CRITICAL ASSESSMENT OF INTERFERENCE MICROSCOPY AS A TECHNIQUE FOR MEASURING LIGNIN DISTRIBUTION IN CELL WALLS

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ABSTRACT

This report investigates sources of experimental error involved in the quantitative measurement of lignin concentration in the layers of the tracheid cell wall using interference microscopy. The refractive index of lignin in the middle lamella region is found to be 1.604 while in the S2 region it is 1.596. The lower value in the S2 is attributed to either residual carbohydrates or chemical differences in the lignin of the two regions. The refractive index of holocellulose varies among specimens from different parts of the stem and it is recommended that this value be determined on matched samples for each specimen. This value does not vary between earlywood and latewood, or between chlorite and per-acetic preparations. The refractive index of the unlignified middle lamella is 1.516 in both primary and secondary xylem and differs from the expected value of 1.504 for pectin. Specimen orientation is an important consideration when comparing wholewood and holocellulose with a maximum acceptable error of ± 4°.

Keywords: lignin; holocellulose; cell wall; interference microscopy; refractive index; **Pinus radiata**.

INTRODUCTION

There are two ways of making quantitative measurements of cell wall composition on a microscopic scale:

(1) Wood can be microdissected to yield fragments of various cell wall layers which can then be analysed using chemical techniques designed for small samples. The earliest example of this approach is that of Bailey (1936) who laboriously isolated a small quantity of middle lamella material from *Pseudotsuga menziesii* (Mirb.) Franco using a micromanipulator. Meier (1961), Meier & Wilkie (1959), and Côté *et al.* (1968) studied the distribution of carbohydrates in the layers of the cell wall by microdissection of developing tracheids adjacent to the cambium, followed by chemical analysis. Kallmes (1960) studied the distribution of lignin, alpha-cellulose, and hemicellulose across the cell wall of sulfite fibres by removal of the primary wall and the Sl layer of the secondary wall. Iwamida *et al.* (1975) were able to characterise the chemical composition of middle lamella fragments

of spruce (*Abies* spp.) tracheids. Kibblewhite & Brookes (1976) analysed the chemical nature of fines from kraft and bisulfite fibres of *Pinus radiata* D. Don. Since 1980, techniques for isolating cell wall fractions have been refined enabling studies of chemical composition and of the chemical structure of lignin from the various fractions obtained (Hardell *et al.* 1980; Hardell & Westermark 1981; Whiting *et al.* 1981; Whiting & Goring 1982a, 1983; Sorvari *et al.* 1983; Westermark 1985).

(2) The alternative to chemical analysis of microdissected specimens is *in situ* analysis of the cell wall either in transverse section or as fragments, using physical techniques. The techniques used include UV microscopy (Scott *et al.* 1969), interference microscopy (Boutelje 1972), and bromination in conjunction with energy dispersive X-ray analysis (bromination EDAX) (Saka *et al.* 1978). UV microscopy and bromination EDAX are restricted to the examination of lignin but interference microscopy can be used to study both lignin and carbohydrates (Boutelje & Hollmark 1972).

This report investigates aspects of the interference microscopy technique in greater detail than that given by Boutelje (1972) in order to assess sources of experimental error.

MATERIALS AND METHODS

Butt discs were collected from 15 freshly felled trees of *Pinus radiata* growing in Kaingaroa, Karioi, and Rotoehu State Forests. Small blocks of wood were removed from Growth Ring 3 (outerwood) of sample discs and boiled in water until saturated. The blocks were then sectioned at a thickness of 100 μm in the radial plane, using a sledge microtome. The sections were dissected into pieces 1 mm wide by 2–3 mm long with a sharp razor blade, taking care to orientate the specimens so that the long axis was parallel to the axial direction in order to simplify orientation during embedding. Specimens were dehydrated in an ethanol series and then extracted in a 2 : 1 (v/v) mixture of benzene and ethanol for 3 days to remove any remaining extractives. Finally, specimens were washed in ethanol and in acetone before being embedded in Spurrs resin.

The remainder of the water-saturated wholewood was divided into matchstick-sized pieces and treated for 6–8 h in a 1 : 1 mixture of glacial acetic acid and hydrogen peroxide at 80–90°C. When specimens were fully bleached they were washed in distilled water, dehydrated in an acetone series, and dissected into shives 2–10 mm long and 1–2 mm wide. The holocellulose shives were then embedded in Spurrs resin. Chlorite holocellulose was also prepared for one specimen, according to the method of Uprichard (1965). The absence of a middle lamella in sectioned material was taken as indicating that delignification was complete.

Further 100-μm-thick sections were treated as follows. Sections were hydrolysed in 72% sulphuric acid v/v at 70°C for 3 h, followed by extensive washing in distilled water. The hydrolysed material was then dissected into specimens 2–10 mm long and 1–2 mm wide with the long axis in the axial direction. Specimens were then dehydrated in an acetone series and embedded in Spurrs resin.

Specimens of differentiating xylem were collected from four trees. Two 16-year-old trees from Rotoehu State Forest were sampled in September 1983, and one 5-year-old tree from Whakarewarewa State Forest Park and one 12-year-old tree from Karioi State Forest were sampled in November 1983. Specimens containing primary xylem were also obtained from shoot tips of a young tree growing in the Forest Research Institute nursery. All of these specimens were dissected into small pieces, dehydrated in an acetone series, and embedded in Spurrs resin.

Embedded material was sectioned in the transverse plane with an LKB ultramicrotome using glass knives at a section thickness setting of 2 μm. Sections were transferred to a clean (ethanol washed) microscope slide and heat fixed to the slide over a flame. Slides were then washed in sodium ethoxide for 5 min to remove the embedding plastic, followed by two washes in ethanol.

Optical path difference (o.p.d.) measurements were made at a temperature of 20°C and a wavelength of 546.1 nm, using a Zeiss Photomicroscope II equipped for quantitative interference microscopy. O.p.d. was calculated from angular readings using the following formula:

$$\text{o.p.d.} = (180 - a) \times 3.034 \qquad (1)$$

where a = analyser rotation

3.034 = a constant (546.1/180)

Optical path difference measurements were made on the same cell wall region after the specimen had been mounted firstly in ethanol then in glycerol.

Refractive index was then calculated from the two o.p.d. measurements using the following formula:

$$N_o = \frac{(\text{o.p.d.}_1\ N_2) - (\text{o.p.d.}_2\ N_1)}{\text{o.p.d.}_1 - \text{o.p.d.}_2} \qquad (2)$$

where N_o = refractive index of the specimen

N_1 = refractive index of medium 1 (1.362 for ethanol)

N_2 = refractive index of medium 2 (1.474 for glycerol)

o.p.d._1 = path difference in medium 1

o.p.d._2 = path difference in medium 2

More detailed information on the theory involved in these calculations has been given by Ross (1967) and Tolansky (1968).

In order to calculate lignin concentration three values are required, the refractive index of wholewood (S2 or cell corner middle lamella, ccml), the refractive index of holocellulose (refractive index of either pectin or the unlignified middle lamella is used for measurements in the cell corner region), and the refractive index of lignin (Boutelje 1972; Boutelje & Hollmark 1972). The refractive indices of lignin, pectin, and the unlignified middle lamella were regarded as constant values during calculations while the refractive indices of wholewood and holocellulose were measured with

replication for each specimen. The lignin concentration v/v was calculated using the following formula:

$$VFL = \frac{NWW - NHC}{NL - NHC} \quad \text{(3)}$$

where VFL = volume fraction lignin
NWW = refractive index of wholewood
NHC = refractive index of holocellulose
NL = refractive index of lignin

For calculations involving the middle lamella, NHC is replaced with values for either pectin or the unlignified middle lamella.

In the following eight experiments each individual site was measured 10 times and five sites were examined for each specimen.

Experiment 1: Specimens from all 15 of the sampled trees were examined. For wholewood, measurements were made in the S2 region of the tangential wall and the ccml region of earlywood tracheids. Measurements were made on matched samples of holocellulose with the same replication, measuring in the S2 region of earlywood cells only.

Experiment 2: A specimen of holocellulose from a single growth ring containing both earlywood and latewood was examined. Refractive index was determined for the S2 region in both earlywood and latewood cells.

Experiment 3: Single specimens of chlorite and per-acetic holocellulose from the same wood specimen were examined. Refractive index was determined for the S2 region in both samples.

Experiment 4: The refractive index of a holocellulose specimen was measured on sections cut at 0° (transverse), 4°, 8°, and 12° tilt in the tangential direction (vertical tracheids are being tilted sideways). Measurements were made on the back tangential wall of the cells examined.

Experiment 5: The refractive index of lignin was determined on transverse sections of hydrolysed wood, measuring in both the S2 and ccml regions.

Experiment 6: The refractive index of the unlignified middle lamella in the cell corner region was determined for differentiating secondary xylem and for mature primary xylem. For the secondary xylem specimens, measurements were made on cells in the expansion phase of differentiation. Staining with toluidine blue was used to confirm the absence of lignin.

Experiment 7: A 1% w/v solution of commercially available pectin (partially methoxylated polygalacturonic acid, Sigma Chemical Company), was used for measuring refractive index by preparing a thin film on the microscope slide.

Experiment 8: The refractive index of a wholewood specimen was measured at the same point on serial sections after 5, 10, and 15 min treatment with sodium ethoxide. Measurements were made in the S2 region.

RESULTS

Refractive indices of wholewood and holocellulose

The values for the refractive indices of wholewood and holocellulose are shown in Table 1. The refractive indices of wholewood measured in the S2 region and of holocellulose, are highly correlated with a correlation coefficient r = 0.99***. This correlation indicates that the refractive index of holocellulose must be measured on matched samples for calculation of wholewood lignin concentration to avoid this potential source of error.

TABLE 1—Refractive indices of wholewood and holocellulose

Specimen		Wholewood S2 N_0	Percentage lignin v/v	Wholewood ccml N_0	Percentage lignin v/v	Holocellulose N_0
Kaingaroa	1	1.547	22	1.590	86	1.531
	2	1.544	21	1.576	72	1.528
	3	1.548	21	1.587	83	1.533
	4	1.544	20	1.583	79	1.529
	5	1.547	21	1.596	92	1.532
	6	1.548	20	1.585	81	1.534
	7	1.548	23	1.580	76	1.531
	8	1.555	21	1.586	82	1.542
	9	1.548	20	1.589	85	1.534
Karioi	1	1.545	21	1.587	83	1.529
	2	1.545	21	1.590	86	1.529
	3	1.545	20	1.586	82	1.530
Rotoehu	1	1.538	21	1.581	77	1.520
	2	1.547	21	1.596	92	1.532
	3	1.549	21	1.595	91	1.534

Measurements of angular rotation (a in Equation 1) were generally within a range of 1° yielding a range of refractive index repeats of ± 2–3 × 10^{-3}. Repetition among sites yielded a similar range of variation. In order to minimise the statistical error in each lignin concentration value, mean values of refractive index repeats for each of the five sites were ranked according to magnitude and the resulting matched pairs (for example wholewood (S2) and holocellulose) were used to calculate lignin concentration. Lignin concentration values calculated in this way varied over a range of 2–3% for each specimen in both S2 and ccml regions giving an approximate least significant difference of ± 1–1.5%.

Refractive index of holocellulose from earlywood and latewood

No significant difference was found between the refractive index of holocellulose in earlywood and latewood cells. Earlywood had an average value of 1.528 with a between-site range of 1.526–1.530. Latewood had an average value of 1.529 with a

between-site range of 1.527–1.530. This result indicates that it is not necessary to measure the refractive index of holocellulose in both earlywood and latewood when calculating wholewood lignin concentration in these areas.

Refractive indices of chlorite and per-acetic holocellulose

No significant difference was found between the refractive indices of per-acetic and chlorite holocellulose. Chlorite holocellulose had an average refractive index of 1.529 with a between-site range of 1.528–1.530. Per-acetic holocellulose had an average refractive index of 1.528 with a between-site range of 1.526–1.530.

Variation in refractive index of holocellulose with orientation

The variation in refractive index of holocellulose with orientation is shown in Fig. 1. Refractive index varies in a linear fashion with a rate of change of 5×10^{-4} per degree.

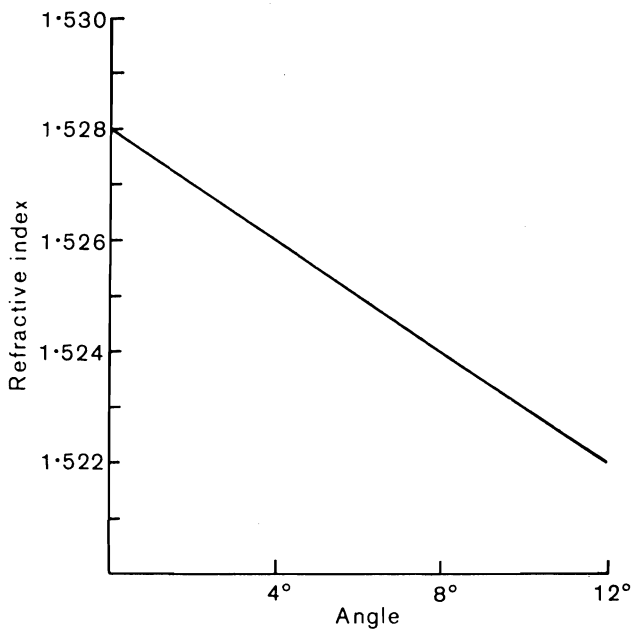

FIG. 1—Relationship between the refractive index of holocellulose and specimen orientation. The angle given represents the angle between the sectioning face and the transverse plane.

Refractive index of lignin

The refractive index of lignin in the S2 layer of the secondary wall in hydrolysed wood had an average value of 1.596 with a between-site range of 1.594–1.599. The refractive index of lignin in the ccml region had an average value of 1.604 with a between-site range of 1.602–1.606.

Refractive index of the unlignified middle lamella

The mean values for the refractive index of the unlignified middle lamella in secondary xylem varied between trees with a range of 1.515–1.521. The value for the primary xylem specimen was 1.516 indicating a similar composition to the secondary xylem.

Refractive index of pectin

The refractive index of pectin was found to be 1.504 indicating a considerable difference from the values for the unlignified middle lamella.

Effect of sodium ethoxide treatment on refractive index

Sodium ethoxide treatment produced no detectable change in refractive index of the specimen examined for treatment times of up to 15 min. The optimal treatment time for removal of plastic from 2-μm sections was found to be 5 min.

DISCUSSION

Variation in refractive index between specimens of holocellulose is probably due mainly to differences in microfibril angle, although differences in hemicellulose concentration and cellulose crystallinity may also contribute (Hermans 1946). Combining the results of Experiment 1 with those of Experiment 4 would suggest a variation in S2 microfibril angle of approximately 44°. Harris & Meylan (1965) have reported a range of about 30° for mean microfibril angle in *P. radiata,* measured by X-ray diffraction. Considering sample differences and the difference in measuring techniques the agreement between these estimates is quite good.

Because the values of the refractive indices of wholewood and holocellulose are highly correlated, it is essential to calibrate wholewood measurements by measuring matched samples of holocellulose in order to avoid the considerable error that would otherwise result from using an average value of refractive index for holocellulose to calculate lignin concentration. Recalculating the S2 lignin concentration using the mean refractive index of holocellulose yields a mean value of 21% which is the same as for the values given in Table 1. However, the range of values increases from 20–23% to 10–33%.

From Experiment 2 there is no indication of a difference in refractive index between earlywood and latewood cells in the same growth ring. On this basis it is only necessary to measure refractive index in earlywood holocellulose, a considerable saving in effort.

Paakkari & Serimaa (1984) have examined the difference in microfibril angle of the S2 layer between earlywood and latewood in *Pinus sylvestris* L. They found a difference of 0.3° which, using the result of Experiment 4, would give an expected change in refractive index of 1.5×10^{-4} which is too small to measure with the equipment used in the present study.

The comparison of chlorite and per-acetic holocellulose carried out in Experiment 3 failed to reveal any difference in chemical composition as reflected in refractive

index. This is a surprising result in the light of a recent investigation by Maekawa & Koshijima (1983). These workers found significant differences in the chemical composition of chlorite and per-acetic holocellulose. A possible explanation for the failure to detect an expected difference in refractive index between the two types of holocellulose, is the dependence of the sensitivity of interference microscopy on the difference in refractive index between the components of the object being examined. The two components of holocellulose, alpha-cellulose and hemicellulose, may both have similar refractive indices. This will mean that only large extraction of hemicellulose, or considerable degradation of cellulose would be detectable. An alternative explanation may be that the difference measured by Maekawa & Koshijima (1983) was the result of extraction from the outer wall layers (S1 and primary wall) in which case the S2 may have been unaffected. Because per-acetic holocellulose is easier to prepare, its use is recommended rather than chlorite holocellulose. Results for chlorite and per-acetic holocellulose should be comparable.

The results of Experiment 4 indicate that orientation differences between wholewood and matched holocellulose are unlikely to result in any major error. A difference in orientation of 4° was clearly visible when comparing the sectioning face with the specimen and it is considered unlikely that orientation differences would exceed this value which leads to a possible error of $\pm$ 2% in lignin concentration. It is worth pointing out that orientation differences are not a problem for measurements in the middle lamella which is not birefringent. Thus measurements in the middle lamella region would be expected to be more accurate than those in the S2 region.

The refractive index of lignin may vary depending on the method used to isolate it. In the present work, sulphuric acid lignin (Klason lignin) was used so that results would be compatible with lignin content measurements based on the Klason technique. However, it is known that sulphuric acid lignin is not chemically the same as native lignin (Sjöström 1981). It is not known to what extent acid hydrolysis results in changes in the refractive index of lignin. The value of 1.604 found for the ccml region agrees closely with the value of 1.603 given by Frey (1959). In the present material the value of the refractive index of lignin measured in the S2 region differed from that in the ccml region (1.596 cf. 1.604). The difference may have been due to the presence of residual carbohydrates in the S2 region or to chemical differences between the lignins in the two regions, such as those reported for other species (Hardell *et al.* 1980; Saka *et al.* 1982; Westermark 1985; Whiting & Goring 1982a, b; Yang & Goring 1978, 1980). Using the value of 1.596 to calibrate S2 lignin concentration calculations, results in an over-estimate of about 3%. Until further studies have been carried out it is recommended that 1.604 be used as the calibrating value for both S2 and ccml regions.

The refractive index of pectin has been measured by Wuhrmann & Pilnik (1945) indicating a value of 1.504 which agrees with the value found in the present work.

The lowest average value for the refractive index of the unlignified middle lamella of about 1.515 does not compare favourably with the expected value of 1.504. The reason for the difference is likely to be the presence of hemicellulose and possibly calcium and proteins in the ccml region. Chemical differences between the refined

pectin used in Experiment 7 and the native pectin present in the ccml regions measured in Experiment 6 could also account for some of the difference. The observation that the refractive index of the unlignified ccml seems to vary among some of the specimens is interesting and further experiments are currently under way to investigate this variation.

Boutelje (1972) proposed the use of the refractive index of pectin (polygalacturonic acid) as a calibrating value to be used in the calculation of lignin concentration in the ccml region. Several workers have examined the carbohydrate composition of the middle lamella and indicated the presence of arabinan, galactan, polygalacturonic acid, and xylan (Meier & Wilkie 1959; Meier 1961; Côté *et al.* 1968; Burke *et al.* 1974; Iwamida *et al.* 1975; Hardell & Westermark 1981; Whiting & Goring 1983). While it is unlikely that the value of 1.516 found in the present work is due to lignification, because of the agreement with the unlignified middle lamella of the primary xylem, it is possible that the higher values of 1.521 may be due to the onset of lignification. Using the value of 1.516 instead of 1.504 results in a 2–3% decrease in the estimated wholewood lignin concentration. This level of error is acceptable and until further studies have been made it is recommended that the value of 1.504 be used. The relationship between the calibrating value for the refractive index of the carbohydrate fraction and the resulting lignin concentration is shown in Fig. 2. Even the value of 1.521 does not result in more than 3–4% error.

A range of variation in refractive index from 1.504 to 1.521 for the middle lamella calibration, results in an approximate range of 5% in lignin concentration. Assuming

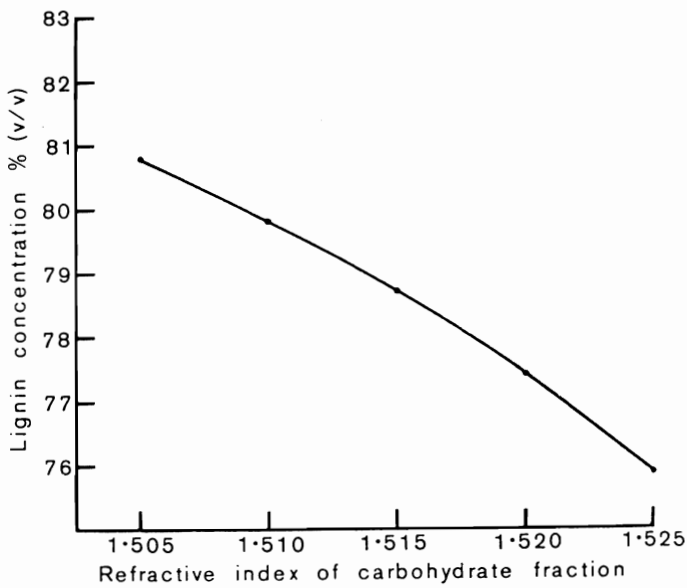

FIG. 2—Relationship between the value used to calibrate for the carbohydrate fraction of the middle lamella and the resulting lignin concentration. The higher the calibrating value the greater the increase in error.

that this range reflects the maximum variation among samples in the composition of the carbohydrate fraction of the middle lamella, then the variation in wholewood refractive index of the ccml region should result in a 5% range in lignin concentration. This is not the case. The observed variation in the refractive index of the wholewood ccml region yields a variation of 20% in lignin concentration. On this basis at least 75% of the variation in refractive index of the wholewood ccml region must reflect real differences in lignin concentration.

The observation that sodium ethoxide treatment has no significant effect on refractive index, indicates that this solvent is not having an adverse effect on the cell walls of specimens. Alternative treatments to remove the embedding resin include acetone and chloroform, although in the author's experience these solvents are less effective with the additional inconvenience of having to treat specimens in a fume hood when using chloroform.

In conclusion, interference microscopy should provide an accurate technique for the determination of lignin concentration in the secondary wall and middle lamella provided that certain guidelines are followed to avoid sources of error. The refractive index of holocellulose must be measured on matched samples but only earlywood cells need be examined for calibration of both earlywood and latewood. The section for the holocellulose calibration must be cut at the same orientation ($\pm 4°$) as the wholewood section. The calibrating value for the refractive index of lignin is 1.604 for both the S2 and middle lamella. For the middle lamella the main source of error is likely to be the uncertainty in the value for the refractive index of the carbohydrate fraction in this region.

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