1988. *In:* Wood, Willis A.; Kellogg, Scott T., eds. Methods in enzymology — Biomass, part b, lignin, pectin, and chitin. Sari Diego, CA: Academic Press, Inc.: 87–101. Vol. 161.

[12] Lignin Determination

By T. KENT KIRK and JOHN R. OBST

Introduction

Lignin is a natural plastic containing carbon, hydrogen, and oxygen. Composed of phenylpropane units, lignin is heterogeneous and chemically complex. It is intimately associated with, and to some extent covalently bonded to, plant cell wall hemicelluloses. Because of these structural features, lignin is difficult to measure quantitatively. The methods commonly used are imperfect, and the researcher must have a clear understanding of the plant material being analyzed and of the limitations of the analytical method being used. The structure of lignins is discussed extensively by Sarkanen and Ludwig[1] and by Adler,[2] and various lignin determination procedures are discussed in detail by Browning.[3] (As is common in lignin chemistry, we refer to lignin in the singular in the remainder of this chapter; the reader should recognize, however, that within a fundamental structural principle, lignin differs among plant species, among plant parts, and even within plant cell walls.)

The heterogeneity of lignin is the result of its formation via a random free radical coupling of three different-*p*-hydroxycinnamyl alcohols: *p*-coumaryl, coniferyl, and sinapyl alcohols (Fig. 1). Phenoxy radicals are produced in the lignifying cell by the peroxidase-catalyzed one-electron oxidation of the alcohols. The radical from each of these precursors exists in several mesomeric forms, and coupling occurs between almost all of them, leading to over 12 different interunit C–C and C-O-C linkages for each alcohol. Further heterogeneity results by coupling between radicals derived from the three different precursors. Some intermonomer linkage types

[1] K. V. Sarkanen and C. H. Ludwig (eds.), "Lignins." Wiley (Interscience), New York, 1971.
[2] E. Adler, *Wood Sci. Technol.* **11,** 169 (1977).
[3] B. L. Browning, "Methods of Wood Chemistry," Vol. II. Wiley (Interscience), New York, 1967.

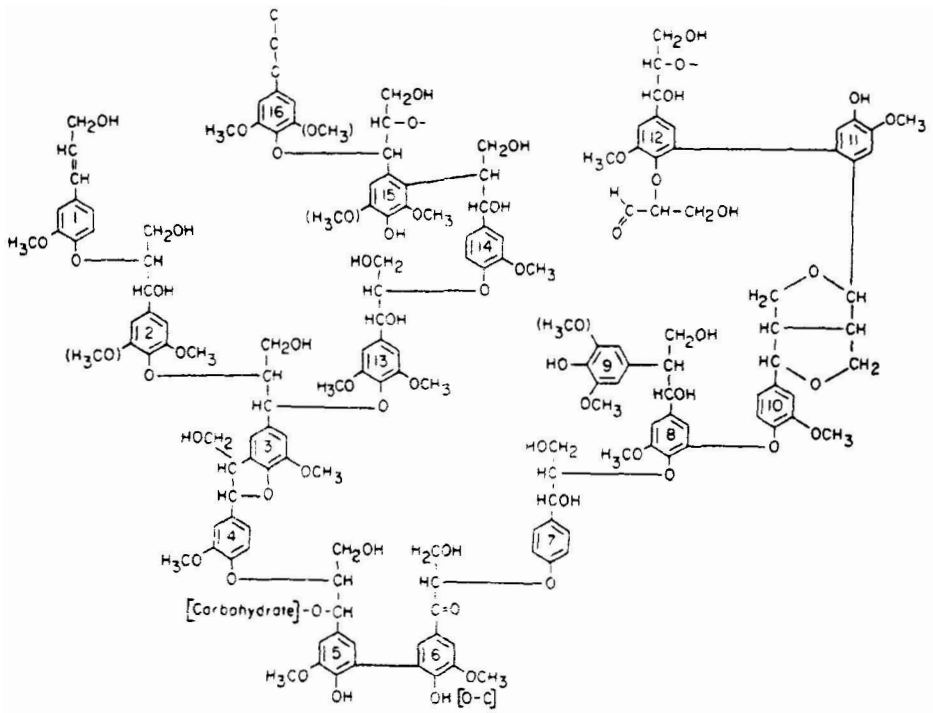


FIG. 1. Schematic structural formula for lignin. adapted from Adler.[2] *This structure is intended to illustrate the various interunit linkages. It is not a quantitatively accurate depiction of the various substructures.*

dominate (those shown in Fig. 1), and consequently, have the greatest influence on the chemical and biochemical reactivities of lignin. Angiosperm lignins typically are formed from all three of the cinnamyl alcohols, whereas most gymnosperm lignins are derived primarily from coniferyl alcohol. *p*-Coumaryl alcohol usually makes only a small contribution to lignin. Figure 1 contains units derived from the three precursors. Units derived from coniferyl alcohol are termed guaiacyl and those from sinapyl alcohol are termed syringyl; similarly, lignins are referred to as being of a guaiacyl, guaiacylsyringyl, orguaiacylsyringyl-*p*-hydroxyphenyl type.

In the following chapter, we have summarized the most widely used methods for determining lignin. Researchers should always include known lignin samples (see chapter [1] in this volume) or samples of known lignin content as controls in any procedure. The procedures that are described were developed for plant tissues, but should be adaptable to isolated lignins in reaction mixtures.

It is important to keep in mind that microbial or enzymatic modification of lignin will affect the results of qualitative and quantitative analyses.

Consequently, researchers must use caution in interpreting results where such changes have taken place.

We begin by describing some qualitative procedures.

Qualitative Determinations

Through the years, many color stains have been reported for lignin[3] The two most widely used are the Wiesner (phloroglucinol-HCl) method and the Mäule method. The basis for both color reactions has been elucidated,[4,5] and both are fairly specific for certain structures in lignin. These stains generally provide an indication that lignin is present or absent, but should not be the sole basis for conclusions.

The best methods for determining whether lignin is present in samples are based on chemical degradations to known lignin-derived products. Three such procedures are described in the following sections, and a fourth promising new method is referenced. These procedures can also be used to gain information about the structure of the lignin in the samples. Before describing these procedures, we consider sample preparation, which can of course markedly affect the outcome of analysis.

Sample Preparation

Preparation of wood samples for analysis has been described by Browning.[3] In general, plant materials are air-dried, ground to pass a 40-mesh screen, and then are dried in a vacuum oven at <50°. The investigator must decide what subsequent steps, if any, might be desirable to remove nonlignin contaminants. Wood is usually extracted with organic solvents (see chapter [1] in this volume) prior to analysis. Dried and ground herbaceous samples might be extracted with neutral and/or acid detergent (see Goering/Van Soest procedure below) or treated with protease.[3] Finally, the air-dry sample is freed of solvents in a vacuum oven at <50°.

Isolated lignin or other lignin-containing samples, too, may first be purified by appropriate procedures if desired (see chapter [1] in this volume).

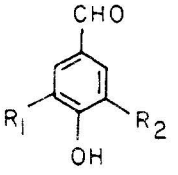
Nitrobenzene Oxidation.[6] A commonly used procedure for determining the presence of lignin is nitrobenzene oxidation. Vanillin (1) is produced from guaiacyl lignins and vanillin plus syringaldehyde (2) from guaiacyl/

[4] D. Fengel and G. Wegener, "Wood Chemistry, Ultrastructure and Reactions." de Gruyter, Berlin, Federal Republic of Germany, 1984.

[5] G. Meshitsuka and J. Nakano, *Mokuzai Gakkaishi* **25,** 588 (1979).

[6] B. Leopold, *Acta Chem. Scand.* **6,** 38 (1952).

syringyl lignins. Total yields of these two products may be as much as 25% by weight of the lignin present. From both angiosperm and gymnosperm lignin, several other products are also formed, of which *p*-hydroxybenzaldehyde (3) can sometimes be a major one. Production of vanillin (and syringaldehyde) provides a good indication that lignin is present; however, certain lignans, flavanoids, condensed tannins, and perhaps suberins can interfere by giving the same products. Compounds closely related to vanillin and synngaldehyde may also be produced from such nonlignin components and care must be taken in separating and identifying these products.



1 $R_1 = OCH_3$, $R_2 = H$

2 $R_1 = R_2 = OCH_3$

3 $R_1 = R_2 = H$

PROCEDURE. The dried and weighed sample, containing approximately 15–25 mg of lignin. is sealed, together with 0.5 ml nitrobenzene and 5 ml 2 *M* NaOH, in a stainless-steel tubing bomb (approximately 1.3 cm i.d. × 3.1 cm long). The tube is tumbled in an oil bath at 170° for 2.5 hr. After cooling, the contents of the bomb are washed with 100 ml of water into a beaker containing an internal standard for gas chromatographic (GC) analysis (approximately 5 mg of accurately weighed *o*-vanillin, 3-hydroxy-4-methoxybenzaldehyde, is convenient).

The alkaline solution is washed twice with 50-ml portions of chloroform in a separatory funnel. The aqueous phase in the funnel is then acidified with concentrated HCl to approximately pH 5 and is extracted three times with chloroform (40 ml each time). The combined chloroform extracts are dried over anhydrous magnesium sulfate or sodium sulfate, are filtered, and are evaporated at reduced pressure to approximately 4 ml.

The major products from hardwood lignins, vanillin **(1),** syringaldehyde **(2),** and a little *p*-hydroxybenzaldehyde **(3),** are conveniently determined quantitatively by GC. We have used a 60-m "DB-5" fused silica capillary column (J & W Scientific, Inc., Rancho Cordova, California). (This column gives efficient separations; less efficient columns might require a different internal standard than *o*-vanillin.)

Methylation - Oxidation.[7] Oxidation of lignin, after methylation, is another widely used procedure. It is more diagnostic for lignins than the nitrobenzene procedure, because it yields more identifiable products characteristic of lignin. The major products are veratric acid from guaiacyl units and tri-O-methylgallic acid from the syringyl units. As described below, the presence of additional products provides very strong evidence that the sample contains lignin. Samples are methylated and then are oxidized sequentially with $KMnO_4$ and H_2O_2, and the resulting substituted benzoic acids are separated and identified as their methyl esters. Pretreatment of the lignin with a pulping reagent or other depolymerizing reagent can be used to increase the yields of products.[8] The procedure is complemented by GC and GC/mass spectrometry (GC/MS).

A variation of the procedure, in which the sample is ethylated instead of methylated,[9] can provide information about microbial or enzymatic modification, namely, ring hydroxylation or demethylation of aromatic methoxyl groups.

PROCEDURE.[7] *i. Methylation.* Methylation with dimethyl sulfate is described in this section. Diazomethane methylation can also be used and has the advantage of creating essentially no residue from which the sample must be separated. (Diazomethane must be used with caution! It is explosive.) However, it is more difficult to methylate lignin quantitatively with diazomethane. For ethylation, diethyl sulfate or diazoethane can be used.[9]

For methylation with dimethyl sulfate, the weighed sample, containing 25 - 50 mg of lignin, is suspended in 10 ml of a mixture of dioxane–water (5:3) in a 50-ml round bottom flask. The suspension, heated to 65°, is maintained under a slow stream of N_2 and is stirred efficiently with a magnetic stirrer. Over 1 hr, dimethyl sulfate and 25% NaOH are added alternately and dropwise.[10] The solution is maintained above pH 10. Complete methylation is indicated by a negative test with diazotized sulfanilic acid,[11] diazotized *p*-nitroaniline (available commercially as the te-

[7] M. Erickson, S. Larsson, and G. E. Miksche, *Acta Chem. Scand.* 27, 127 and 903 (and references cited therein) (1973).

[8] The yields of products can be more than doubled by depolymerizing the lignin. One procedure for doing this is acidolysis; acidolysis is described in this chapter. but following acidolysis, the entire sample, not only solvent extractables, must be recovered.[9] Another procedure for depolymerizing lignin is kraft pulping, which is described in chapter [1] of this volume. Its use with methylation–oxidation analysis is described by Erickson *et al.*[7]

[9] T.K. Kirk and E. Adler, *Acta Chem. Scand.* **24,** 3379 (1970).

[10] An improvement in the methylation procedure[7] employs a pH stat connected to magnetic valves controlling base addition. The procedure involves a single addition of dimethyl sulfate at the outset, and it results in more reproducible methylation with use of lower volumes of reagents.

[11] B. N. Ames and H. K. Mitchell. Jr., *J. Am. Chem. Soc.* **74,** 252 (1952).

trafluoroborate salt), or other sensitive phenol reagents.[3] The sample is transferred to a centrifuge tube, is acidified to pH 4 with H_3PO_4, is diluted with an equal volume of water, and is centrifuged. The residue is washed with 1% Na_2SO_4 and again recovered by centrifugation. The supernatant and the washings are combined and extracted three times with chloroform–acetone (1 : 1, v : v); each extraction is with a volume of organic solvent equal to the volume of the aqueous solution. The chloroform–acetone solution is back-washed with a few milliliters of water and is dried over anhydrous granular Na_2SO_4. and solvents are removed by vacuum evaporation. The residue from centrifugation is combined with the extract from the aqueous phase for oxidation.

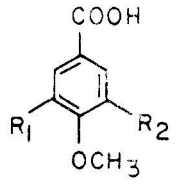
ii. Oxidation. The methylated samples are oxidized in two steps. The first is with permanganate at high pH. and the second is a milder oxidation with alkaline hydrogen peroxide to convert arylglyoxvlic acids formed in the first oxidation to the corresponding aromatic acids. In the permanganate oxidation, a minimum of permanganate is used and is regenerated with periodate; this avoids accumulation of MnO_2, which can interfere with sample oxidation and product recovery.

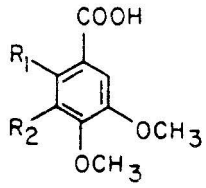
The combined methylated sample from above is dissolved/suspended in 40 ml of *tert*-butanol : water (3 : 1, v : v) in a 250-ml three-necked round bottom flask. With stirring, 40 ml of 0.5 *M* NaOH is added. Then 20 ml of 0.06 *M* $NaIO_4$ is added, followed by 20 ml of 0.03 *M* $KMnO_4$. Finally, an additional 30 ml of the periodate solution is added. *The order ofaddition is important.* The flask is heated during about 1 hr to reflux temperature, and the contents are refluxed for 5 hr. When boiling first occurs, 25 ml of additional periodate solution is added. The. oxidation is stopped by adding a few milliliters of ethanol, which causes the solution to become brown; boiling is continued for 15 min. and the hot solution is filtered through a layer of Celite The cooled filtrate is extracted twice with diethyl ether (50 ml each time). The aqueous phase is neutralized with 2 M H_2SO_4 (about 10 ml), and the solution is concentrated to about 30 ml by rotary vacuum evaporation. Solid Na_2CO_3 (0.9 g) is added, followed by 5 ml of 30% H_2O_2. The solution is heated at 50° for 10 min. Excess H_2O_2 is destroyed with permanganate solution (added dropwise until the purple color persists), and the permanganate is destroyed with a few crystals of $Na_2S_2O_5$. The solution is acidified to about pH 3 with 2 *M* H_2SO_4 and is extracted with 1 : 1 (v: v) acetone: chloroform (3 × 60 ml) and then with chloroform (30 ml). The combined extracts are back-washed with a few milliliters of saturated NaCl. The solution is dried with anhydrous Na_2SO_4 and solvents are evaporated to about 5 ml. Methanol (approximately 5 ml) is added, and the solution is methylated with excess diazomethane in ether.

Evaporation of solvents leaves a yellow oil, which is dissolved in acetone for GC.

iii. Gas chromatography and identification of products. Samples are analyzed with a nonpolar column, such as a methylated silicone elastomer, or with a capillary column. We have used 5% silicone elastomer OV- 101 (Analabs, Inc. North Haven, Connecticut) on Chromosorb G, acid-washed and treated with dimethyldichlorosilane, 80 - 100 mesh. The lignin-derived aromatic acid methyl esters are tentatively identified from their retention times by comparison with those of authentic standards. Mass spectrometry (GC/MS) is recommended to positively identify the products. The gas chromatographic procedure can be used quantitatively with a flame ionization detector by including a suitable internal standard such as pyromellitic acid tetramethyl ester.[7]

The major products from angiosperm lignins are veratric acid **(4)** and tri-O-methylgallic acid **(5);** with some samples, such as aspen wood and grasses, anisic acid **(6)** is also a major product. Products **4** and **5** are obtained in about 2–3% yield based on the lignin in the sample, and their presence provides good evidence that lignin is present. Strong evidence for lignin is provided by the identification of additional products, especially **10 –12** (numbers following structure numbers refer to approximate percentage yield from lignin): isohemipinic acid (**7,** 0.4); metahemipinic acid (**8,** 0.3); 3,4,5-trimethoxyphthalic acid (**9,** 0.2); 5,5'dehydrdveratc acid (**10,** 0.39; 3',4,5-trimethoxy-3,4'-oxydibenzoic acid (**11,** 0.2); and 3',5',4,5-tetramethoxy-3.4'-oxydibenzoic acid (**12,** 0.8). Reference standards of compounds 4 and 5 are available commercially; the others must be synthesized (see Ref. 7 and citations therein).





4 $R_1 = OCH_3$, $R_2 = H$

5 $R_1 = R_2 = OCH_3$

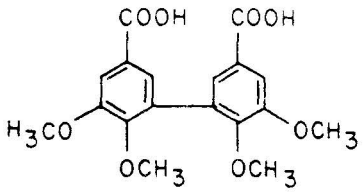
6 $R_1 = R_2 = H$

7 $R_1 = H$, $R_2 = COOH$

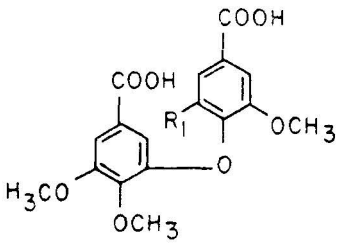
8 $R_1 = COOH$, $R_2 = H$

9 $R_1 = COOH$, $R_2 = OCH_3$

Acidolysis. Acidolysis provides a relatively easy and reliable procedure for qualitatively determining lignin. Lignin-containing samples are dehy-



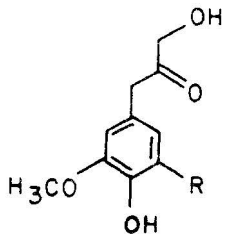
10



11 $R_1 = H$

12 $R_1 = OCH_3$

drated/hydrolyzed in hot dioxane/HCl solution, which results in formation of two ketols (among several other products) that are diagnostic of the major interunit linkage in lignin (the arylglycerol-β-aryl ether linkage, found between units 1 and 2, 2 and 3, 4 and 5, etc. in Fig. 1). These two products are 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-2-propanone (13)



13 R = H

14 $R = OCH_3$

from appropriately linked guaiacyl units and the corresponding 3-hydroxy-1-(3.5-dimethoxy-4-hydroxypheayl)-2-propanone (14) from syringyl units. Yields of these two products vary from 4 to 10% each. The compounds are readily separated and estimated by GC and are identified by MS. It is important to note that the yield of products **9** and **10** is especially sensitive to any structural modification in the units from which they are derived.

PROCEDURE.[12] *i. Acidolysis.* The sample is dissolved or suspended in purified[13] *p*-dioxane–water (9 : 1) containing 0.2 *M* HCl and is refluxed under N_2 for 4 hr. Sufficient sample is required to yield enough of products **13** and **14** for detection by GC (see below). We typically use samples

[12] K. Lundquist, *Acta Chem. Scand.* **27,** 2597 (1973), and references cited therein.
[13] A. I. Vogel. "Practical Organic Chemistry," 3rd Ed. Wiley, New York, 1966.

containing approximately 25 mg of lignin, but much less can be used. A few milliliters of acidolysis reagent are adequate; the amount must only be sufficient to supply HCl in excess. The cooled mixture is diluted with 0.4 *M* $NaHCO_3$ to raise the pH to approximately pH 3 and then is extracted three times with chloroform–acetone(1 : 1, v: v), each time with a volume equal to the volume of the solution. The extract is washed with a small volume of water and is dried over anhydrous Na_2SO_4, and solvents are removed by rotary vacuum evaporation to give a brown-yellow oil. Ketols **13** and **14** are separated—and are quantified if desired—as their trimethylsilyl derivatives by GC. For quantitation, we have used methyl arachidonate as the internal standard.

ii. Derivatization and analysis. A sample of the oil from above is dissolved in 50–100 µl of dioxane containing 10% pyridine, 50 µl of *N,O*-bis(trimethylsily1)acetamide [or *N,O*-bis(trifluoromethylsilyl)acetamide] is added, and the container is heated briefly (50–60°) and is stoppered. Samples are then analyzed within 3–4 hr using the same gas chromatographic conditions as used for the methyl esters of products **4** - **12** above. A precolumn filter of fine glass wool is desirable to trap nonvolatiles.

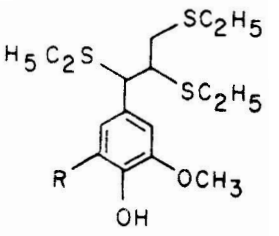
Reference ketols **9** and **10** are not available commercially, but can be synthesized.[12] However, as an alternative, it is suggested that preextracted birch sapwood (see Sample Preparation above for *Methylation - Oxidation)* be used as a reference sample: the major monomeric acidolysis products from birch lignin are ketols **9** and **10;** their identity can be confirmed by GC/MS. Note that both ketols form the di- trimethylsilyl derivatives under the conditions used here, but on prolonged reaction with the trimethylsilylating reagent they can form the tri-TMS derivatives. [14]

Two alternative methods have been described for analyzing the acidolysis products: GC or GC/MS, following reduction - acetylation,[15] and HPLC of the nonderivatized products.[16]

Thioacidolysis.[17] This procedure employs dioxane - ethanethiol, 9 : 1, with 0.2 *M* BF_3 etherate to degrade and derivatize uncondensed arylglycerol-*p*-aryl ether structures (such as units 2 and 7 in Fig. 1), with formation of 1-(4-hydroxy-3-methoxyphenyl)-1,2,3-(tristhioethyl)propane **(15)** from guaiacyl units and the corresponding compound **(16)** from syringyl units. These products are separated and quantitated by HPLC or, as TMS derivatives, by GC. Both analytical methods resolve the threo and erythro

[14] K. Lundquist and T. K. Kirk, *Acta Chem. Scand.* **25,** 889 (1971).
[15] P. Kristersson, K. Lundquist, and A. Strand, *Wood Sci. Technol.* **14,** 297 (1980).
[16] C. Lapierre, C. Roland,:, and B. Monties, *Holzforschung* **37,** 189 (1 983).
[17] C. Lapierre, B. Monties, and C. Rolando, *J. Wood Chem. Technol.* **5,** 277 (1985); C. Lapierre, B. Monties, and C. Rolando, Holzforschung **40,** 47 (1986).

stereoisomers of **15** and **16.** Like acidolysis, thioacidolysis is diagnostic for lignin (with the same slight reservations) and reportedly has the advantages of giving higher yields and fewer products.[17] It has the disadvantage of yielding sugar and other derivatives that must be separated from **15** and **16**



15 R=H

16 R=OCH3

when plant tissues rather than isolated lignins are analyzed. Such separation, however, is apparently accomplished by the procedures already described[17] (C. Lapierre and B. Monties, personal communication).

Quantitative Procedures

Both chemical and physical methods have been described for quantitatively determining lignin. The chemical methods are the best, but as we pointed out initially, they must be used with a full knowledge of the substrates being analyzed and of the limitations of the procedure being used. For measuring the products formed from lignin in enzymatic or microbial degradation, ^{14}C-labeled lignin is often the preferred substrate (see chapters [3] and [8] in this volume).

Chemical Methods

Klason or 72% H_2SO_4 Method. The most widely used method for lignin determination by wood chemists is probably the simplest and overall the most reliable, despite its limitations. Samples are digested with 72% sulfuric acid, then with dilute sulfuric acid, to hydrolyze and solubilize the polysaccharides; the insoluble residue is dried and weighed as lignin. The major disadvantages of the procedure are as follows: (1) other components, including proteins and suberins, may condense and analyze as Klason lignin and (2) some lignins, notably those rich in syringyl residues (angiosperm tissues), are partially solubilized. (Acid-soluble lignin from angiosperms can be estimated from the UV absorbance of the hydrolysate.[3]) The

method gives good reproducibility with both gymnosperm and angiosperm tissues.

PROCEDURE.[13,18] The sample should be ground to pass at least a 20-mesh screen and should be freed of extractive components and of solvents before analysis (see description of Sample Preparation under Qualitative Determinations above and also that under the *Goering–Van Soest Method* below).

Approximately 200 mg of sample is accurately weighed to the nearest 0.1 mg into a shell vial or small beaker. One milliliter of 72% H_2SO_4 (conveniently determined by specific gravity) is added for each 100 mg of sample. The mixture is placed in a water bath at 30 ± 0.5° and is stirred frequently to assure complete solution. After exactly 1 hr, it is diluted and transferred quantitatively to a 125-ml Erlenmeyer flask, using 28 ml of water for each 1 ml of acid. Secondary hydrolysis is in an autoclave at 120° for 1 hr. The hot solution is filtered through a tared Gooch, alundum, or fritted glass crucible, and the Klason lignin residue is washed with hot water to remove the acid. The crucibles containing the samples are then dried to constant weight at 105° and are weighed to the nearest 0.1 mg. Lignin is expressed as a percentage of the original sample. The lignin can be ashed, and the lignin content can be corrected for acid-insoluble inorganics if these are suspected or known to be present. Also, an indication of the punty of the lignin can be obtained by having it analyzed for methoxyl content. Methoxyl contents of Klason lignin from a variety of plant tissues are given by Brauns.[19] Klason lignin contents of many tissues are given by Brauns[19] and also by Pettersen.[20]

The filtrate from the Klason analysis may be used for a total carbohydrate determination. After dilution to a suitable volume, a portion is neutralized with $CaCO_3$ to the methyl orange end point and total sugar content is determined.[21]

Goering–Van Soest Method. This method is also widely used, especially for analysis of cereals, forages, and feed ingredients which often have high contents of protein and other nonlignocellulosic components. In this method, samples are pretreated with acid detergent to remove protein, hemicelluloses, and other components from the cellulose and lignin, and the lignin is determined by the Klason method described above or by

[18] M. J. Effland, *Tappi* **60,** 143 (1977).

[19] F. E. Brauns, "The Chemistry of Lignin." Academic Press, New York, 1352; F. E. Brauns and D. A. Brauns, "The Chemistry of Lignin," Suppl. Vol. Academic Press, New York, 1960.

[20] R. C. Pettersen, in "The Chemistry of Solid Wood'' (R. M. Rowell, ed.), p. 57. Am. Chem. Soc. Press, Washington, D.C 1984

[21] R. C. Pettersen. V. H. Schwandt, and M. J. Effland, *J. Chromaogr. Sci.* **22,** 478 (1984).

difference after removing it from the cellulose via permanganate oxidation - solubilization. In using the latter procedure, care must be taken to assure sample penetration by the permanganate. It is likely, too, that some lignin might be removed in the pretreatments. Nonlignin components, such as condensed tannins and suberin, can analyze as lignin, although correction for the latter can be made, as described below.

PROCEDURE.[22] *i. Acid–detergent fiber preparation.* The sample of known weight (0.3 - 1 g dry weight basis) and moisture content, ground to pass a 40-mesh screen, is placed in a beaker fitted with an apparatus suitable for refluxing." Acid–detergent solution (100 ml) is added at room temperature, and the mixture is heated to boiling in 5 – 10 min. [Acid–detergent solution is prepared by dissolving 20 g of cetyl trimethylammonium bromide (CTAB, technical grade) in 1 liter of 0.5 *M* H_2SO_4.] Heat is reduced after boiling begins to avoid foaming, and the sample is refluxed 1 hr. The sample is then filtered in a tared, 50-ml coarse porosity Gooch crucible and is washed thoroughly with hot (90 – 100°) water. The sample is then repeatedly washed with acetone until no more color is removed. Acetone is allowed to evaporate, and the sample is then freed of solvents in a vacuum oven or is dried overnight at 100 – 105° and is weighed.

ii. Lignin determination. Lignin can be determined on the above sample by the Klason method described above, with a correction for acid-insoluble ash. If cutin is present, it will analyze as lignin; the following alternative lignin determination method can be extended to estimate and correct for cutin. (Note: We have found that the Klason method underestimates the value of lignin in hardwood samples that have been acid–detergent treated, suggesting that the following method might be more accurate.)

This second method for lignin determination involves removing the lignin by its selective chemical oxidation and extraction. The crucible containing the dry acid–detergent fiber is placed in a shallow pan containing cold water to a depth of about 1 cm; fibers should not be wet. About 25 ml of a 2 : 1 (v : v) mixture of saturated potassium permanganate and lignin buffer solution is added, and the level of water in the pan is increased to reduce flow of the solution out of the crucibles. [Saturated $KMnO_4$ is prepared by dissolving 50 g of $KMnO_4$ and 0.05 g of Ag_2SO_4 in 1 liter of distilled water. The lignin buffer is prepared by dissolving 6 g of $Fe(NO_3)_3 \cdot 9H_2O$ and 0.15 g of $AgNO_3$ in 100 ml of distilled water, combining with 500 ml of acetic acid and 5 g of potassium acetate, and adding 400 ml of *tert*-butanol.] The sample is maintained at 20–25° for 90 ± 10 min; more mixed permanganate solution is added if needed to keep the sample wet, and a glass rod is used to assure good mixing.

[22] H. K. Goering and P. J. Van Sorst, "Foiage fiber Analysis," Agric. Handb. No. 379. ARS/U.S. Dept. Agirc., Govt Printing Office, Washington, D.C. 1970.

The treating solution is removed from the sample by suction filtration. and the crucibles are placed in a clean pan and are filled about half full with demineralizing solution. Care must be taken to avoid foaming. (Demineralizing solution is made by dissolving 50 g of oxalic acid dihydrate in 700 ml of 95% ethanol, adding 50 mi of concentrated HCl and 250 ml of distilled water. and mixing.) After about 5 - 15 min, the demineralizing solution is removed by filtration, fresh demineralizing solution is added. and the process is repeated until the sample is white. Care must be taken to ensure that all mineral deposits on the glass crucible are treated with demineralizing solution. The sample is washed thoroughly in the crucible with 80% ethanol and then with acetone. Finally, it is dried at 100° and is weighed. The weighed residue is cellulose plus cutin (and ash).

A correction for ash can be made as described above for Mason lignin. If cutin is suspected to be present, a correction for it can be made by treating the sample with the Klason procedure described above to dissolve the cellulose. The Klason "lignin" thus determined estimates cutin. With or without this step, the residue is corrected for ash.

Other Methods. A number of other procedures have been described for quantitatively estimating lignin. Some of the most useful are briefly described in the following sections.

KAPPA METHOD.[23] A method related to the above permanganate procedure is widely used to determine lignin in chemical pulps and might be useful in certain biochemical studies. The procedure involves oxidizing the lignin in a measured amount of pulp with a known quantity of standard permanganate under acidic conditions. The unused permanganate is then determined by titration with sodium thiosulfate solution, and the lignin content (kappa number) is calculated. The method is simple and provides reproducible results.

THIOGLYCOLIC ACID METHOD.[3] This procedure has not been as widely used as the above procedures, but might also have some advantages in certain studies. The procedure involves acid-catalyzed derivatization of lignin with thioglycolic acid to produce base-soluble, acid-insoluble lignin thioglycolate. The method has the advantage over the Klason method that the isolated lignin is much less modified (aside from being derivatized), is soluble, and can be analyzed more readily. Interference by nonlignin components is apparently minimal, but methoxyl and other analyses of the isolated lignin thiogIycolates should be used to verify purity. Correction for the thioglycolate moieties is baed on a sulfur analysis.

CHLORINE CONSUMPTION METHOD.[3] Lignin reacts readily with chlorine, so that consumption of chlorine by a sample can form the basis for an

[23] Technical Association of the Pulp and Paper Industry, "Kappa Number of Pulp," TAPPI Official Standard OS-76. TAPPI, Atlanta, Georgia, 1976.

estimation of lignin. The method has been especially used in measuring lignin in pulps. but has also been used to measure residual isolated lignin in microbial degradation studies.[24]

METHODS BASED ON CHEMICAL DEGRADATION PRODUCTS. Browning[3] has described a procedure for estimating lignin in samples by quantifying the yield of vanillin or vanillin plus syringaldehyde, following nitrobenzene oxidation (described above as a qualitative procedure). Similarly, the methylation - oxidation procedure can be adapted to lignin determination by using special methylation apparatus[10] and precautions in the methylation and the oxidation procedures.[7] It is possible, too, that the new thioacidolysis method. described above as a qualitative method, can be adapted for quantitative analysis.

Physical Methods

Physical methods for estimating lignin in samples have not yet been developed into generally useful, reliable methods. A method for estimating lignin by ultraviolet (UV) spectroscopy following solubilization by acetylation and dissolution in acetic acid has been reported.[25] The method suffers from two notable disadvantage: (1) it is of course very sensitive to changes in the molar extinction coefficients of the UV chromophores of lignin and (2) it is not specific for lignin [any nonlignin aromatic (or other UV-absorbing) component in the sample will interfere].

Near-infrared (NIR) spectroscopic procedures are being developed for the prediction of digestibility and the estimation of composition of plant materials. At this time, however, these empirical methods have only limited utility for quantifying lignin for research purposes.

A promising technique is ^{13}C NMR. This spectroscopic technique is now widely employed for the study of lignin. The >40 signals in the spectra of milled wood lignins from gymnosperm and angiosperm tissues have been assigned.[26] These assignments are useful in qualitatively identifying an isolated material as lignin 2nd allow classification of the lignin as a guaiacyl-, guaiacylsyringyl, or guaiacylsyringyl-*p*-hydroxyphenyl type.

Research on quantitative ^{13}C WMR of isoiated, soluble lignins is now coming to fruition. Data acquisition and processing parameters have been determined which enable precise and accurate quantitative measurements within the lignin spectrum.[27] Solid-state, cross polarization-magic angle spinning ^{13}C NMR has the potential to yield quantitative measurements of

[24] T. Hiroi and K.-E. Eriksson, *Sven. Paperstidn.* **79,** 157 (1976).
[25] D. B. Johnson, W. E. Moore, and L. C. Zank, *Tappi* **44,** 793 (1961).
[26] H. H. Nimz, D. Robert. O. Faix, and M. Nems, *Holzforschung* **35,** 16 (198 1).
[27] J. R. Obst and L. L. Landucci. *Holzforschung,* **40,** 87 (Supplement) (1986).

the lignin content of lignocellulosic materials.[28] The resolution of lignin peaks in solid-state ^{13}C NMR is usually inferior to that obtained with lignin solutions. However, advances continue, and the technique has the potential to become a highly sensitive research tool in studies of lignin biochemistry.

[28] J. F. Haw, G. E. Maciel, and W. A. Schroeder, *Anal Chem.* **56,** 1323 (1984).