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Extractive concentrations and cellular level distributions change radially from outer to inner heartwood in Scots pine

Radial changes in heartwood extractives

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Abstract

The heartwood of many wood species is rich in extractives, which improve the wood material's resistance to biological attack. Their concentration is generally higher in outer than inner heartwood, but the exact radial changes in aging heartwood remain poorly characterised. This investigation studied these radial changes in detail in Scots pine (*Pinus sylvestris* L.), using radial sample sequences prepared from three different trees. Stilbene and resin acid contents were first measured from bulk samples, after which the extractive contents of individual heartwood annual rings were investigated using Raman spectroscopy and fluorescence microscopy. Raman imaging and fluorescence microscopy were also used to study the cellular level distributions of extractives in different annual rings. Although there were substantial differences between the trees, the content and distribution of stilbenes seemed to follow a general radial trend. The results suggest that stilbenes are absorbed into heartwood tracheid cell walls from small stilbene-rich extractive deposits over several years and then eventually transform into non-extractable compounds in aging heartwood. Resin acids followed no consistent radial trends, but their content was strongly connected to the frequency of large extractive deposits in latewood tracheid lumens. The results highlight the variability of heartwood extractives: their content and distribution vary not only between trees but also between and even within the annual rings of a single tree. This high variability is likely to have important effects on the properties of heartwood and the utilisation of heartwood timber.

Keywords: durability, extractives, pinosylvins, phenolic, terpenoid

39 **Introduction**

40 Heartwood is dead tissue found in the inner layers of the tree trunk, formed from aging sapwood
41 in a process where the innermost sapwood parenchyma cells die. Heartwood formation is
42 characterised by the accumulation of heartwood extractives (Taylor et al. 2002), which are a
43 large and diverse group of mostly small molecular weight compounds. Heartwood extractives
44 often possess antifungal and other bioactive properties, and in many tree species, their presence
45 increases the heartwood's resistance to degradation by wood decaying fungi (Hillis 1987,
46 Taylor et al. 2002).

47 The conversion of sapwood to heartwood takes place in a narrow zone between the two, called
48 the transition zone. The heartwood extractives are either synthesised directly in the transition
49 zone by parenchyma cells, or their precursors are synthesised in the aging sapwood by the
50 parenchyma cells and then converted to heartwood extractives in the transition zone (reviewed
51 by Kampe and Magel 2013, Celedon and Bohlmann 2018). Recent evidence suggests that a
52 small portion of cells may also survive the heartwood formation process and continue
53 synthesising extractives in the heartwood (Celedon and Bohlmann 2018). After synthesis and
54 parenchyma cell death, the extractives are released into the surrounding wood tissues.
55 Microscopic investigations have revealed the progressive spread of heartwood extractives from
56 ray parenchyma cells into surrounding tracheids (Kuroda et al. 2014) and the movement of
57 extractives from ray parenchyma to tracheids/fibres through pit connections (Nagasaki et al.
58 2002, Zhang et al. 2004). Once deposited into the heartwood, the extractives can be found in
59 the cell walls and middle lamellae of heartwood tracheids and as small deposits or large
60 occlusions in the lumens of tracheids, ray parenchyma and axial parenchyma (Nagasaki et al.
61 2002, Zhang et al. 2004, Matsushita et al. 2012, Kuroda et al. 2014, Belt et al. 2017, Felhofer
62 et al. 2018).

The concentration of heartwood extractives generally increases rapidly at the heartwood-sapwood border and then decreases from outer to inner heartwood (Bergström et al. 1999, DeBell et al. 1999, Nagasaki et al. 2002, Gierlinger and Wimmer 2004, Ekeberg et al. 2006). The decrease has been attributed both to increasing extractive formation with tree age (DeBell et al. 1999, Gierlinger and Wimmer 2004) and to secondary reactions (oxidation, polymerisation, binding to lignin) in aging heartwood that decrease the concentration of the original extractives over time (Helm et al. 1997, Bergström et al. 1999, Shimizu et al. 2017). However, most of the information on radial changes in extractive content have been obtained using bulk measurements, which means that the data is low in spatial resolution. Furthermore, no information is available on the radial changes in the cellular level distributions of extractives.

The current investigation set out to study the radial changes in heartwood extractives in detail, using Scots pine (*Pinus sylvestris* L.) as the example wood species. Chromatographic bulk chemical analysis, Raman spectroscopy, confocal Raman spectroscopy imaging, and fluorescence microscopy were used to monitor extractives in radial sample sequences prepared from three different trees. The objectives of the investigation were to determine how the (i) concentrations and (ii) cellular level distributions of extractives change from the heartwood-sapwood border to the innermost heartwood annual rings and to (iii) obtain clues about the processes governing these changes. These variations are important to understand because both extractive content and distribution affect many important heartwood properties, including decay resistance (Hillis 1987, Taylor et al. 2002).

85 **Materials and methods**

86 **Sample preparation**

87 Scots pine (*Pinus sylvestris* L.) wood samples were prepared from three bottom logs obtained
88 from three different trees grown in eastern (logs A and B) or southern Finland (log C). Log A
89 had 122 annual rings (65 heartwood rings), log B 74 annual rings (32 heartwood rings) and log
90 C 74 annual rings (25 heartwood rings). The logs were stored frozen protected from light and
91 desiccation until use. To prepare radial sample sequences for chemical analysis and imaging, a
92 disc approx. 50 mm in thickness (longitudinal) was cut from each log, after which a 15 mm
93 wide (tangential) strip was cut from bark to bark through the pith. After visually identifying
94 the heartwood-sapwood border based on the moisture difference, 15 mm wide (radial)
95 heartwood blocks were cut from the strips starting from the first annual ring designated as
96 heartwood (heartwood ring 1) and continuing towards the pith. The pith itself was excluded
97 from sampling. A total of nine heartwood samples covering heartwood rings 1-56 were
98 prepared from log A, three covering heartwood rings 1-27 from log B and three covering
99 heartwood rings 1-21 from log C. One 15 mm wide block was also cut from each strip from
100 the sapwood side of the border. Finally, all the prepared blocks were cut across the grain to
101 produce one 15 mm thick imaging sample and one 30 mm thick chemical analysis sample.
102 Sample preparation and the prepared samples are shown in Figure 1.

103 [Figure 1]

104

105 **Extraction and extractive analysis**

106 The chemical analysis samples were cut into thin sticks with a razor blade, freeze dried and
107 ground to a powder in a Wiley mill (mesh 20). Portions (0.15 g) of each powder were then
108 solvent extracted with MeOH (2 x 5 ml) for 30 min at 45°C in a sonicator. Three replicate

extractions were performed per sample. The extraction solvent contained 0.1 mg/ml of diethyl stilbestrol as internal standard. After extraction, 25 µl aliquots of each extract were placed into a vial and the solvent evaporated under vacuum. The dry extracts were then dissolved in 75 µl of pyridine and trimethylsilylated at 70°C for 20 min after the addition of 25 µl of N,O-Bis(trimethylsilyl)trifluoroacetamide with 10% chlorotrimethylsilane. The composition of the extracts was analysed by GC-FID (Shimadzu GC 2010 Plus) and GC-MS (Shimadzu GCMS-QP2010 SE). Both analyses used a HP-5 column (30 m x 0.23 mm i.d., 0.25 µm film thickness), helium as the carrier gas (1 ml/min), and the following oven temperature program: 2 min at 100°C, 10°C/min to 200°C, 5°C/min to 280°C, and 5 min at 280°C. Mass spectra (50-700 m/z) were recorded at 70 eV. The extractive compounds were first identified by GC-MS and then quantitatively analysed by GC-FID using the known concentration of internal standard for quantitation.

Raman spectroscopy and imaging

Cross sections approx. 25 µm in thickness were cut from the imaging samples using a rotary microtome. The cross-sections were placed on microscope slides with a drop of deionized water, covered with glass coverslips (0.17 mm thickness) and edge-sealed with nail polish. Raman images and spectra were acquired using a WITec alpha 300 RA Raman microscope equipped with a 532 nm frequency doubled Nd:YAG laser (used at 30 mW) and a DU970-BV EMCCD camera behind a 600 lines/mm grating.

Raman images were acquired from all heartwood samples, from the earlywood and latewood regions of selected annual rings. The images were acquired using a 100x oil objective (NA 1.25, coverslip correction 0.17 mm) and a 0.3s integration time. The image size was 25x25 µm² with 100 lines per image and 100 points per line. False-colour images describing the

distribution of heartwood stilbenes were generated by calculating the integrated intensity of the stilbene C=C stretch band at 1628-1645 rel. cm^{-1} (see Figure S1 available as Supplementary Data at *Tree Physiology* online). Background was subtracted from the integrated intensity by setting the spectral intensity to zero at four wavelengths above and below the wavenumber range of interest.

Single spectra were also collected from all samples, from four separate locations on both the latewood and earlywood regions of every other annual ring in heartwood rings 1-30 and from every fifth annual ring after ring 30. Sapwood spectra were obtained from the two closest annual rings to the heartwood-sapwood border. The spectra were obtained from the secondary cell wall region using a 20x air objective (NA 0.4), a 0.3s integration time and ten accumulations per spectrum. The integrated intensity of the stilbene band was calculated for every spectrum. Due to variations in the overall intensity of the individual spectra, the intensities were normalised by dividing with the integrated intensity of the aromatic ring stretch band at 1580-1620 rel. cm^{-1} . All data processing and integrations were performed using WITec suite 4 software.

Fluorescence microscopy

Cross sections approx. 25 μm in thickness were again cut from the imaging samples using a rotary microtome and placed on microscope slides. Most of the cross sections were directly embedded in water and sealed with nail polish as above, but some were first extracted with acetone (5 x 200 μl) on the slides and then embedded in water. The native and extracted cross sections were observed with an Olympus BX53 microscope using a 20x air objective (NA 0.5) and UV excitation (330-385 nm excitation filter, 420 nm long pass emission filter). Fluorescence images sized 352 x 264 μm^2 were collected from all samples, from the earlywood

and latewood regions of every other annual ring in heartwood rings 1-30 and from every fifth annual ring after ring 30. Sapwood images were obtained from the two closest annual rings to the heartwood-sapwood border. Two to four images were collected from both earlywood and latewood in each ring using a QImaging Micropublisher RTV 3.3 camera. Image acquisition was controlled by ImagePro software. Constant image settings were used for all samples. The fluorescence intensity of each image was calculated by first calculating the total intensity of all pixels where the intensity exceeded a threshold value and then dividing that with the number of pixels where the threshold was exceeded to exclude empty cell lumens.

Results

Extractive content of bulk samples

Scots pine heartwood extractives consist of stilbenes, resin acids, free fatty acids and small amounts of other compounds, while the sapwood extractives consist primarily of triglycerides and smaller amounts of other compounds such as resin acids (Willför et al. 2003). To investigate the radial changes in extractives, the bulk concentrations of extractives were first determined chromatographically in the radial sample sequences obtained from the three logs. Since stilbenes and resin acids are the most abundant extractives found in heartwood and the ones connected to decay resistance (Harju et al. 2002, Venäläinen et al. 2004, Leinonen et al. 2008), the analysis focused on these compounds. Their total concentrations are given in Figure 2, while the concentrations of the individual compounds can be found in Table S2 (available as Supplementary Data at *Tree Physiology* online). Other compounds found in the heartwood extracts are summarised in Figure S3 (available as Supplementary Data at *Tree Physiology* online).

[Figure 2]

180 The total concentration of stilbenes ranged between 0.6 and 19.2 mg/g in the heartwood
181 samples, with similar maximum concentrations (13.9-19.2 mg/g) recorded in all three logs. The
182 radial sampling sequence revealed that the stilbene content of log A increased from the first
183 heartwood sample to the second and then decreased from the third sample onwards. The
184 heartwood stilbene content also decreased from the second to the third sample in log B but
185 increased in log C, which contained fewer annual rings in the first two samples than logs A and
186 B. No stilbenes were detected in the sapwood samples.

187 The concentration of resin acids showed more log to log variation than that of the stilbenes and
188 revealed no consistent radial trends. In log A the resin acid concentration peaked in the first
189 heartwood sample and remained low (3.8-7.6 mg/g) in the subsequent samples, whereas in log
190 B the concentration was low in the first heartwood sample (4.2 mg/g) and increased towards
191 the third and final sample (25.1 mg/g). Log C had a higher resin acid content than the other
192 logs, with a maximum concentration of 60.8 mg/g in the second heartwood sample. The resin
193 acid content of all sapwood samples was low (1.2-3.3 mg/g).

194 In addition to stilbenes and resin acids, the heartwood samples also contained fatty acids and
195 other compounds detectable by GC (see Figure S3 available as Supplementary Data at *Tree*
196 *Physiology* online). The fatty acid content of log A (maximum content 13.1 mg/g) was higher
197 than that of logs B and C (maximum content <5 mg/g). The non-fatty acid compounds detected
198 in log A appeared in the older heartwood samples and increased in concentration towards the
199 pith. These compounds were identified as monosaccharides, primarily arabinose and galactose,
200 likely derived from heartwood arabinogalactan (Fischer and Höll 1992, Willför and Holmbom
201 2004). Small amounts of other unidentified compounds were detected in the log B and C
202 samples.

203

Extractives in different annual rings

Cellular level distributions

To further investigate the radial changes in heartwood extractives, Raman and fluorescence images were collected from different annual rings and analysed to explore the changes in extractive content and cellular level distribution. Raman imaging was used to visualise stilbenes, which produce a unique band at 1628-1645 rel. cm^{-1} in the Raman spectrum of heartwood (Holmgren et al. 1999, Belt et al. 2017, Felhofer et al. 2018, see also Figure S1 available as Supplementary Data at *Tree Physiology* online). The stilbene band is absent in sapwood (Belt et al. 2017, Felhofer et al. 2018, see also Figure S1). False-colour Raman images from selected heartwood annual rings produced by integrating the stilbene band are given in Figure 3. Fluorescence microscopy was used to visualise all UV-fluorescent heartwood compounds, which include stilbenes and resin acids. Stilbenes produce blue fluorescence under UV-excitation, although the excitation wavelengths used in this experiment (330-385 nm) are higher than their excitation maximum at approx. 320 nm (Harju et al. 2009, Antikainen et al. 2012). Resin acid mixtures have been shown to produce blue/green fluorescence under UV-excitation (Donaldson et al. 2019), but the fluorescence properties of the individual acids remain poorly understood. Fluorescence images from selected annual rings are shown in Figure 4.

[Figure 3]

[Figure 4]

Raman imaging indicated the presence of stilbenes in heartwood tracheid cell walls, middle lamellae and lumens in all three logs. Both small deposits and large lumen-filling deposits were present in the latewood tracheid lumens, while only small deposits were found in the earlywood tracheid lumens. The Raman spectra of the small and large deposits showed that the small

deposits consisted mostly of stilbenes, while the large deposits also contained resin acids and/or fatty acids (see Figure S4 available as Supplementary Data at *Tree Physiology* online). Fluorescence imaging also showed the presence of deposits in heartwood tracheid lumens as well as in the lumens of ray cells. The tracheid and ray cell deposits (as well as resin canals, see Figure S5 available as Supplementary Data at *Tree Physiology* online) were brightly fluorescent, while the tracheid cell walls and middle lamellae showed weaker fluorescence. The fluorescence signal from the cell walls and middle lamellae is likely to include contributions from lignin, which is also autofluorescent under UV excitation (Donaldson et al. 2010). In agreement with Raman imaging, the fluorescence images showed both small and large deposits in latewood tracheid lumens and only small deposits in earlywood tracheids. The colour of fluorescence in both the tracheid and ray cell deposits ranged from dark purple-blue to blue to bright blue-green (see Figure S5 available as Supplementary Data at *Tree Physiology* online), indicating variation in their composition.

241

242 Changes in Raman and fluorescence intensity

The Raman and fluorescence images collected from different radial positions on the three logs generally shared the same features, but there was substantial variation in the Raman/fluorescence intensity and the frequency at which the features appeared in each image. To investigate the changes in intensity and thus extractive content across the annual rings, Raman and fluorescence intensity data were extracted. Data for comprehensive Raman intensity analysis was obtained by collecting individual Raman spectra from earlywood and latewood secondary cell walls on every other (heartwood rings 1-30) or every fifth (rings 30+) annual ring. Raman spectra were also obtained from sapwood. The intensity of the stilbene band at 1628-1645 rel. cm^{-1} was integrated, and due to variations in the overall intensity of the spectra, the stilbene band intensities were normalised by dividing with the integrated intensity

of the aromatic ring stretch band (1580-1620 cm^{-1}), which contains contributions from both lignin and stilbenes (Felhofer et al. 2018). Fluorescence intensity data was extracted from the heartwood and sapwood fluorescence images by calculating the total intensity of all pixels where the intensity exceeded a threshold value and then dividing that with the number of pixels where the threshold was exceeded. This procedure was used to exclude empty cell lumens and to minimise the effects of differences in cell number and size. The Raman and fluorescence intensity data are presented in Figure 5.

[Figure 5]

The local stilbene concentrations revealed by the Raman intensity data were correlated ($R^2=0.768$) with the chromatographic bulk measurement results (see Figure S6 available as Supplementary Data at *Tree Physiology* online). The differences between the Raman intensity and bulk results were at least partly due to differences between the methods: the bulk measurements measure the stilbene content of the entire sample block, whereas the Raman method measures the intensity from isolated secondary cell wall locations on the sample surface. The Raman intensity data provided more detailed information on the radial variations in stilbene content than the bulk measurements and revealed notable differences between the three logs. In log A the intensity increased progressively in the first heartwood rings, reached its maximum around the tenth ring and then began to decrease, whereas in log B the intensity remained low in the first heartwood rings, rapidly increased around the tenth ring and then fluctuated around this value. In log C the intensity continued to increase until approx. the 20th heartwood ring. The stilbene band was absent in all sapwood spectra.

The radial trends revealed by the fluorescence intensity data were different from those shown by the Raman intensity measurements. The difference between the Raman and fluorescence results was particularly noticeable in the case of log A, where the fluorescence results indicated

277 that the extractive-derived intensity continued to increase until the 50th heartwood annual ring
278 instead of decreasing after the tenth ring. In log B the fluorescence intensity results showed a
279 moderate increase in intensity from the 13th annual ring to the 21st instead of the rapid increase
280 around the tenth annual ring as indicated by the Raman intensity data. Log C was the only log
281 where the radial patterns derived from Raman and fluorescence data were similar. The
282 fluorescence intensity results did not correlate with the chromatographically determined
283 stilbene content nor with resin acid content (see Figure S6 available as Supplementary Data at
284 *Tree Physiology* online), suggesting the presence of other fluorescent compounds in Scots pine
285 heartwood.

286 To investigate the nature of the increasing fluorescence, new sections were produced from the
287 heartwood samples and solvent extracted on the microscope slides. Fluorescence images were
288 then collected from approximately every tenth annual ring on the extracted sections and
289 fluorescence intensity data extracted as before. The post-extraction fluorescence intensities are
290 shown in Figure 6, while Figure S7 (available as Supplementary Data at *Tree Physiology*
291 online) provides a direct comparison between the fluorescence intensities of sapwood and
292 native and extracted heartwood. Although the applied extraction procedure appeared to
293 efficiently remove all extractives deposits from ray and tracheid lumens, the fluorescence
294 intensity of the extracted heartwood samples was never reduced to the level of sapwood. In all
295 three logs, the intensity of non-extractable heartwood fluorescence increased towards the pith.
296 In the innermost annual rings the increased non-extractable fluorescence may be due to juvenile
297 wood: juvenile wood shares characteristics with compression wood, which has been shown to
298 have a higher fluorescence intensity than normal wood due to differences in lignin structure
299 (Donaldson et al. 2010). However, since juvenile wood is limited to the 20 or so innermost
300 annual rings in Scots pine (Sauter et al. 1999), its presence cannot account for the increasing
301 non-extractable intensity of rings 20 and 30 in log A.

[Figure 6]

Changes in deposits

In addition to variations in Raman and fluorescence intensity, the images collected from different annual rings on the three logs (Figures 3 and 4) showed variations in the distribution of extractives, particularly in the occurrence of different types of extractive deposits. Thus, to investigate the radial changes in the distribution of extractives, we analysed the deposits seen on the fluorescence images collected from different annual rings. The small and large deposits found in tracheid lumens were analysed by calculating their frequency (what percentage of cells in each image contained a deposit), while the deposits found in rays were analysed by estimating the degree of ray filling on a scale of 1-5 (1 = 0-20% filling, 2 = 20-40% filling, 3 = 40-60% filling, 4 = 60-80% filling, and 5 = 80-100% filling). Ray filling was estimated by measuring the length of each ray and the approximate total length of the fluorescent material within the ray. The deposit frequencies and ray fill ratings at different annual ring intervals are given in Figure 7. Annual ring intervals rather than individual rings were used in deposit analysis to highlight the overall trends rather than variations between individual rings.

[Figure 7]

The small deposits found in earlywood and latewood tracheid lumens showed a relatively consistent radial trend in all three logs. Their abundance increased from the first annual ring interval (heartwood rings 1-9) to the second (rings 10-19) and then began to decline. Earlywood had a higher frequency of small deposits than latewood and showed a more prominent radial trend. In contrast to the small deposits, the large latewood deposits and the ray filling degree showed no consistent radial trends. The frequency of large deposits was highest in log C, followed by rings 10-19 and 20-29 in log B and rings 1-9 in log A, while the ray filling degree

declined towards the pith in log A, increased in log B and remained constant in log C. Both the small and the large deposits showed substantial local variation in all logs, with some annual rings containing areas of both high and low deposit abundance (see Figure S8 available as Supplementary Data at *Tree Physiology* online). The ray filling degree showed substantial local variation as well, and in many fluorescence images, completely full rays could be seen adjacent to completely empty rays.

Discussion

The chromatographic bulk measurements (Figure 2) revealed that the stilbene content of heartwood increased from the first sample at the heartwood-sapwood border to the second in all three logs. The stilbene content of log A then decreased from the third sample onwards, reaching very low concentrations in the innermost samples. The radially decreasing stilbene content of log A is in agreement with previous reports (Venäläinen et al. 2003), which have shown that the stilbene content of old Scots pine logs is lower in inner than outer heartwood. The low stilbene content of the innermost heartwood samples in the older log A but not in the younger logs B and C suggests that the lower stilbene content of inner heartwood may be due to secondary reactions in aging heartwood rather than to increasing production with increasing tree age, although more data is needed to confirm this hypothesis.

The concentration of resin acids in turn showed high variation between and within the logs and revealed no consistent radial trends. The high variation and the lack of consistent trends suggest that the resin acid content of Scots pine heartwood is controlled by a different mechanism than its stilbene content. Transcriptomic analyses have shown that stilbenes are synthesised directly in the transition zone, while resin acids are synthesised primarily in the sapwood and then

transported into heartwood (Lim et al. 2016). The processes controlling the synthesis and transport of resin acids are unknown, but the data collected here suggest that their activity can vary substantially during a tree's life. Although the concentration of resin acids showed no consistent radial trends, potential secondary reactions cannot be ruled out since their effects might be masked by the other variations.

Raman images collected from different annual rings on the three logs (Figure 3) revealed the presence of stilbenes in heartwood tracheid lumens, cell walls and middle lamellae, in agreement with previous reports (Belt et al. 2017, Felhofer et al. 2018, Belt et al. 2019). Although all the images collected from different radial positions showed the same general cellular level distributions, there were substantial variations in the overall Raman intensity and extractive deposit frequency. The radial variations in cell wall stilbene content were investigated in detail by extracting stilbene band intensity data from single Raman spectra collected from different annual rings (Figure 5a-c). Although there were substantial differences between the three logs, the Raman intensity data clearly showed that the cell wall concentration of stilbenes increased for some 10-20 annual rings after their first appearance. The increasing concentration is likely to be due to continued deposition of stilbenes into the cell walls, although continued synthesis due to residual enzyme activity cannot be excluded. Small amounts of active phenol-oxidising enzymes have been previously extracted from the heartwood of pines (Shain and Mackay 1973, Fagerstedt et al. 1998). Cell wall stilbene content declined substantially in the inner heartwood rings in log A but not in logs B and C, presumably due to the relatively young age of their inner heartwood.

Fluorescence images collected from different annual rings (Figure 4) also showed the presence of extractives in tracheid lumens, cell walls and middle lamellae, as well as in the lumens of ray cells. Analogous to the Raman intensity analysis, fluorescence intensity data was extracted from the images to obtain information on the radial variations in all UV-fluorescent compounds

(Figure 5d-f). However, the radial patterns revealed by the fluorescence intensity data were different from the Raman intensity data, particularly in the case of log A. Further fluorescence intensity data obtained from images collected from solvent extracted sections (Figure 6) showed that the inner heartwood rings contained increasing amounts of non-extractable fluorescent material. In combination with the decreasing stilbene content of log A (Figures 2 and 5) and the lack of potential stilbene conversion products in the log A extracts (Figure S3), the data suggest that the stilbenes are converted to non-extractable products in aging heartwood. Non-extractable non-lignin polymers (Dellus et al. 1997a,b) and lignin-bound extractives (Helm et al. 1997) have been previously detected in the heartwood of other wood species.

The variations in the frequency of extractive deposits were investigated by counting the small and large tracheid deposits found on the fluorescence images and by estimating the degree of ray filling (Figure 7). The abundance of the small stilbene-rich deposits (see Figure S4) varied between and within annual rings, but there was a relatively consistent radial trend: their frequency increased from the first investigated annual ring interval to the second and then began to decline. Their decreasing frequency suggests that stilbenes spread in the heartwood in the form of droplets, which are then absorbed into the wood material over time. The absorption of extractives into the cell walls from droplets agrees with the Raman intensity data (Figure 5), which showed that the cell wall stilbene content increased for 10-20 annual rings depending on the log. Thus, the small deposit data shows that it was not only the concentration of stilbenes but also their distribution that continuously changed in the heartwood from the outermost to the innermost annual rings.

In contrast to the small deposits, the large deposits found in latewood showed no consistent radial trends. Their frequency suggests that their numbers are connected to local resin acid content (see Figure 2b) rather than to radial position in the log. The large deposits contain both

stilbenes and resin acids (see Figure S4), which means that the local concentration of resin acids also influences the cellular level distribution of stilbenes. The ray deposits also showed no consistent radial trends, and like the large deposits, their trends suggest that ray filling is connected to local resin acid content. However, since the ray fill ratings were not higher in log C than the other logs, it appears that the ray filling degree is not simply a function of resin acid content.

The radial trends in stilbene content and distribution are summarised in Figure 8. Although there were differences between the three trees, the results of this investigation suggest that stilbenes undergo changes in the radial direction from the heartwood-sapwood border to inner heartwood. However, it should be noted that extractive distributions, and most likely also concentrations, vary not only from one annual ring to another but also within annual rings (see Figure S8). The samples used in this investigation were only 15 mm wide, and it is reasonable to assume that even greater variation would be seen within annual rings if the entire tree cross-section was examined. The high variation in extractive content and distribution in the radial direction and within annual rings is likely to have important effects on extractive-dependent heartwood properties such as resistance to biological attack. Even if the overall extractive content of a piece of heartwood is high, the sample may be susceptible because the areas of high extractive content are unable to afford protection to the areas of low extractive content.

[Figure 8]

Conclusions

The analyses performed on three Scots pine trees revealed some interesting trends in the content and cellular level distribution of Scots pine heartwood extractives. The data indicated

422 that the stilbene content of heartwood increases for several annual rings from the heartwood-
423 sapwood border and then begins to decrease, along with a decrease in the abundance of
424 stilbene-rich deposits and an increase in non-extractable fluorescence. Resin acids showed no
425 consistent trends, but their content was found to be strongly linked to the frequency of large
426 deposits in latewood tracheid lumens. The results of this experiment reveal the high variability
427 of Scots pine heartwood extractives: extractive content and distribution vary not only from one
428 tree to another but also from one annual ring to another in the radial direction and even within
429 individual annual rings. This high variability has important effects on the properties and
430 utilisation of heartwood, because it means that any given piece of heartwood is likely to show
431 substantial variation in its properties. The effect is particularly significant in terms of biological
432 resistance because the areas of low extractive abundance are likely to act as points of weakness
433 in the heartwood defences.

434 **Supplementary Data**

435 Supplementary Data.docx

436 **Conflict of Interest**

437 None declared

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Authors' Contributions

T.B. designed the study, M.V. acquired the wood materials and M.A. prepared the samples, T.B. performed the experimental work, T.B. analysed the data and wrote the manuscript with contributions from all co-authors, L.R. supervised the work.

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