JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Microanalytical Method for the Characterization of Fiber Components and Morphology of Woody Plants

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Microanalytical techniques were developed which allow the rapid characterization of fiber components and morphology of loblolly pine in a large number of samples. These techniques consist of extractives removal, holocellulose preparation, α-cellulose and lignin content determination, and fiber length and coarseness analyses. Greater than 95% of the nonvolatile extractives from an increment core sample of loblolly pine was removed by four successive two-day acetone extractions. Fiber morphology and α-cellulose content was determined from holocellulose prepared from only 100 mg of wood. Similarly, a microanalytical acetyl bromide method was developed that enabled the accurate determination of lignin content from less than 50 mg of wood. Through the development of these microanlytical methods, it is possible to accurately and rapidly analyze fiber morphology and chemical components in a large number of increment core samples.

KEYWORDS: Loblolly pine; extraction; holocellulose preparation; α-cellulose preparation; fiber length and coarseness; acetyl bromide method; lignin; softwood

INTRODUCTION

The Southeast U.S. is the largest supplier of industrial wood to the world, providing about 15% of the world's industrial roundwood (*1*) and almost 60% of the U.S. harvest (*2*, *3*). The region is gradually losing its global competitiveness as a result of decreasing harvest from the natural forests and increasing competition from the Southern Hemisphere. *In order for the Southeast U.S. to meet future demands while maintaining global competitiveness, more wood with targeted characteristics has to be produced more efficiently on less land*. One viable solution to meet the future industrial wood demands is to greatly increase the productivity of the current pine plantations, leaving natural forests to be managed at low intensity, primarily for sawtimber, conservation, aesthetics, and recreational ends. The productivity of the current pine plantations, which occupy less than 20% of the total forest area, must be increased two- to three-fold. This can be done through intensively managed plantation forests of genetically superior trees resulting from intensive research efforts involving biotechnology, tree breeding, and silvicultural practices.

The use of intensively managed pine plantation forests as the major source of softwood pulpwood would result in a great increase in the proportion of juvenile wood utilized by the pulp mill. It is well documented that the properties of juvenile wood are quite different from those of mature wood (*4*). Compared with mature wood, juvenile wood of loblolly pine has higher lignin content, shorter average fiber length, smaller cell-wall diameter, a thinner cell wall, higher compression wood content, higher microfibril angle, and lower specific gravity. The thinner cell wall and smaller cell-wall diameter contribute to lower refining energy requirements and better fiber bonding, thus better tensile and burst strength and better printability, making juvenile wood an ideal raw material for specific grades such as printing and writing paper, mechanical pulp, and linerboard. However, the higher lignin content, lower cellulose content, and lower specific gravity decrease the pulp yield to the extent that it significantly impacts pulp production costs.

During the past two decades there has been a gradual but steady increase in juvenile wood content in the raw material furnish of the pulp mill. The trend is expected to continue through the next decade. Given the impact of fiber components and morphology of juvenile wood on processing and end product properties, it is surprising that no systematic study has been carried out to assess the natural variations of these traits in juvenile wood, let alone taking advantage of recent advances in molecular biology and biotechnology. The promise of biotechnology lies in the potential to make major genetic changes through genetic selection or directed modification in a short period of time. To accomplish this, analytical techniques to identify differences in fiber components and morphology need to be developed, specifically microanalytical techniques that enable rapid and accurate characterization of thousands of samples using milligrams of material. Although several spectroscopic methods, e.g., FT-Raman, near-IR, etc., exist which permit rapid structural characterization of plant materials, these techniques are not fully developed, and more importantly require time-consuming wet chemistry to establish calibration sets and confirm their accuracy and precision. In this paper we describe the successive procedure developed for the easy and rapid

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10.1021/jf011173q CCC: $22.00
Published on Web 02/02/2002

analysis of fiber morphology and major chemical components of softwoods. The microanalytical methods developed include extractives removal, holocellulose preparation, α-cellulose and lignin content determination, and fiber length and coarseness analyses.

MATERIALS AND METHODS

Materials. Wood samples were from an 11−12-year old (juvenile) and a 33-year old (mature) loblolly pine. Milled wood lignin (MWL) was prepared from the 33-year old pine using the method of Björkman (*5*). The juvenile pine was received as 12-mm increment cores, whereas the mature pine was a 30-cm bolt from the North Carolina State University Department of Forestry tree breeding program.

Acetone (ACS grade), sodium chlorite (80%, tech grade), acetic acid (ACS grade), sodium hydroxide (ACS grade), acetyl bromide (99%), perchloric acid (70%, ACS grade), and sulfuric acid (ACS grade) were purchase from Aldrich Chemical Co. and used as received.

Microanalytical Methods Developed. *Removal of Extractives.* The 12-mm increment cores taken in the field were immediately cut at the pith into two sections, debarked, and placed in a capped test tube (50 mL) in acetone. The samples were then transported to the lab where, after 2 days at room temperature, the acetone was drained and replaced. The extraction procedure was repeated at 48-h intervals for a total of six times. After each extraction, the acetone was concentrated under reduced pressure, and the amount of nonvolatile extractives was determined gravimetrically.

Preparation of Wood Samples. Wood meal samples of both the juvenile pine and mature pine wood was prepared using a Wiley mill, and ground to pass various mesh screens. To minimize the modification of the wood fibers arising from the milling procedure, thin wafers from both the spring and summer wood portions of the 3-year-old and 8-year-old rings of juvenile pine were prepared by cutting these portions parallel to the fiber using a sharp knife or microtone. These samples represent the juvenile and juvenile-mature wood transition zone, respectively. The thin wafers were then used to make holocellulose and to determine lignin content (discussed below).

Preparation of Holocellulose. Holocellulose was isolated from the extractive-free wood wafers and wood meals prepared as described above. Approximately 100 mg (OD) of wood was suspended in 2 mL of deionized water in a round-bottom flask (10 mL) with a ground glass stopper. The reaction flask was then submerged in a water bath maintained at 90 °C. The reaction was initiated by adding 0.5 mL of sodium chlorite solution which had been prepared by dissolving 200 mg of 80% sodium chlorite into 2 mL of deionized water and 0.2 mL of acetic acid. At 30-min intervals 0.5 mL of sodium chlorite/acetic acid solution was added to the reaction, for a total of 2 mL. At the end of 2 h (total of 4 additions), the reaction was cooled in a cold water bath, and filtered using a sintered glass filter (coarse). The resulting holocellulose was thoroughly washed with deionized water (3 × 50 mL), dried in an oven at 105 °C, and the yield of holocellulose was determined.

To simplify the reaction protocol and enable more sample analyses to be performed, the reaction can be simplified to a single addition of the sodium chlorite/acetic acid solution. Specifically, the 100 mg (OD) of wood is suspended in 4 mL of deionized water at 90 °C, and reacted with 200 mg of 80% sodium chlorite and 0.8 mL of acetic acid for 1 h. The reaction is then filtered, washed, and dried in the same manner as outlined above.

Determination of α-Cellulose Content. A 50-mg portion of the oven-dried holocellulose was weighed into a 10-mL beaker and left to stand at room temperature for 30 min to allow moisture equilibration, at which time 4 mL of 17.5% sodium hydroxide was added to the beaker and left reacting an additional 30 min at ambient temperature. Deionized water (4 mL) was then added, and the mixture was stirred for one minute with a glass stir rod and left for another 29 min. After a total reaction time of 60 min, the fiber suspension was filtered using a sintered glass filter (coarse), washed thoroughly with deionized water (3 × 30 mL), and soaked in 1.0 M acetic acid solution for 5 min. The neutralized α-cellulose was then thoroughly washed with deionized water (3 × 30 mL), and the yield was determined after the sample was oven-dried at 105 °C.

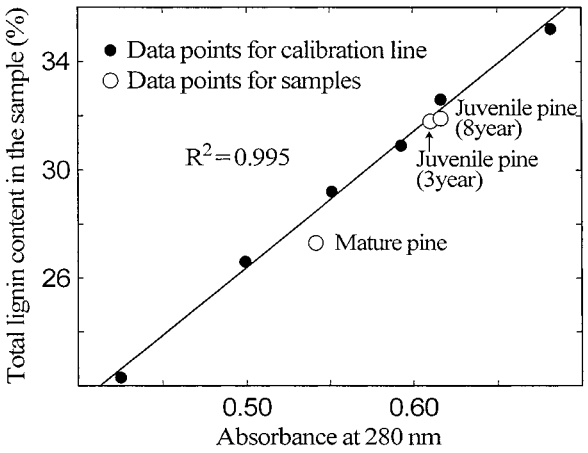

Figure 1. Calibration line used for the determination of lignin content in juvenile pine and data points for actual samples. The calibration line was prepared from known amount of holocellulose and MWL from mature pine. R^2 for the calibration line is 0.995. ●, Data points for calibration line (data points obtained from the mixtures); ○, data points for samples J3, juvenile pine 3-year-old rings; J8, juvenile pine 8-year-old rings; and M, mature pine.

Determination of Fiber Length and Coarseness. Fiber length and coarseness was determined on the isolated holocellulose using a fiber quality analyzer (FQA, Op Test Equipment, Inc., Hawkesbury, ON) and Kajaani FS-200 (Valmet Automation, Inc., Norcross, GA). For FQA analysis, exactly 10−16 mg of the oven-dried holocellulose (it is critical to record the exact weight) was suspended in 20 mL of deionized water and defibrated for 5 min. The liberated fibers were then quantitatively transferred into a 400-mL beaker using 50−100 mL of deionized water. The fiber suspension was diluted with an appropriate volume of water so that a total of 200 mL of deionized water had been added. The suspension was vigorously stirred, and 10 mL of the suspension was extracted using a glass pipet and subjected to fiber length and coarseness analysis. For the Kajaani fiber analysis, 3−4 mg of fibers is required (versus the 0.5−0.8 mg for FQA), therefore 60−80 mg of wood is required.

Determination of Lignin Content. The lignin content of the various wood samples was determined using a modified acetyl bromide method. Accordingly, 10 mL of freshly prepared 25% (w/w) acetyl bromide/acetic acid solution was added to a 20-mg (OD) wood wafer or wood meal sample in a 25-mL Erlenmeyer flask with a ground glass stopper. This was immediately followed by the addition of 4 mL of 70% (w/w) perchloric acid solution. The flask was stoppered and sealed with Teflon tape, quickly shaken, and placed into a water bath maintained at 70 °C. The reaction was left for 30 min, with intermittent shaking at 10 min intervals. At the end of 30 min, the reaction was stopped by cooling the flask in cold water. The reaction mixture was then quantitatively transferred, using acetic acid, to a volumetric flask (250 mL) containing 10 mL of 4.0 M sodium hydroxide and 50 mL of acetic acid. The volume was made up to 250 mL with acetic acid, and the UV absorbance at 280 nm of this acetic acid solution was determined. NOTE: It is extremely important that the UV analysis be recorded within 10 min after the beginning of the dilution.

The determination of lignin content was conducted using a calibration line made by subjecting known amounts of holocellulose and MWL (both from mature pine) to the acetyl bromide method described above. As a result, the gram extinction coefficient of lignin treated by acetyl bromide is not needed. The calibration line is shown in **Figure 1**. The calibration line shows the correlation between the total lignin content in the mixtures and the UV absorbance at 280 nm obtained by subjecting the mixtures to this microacetyl bromide method. The total lignin content in the mixtures is calculated from the MWL content using the values obtained by the Klason lignin determination (*6*): 5.1% for holocellulose prepared from mature pine (0.9% for Klason lignin (KL),

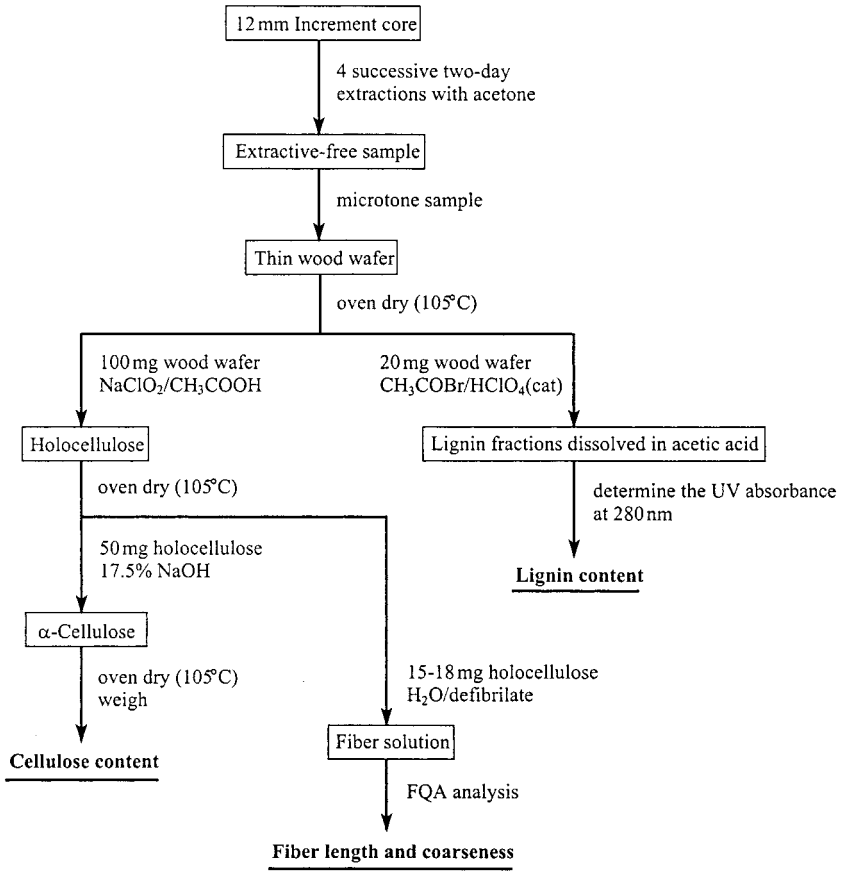

Figure 2. Flowchart of microanalytical method developed.

4.2% for acid soluble lignin (ASL)) and 91.0% for MWL prepared from mature pine (KL 89.9%, ASL 1.1%).

Microanalytical Procedure. Using the microanalytical techniques developed above, a successive procedure for the analysis of fiber components and morphology of softwoods has been established (**Figure 2**). Using this method, fiber morphology and chemical components of increment cores are easily and rapidly analyzed.

RESULTS AND DISCUSSION

Removal of Extractives. To analyze the main chemical components in our wood samples, the extractives had to be removed. Furthermore, as our goal was to survey an enormous number of wood samples, a simple extraction procedure needed to be developed. The conventional extraction procedure, using a Soxhlet apparatus, is extremely tedious and likely not necessary for juvenile wood. In addition, considering the hazards associated with the traditional extraction solvents used (ethanol and benzene), a process modification was warranted. For these reasons, we tried to develop an effective and convenient method for the removal of extractives from increment core wood samples.

The results obtained from repeated acetone extraction are shown in **Table 1**. A total of 1270 mg of nonvolatile extractives was obtained from a single increment core sample. The oven-dried weight of the increment core sample after the extractions was 26.4 g, indicating that the yield of the nonvolatile extractives based on extractive-containing wood was 4.7%. A significant observation was made that four successive two-day soakings with fresh acetone can remove over 95% of the nonvolatile extractives in the increment core sample, and has been adopted as the new method. Moreover, this procedure can be initiated in the field as the increment core samples are being collected, greatly reducing the time required.

Table 1. Amount of Non-Volatile Extractives Obtained by Successive Two-Day Soakings with Acetone

no. of successive two-day extractions	amount of nonvolatile extractives (mg)
1	0802
2	0334
3	0052
4	0021
5	0031
6	0030
total	1270

Table 2. Comparison of Acetone Extraction of 12-mm Increment Wood Cores with Conventional Ethanol/Benzene (1:2) Extraction of Wood Meal

	12-mm increment wood core	wood meal	
sample	acetone extraction (4 × 2 days)	ethanol/benzene (96 h)	ethanol/benzene (24 h)
1	298 mg	305 mg	
2	458 mg		390 mg

Table 2 illustrates the results obtained for both an acetone and an ethanol/benzene (1:2) extraction of two increment wood cores. Included are the results from a conventional ethanol/benzene (1:2) Soxhlet extraction of a corresponding wood meal. No difference was observed in the amount of extractives removed using either the acetone extraction procedure or the conventional ethanol/benzene (1:2) Soxhlet procedure.

Preparation of Holocellulose. To determine α-cellulose content, fiber length, and fiber coarseness, holocellulose has to

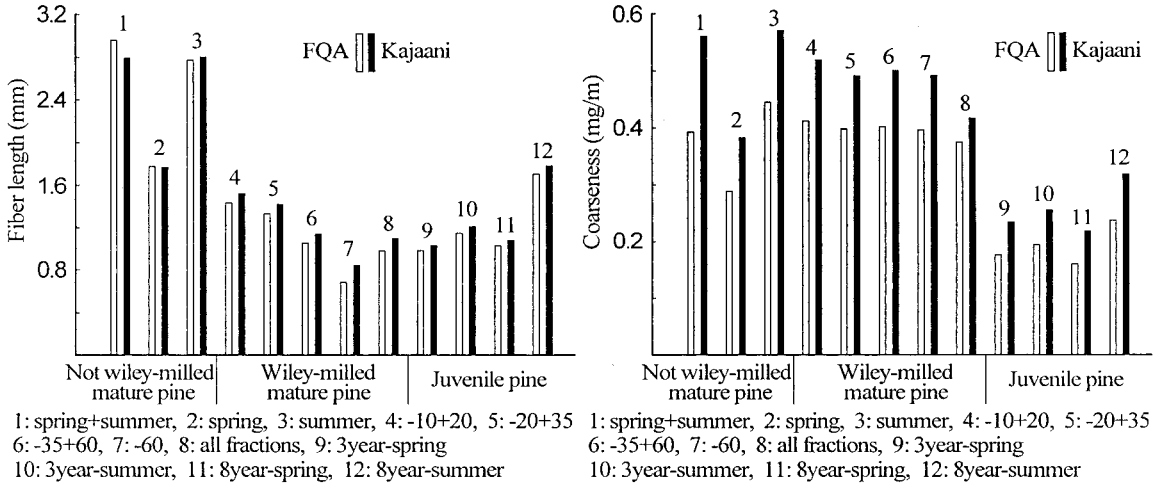

Figure 3. Weight-weighted average of fiber length and fiber coarseness of mature and juvenile pines observed by FQA and Kajaani FS-200.

Table 3. Effect of Kneading in the Early Stage of the Preparation of α-Cellulose on Its Yield

	yield of α-cellulose based on holocellulose	
sample no.[a]	with kneading	without kneading
1	63.2	62.9
2	63.5	64.4
3	64.7	63.9
4	64.3	63.4
average	63.9 ± 0.7	63.7 ± 0.6

[a] Four samples of juvenile pine were used.

be prepared. Traditionally, the preparation of holocellulose requires 5 g of wood meal (*7*). As obtaining 5 g of wood from any ring in a 12-mm increment core is not possible, a micro technique had to be established.

We first ran the conventional holocellulose procedure using 100 mg (OD) of wood sample and the appropriate chemical additions: 3.2 mL of water; 37.5 mg of 80% sodium chlorite; and 10 μL of acetic acid. Analogous to the original method, the sodium chlorite and acetic acid were added 4 times at 60-min intervals and the temperature was maintained at 70−80 °C. The yield of holocellulose obtained was 82.2 ± 1.2%. These results indicate that the holocellulose contains a considerable amount of lignin (5−10% based on mature L. pine), which could possibly affect the following α-cellulose content, fiber length, and fiber coarseness analyses.

In the preparation of holocellulose yields of approximately 70−74% are expected (*7*). Therefore, we adjusted the reaction conditions of time, temperature, and chemical addition to obtain holocellulose yields of 70−74%. Using the protocol outlined above, satisfactory holocellulose yields of 73.5 ± 0.3% and 71.0 ± 0.6% can be obtained using the multiple and single chemical applications, respectively. The holocellulose yield of the single addition microanalytical method is slightly lower than that of the multiple addition method and is likely not an accurate determination of holocellulose content. However, it has the advantage for α-cellulose, fiber length, and fiber coarseness determinations in that a large number of samples can be easily analyzed.

Determination of α-Cellulose Content. In the traditional method for the preparation of α-cellulose, holocellulose is kneaded in the early stage of the treatment with 17.5% sodium hydroxide solution. However, the omission of such a laborious step would greatly increase the ability to perform high-throughput analyses. Therefore, the effect of kneading on the yield of α-cellulose was examined, and the results are shown in **Table 3**. As can be seen, kneading has almost no effect on the yield of α-cellulose. Thus, this process can be eliminated from the protocol.

The α-cellulose content of the thin wood wafers of juvenile pine and the wood meal of the mature pine were 42.4 ± 0.4% and 46.2 ± 0.4%, respectively. These values are in good agreement with reported values for Loblolly pine (*4*).

Determination of Fiber Length and Coarseness. Fiber length and coarseness are very important factors impacting pulp production cost and quality. Results of FQA and Kajaani FS-200 analyses of the holocellulose isolated from the various wood preparations outlined above are shown in **Figure 3** and **Table 4**.

An important factor in the determination of fiber length and coarseness is the particle size of the sample being measured, which is dependent on the techniques used for its isolation. Therefore, we conducted experiments to find the minimum

Table 4. Weight-Weighted Average of Fiber Length and Fiber Coarseness of Mature and Juvenile Pines Observed by FQA and Kajaani FS-200[a]

	fiber length (mm)		fiber coarseness (mg/m)	
	FQA	Kajaani	FQA	Kajaani
mature pine A	2.995 ± 0.082	2.75 ± 0.06	0.374 ± 0.013	0.654 ± 0.075
mature pine B	2.882 ± 0.086	2.74 ± 0.10	0.455 ± 0.023	0.542 ± 0.001
mature pine C	3.005 ± 0.191	2.88 ± 0.07	0.350 ± 0.015	0.488 ± 0.028
juvenile pine 3-year spring	0.980 ± 0.033	1.03 ± 0.02	0.176 ± 0.002	0.235 ± 0.007
juvenile pine 3-year summer	1.148 ± 0.043	1.21 ± 0.02	0.194 ± 0.001	0.256 ± 0.006
juvenile pine 8-year spring	1.028 ± 0.010	1.08 ± 0.02	0.160 ± 0.009	0.218 ± 0.009
juvenile pine 8-year summer	1.705 ± 0.044	1.78 ± 0.01	0.238 ± 0.006	0.319 ± 0.002

[a] Every cell shows the average of three time analyses and the standard deviation.

particle size in which fiber length is not adversely affected. The holocellulose samples prepared from the mature pine wood meal were fractionated by particle size into 6 fractions: not-wiley-milled; wiley-milled −10+20 mesh (pass 10- and retained by 20-mesh screen); −20+35 mesh; −35+60 mesh; −60 mesh; and unfractionated wiley-milled. Holocellulose samples prepared from the thin wafers of the juvenile pine from the 3- and 8-year-old rings were also used. In addition, the not-wiley-milled mature and juvenile pine wafers were divided into spring and summer wood before preparing holocellulose. **Figure 3** shows the average fiber length and coarseness values obtained from FQA and Kajaani FS-200 analyses of the various wood preparations.

The results indicate that particle size of the sample significantly affects their fiber lengths. The larger the particle size becomes, the longer the fiber length. The fiber length of the fraction −10+20 mesh, the largest particle size examined, is even shorter than that of the not-wiley-milled sample. FQA tends to give a little shorter fiber length and a clearly lower coarseness than Kajaani FS-200 for the exactly identical sample.

Table 4 shows the values obtained for three mature pine wood meal samples (not-Wiley-milled) and juvenile pine thin wafer samples for spring and summer wood from years 3 and 8. There is little difference in the fiber length and coarseness between the spring and summer wood in the juvenile pine of the 3-year old ring. However, in the mature pine and the juvenile pine of the 8-year old ring (the transition from juvenile to mature) the fiber length and coarseness values of the summer wood are larger than those observed for the spring wood. Finally, the mature pine wood has a much higher fiber length and coarseness than the juvenile pine wood.

Although similar results are obtainable from both FQA and Kajaani FS-200 analysis, FQA is being adopted as the instrument of choice because of the lower amount of fiber required per analysis (0.5−0.8 mg for FQA versus 3−4 mg for Kajaani FS-200).

Determination of Lignin Content. A microanalytical method for the determination of lignin content was developed based on the modified acetyl bromide method by Iiyama and Wallis (*8*). Again, a method modification was made to make it possible to analyze a large number of samples more easily. Although the modified acetyl bromide method for the determination of lignin content is known to be precise, our modification dictated that we reconfirm its accuracy. In the microanalytical technique developed, the analysis of lignin content required a calibration line which was obtained by subjecting various mixtures of known amounts of holocellulose and MWL (both from the mature pine) to our microanalytical acetyl bromide method. Therefore, the gram extinction coefficient of lignin treated by acetyl bromide was not used.

Figure 1 shows the calibration line and data points of the juvenile and mature pines examined. The total lignin content in the three samples was determined by the Klason method: 31.8% for the 3-year-old ring of juvenile pine (KL 30.6%, ASL 1.2%); 31.9% for the 8-year-old ring of juvenile pine (KL 30.8%, ASL 1.1%); and 27.3% for mature pine (KL 27.0%, ASL 0.3%). Although the calibration line is made by analyzing the mixtures of holocellulose and MWL prepared from mature pine, the data points representing the three samples (mature wood, juvenile wood year 3, and juvenile wood year 8) correlate well with the calibration line. Thus, the microanalytical acetyl bromide method developed here is suitable to make rapid analysis of lignin content in softwoods in a large number of samples.

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Received for review August 30, 2001. Revised manuscript received December 12, 2001. Accepted December 16, 2001. Financial assistance from the North Carolina Biotechnology Center (2000-MRG-1102) is gratefully acknowledged.

JF011173Q