CXCVI. THE DETERMINATION OF LIGNIN

III. ACID PRETREATMENT AND THE EFFECT OF THE PRESENCE OF NITROGENOUS SUBSTANCES

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DURING the past few years there have appeared a number of papers dealing with the determination of lignin, in most cases, in mature woods. Although there is now a measure of agreement as to the difficulties to be overcome, there is no unanimity as to the most satisfactory procedure which must be adopted. The ideal is obviously to obtain full recovery of all the authentic lignin, without contamination by condensed or polymerized residues from other constituents. Most attention has been given to the interference which may be caused by the presence of certain polysaccharides. Errors from this source may be particularly serious if the time of contact with the 72% sulphuric acid is prolonged, or if the temperature during this digestion is above 20°. It has been suggested that such errors may be minimized by control of the conditions of digestion, or by an appropriate pretreatment designed to remove the interfering substances. A vital point is that the pretreatment should not affect the lignin as a whole or any part of it. Ritter *et al.* [1932] showed that significantly lower lignin figures may be obtained if the material is extracted with boiling water for 3 hr. prior to the addition of strong acid. They did not give any explanation of this effect other than that "extractives" which might be converted into insoluble products are removed. Norman & Jenkins [1934, 1] put forward the case for dilute acid hydrolysis as a pretreatment, in order to remove pentose-containing polysaccharides. They stated that the validity of this pretreatment was not proved beyond doubt, since it is not certain that lignin, as existing in the plant and before coming into contact with 72% sulphuric acid, is completely unaffected by hot dilute acids. The principle of a simple acid pretreatment has been accepted by Phillips & Goss [1936], but recently has been challenged by Bamford & Campbell [1936] on the grounds that the result obtained may still be too high through the formation of insoluble products from partially hydrolysed carbohydrates. It has also been questioned by Cohen & Harris [1937] who take the opposing view that the result after such a treatment is too low owing to the removal of "soluble lignin". Bamford & Campbell [1936] do not reject the principle of an acid hydrolysis, but believe that one long treatment is less satisfactory than several brief treatments, by which means the products of hydrolysis are removed as soon as possible.

The following observations bear on some of the issues raised by the authors mentioned and on the more difficult problem of the disturbance caused by nitrogenous substances.

EXPERIMENTAL

(a) *The disturbance caused by non-nitrogenous substances*

The presence of pentose or pentose-containing polysaccharides was shown in a previous paper [Norman & Jenkins, 1934, 1] to cause errors in the lignin determination unless special precautions are taken. It was demonstrated that fur-

furaldehyde is slowly produced in contact with 72 % sulphuric acid, and that the cause of the disturbance is the condensation of this aldehyde with lignin and not necessarily the formation of an insoluble condensation product from the furfuraldehyde itself. Lignin in strong sulphuric acid is apparently more reactive than has been supposed, and lignin-aldehyde products have been obtained from all aldehydes tested. The increment in weight of apparent lignin produced varies with the time of contact with the acid but is quite appreciable in as little as 2 hr. (Table I). Furfuraldehyde is more active than formaldehyde, acetaldehyde and

Table I. *Condensation of aldehydes with lignin*

Yield of apparent lignin (ash-free) from 0.8 g. oat straw. 15 ml. 72 % acid for time stated; temp. < 20°, diluted to 600 ml. and boiled 2 hr.

Aldehyde (ml.)	mg. apparent lignin	
	2 hr.	16 hr.
None	138	159
Furfuraldehyde 0.025	154	194
0.05	156	225
0.1	186	294
0.2	214	374
Formaldehyde (40 %) 0.5	176	179
1.0	173	171
Benzaldehyde 0.05	150	168
0.2	152	172
1.0	162	201

benzaldehyde, and in any case itself gives an insoluble condensation product when the combining power of the lignin is satisfied. Aldehydes are not the only compounds to condense with lignin under these conditions; ketones and phenolic substances may also be retained. Even if the lignin has taken up aldehyde to its full ability, phenol may still combine (Table II).

Table II. *Condensation of phenol and formaldehyde with lignin*

Yield of apparent lignin (ash-free) from 1 g. oat straw. 15 ml. 72 % acid for 2 hr.; temp. < 20°, diluted to 600 ml. and boiled 2 hr.

Substance added	mg. apparent lignin
None	176
0.1 ml. 40 % formaldehyde	225
0.2 ml. 40 % formaldehyde	225
0.1 g. phenol	194
0.1 g. phenol + 0.2 ml. formaldehyde	280

Ritter & Barbour [1935] have shown that tannins of the catechol type may cause interference in the lignin determination, presumably through some similar

Table III. *Condensation of tannic acid with lignin*

Yield of apparent lignin (ash-free) from equivalent amounts of water-extracted and acid-hydrolysed wheat straw. Conditions as in Table I

Tannic acid added mg.	mg. apparent lignin from	
	899 mg. water-extracted straw, 2 hr.	623 mg. acid-hydrolysed straw, 16 hr.
None	173	166
50	189	176
100	205	196
200	209	201

condensation. Tannic acid also produces substantial increases in apparent lignin (Table III).

These observations make it probable that any phenolic substance present in the plant material, or any aldehydic substance produced by the action of the concentrated sulphuric acid, may condense with the lignin and give an erroneously high result. The substance in question may not itself give an insoluble residue with 72 % acid, and the absence of such a residue under any chosen conditions of digestion is not conclusive evidence that no interference is caused if lignin is present. For example Bamford & Campbell [1936] sought conditions of digestion that would give minimum yields of lignin and recommended 5–6 hr. at 10°. Xylose treated under these conditions gave only 0.07 % of insoluble residue. The experiment should however have been conducted in the presence of lignin, since it is quite possible that soluble products capable of condensing with the lignin might be formed. If this is done it is by no means so certain that the effect of the pentose is negligible, though the results were not consistent when a variety of materials was examined.

Table IV. *Effect of addition of xylose to lignin determination*

Yield of apparent lignin % from alcohol-benzene-extracted materials. 0.75 g. xylose added to 0.75 g. material + 15 ml. 72 % sulphuric acid. Digestion 6 hr. at 10°, diluted to 600 ml. and boiled 2 hr.

Material	Alone	With xylose
Oak	26.2	26.9
Hydrolysed oak*	32.2	33.2
Silver fir	31.1	32.0
Hydrolysed silver fir	36.9	38.2
Hay	14.7	16.2
Hydrolysed hay	27.0	26.2
Wheat straw	17.9	17.7
Hydrolysed wheat straw	25.3	25.4
Jute	12.2	12.2
Pine	26.8	27.5

* All hydrolyses were effected with 5 % H_2SO_4 for 1 hr. The hydrolysed samples are not expressed on original basis.

In four cases out of six the addition of xylose to an unhydrolysed material resulted in an increase in apparent lignin. The increments are not large considering the high proportion of pentose added. A comparison of the hydrolysed with the unhydrolysed materials does not suggest that the pentose-containing polysaccharides of the latter are causing any serious error in the determination, though it cannot be said with confidence that they are wholly without effect. Conditions which result in complete solution of the cellulose without at the same time causing breakdown of pentoses may be impossible to find, so that the choice may have to be made of conditions most nearly approximating to that ideal.

(b) *Acid hydrolysis as a pretreatment*

The use of a single dilute acid hydrolysis as a pretreatment to the determination of lignin has been criticized by Bamford & Campbell [1936] as ineffective, because with some abnormal woods results higher than on the untreated material were subsequently obtained. They say, however, that "greater accuracy may conceivably be introduced... provided that the products of hydrolysis are removed as soon as possible" and suggest that several brief acid treatments may be more satisfactory. A very wide range of plant materials has been examined

in this laboratory during the past 3 years, and in no single case has the lignin figure obtained after hydrolysis been higher than that on the untreated material. In the aberrant woods mentioned by Bamford & Campbell [1936] the pentose-containing substances seem to have been peculiarly resistant to hydrolysis. If the acid hydrolysis were intended to prevent any possible pentose disturbance, as was presumably the case, then the treatment was futile, and a lower lignin figure could hardly have been expected. The fact that slightly higher figures were recorded in some cases may have been due either to the formation of some non-lignin insoluble product, as suggested by Campbell, or to the condensation of some soluble product with the lignin or possibly to both. The former is to be deduced from the deposition of an insoluble residue from the filtered acid extract on further boiling as recorded by Bamford & Campbell [1936] or on standing for some days [Cohen & Harris, 1937] as has also been observed here. The second possibility has been demonstrated by the addition of aldehydes to the acid used for hydrolysis (Table V).

Table V. *Effect of addition of aldehydes to the acid hydrolysis*

Yield of apparent lignin from 1 g. straw after hydrolysis with 5% sulphuric acid.

Digestion conditions as in Table I

Aldehyde added	mg. apparent lignin
None	150
1 ml. furfuraldehyde	159
2 ml. furfuraldehyde	175
2 ml. 40% formaldehyde	157

The statement that slightly lower yields of lignin are obtained by successive brief treatments with acid instead of one longer treatment has been confirmed on some materials. The important proviso must be added that the total material removed by the brief treatments must be roughly equal to that extracted by continuous hydrolysis. After four extractions with 100 ml. 5% sulphuric acid for successive periods of 5, 10, 15 and 20 min., the lignin yields from oat straw and oak were lower than if one continuous hydrolysis were given (Table VI).

Table VI. *Effect of discontinuous acid hydrolysis on apparent lignin content*

mg. of apparent lignin from 1 g. alcohol-benzene-extracted material. All results expressed on original materials. Digestion conditions as in Table I for times stated

Treatment	Oat straw				Oak			
	Residue	Furfur- aldehyde yield	2 hr. digestion	16 hr. digestion	Residue	Furfur- aldehyde yield	2 hr. digestion	16 hr. digestion
1. None	100	15.6	17.4	19.4	100	14.4	23.7	26.9
2. 100 ml. 1% acid for 2 min. 5 times	85.0	14.8	16.0	18.3	86.1	14.1	20.2	22.8
3. 100 ml. 5% acid for 2 min. 5 times	79.5	12.8	15.6	16.6	83.7	12.1	20.6	22.5
4. 100 ml. 5% acid for 5, 10, 15 and 20 min.	62.9	5.8	14.5	14.6	73.4	7.1	20.0	20.7
5. 100 ml. 5% acid for 1 hr.	61.9	4.5	15.8	15.9	70.1	6.3	20.4	21.1

If the difference between continuous and intermittent hydrolysis is due to one or both of the possibilities mentioned above, the effect should be enhanced by using the acid extract of one portion to hydrolyse another portion. Amounts of 3 g. were treated for 5 min. with 100 ml. 5% sulphuric acid and filtered, the extract then being employed to hydrolyse an equal portion of the same material for a period of 1 hr. Lignin determinations were carried out on portions of the residues, the lignin yield in most cases being found to be appreciably higher than if a simple or intermittent hydrolysis were given.

Table VII. *Effect of boiling with previous acid extract*

Treatment	Details as Table VI								
	Wheat straw			Wheat plants			Pine		
	Residue	2 hr. digestion	16 hr. digestion	Residue	2 hr. digestion	16 hr. digestion	Residue	2 hr. digestion	16 hr. digestion
1. 5% acid for 1 hr.	62.8	15.3	15.5	50.1	13.1	13.1	80.8	27.1	27.5
2. Boiled for 1 hr. with 5% acid extract 5 min.	63.4	16.0	16.5	51.9	13.6	13.5	81.2	27.1	27.6
3. 5% acid for 5 min. then 55 min.	62.0	14.8	14.8	50.8	13.0	13.4	80.6	26.8	27.3
4. 5% acid for 5 min. then 10 and 45 min.	61.1	14.0	14.6	51.4	12.4	13.0	82.4	26.7	27.6

The filtered acid extract of most materials on standing for days or weeks deposits a brownish black precipitate, instantaneously soluble in alkali to give a deep-coloured solution. Bamford & Campbell [1936] mention the formation of a similar precipitate on continued boiling of an acid extract and presumably consider it to be formed from the hydrolysed carbohydrates. Cohen & Harris [1937] prefer to think that a portion of the lignin of woods is soluble in dilute acid or even in hot water and that the flocculent precipitate which separates out from acid extracts on standing is a polymerized insoluble form of this soluble lignin.

They state that this precipitate from an acid extract of maple wood had a methoxyl content precisely similar to that of the true lignin residues, and moreover gave the characteristic colour changes on chlorination and addition of sulphite. If this view is correct the use of any acid hydrolysis treatment is to be avoided except as a means of distinguishing between "soluble" and "insoluble" lignin. Their results suggest that the cleavage between the two may be clear cut, since prolonged acid hydrolysis did not give lower lignin figures than a 2 hr. treatment. The conception of a separate water-soluble form of lignin is novel and will require to be substantiated by preparation and characterization. Its existence would provide an explanation for the considerable differences in apparent lignin yield under the same digestion conditions when an aqueous extraction is employed as pretreatment, as in the Ritter-Seborg-Mitchell method, and "soluble" lignin would take the place of the interfering "extractives" postulated by them. At the moment the evidence for its existence is not extensive and depends largely on a colour test which may not be as specific as has been supposed. It is not necessary to carry out a prolonged aqueous treatment to obtain an extract which will give a red colour with sulphite after chlorination. Wood or straw treated with warm water for 2 or 3 min. will give an extract with this property. If excess of baryta is added to the filtered aqueous extract of mature straw, a bulky olive-brown precipitate is obtained which settles

readily. This may be washed by decantation with water containing baryta and finally with boiling water. The dried product suspended in water gives a red colour on addition of sulphite after chlorination, and might be said on these grounds to contain lignin. Acid permanganate is rapidly decolorized, and with ferric chloride a brown-purple colour is obtained. The product contains about 30 % Ba. If treated with 72 % sulphuric acid a light brown insoluble residue also giving the lignin colour test is obtained. The yield of ash-free residue in 16 hr. was about 30 %, when expressed on the Ba-free product. This crude product has not yet been fractionated but must be considered to contain either lignin, some lignin-former or precursor, or some substance, probably phenolic, giving positive lignin tests and forming an insoluble residue with strong sulphuric acid.

In view of the results obtained by Cohen & Harris [1937] and the observations above, the water-soluble and acid-soluble fractions of woods and straws would repay further investigation. The information available at present does not seem to warrant the immediate rejection of acid hydrolysis as a pretreatment in the lignin determination, although doubts as to its suitability are certainly raised.

(c) *The effect of the presence of nitrogenous substances*

The lignin residues from many plant materials containing protein may retain appreciable amounts of nitrogen combined with the lignin. The nitrogen is not present as protein, but probably as protein degradation products of variable size. The magnitude of the error thus caused varies with the conditions of digestion and is generally more or less proportional to the protein initially present [Norman & Jenkins, 1934, 2]. So far no means of avoiding this disturbance has yet been found; most expedients which have been tried have resulted also in an effect on the lignin itself. Hydrolytic pretreatment with dilute acid is partially successful in minimizing the disturbance and has been employed in work on agricultural materials containing proteins. The increments in apparent lignin caused by the addition of a known amount of protein tend to vary inversely with the digestion time and are not reduced by digestion at low temperatures.

In an attempt to obtain further information as to the nature of the disturbance, the effects of addition of a number of nitrogenous substances of varied type were determined (Table VIII) following two procedures. In the first the Ritter-Seborg-Mitchell method, which involves hot aqueous extraction for 3 hr. and subsequent digestion with 72 % acid for 2 hr., was adopted, and in the second the material was hydrolysed with 5 % acid for 1 hr. prior to 16 hr. digestion. Equivalent amounts of the extracted residues were taken and the nitrogenous substance mixed in before the addition of the 72 % acid. Considerable increments in apparent lignin were produced by the proteins and also by fungus chitin, the nitrogenous group of which is in the form of hexosamine. The ratios of increment in lignin over increase in nitrogen were variable and in nearly all cases over 10. The addition of simple amino-acids did not result in any large increase in apparent lignin except in the case of tyrosine in which the effect was probably due in part to the condensation of the phenolic residue with the lignin. The water-extracted straw having 2 hr. digestion seemed more affected than the acid-extracted material with a longer digestion period, as has been observed before. This was particularly the case with the more complex nitrogenous substances such as creatinine, guanine and nucleic acid. The general conclusion to be drawn from Table VIII is that the disturbance caused by proteins must be

Table VIII. *Effect of addition of nitrogenous substances*

Apparent lignin from 1 g. wheat straw (=0.899 g. water-extracted or 0.623 g. acid-extracted straw).

Substance added (mg.)	Water-extracted straw, 2 hr. digestion		Acid-extracted straw, 16 hr. digestion	
	Apparent lignin mg.	mg. N in lignin	Apparent lignin mg.	mg. N in lignin
None		1.7	166	1.3
Casein	50	203	190	3.8
"	100	214	201	6.0
Wheat gluten	50	207	186	3.3
"	100	240	204	5.1
Fungus chitin	50	184	168	1.5
"	100	187	165	1.8
"	250	192	178	2.5
"	500	205	192	3.3
Glycine	50	177	166	1.3
"	100	178	166	1.3
"	200	176	170	1.3
Alanine	50	185	164	1.4
"	100	190	166	1.4
"	200	182	162	1.3
Glutamic acid	50	170	168	1.3
"	100	174	169	1.5
Aspartic acid	50	173	170	1.5
Asparagine	50	178	166	1.4
"	100	177	167	1.6
Tyrosine	50	191	172	2.2
"	100	190	169	2.4
Acetamide	50	172	166	1.2
"	100	179	166	1.3
Biuret	100	176	162	1.9
Betaine-HCl	50	170	164	1.5
"	100	173	168	1.3
Creatinine	50	179	168	1.4
"	100	184	166	1.4
Guanine-HCl	50	177	164	1.2
"	100	183	167	1.3
Yeast nucleic acid	50	180	171	1.4
"	100	195	172	1.4

due to the linkage of large fission products, not necessarily through the amino-group. In many cases these fragments which become attached must have suffered partial deamination, since the nitrogen content of the increment produced is often, though not always, far above 6%. The expedient which must ultimately be adopted may have to be directed more towards the breakdown of the protein molecule into small units, rather than to the removal of amino-groups.

SUMMARY

1. Various aspects of the determination of lignin in plant materials are discussed. Interference is shown to be produced by the condensation of aldehydes or phenolic substances with lignin. Proof of the absence of pentose disturbance under any particular conditions must be given by experiments carried out in the presence of lignin.

2. The observation that after intermittent acid hydrolysis slightly lower yields of lignin are obtained than after continuous hydrolysis is confirmed. This may be due either to the formation of some non-lignin insoluble product, or to the condensation of some soluble product with the lignin.

3. The enhanced yields of apparent lignin caused by the presence of protein must be caused by the condensation of large protein fragments, partially deaminated. The disturbance caused by simple amino-acids and nitrogenous bases is of a much lower order.

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