

Small-scale method to determine the content of wood components from multiple eucalypt samples

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ABSTRACT: *The small-scale method was developed to determine the content of wood components, such as extractives, lignin, holocellulose, α -cellulose, and hemicellulose, for a small amount of multiple eucalypt samples, keeping the compatibility of its results with the standard method. This method was applied to six species of Eucalyptus: E. camaldulensis, E. dalrympleana, E. globulus, E. grandis, E. nitens, and E. trabutii. The results were compared with the standard method by the statistical technique of one-way analysis of variance. Triplicate data from all species showed no difference between the small-scale method and the standard method at a 95% confidence level. This method is also used in the sample preparation for structural analysis of each component, e.g., syringyl/guaiacyl ratio of lignin, neutral sugar ratio of hemicellulose, and degree of polymerization of cellulose.*

KEYWORDS: Alpha cellulose, analysis, bibliographies, dimensions, Eucalyptus, extractives, hemicelluloses, holocellulose, lignins, sampling.

In recent years, forests have seen excessive logging and numerous environmental issues. As a result, planting new trees becomes important, not only for the pulp and paper

industry, but also to sustain life. To support the pulp and paper companies, it is important to select seeds that promote high productivity. In addition, to produce high-yield, high-

quality, and low-cost pulp and paper, we need an objective evaluation method to select these seeds.

Eucalyptus is an appropriate family to evaluate because it has a high growth rate (1), it is a large and diverse genus, and it has considerable variation in wood characteristics (2). However, our objective is to select elite seeds without cutting trees to collect the seeds, including artificially cross-pollinated seeds, for quality and efficient breeding. Although one way of accomplishing this is to use an increment core, it is first necessary to establish a methodology to determine multiple samples of wood components, relating to the yield and quality of pulp, with small-scale reproducibility.

In the 1960s, there was considerable interest in devising a method of determining wood components for a small amount of woody samples (3–5). In particular, Erickson evaluated handling a small amount of multiple samples (5); unfortunately, the results from his evaluation were not sufficiently compared to the standard method.

Instrumental analysis has recently advanced with the help of computers. For example, carbon-13 nuclear magnetic resonance (NMR) spectroscopy has been applied to determine lignin content (6), and high-performance liquid chromatography (HPLC) is used to determine the content of cellulose by hydroly-

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I. Content of wood components, by standard and small-scale methods, vs. original wood meal

Species	Extractives, %	Lignin, %	Holocellulose, %	α -cellulose, %	Hemicellulose, %
<i>E. camaldulensis</i>					
Standard	9.0 $\pm$ 0.7	20.7 $\pm$ 0.6	69.9 $\pm$ 0.9	32.3 $\pm$ 0.0	37.6 $\pm$ 0.9
Small-scale	9.8 $\pm$ 0.2	20.4 $\pm$ 0.4	69.5 $\pm$ 1.8	33.6 $\pm$ 0.9	35.9 $\pm$ 0.8
<i>E. dalyrympleana</i>					
Standard	8.1 $\pm$ 0.2	16.9 $\pm$ 0.9	75.3 $\pm$ 0.5	33.9 $\pm$ 0.2	41.4 $\pm$ 0.4
Small-scale	8.8 $\pm$ 0.4	18.6 $\pm$ 0.3	74.6 $\pm$ 0.5	34.9 $\pm$ 1.4	39.7 $\pm$ 1.6
<i>E. globulus</i>					
Standard	4.4 $\pm$ 0.8	19.5 $\pm$ 0.7	79.6 $\pm$ 0.4	41.1 $\pm$ 0.6	38.5 $\pm$ 0.6
Small-scale	3.8 $\pm$ 0.3	18.9 $\pm$ 0.5	80.0 $\pm$ 0.4	43.0 $\pm$ 1.0	37.0 $\pm$ 1.3
<i>E. grandis</i>					
Standard	5.2 $\pm$ 0.6	16.0 $\pm$ 0.6	78.8 $\pm$ 0.8	42.2 $\pm$ 1.1	36.4 $\pm$ 0.3
Small-scale	6.5 $\pm$ 0.3	17.3 $\pm$ 0.9	79.5 $\pm$ 0.5	42.0 $\pm$ 1.0	37.5 $\pm$ 0.5
<i>E. nitens</i>					
Standard	10.0 $\pm$ 0.4	15.9 $\pm$ 1.4	77.1 $\pm$ 0.8	41.0 $\pm$ 1.3	36.1 $\pm$ 0.6
Small-scale	10.3 $\pm$ 0.2	17.8 $\pm$ 0.1	78.6 $\pm$ 1.4	43.4 $\pm$ 0.5	35.2 $\pm$ 1.8
<i>E. trabutii</i>					
Standard	6.5 $\pm$ 0.9	22.6 $\pm$ 1.6	71.9 $\pm$ 0.7	37.6 $\pm$ 1.8	34.3 $\pm$ 1.1
Small-scale	5.8 $\pm$ 0.2	22.9 $\pm$ 0.7	73.3 $\pm$ 0.8	36.9 $\pm$ 0.9	36.4 $\pm$ 0.1

sis of woody samples with 100% trifluoroacetic acid (7). Klason's method and the NMR method exhibited nearly identical lignin content values in wood (6), but the latter has the disadvantage of a long analysis time and is not suitable for multiple samples. The HPLC method for cellulose works well for multiple samples using an auto-sampler. However, this method requires correction of the glucose level in determining cellulose content, because glucose is also produced from glucomannan (8). Our objective is to not only determine the content of wood components, but also to prepare the samples for a structural analysis of wood components. Therefore, the hydrolysis of cellulose is not appropriate for DP (degree-of-polymerization) measurement of cellulose.

Consequently, we have developed a new method to determine the content of wood components (i.e., extractives, lignin, holocellulose, α -cellulose, and hemicellulose) in a small sample (about 200 mg) for multiple eucalypt samples. From the

same sample, a series of the structural analysis was also attempted. Data obtained from our new method were carefully compared with those from classical methods. Although only a small amount of wood sample was examined, reliable and reproducible data were obtained. In the following sections, we describe detailed sequences of extraction procedures for Eucalyptus. Using the new method developed in this study, we may be able to perform quality breeding more effectively, with less damage to trees and less complexity in the lab.

Experimental

Woody samples

Eucalyptus camaldulensis, *E. dalyrympleana*, *E. globulus*, *E. nitens*, and *E. trabutii* samples were taken from trees grown at New Oji Paper Co., Ltd. (Kameyama, Japan); their ages were 35, 2, 36, 2.5, and 35 years, respectively. *E. grandis*, a gift from the Central Research Laboratory of New Oji Paper Co., Ltd. (To-

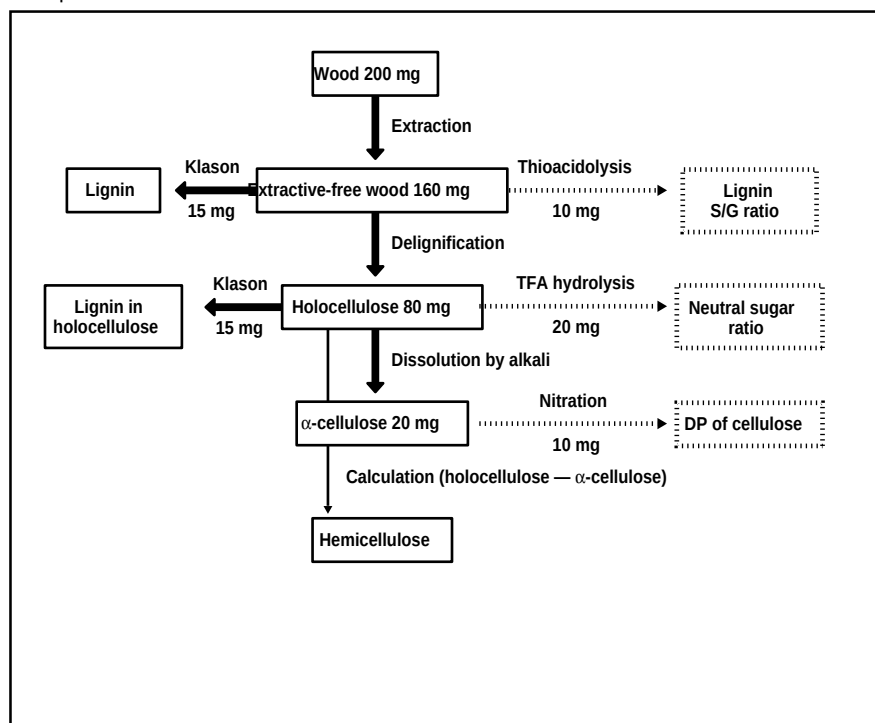
kyo, Japan), was imported as chips from the Republic of South Africa. Each wood meal was prepared with a Wiley mill from highly vacuum-dried woody samples using a 40-mesh pass for the standard method and a 20-mesh pass for the small-scale method.

Content determination of wood components

The standard method was applied (9, 10), and the small-scale method was newly developed based on Erickson's method (5). Hemicellulose was calculated by subtracting α -cellulose from holocellulose. Data are expressed in weight percentage vs. original wood meal. All chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and all glassware was purchased from Asahi Rika-glass Industry Co., Ltd. (Nagoya, Japan).

Extraction length. The extraction length for each solvent was determined by the leveling-off of ultraviolet (UV) absorption in the extraction solvent, which was replenished to give the initial volume with

1. Experimental small-scale method



fresh solvent. UV absorption was determined using a UV spectrophotometer (Hitachi Model 200-20, Tokyo, Japan) with a 1-cm light-pass quartz cuvette at room temperature.

Klason lignin determination.

Klason lignin was determined with 1 g of an extractive-free sample (9).

Standard method. Extractives:

Extractives were determined by the following procedure:

1. Weigh 5 g of a highly vacuum-dried sample with a high-vacuum pump (Shinku Kiko Co., Ltd., Ulvac GCD 050X, Yokohama, Japan) and place in a cellulose extraction thimble (Toyo Roshi Co., Ltd., Tokyo, Japan).
2. Extract with a sequence of 95% ethanol-toluene (1:2 by vol.) for 6 h, 95% ethanol for 4 h, and distilled water for 2 h by Soxtec system (Tacator, Sweden).
3. Squeeze and wash with acetone.
4. Dry in a desiccator under a high vacuum until the pressure falls below 13.3 Pa.

5. Weigh the residue to calculate the extractives content.

Lignin: Lignin content was determined using the following procedure:

1. Weigh 1.5 g of an extractive-free sample and place in a 200-mL flask.
2. Add 75 mL of 0.1 M sodium hydroxide and boil for 1 h.
3. Filter with a preweighed glass filtering crucible (30-mL, medium porosity) and wash with 300 mL of hot distilled water, 50 mL of 10% acetic acid solution, and 300 mL of hot distilled water.
4. Dry under a high vacuum, and weigh the residue to determine the alkali extractives.
5. Weigh 1 g of alkali-extracted sample and place in a 100-mL beaker.
6. Soak the beaker in a 20°C water bath, and add 20 mL of 72% sulfuric acid.

7. After 4 h of occasional stirring, add 765 mL of distilled water and reflux.

8. After 4 h reaction, filter with a preweighed glass filtering crucible (30-mL, medium porosity) and wash with 500 mL of hot distilled water.

9. Dry under a high vacuum and weigh the residue to calculate the lignin content.

Holocellulose: Holocellulose content is determined using the following procedure:

1. Weigh 3 g of an extractive-free sample and place in a 300-mL flask.
2. Soak the flask in a 70°C water bath, and add 180 mL of 0.2% sodium acetate (pH 3.5).
3. Add 1.2 g of sodium chlorite and stir.
4. After 30 min, repeat Step 3.
5. After 45 min, filter with a preweighed glass filtering crucible (30-mL, medium porosity), and wash with 600 mL of cold distilled water followed by 600 mL of acetone.
6. Dry under a high vacuum and weigh the residue.
7. Determine the holocellulose content after measuring the lignin content in holocellulose (see below).

Lignin in holocellulose: The amount of lignin in holocellulose is determined by using the following procedure:

1. Weigh 1 g of holocellulose sample, and follow Steps 5–9 in the previous lignin procedure.

α-cellulose: The amount of α-cellulose is determined by using the following procedure:

1. Weigh 1 g of a holocellulose sample and place in a 200-mL beaker.

II. F values computed by one-way analysis of variance between standard and small-scale methods. F value at 95% confidence interval = 7.71.

Species	Extractives, %	Lignin, %	Holocellulose, %	α -cellulose, %	Hemicellulose, %
<i>E. camaldulensis</i>	2.57	0.26	0.07	4.00	3.95
<i>E. dalyrympleana</i>	6.61	6.54	2.37	1.03	2.28
<i>E. globulus</i>	1.01	1.08	0.91	5.12	2.11
<i>E. grandis</i>	6.56	2.53	1.20	0.13	7.41
<i>E. nitens</i>	1.27	3.92	1.55	5.54	0.48
<i>E. trabutii</i>	1.10	0.07	3.16	0.24	6.46

2. Add 25 mL of 17.5% sodium hydroxide and stir.
3. After 4 min, smash for 5 min.
4. After 16 min, add 25 mL of distilled water and stir for 1 min.
5. After 5 min, filter with a preweighed glass filtering crucible (30-mL, medium porosity), and wash with distilled water until neutral.
6. Add 40 mL of 10% acetic acid and hold for 5 min.
7. Weigh the residue to calculate the α -cellulose content.

Small-scale method. Extractives: The following procedure is used to determine extractives:

1. Weigh 200 mg of a highly vacuum-dried sample, and place in a preweighed glass filtering crucible (2-mL, medium porosity).
2. Extract with a sequence of 95% ethanol-toluene (1:2 by vol.) for 6 h, 95% ethanol for 4 h, and distilled water for 2 h by a Soxhlet apparatus specially made for the crucible.
3. Squeeze and wash with acetone.
4. Dry in a desiccator under a high vacuum until the pressure falls below 13.3 Pa.
5. Weigh the residue with filter to calculate the extractives content.

Lignin: The following procedure is used to determine the lignin content:

1. Weigh 15 mg of an extractive-free sample and place in a 2-mL Eppendorf tube.
2. Add 0.75 mL of 0.1M sodium hydroxide and boil for 1 h, with occasional mixing on a heating block with a dry bath incubator.
3. Filter with a preweighed glass filtering crucible (2-mL, medium porosity), and wash with 4.5 mL of hot distilled water, 0.75 mL of 10% acetic acid, and 4.5 mL of hot distilled water.
4. Dry under a high vacuum, and weigh the residue to determine the alkali extractives.
5. Weigh 10 mg of an alkali-extracted sample and place in a 25-mL flask.
6. Soak the flask in a 20°C water bath, and add 0.2 mL of 72% sulfuric acid.
7. During the reaction, occasionally smash the sample using the head of a disposable transfer pipette. After 2 h, add 10 mL of distilled water and reflux.
8. After 2 h treatment, filter with a preweighed glass filtering crucible (0.8-mL, fine porosity), and wash with distilled water three times.

9. Dry under a high vacuum and weigh the residue to calculate the lignin content.

Holocellulose: Holocellulose content is determined by using the following procedure:

1. Withdraw an extractive-free sample to the point where about 90 mg are left in the same glass filtering crucible as that used for extraction.
2. Wash with acetone and dry under a high vacuum to weigh.
3. Plug the crucible leg with a septum and wrap the joint with labo-sealing tape (3M, St. Paul, MN).
4. Soak the crucible in a 70°C water bath, and add 1.4 mL of 0.2M sodium acetate (pH 3.2).
5. Add 0.1–0.2 mL of 20% sodium chlorite and stir with a small glass rod.
6. After 30 min, repeat Step 5.
7. After 45 min, remove the septum in ice-cold distilled water, and soak the glass crucible for 5 min.
8. Squeeze and wash with ice-cold 1% acetic acid three times and with ice-cold acetone twice.
9. Dry under a high vacuum and weigh the residue.
10. Determine the holocellulose content after measuring the lignin content in holocellulose.

Lignin in holocellulose: Determine the lignin in holocellulose by using the following procedure:

1. Weigh 10 mg of a holocellulose sample and follow Steps 5–9 in the previous lignin procedure.

α -cellulose: The following procedure is used to determine the α -cellulose content:

1. Withdraw a 19–21-mg holocellulose sample from the glass filtering crucible used for holocellulose preparation.
2. Wash with acetone and high-vacuum dry to weigh.
3. Plug the crucible with a septum, and wrap the joint with labo-sealing tape.
4. Soak the crucible in a 20°C water bath, add 0.2 mL of 17.5% sodium hydroxide, and stir.
5. After 5 min, add 0.4 mL of 17.5% sodium hydroxide.
6. After 40 min, add 0.4 mL of distilled water and remove the septum.
7. Squeeze and wash with distilled water three times.
8. Add 0.3 mL of 12% acetic acid and hold for 5 min.
9. Wash with distilled water three times and dry under a high vacuum.
10. Weigh the residue to calculate the α -cellulose content.

Results and discussion

Analysis for Eucalyptus

To improve the previous methods of determining the content of the Eucalyptus wood components, the solvent system for extraction and the length of extraction were re-evaluated. The need to pre-extract for lignin quantification was also examined.

Extraction sequence and length. The extraction sequence of ethanol-toluene (1:2 by vol.) followed

by ethanol and distilled water as previously reported (11) was used in this study. Ethanol extraction is the key step for the treatment of Eucalyptus, since this family contains many catechol tannins (12) that may interfere with subsequent measurements. For more reliable assay, the length for each extraction step was re-evaluated using UV absorption. In ethanol-toluene, the increase in absorbance leveled off after 6 h treatment (4 h in ethanol and 2 h in water).

Pre-extraction for lignin determination. Eucalyptus has been reported to contain polyphenols called kinos, which show resistance to the extractions described above (13). To avoid complications in the lignin determination by the Klason method, pre-extraction with boiling sodium hydroxide is recommended (10). However, this step may cause solubilization of a certain amount of lignin (14). On the other hand, the acetyl bromide method, although useful, may be limited when applied to Eucalyptus (15). Although the loss of lignin during the pre-extraction process may be unavoidable, additional extraction should be done if the error is smaller after the pre-extraction than before (11).

We examined the lignin content of *E. camaldulensis* and *E. globulus* by the Klason lignin method, with and without pre-extraction, using 0.1M sodium hydroxide (10). Duplicate experiments gave an average of 30.1% Klason lignin, without pre-extraction, of *E. camaldulensis* and 24.1% of *E. globulus*. **Table I** shows the triplicate Klason lignin data with pre-extraction: 20.7% for *E. camaldulensis* and 19.5% for *E. globulus*. Since the total amount of extractives, lignin, and holocellulose should be near 100% (16), one can decide if pre-extraction is necessary. *E. camaldulensis* had a total amount of 109.0% without pre-extraction and 99.6% with pre-extraction; *E. globulus* showed 108.1% and 103.5%, respectively. This observation indicates that pre-extraction is required

to achieve a more reliable assay for lignin determination. In this study, extractive-free wood meal was pre-extracted with 0.1M sodium hydroxide for 1 h at the boiling point to determine the lignin content according to Appita Standard Method P2m-73 (10).

Characteristics of the small-scale method

Erickson provides an excellent system to determine the content of wood components for a small amount of multiple samples simultaneously, without transferring the sample, using a filtering crucible (5). However, that experiment lacked sufficient data comparison with the standard method and the consideration to use the samples for other analyses. The main purpose of this study was to design a small-scale assay method that could determine the content of wood components for a small amount of multiple eucalypt samples, keeping the compatibility of results with the standard method, and to finish the sample preparation for a structural analysis of each component [i.e., syringyl/guaiacyl (S/G) ratio of lignin (17), neutral sugar ratio of hemicellulose (18), and degree of polymerization (DP) of cellulose by nitration (19)]. **Figure 1** shows the small-scale method established in this study. Only a 200-mg sample was required to complete all experiments, including structural analysis. The 200 mg of wood meal was obtained from a 5.1-mm-long, 5.0-mm-diam. increment core of 500-kg/m³ basic density wood. Therefore, our method allows easy analysis of the radial variation of wood components and structure using an increment core. The radial variation within a tree has been reported (20–26).

To develop a small-scale method by improving Erickson's method, the following principal instruments were used:

- **A high-vacuum pump with a cold trap and a vacuum gauge.** Instead of using 105°C oven dry-

ing, this gave us uniform and fast drying, and there was a minimal change in the quality of the samples used for structural analysis. For example, holocellulose was dried in only 1 hour.

- **An electrical semi-microbalance with an auto door.** This was accurate in the range of 100 g to 0.01 mg and allowed the determination of 0.1-mg increments of lignin in 10 mg of holocellulose.
- **An Eppendorf repeater.** This is a hand-held repetitive pipetter providing a dispensing range of 1 μ L to 5 mL by seven tip sizes with at least $\pm 1.5\%$ accuracy and 0.6% precision. The repeater significantly reduced the introduction time of liquid chemicals into many samples. For example, the addition of 72% sulfuric acid to 24 samples was achieved in 30 seconds.
- **A 2-mL glass filtering crucible (medium porosity).** A single filter was used for extraction and holocellulose preparation. The weighing of glass filters for each treatment, as in Erickson's method, was omitted, but the capacity and coarseness were changed to achieve more accuracy from a 30-mL crucible (coarse porosity).

Extractives

Extraction was performed on two sets of six independently controlled heaters with a timer, so 12 samples were extracted at once. To fill out solvents, dispensers were used to save time.

Lignin and lignin in holocellulose. In the Klason lignin determination, reaction and reflux times were halved compared to the stan-

dard method. A boiling flask was used for the reaction to avoid the transferring time of a sample solution. During the reaction, the head of a plastic disposable transfer pipette was used to efficiently smash the sample. To wash lignin after the reflux, room temperature distilled water was used instead of hot water, and a 0.8-mL, fine-porosity, glass filtering crucible was used to avoid lignin loss by filtration.

Holocellulose and α -cellulose.

A small-scale assay method developed by Erickson (5) was further improved by plugging the crucible with a septum and labo-tape. This prevented draining of chemicals during the treatment.

Validity of the small-scale method

The results of the content determination of wood components by both standard and small-scale methods are summarized in Table I; they are expressed in terms of the original wood base. Triplicate data show good similarity between the methods and show the species differences. The validity of the small-scale method was verified by a one-way analysis of variance with the standard method (Table II); a 95% confidence level of correlation was obtained. As a result, the small-scale method is acceptable for analyzing Eucalyptus. 

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The authors wish to thank R. Funada (Hokkaido University) and K. Ito (New Oji Paper Co.) for critical suggestions, H. Wariishi (Kyushu University) for English corrections, and R. Noda and T. Furukawa (New Oji Paper Co.) for helping with sample preparations.

Received for review June 27, 1994.

Accepted Oct. 17, 1994.