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# Establishment of the method for preparing cellulose materials “colorless woods” derived from hardwoods with various densities and anatomical structures

Shunsuke Sakai<sup>1</sup>, Masaumi Miyabayashi<sup>1</sup>, Rino Tsushima<sup>2</sup>, Satoshi Nakaba<sup>2</sup>, Ryo Funada<sup>2</sup>, Tatsuki Kurei<sup>1</sup> and Yoshiaki Horikawa<sup>2\*</sup> 

## Abstract

This research reports novel cellulosic materials named “colorless hardwoods”, which have no lignin and almost no hemicellulose, derived from three wood species with different anatomical structures: *Fagus crenata* (diffuse-porous), *Paulownia tomentosa* (semi-ring-porous), and *Zelkova serrata* (ring-porous). To create these colorless hardwoods, we have employed alcoholysis and the Wise treatment, recognized as delignification methods for softwood and bamboo. The process of alcoholysis was carefully optimized based on the progress of the decolorization and the delignification of wood samples. After the optimization of the alcoholysis, the Wise treatment was applied, leading to the successful removal of non-cellulosic components from each of the three hardwood types. The preservation of the wood's morphology has been confirmed by confocal laser scanning microscopy, which showed no alteration in the woody anatomical structure in terms of cell wall shape and cell arrangement. Moreover, X-ray diffraction analyses indicated the retention of microfibril angles and the crystalline structure of cellulose within the cell wall. These results conclusively demonstrate that colorless hardwoods maintain the original hierarchical structure of their respective wood species and differ significantly from typical pulp and cellulose nanofibers in terms of suprastructure. This advancement in wood-based cellulosic materials opens new avenues for application, highlighting their potential for diverse and sustainable uses.

**Keywords** Confocal laser scanning microscopy, Delignification, Hardwood, Hierarchical structure, Microfibril angle

## Introduction

The complex composite materials of woody polymers, comprising oriented-cellulose microfibrils, hemicellulose, and lignin, have resulted in their regularly organized

hierarchical structure fundamental to the growth of massive tree bodies and their enduring presence over centuries. Such a structure supports human life, offering wood materials essential for building and furniture industries. Additionally, the utilization of trees and wood plays a crucial role in sustainable development, characterized by their continuous cycle of consumption and production, long-term availability, and contribution to reducing greenhouse gas emissions [1]. While traditional wood-derived materials like pulp and paper have been long-standing staples, cellulose nanofibers (CNFs) have emerged as materials of interest in recent years due to their lightweight and high-strength properties [2].

\*Correspondence:

Yoshiaki Horikawa  
horikaw@cc.tuat.ac.jp

<sup>1</sup> Department of Symbiotic Science of Environment and Natural Resources, United Graduate School of Agricultural Science, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu, Tokyo 183-8509, Japan

<sup>2</sup> Institute of Agriculture, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu, Tokyo 183-8509, Japan

However, the production of CNFs involves the disintegration of wood's hierarchical structure, resulting in the loss of its inherent orientation [3–6].

Considering the above background, we proposed a novel cellulosic material “colorless wood” inspired by the cellulose architecture of *Cryptomeria japonica* [7]. This material is produced by selectively removing non-cellulosic components while preserving the inherent hierarchical structure of the wood. Such preservation results in the retention of wood's unique properties [8]. Although an effective delignification method for moso bamboo (*Phyllostachys pubescens*) also has been established [9], one for hardwoods remains unexplored. Hardwoods, with their diverse anatomical structures and wide range of densities, present more complex challenges in the production of colorless wood compared to softwoods [10, 11]. Research on cellulosic structural materials using balsa, the lowest-density wood in the world, is active [12, 13], but the preparation methods are difficult to apply to medium- and high-density species, and the resources depend on importation as they are not native to Japan. Production of colorless woods from domestic hardwoods will lead to the development of cellulosic structural materials with various properties derived from their diverse anatomical structures and wide range of densities.

Therefore, we have attempted to create colorless hardwoods by adapting and refining the two-step chemical treatment previously applied to softwoods [7]. We selected three types of hardwoods, characterized as diffuse-porous, semi-ring-porous, and ring-porous, for this study. The optimal treatment conditions for each hardwood species were carefully determined, focusing on the extent of decolorization and the reduction of non-cellulosic components. The samples, processed under these optimal conditions, were then subjected to comprehensive hierarchical structure evaluations to confirm the preservation of their distinctive characteristics. This study aims to promote the development of cellulosic structural materials by creating colorless woods from easy-to-obtain domestic hardwoods with different densities and structures.

## Materials and methods

### Samples and optical microscopic observation

All wood blocks were processed and provided by Mr. Akio Adachi of Kyoto University. Hardwood heartwood blocks from the trunk were used for all wood sample species. The blocks measuring 10 mm in longitudinal (L), radial (R), and tangential (T) directions were procured from three species: *Fagus crenata* (Fc blocks); *Paulownia tomentosa* (Pt blocks); and *Zelkova serrata* (Zs blocks). These species are representative of diffuse-porous, semi-ring-porous, and ring-porous wood, respectively.

Three-dimensional sections of 20–40  $\mu\text{m}$  thickness were cut from the blocks using a sliding microtome (REM-710, Yamatokohki, Saitama, Japan). The sections were thereafter stained with safranin and Astra Blue [14] to examine their microscopic characteristics. Safranin predominantly stains lignin red, while Astra Blue mainly stains polysaccharides in less-lignified cells blue [15]. After washing in water, the stained sections were observed using an optical microscope (BX61, Olympus, Tokyo, Japan).

### Chemical treatments for removing non-cellulosic components

The hardwood blocks were processed using a treatment reported by Horikawa [7], soaked in a 99:1 (w/w) mixture of ethylene glycol and 48.5 wt% sulfuric acid. The samples were then heated in a portable reactor (TVS-N2, Taiatsu Techno, Tokyo, Japan). The Fc and Pt blocks underwent alcoholysis at 150  $^{\circ}\text{C}$  for 1 h. For Fc and Zs blocks, alcoholysis was conducted at 150  $^{\circ}\text{C}$  for 3 h. Additionally, the Zs blocks underwent further alcoholysis at 150  $^{\circ}\text{C}$ , either three times for 1 h or twice for 3 h. Subsequent to these treatments, the samples were washed with ethanol and water. The Wise treatment, involving a sodium chlorite and acetic acid mixture added hourly, was then carried out at 70  $^{\circ}\text{C}$  for 8 h on all alcoholysis-conditioned samples [16]. After a final washing in water, the treated samples were freeze-dried, except for those reserved for confocal microscopy analysis.

The dimensional and mass characteristics of the three freeze-dried hardwood blocks, corresponding to each preparation condition, were precisely measured using a micrometer (CLM1-15QMX, Mitutoyo, Kanagawa, Japan). These measurements were conducted both before and after the two-step chemical treatments to monitor changes in the dimensions and masses of the blocks, and three blocks were used for each condition. Additionally, to accurately determine the dry mass prior to treatment, the average moisture content of each wood species was estimated using other blocks stored under identical conditions.

### Fourier transform infrared (FTIR) spectroscopy

The core section of the hardwood blocks was examined by FTIR spectroscopy. The FTIR spectra were captured using the attenuated total reflection accessory of the FTIR spectrometer (Spectrum Frontier, PerkinElmer, Waltham, MA, USA). These spectra were recorded in the range of 4000–500  $\text{cm}^{-1}$  at a resolution of 4  $\text{cm}^{-1}$ , acquired at eight scans per sample. The FTIR spectra were then refined using the instrument's software package, which included functions for attenuated total reflection correction, baseline correction, and normalization. Particular attention was paid to normalization according

to the top band within the 2000–800  $\text{cm}^{-1}$  range. These measurements used three blocks for each condition.

### Chemical component analysis

To quantify the acid-insoluble fractions and polysaccharides of hardwood blocks before and after the two-step chemical treatments, we employed a hydrolysis protocol as reported by Horikawa [17]. The freeze-dried samples were finely ground using a mill (WC-3L, Osaka Chemical, Osaka, Japan). The ground samples were then subjected to a two-stage sulfuric acid hydrolysis: initially with 72 wt% sulfuric acid at 30 °C for 1 h, followed by 4 wt% sulfuric acid at 121 °C for 1 h. This hydrolysis process yielded a solution containing monosaccharides along with acid-insoluble fractions, which include lignin, ash, and extractives. The neutralized solution was subsequently analyzed using a high-performance liquid chromatography system, equipped with a separation column (Asahipak NH2P-50 4E, Resonac, Tokyo, Japan), a differential refractive index detector, and an autosampler (Prominence, Shimadzu, Kyoto, Japan). The content of monosaccharides in the solution was converted to polysaccharide content using anhydrous correction factors: 0.90 for glucose, mannose, and galactose, and 0.88 for xylose and arabinose. These treatments and analyses used two blocks for each condition.

### Confocal laser scanning microscopy (CLSM)

To analyze the anatomical structure of the hardwood blocks after alcoholysis and the Wise treatment, we conducted a detailed preparation and observation procedure. Initially, the water-saturated cross sections of the hardwood blocks were frozen on the sample stage of the sliding microtome. These sections were then carefully trimmed to a thickness of approximately 2 mm. After slowly defrosting the frozen samples on glass slides in a refrigerator, any excess water was removed. The trimmed samples were stained with Calcofluor White M2R to highlight polysaccharides including cellulose [18]. It is important to note that the actual concentration of Calcofluor White M2R was effectively reduced below 0.25% due to the water content in the samples. The staining process was conducted at room temperature for 20 min. Subsequently, the samples were repeatedly washed in water, followed by the removal of excess water using filter paper. Fluorescence images of the stained samples were captured using a confocal laser scanning microscope (AXR, Nikon, Tokyo, Japan), coupled with an inverted microscope (ECLIPSE Ti2-E, Nikon, Tokyo, Japan). The imaging process utilized excitation wavelengths of 405 nm and an emission range of 419–448 nm. These observations used two blocks for each condition.

### X-ray diffraction (XRD) analysis

The hardwood blocks were trimmed using a razor and subjected to X-ray diffraction (XRD) analysis. The XRD patterns, both before and after chemical treatment, were obtained using a wide-angle X-ray diffractometer (R-Axis RAPID, Rigaku, Tokyo, Japan) operated at 50 kV and 250 mA. The microfibril angles (MFAs) were determined from the (200) reflection of cellulose  $I_\beta$ , employing Gaussian function fitting [8]. These analyses used three blocks for each condition.

Furthermore, X-ray diffractograms in reflection mode were acquired using an automated multipurpose X-ray diffractometer (SmartLab, Rigaku, Tokyo, Japan) with Cu-K $\alpha$  radiation ( $\lambda = 1.5418 \text{ \AA}$ ) at 40 kV and 30 mA. The relative crystallinity index and the full-width at half-maximum (200) of the samples were calculated using non-linear least-squares fitting based on the pseudo-Voigt function, in accordance with methodologies by Wada and Okano [14], and Hirano [19]. These analyses used two blocks for each condition.

## Results and discussion

### Characterization of anatomical features

Figure 1 presents cross-, tangential-, and radial-sections of the Fc, Pt, and Zs blocks stained with safranin and Astra Blue. These hardwood samples exhibited several distinct features, such as vessels, rays, wood fibers, and parenchyma, each unique to the species as described below:

*Fagus crenata*, a diffuse-porous wood, was characterized by vessels that were widely distributed and gradually reduced in diameter and number from earlywood to latewood, as shown in the cross- and radial-sections. This wood type was notable for its uniseriate and multiseriate rays, including considerably broad rays, as shown in the cross and tangential sections. Vessels, rays, and wood fibers were recognized clearly in all the sections.

*Paulownia tomentosa*, a semi-ring-porous wood, comprised vessels that quickly decreased in diameter from earlywood to latewood compared to those of *F. crenata*, as shown in the cross- and radial-sections. Vessels were surrounded by axial parenchyma, forming specific lenticular anatomical structures known as aliform and confluent parenchyma for small vessels in latewood. Similar to *F. crenata*, vessels, multiseriate rays, wood fibers, and parenchyma were observed in all the sections.

*Zelkova serrata*, a ring-porous wood, featured tangential bands formed by large-diameter vessels arranged perpendicularly to the rays in earlywood, as shown in the cross section. Axial parenchyma were localized around clusters of smaller-diameter vessels, while multiseriate rays ran almost regularly spaced. As usual, vessels, rays,

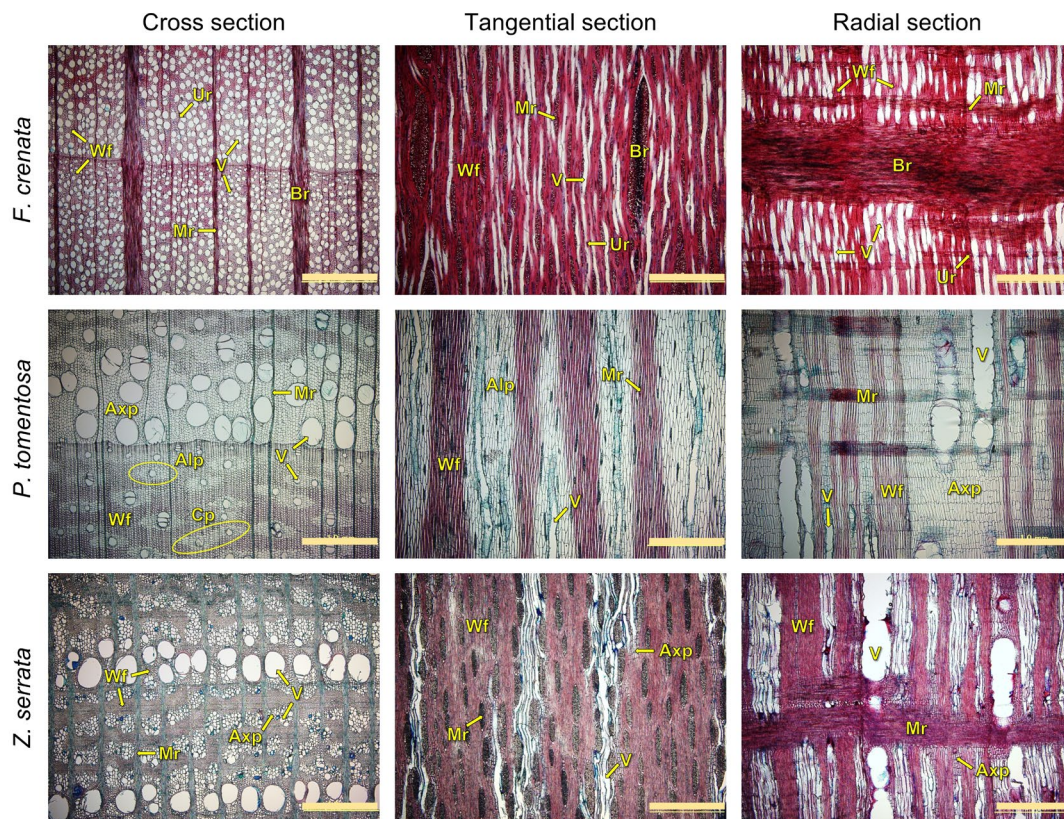


**Fig. 1** Optical microscopic images of samples double stained by safranin and Astra Blue. Scale bars = 1.0 mm. Alp, aliform parenchyma; Axp, axial parenchyma; Br, broad ray; Cp, confluent parenchyma; Mr, multiseriate ray; Ur, uniseriate ray; V, vessel; Wf, wood fiber

wood fibers, and parenchyma were recognized in all the sections.

#### Optimization of delignified conditions

In this study, the Fc and Pt blocks (Fig. 2a, d) were treated by alcoholysis at 150 °C for 1 h, followed by the Wise treatment. This specific combination of treatments was optimized for a softwood from *C. japonica*, as reported in our previous study [7]. While the Fc blocks retained some coloration after treatment (Fig. 2b), the Pt blocks were entirely decolorized (Fig. 2e). Notably, extending the alcoholysis duration from 1 to 3 h resulted in complete decolorization of the Fc blocks following the Wise treatment (Fig. 2c). For the Zs blocks (Fig. 2f), neither the combination of the Wise treatment with alcoholysis at 150 °C for 3 h nor the repeated alcoholysis (three times for 1 h) led to decolorization (Fig. 2g, h). However, when the alcoholysis was conducted twice for 3 h at 150 °C, followed by the Wise treatment, the internal color of the Zs blocks was successfully removed. This outcome highlights that manipulating the conditions of alcoholysis is instrumental in achieving decolorization in hardwood blocks, similarly to softwood blocks.

FTIR spectroscopy was employed to assess the changes in the chemical composition of the hardwood blocks resulting from the decolorization process, as shown in Fig. 3. Untreated hardwood blocks exhibited characteristic FTIR bands at 1730  $\text{cm}^{-1}$  and 1505  $\text{cm}^{-1}$  (Fig. 3a, d, f). The band at 1730  $\text{cm}^{-1}$  was attributed to the C=O stretching in the acetyl groups and carboxylic acids from hemicellulose [20], while the 1505  $\text{cm}^{-1}$  band was linked to the aromatic skeletal vibrations in lignin [21]. Upon examining the FTIR spectra of Fc blocks under two delignification conditions (Fig. 3b, c), it was observed that the band at 1505  $\text{cm}^{-1}$  disappeared in both cases; the band at 1730  $\text{cm}^{-1}$  persisted in the spectrum of the samples that were treated by alcoholysis at 150 °C for 1 h followed by the Wise treatment and remained non-decolorized (Fig. 3b). This finding suggests that the extent of decolorization is indicative of the removal of non-cellulosic components. In contrast, for the completely decolorized Pt blocks, the 1730  $\text{cm}^{-1}$  band was still present (Fig. 3e). This implies that the reduction in band intensity required for decolorization may vary among different wood species. In the case of Zs blocks treated with the Wise treatment combined with alcoholysis at 150 °C for 3 h or three

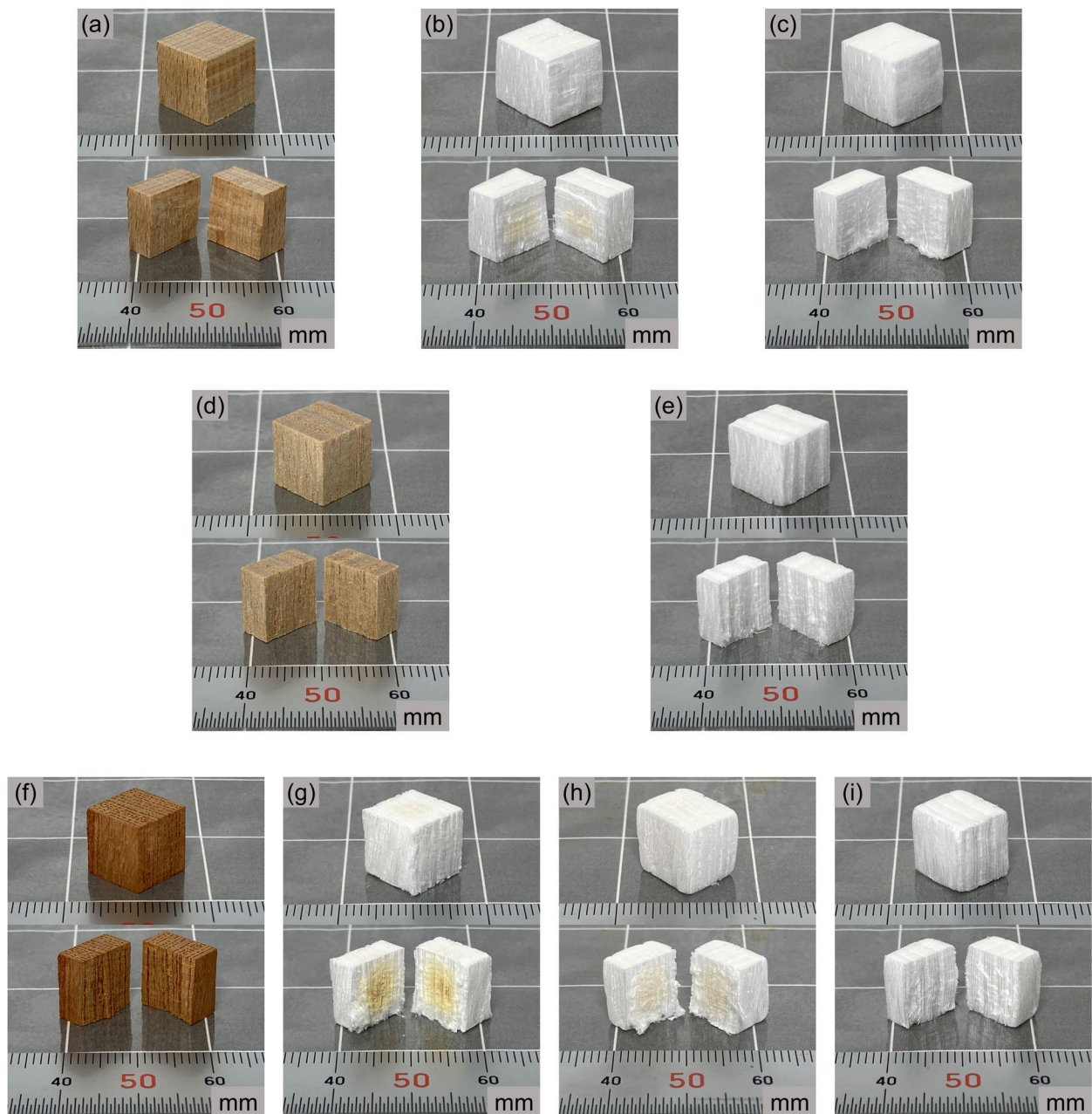


**Fig. 2** Appearance and longitudinal sections of samples before and after the Wise treatment combined with alcoholysis. **a–c** *F. crenata*, **d, e** *P. tomentosa*, **f–i** *Z. serrata*. **a, d, f** Untreated. Alcoholysis at 150 °C **b, e** for 1 h, **c, g** for 3 h, **h** three times for 1 h, **i** twice for 3 h, all followed by the Wise treatment at 70 °C for 8 h

times for 1 h, the  $1730\text{ cm}^{-1}$  band remained unchanged, and a shoulder band at  $1505\text{ cm}^{-1}$  was noticeable (Fig. 3g, h). However, when the alcoholysis was performed at 150 °C twice for 3 h, there was a significant decrease in the absorbance of the  $1730\text{ cm}^{-1}$  band, and the  $1505\text{ cm}^{-1}$  band disappeared in the FTIR spectra of the treated Zs blocks. These results demonstrate that the majority of

non-cellulosic components in the Fc, Pt, and Zs blocks were successfully removed using the two-step chemical treatment.

Table 1 presents a quantitative analysis of the changes in chemical composition of the hardwood blocks, specifically focusing on the removal of non-cellulosic components. The results indicate that the two-step chemical

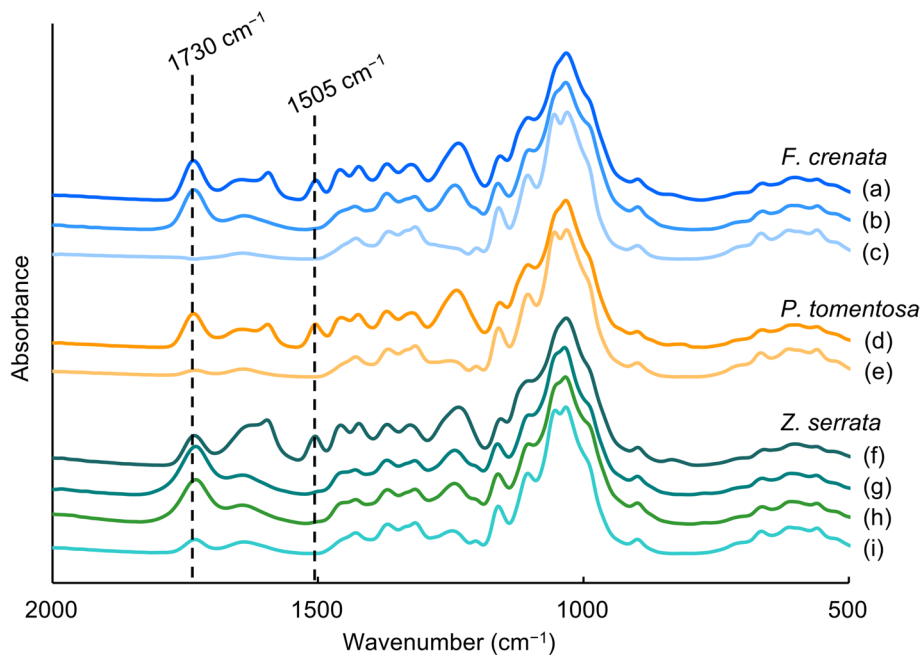


**Fig. 3** FTIR spectra before and after the Wise treatment combined with alcoholysis. **a–c** *F. crenata*, **d, e** *P. tomentosa*, **f–i** *Z. serrata*. **a, d, f** Untreated. Alcoholysis at 150 °C **b, e** for 1 h, **c, g** for 3 h, **h** three times for 1 h, **i** twice for 3 h, all followed by the Wise treatment at 70 °C for 8 h

treatments were largely successful in eliminating most of the acid-insoluble fraction. However, exceptions were noted in the case of Zs blocks treated with the Wise treatment combined with alcoholysis at 150 °C for either 3 h or three times for 1 h, as evidenced by the persistence of the shoulder band at 1505 cm<sup>-1</sup> in their FTIR spectra (Fig. 3g, h). In this study, the components of the acid-insoluble fraction, including lignin, ash, and extractives,

were not individually quantified. The primary focus was on determining whether the acid-insoluble fraction could be effectively removed. Although the actual lignin content appeared to vary slightly among the Fc, Pt, and Zs blocks [22–24], it was observed that the Pt blocks required a shorter duration of alcoholysis for delignification compared to the Fc and Zs blocks. This difference was attributed to the thinner cell walls of Pt blocks, as

**Table 1** Chemical component analysis before and after two-step chemical treatments

|                                      | Concentration (mg/100 mg) |        |       |        |          |          |
|--------------------------------------|---------------------------|--------|-------|--------|----------|----------|
|                                      | Acid-insoluble fraction   | Glucan | Xylan | Mannan | Arabinan | Galactan |
| <i>F. crenata</i>                    |                           |        |       |        |          |          |
| Untreated                            | 23.8                      | 46.0   | 19.6  | 1.1    | 1.0      | 0.6      |
| Alcoholysis 1 h → Wise treatment     | 0.4                       | 96.1   | 10.4  | 0.6    | 0.6      | 0.1      |
| Alcoholysis 3 h → Wise treatment     | 0.9                       | 108.1  | 2.4   | 0.3    | 0.3      | 0.9      |
| <i>P. tomentosa</i>                  |                           |        |       |        |          |          |
| Untreated                            | 28.0                      | 39.1   | 16.4  | 0.3    | 2.5      | 1.3      |
| Alcoholysis 1 h → Wise treatment     | 0.4                       | 85.5   | 0.8   | 0.1    | 1.6      | 1.7      |
| <i>Z. serrata</i>                    |                           |        |       |        |          |          |
| Untreated                            | 40.0                      | 25.7   | 11.3  | 1.3    | 0.8      | 0.7      |
| Alcoholysis 3 h → Wise treatment     | 3.8                       | 63.0   | 8.8   | 0.7    | 1.1      | 0.0      |
| Alcoholysis 1 h × 3 → Wise treatment | 1.5                       | 69.7   | 7.8   | 0.6    | 1.7      | 2.0      |
| Alcoholysis 3 h × 2 → Wise treatment | 0.3                       | 86.6   | 2.5   | 0.0    | 1.9      | 1.4      |

Alcoholysis was performed at 150 °C, and the Wise treatments were conducted at 70 °C for 8 h

indicated by their low density (Table 2). This consideration was supported by the finding that the diffusion of dissolved lignin molecules within the cell wall layer was identified as the rate-determining step in the delignification process using organosolv treatment [25]. Interestingly, despite having similar densities, the Fc and Zs blocks required different alcoholysis conditions. This observation suggests that factors beyond density, such as anatomical structures, may play a significant role in determining the optimal conditions for alcoholysis. Further research is planned to find these factors more thoroughly, employing a diverse range of hardwood samples with varying densities and anatomical structures, especially the distribution and diameter of vessels. In conclusion, the two-step chemical treatment was effective in removing the acid-insoluble fraction, including lignin, under appropriate alcoholysis conditions.

Table 1 further reveals that the reduction in xylan and mannan, major components of hemicellulose, was more pronounced in samples exhibiting a lower absorbance of the band at  $1730\text{ cm}^{-1}$  in Fig. 3. This trend is particularly evident in the case of Fc blocks, where alcoholysis at  $150\text{ }^{\circ}\text{C}$  for 3 h was more effective in removing non-cellulosic components compared to the 1 h condition, despite both conditions achieving delignification. Table 1 also presents data on glucan content, which is predominantly attributed to cellulose, given the low mannan (glucomannan) content in each hardwood species. This information leads to two significant insights for future research. First, the high glucan content in the delignified Fc, Pt, and Zs blocks suggests that they are almost entirely composed of cellulose. Second, calculating absolute glucan amounts by multiplying the glucan contents (as shown in Table 1) by the mass ratios (as indicated in Table 2) provides an estimate of the cellulose amounts before and after treatment.

Based on these calculations, it was deduced that the treated Fc blocks under the 3 h alcoholysis condition, treated Pt blocks under the 1 h condition, and treated Zs blocks under the twice 3 h condition retained 67%, 84%, and 63% of their original cellulose amounts, respectively. Although there is potential for further improvement in preserving cellulose, these chemical treatments were consistent with the study's objective. They reduce lignin and hemicellulose contents to less than 3% and 5%, respectively, without causing structural collapse in the hardwood blocks. Consequently, the optimal alcoholysis conditions for eliminating non-cellulosic components from Fc, Pt, and Zs blocks were determined to be  $150\text{ }^{\circ}\text{C}$  for 3 h, 1 h, and twice for 3 h, respectively. Moreover, the resulting decolorized cellulose samples were defined as colorless hardwoods.

### Structural evaluation of colorless woods

The dimensional stability of colorless hardwoods was examined to verify the preservation of their hierarchical structure (Table 2). This assessment involved comparing the dimensions of colorless hardwoods with those of the original blocks subjected to water saturation and freeze-drying to eliminate any effects attributable to the drying process. Notably, no shrinkage or swelling was observed in the L-direction. This observation aligns with Kurei [26], who reported that degradation of crystalline cellulose in the secondary wall leads to L-directional shrinkage. The absence of L-directional change in colorless hardwoods implies that their cellulose architectures were almost sufficient to maintain the original hardwood morphologies. In contrast, the R- and T-directions of colorless hardwoods exhibited more swelling compared to the original wood blocks, likely due to delignification. It is understood that rays contributed to suppress

**Table 2** Change in dry dimensions, masses, and densities of hardwood blocks

|                                                   | Ratios before and after chemical treatments |               |               |               | Density (kg m <sup>-3</sup> ) |
|---------------------------------------------------|---------------------------------------------|---------------|---------------|---------------|-------------------------------|
|                                                   | Longitudinal                                | Radial        | Tangential    | Mass          |                               |
| <i>F. crenata</i>                                 |                                             |               |               |               |                               |
| Water saturation → Freeze-dry                     | 1.005 (0.001)                               | 0.989 (0.003) | 1.030 (0.013) | –             | 532 (25)                      |
| Alcoholysis 3 h → Wise treatment → Freeze-dry     | 1.002 (0.014)                               | 1.022 (0.009) | 1.138 (0.007) | 0.284 (0.011) | 128 (13)                      |
| <i>P. tomentosa</i>                               |                                             |               |               |               |                               |
| Water saturation → Freeze-dry                     | 1.007 (0.001)                               | 1.006 (0.007) | 1.003 (0.004) | –             | 221 (14)                      |
| Alcoholysis 1 h → Wise treatment → Freeze-dry     | 1.000 (0.001)                               | 1.023 (0.005) | 1.107 (0.020) | 0.383 (0.004) | 85 (3)                        |
| <i>Z. serrata</i>                                 |                                             |               |               |               |                               |
| Water saturation → Freeze-dry                     | 1.001 (0.000)                               | 0.989 (0.000) | 0.994 (0.002) | –             | 565 (5)                       |
| Alcoholysis 3 h × 2 → Wise treatment → Freeze-dry | 1.016 (0.006)                               | 1.019 (0.008) | 1.099 (0.025) | 0.188 (0.003) | 92 (4)                        |

Alcoholysis was performed at  $150\text{ }^{\circ}\text{C}$ , and the Wise treatments were conducted at  $70\text{ }^{\circ}\text{C}$  for 8 h. Numbers in parentheses indicate standard deviations calculated from 3 samples

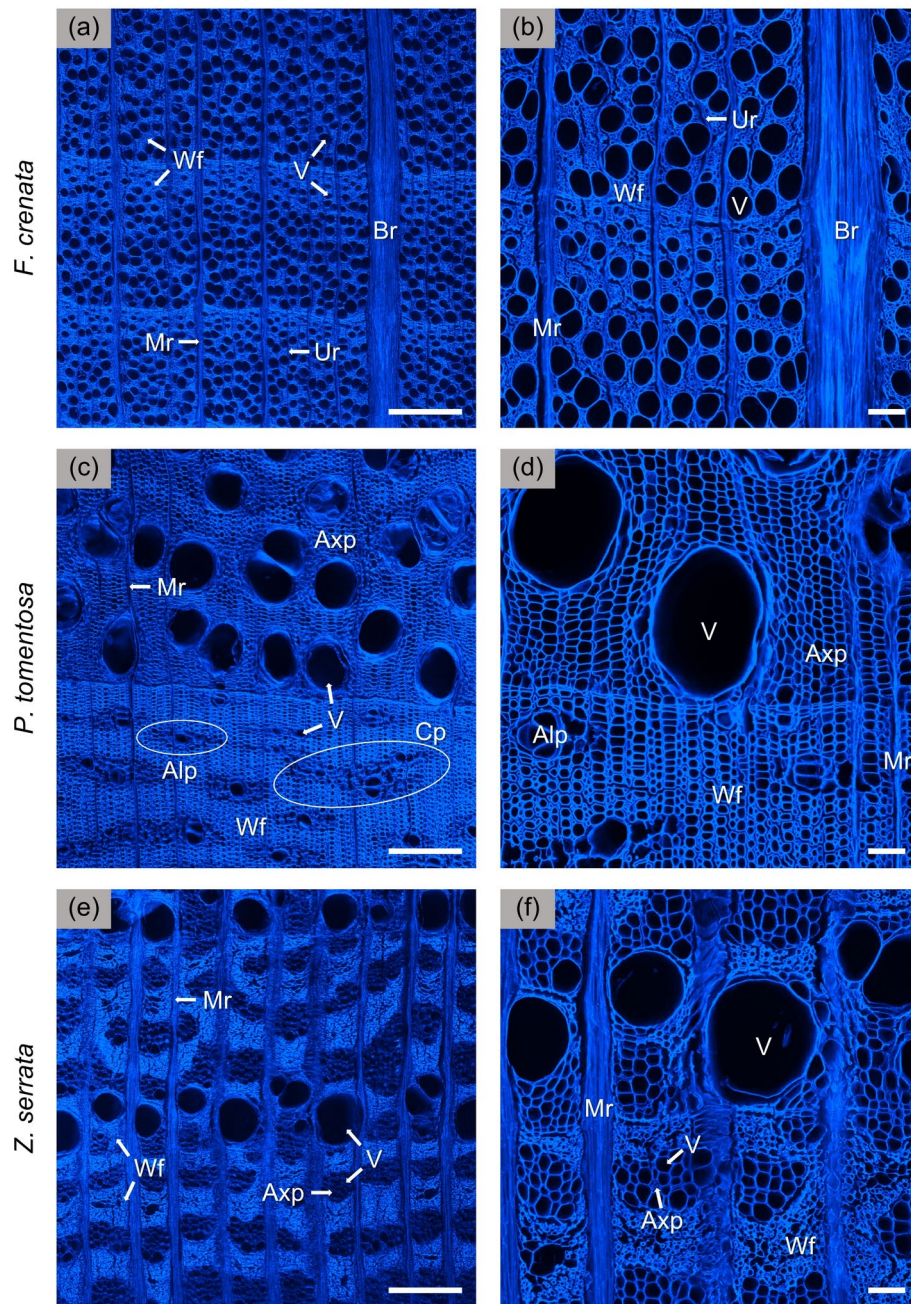


**Fig. 4** CLSM images after the Wise treatment combined with alcoholysis. **a, b** *F. crenata*; **c, d** *P. tomentosa*; **e, f** *Z. serrata*. Alcoholysis at 150 °C **a, b** for 3 h; **c, d** for 1 h; **e, f** twice for 3 h, all followed by the Wise treatment at 70 °C for 8 h. Imaging mode: fluorescent after staining with Calcofluor white M2R. Scale bars = (**a, c, e**) 500  $\mu$ m or (**b, d, f**) 100  $\mu$ m. Alp, aliform parenchyma; Axp, axial parenchyma; Br, broad ray; Cp, confluent parenchyma; Mr, multiseriate ray; Ur, uniseriate ray; V, vessel; Wf, wood fiber

R-directional changes and to promote T-directional ones [27]. In fact, Fc blocks, characterized by broad rays, exhibited more significant different ratios of R- and T-directional change than Pt and Zs blocks. Therefore, the observed dimensional changes, influenced by anatomical structures, were indicative of the retention of

these structures in colorless hardwoods. Importantly, these changes were not destructive and did not fatally impact the dimensional stability of the hardwood blocks.

For evaluating the anatomical structures after the two-step chemical treatments, the traditional observation method using a series of dehydration followed by the

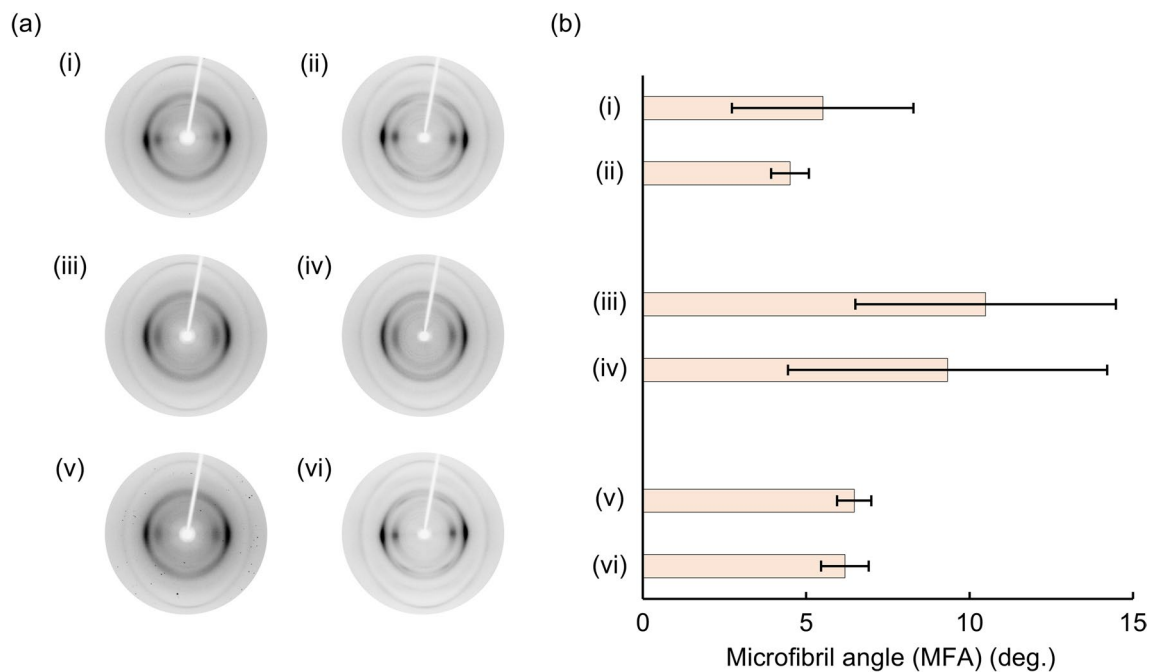


**Fig. 5** **a** Typical X-ray fiber diffraction patterns and **b** microfibril angles calculated from 200-plane diffraction patterns. (i, ii) *F. crenata*; (iii, iv) *P. tomentosa*; (v, vi) *Z. serrata*. (i, iii, v) untreated. Alcoholysis at 150 °C (ii) for 3 h, (iv) for 1 h, (vi) twice for 3 h, all followed by the Wise treatment at 70 °C for 8 h. Error bars indicate the standard deviation calculated from 3 samples

resin-embedding technique has been applied, but the colorless hardwoods were partially deconstructed during this processing. Therefore, a simple ice-embedding combined with a sliding microtome was devised for trimming, and the obtained surfaces were visualized by CLSM. The CLSM images confirmed that the Fc, Pt, and Zs blocks preserved their cell arrangements formed by vessels, rays, wood fibers, and parenchyma shown in the optical microscopic ones (Figs. 1, 4a, c, e). Although magnified CLSM images showed separation between cells, which may be due to the dissolution of lignin in the compound middle lamella by the chemical treatments, the cell arrangements were still largely maintained (Fig. 4b, d, f). Furthermore, the magnified views revealed that the two-step chemical treatment did not affect the cell structures. For distinct microscopic features, widely distributed vessels, uniseriate and multiseriate rays including broad rays, and wood fibers remained in colorless Fc blocks (Fig. 4a, b). In colorless Pt blocks, vessels, multiseriate rays, axial parenchyma involving aliform and confluent parenchyma, and wood fibers were also visualized (Fig. 4c, d). Additionally, the tangential bands formed by large vessels, the clusters of small vessels and axial parenchyma, and multiseriate rays running regularly spaced were observed in colorless Zs blocks (Fig. 4e, f). Thus, it was found that the colorless hardwoods maintained the anatomical structures unique to each wood species.

This observation corroborates the earlier discussion on dimensional changes, affirming that colorless hardwoods successfully retain their original microscopic structures.

To verify the preservation of cell wall layer structure in addition to the microscopic features, we investigated the microfibril orientation of hardwood blocks before and after two-step chemical treatments (Fig. 5). The XRD patterns of colorless hardwoods displayed distinct diffraction spots similar to those of untreated blocks (Fig. 5a), indicating that the microfibril orientation remained unaltered by the treatments. While some samples showed variability in MFAs, as calculated from X-ray fiber diffraction patterns (Fig. 5b), this was attributed to the presence of multiple cells with differing microfibril angles in hardwoods [28]. However, the average MFAs did not significantly change after treatment, suggesting that the colorless hardwoods retained the original microfibril orientation. It is noteworthy that the MFAs of colorless softwoods from *C. japonica* were approximately 3 degrees [7], whereas those of colorless hardwoods from the Fc, Pt, and Zs blocks were higher, particularly for the Pt blocks, which exhibited a difference of approximately 7 degrees. MFAs are known to be closely linked to the mechanical properties of cellulosic materials [8]. Therefore, the development of colorless woods from various wood species with distinct MFAs opens avenues for customizing these materials for specific applications.

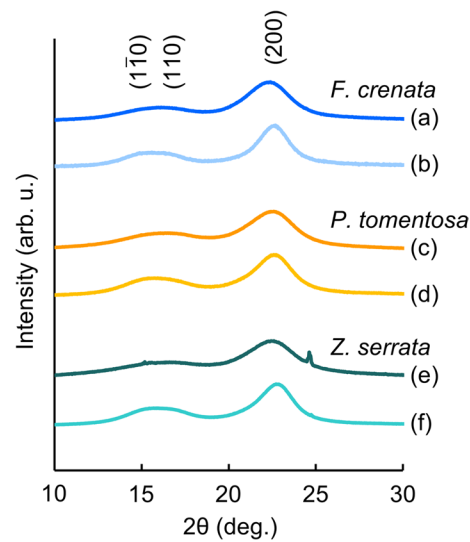


**Fig. 6** X-ray diffractograms. **a, b** *F. crenata*; **c, d** *P. tomentosa*; **e, f** *Z. serrata*. **a, c, e** Untreated. Alcoholysis at 150 °C **b** for 3 h, **d** for 1 h, **f** twice for 3 h, all followed by the Wise treatment at 70 °C for 8 h

The crystalline structures of cellulose in colorless hardwoods were thoroughly analyzed, as shown in Fig. 6. The diffractograms of all samples displayed characteristics of the cellulose I polymorph [29], suggesting that the crystalline structure of cellulose was virtually unchanged before and after chemical treatments. Particularly for Zs blocks, diffraction peaks associated with calcium oxalate crystals were noted at approximately 15 and 24 degrees [30, 31]. The intensity of these peaks in the diffractogram of colorless Zs blocks was observed to be lower than that in untreated Zs blocks. This observation suggests that most of the calcium oxalate was removed during the two-step chemical treatments. Moreover, an improvement in cellulose crystallinity was noted in each colorless hardwood compared to the original blocks. This improvement

was evident from the increase in the relative crystallinity index and a decrease in the full-width at half-maximum of the (200) reflection (Table 3). Such a trend is attributed to the removal of non-cellulosic components, similar to observations made in the case of *C. japonica* [7]. In summary, the colorless hardwoods of Fc, Pt, and Zs are cellulosic materials that maintain a hierarchical structure from anatomical structures to microstructures.

Conclusion

In this study, we successfully demonstrated that the optimal alcoholysis conditions specific to each wood species, followed by the Wise treatment, effectively removed non-cellulosic components, facilitating the preparation of colorless hardwoods. The two-step chemical treatments were applied to a variety of woods, involving diffuse-porous, semi-ring-porous, and ring-porous types, and were achieved without the collapse of their microscopic features. Importantly, both the cellulose orientation and crystallinity were preserved throughout the process. These methods proved to be a versatile approach, capable of maintaining the hierarchical structure of both softwoods and various hardwoods. This versatility is crucial for creating cellulosic materials with distinct properties corresponding to different wood species. Extending the alcoholysis time will enable complete decolorization of large woods. Based on the findings gained from this study, we plan to expand our development of colorless hardwoods to several more wood species and evaluate their mechanical properties. This initiative is driven by a comprehensive understanding of the tailor-made cellulose architectures naturally produced by trees. Our efforts pave the way for the development of innovative wood-derived cellulose materials, which will significantly advance the state of sustainable materials.

**Table 3** Relative crystallinity index and full-width at half-maximum for untreated and colorless hardwoods

|                                                   | Relative crystallinity index | Full-width at half-maximum of (200) (deg.) |
|---------------------------------------------------|------------------------------|--------------------------------------------|
| <i>F. crenata</i>                                 |                              |                                            |
| Untreated                                         | 0.74                         | 2.91                                       |
| Alcoholysis 3 h → Wise treatment → Freeze-dry     | 0.90                         | 2.11                                       |
| <i>P. tomentosa</i>                               |                              |                                            |
| Untreated                                         | 0.67                         | 2.98                                       |
| Alcoholysis 1 h → Wise treatment → Freeze-dry     | 0.81                         | 2.54                                       |
| <i>Z. serrata</i>                                 |                              |                                            |
| Untreated                                         | 0.65                         | 3.20                                       |
| Alcoholysis 3 h × 2 → Wise treatment → Freeze-dry | 0.81                         | 2.38                                       |

Alcoholysis was performed at 150 °C, and the Wise treatments were conducted at 70 °C for 8 h

## Abbreviations

|      |                                    |
|------|------------------------------------|
| Fc   | <i>Fagus crenata</i>               |
| Pt   | <i>Paulownia tomentosa</i>         |
| Zs   | <i>Zelkova serrata</i>             |
| FTIR | Fourier transform infrared         |
| CLSM | Confocal laser scanning microscopy |
| XRD  | X-ray diffraction                  |
| MFAs | Microfibril angles                 |

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## Author contributions

SS drafted the manuscript and carried out the experiments. MM carried out the confocal microscopy. RT supported the preparation of colorless hardwoods. SN and RF contributed to the structural evaluation. TK edited the manuscript and supported the data analysis. YH supervised the entire study. All authors have read and agreed to the published version of the manuscript.

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## Availability of data and materials

All data and materials in this study are included in this published article.

## Declarations

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

All the authors gave consent for publication.

### Competing interests

The authors declare that they have no competing interests.

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