer.
5. Three extractions with phosphate buffer and two extractions with alc-bz. in Blendor

The varying ability of different extractants to remove nitrogen from succulent plant tissues is clearly illustrated in Table VII. At the outset the close agreement between Samples 3 and 4 as compared to Samples 1 and 2 should be noted. Apparently almost all the nitrogenous material removed by the sodium benzoate extractions in Sample 2 was removed from Sample 3 by the extraction with hot 1% hydrochloric acid.

The results for oats picked 34 days after seeding indicate that there is a fraction of the nitrogenous material soluble in ether-water and to some extent, in the phosphate buffer which is insoluble in pure water. However, this substance appears to be soluble in ethanol-benzene. Thus, when an ethanol-benzene extraction is to follow the ether-water this latter has no advantage over pure water, or the phosphate buffer solution. However, the material extracted with ether-water filtered more easily than that extracted with either of the other two reagents and therefore, the use of the former was continued.

The results for the 41 day old oats indicate clearly that , at this stage, the acid hydrolysis is necessary to remove nitrogenous material. Comparison of samples 11 and 12 with 5 and 6 shows that a marked change in the nature of the nitrogenous material in the plants has apparently taken place. Comparison with the results of Samples 1, 2 and 3 in Part "B" of Table VII shows that drying, even at room temperature, apparently causes a change similar to that which occurs with increasing plant age. The results for the air-dried clover are similar to those for the oats.

The results in Table VII indicate clearly then, that whereas 90 - 95% of the total nitrogen of very young plant tissues can be removed by extraction in the cold with mild reagents, acid hydrolysis is necessary to remove this

proportion of the nitrogen from older tissue. In this connection, mention might be made of the work of Osborne (74) on edestin. He found that edestan, the insoluble denaturation product of the globulin edestin, was soluble in hydrochloric acid. He was of the opinion that this was due to combination with the acid to form a soluble salt. Chibnall and Grover (17) claim that the soluble leaf proteins are largely glutelins. These would be insoluble in water and soluble in dilute acid or alkali, and therefore, an acid treatment would be necessary to remove them. If we suppose that the protein fraction of the plant changes to more insoluble types (globulins to glutelins) with age, or that the globulins obtain glutelin-like properties on decreasing hydration then, the increased solubility in hydrochloric acid may be due to the formation of some such salt as Osborne (74) postulates. In addition, it should be noted that any globulins present might be denatured in the Blendor treatment and thus obtain glutelin-like properties.

Table VIII is included in order to compare the amount of nitrogen interference in lignin isolated from oats at different growth stages. Lignin determihationsby methods A, B, and the standard procedure are included.

TABLE VIII

Comparison of nitrogen interference in treatments A, B, and standard method on oats at various growth stages:-

Age	Treatment	% N in orig. Mat'l	% of orig. N in ext'd mat'l	% N in ext'd mat'l	% lignin	% N in lignin	% orig. N in lignin
	A*	4.41±.02(3)	-	-	1.51±.16(3)	3.08±.01(2)	1.05
27 days	B	"	4.83	0.95	2.28±.14(3)	4.71±.11(3)	2.44
	standard	"	9.18	1.77	2.97±.10(4)	6.98±.40(3)	4.71
34 days	B	2.17-.01(2)	7.22	0.55	1.91±.12(3)	2.61±.15(3)	2.30
	standard	"	9.75	0.72	3.93±.04(3)	3.46±.04(3)	6.27
	A	1.81-.01(2)	4.37	0.27	4.77±.15(5)	1.50±.02(3)	3.96
41 days	B	"	6.74	1.32	5.51±.02(3)	2.04±.03(2)	6.22
	standard	"	10.33	0.48	6.81±.10(3)	2.34±.15(3)	8.81
	A	1.11-.01(2)	10.81	0.34	5.62±.13(6)	1.82±.15(3)	7.59
56 days	B	"	9.05	0.29	6.14±.14(3)	1.36±.05(2)	7.52
	standard	"	18.05	0.56	6.90±.05(3)	2.71±.03(2)	16.85
60 days	A	1.33-.12(3)	8.37	0.21	6.62±.16(6)	1.36±.04(3)	8.03
	A	0.42-.01(2)	12.58	0.11	10.88±.11(8)	0.48±.04(4)	12.30
**75 days	B	"	31.20	0.28	9.91±.18(3)	0.96±.04(2)	22.65
	standard	"	33.00	0.29	10.61±.19(3)	1.22±.04(2)	30.81

A. Fresh material - Blendor treatment.
B. Treatment A on air-dry material.

*This sample received two extractions with ether-water and two extractions with sodium benzoate in the Waring Blendor instead of the usual three extractions with ether-water. However, data in Table VII, (Part A) show that this treatment is fairly closely comparable to treatment A.

**The grain was not included in samples analyzed at this stage.

At the outset it should be noted that treatment "B" resulted in removal of a higher percentage of the nitrogen from oats at all the growth stages than did the standard method. The difference in the results obtained by the two methods is rather surprising. Studies on changes in the order of the pre-extractions have already been reported. It was found that reversal of the order of the 1% hydrochloric acid and ethanol-benzene extractions made no consistent difference, either in the amount of lignin isolated, or, in its composition. In addition, it has been shown that ether-water followed by ethanol-benzene does not extract any more of the nitrogenous material from plant tissue than do extractions with ethanol-benzene and distilled water. Therefore, the difference in the results of treatment "B" and the standard method must be due to the fact that the water extraction in the standard method was carried out at 100 deg. C. This is in line with the findings of Osborne (73) on edestin. He studied the amount of edestan formed by contact with water at different temperatures. At 20 deg. C., 4.35% of the edestin had been converted to edestan after 6 hours. At 50 deg. C. 29.00% of the edestin had been changed. These results indicate that water at 100 deg. C. might render a considerable portion of the protein much more difficulty extractable than if the treatment was carried out at room temperature.

It was thought that the possibility of enzymatic removal of nitrogen should be explored. In view of the

known action of ethylene in the ripening of fruit, it was considered possible that it might enhance natural enzyme action, and thus increase protein hydrolysis in the plant cell. The experiment was carried out on 56- and 60-day old oats.

Samples of the oats were treated as follows - One sample was incubated for 40 hours at 40 deg. C. in a closed jar containing ethylene. It then received treatment "A". A second sample was extracted once in the Waring Blendor with ether-water, and was then incubated with ethylene-saturated water under the same conditions. After the incubation, this sample was filtered, and given the 1% hydrochloric acid and ethanol-benzene extractions as described for treatment "A". The third and fourth samples were treated in exactly the same way as the second one, except that in one case the ethylene solution contained 2% pepsin and in the other it was buffered and takadiastase was added. The pepsin solution was made up as described by Crampton and Maynard (25), i.e. 2% pepsin in 0.1N hydrochloric acid. The takadiastase was dissolved in a sodium acetate buffer (p^H4.8) and contained 12.5 grams of the enzyme per litre. This was the concentration of takadiastase recommended by Denny (28) for hydrolysis of potato starch. The acetate buffer was prepared as described by Clark (21).

No definite conclusions could be drawn from the results of this experiment, which therefore, have not been included.

GENERAL SUMMARY OF PART A:

1. Determination of lignin on fresh material results in a lower value than is obtained if the material is dried, even at low temperatures. This was true for all the samples studied, except mature straw.

2. The modified procedure (treatment "A") is more efficient for removal of interfering nitrogenous material from air-dry samples than is the standard treatment.

3. High temperature drying of pre-extracted material causes a high lignin value. This indicates that all the material capable of interfering is not removed by the usual pretreatments.

4. Reversal of the usual order of the 1% hydrochloric acid and ethanol-benzene extractions apparently has no effect on the amount of apparent lignin isolated, or on its composition.

5. The introduction of reducing conditions throughout the lignin determination, and at various stages in it, failed to cause an appreciable difference in the apparent lignin content of plant tissue, or in its composition.

6. Continuous acid extraction did not appear markedly to alter the apparent lignin content of succulent tissues, although it appeared to change somewhat the composition of the lignin fraction isolated.

7. Extraction with dilute mineral acid is unnecessary for removal of interfering substances from young tissues, but seems to be necessary to remove the nitrogenous material from older tissues.

8. Ether-water extractions of fresh material will remove 95% or more of the nitrogen from young tissues. They are not as efficient as this for older tissues, and in the latter case, acid extraction is necessary to remove this proportion of the nitrogenous material.

9. Treatment with ethylene, takadiastase or pepsin failed to remove more of the interfering materials from oat plants than is removed by the standard pretreatments for the lignin determination.

PART B.

Studies on the Chemical Nature of Plant Lignin.

Historical Review

(a) General Theories of Lignin Structure.

Work on the chemical constitution of lignin has been largely carried out on material isolated from wood. In spite of a tremendous amount of research the true chemical nature of this material can still be only speculated upon. Recent reviews (31, 33, 51, 79) on lignin chemistry have emphasized the essentially aromatic nature of the lignin molecule. The above authors all admit the possibilities of the suggestion of Klason (54) that lignin is closely related to coniferyl alcohol which is present as the glucoside coniferin in the sap of many plant tissues.

The problem of discovering the chemical structure of the lignin molecule has been attacked in several ways. Lignin isolated by the traditional concentrated acid procedures is highly modified during the isolation process, and therefore, is of little value for the study of the nature of lignin in situ.

Many attempts have been made to isolate so called 'native' lignin from wood. Freudenberg's work has been largely carried out on lignin isolated by alternate extraction of wood meal with dilute sulphuric acid and cuprammonium hydroxide. Hibbert et al (51) have used alcoholysis methods

to remove lignin fractions from wood. Such procedures involve the use of anhydrous alcohols containing small amounts of hydrogen chloride. Alkaline oxidation (25a) of lignin has led to isolation of aromatic products. Gymnosperms appear to yield only 25% of vanillin on alkaline degradation; whereas maple and aspen woods yielded 46 and 48% respectively, of mixtures of vanillin and syringaldehyde. It was considered that these aldehydes were derived from propylphenyl units similar to those isolated by the alcoholysis procedures.

High pressure hydrogenation methods have also been employed to study lignin structure. Spruce and maple woods (37) were completely liquefied by this technique. The products obtained were 4-n-propylcyclohexanol and 3-(4-hydroxycyclohexyl) propanol-1.

Ultraviolet absorption spectra of lignin solutions have been studied (77, 78) with a view to obtaining some knowledge of the groupings present in the lignin molecule.

Hibbert (51), Freudenberg (33), and Erdtman (31) are all agreed that the principal lignin building unit is a propylphenyl grouping. However, they differ considerably in their opinions as to how such units are linked together to form lignin. Freudenberg (33) believes that lignin is a mixture of polymers of the dehydrodiisoeugenol type. Hibbert et al (30, 51), on the other hand, are of the opinion that native lignin is a series of polymers of dehydrodi-

(oxyconiferyl alcohol) and dehydrodi- (oxysyringyl alcohol). The hypothetical polymers which Freudenberg (33) proposes are characterized by the presence of terminal methyl groups in the propyl side chains. Hibbert (30, 51) believes that the terminal groups of the side chain are either primary alcohols or ether linkages. Hibbert (51) has also pointed out the similarity in structure between substances isolated from the ethanolysis products of wood and units in Szent-Gyorgyi's (90) ene-diol plant oxidase system. Hibbert (51) believes that propylphenols are formed in both lower and higher plants so that they may function as repiratory catalysts in a system similar to Szent-Gyorgyi's.

Creighton et al (26, 27) have studied alkaline nitrobenzene oxidation of a large number of plant species. They showed that gymnosperms yielded only vanillin while the angiosperms gave syringaldehyde in addition. They suggest a taxonomic classification of plants on this basis. They (27) succeeded in isolating p-hydroxybenzaldehyde from the alkaline oxidation products of several monocotyledonous but from no dicotyledonous species. They suggest that this may be a distinguishing feature between the lignins of monocotyledons and dicotyledons.

MacInnes (59) studied the ethanolysis products obtained from fir, redwood, rye straw, corn stalks, bamboo, jute, and red oak. He found that lignin present in angiosperms contains both syringyl and guaiacyl nuclei

while that of gymnosperms contains only the guaiacyl nucleus.

(b) Ultraviolet Absorption Spectra of Lignin Solutions.

Ultraviolet absorption spectra have now been used for a considerable time to predict the structure of organic compounds. The measurements are made on a spectrograph. The calculations of the extinction coefficients are based on the Lambert-Beer law usually expressed mathematically as

$$I = I_0 10^{-kcd}$$

where I_0 = incident intensity

I = transmitted intensity

c = concentration

d = thickness in centimetres

k = specific extinction = E/cd

where $E = \text{Log } I_0 / I$

A complete discussion of chemical spectroscopy is contained in Brode's (10) recent book. The light absorption is due to resonance and therefore it can depend either on an incomplete inner electron shell or on unsaturation in the molecule. The characteristic absorption spectra of many different organic groupings have been determined by the use of simple molecules. The nature of the absorption of those groupings likely to be present in lignin solutions is discussed below.

1. The carbon-carbon single bond. This grouping absorbs only in the far infrared and in the extreme ultraviolet and is therefore of little importance in the present work. When

it separates two chromophoric groups it shields one from the other and the absorption curve shows the independent effects of the two rather than the effect of a single compound resonator.

2. The ethylenic double bond. This group absorbs only in the extreme ultraviolet at about 1950 Å. The presence of other chromophoric groups or unsymmetrical substitution on this group may change the shape or location of this band.

3. The carbonyl group. Aldehydes show an absorption band at about 2700 Å. Enolization produces a very marked effect on this.

4. The alcohol grouping. As this group involves only a single bond linkage it is transparent through the visible and photographically available ultraviolet.

5. The ether linkage. This group is a single bonded non-resonator which may act as a shield between chromophores.

6. The benzene nucleus. Benzene derivatives have a characteristic banded appearance. Benzene itself exhibits seven bands between wave lengths of 2300 and 2750 Å. Substitution in the benzene ring produces damping effects which may largely obliterate the fine structure of the resonance.

The first work on the absorption spectra of lignin solutions was carried out by Herzog and Hillmer (47). They compared curves obtained with coniferin and isoeugenol with

those from lignin-sulphonic acid and sulphite liquor. The general shape of the curves obtained was similar but the relative positions of the various maxima varied to a considerable extent. In a later paper (48) they compared lignin absorption curves with those obtained from polymerized coniferyl alcohol. They found better agreement in this case.

Hagglund and Klingstedt (41) compared the absorption curves obtained from methyl, ethyl, amyl, and alkali lignins as well as from lignin-sulphonic acid. They found that all these substances were very similar to one another, and therefore concluded that they all possess the same skeletal structure. They found that for lignin obtained from spruce, pine, birch, and beech, the principal maxima occurred between 2740 and 2940 Å.

Stamm et al (89) studied the absorption spectra of lignins from a number of woods. They found that softwood lignins gave maxima at from 2810 to 2850 Å while hardwood lignins gave maxima between 2740 and 2760 Å.

Glading (35) prepared native lignin, phenol native lignin, glycol lignin, thiophenol lignin, thiolignin, and alkali lignin from spruce wood. Lignin derivatives were also prepared. Absorption curves of all these materials were determined and from them he assumes that the introduction into lignin of substituent groups having no significant absorption merely serves to reduce the absorption maxima of the lignin. He also compared lignin curves with those of

flavanone and quercetin. His results indicate that the general form of the lignin curve is given by substances with a coumarin type of ring structure.

Patterson and Hibbert (77, 78) studied the ultraviolet absorption spectra of ethanol lignins and also of compounds known to be related to lignin. Compounds containing the vanilloyl or veratroyl group gave absorption bands of 980f, 1070f, and 1300f; syringoyl derivatives have maxima at 980f and 1300f; and trimethoxybenzoyl derivatives gave maxima at 1070f and 1300f. By comparison of these results with those for ethanol lignins they conclude both that the spectra of amorphous lignins can be satisfactorily explained on the assumption that they are derived from substances known to be lignin precursors, and that the curves indicate the aromatic nature of lignin. In addition, they suggest from the evidence obtained that a carbonyl group or ethylenic double bond is present in conjugation with the aromatic nucleus.

Ward (95) studied the absorption spectra of indole derivatives. He found that many such compounds show a maximum at about 2800 Å. He reports that aliphatic aldehydes give curves which rise to a peak at about 2600 Å. This was in agreement with earlier work. In a second investigation (96) he reports on the absorption of a number of amino acids. He found that phenylalanine, tyrosine, and tryptophane gave marked absorption bands between 2100 and 3000Å, while alanine, histidine, glutamic acid, and cystine gave only general

absorption. Tryptophane and tyrosine gave maxima at about 2800 Å, while phenylalanine gave a maximum at about 2600 Å. It seems possible that humin-like material formed from these aromatic amino acids might have absorption similar to that of the acids themselves and could therefore interfere in the determination of lignin absorption spectra.

Spiegel-Adolf and Krumpel (88) studied the ultraviolet absorption spectra of serum albumin samples treated in various ways. In every case they obtained a maximum at about 2675 Å.

Marchlewski and Nowotnowna (62) studied the ultraviolet absorption of a large number of amino acids and some proteins. The only substances which gave appreciable absorption above 2500 Å were tryptophane, tyrosine, phenylalanine, and a sample of keratin prepared from wool.

The absorption spectra of sugar solutions have been studied by several workers. Marchlewski and Urbanczyk (63) report that glucose, galactose, maltose, arabinose, and rhamnose show maxima between 2650 and 2700 Å. Goos et al (38) report that certain methylated monosaccharides show strong absorption in the region of 2800 Å.

It appears, then, that the ultraviolet absorption maxima of lignin are quite definitely established and that they are little affected by the method of isolation of the lignin or by substitution in the lignin molecule. Interference in the determination of the absorption spectra of

plant lignin would probably come mainly from proteinaceous materials especially those containing aromatic amino acids. Carbohydrates which might interfere should be largely removed in the pretreatment extractions.

(c) Studies on the nature of lignin by high pressure hydrogenation.

Preliminary work on the high pressure hydrogenation of lignin was carried out by Harris, D'Ianni, and Adkins (44). They hydrogenated methanol aspen lignin in dry dioxane over copper-chromium oxide. Hydrogen was absorbed at 250-260 deg. C. under 200-300 atmospheres pressure during a period of about eighteen hours. From the hydrogenation products 4-n-propylcyclohexanol-1 and 4-n-propylcyclohexane-diol-1,2 were identified. Bowden and Adkins (4) more recently identified 3-(4-hydroxycyclohexyl) propanol-1 from the reaction products of a similar experiment. Harris et al (45, 46) have shown that wood as well as lignin can be hydrogenated over Raney nickel in alkaline solution. This was accomplished at more moderate pressures than the earlier hydrogenations. They claim that 18% of the original maple wood (containing 22.5% Klason lignin) was obtained as identified hydroaromatic compounds.

Hibbert (50) has reported that hydrogenation of maple ethanol lignin yielded 12% of 4-n-propylcyclohexanol-1 and 3% of 3-(4-hydroxycyclohexyl) propanol-1. Bower et al

(6,7) studied the hydrogenation of maple wood and the course of formation of native lignin in spruce buds as indicated by the hydrogenation products obtained. By use of a more efficient fractionation technique than had been formerly employed 3-cyclohexyl-1-propanol was obtained from the hydrogenation products of both maple wood and maple ethanol lignin. These workers (7) failed to isolate propylcyclohexyl derivatives from the hydrogenation products of 2.5-3.0 weeks old spruce buds although they did isolate 4-n-propylcyclohexanol from 3.5-4.0 weeks old material. From this result it is claimed that young spruce buds do not contain any lignin. They base this conclusion on results obtained (4a) on the high pressure hydrogenation of maple holocellulose. Holocellulose containing no Klason lignin was found to yield about one third as much fractionable water insoluble oil as the entire wood. The oil yielded by the holocellulose did not contain the propylcyclohexyl derivatives that have been identified in the material isolated from wood. Therefore, they assume that the material from the holocellulose is of carbohydrate origin. In spite of this evidence it seems possible that in very young material more reactive compounds are present than in the older tissue and that these might have resulted in a mixture of similar propylcyclohexyl derivatives each of which was present in a quantity too small to be separated from the others by the fractionation method used.

EXPERIMENTAL

(a) Studies on the Nature of the Nitrogen Complexes present in Extracted Material and Isolated Lignin.

In view of the difficulty of isolating nitrogen-free lignin from plant tissue it was decided to study the nature of the combination of the nitrogen in both the pre-extracted material and in the isolated lignin. Clover harvested May 28, 1942 was used in these experiments.

The first method of attack involved dry distillation of both the extracted material and the isolated lignin. This was carried out according to the method of Guest and McFarlane (40). Neither of the distillates gave a positive test with isatin or with p-dimethylaminobenzaldehyde. The negative test with isatin indicates the absence of the pyrrole group while the negative test with p-dimethylamino-benzaldehyde indicates the absence of pyrrolines, pyrrolidines, and indoles. Biuret and Millon's tests were negative on both distillates. These latter show the absence of peptide linkages and hydroxyphenyl groups in the distillates.

In view of the known failure of the standard Kjeldahl method to detect nitrogen present in many types of combination, determinations were carried out involving the reduction procedure described by Friedrich et al (34). The reduction made no difference in the apparent nitrogen content of the lignin. This indicates that the material as

isolated does not contain hydrazines, osazones, or oximes.

Murexide tests were carried out on both the extracted material and the isolated lignin. No reaction was obtained and therefore the absence of purines is indicated.

A portion of the pretreated material was extracted with cold 10% sodium hydroxide. The extract gave no Biuret or Millon reaction.

Van Slyke amino nitrogen determinations were carried out on the products obtained from dry distillation of both lignin and the pre-extracted material; on sodium sulphite solutions of both chlorinated lignin and chlorinated extracted material; and on the sodium hydroxide extract of the pretreated sample. In all cases the results obtained were a little higher than the blank but the differences were very small and the results were quite erratic. In this connection mention might be made of the work of Traub (92) on apple twigs. He found that for mature tissue, inner xylem, and pith of 2-3 year old apple twigs, the amino nitrogen makes up from 13 to 60% of the total nitrogen at different parts of the growing season. Thus, in apple twigs at most growth stages there is a very high proportion of non-amino nitrogen present.

It is possible that the reason no amino groups are detected and that the nitrogen is so tenaciously held is that condensation of the type of Maillard's reaction have occurred. Weast and McKinney (97) have recently shown that this reaction

produces compounds similar to those obtained in sun-dried fruits on non-enzymatic darkening. It may be that a similar reaction occurs in grass on air-drying. The dark colour produced would in this case be masked by the pigments present. They (97) found that 98% of the nitrogen in the dark compounds was of a humin nature. If artifacts interfering in the lignin determination are of a similar nature, then they, too, would be expected to contain a high proportion of insoluble humin nitrogen.

These studies show little of the actual nature of the nitrogen combinations in lignin. However, they do emphasize the fact that the nitrogen is very unreactive and very tenaciously held.

(b) Studies on the Ultraviolet Absorption Spectra of isolated Plant Lignin.

In view of the characteristic nature of lignin absorption spectra in the ultraviolet (77, 78) it was thought desirable to employ this method for study of the nature of the lignin isolated from plant tissues. Accordingly studies were carried out on the lignin isolated from both oats and timothy harvested at various growth stages.

Preparation of Material.

Oats harvested on June 30, July 7, and August 17, 1943 and timothy harvested on May 28 and July 15, 1942 were used in these studies. The material was prepared according to

treatment "B" (see page 22) and the standard method (see page 16). Samples on which absorption spectra were run were filtered on Gooch crucibles using diatomaceous earth as a filter-aid. These were then placed (while still wet) in a closed jar connected with a chlorine generator. The samples were left in this moist atmosphere for from one to three days. They were then removed and leached with a 2% solution of sodium sulphite. After no more colour was removed in the leachings the samples were re-chlorinated for several hours and the leaching process was repeated. The chlorination and sulphite extractions were repeated until the leachate was colourless. This usually required three chlorinations and sulphite extractions. The bulk of the lignin was dissolved on the first treatment. The crucibles were then dried at 105 deg. C, weighed, ashed, and reweighed in order to determine the amount of organic matter which remained undissolved. The amount of material dissolved was calculated by subtracting the amount of undissolved organic matter from the known yield of ash-free lignin from a half gram sample of the pre-extracted material. The sulphite solutions obtained were diluted with distilled water to the concentration required for the spectrographic analysis.

The absorption curves are compared with a similar curve for wood lignin. The wood lignin was prepared by the

72% sulphuric acid method from a sample of maple wood-meal which was obtained from Dr. H. Hibbert, then of the Division of Industrial and Cellulose Chemistry of McGill University. The material had been pretreated by extraction for 24 hours with ethanol-benzene, 24 hours with ethanol, and 12 hours with hot water. The lignin was dissolved in the same way as for the oat and timothy samples.

Determination of absorption spectra.

In view of the results of Stamm et al (89) it was assumed that sulphite solutions gave no absorption in the range studied. They found that solutions which were a few days old gave no absorption above 2500 Å and transmitted light down to 2320 Å. Fresh sulphite solutions did absorb light of the same wave lengths as lignin.

The spectrographic measurements were made with a large quartz spectrograph, Littrow mounting. The prism of this instrument is a 30° quartz and the lens has a focal length of 1800 mm. The dispersion was approximately 2.5 Å per mm. at λ2500 and 25.0 Å per mm. at λ5500. The spectrograph was fitted with a Bellingham and Stanley single-disc rotating sector photometer (1936 design). The sector speed was 100 revolutions per minute. Illumination was effected by a condensed spark between tungsten-steel electrodes set 4 mm. apart. The spectrograms were recorded on Eastman No. 33 (8 x 10 in.) plates. The plates were examined visually and the points of equal blackening were

marked. The wave lengths of the cross-over points were determined by measuring the distance of each from a standard line at the end of the plate and reading of the corresponding wave length from a graph prepared for the purpose from a standard plate. The wave lengths determined were than plotted against the extinction of a 1% solution in a 1 cm. cell.

TABLE IX

Ultraviolet Absorption Data on Plant Lignins.

Part A - Oat Lignin.*

Date Harvested	% Lignin	% N. in Lignin	% Methoxyl in lignin	% Sol. in sulphite	% Purity of sulphite sol. mat'l.	Total Purity
June 30	2.28	4.71	6.16	77.6	60.8	47.2
July 7	1.91	2.61	9.13	76.0	78.1	59.3
July 7(a)	3.93	3.46	10.18	80.0	79.3	63.4
Aug. 17**	9.91	0.96	16.32	89.6	90.3	80.8

* All samples except July 7(a) were prepared by treatment B described in the previous section. July 7(a) was prepared by the standard method.

** The grain was removed from this sample and the remainder of the plant was analyzed.

Part B - Timothy lignin.*

Date Harvested	% Lignin	% N. in Lignin	% Methoxyl in lignin	% Sol. in sulphite	% Purity of sulphite sol. mat'l.	Total Purity
May 28	6.38	4.00	7.47	75.8	76.9	58.2
Jul 15	8.32	2.01	12.68	70.1	107.0	75.1

*Prepared by standard method.

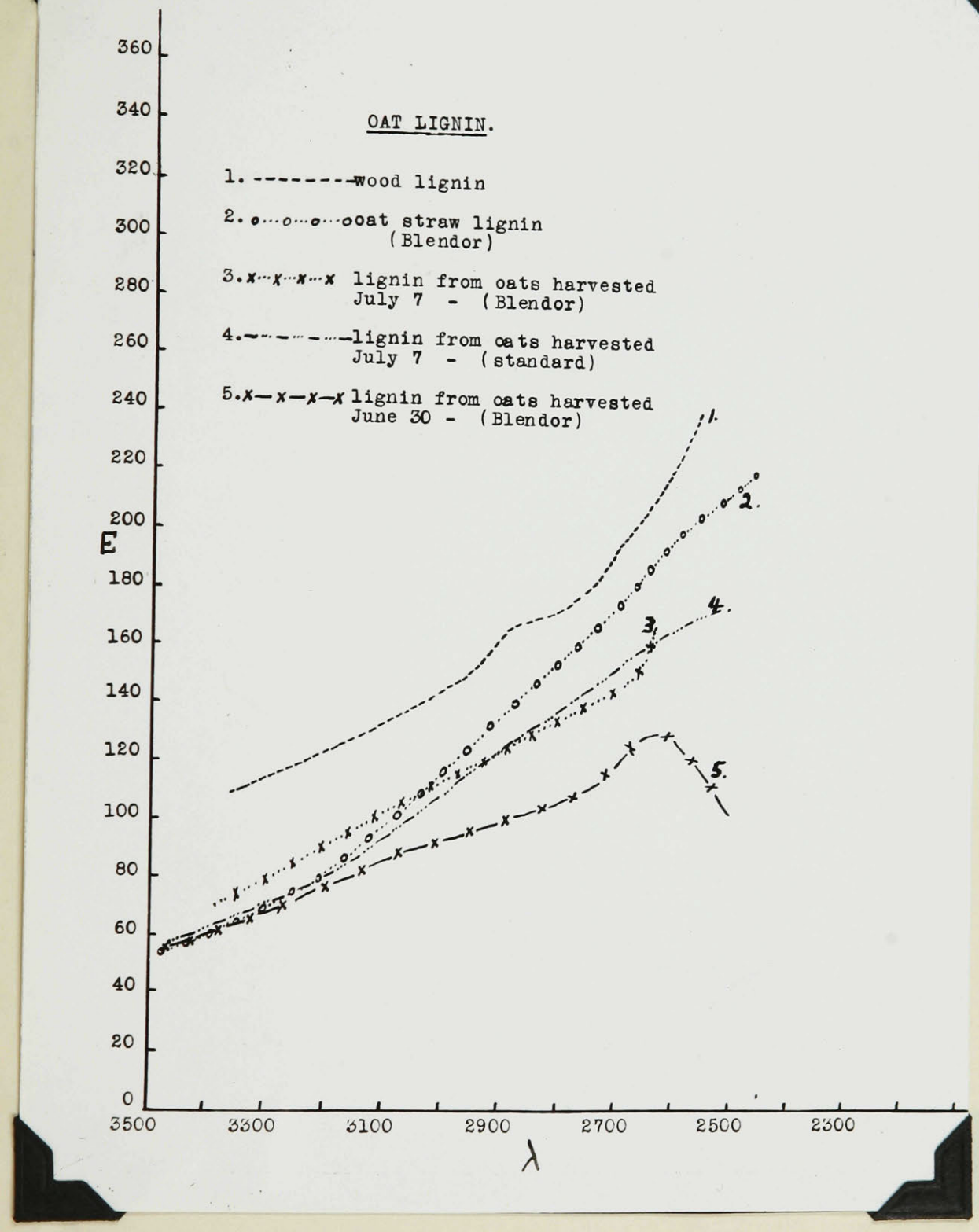

OAT LIGNIN.
1. --------wood lignin
2. o···o···o···oat straw lignin (Blendor)
3. x···x···x···x lignin from oats harvested July 7 - (Blendor)
4. -·-·-·-·-lignin from oats harvested July 7 - (standard)
5. x—x—x—x lignin from oats harvested June 30 - (Blendor)
E
λ
360
340
320
300
280
260
240
220
200
180
160
140
120
100
80
60
40
20
0
3500
3300
3100
2900
2700
2500
2300

Figure I.

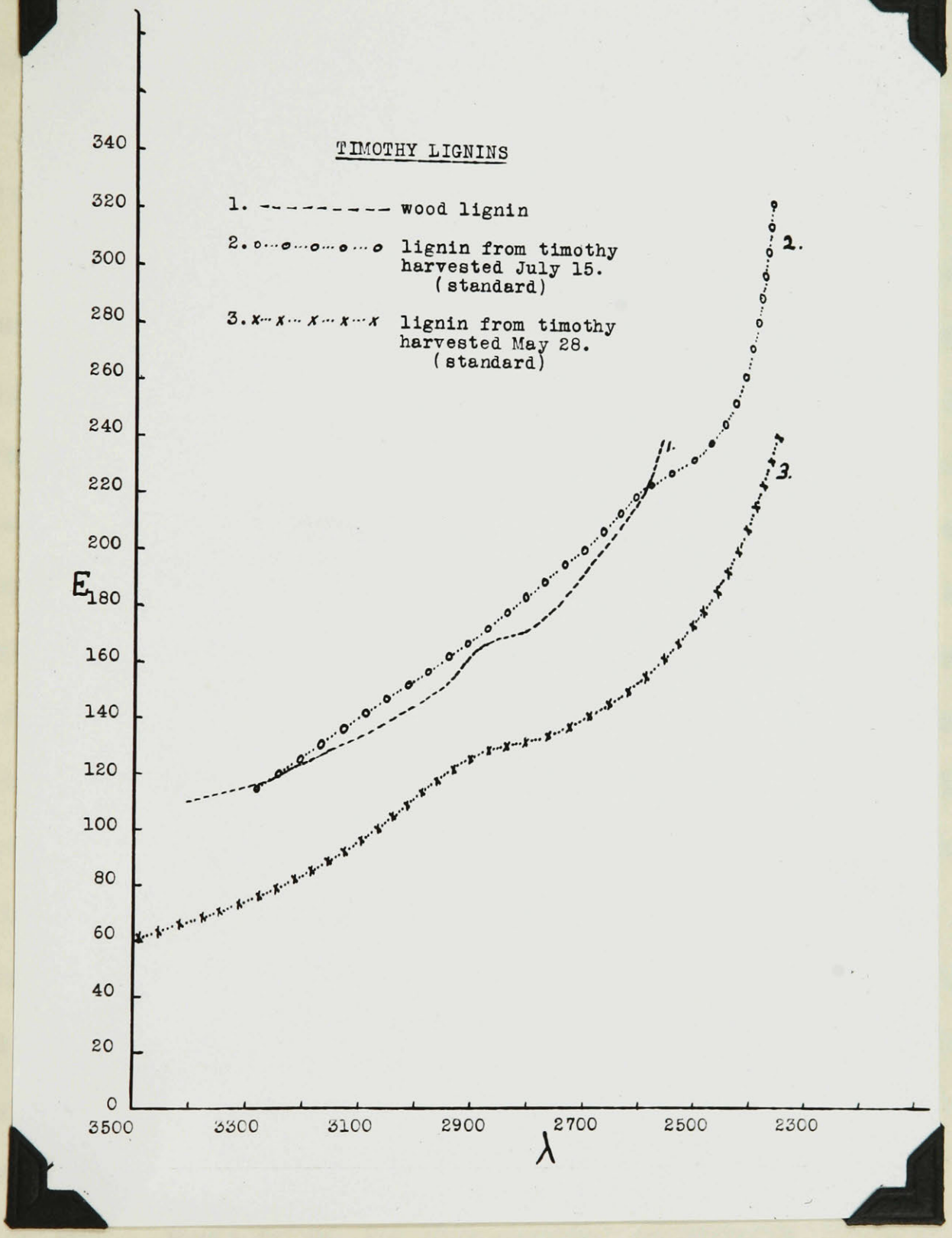

TIMOTHY LIGNINS
1. ---------- wood lignin
2. o···o···o···o···o lignin from timothy harvested July 15. (standard)
3. x···x···x···x···x lignin from timothy harvested May 28. (standard)
E
340
320
300
280
260
240
220
200
180
160
140
120
100
80
60
40
20
0
3500
3300
3100
2900
2700
2500
2300
λ
1.
2.
3.

Figure II.

Discussion of results:

The results of these experiments are contained in Table IX. The corresponding ultraviolet absorption curves are contained in Figures I and II. The extinction coefficients are all calculated for a 1% solution.

It will be noted that Table IX contains a column entitled, "percentage purity of sulphite soluble material", and a second column entitled "total purity". The former results were calculated by comparing the extinction coefficients for the different solutions with the extinction coefficient of wood lignin at 2800 Å. It is assumed that wood lignin is 100% pure and that the extinction coefficients of the plant lignins at this point are directly proportional to their purity. At the same time, isolated plant lignin is only partially soluble in the sulphite solution, while wood lignin is completely soluble. Therefore the total purity of the isolated plant lignin is obtained by multiplying the percentage solubility in sulphite solution by the percentage purity of the sulphite soluble fraction, both of these values being calculated as described above.

Referring to the curves in Figure I it will be noted at the outset that none of the curves for oat lignin have even a trace of a break at the 2800 Å region found (35, 41) to be so characteristic for lignin isolated from wood in many ways. Each of the curves for oat lignin is the average

of the results obtained from two or more samples. The absence of the maximum in the 2800 Å region can be explained in one of two ways. Either plant lignin does not contain the grouping responsible for the maximum or the lignin is contaminated by condensation with its reactive groups or by the presence of material which absorbs light in the same region of the spectrum and thus masks the effect. Patterson and Hibbert (77) explain the maximum at 2800 Å as being due to 'meta-position freedom' in the lignin molecule. The hydrogen atom occupying this position is claimed by these workers to be extremely labile and therefore it would seem logical to assume that if condensations with nitrogenous material occur during the isolation of lignin from succulent plant tissues they might occur at this point. In this case the isolated lignin would not show a maximum in the 2800, Å region.

The second point to be noted from Figure I is that with increasing age a lignin with greater light absorption in this region is obtained. In addition, Table IX shows that the sulphite solubility of the lignin isolated from straw is greater than the solubility of that isolated from young tissue. These effects are due either to the fact that lignin from young tissue has a different structure than that from the older material, or to the fact that lignin from young tissue is more highly diluted with non-absorbing material than is that from older tissue.

Note should be made of the results obtained from the July 7 oats. From Table IX it will be seen that the standard treatment resulted in isolation of twice as much lignin as treatment "B". In addition, the lignin isolated by the standard method contained a higher percentage of methoxyl than that isolated by treatment "B", although the nitrogen content of the former was also considerably higher. The results also show that the sulphite solubility, the percentage purity of the sulphite soluble fraction, and therefore the total purity of the isolated lignin is greater in the case of the material isolated by the standard procedure than for that isolated by treatment "B". At the outset this result is difficult to explain, and further investigation of this point seems to be indicated. The essential difference between treatment "B" and the standard method is that in the former case the treatment with hot 1% hydrochloric acid is preceded by cold ether-water instead of a hot water extraction. This must therefore be the cause of the discrepancy in the results. It would appear that treatment "B" allows removal of some simple lignin building units which are probably condensed and thus rendered insoluble by the standard treatment. Either the ether-water extraction of treatment "B" removes some components or it leaves them uncondensed and thus allows their removal by the hot 1% hydrochloric acid extraction which follows it.

It is impossible to say which of these possibilities is true. It is indicated, however, that an attempt should be made to identify lignin precursors and, or, break down products in the water and hydrochloric acid extracts. This task would be extremely difficult owing to the large amount of carbohydrate and nitrogenous material present in the extracts.

Mention must also be made of the maximum which is obtained for oats harvested June 30, 1943. The cause of this is difficult to explain. However, Ward (95) draws attention to the fact that certain aldehydes show absorption maxima at about 2600 Å. Creighton and Hibbert (27) have claimed that p-hydroxybenzaldehyde is derived from monocotyledon lignin on oxidation with alkaline nitrobenzene. It is possible that in lignin from young tissue an aldehyde group might be free and might therefore cause the absorption maximum observed.

Ward (96) claimed that phenylalanine gives an absorption maximum at about 2600 Å and Spiegel-Adolph and Krumpel (88) have shown that serum albumin also gives a maximum in this region. The sample of lignin under discussion contained a relatively high percentage of nitrogen and if this is present as a derivative of aromatic amino acids these might cause the maximum at 2650 Å.

It is impossible to say whether an aldehyde or a nitrogenous compound caused this effect but it was likely due to one or both of them.

The results for timothy are, in general, similar to those for oats. The lignin from the older timothy shows a higher total purity than that from the young material even though its sulphite solubility is lower. This latter fact is rather surprising in view of the results obtained for oats.

The timothy curves are shown in Figure II. It will be noticed that the young timothy lignin shows a definite break in the 2800 Å region and that the general shape of the curve is very similar to that of the wood lignin. However, the absorption curve for lignin from the older timothy does not show a maximum in the 2800 Å region. It is difficult to see how such could come about unless the lignin from young material possesses 'meta-position freedom' while this group is blocked by methylation or other condensation reactions in the older material.

It should also be noted that the curve for the lignin from old timothy falls slightly above that for wood. On the basis of the method used for calculation this means that the lignin in this particular sulphite solution was 107% pure. It is doubtful whether the timothy curve is significantly above the wood curve and therefore the method of calculating purity cannot be condemned on this evidence

alone. However, it does serve to illustrate the limitations of the method and shows that small differences in purity calculated by it should not be considered to be significant.

Mention should be made of the fact that it was extremely difficult to obtain the same sulphite solubility on duplicate lignin samples. In general, where less lignin was dissolved the material in solution was purer than if the solubility was greater. This would indicate that the most difficultly soluble material has low light absorption as compared with the readily soluble part. The true lignin is probably dissolved first, while artifacts formed from carbohydrates and humin-like materials probably constitute the more difficultly soluble part.

(c) Studies on the high pressure hydrogenation of pretreated plant tissues.

Bower et al (7) have recently claimed that young spruce buds contain no lignin. They base this claim on the fact that they were unable to identify the phenylpropane units, which they consider to be lignin building units, in the hydrogenation products of young spruce buds. In view of this claim it was thought that high pressure hydrogenation of succulent plant tissue might yield valuable information regarding the nature and amount of its lignin. Accordingly this investigation was undertaken.

The materials used in this investigation were timothy harvested on May 28, 1942, timothy harvested on July 15, 1942, and oat straw harvested August 17, 1943. All the samples were pretreated according to the standard A.O.A.C. method (1) except that they were air-dried at room temperature. Hydrogenations were carried out on the extracted samples.

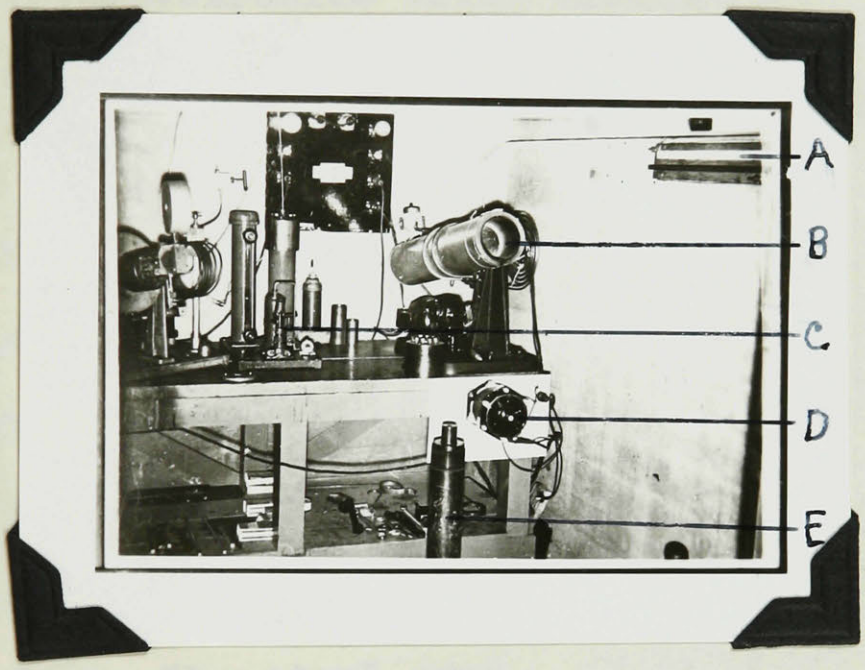
Hydrogenation apparatus and technique.

A high-pressure hydrogenator (No. 406-01a, catalogue #406; American Instrument Company) of 2500 cc. capacity was used for all the experiments carried out. A type 100 Variac manufactured by the General Radio Company was used to control the temperature. The necessary hydrogen pressure was obtained through the use of a hydrogen "booster" pump (#406-135, American Instrument Company). A slide-wire

potentiometer calibrated to read temperature directly was used to measure voltages from a copper-constantan thermocouple, the hot junction of which was inserted in a well in the bomb.

The actual arrangement of the hydrogenation equipment is clearly illustrated in the following photograph.

Hydrogenation Equipment.



A. Slide-wire-potentiometer.

B. Heater for bomb.

C. "Booster" pump.

D. Variac.

E. Bomb.

In view of the fact that it was desired to compare the hydrogenation products with those obtained by Bower et al (7) the technique used approached that which he employed as nearly as possible. Dioxane which had been dried by refluxing with sodium was used as the solvent. Copper-chromium oxide was used as the catalyst. This was prepared according to the method of Connor, Folkers, and Adkins (24).

The charge, consisting of the material to be hydrogenated, the catalyst, and the solvent were placed in the bomb. The bomb was flushed out three times with hydrogen from the low pressure tank (500-800 lbs.) and the hydrogen pressure in the bomb was raised to 3000 lbs. by means of the "booster" pump. The bomb was then set rocking and the heat was turned on. By means of the Variac the temperature was kept at about 280 deg. C during the hydrogenation period. Temperature and pressure readings were taken during the hydrogenation of the young timothy sample and the hydrogen absorption curves for this material are given in Figure III. (Page 91a).

The amount of solvent used for each hydrogenation was governed, to some extent, by the nature of the material. In the case of the young timothy 125 gms. of the extracted material, 75 gms. of the catalyst, and 1000 cc. of dioxane were used. For the older timothy a 130 gm. sample of the extracted material was placed in the bomb along with 80 gms.

of the catalyst, and 1300 cc. of dioxane. For the oat straw a 130 gm. sample, 1000 cc. of the solvent, and 80 gms. of the catalyst were used.

It was discovered at the outset that the catalyst was red after the first hydrogenations had been carried out. In order to obtain black (unpoisoned) catalyst on completion of the hydrogenation procedure it was found necessary to rehydrogenate twice. Even after this treatment the catalyst was still slightly red in the case of the young timothy. On completion of each hydrogenation the bomb was allowed to cool. The charge was removed and the catalyst was separated by centrifuging. The liquid was then returned to the bomb and a fresh charge of catalyst was added. As dry dioxane was used for rinsing the material out of the bomb the total volume of the charge varied from one hydrogenation to the next. In the case of the young timothy the bomb temperature was maintained at 280 deg. C for 18 hours during the first hydrogenation and for 13 hours during each of the two succeeding ones. In the case of the older timothy the bomb was accidently shut off during the first hydrogenation. It was therefore opened, 30 gms. more catalyst was added and the hydrogenation was continued for 14½ hours. The two succeeding hydrogenations were carried out for 13 and 14½ hours respectively. The oat straw was hydrogenated for 13½, 19, and 12 hour periods. It was noted that after the first hydrogenations the colour of the dioxane solutions varied

from greenish-yellow to black. After the final hydrogenation a water-clear solution was obtained in every case.

Technique of isolation and separation of hydrogenation products.

The dioxane was distilled out of the reaction mixture at atmospheric pressure. All the material which boiled up to 110 deg. C was removed. The residue was transferred to a small, weighed distilling flask with dioxane which was then boiled off. Glass wool was used in the distilling flask to prevent bumping. The material in the flask was weighed and it was then distilled into a tared test tube. A Wood's metal bath was used to heat the flask during the distillation. The distillable oil was then weighed. The treatment of the different samples varied somewhat from this point and therefore they will be discussed separately.

Young Timothy:

The material obtained from this sample began to distill over at a pressure of 30μ and a bath temperature of 220 deg. C. The vapor temperature was 100 deg. C in this case. The bath temperature was raised to 345 deg. C and the pressure was lowered to about 10μ before all the distillable material was removed. The temperature of the condensing vapor rose to 250 deg. C. The first material which distilled was a clear colourless oil. This was followed by a light yellow oil and then by a heavy yellow oil as the temperature was raised.

The distillate obtained was weighed and dissolved in ethyl ether. It was found that on standing a white crystalline material settled out of this solution. The crystals were centrifuged out of the ether solution, dried, and weighed. An attempt was made to purify them by re-crystallization from cold ethanol. However, the yield was very small and therefore the purification could not be completed.

The ether solution was then shaken up with water in a separatory funnel. The ether and water layers were separated. Both solvents were evaporated off and the weight of the residues was determined. The ether-soluble fraction consisted of about 6 gms. of material at this point and this was considered to be an insufficient quantity to effect a satisfactory fractionation. A sample similar to the one described above had been prepared by a co-worker, Mr. F. J. Sowden, and therefore the residues were combined at this point in order to obtain sufficient material for an efficient fractionation.

The fractionation of the ether-soluble material was carried out in the original column described by Bower and Cooke (5). Refractive indices were taken on the different fractions obtained using an Abbe refractometer the temperature being held at 25 deg. C by means of an ultrathermostat. The refractive indices were plotted against the weight in grams of the material distilled. The fractionation curves

are shown in Figure IV (page 91b). Carbon and hydrogen determinations were carried out on the samples marked I and II on the fractionation curve for young timothy. These fractions were chosen as they are in the only 'flats' shown on the curve and are therefore the samples most likely to be pure. The semimicro method described by Niederl and Niederl (66) was used for the carbon and hydrogen determinations.

The last portion of the material which passed through the column crystallized on cooling. It was found that these crystals were soluble in warm acetone and could be readily recrystallized from cold acetone. The separation of the fractionatable material from the residue was quite complete. Consequently, the material in the column was almost all in the bottom half. This material was removed by refluxing with acetone. On cooling white crystals settled out of the acetone. Both crystal samples were purified by four recrystallizations from acetone at 0 deg. C. The carbon and hydrogen contents of these were determined in the same way as on the oils. The set of crystals which distilled are sample I in Table X. Those which remained in the column are sample II.

The residue which remained in the flask after fractionation was soluble in hot acetone but was a dark gummy substance from which pure crystals could not be separated without great difficulty. Repeated crystallization from cold acetone was of little value; decolorizing carbon failed to remove the brown color; and the material was too

high boiling to distill in a Perry-Widmer flask (80). In view of the obvious difficulties involved in purification this material was discarded.

Older Timothy:

This material was treated in almost the same way as the younger sample. The conditions at the conclusion of the vacuum distillation were bath temperature 380 deg.C, vapor temperature 220 deg. C, and pressure 14 μ. The first material to distill was a colourless oil which was followed by a yellow oil as the temperature was raised. The technique used to separate the crystals and water soluble material from the ether solution of the distillate was the same as for the younger sample. In this case, however, the water soluble portion was removed before instead of after the crystals. This sample contained only a small amount of crystalline material which was recrystallized from cold ethanol. There was not sufficient of the material to warrant completion of the purification. Fractionation of the water insoluble portion was carried out in the same way as for the young timothy. No crystalline material passed through the column. The residue was a brown amorphous solid which could not be fractionated by crystallization from ethyl ether, petroleum ether, ethanol, or chloroform. This material was not purified. Carbon and hydrogen determinations were carried out on the oil samples marked III, IV, and V on the fractionation curve (Figure IV).

Oat Straw:

In the preliminary vacuum distillation of this material a clear, colourless oil began to distill over at a bath temperature of 130 deg. C, vapor temperature of 85 deg. C, and pressure of 600μ. This was followed by a yellow oil becoming heavier and more viscous as the bath temperature was raised to 360 deg. C, and the pressure was lowered to 50μ. The final vapor distilled over at 250 deg.C. In this case no amorphous solid material settled out of the distillate as it did with the timothy samples. As the ether solution of the distillate was spilled no fractionation of this material could be carried out. As no more of the original material was available the experiment could not be repeated.

TABLE X

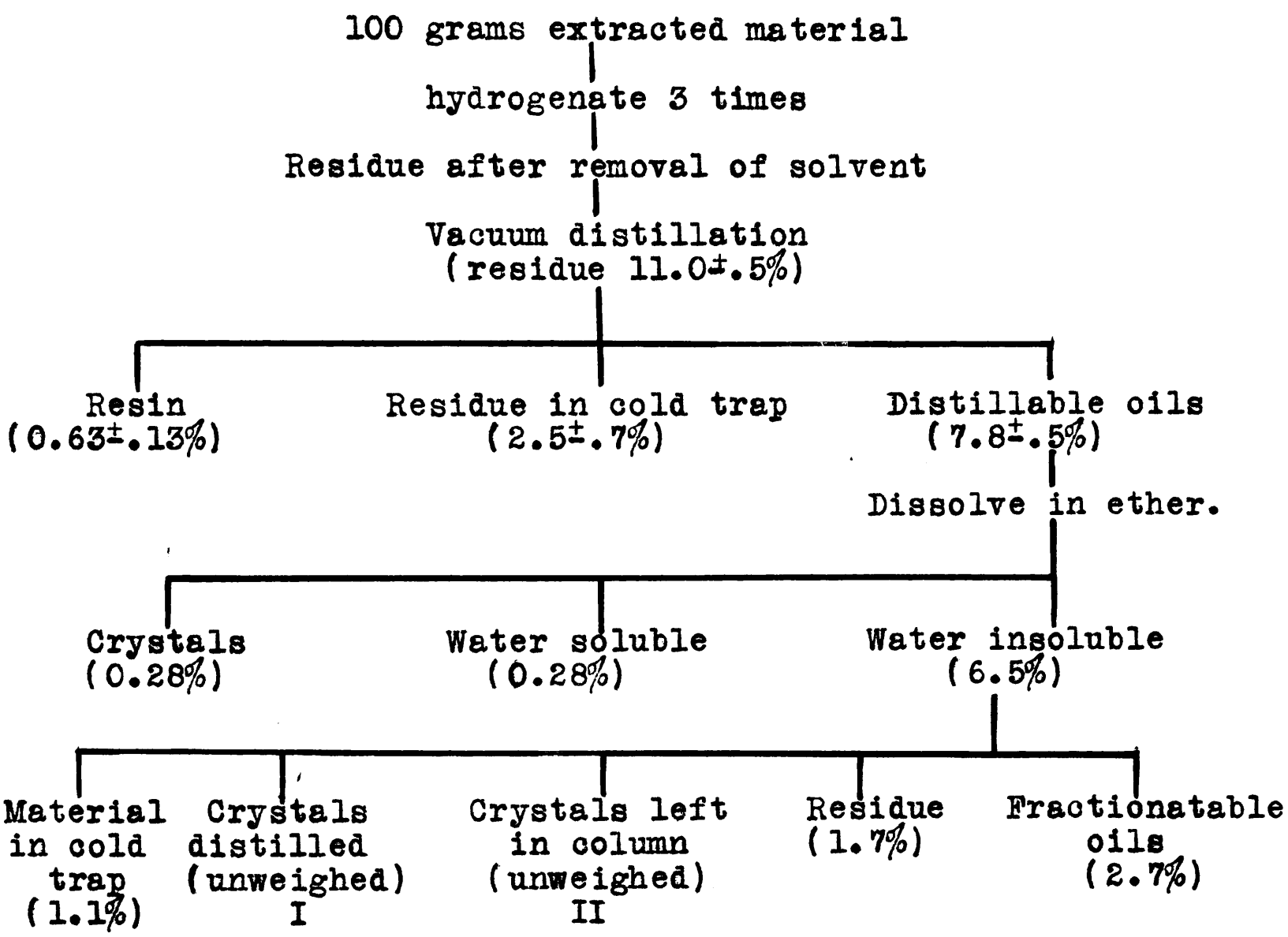
Part A - Oil Samples.

Sample No.	Ref.Index	% Carbon	% Hydrogen
I	1.4635	73.2±1.3(4)	12.6±0.3(4)
II	1.4810	78.7±0.3(2)	13.4±0.0(2)
III	1.4642	74.1±0.2(2)	12.7±0.1(2)
IV	1.4833	76.7±0.2(2)	13.4±0.1(2)
V	1.4847	77.2±0.3(2)	12.8±0.1(2)

Part B - Crystal Samples.

Sample No.	Melting Point	% Carbon	% Hydrogen
I	55° C	80±4±0.1(2)	14.3±0.1(2)
II	95±97° C	74.7±0.2(2)	13.3±0.1(2)

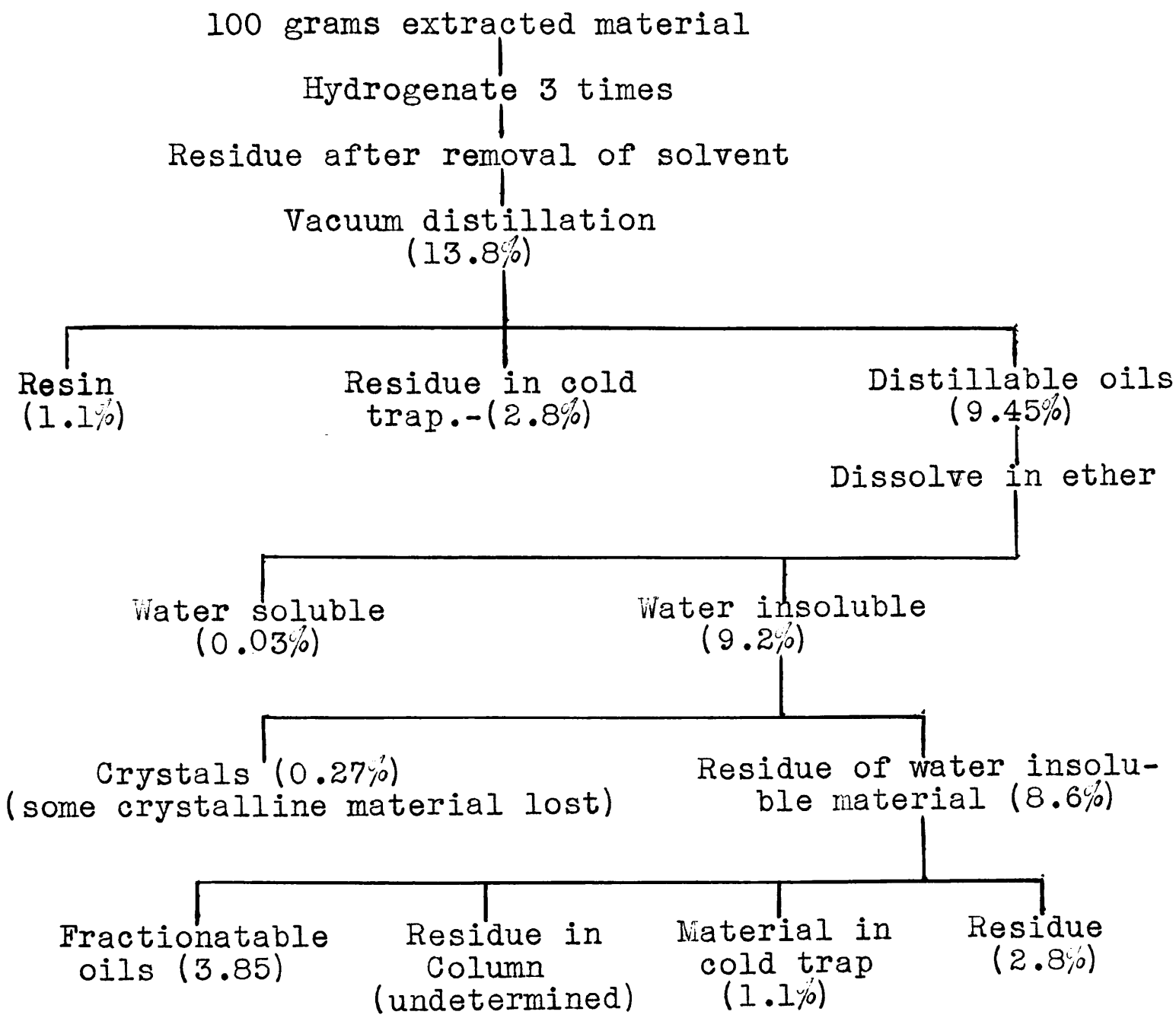
Flow Sheet I (Young Timothy)



Klason lignin = 18.4% of extracted material

Fractionatable oils = 14.7% of Klason lignin.

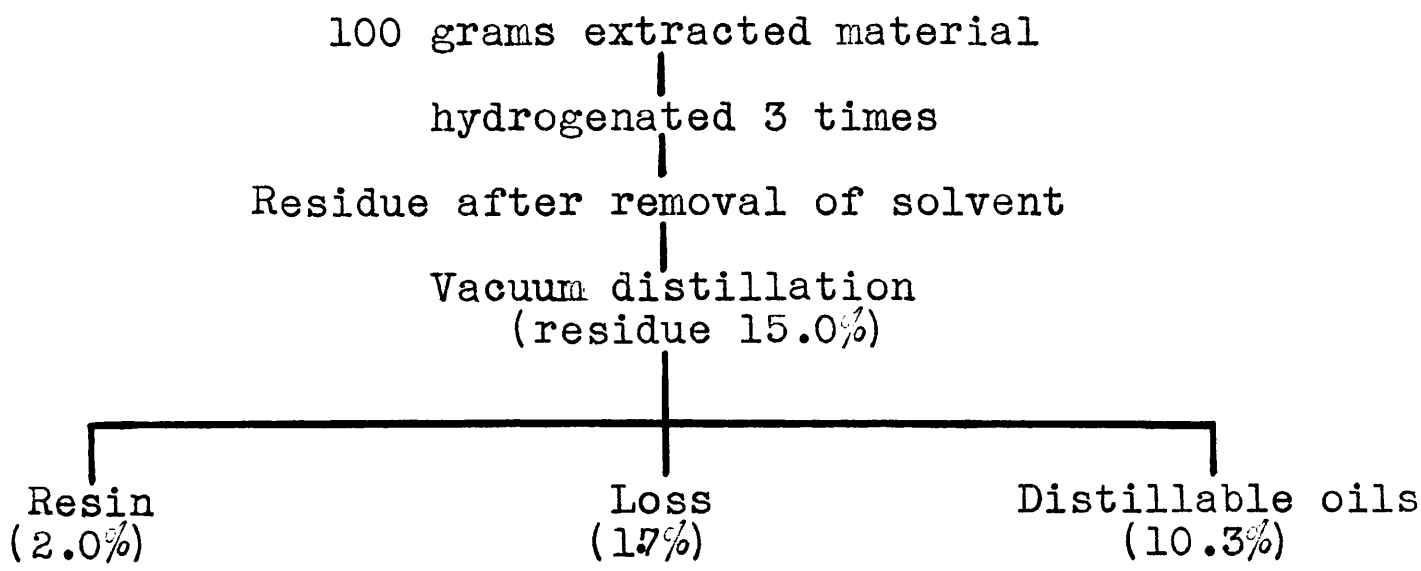
Flow Sheet II (Old Timothy)



Klason lignin = 22.6% of extracted material.

Fractionatable oils = 16.8% of Klason lignin.

Flow Sheet III (Oat Straw)



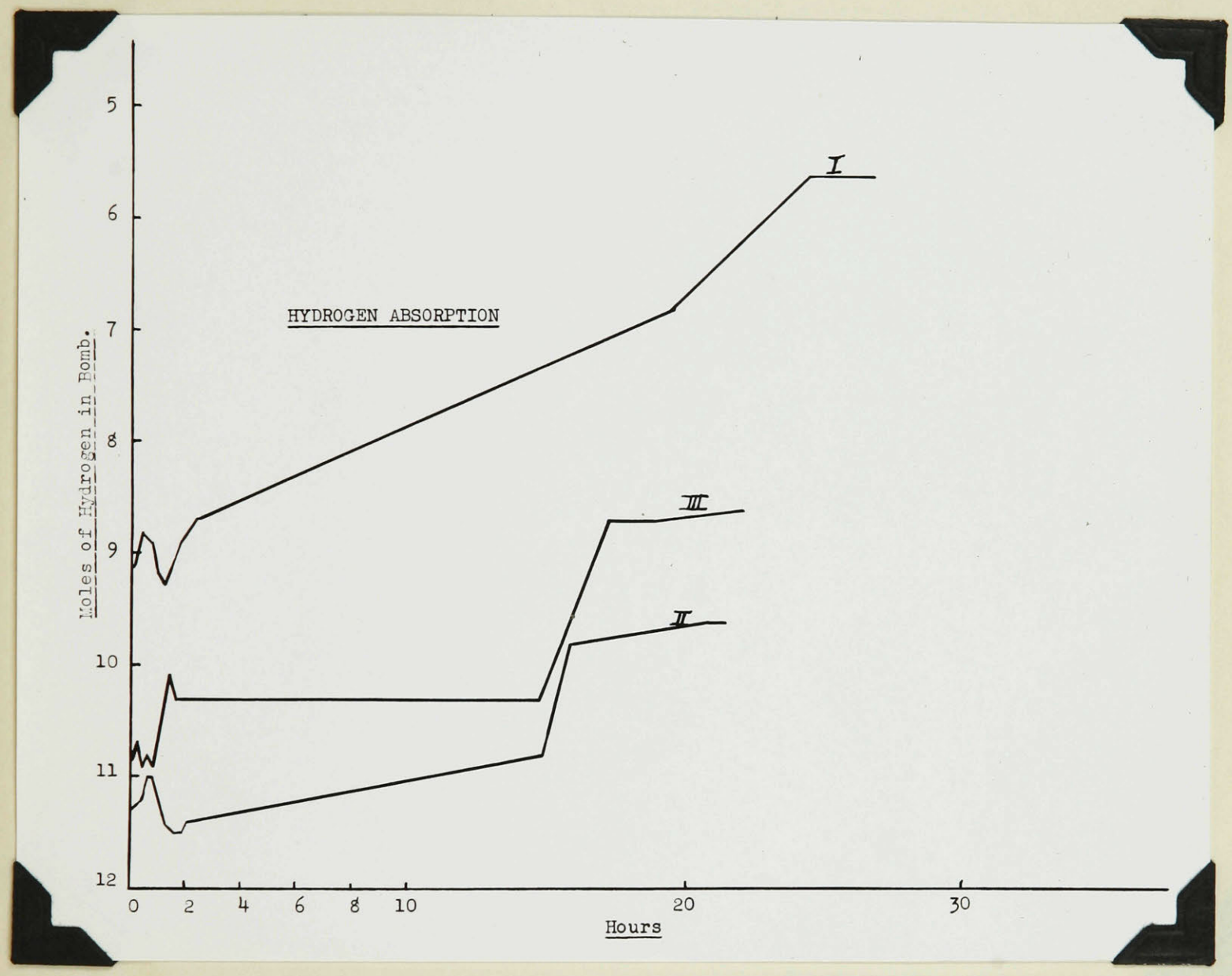

HYDROGEN ABSORPTION
Moles of Hydrogen in Bomb.
5
6
7
8
9
10
11
12
0
2
4
6
8
10
20
30
Hours
I
II
III

Figure III.

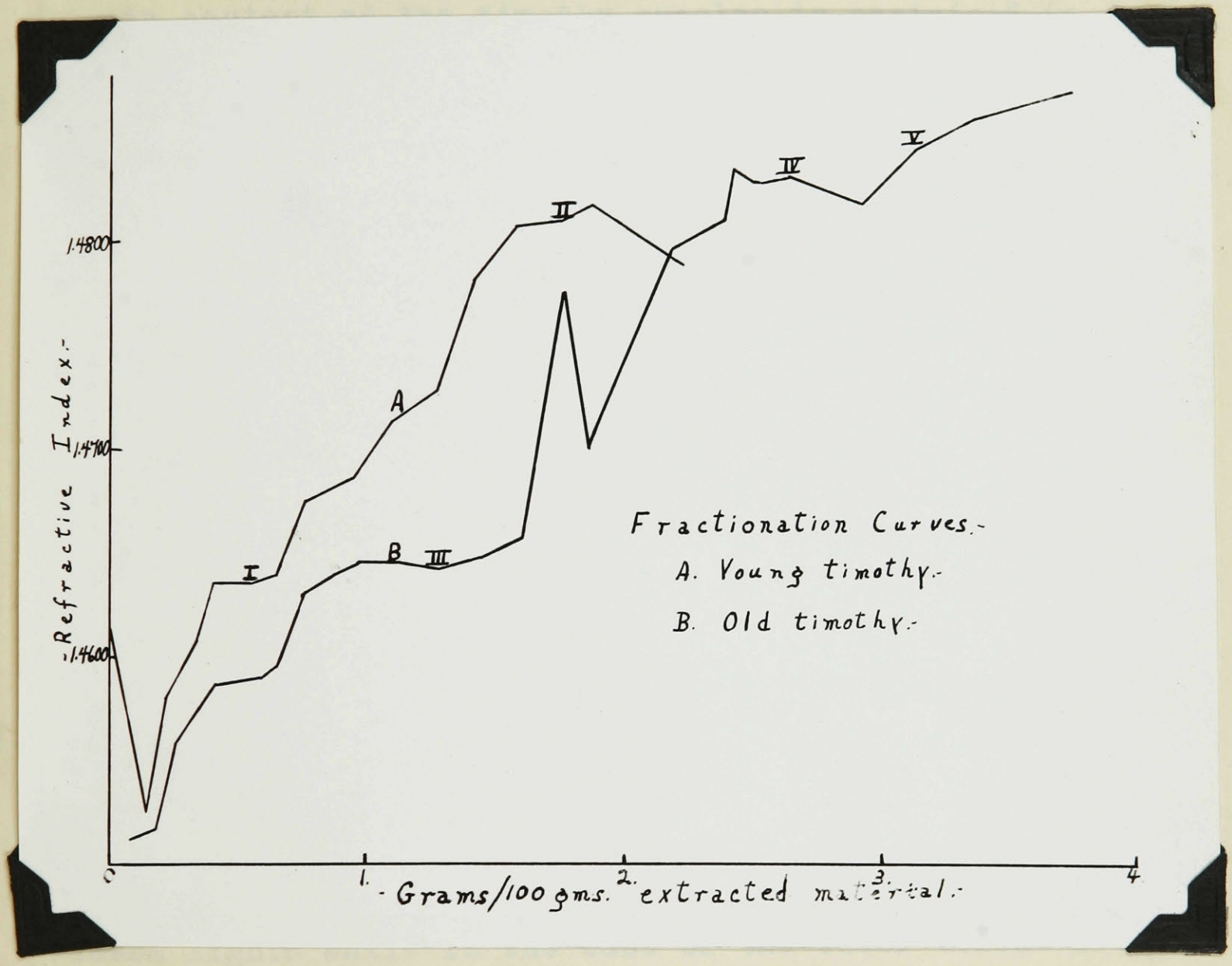

1.4800
1.4700
1.4600
Refractive Index.-
I
II
III
IV
V
A
B
0
1.
2.
3.
4
Grams/100 gms. extracted material.-
Fractionation Curves.-
A. Young timothy.-
B. Old timothy.-

Figure IV.

Discussion of results.

The results of the three hydrogenations are contained in flow sheets I, II, and III. Data on the lignin content of the timothy samples is contained in Table IX (page 70). All values given on the flow sheets are expressed as percentages of extracted material. As the fractionation of the material from young timothy was carried out on the residue from two samples, it was thought wise to average the results for the treatments up to this point. Therefore the results are followed by the average deviation from the mean.

At the outset, the close similarity between the results obtained for the two timothy samples should be noted. If we take the amount of fractionatable oil obtained as being indicative of the amount of lignin present in the original material then we see that in the case of the young sample these were equivalent to 14.7% of the Klason lignin while in the case of the older tissue 16.8% of the Klason lignin would be accounted for. There is, of course, no proof that the fractionatable oils were obtained entirely from lignin, but if we assume that they were, then, on this basis, the Klason lignin from young timothy is about 88% as pure as that from older tissue. On the basis of its ultraviolet absorption, lignin from young timothy was only 78% as pure as that from the older material.

The crystals which separated from the ether solution of the distillate in each case were probably quite similar in composition to those which remained in the residue from fractionation. It seems that the hydrogenation product is composed first, of a mixture of fractionatable oils with quite similar properties; secondly, of a mixture of similar crystalline substances; and lastly, of a resinous material of very high boiling point.

As nothing was known concerning the nature of the materials hydrogenated the hydrogen absorption is not reported for the old timothy or for the straw. The graphs in Figure III show the amount of hydrogen absorption during the three hydrogenations carried out on young timothy. These are included to draw attention to the fact that hydrogen absorption was apparently very great after the bomb started to cool. This marked absorption occurred as the temperature fell through the range 280 deg. C to 200 deg. C. This effect is very difficult to explain. It is difficult to see how the reaction rate could be slowed by increasing the temperature unless there was some substance present in the sample which poisoned the catalyst at 280 deg. C but allowed it to act again as the temperature was lowered. On the other hand, it may be that some reaction which must take place before further hydrogen is absorbed does not go at the higher temperature. In any event there is no information available as to the actual cause of this effect and the

necessity for further investigation is indicated.

The fractionation curves in Figure IV indicate that the distillable oils were a mixture of similar materials. The curve obtained for young timothy is very closely similar to that obtained by Bower et al (7) for young spruce buds. On the basis of this curve he says that young spruce buds contain no lignin. The validity of such an argument is questionable, as failure to isolate known compounds from young spruce bud hydrogenation products does not necessarily mean that these were absent, but rather would indicate that a mixture of similar compounds was obtained and that each was present in too small a proportion to be separated from the remainder in the fractionating column used.

It is felt that the fractions labelled I and III may well have been 4-n-propylcycylohexanol. The refractive indices (1.4635 and 1.4642) are very close to the refractive index of 4-n-propylcyclohexanol reported by Bower (7). This compound theoretically contains 76.0% carbon and 12.8% hydrogen which does not check exactly with the carbon and hydrogen contents of fractions I and III as reported in Table X, Part A. However, the fractions obtained may not have been absolutely pure. At the outset it can be stated that all of the oil samples as well as crystal sample II have carbon and hydrogen contents which correspond closely to that of a phenylpropyl derivative containing one hydroxyl

group. Crystal sample I is apparently of a different nature. It corresponds more closely in composition to a dimer composed of two phenylpropyl units joined by an oxygen bridge.

In general it can be stated, then, that hydrogenation of plant tissues yields distillable oils of the same nature as those obtained from wood, but to a lesser extent. In addition, no long 'flats' are obtained in the fractionation curves indicating that the mixture of oils is more complex than is obtained with wood and that there is present a relatively lower proportion of those which have been identified. The isolation of crystalline substances was apparently more easily accomplished with the young material than with the more mature. In any event Creighton et al (27) have shown that the same products cannot be expected from monocotyledons as from dicotyledons and thus we would not expect to obtain compounds exactly similar to those given by woods.

General Summary.

1. Treatment of fresh material generally results in a lower lignin value than is obtained with air-dry samples.

2. When air-dry material is prepared in the Waring Blendor by the method used for fresh material a lower lignin value is obtained than if the standard method is used. However, the ultraviolet absorption results indicate that the lignin isolated by the latter method is just as pure as that prepared by the former. Thus it would seem that the hot water extraction of the standard method causes condensation of soluble lignin fractions and prevents their removal by the subsequent hydrochloric acid treatment.

3. Reversal of the usual order of the 1% hydrochloric acid and the ethanol-benzene extractions; continuous extraction with hot 1% hydrochloric acid instead of the usual extraction; and the introduction of reducing conditions at various stages throughout the procedure have all been investigated and found to have little or no effect on the apparent lignin content of succulent plant tissues.

4. Experiments with various extractants have shown that extraction of young plant tissue with hot 1% hydrochloric acid is unnecessary to remove interfering nitrogenous material. For older tissue the hydrochloric acid extraction seems to be necessary.

5. The purity of lignin preparations from tissues of different ages has been estimated by the amount of ultraviolet light which their sulphite solutions absorb. The specific absorption of lignin solutions increases with the age of the plant from which the lignin was isolated. The purity of the lignin isolated from plant tissues thus appears to increase regularly as the age of the plant increases.

6. High pressure hydrogenation of timothy from two growth stages produced little, if any, of substances which have been shown to be lignin building units. However, fractionatable oils similar to those from wood were obtained, but to a lesser extent.

To conclude, then, it can be stated that the standard method of determining plant lignin seems adequate for mature tissue. This conclusion is based on the fact that with oat straw several innovations in the procedure caused little change in either the amount of lignin isolated or in its composition. At the same time the age experiment on oats; the ultraviolet absorption studies; and the high pressure hydrogenation experiments all indicate that as a plant matures the lignin isolated from it gradually approaches wood lignin in composition and reactivity. These changes in the amount and composition of isolated lignin are probably due to changes both in the nature of the lignin itself and in the amount and the reactivity of materials which interfere in

its isolation. Thus by variations in the pretreatment procedures and conditions it has been possible to lower the apparent lignin content of young tissues below that indicated by the standard method. In addition, the lignin so obtained contains more methoxyl and less nitrogen than that prepared by the standard treatment. However, the results of the ultraviolet absorption studies indicate that this in itself may not be sufficient on which to base an estimate of the validity of any particular method. These studies also indicate the necessity for studying the pretreatment extracts with the object of determining whether or not they contain soluble lignin precursors or break down products.

- BIBLIOGRAPHY -

1. Association of Official Agricultural Chemists.
Official and tentative methods of analysis. - 5th ed.
A.O.A.C. - Washington, D. C. - 1940.-

2. Bamford K. F. and Campbell W. G. -
Determination of lignin in the analysis of wood. -
Biochem. Jour. - 30: 419, 1936.

3. Bell A., Hawkins W. L., Wright G. F., and Hibbert H.
Occurrence of acetone and syringic aldehyde as degradation products of lignin substances.
J.A.C.S. 59: 598, 1937.

4. Bowden E. and Adkins H.
Synthesis of 3-(4-hydroxycyclohexyl)-1-propanol, a product of the hydrogenation of lignin. -
J.A.C.S. 62: 2422, 1940.

4a. Bower J. R. Jr., McCarthy J. L., and Hibbert H.
Studies on lignin and related compounds. - LXIII
Hydrogenation of wood. (Part 2.)
J.A.C.S. 63: 3066, 1941.

5. Bower J. R. Jr., and Cooke L. M.
An efficient low-holdup laboratory column. -
Ind. Eng. Chem. Anal. Ed. 15: 290, 1943.

6. Bower J. R. Jr., Cooke L. M., and Hibbert H.
Studies on lignin and related compounds. - LXX
Hydrogenolysis and hydrogenation of maple wood.
J.A.C.S. 65: 1192, 1943.

7. Bower J. R. Jr., Cooke L. M., and Hibbert H.
Studies on lignin and related compounds. - LXXI
The course of formation of native lignin in spruce buds.
J.A.C.S. 65: 1195, 1943.

8. Brickman L., Pyle J. J., Hawkins W. L., and Hibbert H.
Structure of the ethanolysis products from spruce and maple wood.
J.A.C.S. 62: 986, 1940.

9. Brickman L., Hawkins W. L., and Hibbert H.
Studies on lignin and related compounds. - XLVIII Identification of vanillin and vanilloyl methyl ketone as ethanolysis products from spruce wood.
J.A.C.S. 62; 2149, 1940.

10. Brode W. R.
Chemical Spectroscopy. 2nd. Ed. -
John Wiley and Sons, Inc. 1943.

11. Buckland I. K., Tomlinson G. H., and Hibbert H.
Occurrence of acetovanillone in waste sulfite liquor from coniferous woods.
J.A.C.S. 59: 597, 1937.

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

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

14. Campbell W. R. and Hanna M. I.
The determination of nitrogen by modified Kjeldahl methods.
J.B.C. 119: 1, 1937.

15. Chibnall A. C. and Schryver S. B.
Investigations on the nitrogenous metabolism of the higher plants. Part 1. The isolation of proteins from leaves.
Biochem. Jour. 15: 60, 1921.

16. Chibnall A. C.
Investigations on the nitrogenous metabolism of the higher plants. Part III. The effect of low-temperature drying on the distribution of nitrogen in the leaves of the runner bean.
Biochem. Jour. 16: 599, 1922.

17. Chibnall A. C. and Grover C. E.
A chemical study of leaf cell cytoplasm. I. The soluble proteins.
Biochem. Jour. 20: 108, 1920.

18. Chibnall A. C.
Protein metabolism in the plant.
Yale Univ. Press. 1939.

19. Clark E. P.
The Viebock and Schwappach method for the determination of methoxyl and ethoxyl groups.
J.A.O.A.C. 15: 136, 1932.

20. Clark E. P.
Preparation of hydriodic acid suitable for alkoxyl and Friedrich-Kjeldahl nitrogen determinations.
Ind. Eng. Chem. Anal. Ed. 10: 677, 1938.

21. Clark W. M.
The determination of hydrogen ions.
Williams and Wilkins Company. 2nd. Ed. 1922.

22. Cohen W. E. and Dadswell H. E.
Chemistry of Australian timbers.
J. Council Sci. Ind. Research. 4: 45, 1931.

23. Cohen W. E. and Harris E. E.
Pretreatment of wood with hot dilute acid. Effect on lignin values.
Ind. Eng. Chem. Anal. Ed. 9: 234, 1937.

24. Connor R., Folkers K., and Adkins H.
The preparation of copper-chromium oxide catalysts for hydrogenation.
J.A.C.S. 54: 1138, 1932.

25. Crampton E. W. and Maynard L. A.
The relation of cellulose and lignin content to the nutritive value of animal feeds.
Jour. Nutrition 15: (4), 1938.

25a. Creighton R. H. J., McCarthy J. L., and Hibbert H.
Aromatic aldehydes from spruce and maple woods.
J.A.C.S. 63: 312, 1941.

26. Creighton R. H. J., Gibbs R. D., and Hibbert H.
Studies on lignin and related compounds. LXXV Alkaline nitrobenzene oxidation of plant materials and application to taxonomic classification.
J.A.C.S. 66: 32, 1944.

27. Creighton, R. H. J. and Hibbert H.
Studies on lignin and related compounds. LXXVI Alkaline nitrobenzene oxidation of corn stalks. Isolation of p-hydroxybenzaldehyde.
J.A.C.S. 66: 37, 1944.

28. Denny F. E.
Improvements in methods of determining starch in plant tissues.
Contrib. from Boyce Thompson Inst. 6: 129, 1934.

29. Dorland R. M., Hawkins W. L., and Hibbert H.
Studies on lignin and related compounds. XLVI. The action of ozone on isolated lignins.
J.A.C.S. 61: 2698, 1939.

30. Eastham A. M., Fisher H. E., Kulka M., and Hibbert H.
Studies on lignin and related compounds. LXXIV. Relation of wood ethanolysis products to the Hibbert series of plant respiratory catalysts. Allylic and dismutation rearrangements of 3-chloro-1-(3,4-dimethoxyphenyl)-2-propanone and 1-bromo-(3,4-dimethoxyphenyl)-2-propanone.
J.A.C.S. 66: 26, 1944.

31. Erdtman H.
Some recent developments in lignin chemistry.
Svensk Papperstidn. 44: 243, 1941.
(English) Pulp and Paper Mag. of Canada. 43: 253, 1942.

32. Freudenberg K., Harder M., and Markert L.
Lignin and cellulose. VII. Remarks on the chemistry of lignin.
Ber. 61 B: 1760, 1928.
Abstracted in Chem. Abs. 23: 276, 1929.

33. Freudenberg K.
Polysaccharides and lignin.
Ann. Rev. of Biochem. 8: 81, 1939.

34. Friedrich A., Kühaas E., and Schnurch R.
The general application of the micro-Kjeldahl determination.
Z. physiol. Chem. 216: 68, 1933.

35. Glading R. E.
The ultraviolet absorption spectra of lignin and related compounds.
Paper Trade J. 111: (TAPPI) 288, 1940.

36. Godard H. P., McCarthy J. L., and Hibbert H.
Hydrogenation of wood.
J.A.C.S. 62: 988, 1940.

37. Godard H. P., McCarthy J. L. and Hibbert H.
Studies on lignin and related compounds. LXII. High pressure hydrogenation of wood using copper chromite catalyst. (Part I).
J.A.C.S. 63: 3061, 1941.

38. Goos F., Schlubach H. H., and Schroter G. A.
Absorptionsmessungen im Ultraviolett mit Thermosaule und ibre Anwendungen auf Probleme der Zucker-Chemie.
Ztschr. f physiol. chem. 186: 148, 1930.

39. Goss M. J. and Phillips M.
Studies on the quantitative estimation of lignin. I. Factors affecting the determination by the fuming hydrochloric acid method.
J.A.O.A.C. 19: 341, 1936.

40. Guest G. H. and McFarlane W. D.
A study of the colorimetric determination of pyrrole with isatin and the application of the method to biological materials.
Can. Jour. Res. - B. 17: 133, 1939.

41. Hägglund E., and Klingstedt F. W.
Uber das Absorptionsspektrum der Ligninderivate im Ultraviolett.
Z. physik. Chem. 152: 295, 1931.

42. Hägglund E.
Advances in lignin chemistry.
Zellstoff u Papier 16: 570, 1936.
Abstracted in Chem. Abs. 31: 2418, 1937.

43. Harris E. E. and Mitchell R. L.
Effect of various pre-extractions on the lignin determination of wood.
Ind. Eng. Chem. Anal. Ed. 11: 153, 1939.

44. Harris E. E., D'Ianni J., and Adkins H.
Reaction of hardwood lignin with hydrogen.
J.A.C.S. 60: 1467, 1938.

45. Harris E. E., Saeman J., and Sherrard E. C.
Hydrogenation of lignin in aqueous solutions.
Ind. Eng. Chem. 32: 440, 1940.

46. Harris E. E.
Hydrogenation of lignin.
Paper Trade J. 111: (TAPPI) 297, 1940.

47. Herzog R. O. and Hillmer A.
The ultra-violet absorption spectrum of lignin.
Ber. 60 B, 365, 1927.
Abstracted in Chem. Abs. 21: 1407, 1927.

48. Herzog R. O. and Hillmer A.
The ultra-violet absorption spectrum of lignin and of substances with the coniferyl group.
Z. physiol. Chem. 168: 117, 1927.
Abstracted in Chem. Abs. 21: 3204, 1927.

49. Hewson W. B. and Hibbert H.
Studies on lignin and related compounds. LXV. Re-ethanolysis of isolated lignins.
J.A.C.S. 65: 1173, 1943.

50. Hibbert H.
Status of the lignin problem.
Paper Trade J. 113: (TAPPI) 35, 1941.

51. Hibbert H.
Lignin.
Ann. Rev. of Biochem. 11: 183, 1942.

52. Hilpert R. S., and Littmann E.
The resinification of sugars by acids and its bearing on the determination of lignin.
Ber. 67B. 1551, 1934.
Abstracted in Chem. Abs. 28: 7251, 1934.

53. Holcomb R.
The analysis and composition of the meat of the domestic fowl.
Can. Chem. and Met. 18: 182, 1934.

54. Klason P. Svensk Kem. Tid. 9, 135, 1897.

55. Leger F. and Hibbert H.
The isolation of guaiacol from waste sulphite liquor.
Can. Jour. Res. B. 16; 68, 1938.

56. Leger F. and Hibbert H.
Studies on lignin and related compounds. XXXIV. Acetovanillone and acetosyringone as degradation products of lignin sulfonic acids.
J.A.C.S. 60: 565, 1938.

57. Lugg J. W. H.
The representativeness of extracted samples and the efficiency of extraction of protein from the fresh leaves of plants; and some partial analyses of the whole proteins of leaves.
Biochem. Jour. 33: 110, 1939.

58. MacDougall D. and DeLong W.A.
Effect of initial drying temperature on the apparent lignin content of plant tissues.
Can. Jour. Res. B. 20: 40, 1942.

59. MacInnes A. S.
A comparison of the lignins from plant materials by ethanolysis.
Ph.D. thesis. (McGill) April, 1941.

60. Mahood S. A. and Cable D. E.
The chemistry of wood. IV. The analysis of the wood of Eucalyptus globulus and Pinus monticola.
Ind. Eng. Chem. 14: 933, 1922.

61. Manning K. R. and DeLong W. A.
The lignin content of some common vegetables, with observations on methods for the determination of lignin.
Sci. Agric. 22: 69, 1941.

62. Marchlewski L. and Nowatnowna A.
L'absorption de la lumiere violette par les substances organiques (VII).
Bull. soc. chim. 39: 159, 1926.

63. Marchlewski L. and Urbanczyk W.
Zur Kenntnis reduzierender Zucker. III.
Biochem. Ztschr. 262: 248, 1933.

64. Morrow C. A.
Biochemical laboratory methods for students of the biological sciences.
John Wiley and Sons Inc. 1927.

65. Mueller W. J. and Herrmann W.
The determination of lignin in wood and wood pulps.
Tech. Wiss. Teil, Papierfabr. 24: 185, 1926.
Abstracted in Chem. Abs. 20: 2746, 1926.

66. Niederl J. B. and Niederl V.
Micromethods of quantitative organic elementary analysis.
John Wiley and Sons Inc., 1938.

67. Norman A. G. and Jenkins S. H.
A new method for the determination of cellulose based upon the removal of lignin and other incrusting materials.
Biochem. Jour. 27: 818, 1933.

67a. Norman A. G. and Jenkins S. H.
The determination of lignin. I. Errors introduced by the presence of certain carbohydrates.
Biochem. Jour. 28: 2147, 1934.

68. Norman A. G. and Jenkins S. H.
The determination of lignin. II. Errors introduced by the presence of proteins.
Biochem. Jour. 28: 2160, 1934.

69. Norman A. G.
The composition of forage crops. I. Rye grass.
Biochem. Jour. 30: 1354, 1936.

70. Norman A. G.
The determination of lignin. III. Acid pretreatment and the affect of the presence of nitrogenous substances.
Biochem. Jour. 31: 1567, 1937.

71. Norman A. G.
The biochemistry of cellulose, the polyuronides, lignin, etc.
Oxford University Press, 1937.

72. Norris F. W. and Schryver S. B.
The pectic substances of plants. Part III. The nature of pectinogen and its relation to pectic acid.
Biochem. Jour. 19: 676, 1925.

73. Osborne T. B.
A hydrolytic derivative of the globulin edestin and its relation to Weyl's albuminate and the histon group.
J.A.C.S. 24: 28, 1902.

74. Osborne T. B.
The basic character of the protein molecule and the reactions of edestin with definite quantities of acids and alkalies.
J.A.C.S. 24: 39, 1902.

75. Ost H. and Wilkening L.
Saccharification of cellulose.
Chem. Ztg. 34: 461, 1910.
Abstracted in Chem. Abs. 4: 1888, 1910.

76. Paloheimo L.
Contributions to lignin determination by acid hydrolysis.
Biochem. Z. 214: 161, 1929.
Abstracted in Chem. Abs. 24: 803, 1930.

77. Patterson R. F. and Hibbert H.
Studies on lignin and related compounds. LXXII. The ultraviolet absorption spectra of compounds related to lignin.
J.A.C.S. 65: 1862, 1943.

78. Patterson R. F. and Hibbert H.
Studies on lignin and related compounds. LXXIII. The ultraviolet absorption spectra of ethanol lignins.
J.A.C.S. 65: 1869, 1943.

79. Percival E. V. G.
The lignin problem.
Annual Report on the progress of chemistry for 1942. page 142 (London) 1943.

80. Perry S. and Hibbert H.
The quantitative analysis of mixtures of polyoxyethylene glycols by fractional distillation.
J.A.C.S. 62: 2561, 1940.

81. Phillips M.
The quantitative determination of methoxyl, lignin, and cellulose in plant materials.
J.A.O.A.C. 15: 118, 1932.

82. Phillips M. and Goss M.J.
Composition of the leaves and stalks of barley at successive stages of growth, with special reference to the formation of lignin.
J. Agr. Res. 51: 301, 1935.

83. Phillips M., Goss M. J., Davis B. L., and Stevens H.
Composition of the various parts of the oat plant at successive stages of growth, with special reference to the formation of lignin.
J. Agr. Res. 59: 319, 1939.

84. Phillips M.
Studies on the quantitative estimation of lignin. IV. Effect of certain proteins on the determination of lignin by the fuming hydrochloric acid method.
J.A.O.A.C. 22: 422, 1939.

85. Ritter G. J. and Barbour J. H.
Effect of pretreatments of wood on the lignin determination. Distribution of methoxyls in wood.
Ind. Eng. Chem. Anal. Ed. 7: 238, 1935.

86. Samsel E. P. and McHard J. A.
Determination of alkoxyl groups in cellulose ethers.
Ind. Eng. Chem. Anal. Ed. 14: 750, 1942.

87. Shrikhande J. G.
Estimation of lignin in tannin materials.
Biochem. Jour. 34: 783, 1940.

88. Spiegel-Adolf M. and Krumpel O.
Physikalisch-chemische Untersuchungen bestrahlter Proteine. II. Absorption des lichtdenaturierten Seralbumins im Ultraviolett.
Biochem. Ztschr. 190: 28, 1927.

89. Stamm A. J., Semb J., and Harris E. E.
Absorption spectra of lignin solutions.
J. Phys. Chem. 36: 1574, 1932.

90. Szent-Györgyi A.
Cell respiration.
Ber. 72: A, 53, 1939.

91. Tomlinson G. H. and Hibbert H.
Studies on lignin and related compounds. XXIV.
The formation of vanillin from waste sulphite liquor.
J.A.C.S. 58: 345, 1936.

92. Traub H. P.
Regional and seasonal distribution of moisture, carbohydrates, nitrogen, and ash in 2-3 year portions of apple twigs.
University of Minnesota, Tech. Bull. 53, 1927.

93. Urban H.
Fir wood.
Cellulosechem. 7: 73, 1926.
Abstracted in Chem. Abs. 20: 3080, 1926.

94. Waksman S. A. and Iyer K. R. N.
Contribution to our knowledge of the chemical nature and origin of humus. IV. Fixation of proteins by lignins and formation of complexes resistant to microbial decomposition.
Soil Sci. 36: 69, 1933.

95. Ward F. W.
The absorption spectra of some indole derivatives.
Biochem. Jour. 17: 891, 1923.

96. Ward F. W.
The absorption spectra of some amino acids. The possible ring structure of cystine.
Biochem. Jour. 17: 898, 1923.

97. Weast C. A. and MacKinney G.
Nonenzymatic darkening of fruits and fruit products.
Ind. Eng. Chem. 33: 1408, 1941.

98. West K. A. and Hibbert H.
Studies on lignin and related compounds. LXIV.
Synthesis and properties of 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-1-propanone.
J.A.C.S. 65: 1170, 1943.

99. Willstatter R. and Zechmeister L.
Hydrolysis of cellulose.
Ber. 46: 2401, 1913.
Abstracted in Chem. Abs. 7: 3412, 1913.