CCLXXXIII. THE DETERMINATION OF LIGNIN.

I. ERRORS INTRODUCED BY THE PRESENCE OF CERTAIN CARBOHYDRATES.

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(*Received October 29th, 1934.*)

The determination of lignin, though apparently a simple matter, is one of the least satisfactory of the analyses commonly carried out on plant materials and woods. Practically all the methods generally used involve the solution and hydrolysis of all other plant constituents and the assumption that the residue after such treatment is exclusively lignin. Various workers from time to time have pointed out that this is not the case, but in spite of this vital objection, considerable reliance has been placed on figures so obtained. There has similarly been some conflict as to the precise procedure to be adopted, inasmuch as different values are given by different methods. The reasons for these differences have not usually been given and are little understood.

In this work an attempt has been made to study some of the factors which disturb the lignin determination, in order that the best possible method may be selected for any particular material. It is not necessarily to be expected that one procedure will be found universally suitable for all classes of material.

The generally accepted methods employ a strong mineral acid to dissolve cellulose and other constituents, either 72 % sulphuric acid in some modification or variation of the Ost and Wilkening [1910] method, or fuming hydrochloric acid (42 %), following the procedure of Willstätter and Zechmeister [1913]. Other things being equal, the 72 % H_2SO_4 is less unpleasant to handle and is consequently most frequently employed. The sulphuric acid method has been used exclusively in these investigations, and no attempt has been made to compare the results obtained with those given by the concentrated HCl method as developed by Phillips [1932]. Probably the best-known modification of the Ost and Wilkening procedure is that employed for many years by the U.S. Forest Products Laboratory in which the material, after alcohol-benzene extraction, is treated at room temperature for 17 hours with 72 % sulphuric acid, the mixture then being diluted till the acid concentration is 3 % and boiled for 2 hours. The lignin is filtered off and weighed. Sherrard and Harris [1932] more recently showed that the amount and properties of the apparent lignin obtained by this procedure are altered by variations in temperature during the 72 % acid treatment, and accordingly the method was considerably modified by Ritter *et al.* [1932]. Following the initial alcohol-benzene extraction they introduced a treatment with boiling water for 3 hours. This is stated to cause the lignin residue to be "lighter in colour, the yield lower, and filtration and washing facilitated." No evidence is given as to the nature of the material removed, though it is presumed to be either insoluble in sulphuric acid or converted into insoluble products by the acid treatment. The subsequent 72 % acid treatment is continued for only 2 hours and the temperature maintained at 20°, it being shown that higher temperatures cause caramelisation. After dilution to 3 % acid, the solution is boiled for 4 hours and filtered as before. Peterson *et al.* [1932] similarly showed that the temperature should not be allowed to be high during the 72 % acid treatment, and in their case recommended an 18-hour treatment at 4°, though there was little difference between the results obtained at 4 and 15°. Cohen and Dadswell [1931] were unable to obtain satisfactory results for certain Australian woods, particularly Eucalypts, by existing methods and proposed a pretreatment of the wood with $N/8$ sodium hydroxide at 100° for 80 minutes to remove extraneous matter causing high results. It has not been conclusively shown that such a treatment does not at the same time remove some lignin.

Recently Phillips [1934] has compared on a number of different materials the original U.S. Forest Products Laboratory method, that of Peterson *et al.* [1932], an older method of Schwalbe [1925] employing a mixture of 72 % H_2SO_4 and concentrated HCl, and his own method [1932] which makes use of 42 % HCl. Unfortunately the later procedure of Ritter *et al.* [1932] was not included. Further, all the materials received a pretreatment by extraction with hot water, a process not a part of any method but that of Phillips [1932]. He ranked the methods according to yield, the lowest being that of Phillips [1932], and then in order, Schwalbe, Peterson *et al.* and the U.S. Forest Products Laboratory method, the last giving very considerably higher figures in most cases.

Some of these methods and modifications will be discussed later in the light of the experimental work here presented. In several cases changes in procedure recommended by various workers seem to have been made without any clear appreciation of the reasons underlying their effects.

EXPERIMENTAL.

(*a*) *Effect of sugars on apparent lignin yields.*

In a paper on the determination of cellulose, Norman and Jenkins [1933] remarked that cellulose preparations from straws giving no test for lignin often yielded about 3 % apparent lignin and showed that this figure could be reduced by treating the cellulose in such a way that the cellulosan fraction was to a great extent removed. They presumed that some form of condensation occurred in the presence of the 72 % acid with the production of an insoluble and unhydrolysable product weighed as lignin, and gave evidence to show that xylose units were responsible for this phenomenon. This has been confirmed. Table I shows the effect of the addition of xylose in various amounts on the apparent lignin content of hydrolysed straw, so treated as to remove practically all hemicellulose material.

All results in this, and later Tables, are the average of two or more closely agreeing determinations, and all lignin determinations are corrected for ash. The straw and other materials used were finely ground and passed through a 60-mesh sieve.

It appears that the disturbance produced by the addition of the sugar is very appreciable but not proportional to the increments added, or necessarily identical in different experiments.

Table I. *Addition of xylose to hydrolysed oat straw* (*5 % acid for 1 hour*).

16 hours with 72 % acid, temp. <20°; diluted to 3 % and boiled for 2 hours.

	Apparent lignin content % of straw	Increase per 0·1 g. xylose added
Straw A (0·5 g.) alone	22·11	—
,, ,, +0·1 g. xylose	24·98	2·87
,, ,, +0·25 g. ,,	29·00	2·76
,, ,, +0·5 g. ,,	34·50	2·48
Straw B (0·5 g.) alone	28·00	—
,, ,, +0·25 g. xylose	31·16	1·26
,, ,, +5 g. ,,	34·10	1·22
,, ,, +1·0 g. ,,	36·94	0·89
,, ,, +2·0 g. ,,	40·54	0·63

The following other sugars were tested, alone and in the presence of cellulosic material: arabinose, glucose, mannose, galactose, fructose and sucrose. Of these only arabinose, fructose and sucrose gave rise to any apparent lignin, and the amount from arabinose was very small. From filter-paper *plus* 1 g. sucrose, treated with 72 % H_2SO_4 for 16 hours, 22 mg. apparent lignin were obtained after dilution and boiling; from 1 g. fructose, similarly treated, 48 mg. and from 1 g. arabinose, 8 mg. Table II gives some observations on the addition of sucrose and arabinose to straw, the sucrose presumably causing a disturbance in the apparent lignin content by reason of its fructose unit.

Table II. *Addition of sucrose and arabinose to straws.*

16 hours with 72 % acid, temp. <20°; diluted to 3 % and boiled for 2 hours.

	Apparent lignin content % of straw	Increase per 0·1 g. sugar added
0·5 g. straw (hydrolysed bean straw)	22·1	—
,, ,, +0·5 g. sucrose	25·7	0·72
,, ,, +1·0 g. ,,	28·0	0·59
0·5 g. straw (oat straw)	18·5	—
,, ,, +0·5 g. arabinose	18·9	—
,, ,, +1·0 g. ,,	18·9	—

The disturbance produced by sucrose, though considerable, is not as great as that caused by xylose and likewise is not proportional to the amount present. That given by arabinose is very small. An explanation of this difference will be given later.

Very recently Hilpert and Littman [1934] have recorded the same observations, that these sugars with strong acid yield insoluble products, but in their experiments the time of contact was 48 hours, and the residues were consequently higher.

Since free xylose, or its fission products, in the presence of concentrated sulphuric acid condenses to form an insoluble material, it was reasonable to

Table III. *Addition of xylan to oat straw.*

16 hours with 72 % acid, temp. <20°; diluted to 3 % and boiled for 2 hours.

	Apparent lignin content of straw %
0·7 g. straw alone	19·71
,, ,, +0·3 g. xylan	22·07

suppose that the polysaccharide xylan would also do so. This was proved by testing a crude xylan preparation obtained by the cold alkaline extraction of a prepared straw cellulose (Table III).

Similarly, increases in apparent lignin were obtained when a crude hemicellulose sample containing about 50 % pentose, prepared from oat straw, was employed. Like practically all hemicellulose preparations this was not absolutely free from lignin, which was determined by boiling with 3 % acid for 2 hours. The insoluble residue, after correction for ash, was regarded as lignin and found to be 4·27 %.

Table IV. *Addition of crude hemicellulose to hydrolysed oat straw (5 % acid, 1 hour).*

16 hours with 72 % acid, temp. <20°; diluted to 3 % and boiled for 2 hours.

	Apparent lignin g.	Lignin in hemicellulose g.	Apparent lignin from straw g.	Apparent lignin on straw %
0·5 g. straw alone	0·1317	—	0·1317	26·34
,, ,, +0·1 g. hemicellulose	0·1450	0·0043	0·1407	28·14
,, ,, +0·3 g. ,,	0·1525	0·0128	0·1397	27·94
,, ,, +0·5 g. ,,	0·1691	0·0213	0·1478	29·56
0·3 g. hemicellulose alone	0·0203	0·0128	—	—

Unfortunately it was not possible to test the effect of the uronic acids upon lignin yields, and until this is done they must, from their very nature, be regarded as likely causes of disturbance. Additions of pectin, which contains nearly 80 % of tetragalacturonic acid anhydride, did not produce any increase in apparent lignin.

(*b*) *Effect of duration of acid treatment on apparent lignin yields.*

In all the experiments quoted above, the 72 % acid was allowed to act for 16 hours at a temperature less than 20°. The work of Sherrard and Harris [1932] on the effect of temperature has shown that if this temperature be exceeded serious errors are introduced owing, no doubt, to the accelerated caramelisation of carbohydrate material. However, by varying the time, it has been shown that the disturbing effect of the sugar increases with the period of contact, and that for a period of 2 hours, the time now recommended by Ritter *et al.* [1932], it is small though appreciable, as will be seen in Table V.

It is noteworthy that Dore [1920] many years ago recommended the use of the short time of contact with acid of 3½ hours.

Table V. *Effect of duration of acid treatment on the lignin yield of xylose added to hydrolysed oat straw (5 % acid for 1 hour).*

Varying times with 72 % acid, temp. <20°; diluted to 3 % and boiled for 2 hours.
0·5 g. straw + 0·5 g. xylose.

		Apparent lignin g.	Increase due to xylose and time mg.
Straw alone	16 hours	0·1105	—
,, +xylose	1 hour	0·1178	7·3
,, + ,,	2 hours	0·1172	6·7
,, + ,,	4 ,,	0·1206	10·1
,, + ,,	7 ,,	0·1380	27·5
,, + ,,	16 ,,	0·1725	62·0
Straw alone	2 ,,	0·1111	—

A similar effect of the time of standing with 72 % acid may be observed with any material containing pentose, as for example a hardwood. In Table VI is given the apparent lignin content of Danzig oak at various periods.

Table VI. *Effect of duration of acid treatment on apparent lignin content of Danzig oak.*

Results expressed on original material, extracted with alcohol-benzene before use.
Varying times with 72 % acid, temp. <20°; diluted to 3 % and boiled for 2 hours.

Duration of acid treatment hours	Apparent lignin %
1	23·6
2	24·0
4	23·8
7	26·1
16	28·8

Further evidence of the fact that at 2 hours the disturbance due to pentose is small was obtained by adding a hemicellulose preparation to hydrolysed oat straw, and treating the mixture for only 2 hours with the 72 % acid. These results given in Table VII should be compared with Table IV in which the time of contact was 16 hours.

Table VII. *Addition of crude hemicellulose to hydrolysed oat straw (5 % acid, 1 hour).*

2 hours with 72 % acid, temp. <20°; diluted to 3 % and boiled for 2 hours.

	Apparent lignin g.	Lignin in hemicellulose g.	Apparent lignin from straw g.	Apparent lignin on straw %
0·5 g. straw alone	0·1277	—	0·1277	25·54
,, ,, +0·1 g. hemicellulose	0·1325	0·0043	0·1282	25·64
,, ,, +0·3 g. ,,	0·1455	0·0128	0·1327	26·54
,, ,, +0·5 g. ,,	0·1544	0·0213	0·1331	26·62
0·3 g. hemicellulose alone	0·0141	0·0128	—	—

(*c*) *Effect of removal of hydrolysable constituents.*

In the case of any normal plant material containing perhaps a considerable amount of xylan in the cellulosan fraction of the cellulose and xylose in the encrusting hemicelluloses also, the disturbance due to pentose may apparently be minimised or much reduced either by removing that fraction or by shortening the period of exposure to acid to 2 hours, as Ritter *et al.* [1932] have done, or by a combination of these two expedients. The hydrolysable constituents, amongst them the pentose-containing polysaccharides, have been progressively removed from a number of materials by mild acid treatment, and the apparent lignin obtained after standing for 16 hours with 72 % acid has been compared with that obtained after standing for only 2 hours. In the latter case boiling after dilution to 3 % was continued for 4 hours, as recommended by Ritter *et al.* [1932], though there does not seem to be any obvious advantage to be gained by so doing. It should be stated that, in general, the materials having the longer exposure to the strong acid, settled and filtered the more rapidly. The results are summarised in Table VIII. There is a steady fall in the apparent lignin content of these materials as the hydrolysable polysaccharides are progressively removed and, as would be expected, this fall is greater in magnitude when the longer period of contact is employed, for this gives rise to more apparent lignin from the hydrolysable substances than the shorter contact. The figure on the untreated material at 2 hours is always considerably lower than that at 16 hours, but this difference disappears when the major part of the hydrolysable constituents has been removed, and after treatment for 1 hour with either 3 or 5 % acid, the figures are not significantly different whichever length of treatment is given.

Table VIII. *Comparison of 16-hour and 2-hour treatments of acid-hydrolysed materials.*

Results expressed as percentages of original material, extracted with alcohol-benzene before hydrolysis. All treatments given at the boil.

				Apparent lignin	
Treatment		Residue	Furfuraldehyde yield	16 hours	2 hours
Oat straw A:					
Untreated		—	Not determined	18·48	15·29
Water	1 hour	80·00	,,	14·79	12·49
0·5 % H_2SO_4	½ ,,	73·20	,,	13·69	12·74
1·0 % ,,	1 ,,	61·26	,,	12·24	12·75
5·0 % ,,	1 ,,	54·47	,,	12·04	12·12
5·0 % ,,	5 hours	50·28	,,	11·94	12·13
Beech wood:					
Untreated		—	,,	23·31	21·47
Water	1 hour	94·08	,,	22·09	20·70
0·5 % H_2SO_4	½ ,,	92·04	,,	21·99	20·27
1·0 % ,,	1 ,,	84·80	,,	21·04	19·37
5·0 % ,,	1 ,,	70·57	,,	19·59	19·23
5·0 % ,,	5 hours	65·15	,,	18·68	19·05
Oat straw B:					
Untreated		—	17·88	16·22	14·82
Water	1 hour	80·05	15·81	13·94	13·41
0·5 % H_2SO_4	½ ,,	72·69	12·46	13·53	13·02
1·0 % ,,	1 ,,	60·71	7·25	12·14	12·05
3·0 % ,,	1 ,,	56·56	6·98	11·54	11·52
5·0 % ,,	1 ,,	51·93	3·36	11·90	11·84
Oak (Danzig):					
Untreated		—	12·70	28·37	25·41
Water	1 hour	87·58	12·17	22·53	21·82
0·5 % H_2SO_4	½ ,,	78·47	8·50	21·46	20·61
1·0 % ,,	1 ,,	76·74	8·17	22·25	20·95
3·0 % ,,	1 ,,	70·03	5·73	21·28	20·51
5·0 % ,,	1 ,,	67·22	4·68	21·01	20·59
Bean straw:					
Untreated		—	10·74	17·03	16·56
Water	1 hour	77·77	9·78	16·58	15·06
0·5 % H_2SO_4	½ ,,	71·63	9·09	15·57	15·40
1·0 % ,,	1 ,,	64·65	7·87	14·42	14·34
3·0 % ,,	1 ,,	58·83	5·37	14·46	14·55
5·0 % ,,	1 ,,	56·80	5·28	15·54	14·35

Now, in their method, Ritter *et al.* [1932] employ an extraction with boiling water for 3 hours prior to treatment for 2 hours with the concentrated acid. The lower results obtained after this pretreatment they explain as being due to the removal of "extractives," insoluble in alcohol-benzene and insoluble in sulphuric acid or converted into insoluble products by the acid treatment. It seems however that a treatment for 3 hours with boiling water ought to be considered to be in part hydrolytic in action in addition to effecting extraction. They give no figures for the amount removed from the various woods by this treatment, but beyond doubt it is quite considerable and includes some encrusting hemicellulose.

The actual amount will vary widely from material to material according to the quantity and resistance of the hemicellulose constituents. In the five samples given in Table VIII, all of which were mature, boiling with water for 1 hour removed from 6 to 23 %. Table IX gives a comparison of the loss on treating several materials for 15 minutes and 3 hours with water at 100° and the furfuraldehyde yield of the material after the latter treatment.

Table IX. *Loss on treatment with water at 100°.*

Material	Furfuraldehyde yield %	Residue 15 mins. %	Residue 3 hrs. %	Furfuraldehyde yield on original basis %
Oak	15·2	94·3	92·3	14·4
Oat straw	17·6	80·4	77·2	14·2
Hay	12·1	72·4	69·9	11·1

The conclusion appears inevitable that the "extractives" removed by prolonged boiling of woods or mature plant materials are partly of a hemicellulosic nature and may contain a considerable portion of the "pentose not in cellulose."

The differences obtained by Ritter *et al.* [1932] between untreated and water-extracted woods may therefore be ascribed, at least in part, not to hypothetical "extractives" but to a pentose disturbance, the actual disturbance being comparatively small because the time of standing is only 2 hours. In Table V the increase in 2 hours produced by 0·5 g. xylose added to 0·5 g. hydrolysed straw was only 6 mg. or about 5 % of the lignin although the ratio of pentose to lignin present was about 5, a ratio which would never occur in mature materials but which might be found in slightly lignified materials. The conditions chosen by Ritter *et al.* [1932] involving prolonged water-extraction and brief acid treatment therefore are such that the pentose disturbance is quite small, but it would be incorrect to say that it does not occur or is negligible in all cases. In Table X are given some results bearing on this point, since it provides a comparison of the effects of the addition of pure xylan (prepared by the cold alkaline extraction of prepared oat straw cellulose) to straw untreated and to straw boiled for 3 hours with water.

Table X. *The addition of xylan to untreated and water-extracted oat straw.*

Various times with 72 % acid; temp. <20°; diluted to 3 % and boiled for 2 hours.

	Time in hours	Apparent lignin on straw %
0·8 g. straw boiled 3 hours (residue 0·611 g.)	2	13·36
0·8 g. straw + 0·5 g. xylan together boiled 3 hours (combined residue 0·632)	2	13·39
0·8 g. straw boiled 3 hours (residue 0·611 g.), then 0·5 g. xylan added	2	14·50
0·7 g. straw alone	2	16·63
,, ,, + 0·3 g. xylan	2	16·77
,, ,, alone	16	19·71
,, ,, + 0·3 g. xylan	16	22·07

While untreated straw had an apparent lignin content of 16·63 % the water-extracted straw gave a figure of only 13·36 %. The addition of xylan to either produced only a slight increase. In the case of the untreated straw this was presumably because the pentose disturbance was already high, and as shown in Table I there is no linear relationship, increasing amounts causing smaller and smaller increments in disturbing condensation products. In the case of the water-extracted mixture, on the other hand, the increase was slight because practically all the added xylan went into solution during the aqueous extraction, only 21 mg. out of the 0·5 g. originally present remaining. The xylan, as prepared, is not easily soluble in water but is apparently almost completely dissolved by this prolonged boiling, indicating again the hydrolytic effect of such a treatment. When, however, the xylan was added after the water-extraction of the straw, there was a considerable increase in apparent lignin even at 2 hours. It has been claimed that the Ritter *et al.* [1932] method is entirely unaffected by the presence of carbohydrates, but no direct or conclusive evidence is offered by them upon this important point. Nevertheless from this work it seems that the product obtained by their procedure will contain a minimum of condensation products and is certainly purer than that given under the best conditions by any other existing method employing 72 % acid. The question arises, however, as to whether it might not be improved and rendered more accurate by substituting an acid hydrolysis for the prolonged boiling with water, and thus rendering more certain and more complete the removal of encrusting hemicelluloses and cellulosans which, containing pentose as they do, may cause the lignin figure to be too high, even with only 2 hours' contact with strong acid. The crucial point is whether lignin, as it exists in the plant material, is affected by treatment with hot dilute acids. The assumption might be made that it is resistant to boiling with 3 % H_2SO_4, after treatment with 72 % acid, since this is normally a part of the lignin determination. Conceivably, however, before such treatment it may be more susceptible, and consequently to introduce such a pretreatment may result in lowering the lignin yield. At present there is no satisfactory evidence upon this point, and indeed, the planning of any conclusive experiments to decide the matter presents many difficulties. The fate of the methoxyl groups is not a satisfactory index of changes in the lignin, inasmuch as Ritter and Kurth [1933] by isolating a lignin-free "holocellulose" fraction (cellulose + hemicelluloses) showed that a portion of the methoxyl and most of the acetyl groups were associated with these polysaccharides rather than with the lignin. If there is any loss of lignin by pretreatment with dilute acid it would not appear to be large, for, as will be seen in Table VIII, there was no significant difference between materials treated with 3 % acid for 1 hour, 5 % acid for 1 hour, or subjected to the unnecessarily long treatment of 5 % acid for 5 hours, when the lignin was determined after standing for 2 hours with 72 % acid. With a 16-hour period, rather curiously, the last treatment gave lower figures. To sum up this series and many other pretreatments, it seems that for some materials a pretreatment for 1 hour with 1 % acid followed by a 2-hour treatment with the 72 % H_2SO_4 is sufficient to give minimum results, and for others, particularly straws, pretreatment with rather stronger acids is necessary. If 3 or 5 % acid be employed it is of little moment whether the duration of contact with 72 % acid be 2 or 16 hours.

From information obtained from the U.S. Forest Products Laboratory it is suggested, not that lignin is affected by the mild acid hydrolysis, but that there is present a small amount of some product intermediate between carbohydrate and lignin which is acid-soluble or hydrolysable, but which is further condensed by the strong acid treatment, so that it then becomes resistant to dilute acid. Further, since this product is rendered soluble, like lignin, by chlorination, it should properly be regarded as lignin, and therefore acid pretreatment methods which remove it are inadmissible. This is an interesting alternative suggestion, but as yet there does not seem to be any experimental evidence directly supporting it. The validity of an acid pretreatment must therefore remain undecided until the question of the action of dilute acids on lignin has been settled. Investigations along this line are proceeding.

(*d*) *The lignin content of various plant materials.*

In view of the observations recorded above, it is clear that the recorded figures for lignin content of all classes of plant materials are too high to a greater or less extent, with the exception of those few determined since 1932 by the method of Ritter *et al.* [1932]. The error will vary considerably according to the pentose content of the material in question and the temperature and time of treatment with 72 % acid. It has frequently been observed that analyses add up to more than 100 % and this may be ascribed almost always to the excessive lignin figure. Phillips [1934] has recently criticised the indiscriminate application of methods developed for woods to other classes of plant materials, but in so far as mature materials are concerned, it has been shown herein that the errors are inherent in the method itself and not caused by any wide difference in composition between woods and "non-woods." Table XI contains analyses made on a very wide range of woods and plant materials, the lignin figure being recorded before and after extraction for 1 hour with 5 % H_2SO_4. The final column contains the percentage reduction in apparent lignin produced by this treatment, which may amount to one-quarter or even one-third in special cases. As might be expected from their higher pentose contents, the reduction in the case of some hard woods is greater than that for soft woods. It will be noted

Table XI. *The lignin content of various materials before and after extraction with 5 % H_2SO_4 for 1 hour.*

16 hours with 72 % acid, temp. <20°; diluted to 3 % and boiled for 2 hours.
All results expressed on 100 g. original material before alcohol-benzene extraction.

Material	Apparent lignin %	Loss of original material on hydrolysis %	Lignin after hydrolysis %	Reduction in apparent lignin figure %
Oak	28·80	34·41	20·24	29·9
Beech	23·30	29·43	19·50	16·3
Basswood	22·82	27·92	17·98	21·1
Teak	28·98	34·42	26·95	7·2
Mahogany	27·91	23·38	25·77	7·5
African walnut	32·87	18·39	30·33	7·9
Deal	25·87	26·81	23·42	9·6
Sitka spruce	26·18	22·10	23·46	10·3
Pine needles	26·85	51·49	23·91	11·2
Almond hulls	30·49	55·93	22·21	27·2
Banana leaves	19·67	41·99	16·72	15·2
Coconut husk	40·86	28·09	34·66	15·2
Moss	21·99	50·19	16·21	26·3
Flax straw	24·94	30·37	21·79	12·4
Oat straw	18·48	45·53	12·04	34·8
Wheat straw	19·31	38·72	13·90	28·0
Barley straw	16·70	52·13	10·53	37·1
Mature hay	18·50	46·26	14·10	23·8
Bracken	30·28	54·55	23·67	21·8
Mustard plants	12·41	59·59	9·43	24·0
Maize plants	11·14	57·72	7·81	29·9
Barley plants	6·13	68·12	3·78	38·4
Lucerne tops	10·93	68·01	8·20	24·8
Lucerne roots	11·49	58·95	8·67	24·8

that all but the last five materials are mature "lignified" materials; the remaining samples were younger and considerably higher in nitrogen. For reasons which will appear in the next paper these samples are subject to an additional and different error, but the figures are quoted for comparison.

In a few samples the lignin content was determined also by the full Ritter *et al.* [1932] method and these results are given in Table XII for comparison with Table XI. The results fall midway between the apparent lignin at 16 hours and that obtained after a hydrolytic pretreatment.

Table XII. *The lignin content of various materials by the Ritter, Seborg and Mitchell method.*

2 hours with 72 % acid, temp. < 20°; diluted to 3 % and boiled for 4 hours. All results expressed on 100 g. original material.

	Lignin %		Lignin %
Basswood	20·44	Sitka spruce	25·38
Teak	27·10	Flax straw	23·14
Deal	25·78	Wheat straw	16·62

(*e*) *Implications of the observations recorded above.*

In two directions at least there are to be found implications of the data presented. The first lies in the preparation of lignin in bulk for ultimate analysis, constitutional studies or the preparation of derivatives. If the sulphuric acid method of preparation be employed, as it is by a number of workers, precautions must be taken to avoid the formation of condensation products from pentose groupings which, under bad conditions, might amount to 25–30 % of the preparation. The presence of such a fraction would have a serious effect on the figures for ultimate analysis, and might interfere with chemical reactions. Sherrard and Harris [1932] have studied the conditions of preparation of lignin from sugar maple and have introduced various modifications which they claim yield lignin free from carbohydrate. It is by no means certain, however, that the product is entirely free from these disturbing condensation products, for the acid treatment recommended is for a period of 15 hours from the time when the material goes into a thin solution, and no pretreatment other than an aqueous extraction is carried out. To avoid any possibility of the presence of such a fraction, a pretreatment with dilute acid would be advisable and the shortening of the period of contact with 72 % acid to 2 or 3 hours. A light friable product may be obtained by this means.

A second important application of these experiments lies in the studies of microbiological decomposition of plant materials, and in digestibility trials, in both of which lignin determinations may be carried out on materials which are changing as regards their other constituents. In the decomposition of such a material the hemicelluloses are rapidly fermented away, and the consequent fall in pentose groupings may produce an accompanying fall in the apparent lignin content. The effect therefore resembles that produced by progressive acid hydrolysis, as in Table VIII, in which the apparent lignin content falls without there being necessarily any loss of true lignin. Unless precautions are taken to minimise the pentose disturbance, there may appear to be a wholly illusory loss of lignin. Various conclusions as to the decomposibility of lignin require confirmation for this reason. Some experiments on this point are in progress.

(*f*) *The nature of the disturbing material.*

Throughout this paper, the substance formed from pentose in the presence of 72 % sulphuric acid has been referred to vaguely as a condensation product. Some evidence has been obtained which points to the view that the condensation is between the lignin molecule and furfuraldehyde, the latter being produced from pentose by dehydration. That furfuraldehyde will combine with phenolic bodies is well known, indeed its determination ordinarily depends on the production of an insoluble phloroglucide with phloroglucinol. Insoluble "resins" have been manufactured commercially by the condensation of furfuraldehyde with higher phenols. Since there is general agreement that the lignin molecule contains a phenolic grouping, and since Ross and Hill [1929] have shown that lignin undergoes a definite reaction with formaldehyde, it seems not unreasonable to suppose that furfuraldehyde also might condense with lignin. It was later found that the condensation of furfuraldehyde with isolated alkali lignins had been the subject of a patent by Phillips [1930]. The possibility of condensation *in situ* was confirmed by the addition of increments of redistilled furfuraldehyde to a lignin determination on straw, and the results are given in Table XIII. The furfuraldehyde was added half an hour after the straw had gone into solution. The precipitates obtained after dilution and boiling were very black, flocculated well and settled rapidly.

Table XIII. *The addition of furfuraldehyde to lignin determinations.*

16 hours with 72 % H_2SO_4, temp. <20°; diluted to 3 % and boiled for 2 hours.

Material	Apparent lignin content g.	Increase per 0·025 ml. addition g.	
0·8 g. straw alone	0·1587	—	
,, ,, +0·025 ml. furfuraldehyde	0·1943	0·0356	Average 0·034
,, ,, +0·050 ml. ,,	0·2248	0·0305	
,, ,, +0·075 ml. ,,	0·2617	0·0369	
,, ,, +0·100 ml. ,,	0·2942	0·0325	
,, ,, +0·200 ml. ,,	0·3738	0·0199	

The results show that there is an apparent increase even larger than the quantity of furfuraldehyde added (sp. gr. 1·16). Up to the addition of 0·1 ml. of furfuraldehyde the increase is approximately proportional to the amount added, the pipetting of such small quantities not being very accurate. Above 0·1 ml. further additions did not give so large an increase. Controls of the same quantities of furfuraldehyde treated alone yielded no weighable precipitate, though there was the formation of a floating film with 0·2 ml. Larger quantities, however, gave a black granular precipitate, for example, 1 ml. with 15 ml. 72 % acid, yielded 46 mg. of precipitate after dilution and boiling. Table XIII shows that for each addition of 0·025 ml. furfuraldehyde there was an average increment in precipitate of 0·034 mg., a clear indication of a linkage between the aldehyde and lignin and some further reaction the nature of which is not understood.

To support the view that the pentose disturbance is due to furfuraldehyde production and subsequent condensation, the presence of furfuraldehyde was detected and the amount determined in experiments in which xylose was treated with 72 % sulphuric acid alone. After standing and dilution to 3 % the solution was steam-distilled, 300 ml. distillate being collected from 600 ml. solution. It is not known whether under these conditions the recovery is quantitative. To the distillate were added 150 ml. concentrated HCl to bring the acid concentration to 12 %. Phloroglucinol was added and the precipitate weighed after standing overnight. The residue after steam-distillation was boiled for a further period of 1$\frac{3}{4}$ hours and filtered.

Table XIV. *Furfuraldehyde yield from xylose and arabinose with 72* $^0/_0$ H_2SO_4.

	Time with 72 % acid hours	Phloroglucide mg.	Furfuraldehyde mg.*	Residue mg.
1 g. xylose	16	19·9	13·0	39·7
,, ,,	11	12·1	8·9	11·6
,, ,,	7	9·9	7·8	10·0
,, ,,	4	16·0	10·9	5·9
,, ,,	2	5·0	5·3	None
,, ,,	24	37·1	21·9	49·4
,, arabinose	16	5·1	5·3	4·1

* Calculated from the formula $(a + 0{\cdot}0052) \times 0{\cdot}517$.

The amount of furfuraldehyde present seems to change with time, presumably by transformation into insoluble residue. The nature of this residue is unknown, but it is possibly a compound formed by slow dehydration and condensation of the furfuraldehyde. Meunier [1929] has stated that furfuraldehyde may condense with loss of water to give brown derivatives.

From these experiments it seems that the insoluble residue formed from xylose alone in the presence of 72 % sulphuric acid may have no part in the increase in apparent lignin caused by xylose when added to straw. That this might be the case was suspected earlier, since the increase found is always so much greater than would be expected from the residue obtained from xylose alone.

The probability is therefore that the disturbance caused by pentose is due to the production of furfuraldehyde and the more or less rapid condensation of this with the lignin present, to give an insoluble phenol-furfuran "resin," which is very stable and from which the furfuraldehyde cannot readily be split off. In the event of there being present furfuraldehyde in excess of the combining power of the lignin, dehydration of the former might occur with the formation of another insoluble precipitate. This however is an unlikely occurrence inasmuch as the amount of lignin usually present in mature materials could combine with far more aldehyde than would be produced from the pentose there, and the first reaction therefore takes precedence.

The observed difference between the effects of addition of xylose and arabinose may be explained by the fact, already known, that the production of furfuraldehyde from these two sugars takes place at different rates, and that the theoretical equation does not adequately represent the changes taking place. Since the uronic acids may be dehydrated to yield furfuraldehyde it is possible that they too might interfere in this way in the lignin estimation. This has not been verified. The disturbance due to fructose and consequently to sucrose may also provisionally be ascribed to the production of hydroxymethylfurfuraldehyde or possibly to β-hydroxy-γ-methylfurfuraldehyde which is produced from fructose under certain conditions of dehydration.

SUMMARY.

1. Some of the disturbing factors concerned in the determination of lignin by the 72 % sulphuric acid method have been investigated, and their effects in various recommended procedures have been studied.

2. Certain sugars, particularly xylose and fructose, give an insoluble residue on standing with 72 % sulphuric acid and increase the apparent lignin figure when added to plant materials. Arabinose does so also, to a slight extent, and sucrose, by reason of its fructose constituent. Polysaccharides containing pentose sugars produce a similar effect.

3. The disturbance caused by the presence of such carbohydrates increases with the time of contact with 72 % acid. At 2 hours the effect is small.

4. In plant materials the effect of the presence of xylose in the hemicellulose may be almost excluded by a hydrolytic pretreatment with dilute mineral acids, or minimised by reducing the time of contact to 2 hours, as in the Ritter-Seborg-Mitchell method. The validity of an acid pretreatment is not proved, since the action of dilute acids on lignin is not known.

5. Because of the presence of xylose, the figures generally quoted for lignin are, in most cases, too high. The lignin content determined after acid pretreatment is given for a wide range of plant materials and woods.

6. The disturbance due to pentose is caused by the slow production of furfuraldehyde and its condensation with lignin to form an insoluble phenol-furfuran resin. Furfuraldehyde itself may give an insoluble product by dehydration and condensation, but the former reaction probably takes precedence as long as there are phenolic groups on the lignin unsatisfied.

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