



CELL WALL SYMPOSIUM



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# Imaging of Lignin and Cellulose In Hardwood using Fourier Transform Infrared Microscopy – Comparison of two Methods<sup>†</sup>

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### Abstract

Fourier transform infrared microscopy combined with a focal plane array detector was used to illustrate the distribution of cellulose and lignin in wood of European beech (*Fagus sylvatica* L.). Two independent methods were used for data analysis: (a) an integration; and (b) a correlation method. In method (a), the typical range of wave-numbers for cellulose (1390-1350  $\text{cm}^{-1}$ ) or lignin (1530-1490  $\text{cm}^{-1}$ ) was integrated in each spectrum and the distribution of the measured intensities was recorded. For the correlation method (b), reference Fourier transform infrared spectra for isolated beech lignin and cellulose were generated. The spectra obtained with the focal plane array detector in beech wood were correlated with reference spectra for cellulose or lignin, respectively illustrating the tightness of the correlation in each measuring point in the wood cross-section. Both methods gave similar results, but the integration method yielded higher resolution than the correlation method. Since structural properties of wood such as wall thickness and lumen width affected the results, the optimum method needs to be evaluated for each species separately.

**Keywords:** Cell wall; Cellulose; Fourier transform infrared imaging; FPA; FTIR spectroscopy; Hardwood; Lignin;

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### Introduction

Wood is primarily composed of cellulose, lignin, hemicelluloses and minor amounts of extractives (Sjöström, 1998; Fengel & Wegener, 2003). Cellulose ( $\text{C}_6\text{H}_{10}\text{O}_5$ )<sub>n</sub>, the major component, is a linear polymer consisting of (1→4)-linked  $\beta$ -D-glucose monomers. The cellulose molecules are arranged into fibrils, which are organised into elements that make up the cell wall of wood fibres. Most of the cell wall cellulose is crystalline (Fengel & Wegener, 2003). Various methods are known for the determination of cellulose, all requiring extraction steps. However, none of these methods yield cellulose in its native state (Fengel & Wegener, 2003). *In situ* analysis requires imaging techniques to study intact wood. For

example, cellulose fibrils in the S2 layer of *Picea abies* (Norway spruce) were localised by position-resolved synchrotron X-ray microdiffraction (Lichtenegger et al., 1999). The anisotropy of cellulose in cell walls of *P. mariana* (black spruce) was analysed by Raman imaging, revealing differences of the S2 and S1 layers, depending on the orientation of fibrils (Agarwal et al., 2007). However, analysis of the quantitative distribution of cellulose in different cell types is not possible by these techniques. Calcofluor and, more recently, fluorescent dyes specifically binding to cellulose have been used to quantify and image lignin distribution (Chaffey, 2002; Pinto et al., 2008). Furthermore Fourier Transform Infra-red (FTIR) microscopy has

been applied for studying cellulose distribution in plant tissues (Labbé et al., 2005; Naumann et al., 2007).

Lignin is a complex three-dimensional phenolic polymer, derived from three hydroxycinnamyl alcohols differing in the degree of methoxylation: *p*-coumaryl (4-hydroxy-cinnamyl), coniferyl (3-methoxy-4-hydroxy-cinnamyl, also called G-units) and sinapyl alcohol (3,5-dimethoxy-4-hydroxy-cinnamyl also called S-units) (Donaldson, 2001; Anterola & Lewis, 2002; Boerjan et al., 2003). Conifers contain mainly G-lignin, whereas hardwoods contain GS-lignin (Fengel & Wegener, 2003). To investigate lignin distribution, various microscopic methods have been developed such as microautoradiography, ultra-violet (UV) absorbance, interference microscopy, fluorescence microscopy or transmission electron microscopy (e.g. Fergus, 1969; Saka & Thomas, 1982; Donaldson, 1985; Donaldson, 2001). These have shown that lignin is accumulated particularly in the middle lamellae and in the secondary cell wall. Additionally, UV-microspectrophotometry and histochemical staining under acidic conditions with Wiesner and Mäule reagents have been used for localisation of lignin in wood tissue (Adler, 1948; Jensen, 1962; Iiyama & Pant, 1988; Möller et al., 2005; Schmitt et al., 2006). However, most of these methods do not distinguish between different lignin constituents and/or do not allow quantification.

FTIR Spectroscopy in combination with microscopy has the potential to resolve and quantify the distribution of organic components in tissues. Infrared radiation is absorbed by molecular bonds in the sample, such as C–H, O–H, N–H, C=O, C–C, resulting in bending, stretching, and twisting of the bonds and leading to characteristic transmittance and reflectance patterns at certain wavenumbers (Günzler & Gremlich, 2002). Combined with microscopy and imaging techniques, spatial resolution of the chemical composition is achieved (Salzer et al., 2000; Naumann et al., 2005; Naumann & Polle, 2006). FTIR Microscopy was used e.g. to detect fungi in *Fagus sylvatica* L. (beech) (Naumann et al., 2005) and for evaluation of differences in the chemical composition of transgenic aspens (Labbé et al., 2005). However, there are also significant methodological limitations with respect to the resolution (Naumann et al., 2007).

Beech wood is a heterogeneous material composed of fibres, vessels and parenchymatic cells (Fengel & Wegener, 2003). In the present study we used beech wood to image the distribution of cellulose and lignin. The main goal of this study was to compare the suitability of two different methods for image generation with FTIR spectroscopy: (a) an integration method; and (b) a correlation method. For the integration method, a wave-number range (which is considered typical for a chemical component in the sample, e.g. for lignin or cellulose) is integrated. The area obtained is documented for each measured point in the wood

specimen analysed. The resulting image illustrates the quantitative distribution of the component in question. This method has been successfully used to document the lignin distribution in *Populus x canescens* (grey poplar, Labbé et al., 2005, Naumann & Polle, 2006). However, a problem with this integration method is that peaks in the FTIR spectra are usually the result of overlapping components and, thus, true quantification is difficult. With the correlation method, specificity for a given component should be improved by correlation of the measured spectra with the spectrum of a reference compound. This method has worked well for imaging of fungi in wood (Naumann et al., 2005) but has not yet been applied to wood itself. Here we tested whether the correlation method is suitable to map the distribution of cellulose and lignin in beech wood and gives similar results as the integration method.

## Materials and Methods

### Wood material

Wood was obtained from three 56-year-old beech trees harvested in 2006. A disk excised at the stem base was used to cut wood blocks of 10 mm length x 10 mm width x 20 mm height in the area of the fifth year ring near the bark. Parts of the blocks were milled to a fine powder using a ball mill (MM2, Retsch, Haan, Germany). The wood was used to generate FTIR spectra and for the isolation of acid insoluble Klason lignin (see below). Further materials of the same blocks were used to prepare cross sections for FTIR microscopic analysis. For the latter purpose, 7- $\mu$ m-thick specimen were cut in cross-sectional direction with a sledge-microtome (Reichert-Jung, Heidelberg, Germany). The sections were dried on microscope slides at room temperature, covered with a cover slip on which a lead block was placed to keep the sections flat.

### Reference Materials

Cellulose (Cotton linters, Buckeye, Memphis, USA) and lignin from the beech wood were used as reference samples. The beech lignin was prepared using powdered beech wood from above by the method of Dence (1992) as described in detail previously (Müller et al., 2007). Briefly, milled powder was washed twice in phosphate buffer and four times in methanol. Subsequently, the sample was incubated in ethanol/cyclohexane (v/v 1 : 2) for 6 h at 60 °C and centrifuged. The pellet was washed twice in ethanol/cyclohexane and subsequently in acetone and dried. The residual material was extracted in 72% sulfuric acid to obtain Klason lignin. The purity was checked by determining the carbon and nitrogen contents and by FTIR analysis (see later sections).

## FTIR spectroscopy

FTIR attenuated total reflectance (ATR) spectra of beech wood, and lignin and cellulose reference samples were recorded in the wavenumber range from 4500-600  $\text{cm}^{-1}$  with an Equinox 55 spectrometer (Bruker Optics, Ettlingen, Germany) including a deuterium-triglycinesulfate-detector and an attached ATR-unit (DuraSampIR, SensIR Europe, Warrington, England). A resolution of 4  $\text{cm}^{-1}$  and a number of 32 scans per sample was used. Six lignin samples (two per tree) were powdered. Three replicates per lignin sample were analysed, yielding 18 spectra of which a mean reference spectrum was calculated (OPUS 6.5 software, Bruker Optics, Ettlingen, Germany). Unlike lignins, which show strong species dependent differences, cellulose FTIR spectra of different source were highly similar (Müller et al., 2007). Therefore, cellulose of cotton linters was used as the reference. The reference spectrum was obtained as the mean of nine FTIR measurements on pressed cellulose (OPUS 6.5 software, Bruker Optics, Ettlingen, Germany).

## FTIR microscopy

FTIR transmission spectra were recorded with the FTIR spectrometer Equinox 55 combined with the IR microscope Hyperion 3000 (Bruker Optics, Ettlingen, Germany), including a focal plane array (FPA) detector (Bruker Optics, Ettlingen, Germany), in the wavenumber range from 3900-900  $\text{cm}^{-1}$ . The detector consists of 64 x 64 detector elements, each with a dimension of 4  $\mu\text{m}$  x 4  $\mu\text{m}$  and simultaneously records 4096 spectra without moving the sample, thereby, covering a total sample area of 256  $\mu\text{m}$  x 256  $\mu\text{m}$ . A spectral resolution of 8  $\text{cm}^{-1}$  and a number of 10 scans per measurement were used. The sections were placed on a round potassium bromide (KBr) window (diameter: 13 mm; height: 2 mm) used as a sample holder. For data evaluation, the borders for spectral integration were set from 1530-1490  $\text{cm}^{-1}$  for lignin (Faix, 1991) and from 1390-1350  $\text{cm}^{-1}$  for cellulose (Pandey & Pitman, 2003), respectively. A straight baseline was drawn connecting the local minima at the defined wavenumbers and the area above the baseline was calculated as illustrated in Figure 1A. For visualisation the measured areas were documented by a colour code from blue (low concentration) to pink (high concentration). For a given cross section, the scale was automatically adjusted between the lowest and highest peak area.

For the correlation method (also called trace-computation), mean ATR spectra of beech Klason lignin and cotton cellulose were used as the reference, respectively (Figures 1B and 1C). ATR and FPA Spectra were baseline-corrected in the fingerprint-region (1800-900  $\text{cm}^{-1}$ ) and used for correlation analysis (Opus software, trace computation). For each of the 4096 spectra of the FPA data set a correlation

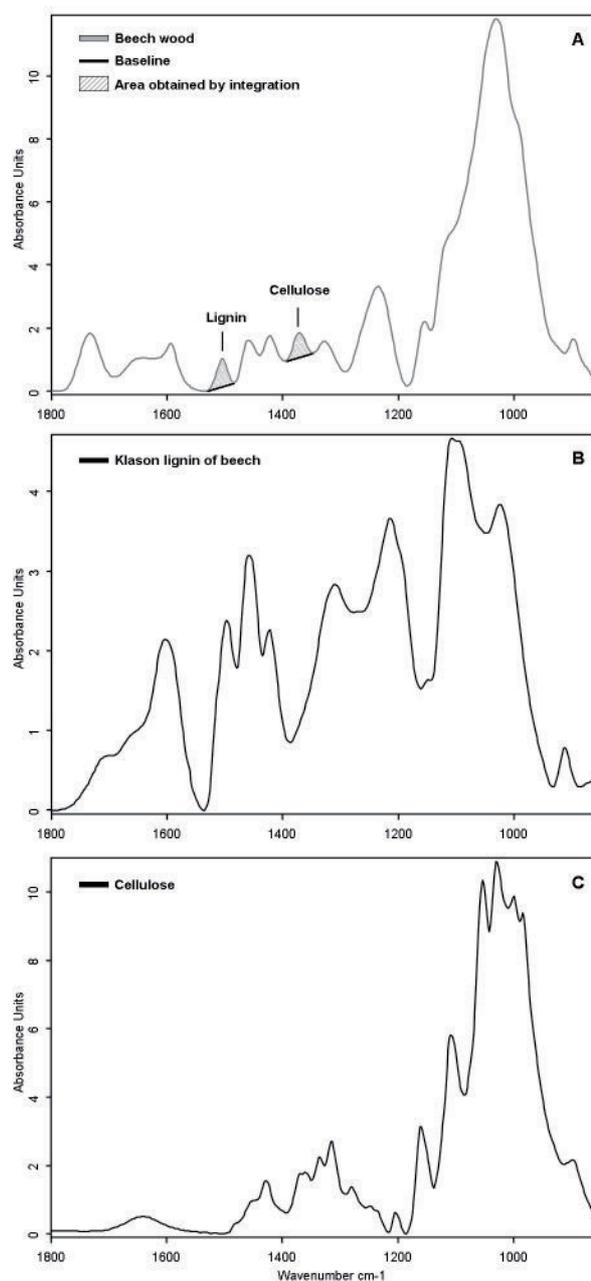


FIGURE 1: Principal of the integration method, exemplarily shown for a mean FTIR-ATR spectrum of European beech (A) ( $n=120$ ). The areas below the spectral bands, obtained after integration, are hatched in grey colour. For the correlation method the mean ATR spectra of 18 beech Klason lignin ATR spectra (B) as well as of 9 cellulose spectra (C) were used and correlated with the FPA data set, respectively. The ATR spectra are given in the wavenumber range from 1800-850  $\text{cm}^{-1}$ .

with the reference spectrum was calculated and the distribution of these correlation data was plotted. No correlation is indicated by blue and high correlation by pink colour.

### Comparative analysis of FTIR and FPA spectra

FTIR Spectra of beech wood, lignin and cellulose (Figure 1) illustrate the suitability of the spectral regions from 1530-1490  $\text{cm}^{-1}$  for lignin analysis and from 1390-1350  $\text{cm}^{-1}$  for cellulose analysis. These regions are useful because cellulose has no absorbance around 1500  $\text{cm}^{-1}$  and lignin shows a local minimum in the cellulose region from 1390-1350  $\text{cm}^{-1}$ . The maximum absorbance of cellulose is in the region from 1000-1100  $\text{cm}^{-1}$  (Figure 1B). Unfortunately, this region was not suitable for cellulose measurements because lignin forms a relatively strong background in this area (Figure 1C). Furthermore, FPA spectra were severely degenerated in this region (Figure 2). This was due to the thickness of the wood sections, whereby signals for compounds with strong absorbance deviate from linearity. The quality of the spectra, thus, depends on the thickness of the sections and increases in thin preparations. However, due to humidity, very thin sections roll up and undulate, rendering the record of a plane section area impossible. Therefore, a compromise was reached by using the thinnest sections that did not deform and excluding wavenumbers between 1150 and 900  $\text{cm}^{-1}$  from the analysis.

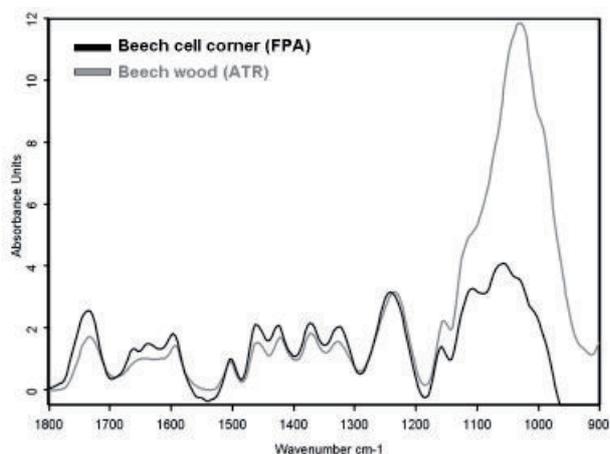


FIGURE 2: FTIR-ATR spectrum of beech solid wood and a FPA spectrum extracted from a cell corner of the FTIR-FPA image, recorded of the solid wood section of beech (see Figure 3). For the ATR spectrum, the mean of 120 spectra, for the FPA spectrum the mean of 5 spectra are given in the wavenumber range from 1800-900  $\text{cm}^{-1}$ .

### Imaging of cellulose and lignin by FTIR microscopy

Light microscopic images illustrate the anatomy of beech (Figure 3A). The corresponding FPA images reflect the spatial distribution of cellulose after evaluating all spectra by integration for the wavenumber range between 1390 and 1350  $\text{cm}^{-1}$ , tentatively assignable to cellulose (Figure 3B). The image obtained by correlating the FPA data set with the averaged ATR spectrum of cellulose is shown in Figure 1C. The colour scale from blue to pink indicates increasing cellulose content. Comparing the image produced by the two different evaluation methods indicates more structural details after application of the integration method. A drawback of both methods was that cells with small lumina, as for example beech fibres, were too small to be resolved. The size of the single detector elements (4  $\mu\text{m}$  x 4  $\mu\text{m}$ ) defines the limit of the spatial resolution (Naumann & Polle, 2006). Furthermore, the resolution of FTIR images depends on the step width of the radiation (Naumann & Polle, 2006). At 900  $\text{cm}^{-1}$ , theoretically a maximum spatial resolution of 7  $\mu\text{m}$  is possible. With increasing wavenumbers, the spatial resolution gradually increases. At 1500  $\text{cm}^{-1}$  and higher, a local resolution of up to 4  $\mu\text{m}$  is achievable (Naumann et al., 2007). Because of these limitations small structures cannot be resolved. Furthermore, it must be considered that if a detector element measures in the lumen and a little bit of the adjacent cell wall, the mean signal of this area will be shown. This means that cell lumina below 16  $\mu\text{m}^2$  cannot be resolved. This drawback affects the relative quantification of cellulose and lignin in images containing cells with large differences in cell lumina as in beech wood. The lumina of fibre cells or late wood cells were not resolved, whereas the large lumina of vessels were clearly distinguished from other parts by bluish colour, indicative of the absence of cellulose (Figures 3B & 3C).

Overall, both Figures 3B and 3C suggest that the distribution of cellulose was quite homogenous as shown by large areas of green colouration. However, areas with apparently slightly higher accumulation of cellulose were indicated by the yellowish colouration in the image obtained by the integration method (Figure 3B). The image obtained by the correlation method (Figure 3C) showed less contrast than that obtained by the integration method (Figure 3B). In particular, even relatively broad cell files between two adjacent vessels were not clearly separated by correlation method. Therefore, the integration method is better suitable for the documentation of cellulose distribution in beech wood.

Figure 4A illustrates the same cross section used previously for cellulose analysis (Figure 3A). The FPA image in Figure 4B reflects the tentative spatial distribution of lignin based on integration of the wavenumber range between 1530 and 1490  $\text{cm}^{-1}$

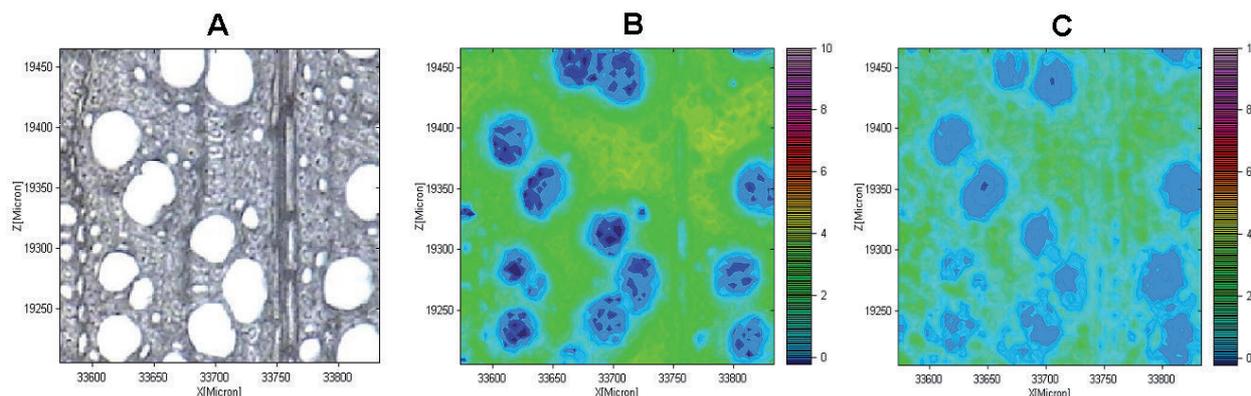


FIGURE 3: Light microscopic view of a beech cross-section (A). The spectra of the corresponding FPA images were integrated for the wavenumber range 1390-1350  $\text{cm}^{-1}$  to show cellulose distribution (B), or were correlated with the FTIR-ATR reference spectrum of cellulose in the wavenumber range of 1800-1150  $\text{cm}^{-1}$  (C). The colour scale represents increasing cellulose content from blue (low) to pink (high).

in same cross section used previously for cellulose analysis. Figure 4C shows the corresponding image for the correlation method based on Klason lignin isolated from European beech as the reference. The resolution obtained by the integration method was higher than that obtained by the correlation method since more details were visible in Figure 4B than in Figure 4C. For example, the wood ray was more clearly apparent in Figure 4B than in Figure 4C. Still, it is noteworthy that both methods show similar trends of lignin densities. The lignin distribution in the measured area was heterogeneous and more concentrated in cell walls in the upper part of the section indicated by stronger reddish colouration by both methods (Figures 4B & 4C). Heterogeneous lignin distribution has also observed in spruce and

birch by UV microscopy or by transmission electron microscopy coupled with an energy dispersive X-ray analyser (TEM-EDXA) revealing higher lignin contents in rays than in the secondary cell wall of tracheids or fibres (Fergus et al., 1969; Eriksson et al., 1988). Overall, the integration method yields more structural details than the correlation method and therefore, is superior for mapping chemical constituents in woods.

## Conclusions

FTIR Spectroscopy in combination with microscopy enables the localisation of cellulose and lignin in different tissues of beech wood. However, the localisation of varying concentrations of lignin or

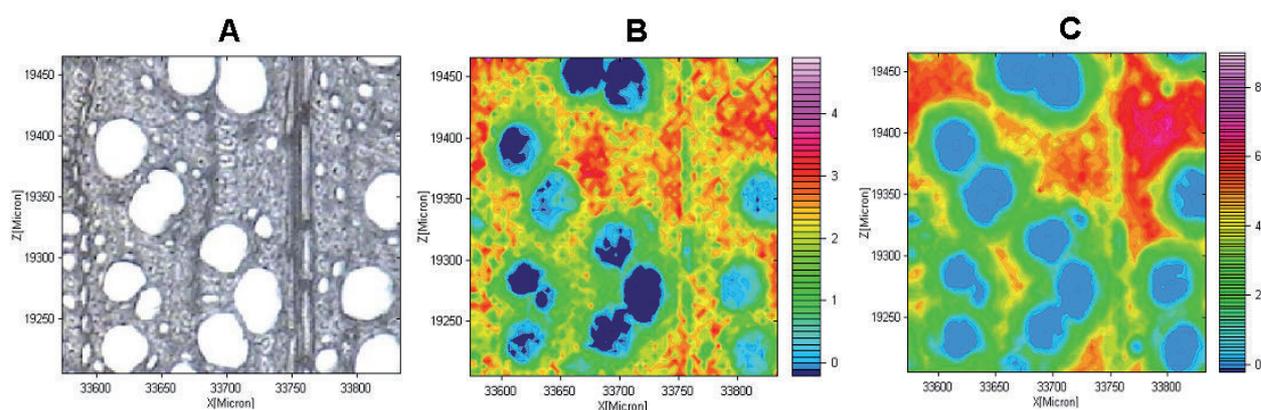


FIGURE 4: Light microscopic view of a beech cross-section (A). The spectra of the corresponding FPA images were integrated for the wavenumber range 1530-1490  $\text{cm}^{-1}$  to show lignin distribution (B), or correlated with the FTIR-ATR reference spectrum of Klason lignin in the wavenumber range of 1800-900  $\text{cm}^{-1}$  (C). The colour scale represents increasing lignin content from blue (low) to pink (high).

cellulose within a single cell wall was not possible but small-scale tissue in-homogeneities can be distinguished. The comparison between two imaging methods revealed that cellular structures were better discernable with the integration than with the correlation method. However, tissue densities affected the signals suggesting that FTIR imaging has to be optimised for each species. We have shown that these problems can be detected by comparison of FPA with ATR spectra which uncover wavenumber regions where FPA spectra degrade. For imaging to be successful, the degraded wavenumber regions have to be excluded or the thickness of the specimen has to be decreased.

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