Interactions between Scots pine heartwood extractives and wood decaying fungi

Tiina Belt
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A doctoral dissertation for the degree of Doctor of Science in Technology to be presented with due permission of the Aalto University School of Chemical Engineering for public examination and debate in Auditorium KE2 (Komppa Auditorium) at the Aalto University School of Chemical Engineering (Espoo, Finland) on the 23rd of November, 2018, at 12 noon.

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### Abstract

The heartwoods of many wood species have natural resistance to degradation caused by wood decaying fungi. Many factors can contribute to the natural durability of heartwoods, but the most significant one is usually the presence of biologically active extractives in the wood material. This thesis investigated the interactions between heartwood extractives and wood decaying fungi in detail, using Scots pine as the wood material. The primary objectives of the thesis were to study the mechanisms of action of extractives and to explore the ways in which the extractives interact with fungi within the structure of wood.

The mechanism of action studies focused on the antioxidant activity of extractives and on their ability to inhibit the enzymatic hydrolysis of wood polysaccharides. The antioxidant activity measurements showed that Scots pine heartwood extractives are active antioxidants, which means that they may be able to interfere with the radical-based degradative mechanisms used by wood decaying fungi, particularly brown rots. The extractives could also inhibit the action of hydrolytic enzymes, suggesting that they may be able to prevent the conversion of wood polysaccharides to digestible sugars. However, substantial hydrolase inhibition was only seen with a white rot enzyme preparation, which contained some enzyme(s) capable of modifying the heartwood extractives.

The interaction studies used confocal Raman spectroscopy imaging to visualise the cellular level distributions of extractives and other chemical components in intact and decaying heartwood. The studies revealed that the phenolic pinosylin were present throughout the heartwood tissues, suggesting that they have good ability to interact with fungi and their degradative agents during decay. However, the studies on decaying heartwood showed that pinosylin were extensively degraded during incipient decay. The hydrophobic resin acids were only detected in the lumens of some tracheids and ray cells, but they were found to have higher resistance to degradation than the pinosylin. Interestingly, the resin-rich extractives deposits found in tracheid lumens appeared to create local areas of reduced degradation in the decaying heartwood.

The results of this thesis provide new information on the ways in which extractives can interact with fungi and contribute to natural durability. The results also increase our understanding of the origins of natural durability in Scots pine, the most abundant wood species in Finland.

### Keywords
extractives, heartwood, pinosylin, Raman imaging, resin acid, wood decay

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Tiina Belt
Helsinki, October 2018
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# List of Abbreviations

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<th>Description</th>
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<tbody>
<tr>
<td>ABTS</td>
<td>2,2’-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)</td>
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<tr>
<td>AE-HW</td>
<td>acetone extracted heartwood powder</td>
</tr>
<tr>
<td>AE-KW</td>
<td>acetone extracted knotwood powder</td>
</tr>
<tr>
<td>AE-SW</td>
<td>acetone extracted sapwood powder</td>
</tr>
<tr>
<td>BHA</td>
<td>butylated hydroxyanisole</td>
</tr>
<tr>
<td>CC</td>
<td>cell corner</td>
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<tr>
<td>CW</td>
<td>cell wall</td>
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<tr>
<td>GC</td>
<td>gas chromatography</td>
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<tr>
<td>GC-MS</td>
<td>gas chromatography-mass spectrometry</td>
</tr>
<tr>
<td>HAE</td>
<td>heartwood acetone extract</td>
</tr>
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<td>HE-HW</td>
<td>hexane extracted heartwood powder</td>
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<tr>
<td>HME-HW</td>
<td>hexane + methanol extracted heartwood powder</td>
</tr>
<tr>
<td>HME</td>
<td>heartwood methanol extract</td>
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<tr>
<td>HPAEC</td>
<td>high performance anion exchange chromatography</td>
</tr>
<tr>
<td>HW</td>
<td>heartwood</td>
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<tr>
<td>KAE</td>
<td>knotwood acetone extract</td>
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<tr>
<td>KME</td>
<td>knotwood methanol extract</td>
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<tr>
<td>KW</td>
<td>knotwood</td>
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<tr>
<td>MHW</td>
<td>middle heartwood</td>
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<tr>
<td>ML</td>
<td>middle lamella</td>
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<tr>
<td>OHW</td>
<td>outer heartwood</td>
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<tr>
<td>PC</td>
<td>principal component</td>
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<tr>
<td>PCA</td>
<td>principal component analysis</td>
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<tr>
<td>PS</td>
<td>pinosylvin</td>
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<tr>
<td>PSM</td>
<td>pinosylvin monomethyl ether</td>
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SW  sapwood
TZ  transition zone
UE-HW  unextracted heartwood powder
UE-KW  unextracted knotwood powder
List of Publications

This doctoral dissertation consists of a summary and the following publications, which are referred to in the text by their numerals


Author’s Contribution

**Publication I:** Antioxidant activity of Scots pine heartwood and knot extractives and implications for resistance to brown rot

The author had primary responsibility in planning the research, interpreting the results and writing the manuscript. The author performed all of the laboratory experiments. TH provided feedback and revised the manuscript. LR supervised the work.

**Publication II:** Inhibitory effects of Scots pine heartwood extractives on enzymatic holocellulose hydrolysis by wood decaying fungi

The author had primary responsibility in planning the research and writing the manuscript. The author performed all of the laboratory experiments with support from FM and interpreted the results with FM. FM and TH provided feedback and revised the manuscript. LR supervised the work.

**Publication III:** Cellular level distributions of Scots pine heartwood and knot heartwood extractives revealed by Raman spectroscopy imaging

The author had primary responsibility in planning the research and writing the manuscript. The author prepared the samples and performed the GC-MS analyses. TK performed Raman imaging and wrote parts of the manuscript. The author interpreted the results with TK and TH. TK and TH provided feedback and revised the manuscript. LR supervised the work.

**Publication IV:** Cellular level chemical changes in Scots pine heartwood during incipient brown rot decay

The author had primary responsibility in planning the research and writing the manuscript. The author and MA prepared the samples, and the author performed the decay testing and the bulk chemical analyses. MA performed Raman imaging and MM conducted the PCA. The author interpreted the results with MA, MM and TH. MA, MM and TH provided feedback and revised the manuscript. LR supervised the work.
1. Introduction

Wood decaying fungi are natural recyclers of woody biomass, breaking down the wood of dead trees and returning the nutrients into the forest ecosystem. Their wood degrading abilities are essential to the health of forests, but they can cause significant problems in the built environment, where wood products are expected to remain in service for a long time. Many methods of wood preservation and modification have been developed to increase the decay resistance of wood products (Hill 2011, Schultz et al. 2007a), but naturally decay resistant wood can also be found in the heartwood (HW) of many trees. Although many factors can contribute to the natural durability of HW, the primary reason is the presence of antifungal HW extractives (Taylor et al. 2002). Natural durability and the properties of many extractives have been extensively investigated, yet our understanding of the interactions between extractives and wood decaying fungi remains incomplete.

The main objective of this thesis was to investigate the interactions between wood extractives and wood decaying fungi. The wood species investigated was Scots pine, which is an important species in Finland and northern Europe and produces HW that is classified as moderately or slightly durable on average (EN 350 2016, Van Acker et al. 2003). The thesis consists of four papers, each studying a different aspect of the interaction between pine extractives and wood decaying fungi. Paper I and Paper II investigated the potential non-biocidal mechanisms of action of extractives. The mechanisms of action were investigated because although many extractives are known to be antifungal, surprisingly little is known about the various mechanisms by which they might prevent fungal growth. The extractives may, for example, function as antioxidants as suggested by Schultz and Nicholas (2000), interfering with the radical-based wood degrading mechanisms used by fungi (Hammel et al. 2002). To determine whether antioxidant activity is a relevant mechanism in Scots pine, the antioxidant properties of its HW and knotwood (KW) extractives were studied in Paper I, with a focus on properties relevant to the inhibition of brown rot decay. The non-biocidal properties of wood extractives might also involve inhibition of enzymatic wood degradation; this mechanisms was explored in Paper II by examining the inhibition of carbohydrate-hydrolysing enzymes by Scots pine HW extractives.

In addition to studying the mechanisms of action, the thesis examined the ways in which the extractives and fungi interact within the wood structure. To
accomplish this, **Paper III** first determined the cellular level distributions of extractives in Scots pine HW and KW. The cellular level distribution of extractives is important in decay resistance, because it is likely to influence how well the extractives can interact with fungi and their radical and enzyme agents during decay. **Paper IV** then examined the cellular level chemical changes taking place in HW during the initial stages of brown rot decay. Even though Scots pine HW has some resistance to decay, it is still degradable by fungi, and a study of the initiation of decay can give information on how fungi are able to overcome the HW defences and how extractives resist the spread of degradation. The focus of **Paper IV** was particularly on the degradation of extractives during decay and on the relationship between the distribution of extractives and the degradation of the wood cell wall components. The topics of this thesis and their relationships are summarised in Figure 1.

![Figure 1. Topics covered in this thesis and their relationships](image)
2. Background

2.1 Composition of wood

2.1.1 Wood cell walls

Wood is a physically and chemically complex cellular material composed primarily of three different polymers: cellulose, hemicelluloses, and lignin. Cellulose is the most abundant of these polymeric components, accounting for 40-50% of the weight of wood. It is a carbohydrate composed of (1→4)-linked β-D-glucose units that are assembled into linear chains; the number of molecules per chain (degree of polymerisation) varies greatly from one plant source to another, but in wood the number is in the region of 10,000. The cellulose chains are intra- and intermolecularly linked by hydrogen bonds, forming cellulose microfibrils that give the cell walls their mechanical strength. The cellulose microfibrils contain both amorphous and crystalline regions that differ in their susceptibility to degradation (Fengel and Wegener 2011, Sjöström 1993).

Hemicelluloses are fully amorphous, branched polysaccharides that account for 20-30% of the weight of wood. They are heteropolymers and consist of D-glucose, D-mannose, D-xylose, D-galactose, L-arabinose, and small amounts of L-rhamnose and uronic acids in various proportions. In softwoods, the principal hemicellulose is galactoglucomannan, which is composed of linear chains of (1→4)-linked β-D-glucose and β-D-mannose units with single (1→6)-linked α-D-galactose units as side-chains. Softwoods also contain arabinogalacturonoxylan, and small quantities of other hemicellulosic polysaccharides. Hardwoods, on the other hand, contain glucuronoxylan as the major hemicellulose. Glucuronoxylan consists of a backbone of (1→4)-linked β-D-xylose units, most of which carry acetyl groups, and 4-O-methyl-α-D-glucuronic acid sidechains. Hardwoods also contain glucomannan and small amounts of other hemicelluloses. (Sjöström 1993)

The third wood constituent, lignin, is not a carbohydrate polymer. Instead, it is an amorphous aromatic polymer composed of three monomeric units: p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol. When incorporated into the lignin polymer, these monomers form p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units, respectively. Softwood lignin is composed almost entirely of G units, whereas hardwood lignin contains both G and S units. Lignin is produced by radical coupling of the monomers, yielding a polymer with a variety of linkages between the monomers and no defined molecular formula. Due to the abundance of ether linkages between the lignin monomers, most of the
lignin polymer is non-phenolic and highly recalcitrant. Lignin reinforces the wood structure and makes the cell walls resistant to microbial degradation. (Fengel and Wegener 2011, Sjöström 1993).

In the wood cell wall, the polymeric wood components exist as a composite structure in which the cellulose microfibrils are embedded in a hemicellulose-lignin matrix. The structure of the wood cell wall is lamellar, and it consists of three different layers: the primary cell wall, the secondary cell wall, and the middle lamella. Closest to the cell lumen is the secondary cell wall, which is the thickest cell wall layer and can be further divided into an outer layer (S1), a middle layer (S2), and an inner layer (S3) based on differences in chemical composition and microfibril angle. Most of the secondary cell wall is comprised of the S2, which contains a high proportion of cellulose and relatively little lignin; the S1 and S3 are thinner and the S1 in particular contains less cellulose and more lignin than the S2. Compared to the secondary cell wall, the primary cell wall is very thin and has little influence on the properties of wood. Outside the two cell wall layers lies the middle lamella, which surrounds the wood cells and binds them together. The middle lamella is composed mostly of lignin, with small amounts of cellulose and hemicelluloses as well as pectins that are found mostly in the middle lamella (Fengel and Wegener 2011).

2.1.2 Wood extractives

In addition to the polymeric cell wall components, wood also contains small molecular weight constituents known as extractives. The term extractives is used for these compounds because they can typically be extracted from wood using organic solvents, although it should be recognised that not all extractives are actually solvent-extractable. The extractives are a diverse group of thousands of different molecules, but for the purposes of classification, they can be roughly divided into phenolics, terpenes and terpenoids, and fats and waxes.

The phenolic extractives typically encountered in trees include flavonoids, condensed tannins, lignans, and stilbenes. Some examples of compounds belonging to these groups are given in Figure 2. Flavonoids are a large group of compounds with a C6-C3-C6 carbon skeleton that consists of two phenyl rings connected by a heterocyclic ring. Flavonoids can be further divided into subgroups based on their ring structure and the presence of substituents, and they can occur in wood as their free or glycosylated forms. Compounds belonging to certain flavonoid subclasses can also polymerise to form condensed tannins. Flavonoids and condensed tannins are produced by a variety of wood species (Fengel and Wegener 2011, Hillis 1987).

Lignans are a large and structurally diverse group of compounds with a (C6-C3)-(C6-C3) carbon skeleton composed of two phenylpropanoid units such as coniferyl alcohol linked in a variety of different ways. Compounds linked at the C8 position of the phenylpropanoid sidechain are known as lignans, whereas
compounds linked at other sidechain positions are known as neolignans (Whit- ing 1985). To add further diversity, individual lignans can also oligomerise to form oligolignans (Willför et al. 2004). Lignans are encountered in a variety of tree species, but they are particularly concentrated in the knots of species such as spruce (Holmbom et al. 2003, Willför et al. 2003b). A related group of compounds are norlignans, which are composed of a phenylpropanoid linked to a C6-C2 (phenylethane) structure (Whiting 1985).

![Figure 2. Examples of phenolic wood extractives: the flavonoid catechin and a catechin-based condensed tannin structure, the stilbene pinosylvin, and the lignan nortrachelogenin](image)

Stilbenes are compounds with a C6-C2-C6 carbon skeleton that consists of two aromatic rings connected by a conjugated double bond. 3,5-substituted stilbenes (pinosylvin and its derivatives) are found in pines, whereas more highly substituted stilbenes can be found in some hardwoods (Drewes and Fletcher 1974, Wang and Hart 1983). Glycosides of higher substituted stilbenes are also found in spruce bark, where they are converted to their aglycone forms upon fungal infection (Viiri et al. 2001). Other types of phenolic extractives produced by trees include compounds such as hydrolysable tannins and phenolic acids, but these classes of compounds are not as commonly encountered as the flavonoids, lignans, and stilbenes (Hillis 1987).

Terpenes are a large family of plant metabolites. All terpenes are built from isoprene units, and depending on the number of units, they are known as mono-(2 units), sesqui- (3 units), di- (4 units), and triterpenes (6 units). Terpenes are often intramolecularly linked, forming 6-membered ring structures: monoterpenes contain one ring, whereas di- and triterpenes contain two and three fused rings, respectively. Terpenes are pure hydrocarbons, whereas terpenoids contains functional groups such as OH, C=O, and COOH (Fengel and Wegener 2011). Terpene and terpenoid wood extractives are produced by both hardwoods and softwoods, and in many species, they occur in a special terpenoid secretion.
known as resin (Back 2002). The production of terpenoid resin is characteristic of many softwoods, which produce a resin consisting of a mixture of diterpenoid acids (resin acids) dissolved in volatile mono- and sesquiterpenes (Fengel and Wegener 2011, Phillips and Croteau 1999). Unique terpenoid derivates with a 7-membered ring, known as tropolones, are found in the family Cupressaceae (Haluk and Roussel 2000). The structures of some terpene and terpenoid wood extractives are shown in Figure 3.

![Figure 3. Examples of terpenoid wood extractives: the resin acids abietic and pimaric acid, the monoterpenes \( \beta \)-pinene and \( \delta \)-3-carene, and the tropolone \( \beta \)-thujaplicin](image)

The last group of compounds commonly encountered in wood is fats and waxes. Fats, otherwise known as triglycerides, are the esters of glycerol with three fatty acid chains, whereas waxes are esters of higher alcohols with fatty acids. Fats and waxes occur in both hardwoods and softwoods, with fats being significantly more abundant than waxes, and they can be broken down to yield the individual free fatty acids and alcohols. Fatty acids also exist as esters of sterols, known as steryl esters. Sterols, which are a type of triterpenoid, exist mostly in their esterified form, but they can also be found as free sterols (Fengel and Wegener 2011).

Extractives belonging to these three classes are found in virtually all tree tissues, including the leaves/needles, cones, bark, and xylem. In the xylem, extractives are distributed throughout the whole stem cross-section, but their concentration and composition are far from uniform. Particularly large differences are seen when comparing sapwood (SW) and heartwood (HW), which are the outer and inner layers of xylem, respectively (Figure 4). SW is the portion of tree xylem that contains living cells and usually relatively little extractives. The tracheary elements (tracheids in softwoods, vessels in hardwoods) of SW participate in water conduction and the parenchyma cells are alive, storing reserve materials such as starch, soluble sugars, and fats. HW, on the other hand, contains no living cells or reserve materials, and its tracheary elements no longer conduct water. The HW of many wood species accumulates relatively large
quantities of extractives, which are often coloured and give the HW a darker colour than the surrounding SW.

Figure 4. Cross-section of a Scots pine stem with bark, sapwood, and heartwood. The sapwood appears darker than the heartwood in fresh Scots pine due to its higher moisture content.

The transition of SW to HW takes place in the transition zone (TZ), which is a narrow zone of differentiation that lies between the SW and HW. The SW to HW transition involves a series of metabolic events that leads to the death of the parenchyma cells, along with changes that result in the cessation of water conduction. The formation of HW extractives also takes place during this process, and it can involve both the synthesis of new heartwood extractives and the hydrolysis of existing SW extractives. In the case of new extractives, Magel and coworkers (reviewed in Kampe and Magel 2013, Magel 2000) have shown that parenchyma cells use their reserve materials to synthesise new HW extractives or their precursors, which are then released into the surrounding tissues upon the death of the parenchyma. Two different types of extractives synthesis processes have been identified: in type I, the HW extractives are synthesised directly in the TZ, whereas in type II, the precursors of extractives are synthesised by parenchyma cells in the aging SW and are then converted to the final HW substance in the TZ. The conversion can involve hydrolysis, oxidation, or polymerisation reactions, which may be enzymatically or non-enzymatically catalysed (Kampe and Magel 2013, Magel 2000).

In addition to the synthesis of new compounds, HW formation also involves the release and hydrolysis of various SW extractives. The SW parenchyma cells store fats, waxes, and steryl esters that are hydrolysed during the HW formation process to yield the free fatty acids, fatty alcohols, and sterols (Back 2002). The SW extractives also include terpenes and terpenoids, which are released into the wood tissues during HW formation. Terpenoids are produced by parenchyma cells and by secretory cells such as the resin blisters or resin canal epithelial cells found in resin-producing softwoods (Back 2002, Phillips and Croteau 1999).
Pines, which are the most prolific producers of resin, also transport some of the resin formed in the SW to the HW, where resin acids accumulate in great quantities (Lim et al. 2016).

During the SW to HW transition, the parenchyma cells die and their extractives contents are released into the newly formed HW where they become distributed amongst the HW tissues. The deposition of extractives is believed to proceed via the pits: several researchers have detected the movement of extractives from the parenchyma cells into the lumens of adjacent tracheids or the intercellular spaces between the tracheids (Fengel 1970, Kuo and Arganbright 1980, Streit and Fengel 1994, 1995, Zhang et al. 2004). Extractives also appear to be capable of moving from tracheid to tracheid via pit connections and within the intercellular spaces if they are present (Zhang et al. 2004). Once in the tracheid lumens or intercellular spaces, the extractives begin to impregnate the tracheid cell walls, either from the lumen side or through the middle lamella (Kuo and Arganbright 1980, Zhang et al. 2004). Some extractives also appear to remain within the parenchyma cells: large extractives deposits have been detected in both axial and ray parenchyma in several wood species (Matsushita et al. 2012, Mayer et al. 2006, Nagasaki et al. 2002). The deposition of HW extractives is depicted in Figure 5.

![Figure 5](image.png)

**Figure 5.** The deposition of HW extractives. The movement of extractives from ray parenchyma to tracheid lumens and intercellular spaces (a) and the penetration of extractives into tracheid cell walls and the movement of extractives from tracheid to tracheid (b, c). Note that not all tracheid-to-tracheid pits allow the passage of extractives (Zhang et al. 2004). Despite the penetration of extractives into cell walls, some extractives still remain in cell lumens or in the middle lamella and intercellular spaces.

While there appear to be clear trends in the deposition and cellular level distribution of HW extractives, it should be noted that most of the information comes from studies utilising nonspecific stains to visualise the distribution of extractives (Fengel 1970, Kuo and Arganbright 1980, Streit and Fengel 1994, 1995, Zhang et al. 2004). Consequently, the chemical identity of the visualised compounds is unknown. Different compounds can have different distributions, as
appears to be the case in Cryptomeria japonica. The diterpene phenol ferruginol of C. japonica was present in ray parenchyma and tracheid cell walls, whereas the lignan agatharesinol appeared almost exclusively in ray parenchyma and only occasionally in the lumens of adjoining tracheids (Kuroda et al. 2014, Nagasaki et al. 2002). At the same time, the axial parenchyma appeared to contain yet another unidentified compound or compounds (Nagasaki et al. 2002).

2.2 Wood decay

2.2.1 Wood decaying organisms

Wood degradation is a process in which the chemical components of wood are degraded and consumed as a source of food by wood-inhabiting organisms. Although many different types of organisms can inhabit wood and cause some level of degradation, the most destructive types of degradation are caused by wood decaying fungi. Wood decaying fungi expand in wood by hyphal growth, secreting enzymes and small molecular mass agents that depolymerise the wood cell wall constituents and provide nutrition to the growing fungus. Wood decay is a complex process, and different fungi have evolved different mechanisms of overcoming the recalcitrance of the wood cell walls. Based on these differences in decay mode, the wood decaying fungi can be broadly classified into brown rot, white rot, and soft rot fungi (Fengel and Wegener 2011).

Brown rot fungi cause a type of decay characterised by extensive carbohydrate removal and lignin modification without lignin mineralisation. During decay, the brown rot hyphae reside in the cell lumens and secret diffusible wood degrading agents into the wood cell walls. The degradative agents cause extensive degradation particularly in the S2 layer of the cell wall, although different types of secondary cell wall degradation patterns are possible (Kim et al. 2015, Schwarze 2007). Early brown rot is characterised by rapid strength loss due to carbohydrate depolymerisation, and in advanced stages of decay, the wood turns into a brittle brown material that forms cuboidal cracks. Brown rots prefer to degrade softwoods, and they are an important cause of damage to structural timber in buildings (Schwarze 2007).

White rots, on the other hand, degrade all wood components. White rots can be further divided into selective and simultaneous degraders: selective white rots preferentially degrade lignin and hemicelluloses in early stages of decay, whereas simultaneous white rots degrade all wood components equally. Some white rots can cause both types of decay. In selective white rot, the fungal hyphae either grow in cell lumens and secret diffusible agents to delignify the cell walls, or they penetrate into the cell walls and begin to delignify the middle lamella. Simultaneous white rots, on the other hand, penetrate the cell walls and degrade them in close proximity to the hyphae, causing localised erosion. White rot, especially the selective kind, often occurs in pockets and causes the wood to
become fibrous and bleached in appearance (Schwarze 2007). White rots typically prefer hardwood substrates; this preference is believed to be due to the higher recalcitrance of softwood lignin (Faix et al. 1985, Highley 1982).

Finally, soft rots are a group of fungi that share similarities with both brown rot and white rot. Soft rots can degrade the wood carbohydrates and lignin at varying rates, although many of them are similar to brown rots in that their ability to decompose lignin is limited. The defining feature of soft rots is their growth pattern in wood, which involves penetration of the cell walls by fine penetrating hyphae and the formation of cavities within the cell walls. Erosion of the cell walls by hyphae growing in the cell lumen is also characteristic of soft rot. Soft rots attack both softwoods and hardwoods, and they tend to occur in damp or fluctuating conditions. Fungi that normally cause brown or white rot can also sometimes cause soft rot as an alternative form of decay (Schwarze 2007).

2.2.2 Enzymatic wood degradation

Regardless of the type of decay, wood is a challenging substrate to use as a source of food due to its complex chemical and physical composition. To access and convert the wood polysaccharides to digestible sugars, wood decaying fungi must utilise various enzymatic and non-enzymatic degradative processes. The array of enzymes secreted by the different kinds of wood decaying fungi is vast and varies greatly from fungus to fungus. However, most of these enzymes can be divided into cellulolytic, hemicellulolytic, and ligninolytic enzymes based on the wood component they target.

Even though cellulose consists of linear chains of only glucose units, its efficient and complete hydrolysis requires the action of three different types of enzymes: endo-β-1,4-glucanases, celllobiohydrolases (exoglucanases), and β-glucosidases (see Figure 6). Endoglucanases initiate the hydrolysis of cellulose by randomly cleaving the amorphous regions of cellulose, after which the celllobiohydrolases attack the newly revealed cellulose chain ends to liberate cellobiose or cello-oligomers. β-glucosidases then hydrolyse the cellobiose units produced by celllobiohydrolase into glucose (Dashtban et al. 2009, Pérez et al. 2002), which the fungi use for nutrition. Although this classical three-enzyme cellulase system can achieve complete cellulose hydrolysis, recent research has revealed that cellulolytic fungi also produce various cellulose-active oxidoreductases that assist in the breakdown of crystalline cellulose (Horn et al. 2012).

The enzymatic hydrolysis of hemicelluloses to monosaccharides is similar to the hydrolysis of cellulose, except that a greater number of enzymes are needed due to the chemical diversity of hemicelluloses. β-1,4-mannanases and β-1,4-xylanases cleave the backbones of mannans and xylans, similar to the way endoglucanases cleave the amorphous regions of cellulose, and β-mannosidases and β-xylosidases then convert the newly formed manno- and xylo-oligosaccha-
rides to monosaccharides. No enzyme analogous to cellobiohydrolase is involved in hemicellulose degradation; instead, the complete hydrolysis of hemicelluloses requires the action of sidechain-removing accessory enzymes, such as α-galactosidases, α-arabinanases, and various esterases (Pérez et al. 2002).

The enzymatic degradation of lignin differs from that of cellulose and hemicelluloses in several key ways. Unlike cellulose and hemicelluloses, lignin is not attacked to release digestible sugars but to increase the ability of cellulases and hemicellulases to access and hydrolyse the wood polysaccharides. The mechanism of lignin degradation is also very different from that of the polysaccharides: the polysaccharides are primarily degraded by a hydrolytic mechanism, whereas lignin is degraded oxidatively. The enzymatic degradation of lignin is a highly complex process that involves the action of peroxidases, laccases, and several accessory enzymes. The most important peroxidases are lignin peroxidase and manganese-dependent peroxidase. In their catalytic cycle, the peroxidase enzyme is first oxidised by hydrogen peroxide, after which the enzyme in turn oxidises two molecules of its substrate. The substrates of lignin peroxidase include a variety of phenolic and non-phenolic fungal metabolites and lignin structures that are oxidised to radical cations. The substrate of manganese peroxidase is Mn(II), which is oxidised to Mn(III). Mn(III) is a diffusible oxidising
agent that causes degradation of the phenolic lignin moieties, giving rise to lignin derived radicals (Martinez et al. 2005, ten Have and Teunissen 2001). Lignin degradation by peroxidases is a complex cycle of events: the radical species generated by the enzymes initiate non-enzymatic degradative reactions in lignin, and the products of lignin degradation can act as substrates for further peroxidase action or the action of hydrogen peroxide generating enzymes that enable further peroxidase activity (Martinez et al. 2005).

Laccases are enzymes that catalyse the reduction of oxygen to water, along with the oxidation of a substrate. Laccases can oxidise a variety of phenolic substrates, including some lignin model compounds, but they are unable to oxidise non-phenolic lignin structures on their own. However, certain laccase substrates can act as so-called laccase mediators. These substrates are oxidised to stable radicals that can attack lignin and cause non-enzymatic degradative reactions, similar to the peroxidases. Due to their broad substrate range, laccases might also serve functions other than lignin degradation, including the neutralisation of toxic compounds (Baldrian 2006).

All wood decaying fungi use enzymes in wood degradation, but there are significant differences in the composition of enzymes secreted by white rot and brown rot fungi. White rots produce a full set of cellulases and various hemicellulases, and they produce the ligninolytic peroxidases and laccases (Hori et al. 2013, Riley et al. 2014). Some white rots produce all three lignin-degrading enzymes, while others produce only two in some combination (Hatakka 1994). White rots also produce various carbohydrate active oxidoreductases and accessory enzymes for lignin degradation (Hori et al. 2013, Riley et al. 2014). Brown rots, on the other hand, produce a significantly reduced set of carbohydrate active enzymes; most importantly, many of them have lost the ability to produce cellobiohydrolases. Brown rots also lack the genes to produce lignin-degrading peroxidases, although many of them do produce laccases (Riley et al. 2014).

### 2.2.3 Fenton reaction-based wood degradation

In addition to enzyme-catalysed degradation, wood can also be degraded non-enzymatically. Non-enzymatic degradation is based on reactive free radicals that can be generated without the action of extracellular enzymes, although enzymes are commonly involved in radical-based degradation as well in an accessory role. The most extensively characterised wood-degrading radical is the hydroxyl radical (‘OH), which is produced by the Fenton reaction (reaction 1). After the rapid initial ‘OH-producing reaction, the reactants and products of the Fenton reaction enter a cycle of slower reactions that produce other oxygen radicals and generate the original reactants (reactions 2-7).

\[
\text{Fe(II)} + \text{H}_2\text{O}_2 \rightarrow \text{Fe(III)} + '\text{OH} + \cdot\text{OH} \quad (1)
\]

\[
\text{Fe(III)} + \text{H}_2\text{O}_2 \rightarrow \text{Fe(II)} + \text{HOO}^{' + \cdot\text{H}} \quad (2)
\]

\[
\text{HO}^{' + \cdot\text{H}} + \text{H}_2\text{O}_2 \rightarrow \text{HOO}^{' + \cdot\text{H}_2\text{O}} \quad (3)
\]
The hydroxyl radical is the strongest oxidant found in aqueous systems and can react rapidly with virtually any organic molecule, causing hydrogen abstraction in non-aromatic molecules and hydroxylation and ring opening in aromatics (Hammel et al. 2002, Walling 1975). In the case of wood, 'OH attack on cellulose and hemicelluloses causes the formation of ROO' radicals that can undergo further reactions that result in depolymerisation and the production of oxidised sugar residues. Attack on lignin can result in a number of different reactions, including demethoxylation by MeOH elimination, depolymerisation by ether cleavage, hydroxylation by 'OH addition, or C-O-oxidation. The hydroperoxyl (HOO') and wood-derived radicals generated by the Fenton reaction can also cause damage to wood, although they are weaker than the hydroxyl radical (Hammel et al. 2002).

To make use of the Fenton reaction, the fungi must be able to sequester iron from their surroundings. The iron used in the Fenton reaction is naturally present in wood, and it is extracted from insoluble iron complexes by oxalic acid and other organic acids secreted by fungi (Arantes et al. 2012). The iron exists naturally in its inactive, ferric form (Fe(III)), which means that the fungi must reduce it to its ferrous form (Fe(II)) to use it in the Fenton reaction. To accomplish this task, fungi secret small molecular weight phenolics such as hydroquinones that are easily oxidised. Under appropriate degradation conditions, the hydroquinones reduce the iron directly and are oxidised to semiquinone radicals in the process. The semiquinone radicals then reduce more Fe(III) to Fe(II) or O₂ to HOO' and are themselves oxidised to benzoquinones (Arantes et al. 2012, Baldrian and Valaskova 2008). Alternatively, the hydroquinones can be first oxidised to semiquinone radicals by enzymes such as laccase, after which they reduce Fe(III) or O₂ and are oxidised to benzoquinones (Gómez-Toribio et al. 2009, Guillén et al. 2000, Wei et al. 2010). To keep reducing iron for the Fenton reaction, the fungi need to convert the quinones back to their hydroquinone form. Quinone reduction can be accomplished by enzymes such as quinone reductase, an intracellular enzyme, or possibly by cellobiose dehydrogenase, a carbohydrate active oxidoreductase (Arantes et al. 2012, Baldrian and Valaskova 2008).

Use of the Fenton reaction also necessitates the production of hydrogen peroxide. Hydrogen peroxide can be generated in many different ways, including via the reduction of O₂ by the semiquinone radicals. The semiquinones reduce O₂ to HOO’, which then reacts with Fe(II) (reaction 6) or another molecule of HOO’ (reaction 7) to give H₂O₂. Hydrogen peroxide can also be generated from the auto-oxidation of iron, or it can be produced enzymatically by enzymes such as glucose oxidase, alcohol oxidase, or aryl alcohol oxidase. These enzymes are

\[
\begin{align*}
\text{HO}^- + \text{Fe(II)} & \rightarrow \text{Fe(III)} + \cdot \text{OH} \quad (4) \\
\text{Fe(III)} + \text{HOO}' & \rightarrow \text{Fe(II)} + \text{O}_2\text{H}^+ \quad (5) \\
\text{Fe(II)} + \text{HOO}' + \text{H} & \rightarrow \text{Fe(III)} + \text{H}_2\text{O}_2 \quad (6) \\
\text{HOO}' + \text{HOO}' & \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \quad (7)
\end{align*}
\]
Background

produced by many wood decaying fungi, and they use wood degradation products as substrates for $\text{H}_2\text{O}_2$ generation (Arantes et al. 2012, Baldrian and Valaskova 2008). Figure 7 is a summary of a simplified fungal Fenton system in which iron reacts directly with hydroquinones and $\text{H}_2\text{O}_2$ is generated enzymatically by the fungus and via the oxidation of semiquinones.

**Figure 7.** Production of $\cdot\text{OH}$ by a simplified fungal Fenton system. OA, oxalic acid; HQ, hydroquinone, $\text{SQ}^-$, semiquinone radical; BQ, benzoquinone

Radical-based wood degradation is believed to be utilised by all types of wood decaying fungi, at least in incipient decay stages, because the fungal wood-degrading enzymes are too large to penetrate the cell walls of sound wood (Hammel et al. 2002). Wood-degrading Fenton chemistry is particularly extensively utilised by the brown rots, which lack the ability to enzymatically degrade lignin and often produce a reduced set of cellulases (Hammel et al. 2002, Martinez et al. 2009, Wymelenberg et al. 2010). In brown rot, the wood substrate is degraded by the Fenton reaction before hydrolytic enzymes are produced (Zhang et al. 2016, Zhang and Schilling 2017). Fenton degradation is believed to allow the fungal enzymes to penetrate the cell wall and increase the hydrolysability of wood, but recent evidence has shown that it might actually result in the formation of fully soluble carbohydrate degradation products, which then diffuse out of the cell wall (Goodell et al. 2017).
2.2.4 Extractives as decay-preventing agents

The SW of all wood species is generally readily decayed by wood decaying fungi, while the HWs of the same species often have some level of natural resistance to decay. Several theories have been proposed to explain the natural decay resistance of HW, but the primary reason is likely to be the presence of HW extractives (Taylor et al. 2002). Experiments with a number of species have shown that durable wood becomes more susceptible to decay when the extractives are removed, and that susceptible wood becomes more durable if impregnated with the extractives of a durable species (Hart and Hillis 1974, Kamdem 1994, Kirker et al. 2013, Onuorah 2000, Schultz et al. 1995, Smith et al. 1989, Taylor et al. 2006). The antifungal properties of various extractives have also been demonstrated using simple bioassays (Chang et al. 1999, Hart and Hillis 1974, Mihara et al. 2005, Schultz et al. 1995); together, these three types of evidence have led to the consensus that antifungal extractives are primarily responsible for the decay resistance of durable HW.

However, the relationship between extractives and decay resistance is not straightforward. For example, several experiments have shown that extraction does not always fully eliminate the decay resistance of durable HW (Hart and Hillis 1974, Kirker et al. 2013, Smith et al. 1989). While it may also indicate the involvement of factors other than extractives, the persistence of decay resistance, and in some cases HW colour, has led to the assumption that not all extractives are actually removable by solvent extraction (Hart and Hillis 1974, Hart and Shrimpton 1979, Mayer et al. 2006). The non-extractable “extractives” are believed to be polymeric or associated with cell wall components such as lignin. While analyses of such extractives are limited, lignin-bound ellagitannins have been detected in the HW of American chestnut (Helm et al. 1997).

Discrepancies are also occasionally found between the antifungal activity of extractives in simple bioassays and their ability to provide decay resistance when impregnated into susceptible wood. Stilbenes in particular tend to show high antifungal activity in bioassays but fail to improve decay resistance upon impregnation unless relatively high concentrations are used (Hart and Shrimpton 1979, Schultz et al. 1995). Similarly, sometimes little correlation is found between the concentration of a known antifungal compound and the decay resistance of HW, as was the case with the highly antifungal tropolones in Western red cedar HW (Morris and Stirling 2012). The low efficacy in wood of an otherwise antifungal compound could be due to the binding of extractives to cell wall polymers, to differences in fungal decay mode between a nutrient medium and the natural wood substrate, or to an unfavourable distribution of the compound in wood. Yet another source of confusion is the finding that sometimes trees produce HW extractives that appear to have little to no antifungal activity in any test system (Chang et al. 1999, Malterud et al. 1985, Reyes-Chilpa et al. 1998). These compounds may serve a purpose unrelated to microbial resistance, but they might also be involved in decay resistance due to synergistic action with more bioactive extractives. Synergism between extractives has been proposed
(Schultz et al. 1995, Stirling and Morris 2016), but most of the evidence for synergism comes from studies involving artificial or non-wood derived compounds (Hsu et al. 2007, Schultz and Nicholas 2002).

Even though the relationship between extractives and decay is complicated, it is clear that extractives are involved in decay prevention. However, the mechanisms by which extractives accomplish decay prevention are far from fully understood. Direct toxicity to fungal cells is an obvious feature of many extractives, and while relatively little work has been done on the effects of wood extractives on wood decaying fungi, much is known about the interaction between other plant metabolites and plant and human pathogenic fungi. Extensive work on grapevine and its pathogens, for example, has shown that stilbenes have affinity for membrane proteins and function as uncoupling agents of electron transport and photophosphorylation, thus interfering with basic cellular metabolism (Jeandet et al. 2002). Many other compounds disrupt fungal cell membrane integrity and cause ion imbalance, resulting in cell rupture and metabolite leakage. Many compounds also interfere with the function of enzymes involved in basic cellular metabolism or the synthesis of cell wall components or membrane sterols (Valette et al. 2017).

However, decay prevention may also involve mechanisms other than direct toxicity. Extractives may, for example, function as antioxidants, preventing the free radical-induced oxidation of wood cell wall constituents. Antioxidants can scavenge dangerous free radicals by reacting with them, or they can prevent their formation by chelating metals that can catalyse radical-producing reactions such as the Fenton reaction (see section 2.2.3) (Balasundram et al. 2006, Rice-Evans et al. 1997). Metal chelation can also interfere with the production of metal containing enzymes such as laccases. Schultz and co-workers (Schultz and Nicholas 2000, 2002, 2011, Schultz et al. 2004) investigated the effects of antioxidants and metal chelators on wood decay and found that they can improve decay resistance when used in combination with a fungicide. This led them to suggest that the antifungal activity of extractives may involve both direct toxicity and antioxidant activity (radical scavenging and metal chelation). Many wood extractives have been shown to be effective antioxidants (Apetrei et al. 2011, Eklund et al. 2005, Pietarinen et al. 2006, Willför et al. 2003a), but only a few studies have considered their effects on oxidative wood degradation (Binbuga et al. 2008, Donoso-Fierro et al. 2009). All of these studies have also been conducted using isolated extractives in solution, which means that their ability to function in their natural wood matrix remains unknown.

In addition to interfering with oxidative wood degradation, extractives may also inhibit the function of hydrolytic enzymes (see section 2.2.2). Hydrolase inhibition has not been studied as a mechanism of decay prevention by extractives, but studies on enzymatic biomass conversion processes have shown that various biomass-derived phenolic compounds can interfere with hydrolase activity. Many small molecular weight phenolics form complexes with cellulases and hemicellulases, causing their deactivation and thus the inhibition of hydrolysis (Boukari et al. 2011, Sharma et al. 1985, Tejirian and Xu 2011, Ximenes et
Lignin can also inhibit hydrolases by blocking their access to the carbohydrates and causing their unproductive binding onto its hydrophobic surface (Palonen et al. 2004, Rahikainen et al. 2011, Rollin et al. 2011). Due to the similarity between many extractives and other biomass phenolics, it is reasonable to suspect that extractives might have similar effects, although the ability of extractives to function in their wood matrix needs to be confirmed. Some cell wall associated hydrophobic extractives may even behave similarly to lignin.

Although the indirect effects of extractives on wood decaying fungi are not limited to antioxidant activity and hydrolase inhibition, these other effects are likely to be secondary in significance. Extractives can, for example, reduce the rate of fungal degradation by lowering the equilibrium moisture content of wood, but this effect appears to be relatively small (Nzokou and Kamdem 2004) and is likely to be of limited relevance in ground contact where free water exists. Extractives can also form encrustations on pit membranes, hindering the movement of fungi and water, but it is unknown whether these provide any benefit in addition to pit aspiration and other obstructions found in HW (Taylor et al. 2002). Pit aspiration itself appears to provide some decay resistance to HW (Schubert et al. 2011).

### 2.3 Scots pine

#### 2.3.1 Extractives

Scots pine is one of the most common species in northern Europe and accounts for approx. 50% of the total forest stock in Finland. Due to its availability and commercial significance, the chemistry and properties of Scots pine have been extensively investigated, including the composition of its extractives. Scots pine SW extractives are known to consist primarily of triglycerides (fats). Resin acids and steryl esters are present in small amounts and free fatty acids and sitosterol in trace quantities (Willför et al. 2003b). The triglycerides of Scots pine SW are composed almost entirely of C18 acids: stearic acid (C18:0) is a minor component, whereas oleic (C18:1), linoleic (C18:2), and linolenic (C18:3) acids are abundant (Piispanen and Saranpää 2002). Phenolic extractives are absent or present in only trace amounts in the SW (Willför et al. 2003b).

The extractives composition of the wood material changes in the TZ. The most significant change is the appearance of pinosylvins (3,5-substituted stilbenes, see section 2.1.2), which are synthesised in the TZ and reach their peak concentration within a few annual rings (Bergström et al. 1999, Bergström 2003, Lim et al. 2016). Other changes include a drop in the concentration of triglycerides and an increase in that of free fatty acids, which is consistent with the hydrolysis of triglycerides during HW formation (Bergström 2003, Piispanen and Saranpää 2002). The concentration of resin acids increases as well, and the relative proportions of the individual resin acids change (Bergström 2003, Willför et al. 2003b).
The HW extractives consist primarily of resin acids and the newly formed pinosylvins and free fatty acids. The most abundant group of compounds is the resin acids (Fang et al. 2013, Hovelstad et al. 2006, Willför et al. 2003b), which are mostly of abietane-type (abietyc, dehydroabietyc, neoabietyc, palustriic, and levopimariic acids). Pimarane-type resin acids (pimariic, isopimariic, sandaracopimariic acids) are also present in smaller quantities (Willför et al. 2003b). The pinosylvins of Scots pine HW consist primarily of pinosylvin (PS, 3,5-dihydroxystibene) and pinosylvin monomethyl ether (PSM, 3-hydroxy-5-methoxystibene). Other stilbenes such as pinosylvin dimethyl ether are present in trace amounts (Fang et al. 2013, Hovelstad et al. 2006, Willför et al. 2003b). The concentration of pinosylvins peaks in the TZ/outer HW and then declines towards inner HW (Bergström et al. 1999, Bergström 2003). The cause of the decline is unknown, but it may involve oxidation, polymerisation, or reactions with lignin.

As could be expected based on the composition of triglycerides, the free fatty acids of Scots pine HW consist almost entirely of C18 acids. The proportions of the individual acids are very similar to those found in the triglycerides, and they remain relatively unchanged from outer to inner HW, despite a slight drop in total fatty acid concentration (Piispanen and Saranpää 2002). The concentration of intact triglycerides is very minor (Piispanen and Saranpää 2002, Willför et al. 2003b). The HW also contains small amounts of modified resin acids, steryl esters, and free sitosterol (Willför et al. 2003b). Unlike the HW of many other wood species, Scots pine HW is not rich in flavonoids and contains only trace amounts of the flavonoid pinocembrin (Willför et al. 2003b).

In addition to the SW, HW, and TZ, the knots of Scots pine are also of interest in terms of extractives. Pine knotwood (KW), or more specifically knot HW, can contain over 30% of extractives by weight. The most abundant group of compounds in KW is the resin acids, which are mostly of abietane-type like in HW. PS and PSM are abundant, whereas the amount of free fatty acids is similar to or lower than in the HW (Fang et al. 2013, Willför et al. 2003b). In addition to the compounds found in HW, Scots pine KW also contains lignans and oligolignans (Willför et al. 2003b, 2004). The primary KW lignan is nortrachelogenin, and other monomeric lignans are found only in small amounts (Willför et al. 2003b).

The concentration of extractives in any given tissue of Scots pine is highly variable, with variation caused by factors such as tree age, genetics, and environmental conditions (Fries et al. 2000). Extractive concentrations also vary along the length of the tree stem, and from outer to inner HW and along the length of knots (Bergström et al. 1999, Bergström 2003, Hovelstad et al. 2006, Willför et al. 2003b). Typical concentration ranges of the most abundant SW, HW, and KW extractives are given in Table 1. The data in Table 1 are from Willför et al. (2003b) and Fang et al. (2013) and were collected from trees aged 46-137 years.
Table 1. Typical concentrations of important extractives compounds or compound groups in Scots pine SW, HW, and KW. Data from Willför et al. (2003b) and Fang et al. (2013).

<table>
<thead>
<tr>
<th></th>
<th>SW</th>
<th>HW</th>
<th>KW</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS</td>
<td>0.05 - 0.14</td>
<td>2.2 - 7.1</td>
<td>4.3 - 20</td>
</tr>
<tr>
<td>PSM</td>
<td>0 - 0.03</td>
<td>4.2 - 12</td>
<td>5.9 - 57</td>
</tr>
<tr>
<td>Nortrachelogenin</td>
<td>0 - 0.02</td>
<td>0 - 0.11</td>
<td>3.8 - 28</td>
</tr>
<tr>
<td>Resin acids</td>
<td>0.9 - 3.5</td>
<td>16 - 99</td>
<td>88 - 298</td>
</tr>
<tr>
<td>Fatty acids</td>
<td>0.08 - 0.55</td>
<td>2.2 - 15</td>
<td>1.9 - 6.0</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>7.8 - 23</td>
<td>0.14 - 0.39</td>
<td>0.55 - 2.9</td>
</tr>
</tbody>
</table>

2.3.2 Durability

Scots pine HW has been favoured as a construction material in northern Europe due to its decay resistance. Although the HW is typically classified as only moderately or slightly durable, its resistance is still significantly higher than that of other common local species such as spruce or birch. Scots pine SW, like the SW of most species, is classified as nondurable (EN 350 2016, Van Acker et al. 2003).

Although moderately or slightly durable on average, the decay resistance of Scots pine HW is actually highly variable (Harju et al. 2001). The causes of durability have been extensively investigated in Scots pine HW, and the results show that extractives are likely to be the most significant factor in determining mass loss due to decay. Pinosylvinns appear to be particularly strongly linked to decay resistance: their antifungal activity is high (Hart and Shrimpton 1979), and their concentration in wood is correlated with the decay resistance of HW (Harju et al. 2003, Venäläinen et al. 2003, 2004). However, investigations using the HW of various pine species have revealed that pinosylvin content and decay resistance are sometimes poorly correlated, with specimens of low pinosylvin content showing low mass loss and vice versa (Hart and Shrimpton 1979). In the case of Scots pine, particularly poor correlations have been recorded in outer HW, which is where the concentration of pinosylvinns and decay resistance are the highest. Pinosylvin content and decay resistance were significantly correlated in inner HW, but even there, a significant portion of the decay resistance could not be accounted for by pinosylvinns (Venäläinen et al. 2003).

The poor correlation between pinosylvin content and decay resistance, in combination with the reduced antifungal activity of pinosylvinns on woody substrates (Hart and Shrimpton 1979), has led many to suspect that extractives other than pinosylvinns are involved in the decay resistance of pine HW. Resin acids are often cited as a factor in durability, but the evidence on their involvement is conflicting. Some studies have found a higher concentration of resin acids in decay resistant than in susceptible HW, whereas others report no statistically significant correlations between resin acids and mass loss due to decay (Harju et al. 2002, 2003, Venäläinen et al. 2003). Furthermore, tests involving resin acid impregnation have shown limited improvement in decay resistance.
unless very large concentrations are used (Eberhard et al. 1994, Hart et al. 1975). The lack of antifungal effects has led some to suggest that the resin acids’ principal mode of action might be their hydrophobicity (Eberhard et al. 1994). While there is evidence that impregnation of wood with resin acids can reduce water uptake (Schultz et al. 2007b), investigations of natural HW have found no correlation between resin acid content and equilibrium moisture content or water content after wetting (Harju et al. 2002, Venäläinen et al. 2003).

Apart from pinosylvins and resin acids, the above-mentioned equilibrium moisture content and water content after wetting are the only factors identified that seem to be (at least in some cases) correlated with the decay resistance of HW (Venäläinen et al. 2003). Variations in basic density or cell wall polymer composition do not seem to be involved in decay resistance (Harju et al. 2003, Venäläinen et al. 2003), and no additional extractives components have been identified. Synergism between different extractives has been suggested to play a role in the decay resistance of some species (Schultz et al. 1995, Stirling and Morris 2016), but no such effects have been identified in Scots pine HW.

What then controls pine decay resistance in addition to pinosylvins and resin acids? One factor that has received limited attention is the distribution of extractives. Variations in the cellular level distribution of extractives could explain differences in decay resistance, but currently no information is available on the distribution of pinosylvins or other pine HW extractives. Some aspects of the distribution of pinosylvins might also offer some additional insight as to why such otherwise highly antifungal compounds are able to provide only moderate decay resistance to wood (Hart and Shrimpton 1979). Another factor that is yet to be investigated is the decreasing pinosylvins content of aging HW. The pinosylvins content decreases from outer HW to inner HW (Bergström et al. 1999, Bergström 2003), but no information exists on what causes this decrease, what compounds the pinosylvins are converted to, or how these new compounds affect decay resistance.

In addition to the causes of decay resistance, knowledge gaps also exist on how the known antifungal extractives prevent decay. Pinosylvins are known to be toxic to fungi, but their exact mechanism of action is unknown, as are their other potential modes of antifungal activity. It could be assumed that the toxicity of pinosylvins involves similar mechanisms as those of resveratrol and its derivatives, except that the uncoupling reactions caused by these stilbenes are believed to require a 4’-OH (Jeandet et al. 2002), which the pinosylvins lack. Pinosylvins and pinosylvin-containing extracts have been shown to have radical scavenging activity (Pietarinen et al. 2006, Willför et al. 2003a), which means that they may be able to interfere with oxidative wood degradation. However, their ability to function in wood has not been demonstrated. Pinosylvins might also act as hydrolase inhibitors, but so far the stilbene glucoside isorhapontin is the only stilbene that has been investigated (Shibutani et al. 2001). Little is also known about the mechanism of action of resin acids, except that abietic acid has been shown to inhibit the enzymatic hydrolysis of cellulose (Leskinen et al. 2015).
3. Experimental

3.1 Wood and extractives materials

3.1.1 Sample preparation

The wood material used in all studies consisted of freshly felled Scots pine logs that were stored frozen and protected from light until use. HW and SW (Papers I-IV) were obtained from mature logs, whereas KW (Papers I and III) was obtained from younger, branch-rich logs. More details on the wood materials can be found in Papers I-IV.

For Paper I and Paper II, the wood materials were separated into SW, HW, and KW and then ground to a powder in a Wiley mill. The powders were air dried (Paper I) or freeze dried (Paper II), and a portion of each powder Soxhlet extracted with either acetone or sequentially with n-hexane and MeOH. A small aliquot of each extract was taken for composition analysis by GC-MS and GC, while the remaining extracts were evaporated to dryness under vacuum, weighed, and redissolved in EtOH. The extracts and the unextracted and extracted wood powders were used to study the antioxidant (Paper I) and hydrolase inhibitor (Paper II) properties of extractives.

Paper III and Paper IV used solid wood samples. In Paper III, blocks of SW, outer HW, and middle HW were sawn from a strip of wood as shown in Figure 8a, while KW blocks were prepared by cutting the knots out of the surrounding wood material and trimming until only KW remained (Figure 8b-d). The SW and HW blocks were cut in half across the grain: one half was used to cut sample sticks measuring 5 x 5 mm in cross section, while the other half was cut into small pieces and ground in a Wiley mill. The KW blocks were also cut into sticks, with half of the sticks kept as samples and the other half ground in a Wiley mill. The wood sticks and powders were air dried, after which the powders were Soxhlet extracted with acetone to determine the extractives composition of the materials. Half of the sticks were also extracted with acetone to produce extractives-free controls of each type of sample.
Experimental

Figure 8. Sampling of SW, outer HW (OHW), middle HW (MHW) and KW. The position of SW, OHW, and MHW in the wood strip (a), and the processing of knot samples, showing knots in a section of log (b), a knot removed from one section (c), and a final trimmed KW block (d) (Paper III)

In Paper IV, sticks approx. 250 mm in length were sawn from OHW as shown in Figure 9. The sticks were planed to a cross section of 7 x 7 mm, after which each stick was cut to produce a series of final sample sticks measuring 40 mm in length. The sticks were sealed in plastic bags and sterilised by irradiation (dose 25-50 kGy). The sterilised blocks were then used in decay tests without drying or any other further processing.

Figure 9. Sampling of outer HW for Paper IV. Long sticks were cut from the positions indicated on the figure, planed, and cut in length to produce the final samples (Paper IV)

3.1.2 Analysis of extractives

The chemical composition of the used extracts and wood materials was determined using GC (Shimadzu GC 2010 Plus) and GC-MS (Trace 1300 GC/Thermo Scientific ISQ single quadrupole MS). In Paper I and Paper III, GC-MS was
used for both compound identification and quantitation, whereas in Paper II and Paper IV compounds were identified with GC-MS but quantified with GC-FID. To prepare samples for GC/GC-MS analysis, an aliquot of each sample was combined with an aliquot of internal standard solution (heneicosanoic acid) and the solvents evaporated under vacuum. The samples were dissolved in pyridine and silylated at 70°C after the addition of a mixture of N,O-bis(trimethylsilyl)trifluoroacetamide and chlorotrimethylsilane. Further details on sample preparation and the analytical methods can be found in Papers I-IV.

3.2 Antioxidant activity of extractives (Paper I)

The antioxidant activity of extractives was studied using extract solutions and wood powders. The solutions selected for study were the acetone and MeOH extracts of HW and KW and solutions of pure PS and PSM. Two known antioxidants, butylated hydroxyanisole (BHA) and quercetin, served as standards. Unextracted and acetone extracted HW and KW were used as the wood powders, while acetone extracted SW served as an additional extractives-free reference material. The extract solutions and pure compounds were tested at a range of concentrations to calculate their 50% effective concentrations. The wood powders were tested at a concentration of 0.5 and 1% (w/v), and their activities were reported as % of maximum activity.

3.2.1 Free radical scavenging

Free radical scavenging assays were performed using a solution of ABTS’+, which was prepared by reacting 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) with potassium persulfate according to Re et al. (1999). For assay of extracts, the radical solution was diluted with 1:1 EtOH:water to give an absorbance of 0.7 at 734 nm. A 150 μL aliquot of the ABTS’+ solution was mixed with 50 μL of extract solution in a microtiter plate, and the reduction in A734 was recorded after 6 min of incubation at room temperature. Pure EtOH was used in place of extract in the reference. For assay of wood powders, the ABTS’+ solution was diluted to 0.7 A734 with water and mixed with the wood powders in 1.5 mL capped tubes. The tubes were incubated at room temperature for 6 min in an inverting mixer, after which the solutions were filtered into cuvettes through syringe filters and the A734 values read immediately. Pure ABTS’+ solution was used as reference. In both assays, scavenging activity (%) was calculated as follows:

\[
\text{ABTS’+ scavenging (\%)} = 100 \times \frac{(A_{\text{ref}} - A_{\text{sample}})}{A_{\text{ref}}}
\]

where \(A_{\text{ref}}\) is the absorbance of the reference and \(A_{\text{sample}}\) is that of the sample.
Iron binding assays were performed with ferrozine according to Dinis et al. (1994). For assay of extracts, 100 µL of each solution was first mixed with 50 µL of freshly prepared 0.1 mM FeCl₂ in a microtiter plate, followed by 50 µL of 1 mM ferrozine. FeCl₂ and ferrozine were both prepared in 1:1 EtOH:buffer (0.1 M acetate, pH 4) to aid solubilisation and to provide an acidic environment similar to brown rotting wood. The absorbance of the samples at 562 nm was measured after 10 min of incubation at room temperature. Pure EtOH was used in place of extract in the reference, and additional controls containing EtOH:buffer in place of ferrozine were measured for each sample to account for the formation of coloured complexes in some samples. For assay of wood powders, 1.35 mL of 50 mM acetate buffer (pH 4) and 75 µL of 0.5 mM FeCl₂ were mixed with the wood powders in 1.5 mL capped tubes. The tubes were incubated at room temperature for 10 min in an inverting mixer, after which 75 µL of 5 mM ferrozine was added to each tube. The solutions were filtered into cuvettes through syringe filters and the A₅₆₂ values read immediately. Ferrozine and FeCl₂ in buffer were used as a reference, and controls without added iron were measured for each sample to account for potential iron contamination in wood powders. In both assays, Fe²⁺ binding activity (%) was calculated as:

\[
\text{Fe}^{²⁺} \text{ binding (\%) = } 100 \times \frac{[A_{\text{ref}} - (A_{\text{sample}} - A_{\text{control}})]}{A_{\text{ref}}}
\]

where Aₕ₆₂ is the absorbance of the reference, and A_sample and A_control that of the sample and the appropriate control, respectively.

Iron reduction assays were performed similarly to the iron binding assays, except that FeCl₃ was used in place of FeCl₂. For extracts, 100 µL of each solution was mixed with 50 µL of ferrozine, followed by 50 µL of freshly prepared FeCl₃. The samples were incubated at room temperature for 10 min after which the increase in A₅₆₂ was measured. The reference contained FeCl₂ in place of FeCl₃, and pure EtOH in place of extract. Additional controls containing EtOH:buffer in place of ferrozine were measured for each sample. For wood powders, buffer and ferrozine were added to the powders and mixed, followed by the addition of FeCl₃. The tubes were incubated at room temperature for 10 min in an inverting mixer, after which the solutions were filtered into cuvettes and the A₅₆₂ values read immediately. Ferrozine and FeCl₂ in buffer were used as a reference, and controls without any added iron were measured for each sample. In both assays, Fe³⁺ reducing activity (%) was calculated as:

\[
\text{Fe}^{³⁺} \text{ reducing activity (\%) = } 100 \times \frac{(A_{\text{sample}} - A_{\text{control}})}{A_{\text{ref}}}
\]

where Aₕ₆₂ is the absorbance of the reference sample, and A_sample and A_control that of the sample and the appropriate control, respectively.
3.3 Fungal hydrolase inhibition by extractives (Paper II)

Hydrolase inhibition by extractives was studied using the acetone and MeOH extracts of HW and unextracted and hexane extracted HW powders. Hexane + MeOH extracted HW powder (HME-HW) was used as a reference in wood powder studies. Extracts were used at a concentration of 2.5 mg/mL in all experiments, and due to their hydrophobicity, they were added from an EtOH solution (final EtOH concentration 2.5%, v/v) to produce a stable suspension. The wood powders were added at a concentration of 25 mg/mL, because the extractives content of the UEW was approx. 10%.

3.3.1 Enzyme production

Fungal enzymes were produced by cultivating Coniophora puteana and Trametes versicolor in a liquid medium containing (per L) 50 g acetone extracted Scots pine SW powder, 3 g yeast extract, 3 g tryptone, 0.8 g NaH₂PO₄, 0.5 g MgSO₄ x 7H₂O, and 0.4 g K₂HPO₄ x 3H₂O. Five plugs of fungal mycelium from malt extract agar plates were used to inoculate 50 mL batches of culture medium. The fungi were grown for 11 days in the dark at 28°C, after which the enzyme-containing culture medium was separated from the solids by filtration and concentrated in spin columns (10 kDa cut off). The enzymes were maintained on ice or frozen in liquid N₂ and stored at -80°C if not used within a few days. Celluclast 1.5L, a commercial Trichoderma reesei cellulase preparation, was purchased from Sigma-Aldrich and used as received.

3.3.2 Inhibition and deactivation

All hydrolyses were conducted in 2 mL capped tubes in an inverting mixer. In a total volume of 1 mL, each hydrolysis reaction contained 0.1 mg of enzyme and 10 mg of bleached softwood Kraft pulp as substrate in 50 mM acetate buffer (pH 5.0). C. puteana and T. versicolor hydrolyses were performed at 30 °C, whereas Celluclast hydrolyses were performed at 30 and 50 °C to determine if the effects of extractives are dependent on the temperature. Each hydrolysis was run for 24 h, after which the samples were boiled to denature the enzymes. The monosaccharide composition of the boiled and appropriately diluted samples was determined by High-Performance Anion Exchange Chromatography with pulsed amperometric detection (HPAEC-PAD), using a CarboPac PA20 column with water as eluent at 0.37 mL/min. Pure monosaccharides were used as standards for quantitation.

Inhibition of hydrolysis by extractives was studied by combining extractives, enzyme, and substrate at the beginning of hydrolysis. The sugar yields of extract hydrolyses were compared to a reference hydrolysis containing an equal volume of pure EtOH in place of extract, while the yields of wood powder hydrolyses were compared to a reference containing 25 mg/mL of HME-HW. Deactivation
of enzymes by extractives, on the other hand, was studied by first incubating the enzymes with extractives for 24 h at hydrolysis temperature in the absence of substrate, after which the enzyme-extractives mixtures were combined with the substrate to start the hydrolysis. Samples with pure EtOH and HME-HW, also incubated at hydrolysis temperature for 24 h, served as references.

### 3.3.3 Enzyme distribution experiments

To study whether extractives bind or precipitate enzymes, the enzymes were incubated with extractives at hydrolysis temperature for 24 h in the absence of substrate. The samples were then centrifuged and separated into supernatant and solid fractions, which were used to study the distribution of total protein and hydrolytic activity. Total protein was measured only in the supernatant fraction. The supernatants were extracted with 1% (w/v) of polyvinylpolypyrrolidone to remove solubilised phenolics and then filtered through 0.2 μm syringe filters. The protein contents of the filtered supernatants were measured using the Bradford protein assay (Biorad Laboratories, Inc.) according to the manufacturer’s instructions. Hydrolytic activity, on the other hand, was measured in both fractions. The supernatants were again extracted with 1% polyvinylpolypyrrolidone and filtered, while the solids were resuspended in fresh buffer or fresh buffer with 2.5% EtOH. The extracted supernatants and the resuspended solids were then used to hydrolyse the pulp substrate as in section 3.3.2. Similarly treated samples containing pure EtOH and HME-HW served as references in both distribution experiments.

### 3.3.4 Pinosylvins modification experiments

The modification of pinosylvins by the enzyme preparations was studied by incubating the enzymes with extractives in the absence of substrate. Incubation was conducted at hydrolysis temperature, with samples withdrawn for analysis after 0, 4, 8, and 24 h of incubation. Extract-containing reactions were carried out in a total volume of 1 mL, with aliquots withdrawn at the indicated times. The wood powder reactions were also conducted in a volume of 1 mL but in individual tubes: at each time point, a set of tubes was withdrawn, centrifuged, and the supernatants discarded. The solids were washed once with 1 mL of water, and then extracted twice with 1 mL of MeOH in a sonicator (45 °C, 30 min per extraction). The extractive composition of the collected aliquots and extracts were determined by GC.

### 3.4 Cellular level distributions of extractives (Paper III)

The distributions of extractives were studied by confocal Raman spectroscopy imaging. SW, OHW, MHW, and KW were all analysed to provide a wide variety
of different samples. To prepare samples for imaging, thin cross sections (8 μm) were cut from the sample sticks using a rotary microtome. The cross sections were placed on microscope slides with a few drops of water, covered with cover slips and scanned using a Renishaw inVia Raman microscope equipped with a 532 nm laser (used at 20 mW), an oil immersion objective (Nikon, 100×, NA = 1.4), and a 1800 lines/mm grating. A step size of 300 nm was used to scan a measurement area of 30–100 × 30–100 μm. The integration time was 0.2 s. To ensure that the acquired images were representative of the type of sample in question, cross sections were prepared from at least three separate sample sticks per sample type, and each cross section was analysed on three separate locations. After the scan, the measurement software (Wire 4.1) was used to apply a cosmic ray removal filter to the spectra and to perform a baseline correction. Finally, the spectra were exported to Cytospec (v.2.00.01) for analysis and plotted in Origin Pro 8.1.

3.5 Cellular level changes in decaying heartwood (Paper IV)

3.5.1 Decay

Cellular level chemical changes taking place during the decay of HW were studied by exposing irradiation sterilised OHW sticks to the brown rot fungus *Rhodonia placenta*. Decay testing was conducted on 2% malt extract agar plates, which were inoculated with plugs of mycelium obtained from stock cultures of *R. placenta*. The fungus was allowed to grow until the plates were covered with mycelium, after which the sterilised HW sticks were placed on the plates on top of sterile filter paper supports. Five replicate sticks were placed on each plate. The plates were then sealed with parafilm and incubated in the dark at room temperature, with one plate (=five replicate sticks) withdrawn for analysis after 0, 2, 4, 6 and 8 weeks of degradation. The sample sticks were wiped clean of adhering mycelium before use in analyses.

3.5.2 Raman imaging and principal component analysis

At every fungal exposure time point, two of the five replicate sticks were randomly selected for imaging. Thin sections (20-50 μm) were cut from the ends of the sticks using a rotary microtome, after which the sections were placed on microscope slides with a few drops of water, covered with cover slips and edge sealed with nail polish. The sections were scanned using a WITec alpha 300 RA Raman microscope equipped with a 532 nm frequency doubled Nd:YAG laser (used at 30 mW), a 20x air objective (NA = 0.4), and a DU970-BV EMCCD camera behind a 600 lines/mm grating. The size of the scanned area was 70 x 70 μm, with 150 lines per image and 150 points per line. The integration time was 0.3 s. At least two separate scans were acquired from the latewood regions of each section.
For principal component analysis (PCA), the individual Raman images were combined into an image mosaic, which was unfolded into a two-dimensional array with individual pixels as row objects and wavelengths as the corresponding columns. Wavelengths outside 300-3600 \( \text{cm}^{-1} \) were excluded as they mainly consisted of noise. The spectra were baseline corrected with a second-order polynomial to remove fluorescence and normalised based on the length of each spectrum to decrease the intensity differences of the individual images (Afseth et al. 2006, see Paper IV). Additional random spike (cosmic ray) removal was performed using a moving window of 3x3 pixels by replacing outlier spectra with the median spectrum of each window. The pre-processed data were then decomposed into a bilinear combination of pixel scores and wavelength loadings according to principal component (PC) model after mean-centering (Daszykowski et al. 2003). As the largest variation in the Raman images was related to differences between the cell walls and the lumen, pixels representing lumen water were excluded from the final PCA. Data analysis and plotting were performed with the Matlab® (The Mathworks, Inc.), PLS Toolbox (Eigenvector Research, Inc.) and OriginPro (OriginLab Corp.) software packages.

### 3.5.3 Bulk chemical analyses

After cutting cross sections for imaging, the remaining wood material was freeze dried, pooled, and then ground in a Wiley mill (20 mesh). To determine the extractives composition of the material, 0.3 g of the powder was extracted twice with 5 mL of MeOH in a sonicator (45 °C, 30 min per extraction), after which the two MeOH extracts were pooled and processed for analysis by GC (see section 3.1.2). The remaining wood powder was dried overnight at 60°C and then acid hydrolysed according to NREL/TP-510-42618 to determine the polymer composition of the material. The monosaccharide composition of the hydrolysates was determined by HPAEC-PAD, using a CarboPac PA20 column with water as eluent at 0.37 mL/min. Pure monosaccharides were used as standards for quantitation.

Rather than attempting to measure mass loss, fungal activity in the samples was estimated by measuring the ergosterol content of the samples according to the method of Niemenmaa et al. (2006). Briefly, 0.25 g of wood powder was saponified with 3 mL of 10% KOH in MeOH for 60 min at 80 °C. After the addition of 1 mL of water, ergosterol was extracted from the solution with n-hexane (2 x 2 mL), and 3 mL of the combined hexane phases was evaporated to dryness under vacuum. The dry residue was redissolved in 500 \( \mu \text{L} \) of pyridine, silylated at 70 °C for 20 min after the addition of 400 \( \mu \text{L} \) of BSTFA and 100 \( \mu \text{L} \) of TMSC, and then analysed by GC. The column used was HP-5 (30 m x 0.23 mm i.d., 0.25 \( \mu \text{m} \) film thickness), and the oven temperature program was 2 min at 200 °C, 10 °C/min to 300 °C, and 15 min at 300 °C. Helium was used as the carrier gas at 1 mL/min. The ergosterol content of the fungal mycelium scraped off the sample surfaces was determined using the same procedure. The wood ergosterol contents were then converted to mycelium content.
4. Results and discussion

4.1 Non-biocidal properties of extractives

4.1.1 Antioxidant activity

Wood decaying fungi are known to utilise oxidative, radical-based reactions in wood degradation. Radical-based reactions are used by both brown rot and white rot fungi, but they are of particular significance to brown rots, which typically produce a reduced set of carbohydrate active enzymes and no ligninolytic peroxidases (Eastwood et al. 2011, Riley et al. 2014). Due to the dependence of brown rots on oxidative reactions, it has been suggested that wood extractives may contribute to decay resistance as antioxidants, in addition to their biocidal activity (Schultz and Nicholas 2000, 2002, 2011). Many wood extractives have been shown to have antioxidant activity (Eklund et al. 2005, Pietarinen et al. 2006, Wijayanto et al. 2015, Willför et al. 2003a), but their antioxidant properties have rarely been studied from the perspective of wood decay inhibition (Bengtsson et al. 2008, Donoso-Fierro et al. 2009).

In Paper I, the decay-preventing antioxidant properties of extractives were studied using Scots pine heartwood (HW) and knotwood (KW), which are both rich in extractives. The antioxidant properties were first studied using traditional solution-based antioxidant assays; after that, the assays were modified to allow the testing of HW and KW powders to study the properties of extractives in their natural wood matrix. The extractives preparations selected for testing were the acetone and MeOH extracts of HW and KW, and the unextracted and acetone extracted HW and KW powders. The unextracted HW and KW powders (UE-HW and UE-KW, respectively) represent natural HW and KW, whereas the HW and KW acetone extracts (HAE and KAE) represent the total HW and KW extractives. The MeOH extracts (HME and KME), obtained by sequential extraction, represent the more hydrophilic portion of the HW and KW extractives.

The composition of extractives in the extracts and wood powders is summarised in Table 2. The acetone extracts (HAE and KAE) contained a large proportion of resin acids. Pinosylvin (PS) and pinosylvin monomethyl ether (PSM) were also abundant, while free fatty acids were present only in small amounts. The KAE also contained 2.9% lignans, while the HAE contained none. The
MeOH extracts of HW and KW both contained an increased proportion of phenolic extractives (pinoresinols and lignans) compared to the acetone extracts, caused by the preferential solubility of resin acids and fatty acids in hexane in the first extraction step. The HME and KME contained 37.2 and 45.1% of phenolic extractives, respectively; while these do present a significant increase over the acetone extracts, very high phenolics contents such as those reported by Fang et al. (2013) were not obtained, possibly due to the use of air-drying rather than freeze drying to dry the wood powders. Finally, the UE-HW and UE-KW powders contained 8.5 and 12.1% of acetone soluble extractives, respectively. The extractives yields and the composition of extractives were similar to those previously reported, although the UE-KW contained relatively little extractives for a KW sample (Fang et al. 2013, Willför et al. 2003b). All of the extracts and wood powders also contained a notable proportion of unidentified compounds, which might include oxidation products of the other extractives.

Table 2. Composition of extractives in the acetone (HAE, KAE) and MeOH (HME, KME) extracts of HW and KW (% of g/g of extract) and the unextracted HW and KW powders (UE-HW, UE-KW) (mg/g of oven-dry wood) (modified from Paper I)

<table>
<thead>
<tr>
<th></th>
<th>HAE %</th>
<th>HME %</th>
<th>KAE %</th>
<th>KME %</th>
<th>UE-HW mg/g</th>
<th>UE-KW mg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gravimetric yield</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>85.4</td>
<td>121.1</td>
</tr>
<tr>
<td>PS</td>
<td>5.6</td>
<td>14.2</td>
<td>3.5</td>
<td>9.5</td>
<td>4.8</td>
<td>4.2</td>
</tr>
<tr>
<td>PSM</td>
<td>9.9</td>
<td>12.0</td>
<td>14.5</td>
<td>3.3</td>
<td>8.5</td>
<td>17.6</td>
</tr>
<tr>
<td>Lignans</td>
<td>0.0</td>
<td>23.2</td>
<td>2.9</td>
<td>8.0</td>
<td>0.0</td>
<td>3.5</td>
</tr>
<tr>
<td>Resin acids</td>
<td>50.7</td>
<td>25.2</td>
<td>45</td>
<td>13.3</td>
<td>43.3</td>
<td>54.5</td>
</tr>
<tr>
<td>Fatty acids</td>
<td>1.6</td>
<td>0.0</td>
<td>3.1</td>
<td>0.0</td>
<td>1.4</td>
<td>3.8</td>
</tr>
</tbody>
</table>

Many different radicals might be involved in oxidative wood degradation, but most of the research (see reviews by Arantes et al. 2012, Baldrian and Valaskova 2008, Hammel et al. 2002) has focused on the involvement of the hydroxyl radical (·OH), which is produced by the Fenton reaction (Fe(II) + H₂O₂ → Fe(III) + ·OH + ·OH). Because of the involvement of iron in oxidative degradation, the following three properties were selected to study the antioxidant activity of extractives: free radical scavenging, ferrous iron binding, and ferric iron reduction. Free radical scavenging describes the ability of an antioxidant to react with and neutralise free radicals, while ferrous iron binding describes the ability of an antioxidant to form complexes with the reactive form of iron, making the iron unavailable for reaction with H₂O₂. Ferric iron reduction, on the other hand, describes the pro-oxidant potential of an antioxidant. As compounds that readily undergo oxidation, antioxidants may actually reduce iron from its biologically inactive, ferric form to the reactive ferrous form. Wood decaying fungi themselves take advantage of this iron reducing property by secreting small molecular phenolics with high iron reducing activity (Arantes et al. 2012, Hammel et al. 2002).
The radical scavenging, iron binding, and iron reducing activities of the HW and KW extracts are given in Table 3, along with the activities of pure PS and PSM. The extracts and pinosylvincs were compared to two known antioxidants (butylated hydroxyanisole (BHA) and quercetin) (Table 3). Radical scavenging activity was studied using ABTS$^+$, which is a strongly coloured and stable artificial radical. The scavenging assays showed that all wood extracts and pure pinosylvinoms were capable of scavenging the radical, with the MeOH extracts showing higher scavenging activity than the acetone extracts and pure pinosylvinoms higher activity than any of the extracts. The results therefore strongly suggest that the radical scavenging activity of the extracts is primarily due to the phenolic extractives that consist mostly of pinosylvinoms (Table 2). Some previous publications have reported poor radical scavenging activity by phenolics such as pinosylvinoms that contain only meta-substituted OH-groups (Shang et al. 2009, Wang et al. 1999), but this investigation found their scavenging activity to be comparable to that of BHA and quercetin, known antioxidants. The KW extracts were better radical scavengers than the HW extracts, which may be due to the higher proportion of pinosylvinoms in the KAE and KME or to the presence of lignans (Table 2). Many lignans, including nortrachelogenin, have been shown to be effective radical scavengers (Eklund et al. 2005, Willför et al. 2003a).

Table 3. Free radical scavenging, ferrous iron binding, and ferric iron reducing activities of the HW and KW acetone (HAE, KAE) and MeOH extracts (HME, KME), pure pinosylvinoms (PS, PSM), and the antioxidant standards (BHA, quercetin) at 50% effective concentration (μg/mL) (Paper I)

<table>
<thead>
<tr>
<th></th>
<th>ABTS$^+$ scavenging</th>
<th>Fe(II) binding</th>
<th>Fe(III) reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAE</td>
<td>34.3</td>
<td>137</td>
<td>&gt;&gt;500</td>
</tr>
<tr>
<td>HME</td>
<td>12.8</td>
<td>160</td>
<td>&gt;500</td>
</tr>
<tr>
<td>KAE</td>
<td>23.4</td>
<td>124</td>
<td>&gt;500</td>
</tr>
<tr>
<td>KME</td>
<td>11.1</td>
<td>203</td>
<td>&gt;500</td>
</tr>
<tr>
<td>PS</td>
<td>3.3</td>
<td>243</td>
<td>&gt;&gt;500</td>
</tr>
<tr>
<td>PSM</td>
<td>4.9</td>
<td>237</td>
<td>&gt;&gt;500</td>
</tr>
<tr>
<td>BHA</td>
<td>3.5</td>
<td>&gt;500</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Quercetin</td>
<td>2.5</td>
<td>4.7</td>
<td>&gt;&gt;500</td>
</tr>
</tbody>
</table>

All wood extracts and pinosylvinoms were capable of binding ferrous iron, although their activity was much lower than that of quercetin. The relatively low iron binding activity of the extractives is not surprising, considering that neither pinosylvinoms nor resin acids contain ortho- or para-substituted OH or C=O groups that are believed to be required for metal chelating activity (Andjelkovic et al. 2006, Moran et al. 1997, Van Acker et al. 1996). However, the fact that even pure pinosylvinoms showed iron binding activity clearly demonstrates that some interactions with iron are possible even in the absence of typical metal chelating functional group arrangements. It is of interest to note that both acetone extracts showed higher iron binding activity than the phenolics-rich MeOH ex-
tracts. This suggests that non-phenolic compounds such as the resin acids actually have a higher affinity for iron than the phenolics in Scots pine HW and KW, which are lacking in classical metal chelating phenolics such as flavonoids.

The wood extractives and standards demonstrated poor activity in the iron reducing assays. The 50% effective concentration values could not be calculated due to the limited solubility of the acetone extracts, but the measurements performed at a concentration of 500 μg/mL showed that the MeOH extracts had higher iron reducing activity than the other solutions (see Paper I). The KME and HME reduced 24.8 and 9.3% of the added Fe(III), respectively, whereas the acetone extracts, pure pinosylvins, and the standards reduced only a few percent or less. The identity of the iron reducing compound(s) in the MeOH extracts remains unknown, but it is likely to be either a minor hydrophilic extractive compound or an oxidation product of other extractives.

After the assay of extract solutions, the assay procedures were modified to enable the analysis of wood powders. The radical scavenging, iron binding, and iron reducing activities of the UE-HW, UE-KW, and the acetone extracted HW and KW powders (AE-HW and AE-KW, respectively) are presented in Table 4. Acetone extracted sapwood (AE-SW) is given as an additional control. The ABTS\(^{+}\) scavenging assays showed that the UE-HW and UE-KW powders were highly effective radical scavengers, with both powders scavenging approx. 100% of the radical. The extracted HW and KW powders also had strong scavenging activity, which demonstrates that not all of the radical scavenging extractives are removable by simple solvent extraction. Even the AE-SW had notable scavenging activity, suggesting that the wood cell wall polymers contribute to radical scavenging as well. Nonetheless, the scavenging activities of the UE-HW and UE-KW were significantly higher than the activities of the extracted powders, confirming the role of extractives in radical scavenging and demonstrating their ability to interact with radicals even when immobilised on the wood matrix. However, it should be noted that ABTS\(^{+}\) is not a naturally occurring radical, and that the mechanism of ABTS\(^{+}\) bleaching is different from the mechanism of \(^{·}\)OH or HOO\(^{·}\) radical scavenging (MacDonald-Wicks et al. 2006). The ABTS\(^{+}\) assay gives an indication of the reactivity of extractives with radicals, but it is unlikely to give a full evaluation of their ability to scavenge radicals relevant to wood degradation.

In addition to radical scavenging, all of the wood powders also showed ferrous iron binding activity. The activities of the UE-HW and UE-KW were higher than the activities of the extracted powders, demonstrating that extractives are capable of binding iron in their natural wood matrix. The UE-HW was more effective than the UE-KW, which is surprising in light of the higher binding activity of the KAE (Table 3) and the higher extractives concentration of the UE-KW (Table 2). In brown rot decay, the iron used in the Fenton reaction is extracted from wood by organic acids secreted by the fungus. The wood material typically contains some 10 μg/g of iron (Fengel and Wegener 2011), which is significantly lower than the 12 mg/g of iron bound by the UE-HW at a concentration of 1% in this experiment. Consequently, it might be possible for all of the iron present in
wood to be bound by extractives. However, the current experiment does not provide information on how strong the interaction between iron and extractives is, or whether the binding renders the iron incapable of participating in the Fenton reaction. Some researchers report that chelated iron is unable to react with H₂O₂, whereas others show that reactions are still possible (Li et al. 2007, Lopes et al. 1999). The addition of iron chelating compounds to fungicides has been found to improve the decay resistance of wood (Schultz and Nicholas 2002), which shows that iron binding by some chelators can prevent wood degradation.

### Table 4.

<table>
<thead>
<tr>
<th></th>
<th>ABTS⁺⁺ scavenging</th>
<th>Fe(II) binding</th>
<th>Fe(III) reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1%</td>
<td>0.5%</td>
<td>1%</td>
</tr>
<tr>
<td>UE-HW</td>
<td>96.8 (1.0)</td>
<td>93.8 (0.5)</td>
<td>86.7 (1.1)</td>
</tr>
<tr>
<td>UE-KW</td>
<td>99.9 (0.3)</td>
<td>98.7 (2.8)</td>
<td>60.6 (0.9)</td>
</tr>
<tr>
<td>AE-HW</td>
<td>87.9 (0.6)</td>
<td>66.6 (2.2)</td>
<td>35.5 (1.4)</td>
</tr>
<tr>
<td>AE-KW</td>
<td>91.5 (0.9)</td>
<td>75.5 (0.1)</td>
<td>23.2 (2.6)</td>
</tr>
<tr>
<td>AE-SW</td>
<td>61.9 (0.3)</td>
<td>42.3 (0.4)</td>
<td>37.9 (1.9)</td>
</tr>
</tbody>
</table>

Despite the poor iron reducing activity of extractives in solution assays, tests on the wood powders showed that all of the wood materials were capable of iron reduction. The amounts reduced by the extractives-containing powders were higher than those reduced by AE-SW, demonstrating again the involvement of extractives. The UE-KW reduced more iron the UE-HW, which is in line with the results obtained with extract solutions. However, whether the iron reducing activity of extractives has any real pro-oxidant effect is likely to depend on the other properties of the extractives, such as their ability to bind and safely lock away the newly formed, reactive ferrous iron.

The results presented in Tables 3 and 4 clearly demonstrate that Scots pine HW and KW extractives have antioxidant (and pro-oxidant) activity and that they are active in the wood matrix, meaning that they have potential to act as antioxidants during wood decay. However, more information is needed before the role of Scots pine extractives as antioxidants can be confirmed. As discussed above, the simple assays used in this experiment might not accurately describe the ability of extractives to function as antioxidants under real decay conditions. The assays also fail to take into account the stability and nature of the extractives-derived radicals that form when the extractives scavenge free radicals or react with iron. Some antioxidants give stable radical products because they are able to delocalise the charge and undergo further reactions to produce harmless products, whereas others are unstable and may be damaging to wood on their own (Shahidi et al. 1992). Therefore, to make accurate assessments of the various antioxidant properties of extractives, further studies should utilise tests that
better represent real wood decay and take into account the stability of the extractives-derived radicals.

Yet another limitation of antioxidant assays is the fact that they investigate the various anti- and pro-oxidant properties of extractives in isolation. Consequently, the final step in the study of the antioxidant properties should be a realistic degradation scenario. Actual fungal decay experiments such as those utilised by Schultz and co-workers (Schultz and Nicholas 2000, 2002, 2011, Schultz et al. 2004) are not possible due to the biocidal and other potential non-biocidal properties of extractives, but wood degradation experiments mimicking fungal Fenton degradation should be possible to devise. After degrading natural HW or KW, the damage to the underlying wood material should be assessed and compared to the damage sustained by extractives-free wood. Methods ideal for the assessment of oxidative damage to wood include the measurement of cellulose degree of polymerisation or strength loss, although other measures of oxidative degradation may also prove suitable.

4.1.2 Fungal hydrolase inhibition

Oxidative degradation by wood decaying fungi is believed to serve as a means of opening the wood structure to allow the fungal enzymes access to the cell wall polymers (Arantes et al. 2012). Although some evidence exists to show that oxidative brown rot degradation actually results in a full solubilisation of cell wall material, the oxidative step is nonetheless followed by hydrolytic breakdown of the cell wall carbohydrates (Goodell et al. 2017). Consequently, the non-biocidal properties of extractives may also involve the inhibition of enzymatic carbohydrate hydrolysis. Hydrolase inhibition by extractives has not been considered as a mechanism of decay inhibition, but extensive studies on lignin and lignin-derived small molecular weight phenolics have demonstrated that phenolic compounds can inhibit enzymatic hydrolysis (Boukari et al. 2011, Palonen et al. 2004, Rahikainen et al. 2011, Rollin et al. 2011, Sharma et al. 1985, Tejirian and Xu 2011, Ximenes et al. 2011).

To continue the study of the non-biocidal properties of extractives, Paper II investigated the ability of Scots pine extractives to inhibit enzymatic hydrolysis of holocellulose. The extractives were limited to HW, but the fungi used included a representative of both brown rot (Coniophora puteana) and white rot (Trametes versicolor), which differ in their mechanism of wood degradation and might therefore differ in their susceptibility to inhibition by extractives. A commercial Trichoderma reesei cellulase preparation (Celluclast) was also used as a reference. As with antioxidant studies, the extractives materials included a HW acetone extract (HAE), a MeOH extract (HME), and an unextracted HW powder (UE-HW). A hexane extracted HW powder (HE-HW) was included as well to give two matching extract-wood powder pairs.

The composition of the chosen extractive materials is summarised in Table 5. As expected, the HAE consisted mostly of resin acids, with pinosylvins as the
second most abundant component and fatty acids as a relatively minor constituent. The HAE contained more resin acids and fatty acids than the equivalent extract used in Paper I and a notably reduced portion of unidentified compounds. The HME contained 64.4% pinosylvin and only 5.8% resin acids, which makes the extract very rich in phenolics. The higher proportion of phenolics achieved here than in Paper I might be due to the use of freeze drying in sample preparation, which is likely to cause fewer oxidation reactions than the air drying used before. The UE-HW contained 9.8% hexane and MeOH soluble extractives in proportions very similar to the HAE, which confirms that the HAE is a good representation of the total HW extractives. The HE-HW contained only 2.1% MeOH soluble extractives, but it had retained most of the pinosylvin found in the UE-HW.

Table 5. Composition of extractives in the HW acetone and MeOH extracts (HAE, HME) (% g/g of extract) and the unextracted and hexane extracted HW powders (UE-HW, HE-HW) (mg/g of oven-dry wood) (Paper II)

<table>
<thead>
<tr>
<th></th>
<th>HAE</th>
<th>HME</th>
<th>UE-HW*</th>
<th>HE-HW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gravimetric yield</td>
<td>–</td>
<td>–</td>
<td>98.0</td>
<td>20.8</td>
</tr>
<tr>
<td>Pinosylvins</td>
<td>15.0</td>
<td>64.4</td>
<td>15.4</td>
<td>13.4</td>
</tr>
<tr>
<td>Resin acids</td>
<td>75.2</td>
<td>5.8</td>
<td>71.8</td>
<td>1.3</td>
</tr>
<tr>
<td>Fatty acids</td>
<td>3.1</td>
<td>0.0</td>
<td>2.9</td>
<td>0.0</td>
</tr>
</tbody>
</table>

*Sum of the hexane and MeOH extractions

The inhibitory properties of extractives were studied by hydrolysing the Kraft pulp substrate with the enzyme preparations in the presence of extractives. The results (Figure 10) demonstrate that all of the enzyme preparations showed at least some degree of inhibition by extractives. Glucose release from pulp by the brown rot fungus C. puteana was mildly inhibited by all extractives preparations, but the release of xylose (and mannose, data not shown) was not or was only very slightly inhibited. As brown rot fungi such as C. puteana tend to degrade hemicelluloses preferentially (Highley 1987, Irbe et al. 2006), the slight inhibition of glucose release from wood is unlikely to be a significant factor in preventing wood decay.
Figure 10. Inhibition of enzymatic glucose (a) and xylose (b) release from pulp by the HW acetone and MeOH extracts (HAE, HME) and the unextracted and hexane extracted HW powders (UE-HW, HE-HW). Cp, C. puteana; Tv, T. versicolor; Cell., Celluclast. *statistically significant difference to reference (p < 0.05, Tukey’s range test) (Paper II)

Like C. puteana, the commercial cellulase preparation was also only moderately or mildly inhibited. Glucose and xylose release remained unaffected when the hydrolysates were conducted at 30°C, but when the hydrolysis temperature was increased to 50°C, the HAE reduced the glucose and xylose yields to approx. 65% of the reference. The other extractives preparations gave only mild inhibition, which shows that the more hydrophobic extractives are actually more inhibitory at higher temperatures, at least in the case of Celluclast. The brown rot and white rot enzymes could not be tested at 50°C due to denaturation. The inhibitory component in the HAE is likely to be the resin acids, which accounted for 75% of the extract (Table 5). The inhibition of cellulose hydrolysis by abietic acid has been previously documented (Leskinen et al. 2015). However, the inhibitory action of the hydrophobic fraction appears to require a wood-free extract: the UE-HW was less inhibitory than the HAE, even though both hydrolysate reactions contained approximately the same amount of extractives in the same proportions (Table 5).

The hydrolases of the white rot fungus T. versicolor were also inhibited by extractives, but the inhibition behaviour clearly differed from that of the other two enzyme preparations. The T. versicolor hydrolases were strongly inhibited by the HME, with glucose release reduced to 35% and xylose release to 43% of the reference. As the other extractives were mildly inhibitory at best, it can be inferred that the inhibition is strongly linked to the pinosylvins.

In addition to inhibition, the hydrolase deactivating properties of the extractives were also investigated. The enzymes were first incubated with extractives for 24 h in the absence of substrate and then combined with substrate to start the actual hydrolysis. The extractives preparations studied were limited to the HME and UE-HW, which represented the most inhibitory and the most natural extractives preparations. The results of the deactivation hydrolysates are given in Figure 11. Pre-incubation with extractives had only a small effect on sugar release for C. puteana and Celluclast hydrolases. The HME became slightly more
inhibitory to glucose release by *C. puteana* and to xylose release by Celluclast, but otherwise the sugar release values remained very similar to those obtained without pre-incubation (Figure 10).

**Figure 11.** Deactivation of enzymatic glucose (a) and xylose (b) release from pulp by the HW MeOH extract (HME) and the unextracted and HW powder (UE-HW). Cp, *C. puteana*; Tv, *T. versicolor*; Cell., Celluclast. *statistically significant difference to reference (p < 0.05, Tukey’s range test) (*Paper II*)

The *T. versicolor* hydrolases, on the other hand, showed strong deactivation. Glucose release in the presence of the HME was reduced to 13% of the reference, which is much lower than the 35% obtained without pre-incubation (Figure 10). The UE-HW also became much more inhibitory: without pre-incubation, UE-HW reduced glucose release to 80% of the reference and had no effect on xylose release (Figure 10), whereas with pre-incubation, the glucose and xylose yields were reduced to approx. 55% of the reference. The reduced sugar release in the presence of the UE-HW suggests that extractives may contribute to decay resistance as hydrolase inhibitors. Wood decay is a long process of continued hydrolysis, which means that the extractives are continuously able to deactivate the hydrolases secreted by the fungus.

The results presented in Figures 9 and 10 clearly demonstrate how the behaviour of the *T. versicolor* enzyme preparation differed from that of the other two. While differences in the nature of the hydrolases are possible, the more likely explanation for the differences is that the *T. versicolor* enzyme preparation contained enzymes that were capable of modifying the extractives to more inhibitory compounds. The modification hypothesis is supported by the finding that the extractives preparations changed colour during incubation with the *T. versicolor* enzyme. The HME changed from light brown to pink to orange over the course of several hours, and the UE-HW developed a slight reddish colour as well, although the change was less obvious (Figure 12). Pinosylvins are likely to be the modified compounds, given that the pinosylvins-rich HME showed the strongest hydrolysis inhibition (Figures 10 and 11) and the strongest colour change.
Results and discussion

To confirm the modification/degradation of pinosylvins by *T. versicolor*, the HME and the UE-HW were incubated with the enzyme preparation for 24 h. The changes in the concentrations of PS and PSM over the course of incubation (Figure 13) showed that *T. versicolor* was able to cause extensive degradation of both compounds. PS was degraded more extensively than PSM, and both compounds were degraded to a similar extent in the extract and the wood powder. The experiment was repeated with *C. puteana* and Celluclast, but no changes in the concentration of extractives were detected with these two enzyme preparations. *T. versicolor* produces a number of enzymes that can attack phenolic compounds (Moldes et al, 2004, Schlosser et al, 1997), including laccases that have been shown to catalyse the oligomerisation of resveratrol and its derivatives, the structural analogues of pinosylvins (Nicotra et al. 2004, Ponzoni et al. 2007, Schouten et al. 2002). The *T. versicolor* enzyme preparation used in this experiment had 4.6 U/mg of laccase activity, whereas the other two preparations had no activity at all. However, despite the clear degradation of pinosylvins and the high laccase activity, no oligomers or other degradation products were detected in this experiment.

The fact that pinosylvins are degraded during incubation with the *T. versicolor* enzyme preparation is a strong indicator that the inhibition of *T. versicolor* hydrolases is due to the formation of more inhibitory compounds. But how do the newly formed compounds inhibit and deactivate hydrolases? Research on plant biomass derived phenolic compounds has shown that many phenolics form complexes with enzymes, causing their deactivation (Boukari et al. 2011, Sharma et al. 1985, Tejirian and Xu 2011, Ximenes et al. 2011). In the case of pinosylvins, the radical intermediates of oxidase action might also be responsible for deactivation. Research on resveratrol has shown that enzymatic action on the 3,5-OH structure produces an unstabilised m-semiquinone radical that can attack and deactivate enzymes, becoming bound to the enzyme in the process (Szewczuk et al. 2004, 2005).
Figure 13. Removal of pinosylvins from the HW MeOH extract (HME) or the unextracted HW powder (UE-HW) by the *T. versicolor* enzyme preparation (Paper II)

To study phenolic-protein complex formation as a mechanism of inhibition, the *T. versicolor* enzyme preparation was incubated with the HME and UE-HW for 24 h and then separated into supernatant and solid fractions. Measurement of the supernatant protein content showed a loss of soluble protein, with the HME and UE-HW supernatants containing 28 and 52% of the reference protein, respectively (see Paper II). *C. puteana* and Celluclast, measured as a reference, showed no loss of protein. The supernatant and solid fractions were also used to hydrolyse pulp to determine if the loss of protein was accompanied by a loss of hydrolytic activity. The hydrolysis results (Figure 14) showed that the incubations resulted in a loss of hydrolytic activity and the partitioning of activity between the two fractions. The HME and UE-HW supernatants contained only 10 and 38% of the original glucose-releasing enzyme activity, respectively, which supports the complex formation hypothesis, although irreversible deactivation without complex formation is also possible. Very little cellulase activity could be detected in the solids. A large portion of the xylose-releasing activity was also lost from the supernatant, but unlike cellulase activity, much of the xylanase activity could be recovered from the solids. The ability of xylanases to retain their activity in the solids likely explains why xylose release was generally less inhibited by extractives than glucose release (Figures 10 and 11).

The results presented in Figures 10-14 demonstrate that Scots pine HW extractives have potential to inhibit the enzymatic hydrolysis of wood polysaccharides, at least when the fungus produces laccases or other enzymes capable of modifying the extractives. In this experiment, only the white rot fungus had laccase activity, but laccase production in general is not limited to white rot fungi. Many brown rot fungi have laccase genes and produce laccase activity (D’souza et al. 1996, Riley et al. 2014), which means that hydrolase inhibition can be significant against brown rot fungi as well. However, the overall significance of hydrolase inhibition in decay prevention is likely to depend on a number of factors not considered in this experiment. The toxicity of the modified extractives is likely to be important, as is the ability of the fungus to modify them further. It should also be noted that extractives are modified not only by enzymes but also by the
non-enzymatic, radical-based system. Radical-catalysed oxidation might convert the extractives to compounds that are less inhibitory to hydrolases, or it might convert them to more inhibitory compounds, possibly even making enzymatic oxidation unnecessary for inhibition. Experiments combining non-enzymatic and enzymatic degradation are needed to fully understand the non-biocidal properties of Scots pine HW extractives on wood decay.

![Figure 14](image.png)

**Figure 14.** Residual glucose and xylose releasing enzyme activity in the supernatant and solid fractions of *T. versicolor* after a 24 h incubation with the HW MeOH extract (HME) or the unextracted HW powder (UE-HW) (Paper II)

### 4.2 Cellular level distributions of HW constituents

#### 4.2.1 Natural wood

In addition to being a chemically complex sequence of radical and enzyme catalysed degradative reactions, wood decay is also a physically complex process taking place within the structure of wood. If wood extractives are to interfere with these degradative processes, their distribution in wood needs to be such that they come into contact with fungi or their degradative agents. Despite the obvious importance of distribution on their ability to interact with fungi, relatively few studies have been conducted on the cellular level distribution of extractives. Most of the existing distribution information comes from a limited number of wood species and it has been, in many cases, obtained by using non-specific stains to visualise the distribution of extractives (Fengel 1970, Kuo and Arganbright 1980, Streit and Fengel 1994, 1995, Zhang et al. 2004). As there is evidence to suggest that different compounds can have different distributions (Kuroda et al. 2014, Nagasaki et al. 2002), there is a clear need for more information on the distribution of extractives.

The distribution of extractives in Scots pine is currently unknown. However, knowledge on the distribution could be particularly important in pine, because it might explain why pine HW is only moderately durable on average, despite the high antifungal activity of pinosylvins. In **Paper III**, the distribution of
Scots pine extractives was investigated in pine SW, HW, and KW, which provide a series of different types of samples with an increasing amount of extractives. The HW was further separated into outer heartwood (OHW) and middle heartwood (MHW) to provide more variety. Different types of extractives-containing samples were included in the analysis to see if the distribution of extractives varies from tissue to tissue or changes as the concentration of extractives changes. Comparison of OHW and MHW would also show if the distribution changes as the HW matures and the amount of pinosylvins declines.

First, the composition and amounts of extractives in the chosen wood samples were determined by GC-MS (Table 6). As expected, the SW contained the lowest amount of extractives and no pinosylvins. The amount of resin acids and free fatty acids was low, which means that the extractives are likely to have consisted mostly of triglycerides. The two HW samples contained higher amounts of extractives, with resin acids again as the most abundant component. The MHW sample in particular contained an unusually large amount of resin acids. Pinosylvins were the second most abundant component in the HW samples, and their concentration appeared to be higher in the MHW than the OHW, which is in contrast to previous studies (Bergström et al. 1999, Bergström 2003). The OHW sample was collected from the very border of SW and HW (Figure 8), which means that it might have contained the transition zone and some SW, resulting in a lower overall pinosylvins content. The KW sample used in this experiment contained a very large amount of extractives, 42% by weight. The KW extractives consisted mostly of resin acids and pinosylvins, with fatty acids, lignans, and other extractives present in relatively small amounts.

Table 6. Composition of extractives in SW, outer HW, middle HW, and KW (mg/g of oven-dry wood) (Paper III)

<table>
<thead>
<tr>
<th></th>
<th>SW</th>
<th>OHW</th>
<th>MHW</th>
<th>KW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gravimetric yield</td>
<td>28.7</td>
<td>74.6</td>
<td>228.9</td>
<td>420.0</td>
</tr>
<tr>
<td>Pinosylvins</td>
<td>–</td>
<td>7.3</td>
<td>12.3</td>
<td>66.1</td>
</tr>
<tr>
<td>Resin acids</td>
<td>1.0</td>
<td>42.0</td>
<td>178.6</td>
<td>285.7</td>
</tr>
<tr>
<td>Fatty acids</td>
<td>0.8</td>
<td>3.7</td>
<td>3.7</td>
<td>2.5</td>
</tr>
<tr>
<td>Other</td>
<td>2.7</td>
<td>1.5</td>
<td>0.9</td>
<td>14.8</td>
</tr>
</tbody>
</table>

The actual cellular level distributions of extractives were analysed by confocal Raman spectroscopy imaging. Raman imaging is a process in which a large number of Raman spectra are collected from the sample at pre-defined positions to build a hyperspectral image in which every pixel consists of a whole Raman spectrum. The spectral information can then be processed to produce false-colour images that describe the distributions of components of interest; the simplest way to produce images is to integrate a specific Raman band or bands that arise from a chemical bond on the component of interest. Raman imaging has previously been used to study the distribution of wood cell wall polymers and
compounds inserted into the cell walls (Agarwal 2006, Ermeydan et al. 2012, Gierlinger and Schwanninger 2006, Gierlinger 2014, Hänninen et al. 2011, Keplinger et al. 2015, Merk et al. 2015), but it has not been used to study the distributions of extractives. Non-imaging Raman spectroscopy, on the other hand, has been successfully applied to the analysis of extractives, including those from Scots pine (Bergström 2003, Holmgren et al. 1999, Bergström et al. 1999, Nuopponen et al. 2004a, 2004b).

In this experiment, the unextracted and extracted SW, HW, and KW samples were scanned to produce the hyperspectral images described above. False-colour images were then produced by integrating over 1550-1700 1/cm (Figure 15a-h), and average Raman spectra were extracted from cell wall (CW) and cell corner (CC) regions (Figure 15i) identified on the images. All of the CW and CC spectra showed typical features of wood, with strong lignin-derived bands at 1600 and 1657 1/cm and lignin- and cellulose-derived bands in the area between 1500 1/cm and 1100 1/cm (Agarwal and Ralph 1997, 2008). Comparison of the HW and KW spectra with those of the SW revealed the presence of two additional bands at 1635 and 995 1/cm, which correspond to the known C=C stretch and 1,3,5-substituted aromatic ring vibrations of pinosylvins (Holmgren et al. 1999, Nuopponen et al. 2004b, see also Paper III). These two bands were absent (or nearly absent) in the SW and increased in intensity from OHW to MHW to KW, in accordance with the pinosylvins concentrations measured by GC-MS (Table 6). Their intensity was also greatly reduced by extraction, which provides further support that they arise from extractable compounds. Other researchers have also detected the two bands in Scots pine HW and KW and attributed them to the pinosylvins (Holmgren et al. 1999, Nuopponen et al. 2004b).

Apart from the two pinosylvins bands, no other potential extractives-derived bands were detected in the HW and KW spectra. Abietane-type resin acids (and fatty acids) produce a strong Raman band at approx. 1650 1/cm that has been previously detected in the spectrum of Scots pine HW (Nuopponen et al. 2004a, see also Paper III), but no band was found at 1650 1/cm in this experiment. While it is possible that the absence of the previously detected resin acid band is due to the use of a different excitation wavelength in this experiment, the most likely explanation is that resin acids are not actually present in the CW or CC in significant amounts. The sample used by Nuopponen et al. (2004a) was spun during measurement, which means that the resin acids were detected regardless of their specific cellular location. The resin acid concentration of the MHW and KW samples used in this experiment was very high (Table 6), which means that they are unlikely to go undetected if present.
Figure 15. Raman images (1550-1700 1/cm) of unextracted and extracted SW, OHW, MHW and KW (a-h) and average cell wall and cell corner Raman spectra (i and j), extracted from locations indicated on the Raman images (Paper III)

To analyse the actual cellular level distribution of extractives, false-colour images were produced by integration of Raman bands corresponding to extractives. As no Raman bands corresponding to resin acids could be detected, the analyses were limited to pinosylvins. The distribution of pinosylvins (integration over 985-1009 1/cm) is shown in Figure 16, along with the distributions of cellulose (1065-1102 1/cm), lignin (1104-1173 1/cm), and all wood aromatics (1538-1690 1/cm), which were analysed to confirm the validity of the method.

As SW has been omitted from Figure 16 due to the absence of pinosylvins. The distributions of cellulose and lignin were typical, with lignin showing a high concentration in the CC and the middle lamella (ML) and cellulose in the CW (Agarwal 2006, Gierlinger and Schwanninger 2006, Gierlinger 2014, Hänninen et al. 2011). The high cellulose concentration seen in the S1 is due to the orientation-sensitive nature of the used cellulose band (Gierlinger et al. 2010).

Analysis of pinosylvins distribution showed that they were present in the CW and CC/ML as well as in the lumens of some tracheids. Within each sample, the amount of pinosylvins appeared to increase from the CW to ML to CC. The distribution of pinosylvins therefore resembles that of lignin, suggesting that there might be some interaction between the two components. The binding of pinosylvins to lignin has been suggested (Hart and Shrimpton 1979), and it might be a factor contributing to the relatively low durability of pine HW. However, the similarities in distribution are not necessarily due to an interaction between pi-
nosylvin and lignin. The similarities could be due to the mechanism of pinosylvin deposition during HW formation, which might proceed through intercellular spaces and the ML as suggested in two previously investigated hardwood species (Streit and Fengel 1994, Zhang et al. 2004).

Figure 16. Raman imaging of unextracted and extracted OHW, MHW, and KW, showing the distributions of cellulose (1065-1102 1/cm), lignin (1104-1173 1/cm), lignin + extractives (1538-1690 1/cm), and pinosylvins (985-1009 1/cm) (Paper III)

The intensity of the pinosylvins band increased from OHW to MHW to KW, following the increase in pinosylvins concentration as measured by GC-MS (Table 6). The distribution was similar in all samples, but there appeared to be a stronger-than-average increase in the concentration of pinosylvins on the lumen wall/in the S3 layer. Band intensity was greatly reduced by extraction, but
it is clear that not all pinosylvins were actually removed by simple Soxhlet extraction (see also Figure 15). The existence of “unextractable extractives” has been previously suggested (Hart and Hillis 1974, Hart and Shrimpton 1979, Mayer et al. 2006); these extractives are likely to be bound to lignin or other cell wall components and may be an additional factor in decay resistance.

The pinosylvins found in tracheid lumens existed as small deposits or large occlusions filling the entire lumen. Both types of deposits were very common in the samples (Figure 17 and Figure 18). However, a more detailed analysis of the spectra revealed differences in the composition of the small deposits and filled lumens. The spectra of the small deposits had strong bands at 995, 1597, and 1634 1/cm, which corresponded to the substituted aromatic ring vibrations, aromatic ring stretching, and C=C stretching vibrations of the pinosylvins, respectively. The small shoulder at 1608 1/cm corresponds to an additional C=C stretching band of PSM (Holmgren et al. 1999, Nuopponen et al. 2004b, see also Paper III). The small deposits therefore consisted almost entirely of pinosylvins. The filled lumens, on the other hand, appeared to contain a mixture of pinosylvins and resin acids. In addition to the pinosylvins bands, the material had very strong bands at 1612 and 1649 1/cm, which correspond to the aromatic ring stretch of dehydroabietic acid and the C=C stretching vibrations of abietane-type resin acids, respectively (Nuopponen et al. 2004a, see also Paper III). Deposits of a similar mixed composition were also found within HW and KW rays (see Paper III).

Figure 17. A Raman image (1550-1700 1/cm) of unextracted MHW, showing the abundance of tracheids with small extractives deposits and extractives-filled lumens (unpublished)
The results presented in Figures 15-18 clearly demonstrate that pinosylvins were distributed throughout the HW (and KW) tissues, which means that they should able to interact with fungi and their degradative agents during decay. Resin acids, on the other hand, were only detected in the lumens of ray cells and tracheids, and considering the abundance of the resin-rich deposits, it seems possible that all of the HW and KW resin acids could actually be located within these cell lumens. The significance of these resin-rich deposits in decay resistance is likely to depend on how wood decaying fungi interact with the deposits; if the fungi are able to avoid them entirely, their significance is likely to be minor.

Although the concentration of pinosylvins increased greatly from OHW to MHW to KW, no substantial differences in distribution could be seen between HW and KW or OHW and MHW, apart from a potential increase in concentration around the lumen. However, to gain a comprehensive understanding of extractive distribution and its role in durability, the analysis should be expanded to cover a large set of HW samples differing in their age, position in the trunk and extractive content and durability. The detection of differences that could explain variations in decay resistance would most likely also benefit from the
analysis of larger sample areas, in addition to the analysis of a variety of different samples. Research into the cellular level distribution of Scots pine extractives should be continued to further our understanding of natural durability. However, the research would most likely benefit from the use of other methods in addition to Raman imaging. Raman imaging is a time consuming and qualitative method best suited for the analysis of a limited number of small samples.

4.2.2 Decaying heartwood

The results presented above (Papers I-III) clearly show that Scots pine HW extractives have multiple potential mechanisms of action against wood decaying fungi and that at least the pinosylvins are distributed in the HW in such a way that they can easily interact with the fungi and their degradative agents during decay. However, despite the presence of these multifunctional and well-distributed extractives, Scots pine HW is still susceptible to decay. The decay of Scots pine HW has been extensively investigated, with most studies focusing on the extent of degradation after fungal exposure and on the correlations between degradation and the properties of the undegraded material (e.g. Gref et al. 2000, Harju et al. 2002, 2003, Jebrane et al. 2014, Venäläinen et al. 2003, 2004). The initiation and progress of decay, on the other hand, appear to have attracted very little attention. It is known that Scots pine HW extractives are degradable by various wood decaying fungi (Karppanen et al. 2008, Loman 1970, Martínez-Iñigo et al. 1999), but very little information is available on the relationships between the degradation of extractives and wood cell wall material, and on the way these changes take place on a cellular level.

To understand natural durability and the interactions between extractives and fungi, one should also understand how the fungi are able to overcome the extractive defences of HW and how the HW eventually decays. To provide information on this topic, Paper IV investigated the onset and progress of decay in Scots pine HW exposed to the brown rot fungus *Rhodonia placenta*. The objective was to analyse the chemical changes caused by brown rot decay and to determine how these changes take place on a cellular level.

The chemical composition of the decaying HW samples was first determined using bulk chemical analyses. The composition of extractives and cell wall polymers were determined by GC and acid hydrolysis, respectively, while the mycelium content of the samples was estimated by measuring fungal ergosterol. The chemical analyses showed that the most significant change in the composition of HW was the degradation of pinosylvins (Figure 19a). PS and PSM were both lost from the samples at approximately equal rates, and by week 8, the HW material contained less than 25% of the PS and PSM present in the undegraded material. The amount of resin acids in the HW also decreased, from 81.8 mg/g to 69.4 mg/g at week 8, but their degradation did not follow a similar consistent trend as that of the pinosylvins. The differences in the extent of degradation likely reflect differences in the toxicity and chemistry of pinosylvins and resin
The removal of the more toxic pinosylvins may be a requirement for fungal colonisation, and their stronger reactivity with radicals (Table 3 in section 4.1.1) is also likely to result in a faster rate of degradation by *R. placenta*, which uses radicals particularly in the early stages of decay (Cohen et al. 2002, Martinez et al. 2009).

In addition to the degradation of pinosylvins, changes could also be seen in the polymer composition of the wood material (Figure 19b). The most noticeable change was the degradation of hemicellulosic sugars, which is a characteristic feature of incipient brown rot decay (Curling et al. 2002, Irbe et al. 2006). Relative to their concentration in undegraded wood, the most extensively degraded sugars were arabinose and galactose, followed by xylose and mannose. Glucose was not appreciably degraded, while the relative amount of Klason lignin increased due to selective carbohydrate removal. The overall change in the polymer composition of wood was small, which shows that the wood material was still in early stages of decay. However, given that brown rot is known to cause extensive disruption of the cell wall structure before large changes in composition (e.g. Curling et al. 2002, Howell et al. 2009), it is likely that the properties of the cell wall material were already significantly altered. Nonetheless, the HW material appeared to have strong resistance to degradation by *R. placenta*, most likely due to the high initial concentration of pinosylvins. However, given the degradability of pinosylvins, it is likely that higher rates of wood degradation would have been seen in later stages of decay. Experiments with wood artificially impregnated with pinosylvins have shown that the high initial resistance to *R. placenta* is lost in later stages of decay when the fungus has eliminated all of the toxic pinosylvins (Lu et al. 2016).

![Figure 19](image)

**Figure 19.** Chemical composition of HW degraded by *R. placenta*. Changes in pinosylvins and fungal mycelium content (a) and the amounts of Klason lignin and carbohydrate monomers (b) over eight weeks of degradation *(Paper IV)*

To analyse the cellular level distributions of these changes, the HW samples were subjected to confocal Raman spectroscopy imaging, which was previously used to investigate the distributions of extractives in intact wood (section 4.2.1).
However, the integration approach used earlier was not sensitive enough to
detect the slight spectral alterations caused by incipient brown rot decay. This was
most likely due to the increased noisiness of the spectrum and the fact that the
differences between the cell wall layers were greater than the differences be-
tween the images collected at different stages of decay. To improve the sensitiv-
ity of the method, the Raman imaging approach was combined with principal
component analysis (PCA). PCA is a multivariate analytical method in which the
complex set of spectral information is reduced to just a few uncorrelated principal
components (PCs) that describe variation in the data. The loading plots cor-
responding to each PC show the spectral variations described by the PCs, while
the corresponding scores show how well the PCs describe the differences be-
tween the individual data points. In the case of image data, these scores can also
be refolded back to image dimensions, producing false-colour images that de-
pict variations in the samples. PCA is more sensitive than integration, because
PCA uses the whole spectrum to look for difference between pixels rather than
just one band.

The loading plots corresponding to the first three PCs are given in Figure 20.
Together, these three PCs accounted for 69% of the total spectral variation
within and between samples, with further PCs accounting for only a few percent
or less. PC 1 (41% of total variation) was dominated by a strong positive band at
2890 1/cm, which corresponds to CH and CH2 stretching vibrations (Blackwell
et al. 1970, Edwards et al. 1994). Most of the signal intensity at 2890 1/cm is
derived from cell wall carbohydrates, although lignin, resin acids and fatty acids
also produce CH/CH2 stretching bands in this region (Agarwal and Ralph 1997).
PC 1 had additional positive bands at 1600 and 1637 1/cm, which correspond to
aromatic ring stretching and the C=C stretch of pinosylvins, respectively
(Holmgren et al. 1999, Nuopponen et al. 2004b, see also Paper III). However,
these additional bands were small compared to the CH/CH2 stretching band,
which means that the first PC describes mainly the distribution of cell wall car-
obhydrates and other CH/CH2 stretch-producing material.

PC 2 (19% of total variation) contained the same bands as PC 1. However, the
CH/CH2 stretching band (2880 1/cm) was negative in PC 2, and the aromatic
ring stretch and pinosylvins C=C stretch bands were significantly stronger than
in PC 1. The aromatic ring stretch band is usually considered a lignin-derived
band, but in pine HW, it also includes a contribution from the pinosylvins,
which produce aromatic ring stretching bands in the same region (Agarwal and
The second PC therefore describes the distribution of lignin and pinosylvins
(positive) and carbohydrates and other CH/CH2 stretch-producing material
(negative). PC 3 (9% of total variation) showed a strong positive band at 1589
1/cm and smaller positive bands at 1328, 1269 and 1139 1/cm, which can all be
attributed to lignin (Agarwal and Ralph 1997). An additional positive band was
seen at 380 1/cm, derived from crystalline cellulose (Agarwal et al. 2010). The
pinosylvins C=C stretch band appeared on the negative side, along with the
broad OH stretching band (3000-3600 1/cm), which includes contributions
from water (Carey and Korenowski 1998).
Figure 20. Loading plots corresponding to PC 1 (a), PC 2 (b) and PC 3 (c) (Paper IV)

The score images corresponding to PCs 1 and 2 are shown in Figure 21. PC 3 has been omitted, because the presence of so many different bands in the loading plot resulted in difficulties in chemically interpreting the score changes. In the case of PC 1, the pixels with positive score values are those in which the Raman spectrum had strong contributions from CH and CH$_2$ stretching vibrations, while the opposite is true for the pixels with negative score values. The PC 1 scores revealed a distribution typical of carbohydrates in the undegraded sample, confirming that the CH/CH$_2$ stretching vibrations are indeed strongly linked to carbohydrates. However, the PC 1 scores also picked up the presence of extractives deposits (see Figure 17 and Figure 18 in section 4.2.1) in the week 0, 6 and 8 samples. A more detailed analysis of their Raman spectra (see Paper IV) indicated that the small deposit seen in the week 0 sample contained mostly pinosylvins, while the lumen-filling deposits seen in the week 6 and 8 samples contained mostly (week 6) or only (week 8) resin acids. However, despite the positive PC 1 scores of extractives, PC 1 is likely to represent primarily the cell wall carbohydrates in the CW and ML regions due to the fact that resin acids do not appear to be present in the CW or ML (see section 4.2.1).
Over the course of eight weeks, *R. placenta* caused a gradual degradation of the wood carbohydrates and possibly other CH/CH$_2$ stretch-producing material. Obvious degradation could be seen in the CW regions but also in the ML and CC. Degradation of the ML has been previously identified as a feature of incipient brown rot, characterised by the selective removal of easily digestible polysaccharides such as pectins (Fackler et al. 2010, Kim et al. 2015, Wagner et al. 2015). In the CW regions, the degradation appeared to proceed in a non-uniform manner, with spots of more and less extensive degradation appearing within each CW. Differences in the extent of degradation could also be seen between the CWs of different tracheids in the week 6 and 8 samples. Interestingly, the less degraded tracheids were those whose lumens contained extractives and those adjacent to an extractives-containing tracheid. Based on these findings, it seems possible that the extractives deposits commonly encountered in Scots pine HW (Figure 17 and Figure 18, section 4.2.1) can create localised areas of reduced degradation. The deposits are likely to prevent the growth of fungal hyphae in the affected lumen, which means that the fungal degradative agents need to diffuse greater distances to reach these cell walls, most likely resulting in a reduced rate of degradation. This type of lumen-blocking effect might be one mechanism of action of resin acids against decay, allowing them to contribute to natural durability without their presence in the cell walls. Given the higher resistance of resin acids to degradation by brown rot, their effects might persist in wood after the more toxic pinosylvins have been degraded, although it is likely that the resin acids will eventually be depleted as well.

In the case of PC 2, the positive score values represent pixels with a high contribution from pinosylvins and/or lignin, while the negative score values represent pixels with a high contribution from CH/CH$_2$ stretching vibrations. As expected, the undegraded sample showed a high concentration of pinosylvins and lignin in the ML and CC and a lower concentration in the CW, where the concentration of carbohydrates is higher. Because of the presence of the aromatic ring stretch band in the PC 2 loading vector, it is not possible to tell whether the concentration of pinosylvins is actually higher in the ML/CC regions than in the

![Figure 21. Score images based on the first (a) and the second (b) principal components. Samples from left to right are week 0, 2, 4, 6 and 8 (Paper IV)](image-url)
Results and discussion

CW; however, a high local concentration of pinosylvins could be seen on the lumen walls and in small extractives deposits.

Over the course of decay, *R. placenta* caused extensive degradation of material corresponding to the positive PC 2 loading plot bands. Average Raman spectra extracted from the CW regions of decaying HW (see Paper IV) showed a strong decrease in the intensity of the pinosylvins C=C stretch band, in agreement with the decreasing pinosylvins concentration of HW (Figure 19a). A small decrease could also be seen in the intensity of the aromatic ring stretch band. This decrease is also likely to be due to the degradation of pinosylvins, which produce an aromatic ring stretch band in the same region. Previous research has shown that brown rot degradation results in an increase in the intensity of the aromatic ring stretch band in spruce SW, which is free from phenolic extractives such as pinosylvins (Durmaz et al. 2016). Thus, the increasingly negative PC 2 scores seen in the CW regions of decaying HW are likely to be due to the degradation of pinosylvins. A closer inspection of the score images revealed local spots of lower PC 2 scores within the CWs, but a comparison with the PC 1 score images showed that these spots coincided with spots of more positive PC 1 scores. This finding suggests that the lower PC 2 scores are not due to a lower local concentration of pinosylvins but to a higher local concentration of CH/CH₂ stretch-producing material, most likely carbohydrates.

In addition to the cell walls, pinosylvins were also degraded in the extractives deposits. The lumen-filling deposits of intact Scots pine HW contain a mixture of pinosylvins and resin acids (Figure 18), but the deposits seen in degraded wood contained either very little pinosylvins (week 6) or none at all (week 8) (see Paper IV). Pinosylvins were also degraded in the ML/CC, but the presence of the aromatic ring stretch band in the PC 2 loading plot made it difficult to assess the extent of their degradation within these regions. Raman images showing the distribution of pinosylvins were therefore produced to accompany the PCA score images. Images were produced by integration of the substituted aromatic ring stretch band (990-1003 1/cm, Figure 22a-e) and the C=C stretch band (1630-1645 1/cm, Figure 22f-j), and although the integration images lack the sensitivity and robustness of PCA, they clearly show the extensive removal of pinosylvins from the CW and the ML/CC regions. The images produced according to the C=C stretch band showed more extensive degradation than the substituted aromatic ring images, and they revealed the presence of a more pinosylvins-rich area on the lumen walls. However, the small size of both of the pinosylvins bands means that their integrated intensity is easily affected by changes in the rest of the Raman spectrum. The pinosylvins distribution images shown in Figure 22 should therefore be considered merely indicative.
Figure 22. Distribution of pinosylvins. Raman images obtained by integration over 990-1003 $1/cm$ (a-e) and 1630-1645 $1/cm$ (f-j) in HW degraded for 0 (a,f), 2 (b,g), 4 (c,h), 6 (d,i) and 8 (e,j) weeks by R. placenta (Paper IV)

The results presented in Figures 19-22 clearly show that extractives and their degradation play a central role in the decay of Scots pine HW. Pinosylvins appeared to provide strong initial resistance to the HW, but they were also extensively degraded during the initial stages of decay. It was assumed that the degradation of pinosylvins results in a decrease in the toxicity of the HW extractives, but no information actually exists on how the toxicity of pinosylvins evolves during decay. Resin acids appeared to be quite resistant to degradation within the time frame of this experiment, but it is likely that they will eventually be degraded as well. Future works on Scots pine should determine how long the potential protective effect of the resin-rich deposits persists in decaying HW. Future work on natural durability in general should also consider the role of extractive degradation in more detail. Degradation is not usually discussed in connection with natural durability, but it is possible that the degradability of the extractives and the properties of the various degradation products could play some role in long-term decay resistance.
5. Conclusions and future perspectives

This thesis investigated the interactions between wood extractives and wood decaying fungi. The wood species selected for study was Scots pine, which is an important species in Finland and produces HW that is moderately resistant to decay. The objective of the work was to characterise the poorly understood mechanisms of action of pine extractives and to explore the ways in which extractives and fungi interact within the structure of wood. The interactions between wood extractives and wood decaying fungi have seen very limited research attention, despite extensive studies on the antifungal activity of extractives and the decay resistance of various HWs.

The potential mechanisms of action selected for study were antioxidant activity and enzyme inhibition. The antioxidant assays demonstrated that Scots pine HW and KW extractives have good radical scavenging activity, with pure pinosylvins even showing activities comparable to the antioxidant standards. The HW and KW extractives also showed some ferrous iron binding activity, which is surprising considering the lack of classical iron chelating functional groups on the pine extractive compounds. However, it is unclear how strong the association between pine extractives and iron is, and whether the extractives are capable of rendering the iron inactive and unable to participate in the Fenton reaction. The enzyme inhibition experiments, in turn, showed that Scots pine HW extractives can act as inhibitors of enzymatic holocellulose hydrolysis; however, substantial inhibition was only seen when the enzyme preparation in question contained an enzyme or enzymes that were capable of modifying the HW pinosylvins.

The antioxidant and enzyme inhibition studies both showed that Scots pine extractives are active not only in solution but also in their natural wood matrix, which suggests that the extractives have potential to contribute to decay resistance via these two non-biocidal mechanisms. However, it is unclear how significant these potential mechanisms are compared to the toxicity of the extractives, particularly the pinosylvins. It should also be recognised that these experiments are not representative of real wood decay: the antioxidant assays are simplistic, and the enzyme inhibition experiments did not consider the fact that the enzymatic hydrolysis of wood holocellulose is preceded by radical degradation, which also results in the modification of extractives. Future studies on the non-biocidal properties of extractives should therefore develop degradation methods that are more representative of real wood decay and take into consideration both the oxidative and hydrolytic stages of decay.
To bring a spatial dimension to the interactions between extractives and fungi, Raman imaging was used to study the cellular level distributions of the extractives in HW and KW and the chemical changes taking place in HW during brown rot decay. In the case of undegraded HW and KW, pinosylvins were found in the cell walls, middle lamella and lumens of tracheids, which shows that pinosylvins have potential to interact with fungi and their radical and enzyme agents during decay. However, the degradation experiment revealed that pinosylvins were readily degraded by the brown rot fungus used in the test. Although the overall extent of wood decay remained low during the fungal exposure period, the degradation of pinosylvins was extensive and appeared to proceed in a rather uniform manner within the cellular structure of wood. It appears that the pinosylvins were able to provide strong initial protection against the brown rot fungus, but given their sub-lethal concentration, the fungus was able to reduce their concentration over the course of decay. It is likely that higher rates of wood degradation would have been seen in later stages of decay, after the elimination of pinosylvins was complete.

The cellular level distribution of the resin acids and their behaviour during decay differed significantly from that of the pinosylvins. In undegraded HW and KW, resin acids were detected only in the lumen-filling extractives deposits, which suggests that their ability to interact with fungi and their degradative agents is more limited. However, the degradation experiment indicated a role for resin acids and extractives deposits in decay protection. The combination of Raman imaging and PCA showed that not all tracheid cell walls were degraded to the same extent; the less degraded tracheids were those whose lumens contained resin-rich extractives deposits and those adjacent to an extractives-containing tracheid. It is likely that these extractives deposits prevent the growth of fungal hyphae in the affected lumen, meaning that the fungal degradative agents need to diffuse greater distances to reach these cell walls. As the resin acids showed much higher resistance to degradation than the pinosylvins, it is possible that their protective effects will persist in wood after the more toxic pinosylvins have been degraded and their stronger initial protection has been lost.

The results of this thesis provide new information on the natural durability of Scots pine HW and on the ways in which pine extractives interact with wood decaying fungi. However, the interactions between wood extractives and fungi are still not fully understood. One of the more interesting aspects of these interactions is the degradation of extractives by fungi. It is clear that the extractives are degraded by fungi, but what remains unknown is how they are degraded and how their properties evolve over the course of degradation. It should also be appreciated that the results of this thesis are only applicable to Scots pine. Other wood species produce different types of HW extractives, and their interactions with fungi might be very different from the interactions seen in this thesis. To build a comprehensive understanding of natural durability, the interactions between extractives and fungi should be examined in a number of different species, ranging from those with little durability to those that are highly resistant to decay.
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Scots pine heartwood is moderately resistant to degradation caused by wood decaying fungi, primarily due to the presence of antifungal extractives in the heartwood. But how do the heartwood extractives prevent fungal growth? What types of mechanisms of action do they have, and how do they interact with wood decaying fungi within the structure of wood?