94. ESTIMATION OF LIGNIN IN TANNIN MATERIALS

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THE necessity for an investigation of this problem arose when the question of losses of lignin during decomposition of green materials was under examination. Lignin percentages obtained by the 72 % H_2SO_4 method after a pretreatment by 5 % H_2SO_4 looked strikingly high and the results became more suspicious when they were considered along with the decomposition undergone by the tannin materials under investigation. For instance, for materials of lignin content of 39 % to suffer a loss of about 35 % in dry matter during a period of 35 days appeared anomalous because it is known that highly lignified tissues are very slow to decompose. Secondly, such a lignin content in green leafy materials like tea leaf prunings or refuse tea looks absurd when compared with even the most highly lignified tissues like coconut coir or certain woods which only rarely attain such a high degree of lignification. It thus became apparent that 5 % H_2SO_4 hydrolysis as a pretreatment for materials containing tannin is undesirable since instead of removing any extraneous material which interferes with lignin estimation it probably renders it insoluble.

The various substances known to interfere with the determination of lignin in plant materials in general are the pentose groups of the hydrolysable polysaccharides, proteins, substances of an aldehydic or ketonic character and fatty and resinous substances. Waxes and fats are removed by extraction with a mixture of benzene and alcohol. Since no serious objection to the use of acid pretreatment has been raised this has been widely employed for a long time to minimize pentose and protein disturbance [Norman & Jenkins, 1934; and Norman, 1937]. Although acid hydrolysis as a pretreatment for a wide range of materials has been found handy, it can by no means be universally adopted. Exceptions have occasionally been recorded. Bamford & Campbell [1936] obtained an increase in the apparent lignin from wood of beech and African pencil cedar after a treatment with 5 % sulphuric acid. Cohen [1936] found it difficult to estimate the lignin in eucalyptus wood correctly by the common procedure. Ritter & Barbour [1935] observed an interference due to the presence of tannins of the catechol group in lignin determination of certain woods. The question arises whether other tannins, or even quite different substances present in different materials are rendered insoluble in the presence of dilute acid. The existence of such special minor substances affecting the lignin determination calls for further pretreatment. After surveying the literature it was noted that no other tannin materials except those of the catechol group have been studied. It was therefore thought desirable to make comparisons between catechol tannins and those of other groups.

CLASSIFICATION OF TANNIN MATERIALS

As a preliminary to the investigation it was necessary to classify the various tannin materials into certain definite groups. This should give a clue to whether all forms of tannin behave alike in lignin estimations or whether the catechol group is specific with regard to such a hindrance. The present classification is based on the suggestion of Nierenstein [1934] and Haas & Hill [1921] and chemical tests described by Proctor [1903]:

- (1) Condensed tannins $\begin{cases} \rightarrow \text{Catechol tannins} \rightarrow \text{Tea leaf and refuse tea.} \\ \rightarrow \text{Pyrocatechol tannin} \rightarrow \text{Pterocarpus indicus.} \end{cases}$
- (2) Hydrolysable tannins $\rightarrow$ Ellagi-tannins $\rightarrow$ *Terminalias* and *Caesalpinias*.
- (3) Substituted pyrogallol derivatives $\rightarrow$ Pyrogallol tannin $\rightarrow$ *Gordonia imbricata*.

It will thus be seen that the materials under investigation represent a major portion of the various types of tannins given by Nierenstein [1934]. Such a classification should enable one to study the method more closely and to develop a pretreatment which might embrace all groups of tannins.

EXPERIMENTAL

(a) Application of the common method

Results in Table 1 (a) leave no doubt that the pretreatment with 5% H_2SO_4 is faulty and is inapplicable, with one or two exceptions, to such types of materials on account of the high percentages of apparent lignin. Water extraction of the hydrolysed product from a few of the materials was also tried with a view to dissolving out any extraneous matter unaffected. Water does seem to lower the values for apparent lignin, perhaps by extracting some of the substances unattacked by acid. But they still appear to be appreciably high. It is probable that tannin, being a phenolic body, is precipitated in the presence of H_2SO_4 and is thus rendered insoluble in water. It is then ultimately estimated as lignin by the 72% H_2SO_4 method.

Table 1. Apparent lignin in materials after various extractions as pretreatments for lignin estimation

Results expressed on 100 g. original material on ash free basis

						6	7	8	9
	1	2	3	4	5	(c)			
Material		(a)		(b)		95 % alcohol	Alcohol- H ₂ SO ₄	Alcohol- H ₂ O	Alcohol- H ₂ O- H ₂ SO ₄
		H ₂ SO ₄	H ₂ SO ₄ -H ₂ O	H ₂ O	H ₂ O-H ₂ SO ₄				
Tea leaf		39.84	31.08	34.12	25.40	34.22	29.80	28.31	23.93
Refuse tea		38.70	29.04	32.45	28.44	41.90	29.70	31.47	26.61
<i>Pterocarpus indicus</i>		21.63	—	25.68	21.35	—	—	—	16.94
<i>Caesalpinia Bonduc</i>		30.39	28.97	30.32	27.56	29.07	25.87	26.73	23.85
<i>C. coriaria</i>		25.71	21.81	24.43	20.59	21.64	18.67	17.84	15.50
<i>C. sappan</i>		26.11	—	27.93	25.18	—	—	—	17.70
<i>Terminalia</i>		27.99	—	30.37	27.87	—	—	—	23.70
<i>Belerica</i> Roxb.									
<i>T. Chebula</i>		31.93	—	—	—	—	—	—	—
<i>Gordonia</i> (probably <i>imbricata</i>)		18.92	—	18.98	17.19	—	—	—	12.42

(b) Proposed modification of the common method

The modification is based on the fact that the tannins in these materials are extractable by hot water. In fact tannins in plant materials are estimated in the hot water infusion obtained under certain specified conditions [Shaw, 1935]. There is therefore every reason to suppose that a preliminary treatment with hot water ought to remove any tannins if they are the other interfering substances. The hemicelluloses and proteins can then be hydrolysed in the water-extracted residue by dilute H_2SO_4 thus removing almost all the suspected disturbing factors in lignin determination of tannin materials. Working on such a basis, all the materials were first extracted with hot water. Lignins were first determined on this residue. It will be noted from Table 1 (b) that in general the apparent lignin in the water-extracted residue is practically of the same order as after acid treatment (Table 1 (a)) with the exception of tea leaf and refuse tea, materials which are classified as catechol tannins. Lignins for these two are lower on water-extracted residue than on the hydrolysed product. Thus catechol tannins react in a different way from the other types in that they appear to be insoluble in the presence of dilute acid. Lower apparent lignins in the residue after water extraction of catechol tannins are undoubtedly due to removal of tannin and other water-soluble extraneous substances; but with other materials, on account of the similarity between acid and water treatments, it looks as though the disturbances caused by hemicelluloses and proteins which are minimized by acid hydrolysis mask the lowering in apparent lignin due to tannin water extraction. But such a masking of factors is undesirable in any accurate method of analysis. Therefore, after removing tannins it is equally essential to remove proteins and hemicelluloses which are hardly extractable by water. Evidence of the water-insoluble nature of proteins, at any rate, in tea leaf and refuse tea has been given by Shaw [1934]. Similar results have also been obtained in these laboratories. Dilute H_2SO_4 hydrolysis is therefore adopted for water-extracted residues to get rid of any hemicellulose and protein interference. Some idea of the abundance of proteins in these materials can be obtained from the nitrogen figures quoted in Table 3. Apparent lignins in the hydrolysed products are given in column 5 of Table 1 (b). These values look more reasonable and are perhaps closer to the true lignins in these materials, not only because they are lower, but because they conform to the idea previously mentioned that easily decomposable materials are not high in lignin content. The proposed method, therefore, consists in extracting the tannin material with boiling water for 1 hr. and subsequently hydrolysing with 5% H_2SO_4 for a similar period to get rid of tannin, protein and pentose interference, before proceeding with the 72% H_2SO_4 method of lignin estimation originally described by Ost & Wilkening [1910].

(c) 95% alcoholic extraction as a preliminary to the proposed method

While the above method was under investigation the paper by Ritter & Barbour [1935] became available and it was then considered expedient to try their method of alcohol and water extractions in relation to tannin interference. Accordingly all the materials were extracted with 95% alcohol for 4 hr. in a Soxhlet apparatus as recommended by them. Instead of extracting the residue after alcohol extraction with water alone, it was thought desirable simultaneously to study the effect of hydrolysis with dilute acid, and also the effect of combining both the water and acid extractions on the alcohol-extracted residues. Apparent lignins were then determined by the usual acid method in the four different residues. The results are incorporated in Table 1 (c). The

results with alcohol extraction alone are apparently too high because although alcohol extracts the tannin it has practically no effect on proteins and hemicelluloses. Subsequently water has almost the same effect as dilute H_2SO_4 . This may be explained by considering the fact that any tannin which would ordinarily have been affected by dilute acid has already been extracted by alcohol and hence does not interfere with lignin. Such similarity has been commented upon by Norman [1937, 1]. Judging from the trend of these results it seems that neither of the two individual extractions with water or H_2SO_4 following upon alcoholic extraction is so effective as the combined action of both because the apparent lignins are still appreciably higher than those in column 5 of Table 1 (b). It seems therefore that water and H_2SO_4 treatments following successively upon alcoholic extraction are necessary for a satisfactory working of the proposed method. When alcohol-sulphuric acid figures are compared with water-sulphuric acid in Table 1 it looks as though water is superior to alcohol in its extracting capacity for catechol tannins. Water on this ground would have been sufficient to get over the tannin interference but for the disturbance caused by chlorophyll which is reported later in the paper.

In view of these results the lignin determinations on the remaining species were confined to the residue after the combined alcohol-water-sulphuric acid pretreatment. When such values are compared with those in column 5, Table 1 (b) it will be noted that they are lower in general by a mean of about 4% than those with water-sulphuric acid treatments. Obviously alcohol has extracted something extraneous which was unaffected by the solvent action of water and sulphuric acid and which acted as a disturbing factor in addition to tannins, proteins and hemicelluloses in the lignin determination. Such lower values are possible under two circumstances—(1) alcohol dissolves some of the “soluble lignin”, (2) chlorophyll and other pigments are removed by 95% alcohol. The dissolution of soluble lignin during alcoholic extraction is possible on the evidence given by Mehta [1925] who worked with certain woods. But in the present investigation it is felt that chlorophyll, being ketonic in nature, is the apparent cause for an additional interference which is removed by alcohol. Evidence to this effect has been obtained and is contained in the following section.

(d) Some constituents in tannin materials which cause interference during lignin estimations

Table 2 records data for the possible interfering constituents either reacting independently or in association with others during lignin determinations by the 72% H_2SO_4 method. Commercial tannic acid was the first to be tested because that is the most easily procurable tannin in pure condition. Caffeine, which is largely present in tea leaf and refuse tea, was another which is also easily obtainable. Neither of them independently or conjointly produced any precipitate in presence of 72% H_2SO_4 . But this evidence does not preclude the possibility of tannic acid as a phenolic body or caffeine as a ketonic body condensing with lignin in the presence of strong acid. In fact Norman [1937, 2] obtained substantial increases in the apparent lignin of wheat straw in presence of tannic acid. Since it is known that the tea tannin is different in composition from ordinary tannic acid, it was decided to isolate it in pure condition by Shaw's method [1935] for the present investigation. It will be noted that this tannin produced a precipitate with H_2SO_4 and four times as much with caffeine. The question of chlorophyll interference which was raised in the last section was

tested on two chlorophyll preparations reacting either alone with caffeine or with caffeine and theotannin. One sample was prepared in the laboratory by Willstätter's method and the other one was a B.D.H. solution of water-soluble chlorophyll. Both the samples threw down a precipitate in strong H_2SO_4 . The effects of caffeine and theotannin were the same on chlorophyll although caffeine alone gave no precipitate with H_2SO_4 . When caffeine and theotannin were both present the precipitate with acid was still greater. It is not difficult to explain chlorophyll interference from these results because chlorophyll contains a ketonic group and substances with ketonic or aldehydic groups are known to condense readily with lignin in presence of strong acids. Some condensation products might also have been formed between theotannin and caffeine, the former being phenolic like lignin and the latter a ketonic substance. The resultant product might fall into a group of tannins described by Nierenstein [1934] as caffe-tannins and might consequently be estimated as lignin. Wherever theotannin and caffeine were present together the filtrate after lignin filtration became turbid on standing and started throwing down a precipitate. This precipitation of the filtrate might be attributed to the oxidation products of theotannin which are being slowly brought down. These observations clearly indicate the causes why tea leaf and refuse tea in particular give disproportionately high and erroneous values for apparent lignins if improperly treated. Such a formidable grouping of ketonic, phenolic and quinone bodies in the form of caffeine, chlorophyll and theotannin is rarely met with in other plant tissues, or even in the other tannin materials.

Table 2. *Apparent lignin from different constituents in tannin materials after 72 % sulphuric acid treatment*

Material	Apparent lignin (actually obtained) g.	Remarks
0.2 g. Tannic acid	Nil	Does not dissolve in 72 % H_2SO_4 but goes into solution on dilution
0.2 g. Caffeine	Nil	Dissolves in 72 % H_2SO_4
0.2 g. Tannic acid + 0.1 g. caffeine	Nil	Same as tannic acid
0.2 g. Theotannin	0.0021	Dissolves in 72 % H_2SO_4 with deep yellow colour. Precipitate forms overnight colour changing to reddish yellow. During heating on dilution colour changes to amber
0.2 g. Theotannin + 0.1 g. caffeine	0.0079	Same as theotannin
0.11 g. Chlorophyll (lab. preparation)	0.0610	Gives a clear green solution with 72 % H_2SO_4 . Precipitate forms overnight
0.5 ml. Water-soluble B.D.H. chlorophyll	0.0050	Precipitates at once in 72 % sulphuric acid
0.5 ml. Water-soluble B.D.H. chlorophyll + 0.1 g. caffeine	0.0151	Same as B.D.H. chlorophyll
0.5 ml. Water-soluble B.D.H. chlorophyll + 0.1 g. theotannin	0.0152	Forms precipitate with 72 % H_2SO_4 with amber colour
0.5 ml. Water-soluble B.D.H. chlorophyll + 0.1 g. caffeine + 0.1 g. theotannin	0.0235	Same as B.D.H. chlorophyll and theotannin

(e) *Final procedure for estimation of lignins in tannin materials*

2 g. of the oven-dry material passing through a 80-mesh sieve are extracted in a Soxhlet apparatus with 200 ml. 95 % alcohol for 4 hr. The extracted residue is transferred to a round-bottomed flask of 300 ml. capacity and extracted with

150 ml. boiling water under a reflux for 1 hr. The contents of the flask are then filtered and the residue is transferred back to the flask and hydrolysed with 150 ml. 5% H_2SO_4 for 1 hr. The hydrolysed product is then collected in a weighed sintered glass crucible after being washed free of acid. The residue is weighed and well powdered. 0.2 g. lots are weighed out into 1000 ml. beakers and treated with 20 ml. 72% H_2SO_4 . The powder is well mixed with the acid and allowed to stand overnight, at a temperature varying between 20 and 22° (this is the range between 4 p.m. and 8 a.m. the following morning). The following morning the contents of the beakers are made up to 800 ml. with water and boiled for 2 hr., the volume being maintained by occasional addition of water. It is best to leave the beakers overnight so that the lignins settle down, thereby facilitating filtration. Solutions are invariably cloudy and are difficult to filter. This may be attributed to large amounts of protein in these materials. Such a difficulty was also noted by Norman & Jenkins [1934] with large amounts of protein at the time of studying protein interference in lignin determination. The filtration is done under suction in Gooch crucibles with No. 42 Whatman filter paper. After drying, the precipitates are weighed and ignited and the lignins calculated on an ash-free basis.

(f) *Relationship between lignin, nitrogen and tannin in tannin materials*

When lignins and nitrogen contents of the different materials are arranged in descending order as in Table 3, the rank in respect of nitrogen and lignin coincides in 5 of the 8 examples. This is not a sufficient coincidence to establish a valid correlation but further exploration of a larger number of samples might clarify a relationship between the two if such exists. No relation is noticeable between tannin and lignin. This was unexpected because some evidence exists of relationship between lignin and phenolic residues of tannin, Norman [1933].

Table 3. *Relationship between lignin, nitrogen and tannin in tannin materials*

Material	Lignin % alcohol- $\text{H}_2\text{O}-\text{H}_2\text{SO}_4$	Lignin % $\text{H}_2\text{O}-\text{H}_2\text{SO}_4$	Nitrogen %	Tannin %
Refuse tea	26.61	28.44	3.97	18.30
<i>Pterocarpus indicus</i>	16.94	21.35	3.50	3.47
<i>Caesalpinia Bonduc</i>	23.85	27.56	3.21	4.71
Tea leaf	23.93	25.40	2.97	7.93
<i>Caesalpinia sappan</i>	17.70	25.18	2.73	1.65
<i>Terminalia Belerica</i>	23.70	27.87	2.41	14.03
<i>Caesalpinia coriaria</i>	15.50	20.59	1.99	18.23
<i>Gordonia imbricata</i>	12.42	17.19	0.78	15.31

SUMMARY

1. A number of tannin materials were examined with regard to the question of tannin interference in lignin determinations. They were classified in three major groups so as to obtain information as to whether such interference is specific to any particular group. The results indicate that tannin interference is more a question of degree than of kind although catechol tannins cause the greatest disturbance.

2. 5% H_2SO_4 pretreatment instead of removing the extraneous material actually enhances the apparent lignin.

3. 1 hr. boiling with water to remove the tannin and subsequent hydrolysis with 5% H_2SO_4 to get over pentosan and protein interference bring down the

apparent lignins within reasonable limits. Since 95 % alcohol extraction revealed one new cause of interference in the form of chlorophyll, its value as a preliminary extracting agent is indicated.

4. Vegetable tannins, caffeine and chlorophyll should be added to the list of substances which are known to interfere in lignin determination of green materials.

5. The exceptionally high values of apparent lignins for tea leaf and refuse tea with 5 % H_2SO_4 as the only pretreatment are due to a formidable grouping of phenolic and ketonic groups in the form of theotannin, caffeine and chlorophyll. Such grouping is rarely met with in other plant tissues including other tannin materials.

6. Successive extractions of any tannin material with 95 % alcohol, hot water and 5 % H_2SO_4 make the Ost & Wilkening [1910] method more adaptable to such materials. This procedure is desirable irrespective of the nature of the tannin.

7. No relationship is discernible between tannin content and lignin. A superficial connexion between nitrogen and lignin requires further and more extensive work in order to clarify the position.

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