Application of Histochemical Stains for Rapid Qualitative Analysis of the Lignin Content in Multiple Wood Species

Yan Yang,[a] Yiming He,[a] Li Han,[b] Aifeng Wang,[a] Wei Wang,[a] Rui Zhao,[a] and Bin Li [a,*]

Rapid qualitative analysis was used to determine the influence of the lignin content of wood cell walls on the compression and bending properties of multiple wood species. The lignin type and cell wall content of *Cunninghamia lanceolate*, *Fagus longipetiolata*, *Betula alnoides*, *Fraxinus mandshurica*, and *Tectona grandis* was analyzed *via* histochemical staining, which included: the Mäule staining reaction, the Weisner staining reaction, and a fluorescence reaction. The results showed that the more red the Mäule staining reaction was, the greater the Syringyl lignin (S-type lignin) content was, and the more yellowish-brown the Mäule staining reaction was, the greater the Guaiacyl lignin (G-type lignin) content was. In addition, the more reddish-purple the Wiesner staining reaction was, the greater the lignin content was. The greater the brightness value of the fluorescence reaction was, the greater the lignin content was. Due to the negative correlation between the lignin content of the wood cell wall and the bending and compression properties of the wood, the application of histochemical stains for the analysis of wood lignin content could provide a reference and experimental basis for bending and compression treatments of various woods.

Keywords: Lignin content; Guaiacyl lignin (G-type lignin); Syringyl lignin (S-type lignin); Mäule staining reaction; Weisner staining reaction; Fluorescence reaction

*Contact information: a: School of Architecture and Urban Planning, Nanyang Institute of Technology, Nanyang City, Henan Province, 473000, P.R. China; b: Henan Key Laboratory of Zhang Zhongjing Formulae and Herbs for Immunoregulation, Nanyang Institute of Technology, Nanyang City, Henan Province, 473000, P.R. China; * Corresponding author: yangyanrainy@163.com*

INTRODUCTION

The wood cell wall is primarily composed of cellulose, hemicellulose, and lignins. As a basic unit of the wood cell wall, the lignin content has a strong influence on pulping and paper making processes, bending, and compression, *etc.* In general, the greater the lignin content, the greater will be the consumption of chemicals needed to remove the lignins from wood. This also means that a greater amount of energy generally is required to break the lignin polymers into small molecular structures during the softening process, making the process more difficult (Cui *et al.* 2016). In addition, the lignin content directly affects the degree of hardness for the wood. Wood with a higher lignin content usually is more difficult to bend or compress. The study of lignin content in multiple wood species could provide a preliminary reference and theoretical basis for the specific use of wood.

Lignins contain chromogenic groups, such as carbonyls, carboxyls, and alkenes conjugated with a benzene ring, and chromophore groups, such as phenolic hydroxyls and alcohol hydroxyls. Therefore, the lignin content could be qualitatively or quantitatively determined *via* a chromogenic or chromic reaction (Liu *et al.* 2004). The important colour reactions for the determination of lignin content were the Mäule staining reaction and the

Wiesner staining reaction. The Mäule staining reaction primarily stains syringyl lignins (S-type lignins) in the wood cell wall into a red or reddish-purple, and it stains guaiacyl lignins (G-type lignins) to a yellow or yellowish-brown. The lignin type in softwoods is primarily the G-type lignin, while hardwoods contain both G-type lignins and S-type lignins (Liu *et al.* 2004). Therefore, the Mäule staining reaction can be used to distinguish softwood from hardwood, *i.e.*, the difference between G-type lignin and S-type lignin content (Liu *et al.* 2004; Yang *et al.* 2012). The greater the S-type lignin content, the more reddish or reddish-purple will be the stained wood (Nakagawa e*t al.* 2012). The Wiesner staining reaction stains both S-type lignins and G-type lignins in wood cell walls a reddish-purple (Yang *et al.* 2012). The slightly reddish-purple color of the lignins after the Wiesner staining reaction indicates a greater S-type lignin content, while the dark reddish-purple indicates a greater G-type lignin content (Takabe *et al.* 1992; Nakagawa *et al.* 2012; Cui *et al.* 2016). The lignin content also has been determined *via* fluorescence reaction. Generally, the greater the fluorescence intensity, the greater was the lignin content (Xu *et al.* 2009; Nakagawa *et al.* 2012; Wang *et al.* 2012; Liu *et al.* 2017). Therefore, the authors could judge the lignin content in different cell types and wall structures of the wood cell wall according to the intensity of the fluorescence reaction.

In this study, the Mäule staining reaction, the Weisner staining reaction, and a fluorescence reaction were performed on several common wood species found in the Yunnan province of China. These wood species were qualitatively analyzed to determine the lignin content and obtain data references and a theoretical basis to establish whether these species were suitable for bending and compression treatments or pulping and paper making.

EXPERIMENTAL

Materials

The materials selected and tested were samples from multiple wood species: *Cunninghamia lanceolate* (as control); *Tectona grandis* with poor compression and bending properties; and *Fagus longipetiolata*, *Betula alnoides*, and *Fraxinus mandshurica* with excellent compression and bending properties. They were purchased from the Kung Ming wood market (Yunnan province, China) with no defects and an air dry moisture content of approximately 10%.

Methods

Mäule staining reaction

The Mäule staining reaction was carried out as follows: 10 μm to 20 μm sections were cut with a microtome (SM2000R, Leica, Buffalo Grove, IL). They were treated with a 1% potassium permanganate ($KMnO_4$) aqueous solution for 5 min, washed 3 times with distilled water, then treated with 3 M hydrochloric acid (HCl) for 1 min, washed again 3 times with distilled water, and then mounted in a 29% ammonia water solution. The sections were then observed *via* a light microscope (ECLIPSE 80i, Nikon, Tokyo, Japan) (Takabe *et al.*1992; Yang *et al.* 2010, 2012; Liu *et al.* 2017).

Wiesner staining reaction

The Wiesner staining reaction was carried out as follows: 10 μm to 20 μm sections were cut with a microtome and were treated with a 2% phloroglucin in 95% ethanol

solution for 5 min and then mounted with a 6 M hydrochloric acid (HCl) solution. The sections were then observed *via* a light microscope (Yang *et al.* 2010; Nakagawa *et al.* 2012; Yang *et al.* 2012; Liu *et al.* 2017).

Fluorescence reaction

The fluorescence reaction was carried out as follows: 10 μm to 20 μm sections were cut with a microtome and were stained with 0.001% acriflavine and then dehydrated with an ethanol solution with different gradients (30%, 50%, 70%, 90%, and 100%) then mounted with a 70% glycerol solution. The sections were then observed *via* fluorescence microscope (blue light with 488nm excitation source) (Donaldson *et al.* 2001; Xu *et al.* 2009; Nakagawa *et al.* 2012; Cui *et al.* 2016; Liu *et al.* 2017).

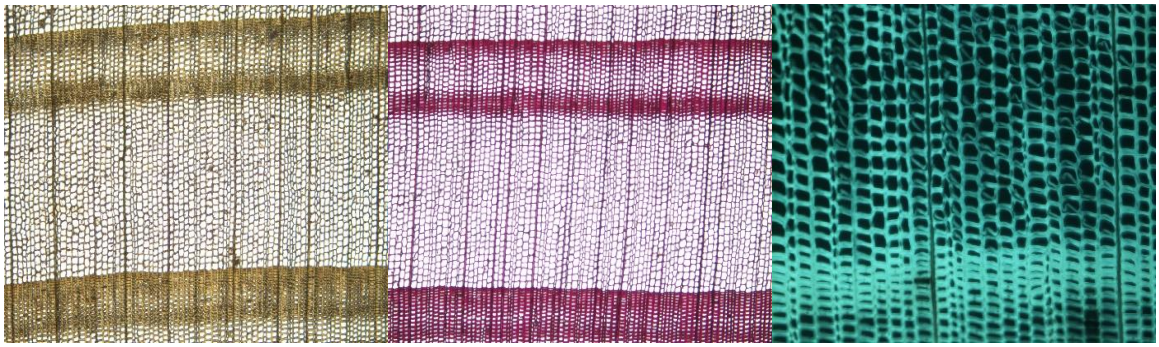
Working assumptions regarding the staining assays

To facilitate interpretation of the experimental findings, two key assumptions were made in this work. The first working hypothesis was that each of the three staining assays was 100% specific to the type or types of lignin, as described above. Though this assumption goes beyond what has been shown by others (Liu *et al.* 2004; Yang *et al.* 2012), it simplifies the interpretation. Second, it was assumed that the depth of staining will be directly proportional to the amount of target compound in the wood, *i.e.* the concentration of S-lignin, G-lignin, or their sum, in the case of the three assays. Again this assumption goes beyond what is claimed in the prior work (Takabe *et al.* 1992; Nakagawa e*t al.* 2012; Cui *et al.* 2016). There is a need for further experimentation to evaluate the precision of the anticipated proportionality. It is reasonable to expect that the depth of staining may depend partly on other factors such as the density of the wood material, details about the microstructure, and whether access to lignin domains are partly blocked by the polysaccharide components of the wood.

RESULTS AND DISCUSSION

Histochemical Staining Reactions of *Cunninghamia lanceolate*

The lignins in softwoods are primarily composed of G-type structure units, while the lignins in hardwoods include G- type and S-type structure units. Figure 1 shows the microstructures of the histochemical staining reactions in wood cell walls for *Cunninghamia lanceolate*. The cell walls of the tracheids and wood rays after the Mäule staining were stained a light yellowish-brown colour, as shown in Fig. 1a. The results showed that the lignins of the cell walls of the tracheids and wood rays were composed of G-type units, and almost no S-type units. The cell walls of the tracheids and wood rays after the Wiesner staining were stained a purple colour, as shown in Fig. 1b. The wood contained very few S-type lignins, so the purple colour should have come from the G-type lignins. Fluorescence microscopy revealed that the wood had a strong green fluorescence phenomena after undergoing the fluorescence reaction, as shown in Fig. 1c, and indicated that there was a high lignin content in the wood. The greater the lignin content in the wood, the more difficult the wood was to bend or compress. In addition, the cell wall layers of the tracheids and wood rays were observed. It was found that a strong green fluorescence phenomena was found in the compound middle lamellar (CML), the cell corners (CC), and the secondary cell walls (S) of the tracheids and wood rays. Thus, the lignin content in all the tracheid and wood ray wall structures was high.



(a) Mäule reaction staining (10X); (b) Miesner staining (10X); and (c) Fluorescence reaction (20X)

Fig. 1. Microstructure images of lignins in *Cunninghamia lanceolate* after histochemistry stainings

Histochemical Staining Reactions of *Betula alnoides*

Figure 2 shows the microstructures after the histochemical staining reactions in the wood cell walls for the *Betula alnoides* samples. The colour of the cell walls of the vessels, wood fibers, axial parenchymas, and wood rays after the Mäule staining reaction was a deeper red (Fig. 2a) than the *Cunninghamia lanceolate* samples (Fig. 1a). The results showed that there was a greater S-type lignin content in the vessels, wood fibers, axial parenchymas, and wood rays. Fergus and Goring (1970a,b) found that there was a greater G-type lignin content in the cell walls of wood vessels in *Betula* spp., while a greater S-type lignin content was found in the cell walls of the wood fibers. Musha and Goring (1975) also found that a greater G-type lignin content was found in the cell walls of the wood vessels of 14 woods. The colours of the cell walls of the wood fibers, axial parenchymas, and wood rays after the Wiesner staining reaction were reddish-purple, as shown in Fig. 2b. Combined with the Mäule staining reaction, as shown in Fig. 2a, the S-type lignins were primarily stained *via* the Wiesner staining reactions to a reddish-purple. Fluorescence microscopy revealed that it had a weaker green fluorescence phenomena after the fluorescence reaction in *Betula alnoides*, as shown in Fig. 2c, than the fluorescence reaction in *Cunninghamia lanceolate*. This indicated that there was a lower lignin content in *Betula alnoides*. Therefore, the difficulties of performing compression and bending processes on this wood species would be decreased, due to the lower lignin content. In addition, the cell wall layers of the wood fibers were observed, and it was found that the brightness was lower for the CML, CC, and S in the cell wall of the wood fibers.

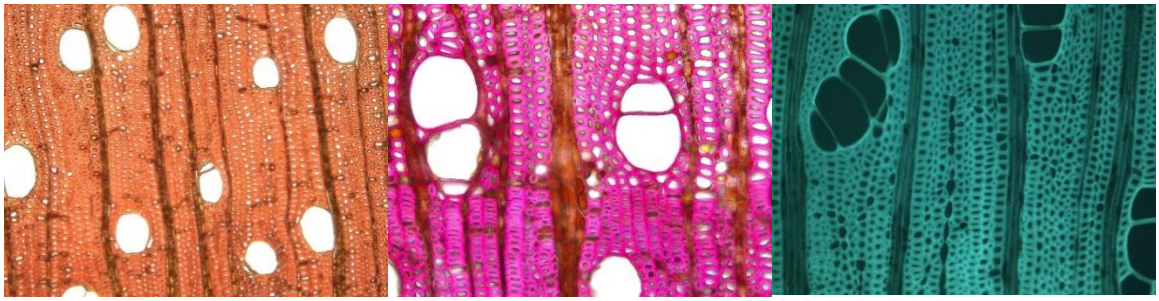


Fig. 2. The microstructure images of the lignins in *Betula alnoides* after histochemistry stainings. (a) Mäule reaction staining (10X); (b) Miesner staining (10X); and (c) fluorescence reaction (20X)

Histochemical Staining Reactions of *Fagus longipetiolata*

Figure 3 shows the microstructures after the histochemical staining reactions in the wood cell walls for the *Fagus longipetiolata* samples However, different type cell walls showed different colours. Among them, the colours of the cell walls of the latewood vessels, wood fibers, and axial parenchymas after the Mäule staining were a deep red, while the earlywood vessels and wood rays were a yellowish-brown, as shown in Fig. 3a. The results showed that there was a greater S-type lignin content in the latewood vessels, wood fibers, and axial parenchymas, while a greater G-type lignin content was found in earlywood vessels and wood rays. When the early and late wood fibers subjected to the Mäule staining reactions were compared, it was found that the early wood fibers were more reddish than the late wood fibers, which indicated that the cell walls of the early wood fibers had a greater S-type lignin content than the cell walls of the late wood fibers. Takabe *et al.* (1992) also found that there was a greater G-type lignin content in the cell walls of the early wood vessels of *Fagus crenata* than the cells walls in late wood vessels. In addition, the S-type lignin content in the cell walls of early wood fibers was greater than the cell walls of late wood fibers.

The colour of the cell walls of the wood fibers, axial parenchymas, and wood rays after the Wiesner staining reaction were a reddish-purple, as shown in Fig. 3b. Combined with the Mäule staining reaction (as shown in Fig. 3a), both the S-and G-type lignins were stained by the Wiesner staining reactions to a reddish-purple.

Fluorescence microscopy exhibited a weaker green fluorescence phenomena after the fluorescence reaction in *Fagus longipetiolata*, as shown in Fig. 3c, than the fluorescence reaction in *Cunninghamia lanceolate*. This indicated that *Fagus longipetiolata* had a lower lignin content. Therefore, the difficulties of performing compression and bending processes on this wood species would be decreased, due to the lower lignin content. In addition, the cell wall layers of the wood fibers were observed. It was found that the brightness of the CML and CC in the cell walls of the wood fibers were much greater than that of the S, which indicated that the CML and CC in the cell walls of the wood fibers had a greater lignin content than the S did.

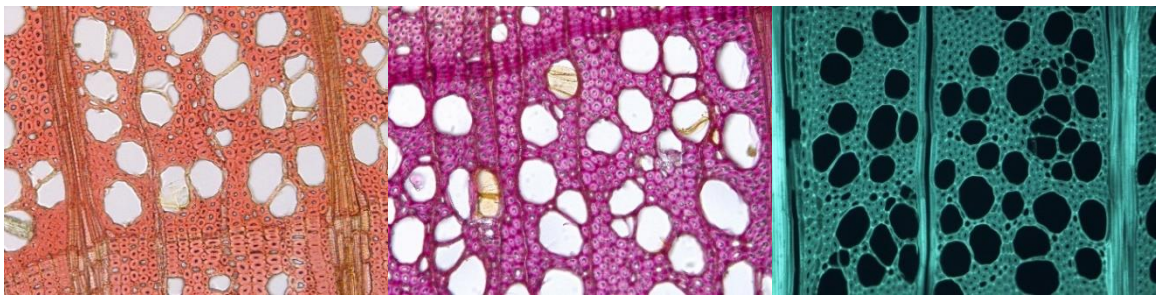


Fig. 3. The microstructure images of the lignins in *Fagus longipetiolata* after histochemistry stainings. (a) Mäule reaction staining (10X); (b) Miesner staining (10X); and (c) Fluorescence reaction (20X)

Histochemical Staining Reactions of *Fraxinus mandshurica*

Figure 4 showed the microstructures after the histochemical staining reactions in the wood cell walls for the *Fraxinus mandshurica* samples. The colours of the cell walls of the vessels, wood fibers, axial parenchymas, and wood rays after the Mäule staining were a deeper red (Fig. 4a) than the cell walls of *Cunninghamia lanceolate* (Fig. 1a). The results showed that there was a greater S-type lignin content compared to the other lignin

types in the vessels, wood fibers, axial parenchymas, and wood rays. The colour of the cell walls of the vessels, fibers, axial parenchymas, and wood rays after the Wiesner staining reaction was a reddish-purple, as shown in Fig. 4b. When combined with the Mäule staining reaction (Fig. 4a), the S-type lignins in the vessels, fibers, axial parenchymas, and wood rays after also being stained by the Wiesner staining reactions turned to a reddish-purple. Fluorescence microscopy revealed that it had a weaker green fluorescence phenomena after the fluorescence reaction in *Fraxinus mandshurica* (Fig. 4c) than the reaction in *Cunninghamia lanceolate* (Fig. 1c), but had a stronger green fluorescence phenomena than the fluorescence reactions of *Betula alnoides* (Fig. 2c) and *Fagus longipetiolata* (Fig. 3c). The brightness of the vessels and axial parenchymas were stronger than the wood rays and fibers, which indicated that the cell walls of the vessels and axial parenchymas had a greater lignin content than the wood rays and fibers. In addition, the cell wall layers of the vessels and axial parenchymas were observed. It was found that the CML, CC, and S layers all showed high brightness, which indicated that the CML, CC, and S layer in the cell walls of the vessels and axial parenchymas had a greater lignin content.

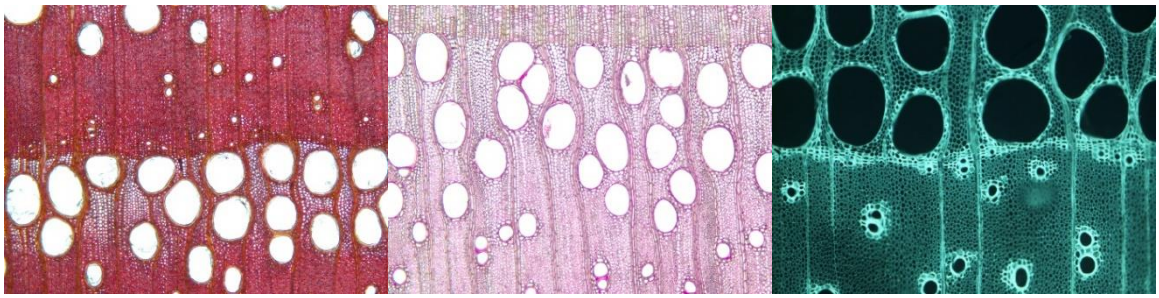
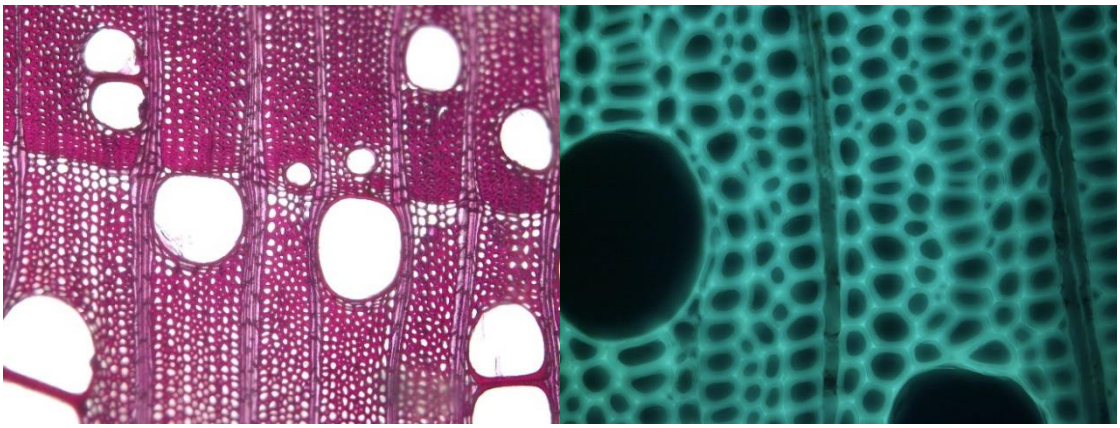


Fig. 4. The microstructure images of the lignins in *Fraxinus mandshurica* after histochemistry stainings. (a) Mäule reaction staining (10X); (b) Miesner staining (10X); and (c) Fluorescence reaction (20X)

Histochemical Staining Reaction of *Tectona grandis*

Figure 5 shows the microstructures after the histochemical staining reactions in the wood cell walls for the *Tectona grandis* samples. The colour of the cell walls of the vessels, wood fibers, axial parenchymas, and wood rays after the Mäule staining were a deeper yellowish-brown (Fig. 5a and 5b). This was observed especially for the colour of the tangential section (Fig. 5b), which was similar to the color of the *Cunninghamia lanceolate* samples after the Mäule staining (Fig. 1a). The results showed that there was a greater G-type lignin content compared to the other lignin types in the cell walls of the vessels, wood fibers, axial parenchymas, and wood rays in *Tectona grandis*. The colour of the cell walls of the vessels, fibers, axial parenchymas, and wood rays after the Wiesner staining reaction were a reddish-purple, as shown in Fig. 5c. When combined with the Mäule staining reaction (Fig. 5a and 5b), the G-type lignins in the vessels, fibers, axial parenchymas, and wood rays were stained *via* the Wiesner staining reactions to a reddish-purple. Fluorescence microscopy revealed that it had a stronger green fluorescence phenomena after the fluorescence reaction in *Tectona grandis* (Fig. 5d) than the fluorescence reactions in *Betula alnoides* (Fig. 2c), *Fagus longipetiolata* (Fig. 3c), and *Fraxinus mandshurica* (Fig. 4c), but it had a similar reaction to *Cunninghamia lanceolate* (Fig. 1c).

(a) Mäule reaction staining (10X) **(b)** Mäule reaction staining (10X)



(c) Miesner staining (20X) **(d)** Fluorescence reaction (40X)

Fig. 5. The microstructure images of the lignins in *Tectona grandis* after histochemistry stainings.

Some scholars have used a chemical analysis method to determine the lignin content of the above-mentioned woods. The lignin content of a branch of *Fraxinus rhynchophylla* was 26% (Nie 2012), *Betula alnoides* had a lignin content of 23.69% (Yang *et al*. 2015;Yang e*t al.* 2016), while the lignin content of *Tectona grandis* was greater than 30.91% (Moya *et al*. 2010; Miranda *et al*. 2011; Lourenço *et al*. 2015). The lignin content of *Tectona grandis* was also determined by the authors in this paper and the result was consistent with the results of Moya *et al*. (2010), Miranda *et al*. (2011), and Lourenço *et al*. (2015).

The authors in this paper found that the bending property of *Tectona grandis* (the ratio of the thickness of the curved wood (*t*) to the minimum radius of the curvature (*r*) was used as the evaluation index, where *t*/*r* was less than 1/8, the datum was unpublished) was much lower than the bending property of *Fraxinus rhynchophylla* (*t*/*r* = 1/2) (Shen *et al.* 2000). The greater the lignin content, the greater the rigidity. Therefore, the bending and compression properties of *Tectona grandis* were poor. There was also evidence that *Ulmus davidiana* var. *japonica* (*t*/*r* = 1/3) (Lu *et al.* 2013) and *Sassafras tzumu* (*t*/*r* = 1/5) (Yao 2014) also had good bending properties

CONCLUSIONS

1. The lignin type in *Cunninghamia lanceolate* was the G-type, and the fluorescence reaction was very strong, which implied that there was a greater lignin content in this wood. The greater lignin content would increase the difficulty of performing compression and bending processes on this wood species.
2. The fluorescence reactions to the lignins in the wood of *Betula alnoides*, *Fagus longipetiolata*, and *Fraxinus mandshurica* were not obvious, which indicated that the lignin contents in the woods was lower than the other tested species. The lower lignin content would reduce the difficulty of performing compression and bending processes on these wood species.
3. *Tectona grandis* contained more G-type lignins, and the overall fluorescence reaction was stronger than other tested species, which indicated that the wood had a greater lignin content. The greater lignin content would increase the difficulty of performing compression and bending processes on this wood species.
4. There was a negative correlation between the lignin content in the wood cell walls and the bending and compression properties of the woods. Therefore, the application of histochemical stains for the analysis of wood lignin content could provide a reference and experimental basis for whether they would be suitable for bending or compression treatments of various wood species.

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