Analysis of the cell-wall compositions of different corewood types of *Pinus radiata* in relation to wood quality

Maree Brennan

A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy, The University of Auckland, 2011

Abstract

The cell-wall compositions of different corewood types of juvenile radiata pine grown straight, tilted or flexed were examined, with a focus on identifying differences in the composition of compression wood and flexure wood compared with normal and opposite wood. The relationships between their cell-wall compositions (lignin, *O*-acetyl and cellulose content and monosaccharide composition) and moisture-related longitudinal dimensional change of the different wood types were examined. The composition of compression wood was different from the non-compression wood types, with compression wood having a higher lignin and galactosyl residue content. Compression wood also had a higher magnitude of longitudinal dimensional change than non-compression wood. Analysis of whole cell-wall gels by two-dimensional nuclear magnetic resonance spectroscopy was done to compare differences in the compositions of the polymers (lignin and polysaccharides) present among the wood types. Compression wood was the only wood type to have detectable proportions of H-units in the lignin, and this wood type had a higher proportion of (1→4)-β-D-galactans than non-compression wood samples. The cell-wall polymer composition of flexure wood was not different from that of normal wood or opposite wood. Analytical pyrolysis followed by gas-chromatography and mass-spectrometry was done on samples of compression wood and opposite wood to identify pyrolysis products that were unique markers of compression wood. Compounds derived from the H-units of lignin, and anhydrosugars from the (1→4)-β-D-galactans of compression wood were identified that were present in significantly different proportions between compression wood and opposite wood. Hydrolysis of compression wood (1→4)-β-D-galactans using highly specific *endo*-(1→4)-β-galactanases to digest extracts of compression wood and ball-milled compression wood was investigated using matrix-assisted laser-desorption ionisation

time-of-flight mass spectrometry (MALDI-TOF MS) to detect the oligosaccharides released. Quantification of *endo*-(1→4)-β-galactanase-hydrolysed lupin seed (1→4)-β-D-galactans using MALDI-TOF MS was demonstrated.

Acknowledgements

Firstly, I thank my supervisor, Professor Philip Harris, for guidance and supervision throughout this project. He has provided much support and assistance, and has been a valuable and comprehensive resource of cell-wall related knowledge. Thanks also go to my secondary supervisor, Dr. Paul McLean, for guidance and time helping with statistical analyses, and for insisting on drinking good coffee. Thank you both for helpful discussions, and for review of my thesis manuscript.

I thank my colleagues at the University of Canterbury: Dr. Clemens Altaner, for his assistance with beginning my practical work, helpful discussions during the project and help with organising a rewarding research trip to Hamburg; Dr. Shakti Chauhan for providing the physical data for the wood samples; Dr. Luis Apiolaza and Professor John Walker for provision of the sample material. I also thank FRST for funding this collaborative research project.

I thank Professor John Ralph for his help with NMR and for many useful discussions. Thanks go to Dr. Jürgen Odermatt and Andreas Klingberg at the University of Hamburg for their help with the pyrolysis project. Thanks also to Martin Middleditch for his valuable assistance with MALDI-TOF MS. Many thanks to Heather Free for running the NMR samples and standards, discussion of NMR assignments and relaying my many questions to others during her trip to Wisconsin. I thank Esther Dale for being an enthusiastic student and for measurement of the cellulose content.

Big thanks to my lab-mates, Forrest and Leona, for making the lab a fun place to come to and for understanding the necessity of "chocolate days". Rob and Glenn, thank you so much for all of the love and support that you have given me. Special thanks to the Pinkest of Panthers, you sneaky, sneaky you.

Finally, I express my immense gratitude to my parents, who have always been so supportive, enthusiastic and understanding. Your encouragement over the last few years is deeply appreciated.

Table of contents

List of figures

List of tables

Abbreviations used

Abbreviations used were those of the "International System of Units" (SI). Standard notations for chemical elements and formulae were used.

°C	degrees Celsius
ε	error
2D NMR	two-dimensional nuclear magnetic resonance
ANOVA	analysis of variance
Ara*f*	arabinofuranosyl
CHO	indicates pyrolysis products derived from polysaccharides
CW	compression wood
Da	Dalton
DMSO	dimethyl sulphoxide
eV	electron volts
FID	flame ionisation detector
FW	flexure wood
G	guaiacyl
g	gram
GalA	galacturonic acid
Gal*p*	galactopyranosyl
Gal*p*A	galactopyranosyluronic acid
GC/MS	gas-chromatography/mass-spectrometry
Glc*p*	glucopyranosyl
Glc*p*A	glucopyranosyluronic acid

G-unit	guaiacyl-derived unit of lignin
H	*p*-hydroxyphenyl
h	hour
H/CHO	indicates pyrolysis products that are phenolic compounds containing no methoxyl groups and no, or a single carbon, sidechain
HPAEC-PAD	high-performance anion-exchange chromatography with pulsed amperometric detection
HSQC	heteronuclear single-quantum correlation
H-unit	*p*-hydroxyphenyl-derived unit of lignin
l	litre
M	molar (mole per litre)
m/z	mass-to-charge ratio
MALDI-TOF MS	matrix-assisted laser-desorption ionisation time-of-flight mass spectrometry
Man*p*	mannopyranosyl
min	minute
NMI	N-methylimidazole
NR	non-reducing
NW	normal wood
*O*Ac	*O*-acetyl
*O*MeGlcA	4-*O*-methylglucuronic acid
OW	opposite wood
pH	potentio hydrogeni
ppm	parts per million
P. radiata	*Pinus radiata* D. Don

R	reducing
RG I	rhamnogalacturonan I
R^2	coefficient of determination
rpm	revolutions per minute
RSE	relative standard error
RT	retention time
$S2_{(L)}$	highly lignified outer S2 layer of the cell wall
Tukey's HSD	Tukey's honestly significant difference
v/v	volume per volume
w/v	weight per volume
Xyl*p*	xylopyranosyl

Chapter 1 - General introduction

1.1 Introduction

The primary functions of wood in a living tree are conduction, mechanical support and storage. Wood with different structural, mechanical and chemical properties can be formed by the tree to fulfil these functions. The properties of wood vary both along the stem (longitudinally), and across the stem (radially). Therefore, it is important to define the terminology that describes where in the stem a piece of wood has come from. In this thesis, in which the analysis of wood of radiata pine (*Pinus radiata*) is reported, the nomenclature of Burdon et al. (2004) will be used, although this is typically only used in New Zealand for radiata pine. Proceeding from the pith to the bark, the innermost wood is termed "corewood" (often thought of as comprising the first ten years of growth in radiata pine), followed by "outerwood". The boundary between these two zones is not always clear and can be considered a 'transitional zone'. The terms "juvenile" wood and "mature" wood refer to the age at which the wood is formed. The juvenile wood is formed when the tree is still young, whereas mature wood forms when the tree is older, therefore these terms cannot be easily described in terms of growth rings. In older trees, the innermost wood in the trunk (termed the heartwood) consists of cells which are no longer living. The newly formed sapwood contains living cells that function in conduction. In temperate climates, wood growth is seasonal, and annual growth can be observed in rings. The growth that occurs in spring forms "earlywood", which consists of cells with larger lumens and thinner walls than those of "latewood" which forms in summer. The typical location of these different regions within a tree is shown in Figure 1-1.

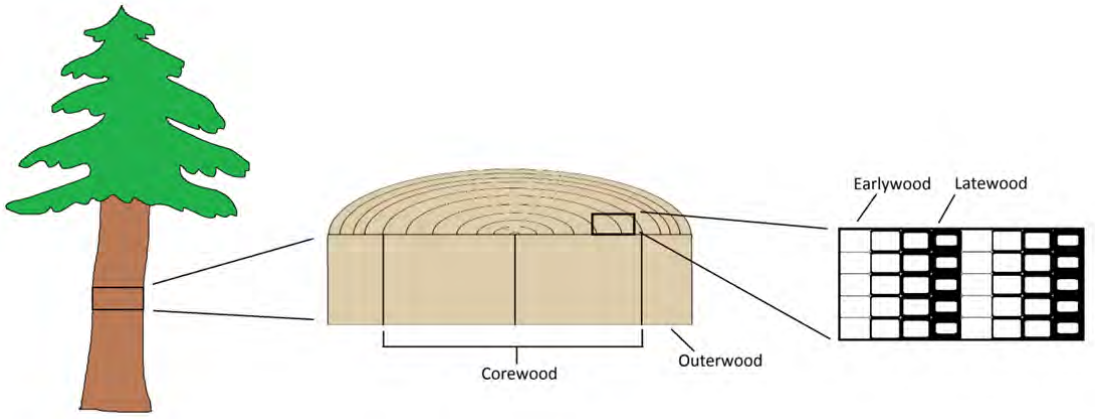

Figure 1-1 Tangential variation of wood produced within a typical coniferous gymnosperm tree. In radiata pine, the corewood is usually considered to be the first 10 years of growth.

While the ability to produce wood with different properties is beneficial for the living tree, it can be a problem for the timber industry, which desires the production of timber that is suitable for structural use, e.g. for building houses. It is desirable to produce good quality timber that is strong (resists rupture), stiff (resists bending) and that does not shrink or move in service (is dimensionally stable) (Walker, 2006). In species that are grown quickly and harvested at a young age, there is a high proportion of corewood. Corewood has undesirable properties for use as structural timber, being less stiff than outerwood and mature wood, with low strength and low dimensional stability. Therefore, wood that contains a high proportion of corewood is considered poor quality wood.

In New Zealand, around 90% of plantation forests are radiata pine (*Pinus radiata*), otherwise known as Monterey pine, with a small proportion of eucalypts (*Eucalyptus* spp.), redwoods (*Sequoia* spp.), poplar (*Populus* spp.) and douglas fir (*Pseudotsuga menziesii*) (Ministry of Agriculture and Forestry, 2007). Radiata pine is a favoured plantation forest species because it produces a large volume of wood very quickly

(Sutton, 1999). Unfortunately, the quality of wood produced from radiata pine is typically low due to the high proportion of corewood in the logs (Cown, 1992). The demand for higher quality wood is increasing (Eastin *et al.*, 2001), and a fundamental understanding of the different wood types produced by the tree, and methods to detect poor quality wood, will aid the forestry sector in meeting this demand.

1.2 Wood

Wood may be classified as either "softwood" or "hardwood", depending on its botanical origin. Softwood refers to wood from gymnosperms, e.g. pines (*Pinus* spp.), spruces (*Picea* spp.), firs (*Abies* spp.), larches (*Larix* spp.) etc. Hardwood refers to wood from angiosperms, e.g. oak (*Quercus* spp.), ash (*Fraxinus* spp.), poplar (*Populus* spp.), eucalypts (*Eucalyptus* spp.) etc.

Wood is secondary xylem tissue that consists of different cell types. In softwoods, 95% of the wood volume is made up of tracheids, which function in both conduction and support. In transverse section, tracheids are square or rectangular in shape, with a width of 15 to 65 µm (Butterfield and Meylan, 1980). They are longitudinally oriented with a length of 3 to 5 mm in mature wood (Thomas, 1991). The tracheid cell walls have pits for facilitating liquid flow. Softwoods also have transversely oriented rays, which are parenchyma cells and are usually uniseriate (one cell wide) (Figure 1-2). These are living cells that store food in the form of starch.

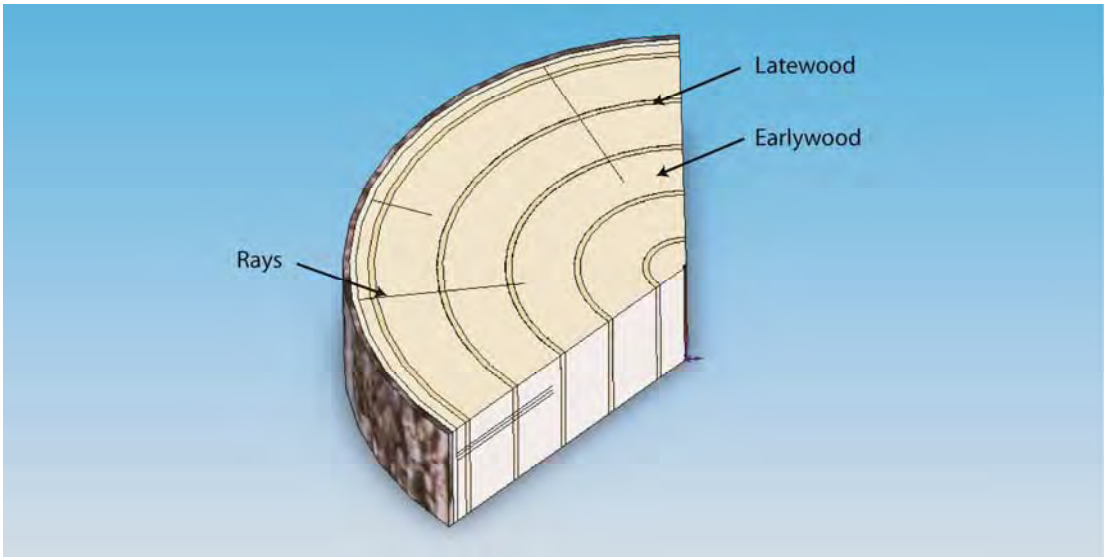

Figure 1-2 Illustration of the cross-section of a typical softwood species. Annual rings are visible due to the transition between latewood and earlywood. Rays are usually uniseriate.

The wood of hardwoods is more complex than that of softwoods. The different cell types have greater variation in size, shape and cell arrangement (Figure 1-3). The bulk of hardwood is composed of fibres which function in support. Fibres can be 0.8 to 2.3 mm long, with a width of 20 µm (Thomas, 1991). Vessels can occupy 6 to 55% of the volume of hardwoods and they function in conduction. Vessels are 0.2 to 1.3 mm long, and can vary greatly in width, from 20 to 300 µm. Unlike softwoods, the ray parenchyma of hardwoods are usually biseriate to multiseriate (Figure 1-3), rather than uniseriate.

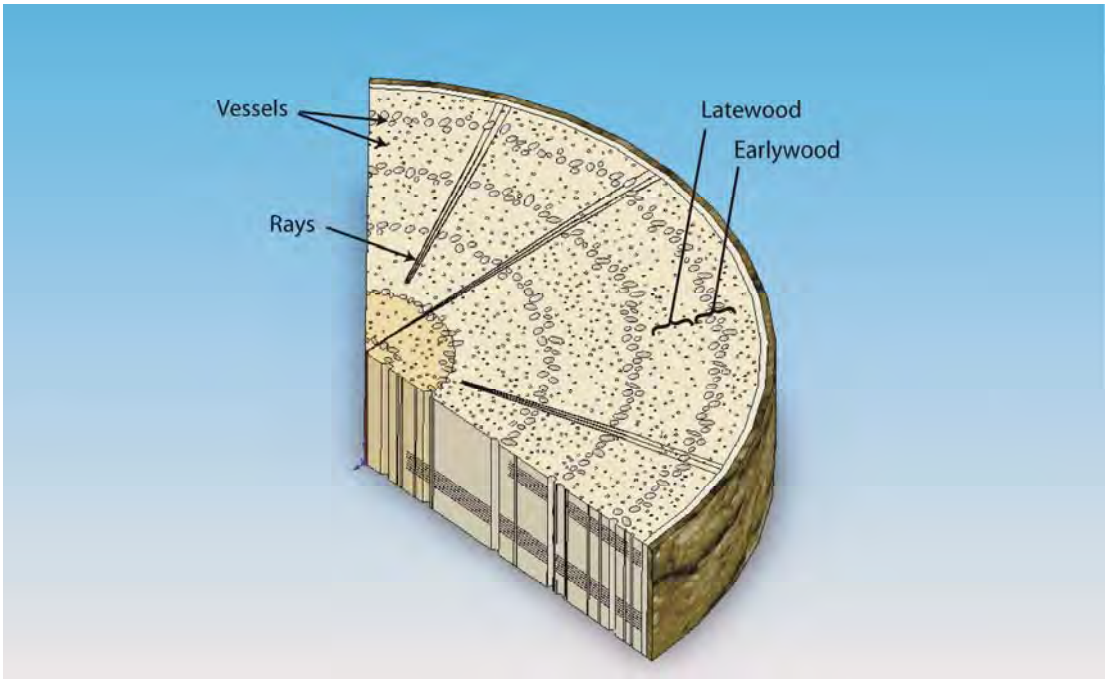

Figure 1-3 Illustration of the cross-section of a typical ring-porous hardwood species. Numerous vessels are present. Ray cells are multiseriate.

1.3 Wood formation and cell differentiation

Trees increase in height by primary growth which occurs by cell divisions in the apical meristem, followed by cell expansion. An increase in tree diameter is accomplished by divisions in the cambium which is a lateral meristem and is termed secondary growth (Larson, 1994). Wood cells develop first by cell divisions in the meristems, followed by cell enlargement, cell wall thickening and lastly lignification of the cell-wall layers.

A schematic of the general structure of plant cells with primary and secondary walls is shown in Figure 1-4. There is a thin, primary wall, which is formed while the cell is still actively growing. Adjacent cells are connected via the middle lamella. The primary walls of adjacent cells and the middle lamella are together referred to as the compound middle lamella. The secondary wall is formed in layers when expansive growth has

finished. Typically there are three secondary wall layers, S1, S2 and S3, with S2 being the thickest (~70 to 80 % of the cell wall area).

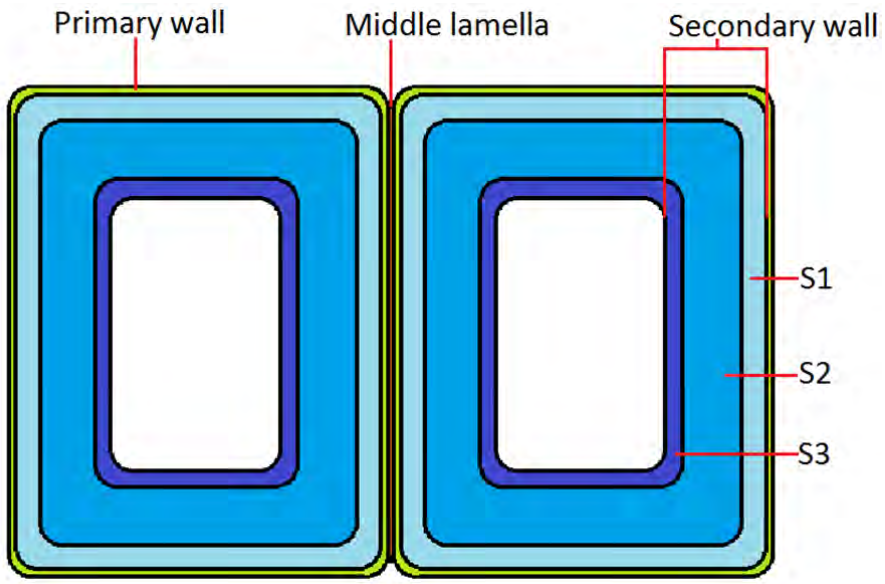

Figure 1-4 General structure of a transverse section of plant cells with a primary wall, onto which is deposited the secondary wall in three layers (S1, S2 and S3). The compound middle lamella includes the primary walls of adjacent cells, and the middle lamella.

Lignification is considered to be the final phase of differentiation of the wood cells. Lignin is first deposited in the cell corners and the middle lamella while the secondary cell wall is forming (Donaldson, 2001). Lignification of the secondary walls begins soon after their deposition, when the middle lamella is at approximately 50% of its final lignin concentration. The middle lamella has a high lignin concentration, with the highest concentration typically occurring in the cell corner middle lamella (Terashima and Fukushima, 1988; Donaldson, 2001). The lignin concentration in the middle lamella is ~ 50%, whereas the lignin concentration in the S2 layer is ~ 20%. As the secondary wall

accounts for a much greater proportion of wood volume, the secondary wall thus contains most of the lignin of wood (Donaldson, 2001).

1.4 Composition of wood cell walls

1.4.1 Cellulose

Cellulose makes up ~40-45% of the dry weight of wood (Goldstein, 1991). Cellulose consists of linear chains of (1→4)-linked β-D-glucopyranosyl residues, and has repeating units of cellobiose (two β-D-glucosyl units flipped 180° with respect to each other) (Figure 1-5). Aggregates of these molecules are called cellulose microfibrils. Because the arrangement of the cellulose molecules is very regular, the cellulose is crystalline.

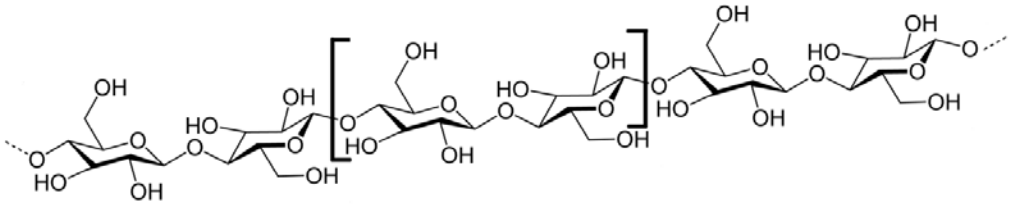

Figure 1-5 Chemical structure of cellulose. The repeating unit is cellobiose, shown in brackets.

1.4.2 Non-cellulosic polysaccharides

Non-cellulosic polysaccharides make up 20 to 30% of dry wood by weight (Whistler and Chen, 1991). Most have at least some side groups.

1.4.2.1 Heteromannans

In softwoods, these are galactoglucomannans, and are the most abundant polysaccharides, accounting for 12 to 25% of the wood (Whistler and Chen, 1991; Harris and Stone, 2008). Galactoglucomannans have a backbone of randomly arranged β-D-mannopyranosyl and β-D-glucopyranosyl residues linked (1→4). Galactoglucomannans have α-D-galactopyranosyl residues attached at C(O)6 to approximately 3% of the mannose and glucose residues (Fu and Timell, 1972). They also have *O*-acetyl groups attached to some mannose residues at the C(O)2 and C(O)3 positions, and in some cases at C(O)3 of the glucose residues (Meier, 1961; Kenne *et al.*, 1975; Lundqvist *et al.*, 2002; Willför and Holmbom, 2004). Softwood heteromannans are therefore commonly referred to as *O*-acetyl-galactoglucomannans (Figure 1-6). Hardwood heteromannans are glucomannans, they do not contain galactosyl residues, and only make up 3 to 5% of the wood (Whistler and Chen, 1991). The glucomannans of the hardwoods aspen and birch contain *O*-acetyl groups at C(O)2 and C(O)3 of some mannose residues (Teleman *et al.*, 2003).

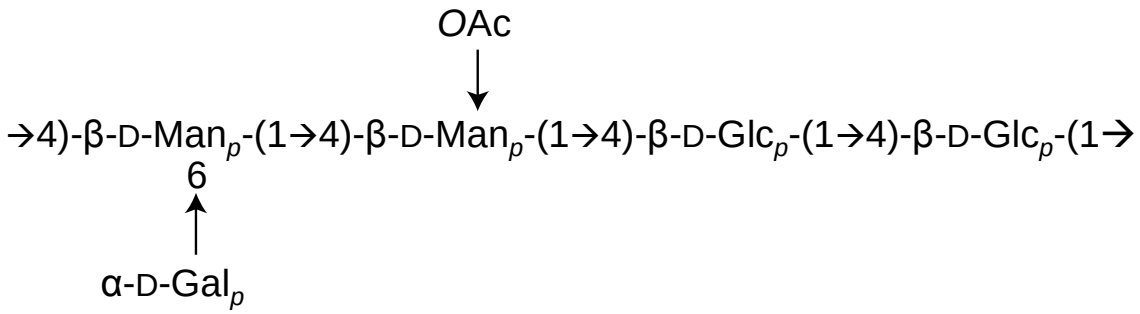

Figure 1-6 Structure of a softwood *O*-acetyl-galactoglucomannan.

1.4.2.2 Heteroxylans

Heteroxylans have a backbone of (1→4)-linked β-D-xylopyranosyl residues with some sidechains attached. In softwoods, these are glucuronoarabinoxylans and are the second most abundant non-cellulosic polysaccharide, making up 7 to 15% of the wood (Whistler and Chen, 1991). Glucuronoarabinoxylans have 4-*O*-methyl-glucopyranosyluronic acid residues attached at C(O)2 of every fifth or sixth xylosyl residue on average, and also have α-L-arabinofuranosyl residues attached at C(O)3 of about every eighth xylosyl residue on average (Shimizu *et al.*, 1978; Whistler and Chen, 1991; Harris and Stone, 2008). Softwood glucuronoarabinoxylans are therefore called 4-*O*-methyl-glucuronoarabinoxylans (Figure 1-7).

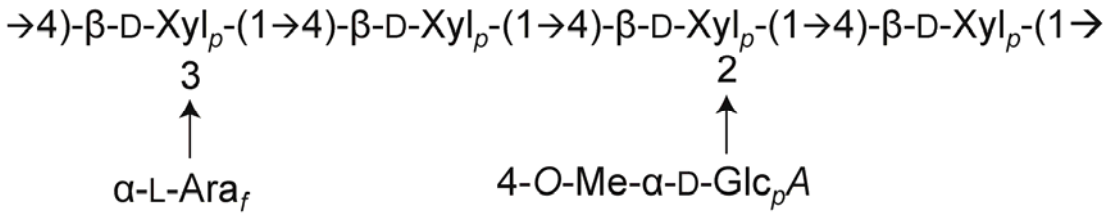

Figure 1-7 Structure of a softwood 4-*O*-methylglucuronoarabinoxylan.

Hardwood heteroxylans (4-*O*-methylglucuronoxylans) make up 10 to 35% of hardwood (Whistler and Chen, 1991). They have fewer 4-*O*-methyl-glucuronopyranosyl residues than softwood heteroxylans, with residues attached to every tenth xylosyl residue on average (Timell, 1964; 1965). They have *O*-acetyl groups attached at C(O)2, and sometimes at C(O)3, to 8 to 17% of the xylosyl residues (Bouveng *et al.*, 1960).

1.4.2.3 Other non-cellulosic polysaccharides

1.4.2.3.1 Glucans and heteroglucans

Xyloglucans are primary wall polysaccharides that consist of a backbone of (1→4)-linked β-D-glucosyl residues, some of which are substituted at C(O)6 with α-D-xylosyl residues, which may be further substituted with other glycosyl residues. The xyloglucans of the primary walls of differentiating xylem in Sugi (*Cryptomeria japonica*) seedlings have been characterised (Kakegawa *et al*., 1998), and have similar structures to those of most eudicotyledons which are fucogalactoxyloglucans (Hsieh *et al.*, 2009; Brennan and Harris, 2011).

(1→3)-β-D-glucans, callose, is present in the walls of the sieve cells of phloem (Fengel and Wegener, 1989). In addition, a (1→3)-β-D-glucan, sometimes called laricinan, has been isolated in the reaction wood of tamarack (*Larix laricina*), red spruce (*Picea rubens*), balsam fir (*Abies balsamea*) and red pine (*Pinus resinosa*) (Hoffman and Timell, 1970; Timell, 1982). Callose has been shown to be deposited onto the walls in the helical grooves of the S2 layer (Altaner *et al*., 2010).

1.4.2.3.2 Pectic polysaccharides

In softwoods and hardwoods, the pectic polysaccharides occur predominantly in the middle lamella and primary wall, and make up less than 1% of the wood. The pectic polysaccharides are complex polymers made up of four different domains. These domains are predominantly homogalacturonan, and rhamnogalacturonan I, with smaller proportions of xylogalacturonan, and rhamnogalacturonan II (RG II) (Harris, 2005).

Homogalacturonan is a linear polymer of 1→4-linked α-D-galactopyranosyluronic acid residues (Figure 1-8).

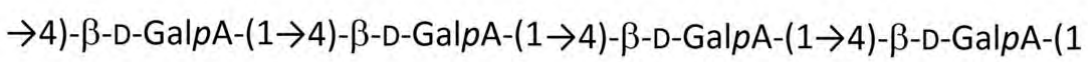

Figure 1-8 Structure of homogalacturonan. Xylogalacturonan and rhamnogalacturonan II have a backbone of this structure, which is further substituted with different side chains.

Rhamnogalacturonan I (RG I) has a backbone of alternating α-D-galactopyranosyluronic acid and α-L-rhamnopyranosyl residues, and the rhamnosyl residues can be substituted with polysaccharides or oligosaccharides rich in galactose and arabinose, specifically: arabinans, galactans and type I arabinogalactans. The galactans are (1→4)-β-D-galactans of the structure shown in Figure 1-10. The arabinogalactan sidechains have a backbone of (1→4)-β-D-galactosyl residues which are substituted with arabinofuranosyl side chains (Clarke *et al.*, 1979; Goellner *et al.*, 2011). A water soluble (1→3),(1→6)-β-D-galactan with arabinosyl side groups is found in large quantities in larch (Figure 1-9), but this is thought to be entirely lumenal and not present in the cell walls (Côté *et al.*, 1966).

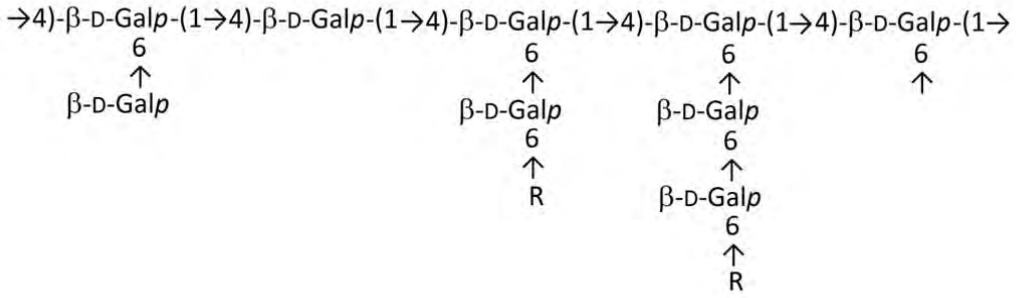

Figure 1-9 Structure of a larch arabinogalactan, redrawn from Ponder and Richards (1997). R = galactan or arabinan sidechain.

Xylogalacturonan has the same backbone structure as homogalacturonan, but has β-D-xylopyranosyl residues attached at C(O)3 to some residues on the galacturonic acid backbone. Rhamnogalacturonan II also has the same backbone structure as homogalacturonan, but is substituted with four different side chains, two of which have complex structures (O'Neill *et al.*, 2004; Harris, 2005).

1.4.2.4 (1→4)-β-D-Galactans

Reaction wood forms in trees response to displacement of a stem or branch from its equilibrium growth position, for example when stems are displaced from the vertical. A (1→4)-β-D-galactan occurs in the reaction wood of both softwoods and hardwoods (Bouveng and Meier, 1959b; Meier, 1962b), its structure is shown in Figure 1-10. In compression wood, high proportions of (1→4)-β-D-galactans are found in the $S2_{(L)}$ layer, whereas they are only found in smaller proportions in the primary walls of normal wood, probably as side chains of the pectic polysaccharide RG I (Altaner *et al.*, 2010; Kim *et al.*, 2010). It is chemically similar to the (1→4)-β-D-galactan sidechains of RG I in the primary

walls of eudicotyledons such as potato (*Solanum tuberosum*) (Harris and Stone, 2008). It is not known whether the secondary walls of flexure wood contain a (1→4)-β-D-galactan.

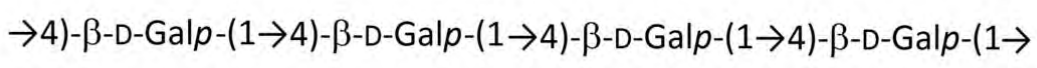

Figure 1-10 Structure of a (1→4)-β-D-galactan which is a sidechain of RG I, and also found in the secondary walls of compression wood.

1.4.3 Lignin

Lignins are a complex class of polymers that are not thought to have strictly defined structure as a macromolecule (Ralph *et al.*, 2004). Lignins are formed by the oxidative polymerisation of hydroxycinnamyl alcohol precursors (monolignols) (Boerjan *et al.*, 2003; Ralph *et al.*, 2004; Ralph *et al.*, 2009). The three monolignols are *p*-coumaryl, coniferyl and sinapyl alcohols (Figure 1-11). In the lignin polymer, the monolignols produce *p*-hydroxyphenyl (H), guaiacyl (G) or syringyl (S) units respectively. Hardwood lignins contain G- and S-units and trace amounts of H-units. Softwood lignins contain mostly G-units, with small proportions of H-units. Occasionally S-units have been reported in the lignin of softwood species (Lewis and Yamamoto, 1990). The lignin of compression wood has a high proportion of H-units compared with normal wood (Bland, 1958).

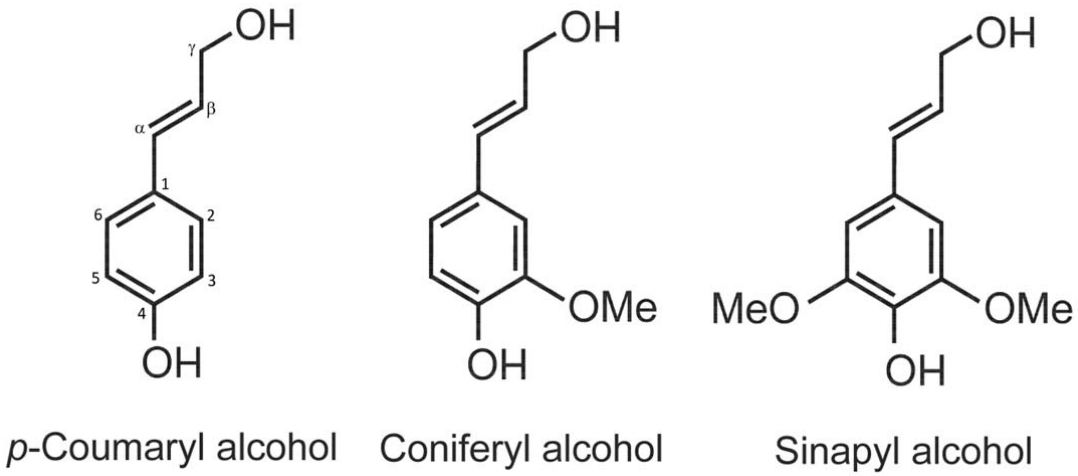

Figure 1-11 The lignin polymer is synthesised from three hydroxycinnamyl alcohol (monolignol) precursors, *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol, which, on incorporation into the lignin polymer, form H-units, G-units and S-units, respectively. The numbering system for the carbons is shown for *p*-coumaryl alcohol.

Growth of the lignin polymer is achieved by formation of bonds between monolignol radicals and phenoxy radicals on the lignin polymer (Boerjan *et al.*, 2003; Ralph *et al.*, 2007). The radicals are formed by dehydrogenation of the monolignols, probably by peroxidases. Monolignols usually couple at their β- positions, to the 4-O- or 5- positions on the lignin polymer. β-O-4 is usually the predominant interunit linkage. Coupling of guaiacyl units via β-O-4- to the polymer yields β-ether structures (Figure 1-12, structure A), whereas coupling of β-5- yields phenylcoumaran (Figure 1-12, structure B). Coupling between lignin oligomers yields units linked 5-5- (Figure 1-12, structure D). β-β- dehydrodimerisations yield resinol units (Figure 1-12, structure C) and β-O-4- dehydrodimerisations yield cinnamyl end groups (Figure 1-12, structure X1).

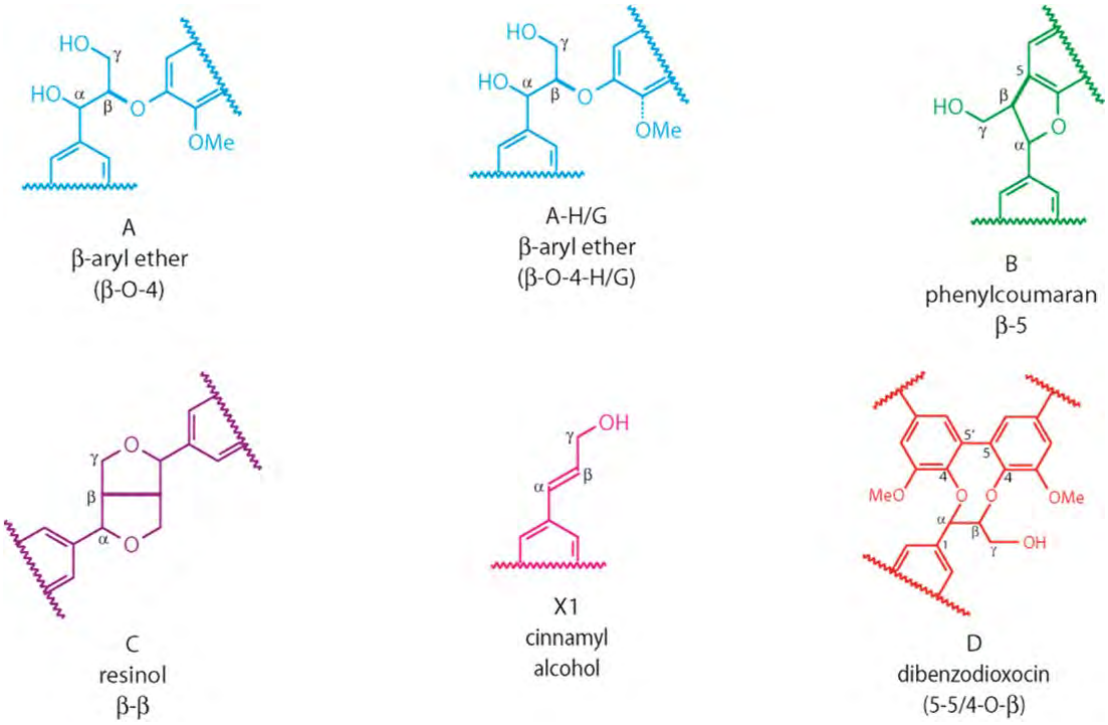

Figure 1-12 Common interunit linkages of softwood lignins. Figures modified from Kim and Ralph (2010).

1.4.4 Glycoproteins and proteoglycans

The cell walls of wood may also contain small proportions of glycoproteins and proteoglycans such as arabinogalactan proteins and extensins. These are a spectrum of molecules that range from those containing small proportions of proteins and large proportions of polysaccharides, to those containing large proportions of proteins with only a small proportion of polysaccharides (Clarke *et al.*, 1979; Ellis *et al.*, 2010; Lamport *et al.*, 2011). Arabinogalactan proteins have a protein core, and are glycosylated with arabinogalactan sidechains (Seifert and Roberts, 2007). The arabinogalactan side chains are type II arabinogalactans which have backbones of (1→3),(1→6)-β-D-galactans which are substituted with arabinofuranosyl side chains (Clarke *et al.*, 1979).

The protein core of arabinogalactan proteins is typically rich in proline, hydroxyproline, alanine, serine and threonine (Seifert and Roberts, 2007). The proteins are thought to be glycophosphatidylinositol anchored in the plasma membrane, which would make them ideally placed for their proposed roles in cell expansion, cell-cell signalling and cell differentiation (Knox, 2006; Driouich and Baskin, 2008). Hydroxyproline-rich, glycoslyated proteins (extensins) have been found in the differentiating xylem of *Pinus radiata* (Altaner *et al.*, 2010), and in the differentiating and mature xylem cells of *Pinus taeda* (Bao *et al.*, 1992).

1.4.5 Low molecular-weight compounds

The low molecular-weight compounds of wood are extractives and ash. Extractives include volatile oils, terpenes, resins, waxes, fatty acids and aromatic compounds (Hillis, 1987). The amount of extractives in wood can vary greatly among species, and within a single tree. For example higher proportions of extractives are often found in the heartwood and branch bases in trees (Hillis, 1987; Fengel and Wegener, 1989). Ash is the term given to the inorganic constituents in wood, mainly potassium, calcium and magnesium. It makes up 0.2 to 0.5% of the wood of softwoods, but this value can be much higher in tropical hardwoods (Fengel and Wegener, 1989).

1.5 Architecture of tracheid cell walls

The primary walls of wood cells consist of cellulose microfibrils embedded in a matrix of non-cellulosic polysaccharides, and lignin once lignification begins. The secondary cell walls of wood have aggregates of cellulose microfibrils embedded in a matrix, and the

matrix contains both non-cellulosic polysaccharides (predominantly heteromannans and heteroxylans) and lignin. The cellulose microfibrils are arranged differently in each of the cell wall layers (Figure 1-13). In softwood tracheids, the primary wall has cellulose microfibrils that are not highly organised. The microfibrils in the S1 layer of the secondary wall are oriented at an angle of ~50° to 70° to the longitudinal axis of the cell (Thomas, 1991). The S2 layer, which is the thickest layer, has microfibrils oriented at ~10° to 40° to the longitudinal axis of the cell (Thomas, 1991). The innermost layer, S3, has microfibrils oriented nearly perpendicular to the cell axis at ~60° to 90° (Thomas, 1991).

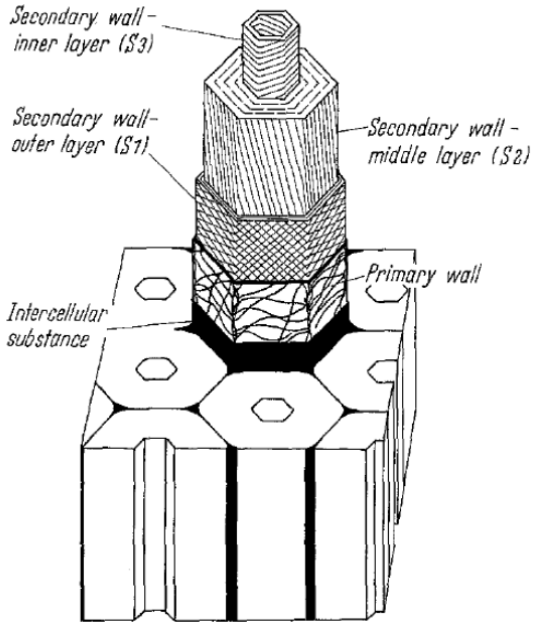

Figure 1-13 Typical structure of a conifer tracheid. Adjacent cells are joined by the middle lamella (intercellular substance). The primary wall is thin, and has irregularly arranged microfibrils. The secondary wall is deposited in three layers, S1, S2 and S3. Figure from Wardrop and Bland (1959).

The non-cellulosic polysaccharides of the secondary walls of softwood tracheids are glucuronoarabinoxylans and galactoglucomanans. The matrix polymers (non-cellulosic

polysaccharides and lignin) associate with each other, and the cellulose microfibrils, through hydrogen bonds (Harris and Stone, 2008). In the cell walls of tracheids, lignin is most tightly associated with glucuronoarabinoxylans (Salmén and Olsson, 1998), whereas the cellulose microfibril aggregates are tightly associated with the galactoglucomannans (Åkerholm and Salmén, 2001). The galactoglucomannans are oriented in parallel with the highly oriented cellulose microfibrils in the secondary walls of softwood tracheids (Åkerholm and Salmén, 2001). In addition, the molecular orientation of softwood galactoglucomannans in the wall may be influenced by the occurrence of acetyl groups (Katz, 1965). There is evidence that the phenylpropane units of lignin are preferentially oriented parallel to the fibre axis, although this is not as evident as the orientation of the non-cellulosic polysaccharides (Åkerholm and Salmén, 2003; Stevanic and Salmén, 2009). Atomic force microscopy has been used to examine the architecture of the secondary cell wall of spruce tracheids (Fahlén and Salmén, 2005), and this is shown schematically in Figure 1-14.

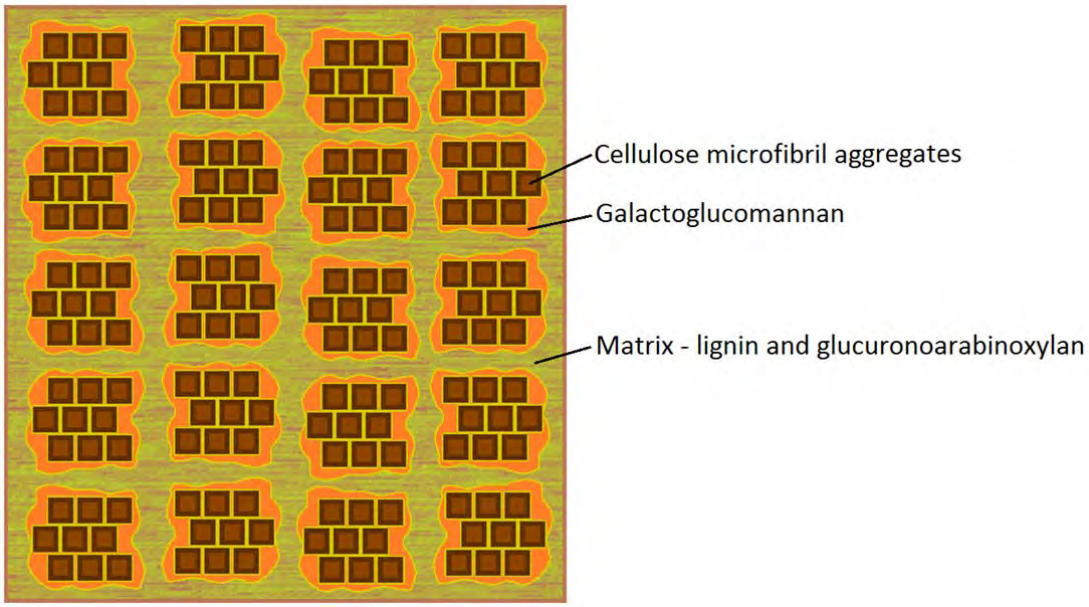

Figure 1-14 Illustration of the architecture of the S2 wall layer of softwoods, redrawn from Fahlén and Salmén (2005) and Harris and Stone (2008). There is a tight association between cellulose and galactoglucomannans. The lignin and glucuronoarabinoxylans are associated in a matrix.

1.6 Reaction wood

Reaction wood is formed in woody plants as a special tissue in reaction to displacement of the stem from the vertical by external environmental factors (e.g., partial uprooting or uneven crown loading) (Scurfield, 1973). In softwoods, reaction wood is formed on the underside of leaning stems and branches and is termed compression wood because it produces a compressive maturation strain. Compression wood has been extensively reviewed by Timell (1986). In hardwoods, reaction wood forms on the upper side of leaning stems and branches, and is called tension wood because it produces a tensile maturation strain. The presence of reaction wood is necessary for the living tree to maintain vertical growth, but it is considered to be a defect in timber and pulp as it has less desirable properties for these purposes. For comparative purposes, the wood formed directly opposite reaction wood is termed opposite wood, and wood that is not reaction

wood is termed normal wood, although it should be noted that formation of all of these wood types is normal for the tree.

1.6.1 Compression wood

Compression wood is frequently present in high proportions in the corewood of plantation softwoods such as radiata pine, contributing to the poor quality of corewood for structural use (Johansson and Kliger, 2002). Compression wood typically has a high microfibril angle compared with normal wood which contributes to the high longitudinal shrinkage of this wood type on drying compared with normal wood (Harris and Meylan, 1965; Meylan, 1968). Uneven distribution of compression wood can cause uneven longitudinal shrinkage throughout a stem, contributing to dimensional instability (Johansson, 2003). Compression wood has lower strength and stiffness compared with normal wood. Therefore every gymnosperm tree, even if it appears straight, is likely to contain at least some compression wood (Timell, 1986).

Compression wood differs anatomically from normal wood. Compression wood tracheids have intercellular spaces (IS) between them as shown in Figure 1-15, and have a rounded appearance compared with normal wood tracheids (Figure 1-13). The cell walls of compression wood tracheids lack an S3 layer, and have a highly lignified outer region in the S2 layer, termed the $S2_{(L)}$. The inner S2 layer contains helical cavities that terminate at the $S2_{(L)}$ layer (Figure 1-15). The cell-wall composition of compression wood is different to normal wood. It contains high proportions of a (1→4)-β-D-galactan (Bouveng and Meier, 1959a), and has a high proportion of *p*-hydroxyphenyl units (H-units) in its lignin (Bland, 1958).

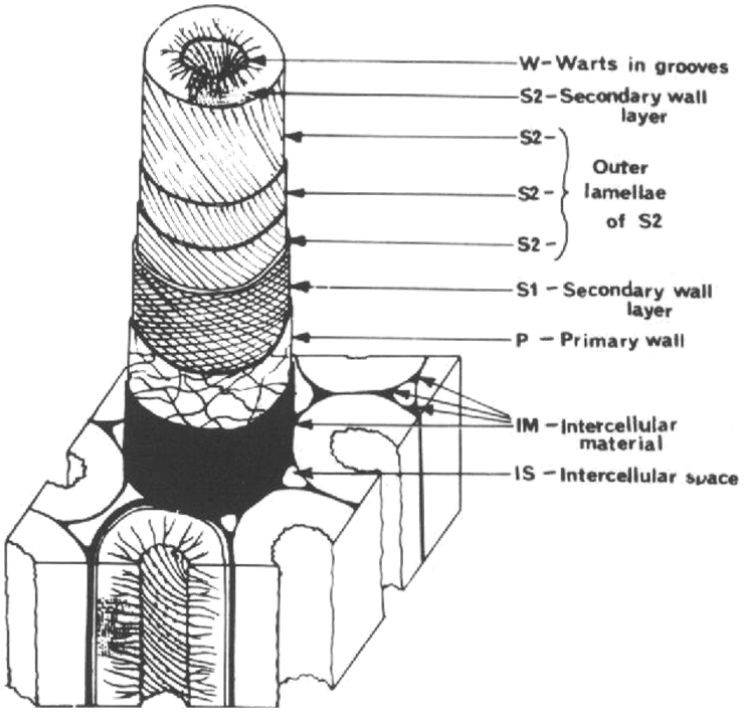

Figure 1-15 Illustration of a typical compression wood tracheid. Compression wood cells are rounded, have intercellular spaces and lack an S3 layer. Figure from Timell (1986).

1.1.1.1 Detection of compression wood

Severe compression wood is often more darkly coloured than normal wood, but mild compression wood is not as easy to detect visually. The characteristic anatomical changes associated with compression wood can be detected by bright-field microscopy. An advantage of microscopy is that the severity of compression wood can be determined using the guidelines of Donaldson (2004), in which mild compression wood has the first two characteristics and severe compression wood has all of the characteristics of the following list:

1) Presence of a highly lignified $S2_L$ layer

2) Presence of intercellular spaces

3) Absence of an S3 layer

4) Presence of helical checks

A compression wood severity indicator, calculated from the ratio of different wavebands, has been reported using scanning Fourier-transform infrared micro-spectroscopy on ground wood from Norway spruce (*Picea abies*) and Sitka spruce (*Picea sitchensis*) that contained compression wood (Altaner *et al.*, 2009). UV fluorescence microscopy has also been used to detect lignin. Radiata pine compression wood has a different lignin fluorescence spectrum than normal wood, possibly due to the presence of H-units in the lignin (Donaldson *et al.*, 2010). The lignin content of compression wood, and the proportions of H-units in the lignin, have been shown to be related to the severity of compression wood in radiata pine (Nanayakkara *et al.*, 2009).

1.6.2 Flexure wood

Flexing mechanical perturbation of stems, for example by wind action, produces a type of wind-affected wood that has been referred to as 'flexure wood'. This has been studied in some detail in *Pinus taeda* and *Abies fraseri* stems, which were mechanically perturbed in the laboratory by flexion backwards and forwards on a single axis (Telewski and Jaffe, 1981; 1986b; a; 1989). The flexure wood of *P. taeda* has a different morphology and anatomy compared with normal wood: increased radial growth in the direction of mechanical perturbation (Figure 1-16), shorter stems and decreased tracheid length (Telewski and Jaffe, 1986a). The increased growth in the direction of perturbation is due to a greater number of cell divisions in the cambium, resulting in the production of more tracheids.

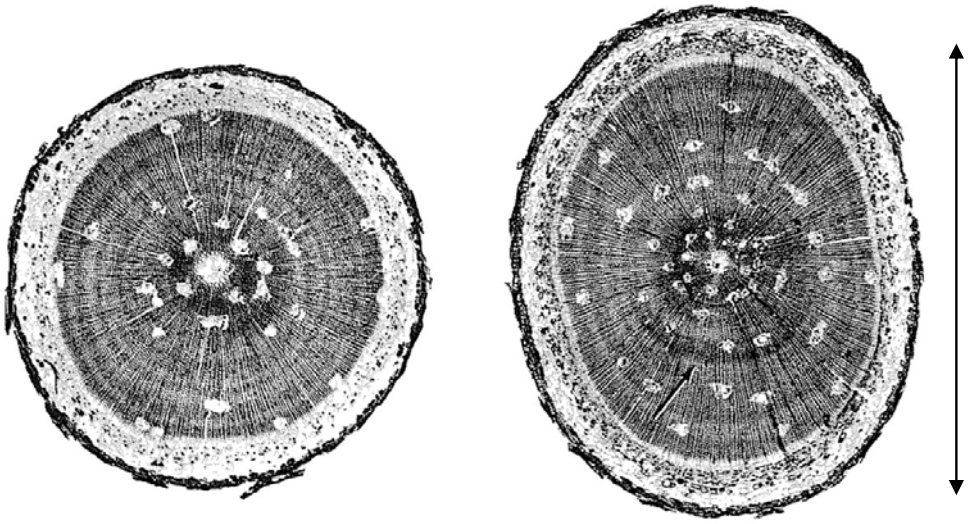

Figure 1-16 Transverse sections of control *Pinus taeda* (left) and mechanically perturbed *P. taeda* (Telewski and Jaffe, 1981). Mechanical perturbation was performed in the direction of the arrow.

Flexure wood has lower stem stiffness than normal wood (Telewski and Jaffe, 1986b), allowing it to withstand a greater degree of bending without mechanical failure. The microfibril angle of flexure wood tracheids is not as high as compression wood tracheids, but is greater than in normal wood tracheids (Telewski, 1989). The action of wind is often postulated to cause compression wood formation due to toppling or causing stems to lean during growth (Nicholls, 1982; Yeh *et al.*, 2005; Moore *et al.*, 2008). Wind-affected wood does have some physical and anatomical similarities with compression wood, but it is not known whether the composition of their cell walls is also similar. To date, knowledge of the chemical composition of flexure wood is that the proportion of solvent-extractable compounds is increased compared to normal wood, whereas the Klason lignin and holocellulose content (cellulose and non-cellulosic polysaccharides) is the same as in normal wood (Telewski and Jaffe, 1981).

1.7 Chemical analysis of wood

Conventional chemical analysis of wood often involves fractionation and isolation of the different components that make up the cell wall to characterise and/or quantify them. The cell-wall polymers exist in a matrix and are often covalently linked with each other, therefore the separation of individual components, without resulting in undesired structural/chemical changes, is difficult. Hence, there is often a trade-off between quantification or structural analysis of the wood polymers.

1.7.1 Cellulose quantification

Cellulose is often analysed as a preparation called α-cellulose which still contains some residual non-cellulosic polysaccharides (Wise *et al.*, 1946; Fengel and Wegener, 1989). This may be isolated from holocellulose (delignified wood), or from milled wood. Treatment of holocellulose with a solution of strong alkali such as sodium hydroxide or potassium hydroxide will remove most of the polysaccharides and residual lignin. Cellulose from milled wood can also be isolated with nitric acid in ethanol, or a mixture of nitric acid and acetic acid (Updegraff, 1969; Brendel *et al.*, 2000). The isolated cellulose can be quantified by weight, or by quantifying the total reducing sugars released after total acid hydrolysis or by quantifying only the glucose released after such hydrolysis (Fengel and Wegener, 1989; Easty and Thompson, 1991).

1.7.2 Non-cellulosic polysaccharides

1.7.2.1 Quantification

Methods for quantitative isolation of specific non-cellulosic polysaccharides are currently unavailable. However, the monosaccharide composition of the polysaccharides can be determined by two-stage sulphuric acid hydrolysis of extracted wood (Saeman *et al.*, 1954), followed by separation and quantification of the monosaccharides. Several methods can be employed to separate and quantify the monosaccharides, the most common being capillary gas-chromatography (GC) of their alditol acetates (Harris *et al.*, 1988) or high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (Lee, 1996; Weitzhandler *et al.*, 2004). Proton nuclear magnetic resonance (NMR) spectroscopy has also been used to determine the monosaccharide compositions of polysaccharides in acid hydrolysates of wood (Kiemle *et al.*, 2004), wood pulps (Çöpür *et al.*, 2003), and polysaccharides isolated from wood (Shin, 2008). Uronic acids can be quantified as total uronosyl residues using a colorimetric assay (Blumenkrantz and Asboe-Hansen, 1973), and individual uronic acids in acid hydrolysates can be separated and quantified using HPAEC-PAD (Smith and Harris, 1995; Weitzhandler *et al.*, 2004).

1.7.2.2 Structural analysis

Fractions of the non-cellulosic polysaccharides can be isolated from milled wood with aqueous alkali solutions without prior delignification of the wood. To isolate particular polysaccharides, however, further fractionation is required (Browning, 1967; Fengel and Wegener, 1989). The structure of isolated polysaccharides can be examined, for example, by determining their monosaccharide compositions to identify and quantify the

component monosaccharides (Harris *et al.*, 1988). The carbon atoms involved in linking the different monosaccharides (linkage composition) is usually determined by methylation analysis (Harris *et al.*, 1984). Isolated plant cell-wall polysaccharides have also frequently been examined by ^{1}H and ^{13}C NMR spectroscopy in one and two dimensions, typically in solution, to obtain information on anomeric carbon configurations and linkage (Dais and Perlin, 1982; Cavagna *et al.*, 1984; Excoffier *et al.*, 1986; Lundqvist *et al.*, 2002; Hannuksela and Hervé du Penhoat, 2004; Moulthrop *et al.*, 2005). Additionally, highly specific enzymes can be used to cleave polysaccharides into oligosaccharides, the structures of which can be further analysed by HPAEC-PAD or matrix-assisted laser-desorption ionisation time-of-flight mass spectrometry (MALDI-TOF) (Lerouxel *et al.*, 2002; Hsieh *et al.*, 2009; Günl *et al.*, 2011).

Compositional analysis of the whole cell-wall using two-dimensional ^{1}H-^{13}C NMR spectroscopy has recently been achieved using solution-state NMR after dissolution of the whole cell wall or formation of a gel (Lu and Ralph, 2003; Yelle *et al.*, 2008; Hedenström *et al.*, 2009; Terashima *et al.*, 2009). For example, the whole cell wall can be swollen in dimethyl sulphoxide (DMSO), and in mixtures of DMSO and pyridine (Kim *et al.*, 2008; Kim and Ralph, 2010; Rencoret *et al.*, 2011). The spectra produced from whole cell-wall gels give detailed information about the structures of both the polysaccharides and the lignin present in the cell walls.

1.7.3 Lignin

1.7.3.1 Quantification

Available methods of lignin quantification all have their limitations (reviewed by Hatfield and Fukushima, 2005). One of the most commonly used methods of determining lignin is to measure the "Klason" lignin content, which is the residue left after two-stage sulphuric acid hydrolysis of wood (TAPPI standard, 1998). The acid-insoluble residue also contains other acid-insoluble compounds such as small amounts of proteins which may form condensation products with the lignin (Norman and Jenkins, 1934). The acid-soluble component of lignin, which occurs especially in hardwoods, can be measured by UV spectroscopy of the hydrolysate.

1.7.3.2 Structural analysis

Lignin preparations can be obtained in a variety of ways, but those that result in the least structural alteration often involved milling. For example, vibratory ball-milling, followed by extraction with dioxane is carried out to yield "milled wood lignin" (Björkman, 1956). The structure of the isolated lignin can then be analysed by methods such as thioacidolysis (Lapierre *et al.*, 1985), or derivitisation followed by reductive cleavage (DFRC) (Lu and Ralph, 1997). Both thioacidolysis and DFRC can be used to measure the releasable β-O-4 units of lignin. The monomers released are derivitised, followed by separation and quantification by gas chromatography.

Characterisation of the structure and composition of isolated lignin in solution has also been determined using solution-state NMR spectroscopy, in particular the use of two-dimensional (2D) ^{1}H-^{13}C NMR spectroscopy provides detailed information about

interunit linkages between lignin monomers (Ralph *et al.*, 1999). Pyrolysis of lignin yields characteristic products of the different lignin units (H-, G- or S-units) which can be separated, identified and quantified using gas chromatography and mass spectrometry (Faix *et al.*, 1990; Serban, 1998).

1.7.4 Acetyl groups

Acetyl groups can be quantified by diffuse reflectance infrared Fourier transform (DRIFT) spectroscopy or solution-state NMR spectroscopy after acid hydrolysis or alkali treatment (Iiyama *et al.*, 1994; Zanuttini *et al.*, 1998). They can also be released as acetic acid using oxalic acid on milled wood and then quantified by gas chromatography (Solar *et al.*, 1987). Two-dimensional NMR spectroscopy can be used to determine which glycosyl residues in the polysaccharides the *O*-acetyl groups are attached to (Teleman *et al.*, 2000; Capek *et al.*, 2002; Teleman *et al.*, 2003).

1.7.5 Low molecular weight compounds

Extractives are low molecular weight compounds that can be extracted by a number of solvents, including dichloromethane, ethanol, water (steam or hot water), ether or a mixture of ethanol and benzene (Easty and Thompson, 1991; TAPPI standard, 1997). The ash content of wood (the inorganic component) is quantified by combusting the wood at temperatures that range from 600 to 850°C and weighing the residue.

1.8 Aims

The aims of this thesis were to:

1. Examine the cell-wall composition (lignin, cellulose and *O*-acetyl content, and monosaccharide composition of the polysaccharides) of different radiata pine corewood types (normal wood, opposite wood, flexure wood and compression wood).
2. To relate these compositions to moisture-related longitudinal dimensional changes (longitudinal swelling and shrinkage)
3. To use gel-state two-dimensional NMR correlation spectroscopy of ball-milled wood swollen with DMSO/pyridine to compare differences in the cell-wall polymer structures (lignin and non-cellulosic polysaccharides) among the different corewood types.
4. To identify chemical indicators of the presence of compression wood by comparing the cell-wall composition of compression wood with opposite wood using pyrolysis followed by gas-chromatography and mass spectrometry.
5. To explore the possibility of using purified *endo*-(1→4)-β-D-galactanases to specifically hydrolyse the (1→4)-β-D-galactans of compression wood to detect and quantify the oligosaccharides released using matrix-assisted laser-desorption ionisation time-of-flight mass spectrometry. This could lead to future development of the method to measure compression wood severity and therefore wood quality.

1.9 References

Åkerholm, M. and L. Salmén (2001). "Interactions between wood polymers studied by dynamic FT-IR spectroscopy." Polymer **42**: 963-969.

Åkerholm, M. and L. Salmén (2003). "The oriented structure of lignin and its viscoelastic properties studied by static and dynamic FT-IR spectroscopy." Holzforschung **57**: 459-465.

Altaner, C. M., E. N. Tokareva, M. C. Jarvis and P. J. Harris (2010). "Distribution of (1→4)-β-galactans, arabinogalactan proteins, xylans and (1→3)-β-glucans in tracheid cell walls of softwoods." Tree Physiology **30**: 782-793.

Altaner, C. M., E. N. Tokareva, J. Wong, A. I. Hapca, J. P. McLean and M. C. Jarvis (2009). "Measuring compression wood severity in spruce " Wood Science and Technology **43**: 279-290.

Bao, W., D. M. O'Malley and R. R. Sederoff (1992). "Wood contains a cell-wall structural protein." Proceedings of the National Academy of Sciences USA **89**: 6604-6608.

Björkman, A. (1956). "Studies on finely divided wood. Part 1. Extraction of lignin with neutral solvents." Svensk Papperstidning **59**: 477-485.

Bland, D. E. (1958). "The chemistry of reaction wood. Part I. The lignins of *Eucalyptus goniocalyx* and *Pinus radiata*." Holzforschung **12**: 36-43.

Blumenkrantz, N. and G. Asboe-Hansen (1973). "New method for quantitative determination of uronic acids." Analytical Biochemistry **54**: 484-489.

Boerjan, W., J. Ralph and M. Baucher (2003). "Lignin biosynthesis." Annual Review of Plant Biology **54**: 519-546.

Bouveng, H. O., P. J. Garegg and B. Lindberg (1960). "Position of the *O*-acetyl groups in birch xylan." Acta Chemica Scandinavica **14**: 742-748.

Bouveng, H. O. and H. Meier (1959a). "Studies on a galactan from Norwegian spruce compression wood (*Picea abies* Karst.)." Acta Chemica Scandinavica **13**: 1884-1889.

Bouveng, H. O. and H. Meier (1959b). "Studies on a galactan from Norwegian spruce compression wood (*Picea abies* Karst.)." Acta Chemica Scandinavica **13**: 1884-1889.

Brendel, O., P. P. M. Iannetta and D. Stewart (2000). "A rapid and simple method to isolate pure alpha-cellulose." Phytochemical Analysis **11**: 7-10.

Brennan, M. and P. J. Harris (2011). "Distribution of fucosylated xyloglucans among the walls of different cell types in monocotyledons determined by immunofluorescence microscopy." Molecular Plant **4**: 144-156.

Browning, B. L. (1967). Hemicellulose isolation and separation. Methods of wood chemistry. New York, Interscience Publishers. **2**.

Burdon, R. D., R. P. Kibblewhite, J. C. F. Walker, R. A. Megraw, R. Evans and D. J. Cown (2004). "Juvenile versus mature wood: a new concept, orthogonal to corewood versus outerwood with special reference to *Pinus radiata* and *P. taeda*." Forest Science **50**: 399-415.

Butterfield, B. G. and B. A. Meylan (1980). Three-dimensional structure of wood: an ultrastructural approach. London, Chapman and Hall.

Capek, P., J. Alföldi and D. Lisková (2002). "An acetylated galactoglucomannan from *Picea abies* L. Karst." Carbohydrate Research **337**: 1033-1037.

Cavagna, F., H. Deger and J. Puls (1984). "2D-N.M.R. analysis of the structure of an aldotriouronic acid obtained from birch wood." Carbohydrate Research **129**: 1-8.

Clarke, A. E., R. L. Anderson and B. A. Stone (1979). "Form and function of arabinogalactans and arabinogalactan-proteins." Phytochemistry **18**: 521-540.

Çöpür, Y., D. J. Kiemle, A. J. Stipanovic, J. Koskinen and H. Makkonen (2003). "^{1}H-NMR spectroscopic determination of carbohydrates and yield in pine and maple pulps." Paperi ja Puu - Paper and Timber **85**: 158-162.

Côté, W. A., A. C. Day, B. W. Simson and T. E. Timell (1966). "Studies on larch arabinogalactan I. The distribution of arabinogalactan in larch wood." Holzforschung **20**: 178-192.

Cown, D. J. (1992). "Corewood (juvenile wood) in *Pinus radiata* - should we be concerned?" New Zealand Journal of Forestry Science **22**: 87-95.

Dais, P. and A. S. Perlin (1982). "High-field, ^{13}C-N.M.R. spectroscopy of β-D-glucans, amylopectin, and glycogen." Carbohydrate Research **100**: 103-116.

Donaldson, L., K. Radotić, A. Kalauzi, D. Djikanović and M. Jeremić (2010). "Quantification of compression wood severity in tracheids of *Pinus radiata* D. Don using confocal fluorescence imaging and spectral deconvolution." Journal of Structural Biology **169**: 106-115.

Donaldson, L. A. (2001). "Lignification and lignin topochemistry - an ultrastructural view." Phytochemistry **57**: 859-873.

Donaldson, L. A., J. Grace and G. M. Downes (2004). "Within-tree variation in anatomical properties of compression wood in radiata pine." IAWA Journal **25**: 253-271.

Driouich, A. and T. I. Baskin (2008). "Intercourse between cell wall and cytoplasm exemplified by arabinogalactan proteins and cortical microtubules." American Journal of Botany **95**: 1491-1497.

Eastin, I. L., S. R. Shook and S. J. Fleishman (2001). "Material substitution in the US residential construction industry, 1994 versus 1998." Forest Products Journal **51**: 30-37.

Easty, D. B. and N. A. Thompson (1991). Wood analysis. Wood structure and composition. M. Lewin and I. S. Goldstein. New York, Marcel Dekker**:** 49-138.

Ellis, M., J. Egelund, C. J. Schultz and A. Bacic (2010). "Arabinogalactan-proteins: key regulators at the cell surface?" Plant Physiology **153**: 403-419.

Excoffier, G., R. Nardin and M. R. Vignon (1986). "Détermination de la structure primaire d'un acide tri-d-xylo-4-*O*-méthyl-D-glucuronique par R.M.N. à deux dimensions." Carbohydrate Research **149**: 319-328.

Fahlén, J. and L. Salmén (2005). "Pore and matrix distribution in the fiber wall revealed by atomic force microscopy and image analysis." Biomacromolecules **6**: 433-438.

Faix, O., D. Meier and I. Fortmann (1990). "Thermal degradation products of wood. Gas chromatographic separation and mass spectrometric characterization of monomeric lignin derived products." Holz als Roh- und Werkstoff **48**: 281-285.

Fengel, D. and G. Wegener (1989). Wood chemistry, ultrastructure, reactions. Berlin, Walter de Gruyter.

Fu, Y.-L. and T. E. Timell (1972). "Polysaccharides in the secondary phloem of scots pine *Pinus sylvestris* L). III. The constitution of a galactoglucomannan." Cellulose Chemistry and Technology **4**: 517-519.

Goellner, E. M., J. Utermoehlen, R. Kramer and B. Classen (2011). "Structure of arabinogalactan from *Larix laricina* and its reactivity with antibodies directed against type-II-arabinogalactans." Carbohydrate Polymers **86**: 1739-1744.

Goldstein, I. S. (1991). Overview of the chemical composition of wood. Wood structure and composition. M. Lewin and I. S. Goldstein. New York, Marcel Dekker.

Günl, M., F. Kraemer and M. Pauly (2011). Oligosaccharide mass profiling (OLIMP) of cell wall polysaccharides by MALDI-TOF/MS. The plant cell wall. Methods in molecular biology. Z. A. Popper. New York, Springer.

Hannuksela, T. and C. Hervé du Penhoat (2004). "NMR structural determination of dissolved *O*-acetylated galactoglucomannan isolated from spruce thermomechanical pulp." Carbohydrate Research **339**: 301-312.

Harris, J. M. and B. A. Meylan (1965). "The influence of microfibril angle on longitudinal and tangential shrinkage in *Pinus radiata*." Holzforschung **19**: 144-153.

Harris, P. J. (2005). Diversity in plant cell walls. Plant diversity and evolution: genotypic and phenotypic variation in higher plants. R. J. Henry. Wallingford, Oxon, UK, CAB International Publishing**:** 201-227.

Harris, P. J., A. B. Blakeney, R. J. Henry and B. A. Stone (1988). "Gas chromatographic determination of the monosaccharide composition of plant cell wall preparations." Journal of the Association of Official Analytical Chemists **71**: 272-275.

Harris, P. J., R. J. Henry, A. B. Blakeney and B. A. Stone (1984). "An improved procedure for the methylation analysis of oligosaccharides and polysaccharides." Carbohydrate Research **127**: 59-73.

Harris, P. J. and B. A. Stone (2008). Chemistry and molecular organization of plant cell walls. Biomass recalcitrance: deconstructing the plant cell wall for bioenergy. M. E. Himmel. Oxford, Blackwell**:** 61-93.

Hatfield, R. D. and R. S. Fukushima (2005). "Can lignin be accurately measured?" Crop Science **45**: 832-839.

Hedenström, M., S. Wiklund-Lindström, T. Öman, F. Lu, L. Gerber, P. F. Schatz, B. Sundberg and J. Ralph (2009). "Identification of lignin and polysaccharide modifications in *Populus* wood by chemometric analysis of 2D NMR spectra from dissolved cell walls." Molecular Plant **2**: 933-942.

Hillis, W. E., Ed. (1987). Heartwood and tree exudates. Springer series in wood science. Berlin, Springer-Verlag.

Hoffman, G. C. and T. E. Timell (1970). "Isolation of a β-1,3-glucan (laricinan) from compression wood of *Larix laricina*." Wood Science and Technology **4**: 159-162.

Hsieh, Y. S.-Y., M. Paxton, C. P. Ade and P. J. Harris (2009). "Structural diversity, functions and biosynthesis of xyloglucans in angiosperm cell walls." New Zealand Journal of Forestry Science **39**: 187-196.

Iiyama, K., T. B. T. Lam, N. Kasuya and B. A. Stone (1994). "Rapid and simple determination of *O*-acetyl groups bound to plant cell walls by acid hydrolysis and ^{1}H-NMR measurement." Phytochemistry **35**: 959-961.

Johansson, M. (2003). "Prediction of bow and crook in timber studs based on variation in longitudinal shrinkage." Wood and Fiber Science **35**: 445-455.

Johansson, M. and R. Kliger (2002). "Influence of material characteristics on warp in Norway spruce studs." Wood and Fiber Science **34**: 325-336.

Kakegawa, K., Y. Edashige and T. Ishii (1998). "Xyloglucan from xylem-differentiating zones of *Cryptomeria japonica*." Phytochemistry **47**: 767-771.

Katz, G. (1965). "The location and significance of the *O*-acetyl groups in a glucomannan from Paraña pine." TAPPI Journal **48**: 34-41.

Kenne, L., K.-G. Rosell and S. Svensson (1975). "Studies on the distribution of the *O*-acetyl groups in pine glucomannan." **44**: 69-76.

Kiemle, D. J., A. J. Stipanovic and K. E. Mayo (2004). Proton NMR methods in the compositional characterization of polysaccharides. 1st International symposium on xylans, mannans and other hemicelluloses, Orlando, Florida, Amer Chemical Soc.

Kim, H. and J. Ralph (2010). "Solution-state 2D NMR of ball-milled plant cell wall gels in DMSO-d_6/pyridine-d_5." Organic and Biomolecular Chemistry **8**: 576-591.

Kim, H., J. Ralph and T. Akiyama (2008). "Solution-state 2D NMR of ball-milled plant cell wall gels in DMSO-d_6." Bioenergy Research **1**: 65-66.

Kim, J. S., T. Awano, A. Yoshinaga and K. Takabe (2010). "Immunolocalization of β-1-4-galactan and its relationship with lignin distribution in developing compression wood of *Cryptomeria japonica*." Planta **232**: 109-119.

Knox, J. P. (2006). "Arabinogalactan-proteins (AGPs) and plant cell development." Foods and Food Ingredients Journal **211**: 1-6.

Lamport, D. T. A., M. J. Kieliszewski, Y. Chen and M. C. Cannon (2011). "Role of the extensin superfamily in primary cell wall architecture." Plant Physiology **156**: 11-19.

Lapierre, C., B. Monties, C. Rolando and L. d. Chirale (1985). "Thioacidolysis of lignin: comparison with acidolysis." Journal of Wood Chemistry and Technology **5**: 277-292.

Larson, P. R. (1994). The vascular cambium: development and structure. Berlin, Springer-Verlag.

Lee, Y. C. (1996). "Carbohydrate analyses with high-performance anion-exchange chromatography." Journal of Chromatography **720**: 137-149.

Lerouxel, O., T. S. Choo, M. Seveno, B. Usadel, L. Faye, P. Lerouge and M. Pauly (2002). "Rapid structural phenotyping of plant cell wall mutants by enzymatic oligosaccharide fingerprinting." Plant Physiology **130**: 1754-1763.

Lewis, N. G. and E. Yamamoto (1990). "Lignin: occurrence, biogenesis and biodegradation." Annual Review of Plant Physiology and Plant Molecular Biology **41**: 455-496.

Lu, F. and J. Ralph (1997). "Derivatization followed by reductive cleavage (DFRC method), a new method for lignin analysis: protocol for analysis of DFRC monomers." Journal of Agricultural and Food Chemistry **45**: 2590-2592.

Lu, F. and J. Ralph (2003). "Non-degradative dissolution and acetylation of ball-milled plant cell walls: high-resolution solution-state NMR." The Plant Journal **35**: 535-544.

Lundqvist, J., A. Teleman, L. Junel, G. Zacchi, O. Dahlman, F. Tjerneld and H. Stålbrand (2002). "Isolation and characterization of galactoglucomannan from spruce (*Picea abies*)." Carbohydrate Research **48**: 29-39.

Meier, H. (1961). "Isolation and characterisation of an acetylated glucomannan from pine (*Pinus silvestris* L.)." Acta Chemica Scandinavica **15**: 1381-1385.

Meier, H. (1962). "Studies on a galactan from tension wood of beech (*Fagus silvatica* L.)." Acta Chemica Scandinavica **16**: 2275-2283.

Meylan, B. A. (1968). "Cause of high longitudinal shrinkage in wood." Forest Products Journal **18**: 75-78.

Ministry of Agriculture and Forestry (2007). "New Zealand forest industry facts and figures 2006/2007." from http://www.maf.govt.nz/statistics/.

Moore, J. R., J. D. Tombleson, J. A. Turner and M. van der Colff (2008). "Wind effects on juvenile trees: a review with special reference to toppling of radiata pine growing in New Zealand." Forestry **81**: 377-387.

Moulthrop, J. S., R. P. Swatloski, G. Moyna and R. D. Rogers (2005). "High-resolution ^{13}C NMR studies of cellulose and cellulose oligomers in ionic liquid solutions." Chemical Communications **12**: 1557-1559.

Nanayakkara, B., M. Manley-Harris, I. D. Suckling and L. A. Donaldson (2009). "Quantitative chemical indicators to assess the gradation of compression wood." Holzforschung **63**: 431-439.

Nicholls, J. W. P. (1982). "Wind action, leaning trees and compression wood in *Pinus radiata* D. Don." Australian Forest Research **12**: 75-91.

Norman, A. G. and S. H. Jenkins (1934). "The determination of lignin. I. Errors introduced by the presence of certain carbohydrates." Biochemical Journal **28**: 2147-2160.

O'Neill, M. A., T. Ishii, P. Albersheim and A. G. Darvill (2004). "Rhamnogalacturonan II: structure and function of a borate cross-linked cell wall pectic polysaccharide." Annual Review of Plant Biology **55**: 109-139.

Ponder, G. R. and G. N. Richards (1997). "Arabinogalactan from Western larch, Part III: alkaline degradation revisited, with novel conclusions on molecular structure." Carbohydrate Polymers **34**: 251-261.

Ralph, J., G. Brunow and W. Boerjan (2007). Lignins. eLS, John Wiley & Sons, Ltd.

Ralph, J., G. Brunow, P. J. Harris, R. A. Dixon and W. Boerjan (2009). Lignification: are lignins biosynthesized via simple combinatorial chemistry or via proteinaceous control and template reaction? Recent advances in polyphenol research. F. Daayf and V. Lattanzio, Wiley-Blackwell**:** 36-66.

Ralph, J., K. Lundquist, G. Brunow, F. Lu, H. Kim, P. F. Schatz, J. M. Marita, R. D. Hatfield, S. A. Ralph, J. H. Christensen and W. Boerjan (2004). "Lignins: natural polymers from oxidative coupling of 4-hydroxyphenyl-propanoids." Phytochemistry Reviews **3**: 29-60.

Ralph, J., J. Marita, S. A. Ralph, R. D. Hatfield, F. Lu, R. M. Ede, J. Peng, S. Quideau, R. F. Helm, J. H. Grabber, H. Kim, G. Jimenez-Monteon, Y. Zhang, H. J. G. Jung, L. L. Landucci, J. J. MacKay, R. R. Sederoff, C. Chapple and A.-M. Boudet (1999). Solution-state NMR of lignins. Advances in lignocellulosics characterization. D. S. Argyropoulos. Atlanta, GA., TAPPI Press**:** 55-108.

Rencoret, J., A. Gutiérrez, L. Nieto, J. Jiménez-Barbero, C. B. Faulds, H. Kim, J. Ralph, Á. T. Martínez and J. C. del Río (2011). "Lignin composition and structure in young versus adult *Eucalyptus globulus* plants." Plant Physiology **155**: 667-682.

Saeman, J. F., W. E. Moore, R. L. Mitchell and M. A. Millett (1954). "Techniques for the determination of pulp constituents by quantitative paper chromatography." TAPPI Journal **37**: 336-343.

Salmén, L. and A.-M. Olsson (1998). "Interaction between hemicelluloses, lignin and cellulose: structure-property relationships." Journal of Pulp and Paper Science **24**: 99-103.

Scurfield, G. (1973). "Reaction wood: its structure and function." Science **179**: 647-655.

Seifert, G. J. and K. Roberts (2007). "The biology of arabinogalactan proteins." Annual Review of Plant Biology **58**: 137-161.

Serban, C. M. (1998). Analytical pyrolysis of natural organic polymers. Amsterdam, Elsevier. **20:** 496.

Shimizu, K., M. Hashi and K. Sakurai (1978). "Isolation from a softwood xylan of oligosaccharides containing two 4-*O*-methylglucuronic acid residues." Carbohydrate Research **62**: 117-126.

Shin, S.-J. (2008). "Conversion factors for carbohydrate analysis by hydrolysis and ^{1}H-NMR spectroscopy." Cellulose **15**: 255-260.

Smith, B. G. and P. J. Harris (1995). "Polysaccharide composition of unlignified cell walls of pineapple [*Ananas comosus* (L.) Merr.] fruit." Plant Physiology **107**: 1399-1409.

Solar, R., F. Kačik and I. Melcer (1987). "Simple semimicro method for the determination of O-acetyl groups in wood and related materials." Nordic Pulp and Paper Research **2**: 139-141.

Stevanic, J. S. and L. Salmén (2009). "Orientation of the wood polymers in the cell wall of spruce wood fibres." Holzforschung **63**: 497-503.

Sutton, W. R. J. (1999). "The need for planted forests and the example of radiata pine." New Forests **17**: 95-110.

TAPPI standard (1997). "T 204 cm-97. Solvent extractives of wood and pulp."

TAPPI standard (1998). "T-222 om-98. Acid-insoluble lignin in wood and pulp."

Teleman, A., J. Lundqvist, F. Tjerneld, H. Stålbrand and O. Dahlman (2000). "Characterization of acetylated 4-*O*-methylglucuronoxylan isolated from aspen employing ^{1}H and ^{13}C NMR spectroscopy." Carbohydrate Research **329**: 807-815.

Teleman, A., M. Nordström, M. Tenkanan, A. Jacobs and O. Dahlman (2003). "Isolation and characterization of *O*-acetylated glucomannans from aspen and birch wood." Carbohydrate Research **338**: 525-534.

Telewski, F. W. (1989). "Structure and function of flexure wood in *Abies fraseri*." Tree Physiology **5**: 113-121.

Telewski, F. W. and M. J. Jaffe (1981). "Thigmorphogenesis: changes in the morphology and chemical composition induced by mechanical perturbation in 6-month-old *Pinus taeda* seedlings." Canadian Journal of Forest Research **11**: 380-387.

Telewski, F. W. and M. J. Jaffe (1986a). "Thigmorphogenesis: anatomical, morphological and mechanical analysis of genetically different sibs of *Pinus taeda* in response to mechanical perturbation." Physiologia Plantarum **66**: 219-226.

Telewski, F. W. and M. J. Jaffe (1986b). "Thigmorphogenesis: field and laboratory studies of *Abies fraseri* in response to wind or mechanical perturbation." Physiologia Plantarum **66**: 211-218.

Terashima, N., T. Akiyama, S. Ralph, D. Evtuguin, C. P. Neto, J. Parkås, M. Paulsson, U. Westermark and J. Ralph (2009). "2D-NMR (HSQC) difference spectra between specifically ^{13}C-enriched and unenriched protolignin of *Ginkgo biloba* obtained in the solution state of whole cell wall material." Holzforschung **63**: 379-384.

Terashima, N. and K. Fukushima (1988). "Heterogeneity in formation of lignin - XI: An autoradiographic study of the heterogeneous formation and structure of pine lignin." Wood Science and Technology **22**: 259-270.

Thomas, R. J. (1991). Wood: formation and morphology. Wood structure and composition. M. Lewin and I. S. Goldstein. New York, Marcel Dekker**:** 7-47.

Timell, T. E. (1964). Wood hemicelluloses: Part I. Advances in carbohydrate chemistry. M. L. Wolfrom and S. R. Tipson. New York, Academic Press. **19:** 247-302.

Timell, T. E. (1965). Wood hemicelluloses: Part II. Advances in carbohydrate chemistry. M. L. Wolfrom and S. R. Tipson. New York, Academic Press. **20:** 410-483.

Timell, T. E. (1982). "Recent progress in the chemistry and topochemistry of compression wood." Wood Science and Technology **16**: 83-122.

Timell, T. E. (1986). Compression wood in gymnosperms. Berlin, Springer-Verlag.

Updegraff, D. M. (1969). "Semimicro determination of cellulose in biological materials." Analytical Biochemistry **32**: 420-424.

Walker, J. (2006). Primary wood processing. Principles and practice. Dordrecht, Springer.

Wardrop, A. B. and D. E. Bland (1959). Biochemistry of wood. London, Pergamon Press.

Weitzhandler, M., V. Barreto, C. Pohl, P. Jandik, J. Cheng and N. Avdalovic (2004). "CarboPac™ PA20: a new monosaccharide separator column with electrochemical detection with disposable gold electrodes." Journal of Biochemical and Biophysical Methods **60**: 309-317.

Whistler, R. L. and C.-C. Chen (1991). Hemicellulose. Wood structure and composition. M. Lewin and I. S. Goldstein. New York, Marcel Dekker**:** 287-319.

Willför, S. and B. Holmbom (2004). "Isolation and characterisation of water soluble polysaccharides from Norway spruce and Scots pine." Wood Science and Technology **38**: 173-179.

Wise, L. F., M. Murphy and A. A. D'Addieco (1946). "Chlorite holocellulose, its fractionation and bearing on summative wood analysis and on studies on the hemicelluloses." Technical Association of the Pulp and Paper Industry **122**: 35-43.

Yeh, T.-F., B. Goldfarb, H.-m. Chang, I. Peszlen, J. L. Braun and J. F. Kadla (2005). "Comparison of morphological and chemical properties between juvenile wood and compression wood of loblolly pine." Holzforschung **59**: 669-674.

Yelle, D. J., J. Ralph and C. R. Frihart (2008). "Characterization of nonderivatized plant cell walls using high-resolution solution-state NMR spectroscopy." Magnetic Resonance in Chemistry **46**: 508-517.

Zanuttini, M., M. Citroni and M. J. Martínez (1998). "Application of diffuse reflectance infrared fourier transform spectroscopy to the quantitative determination of acetyl groups in wood." Holzforschung **52**: 263-267.

Chapter 2 - Cell-wall compositions of juvenile *Pinus radiata* corewood types and their relationships to moisture-related longitudinal dimensional change

2.1 Introduction

The cell-wall composition of wood can be analysed by measuring the Klason lignin content, the monosaccharide composition and acetyl content. Monosaccharide compositions of wood samples give an indication of the compositions of the polysaccharides in the wood. Therefore, the monosaccharide composition can be used to quantitatively determine overall differences in the cell-wall polysaccharide compositions of wood. Such analyses have been used to investigate differences in the cell-wall compositions of economically important softwood species (Willför *et al.*, 2005), and variation in the cell-wall compositions among different spatial locations within softwood stems (Uprichard and Lloyd, 1980; Yeh *et al.*, 2006; Kibblewhite *et al.*, 2010). The monosaccharide compositions of compression woods of varying degrees of severity in radiata pine have also been determined (Nanayakkara *et al.*, 2009). Such comparative studies may also include quantification of the lignin content, *O*-acetyl content and cellulose content of the wood.

Two-stage sulphuric acid hydrolysis (Saeman *et al.*, 1954) hydrolyses the glycosidic linkages between the monosaccharides in all of the cell-wall polysaccharides. The released monosaccharides can be separated and quantified, for example, by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (Floyd, 2005; Willför *et al.*, 2005; Nanayakkara *et al.*, 2009) of the

hydrolysate under alkaline conditions without derivitisation (Lee, 1990; 1996). Capillary gas-chromatography with flame ionisation detection can also be used to determine the monosaccharide composition, but the monosaccharides first have to be converted to volatile derivatives. They are commonly reduced to their alditols and then acetylated to form alditol acetates (Albersheim *et al.*, 1967; Crowell and Burnett, 1967; TAPPI Standard T 249 cm-85, 1985; Harris *et al.*, 1988).

For HPAEC-PAD, until recently, separation of neutral monosaccharides of plant cell walls (arabinose, fucose, rhamnose, galactose, glucose, xylose and mannose) and the uronic acids (4-*O*-methylglucuronic acid, galacturonic acid and glucuronic acid) required the use of two different elution profiles. However, with the development of the CarboPac™ PA20 (Weitzhandler *et al.*, 2004), separation of the neutral monosaccharides and the uronic acids present in sulphuric acid hydrolysates of wood can now be achieved in a single elution program (Currie and Perry, 2006; Lina *et al.*, 2006).

The aim of the work reported in this chapter was to investigate differences in the cell-wall compositions among different corewood types of radiata pine, and relate these to differences in moisture-related longitudinal dimensional changes (longitudinal swelling and shrinkage) of the corewood types. The lignin, *O*-acetyl groups and cellulose content, and the monosaccharide compositions were measured and any compositional differences were related to differences in longitudinal dimensional change. Two different sets of corewood samples from the following trees were examined. “Glasshouse” trees were grown in a glasshouse, straight (producing normal corewood), tilted (producing compression corewood and opposite corewood) and rocked backwards and forwards (producing flexure corewood). “Amberley” trees were field-grown at Amberley (Canterbury) tilted to produce compression corewood and opposite corewood. All of the

wood examined in this thesis is corewood and from this point on will be referred to as "wood" rather than "corewood" for brevity.

2.2 Materials and methods

2.2.1 Glasshouse trees

Seedling trees of two *Pinus radiata* (D. Don) clones, A and K (96047/98015 and 96044 respectively, Arborgen Australasia, TeTeko, Whakatane, New Zealand), were grown in an unheated glasshouse at the University of Canterbury, Christchurch, New Zealand, for 8 months as described by Apiolaza et al. (2011a). Trees were grown upright to produce "normal" wood, tilted by staking at 45° from the vertical to produce compression wood and opposite wood, or rocked in a purpose-built frame for 15 min in every hour at a rate of 24 cycles/min, reaching 22° from the vertical, simulating a wind speed of 10 km h^{-1} to produce flexure wood (Apiolaza, 2009). In total, 47 samples were analysed: 23 from clone A (6 × normal wood, 5 × opposite wood, 5 × compression wood and 7 × flexure wood) and 24 from clone K (6 × normal wood, 6 × opposite wood, 6 × compression wood and 6 × flexure wood). Compression wood and opposite wood came from geometrically opposite parts of the same tree. All of the wood examined was from the lowest portion of clearwood (free of knots or wounds) in the stem.

2.2.2 Amberley trees

A trial of 49 families of radiata pine was grown at Amberley, Canterbury, New Zealand as described in Apiolaza et al. (2011b). All trees were grown tilted at approximately 25°

from the vertical to produce trees containing, in cross-sections, regions of pure compression wood and pure opposite wood which provided wood with a wide range of mechanical properties for analysis. From this trial, 100 samples (50 × compression wood and 50 × opposite wood) were chosen for chemical analysis. Samples were chosen that spanned a wide range of longitudinal dimensional change values, and had only pure compression wood or opposite wood (determined by visual inspection of wet samples). All of the wood examined was from the lowest portion of clearwood (free of knots or wounds) in the stem.

2.2.3 Measurement of moisture-related longitudinal dimensional change

The percent change in length from wet (green or saturated) wood to oven-dry wood or vice versa was measured, and both measurements are here collectively termed longitudinal dimensional change. For glasshouse trees, longitudinal swelling was measured. Stem segments (~15 cm long) were split lengthwise, and the lowest ~4.5 cm of clearwood was taken to measure the longitudinal dimensional change. Stainless steel balls (1 mm diameter, 15 mg) were glued to the centre of each of the cut ends. The samples were dried overnight at 105°C, and cooled in a desiccator over indicating silica gel. The length was measured using a micrometer accurate to ± 0.001 mm, ensuring that the steel balls were placed in the centre of the micrometer ends. The samples were then immersed in water under vacuum for three days at which time they were considered fully saturated. The lengths of the fully saturated samples were then measured as above. The longitudinal dimensional change was measured twice, or three times if the measurements differed by more than 0.1%. The longitudinal shrinkage of the glasshouse

wood samples could be calculated from the change in length from the saturated state to the oven-dry state, and is highly correlated ($R^2 = 0.93$) with longitudinal swelling (Figure 2-1). The mean longitudinal swelling was used as the measurement of longitudinal dimensional change for the glasshouse samples as replicate measurements were made for this longitudinal dimensional change, therefore the precision of the measurements was confirmed.

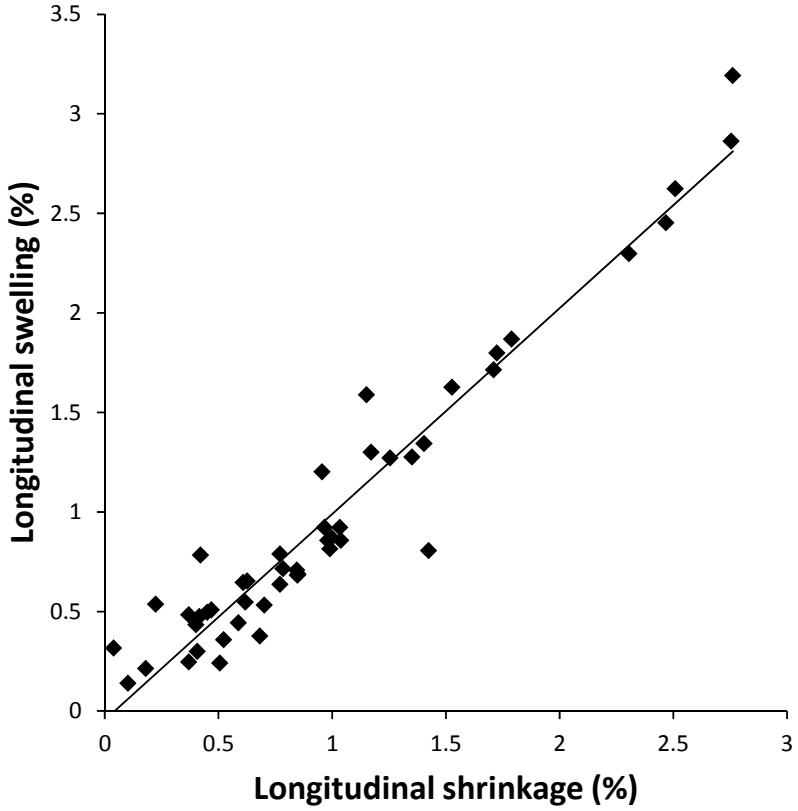

Figure 2-1 Relationship between longitudinal swelling and longitudinal shrinkage measured for the glasshouse wood samples, $R^2 = 0.93$.

The longitudinal shrinkage of the Amberley wood samples was measured at the University of Canterbury as described in (Apiolaza *et al.*, 2011b). Pins were inserted into each end of the wood samples as a reference point for length measurement with a

micrometer. The change in length was measured from the green to oven-dry state (dried at 35°C until constant weight), and could therefore only be measured once.

2.2.4 Chemical analysis

2.2.4.1 Sulphuric acid hydrolysis and Klason lignin content

After the longitudinal dimensional change was measured, the wood samples were milled to pass a 40-mesh screen (422 µm pore size) in a Wiley® mini-mill (Thomas Scientific, Swedesboro, NJ) before extracting with dichloromethane in a Soxhlet extractor for 8 h at 60°C. Extracted wood was dried overnight at 105°C and two replicates (100 mg each) were hydrolysed using the two-stage sulphuric acid hydrolysis method of TAPPI standard T-249 cm-85 (1985), with the exception that reagent volumes were scaled down according to the lower mass of wood hydrolysed in this study. The wood was weighed into a hydrolysis tube and 72% (w/w) sulphuric acid (0.5 ml) added. The mixture was placed in a water bath (30°C) for 1 h, with stirring every 15 min. Water (14 ml) was used to transfer the mixture to a 25 ml volumetric flask which was covered and placed in an autoclave at 121°C (~15 psi) for 1 h. After the hydrolysate had cooled, the volume was brought to 25 ml with water. The hydrolysate was filtered through oven-dried (105°C) glass sinters (porosity 4) of accurately known weight and collected in a vacuum flask for monosaccharide and acetyl quantification. The residue from the hydrolysis was quantitatively transferred into the sinters, washed several times with hot water and dried until constant weight (105°C overnight). The sinter was cooled in a desiccator over indicating silica gel and then weighed to give an indication of the Klason lignin content. Two replicates of each wood sample were hydrolysed.

2.2.4.2 Monosaccharide composition

Neutral monosaccharides and uronic acids were separated and quantified using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Chromatography was done on a Dionex BioLC system (Dionex, Sunnyvale, CA, USA) with an ED50 electrochemical detector and GP50 gradient pump. A CarboPac™ PA20 guard column (3 x 30 mm) and a CarboPac™ PA20 analytical column (3 × 150 mm) were used. Column temperature was kept at 18°C or 25°C by a TCC-100 thermostatted column compartment. Neutral monosaccharides were separated using isocratic elution (1 mM NaOH for 20 min); uronic acids were then separated in the same run using isocratic elution (200 mM sodium acetate in 200 mM NaOH for 5 min). The column was then washed for 10 min with 200 mM NaOH before equilibration for 10 min with 1 mM NaOH. The injection volume was 20 µl, and the flow rate 0.4 ml min^{-1}.

The order of elution of monosaccharides and the *myo*-inositol external standard was confirmed by running solutions of individual monosaccharides and was as follows: *myo*-inositol, arabinose, galactose, glucose, xylose, mannose, galacturonic acid, 4-*O*-methylglucuronic acid and glucuronic acid. The retention time of 4-*O*-methylglucuronic acid was determined by chromatography of a 2 M trifluoroacetic acid hydrolysate of a 4-*O*-methylglucuronoxylan from birch wood (Sigma Aldrich, St Louis, MO, USA) (Smith and Harris, 1995); the detector response of 4-*O*-methylglucuronic acid was assumed to be the same as that of glucuronic acid. Between each six samples, a water blank was run followed by an external standard solution of monosaccharides approximating a softwood hydrolysate. This contained *myo*-inositol or 2-deoxy-D-galactose (10.0 µg ml^{-1}), L-arabinose (5.0 µg ml^{-1}), D-galactose (20.0 µg ml^{-1}), D-glucose (150.0 µg ml^{-1}), D-xylose (30.0 µg ml^{-1}), D-mannose (30.0 µg ml^{-1}), D-galacturonic

acid (5.0 μg ml^{-1}), D-glucuronic acid (5.0 μg ml^{-1}). The *Myo*-inositol or 2-deoxy-D-galactose was used as a retention time reference standard.

2.2.4.3 *O*-Acetyl content

The *O*-acetyl content of wood samples from the glasshouse trees was determined by ^{1}H-NMR spectroscopy in the manner of Iiyama et al. (1994). To the 2-stage sulphuric acid hydrolysate (1800 μl), 100 % ethanol (dried over 3A molecular sieves) (2 μl) and D_2O (99.9 atom % D; Sigma-Aldrich, St Louis, MO, USA) (198 μl) was added. Spectra were acquired on a Bruker Avance DRX-400 spectrometer. The acetyl content was quantified by taking the ratio of the methyl proton signal of released acetic acid at 1.9 ppm and the methyl proton signal of ethanol at 1.0 ppm. Suppression of the large residual water signal was done by presaturation of the solvent proton resonance for (5 s) at 50 Hz bandwidth (Bax, 1985). Quantification was verified using standard solutions of acetic acid in 10% (v/v) aqueous D_2O. The *O*-acetyl contents of the glasshouse wood samples were measured before the Amberley samples were analysed, and was found not to be correlated with longitudinal dimensional change, therefore the *O*-acetyl contents of the Amberley samples were not determined.

2.2.4.4 Cellulose content

As the glasshouse compression wood samples had a lower glucosyl residue content than the non-compression wood samples, the cellulose content of the Amberley wood samples was considered to be of interest in predicting longitudinal dimensional change. The cellulose contents of the Amberley wood samples were determined by isolating the cellulose using the method of Brendel et al. (2000) and determining the weight.

Oven-dried wood (50 mg), milled to pass a 40-mesh screen and extracted with dicholormethane as described above, was weighed into a hydrolysis tube and a 10:1 (v/v) solution of 80% acetic acid and 69% nitric acid (2.2 ml) added. The tube was placed in an autoclave at 121°C (~15 psi) for 1 h. After cooling, the isolated cellulose was washed with 99% ethanol (5 ml), deionised water (5 ml), 99% ethanol (5 ml) and 100% acetone (5ml). The sample was dried to constant weight to determine the weight of the isolated cellulose. The cellulose content of duplicate wood samples was determined. The purity of the isolated cellulose was comparable to commercial Avicel® micro-crystalline cellulose (FMC BioPolymer, Brussels, Belgium). This was determined by two-stage sulphuric acid hydrolysis of Avicel® micro-crystalline cellulose, followed by HPAEC-PAD of the hydrolysate as described above, to determine the proportions of residual monosaccharides other than glucose. Avicel® micro-crystalline cellulose and the cellulose isolated from the Amberley wood samples had similar proportions of only xylose and mannose impurities (~3% xylose and ~2% mannose). Other monosaccharides were not detected.

2.2.5 Statistical analyses

For the glasshouse wood samples, generalised linear models were built to test the effect of clone (A and K), wood type (normal wood, opposite wood, flexure wood and compression wood) and their interaction on the cell-wall composition of the wood and the longitudinal dimensional change. For the Amberley samples, replicates of families were not measured, therefore only the effect of wood type on the cell-wall composition of the wood and the longitudinal dimensional change were examined. The effect of wood type, origin of the wood (glasshouse or Amberley) and the interaction between wood

type and origin on longitudinal dimensional change was examined. The generalised linear models followed the forms:

$$y = Clone + Type + Clone.Type + \varepsilon \quad \text{[Model 1]}$$

$$y = Type + \varepsilon \quad \text{[Model 2]}$$

$$y = Type + Origin + Type.Origin + \varepsilon \quad \text{[Model 3]}$$

Where "*y*" is the content of each of the measured cell-wall components, "*Clone*" is the effect of clone, "*Type*" is the effect of wood type, "*Clone.Type*" is the interaction between clone and wood type, "*Type.Origin*" is the interaction between wood type and its origin and ε is the error. The generalised linear models were then tested using an *F*-test in analysis of variance (ANOVA) ($a = 0.05$). Statistical analysis was done using the statistical analysis software R (R Development Core Team, 2011).

2.3 Results

2.3.1 Differences in cell-wall composition and longitudinal dimensional changes among wood types

The mean lignin content, *O*-acetyl content, and monosaccharide compositions of the two clones of each wood type (normal wood, opposite wood, compression wood and flexure wood) of the glasshouse samples are shown in Table 2-1. The cell-wall compositions of the wood types were significantly different. The cell-wall compositions were not significantly different when any interaction between clone and wood type were taken into

account. The cell-wall compositions of the two clones did not differ significantly, except for the mannosyl residue content, although the *F*-value (5.7) is small compared with the *F*-value for the effect of wood type (40.7), suggesting that if there is a true effect of clone on mannosyl residue content, the effect is much smaller than the effect of wood type.

Post-hoc Tukey's HSD tests showed that the cell-wall composition of compression wood differed markedly from that of the other three wood types. There was no significant difference between the normal wood, opposite wood or flexure wood except for the lignin content, which was different between opposite wood and normal wood, with normal wood having a higher lignin content. Compression wood had a significantly higher lignin content and galactosyl residue content compared with non-compression wood samples. Compression wood also had a significantly lower proportion of *O*-acetyl groups, and smaller proportions of arabinosyl, glucosyl, xylosyl, mannosyl, and 4-*O*-methyglucuronosyl residues compared with the other wood types. Compression wood had smaller proportions of galacturonosyl residues compared with normal wood and flexure wood, but not opposite wood.

Table 2-1 Cell-wall composition[a] and longitudinal dimensional change of glasshouse-grown *P. radiata* wood types

		Lignin	***O*-Acetyl**	**Arabinose**	**Galactose**	**Glucose**	**Xylose**	**Mannose**	**4*O*MeGlcA[b]**	**GalA[c]**	**LDC**
***F*-value**	Clone	2.1	3.3	1.1	0.0	0.6	2.6	5.7	0.2	0.7	2.8
	Type	72.8	9.2	17.8	113.3	26.1	35.0	40.7	8.5	5.2	44.5
	Interaction	0.4	1.1	2.0	0.5	0.4	2.5	0.3	1.0	1.0	0.7
***p*-value**	Clone	0.200	0.100	0.300	0.895	0.452	0.115	0.022	0.699	0.397	0.100
	Type	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.004	<0.001
	Interaction	0.743	0.340	0.129	0.707	0.720	0.071	0.791	0.384	0.423	0.555
NW	A	29.1 ± 1.5	0.9 ± 0.1	2.2 ± 0.4	3.3 ± 1.3	39.8 ± 1.6	10.2 ± 0.9	10.8 ± 0.4	1.3 ± 0.3	0.8 ± 0.2	0.7 ± 0.3
	K	29.0 ± 0.9	1.1 ± 0.1	2.5 ± 0.2	3.5 ± 1.5	38.7 ± 1.8	10.6 ± 0.9	12.0 ± 0.9	1.2 ± 0.1	0.7 ± 0.1	0.8 ± 0.3
	A + K	**29.1 ± 1.0 a**	**1.0 ± 0.1 a**	**2.4 ± 0.3 a**	**3.4 ± 1.3 a**	**39.2 ± 1.7 a**	**10.4 ± 1.0 a**	**11.4 ± 1.0 a**	**1.3 ± 0.2 a**	**0.8 ± 0.2 a**	**0.7 ± 0.3 a**
OW	A	28.0 ± 1.7	1.1 ± 0.1	2.2 ± 0.3	3.0 ± 0.6	40.3 ± 1.6	10.8 ± 0.5	11.9 ± 0.8	1.3 ± 0.2	0.7 ± 0.2	0.6 ± 0.3
	K	27.3 ± 1.2	1.0 ± 0.1	2.0 ± 0.4	2.8 ± 0.8	39.9 ± 1.5	10.4 ± 0.7	12.4 ± 0.5	1.2 ± 0.1	0.7 ± 0.1	0.4 ± 0.2
	A + K	**27.6 ± 1.5 b**	**1.0 ± 0.1 a**	**2.1 ± 0.3 a**	**2.9 ± 0.7 a**	**40.0 ± 1.5 a**	**10.6 ± 0.6 a**	**12.2 ± 0.7 a**	**1.3 ± 0.1 a**	**0.7 ± 0.1 ab**	**0.5 ± 0.3 a**
CW	A	35.2 ± 0.9	0.8 ± 0.1	1.7 ± 0.4	10.3 ± 1.2	33.9 ± 1.7	7.3 ± 0.9	7.9 ± 1.1	0.9 ± 0.2	0.6 ± 0.2	2.3 ± 0.7
	K	34.3 ± 0.9	0.9 ± 0.1	1.6 ± 0.2	10.4 ± 1.5	34.3 ± 1.8	8.5 ± 0.9	8.6 ± 0.9	1.0 ± 0.1	0.5 ± 0.1	1.9 ± 0.5
	A + K	**34.8 ± 1.0 c**	**0.8 ± 0.1 b**	**1.6 ± 0.3 b**	**10.4 ± 1.3 b**	**34.1 ± 1.7 b**	**8.0 ± 1.0 b**	**8.3 ± 1.0 b**	**1.0 ± 0.2 b**	**0.5 ± 0.2 b**	**2.1 ± 0.6 b**
FW	A	29.2 ± 1.5	1.0 ± 0.1	2.6 ± 0.2	3.9 ± 1.0	39.2 ± 1.6	10.1 ± 0.5	11.2 ± 1.2	1.3 ± 0.1	0.8 ± 0.2	0.8 ± 0.3
	K	28.0 ± 1.0	1.1 ± 0.1	2.3 ± 0.2	3.2 ± 1.1	39.3 ± 2.6	10.5 ± 0.7	11.7 ± 1.2	1.3 ± 0.1	0.8 ± 0.2	0.5 ± 0.2
	A + K	**28.7 ± 1.4 ab**	**1.1 ± 0.1 a**	**2.5 ± 0.2 a**	**3.6 ± 1.1 a**	**39.3 ± 2.0 a**	**10.3 ± 0.6 a**	**11.4 ± 1.2 a**	**1.3 ± 0.1 a**	**0.8 ± 0.2 a**	**0.7 ± 0.3 a**

[a]Expressed as mean % oven-dry weight of wood ± standard deviation.

[b]4*O*MeGlcA = 4-*O*-methylglucuronic acid.

[c]GalA = galacturonic acid.

LDC = % longitudinal dimensional change

NW = normal wood, OW = opposite wood, CW = compression wood, FW = flexure wood.

F-values and *p*-values are given for ANOVA of Model 1, testing the effect of clone, wood type and their interaction on each of the cell-wall components and longitudinal dimensional change. Degrees of freedom are the following: clone = 1, type = 3, interaction = 3, residuals = 39. Significantly different wood types (Tukey's HSD) are denoted by different letters.

For the Amberley wood samples, the mean lignin content, cellulose content, and monosaccharide compositions of compression wood and opposite wood samples are shown in

Table 2-2. The proportions of lignin, cellulose and the glycosyl residue contents in the wood samples were significantly different between the two wood types (opposite wood and compression wood). Compression wood had a higher lignin and galactosyl residue content than opposite wood. Opposite wood had a higher cellulose, arabinosyl, glucosyl, xylosyl, mannosyl, 4-*O*-methyl-glucuronosyl and galacturonosyl residue content than compression wood. Trace amounts of glucuronosyl residues were detected in some of the samples from each wood type of both glasshouse and Amberley wood samples, but not in large enough proportions for these to be quantified.

Table 2-2 Cell-wall composition[a] and longitudinal dimensional change of field-grown *P. radiata* (Amberley wood samples)

Wood Type[b]	Lignin	Cellulose	Arabinose	Galactose	Glucose	Xylose	Mannose	OMeGlcA [b]	GalA[c]	LDC
***F*-value**	1152.0	372.6	180.0	1488.4	208.9	537.8	409.6	366.6	191.2	99.1
***p*-value**	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
OW	27.7 ± 1.1	**39.0 ± 2.1**	**1.7 ± 0.2**	3.5 ± 0.7	**39.3 ± 1.9**	**9.3 ± 0.9**	**10.1 ± 0.8**	**1.7 ± 0.2**	**1.1 ± 0.1**	1.4 ± 0.5
CW	**37.5 ± 1.7**	30.2 ± 2.5	1.1 ± 0.2	**12.0 ± 1.4**	33.2 ± 2.3	5.8 ± 0.6	6.8 ± 0.8	1.0 ± 0.2	0.8 ± 0.1	**2.4 ± 0.4**

[a]Expressed as mean % oven-dry weight of wood ± standard deviation.

[b]4*O*MeGlcA = 4-*O*-methylglucuronic acid.

[c]GalA = galacturonic acid.

LDC = % longitudinal dimensional change

OW = opposite wood, CW = compression wood.

F-values and *p*-values are given for ANOVA of Model 2, testing the effect of wood type on each of the cell-wall components and longitudinal dimensional change. Degrees of freedom are the following: type = 1, residuals = 98.

Components found to be significantly higher in ANOVA of the generalised linear model testing the effect of wood type are shown in bold.

The compression wood samples of the glasshouse and the Amberley trees always had a lignin content greater than 32% and a galactosyl residue content greater than 6%, which distinguished them from the non-compression wood samples as shown in Figure 2-2.

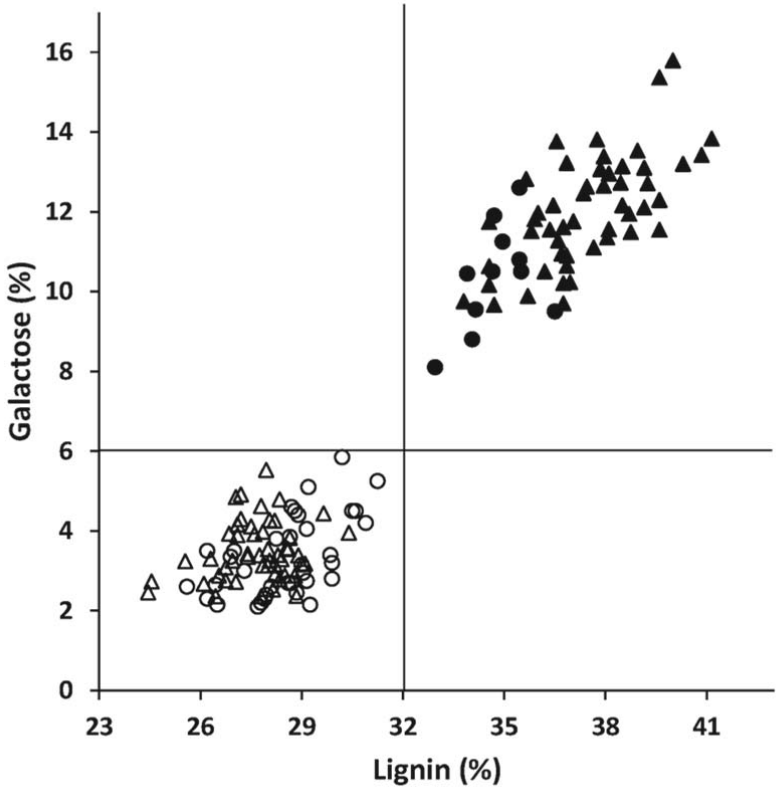

Figure 2-2 Threshold values of 32% lignin content and 6% galactosyl residue content clearly distinguish compression wood samples from non-compression wood samples. ● = Glasshouse compression wood, O = glasshouse non-compression woods, ▲ = Amberley compression wood, △ = Amberley opposite wood.

2.3.2 Differences in longitudinal dimensional change among wood types

The mean longitudinal dimensional change of the two clones of each wood type of the glasshouse samples are shown in Table 2-1. Longitudinal dimensional change was

significantly different between wood types. Longitudinal dimensional change was not significantly different between clone, nor when the interaction between clone and wood type was considered. Post-hoc Tukey's HSD tests showed that compression wood had a significantly higher longitudinal dimensional change than normal wood, opposite wood or flexure wood. Longitudinal dimensional change was not significantly different among normal wood, opposite wood and flexure wood. The mean longitudinal dimensional change of the compression wood and opposite wood samples from the Amberley trees is shown in

Table 2-2. Compression wood had a higher longitudinal dimensional change compared with opposite wood.

Boxplots of the longitudinal dimensional change of the different wood types from the glasshouse and Amberley samples are shown in Figure 2-3. Wood type ($F_{3\,141} = 108.2$, $p < 0.001$), the origin of the samples (glasshouse or Amberley) ($F_{1\,141} = 34.2$, $p < 0.001$) and the interaction between wood type and origin ($F_{1\,141} = 11.0$, $p = 0.001$) had a significant effect on longitudinal dimensional change. Post-hoc Tukey's HSD tests showed that there was no significant difference between the longitudinal dimensional change between the compression wood samples from the glasshouse and Amberley wood samples. Yet the opposite wood samples from the glasshouse and Amberley wood samples had significantly different longitudinal dimensional change. The longitudinal dimensional change of the Amberley opposite wood samples was higher, and showed a higher degree of variation, than the opposite wood samples from the glasshouse trees.

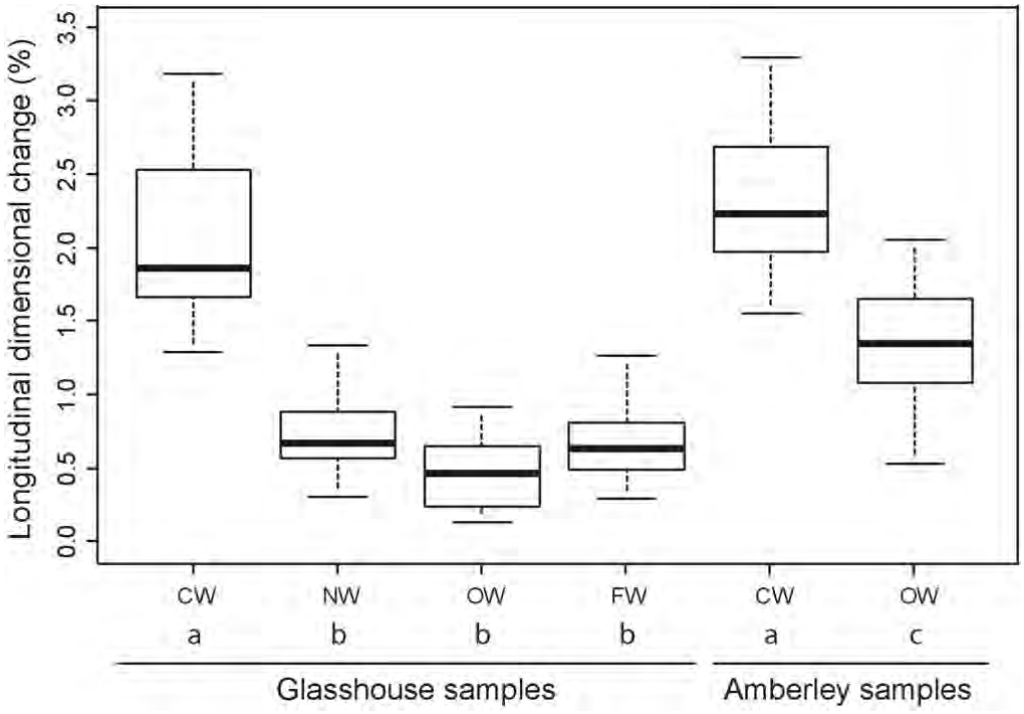

Figure 2-3 Boxplots of the longitudinal dimensional change of the different wood types in the glasshouse samples, and the longitudinal dimensional change of the Amberley samples. CW = compression wood, NW = normal wood, OW = opposite wood, FW = flexure wood. Lower case letters indicate groups with significantly different (post-hoc Tukey's HSD) longitudinal dimensional changes.

2.3.3 Relationships between cell-wall composition and longitudinal dimensional change

The lignin content, *O*-acetyl content and proportions of each of the glycosyl residues of the glasshouse samples were plotted against longitudinal dimensional change (Figure 2-4). Separate clusters of the compression wood samples and the non-compression wood samples were observed. Across all wood types, samples with higher longitudinal dimensional change had higher lignin and galactosyl residue contents, lower *O*-acetyl content and lower arabinosyl, glucosyl, xylosyl, mannosyl and 4-*O*-methylglucuronosyl residue contents. The variation in proportions of the different chemical components and longitudinal dimensional change was greater in compression wood than non-compression wood. There were positive

correlations between lignin content and longitudinal dimensional change, and between the galactosyl residue content and longitudinal dimensional change. The glucosyl residue content of compression wood samples was inversely proportional to longitudinal dimensional change, but this relationship did not hold for the opposite wood samples. Neither the xylosyl residue contents nor the mannosyl residue contents were related to the longitudinal dimensional change. The proportions of arabinosyl, 4-*O*-methyl-glucuronosyl and galacturonosyl residues and *O*-acetyl content were in such small proportions, that, due to limitations in the quantification of these components, relationships with longitudinal dimensional change could not be determined.

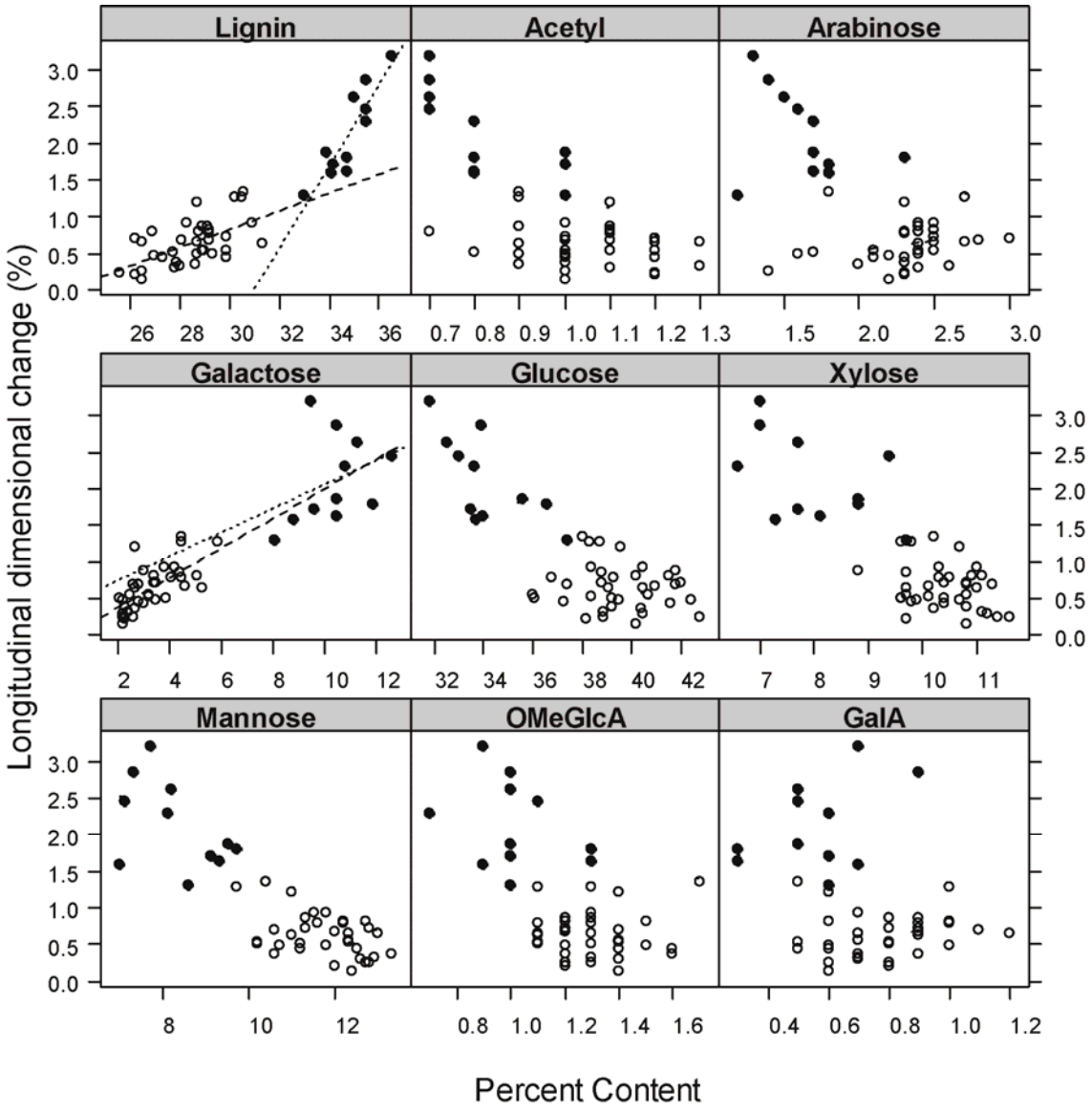

Figure 2-4 Relationships between longitudinal dimensional change and the lignin content, *O*-acetyl content and glycosyl residue contents for compression wood, ●, and non-compression wood, ○, of the glasshouse samples. A linear regression of longitudinal dimensional change and percent content for compression wood samples is shown by a dotted line, a linear regression of longitudinal dimensional change and percent content for non-compression wood samples is shown by a dashed line. OMeGlcA = 4-*O*-methylglucuronic acid, GalA = galacturonic acid.

The proportions of lignin, cellulose and the proportion of each of the glycosyl residues of the Amberley samples were plotted against longitudinal dimensional change (Figure 2-5). Relationships observed across both wood types were similar to those for the glasshouse

data, with samples with higher longitudinal dimensional change having higher lignin and galactosyl residue contents, lower cellulose content and lower arabinosyl, glucosyl, xylosyl, mannosyl and 4-*O*-methylglucuronosyl residue contents. The glucosyl and mannosyl residue contents were inversely proportional to longitudinal dimensional change for the compression wood samples, but there was no relationship between glucosyl or mannosyl residue contents and longitudinal dimensional change for the opposite wood samples. Only the galactosyl residue content was related to longitudinal dimensional change across both wood types. The total proportions of arabinosyl, 4-*O*-methylglucuronosyl and galacturonosyl residue contents were too small for their relationships with longitudinal dimensional change to be determined.

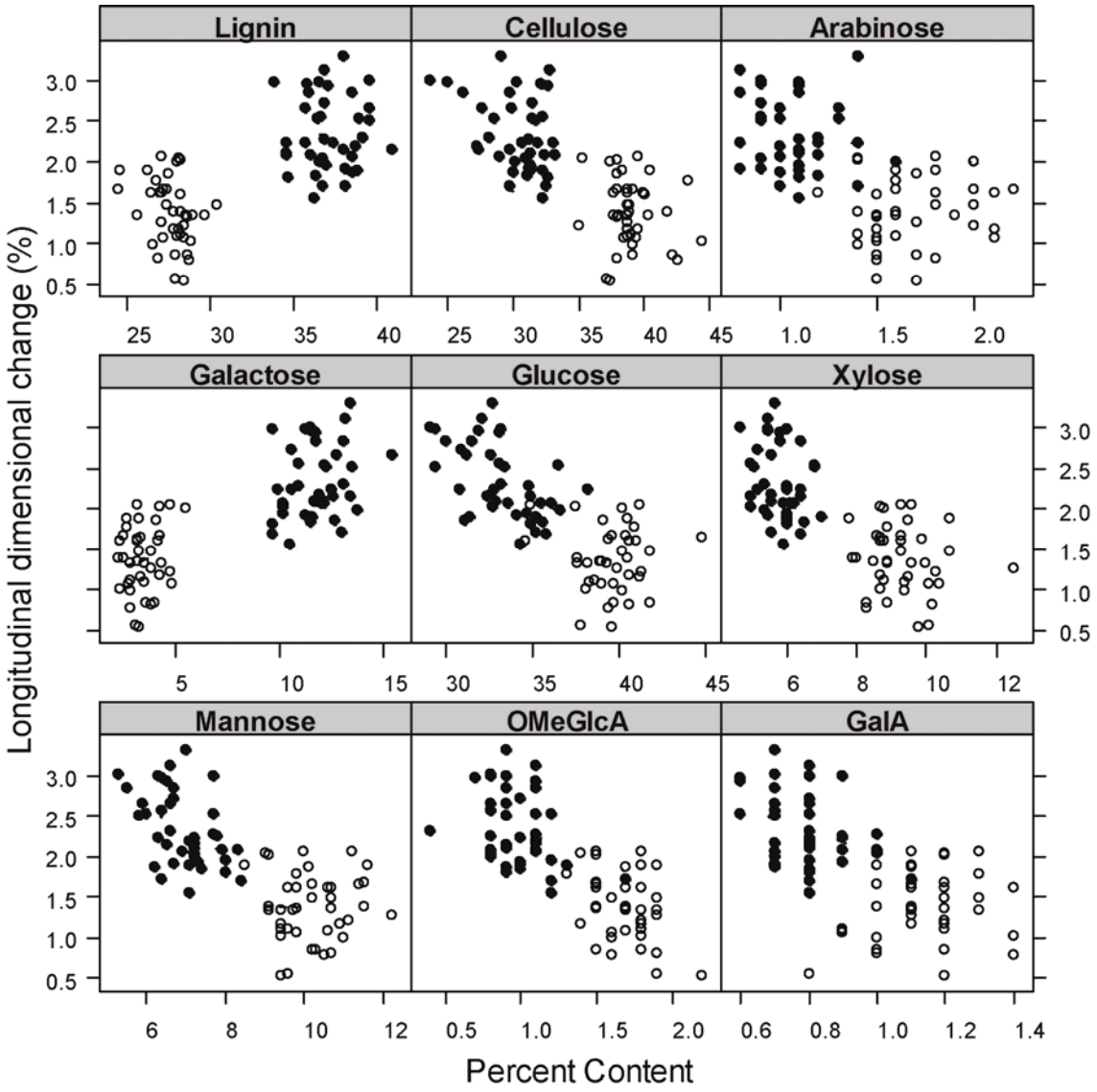

Figure 2-5 Relationships between longitudinal dimensional change and the lignin content, cellulose content and glycosyl residue contents for compression wood, ●, and opposite wood, O, of the Amberley samples. OMeGlcA = 4-*O*-methylglucuronic acid, GalA = galacturonic acid.

In the glasshouse samples the lignin content of compression wood samples and non-compression wood samples were linearly related to longitudinal dimensional change, but the slope of the regression was different between these wood types. To account for the changing slope of regression, the relationship across both wood types was investigated using non-linear regression. The relationship between longitudinal dimensional change and

galactosyl residue content of the glasshouse samples had a similar slope of regression across both compression wood and non-compression wood samples, therefore a generalised linear model of longitudinal dimensional change for galactosyl residue content was investigated. The models that were found to be good fits for describing the longitudinal dimensional change as a function of lignin content or galactosyl residue contents were then applied to the Amberley data. The longitudinal dimensional change of the Amberley samples could not be described well using these models; both models predicted longitudinal dimensional change values too low for the opposite wood, and the non-linear model predicted longitudinal dimensional change values too high for the compression wood. The models are plotted in Figure 2-6 and described in Table 2-3.

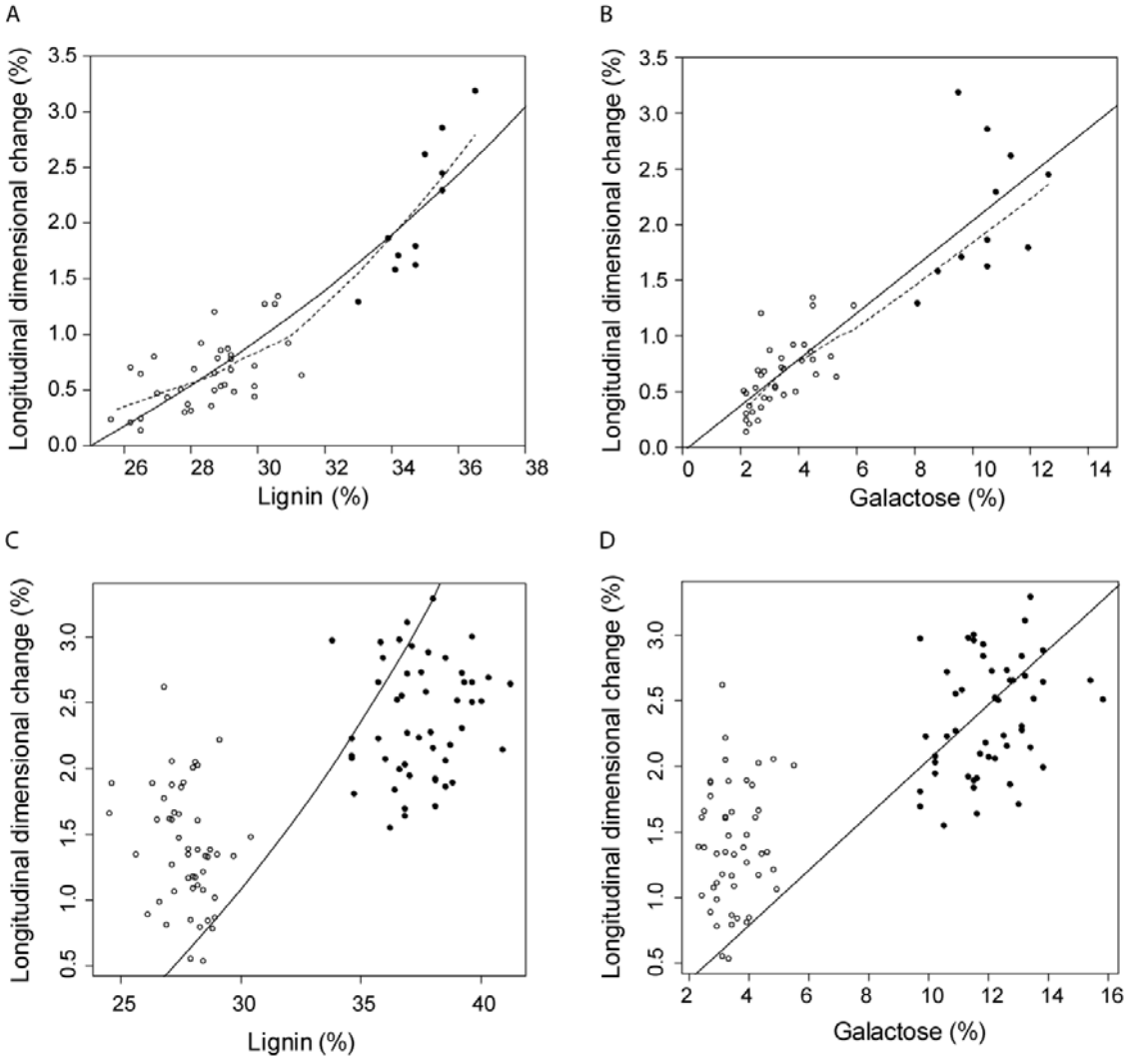

Figure 2-6 (A) Non-linear regression for longitudinal dimensional change and lignin content of the glasshouse samples. (B) Linear regression for longitudinal dimensional change and galactosyl residue content of the glasshouse samples. (C) Non-linear regression for longitudinal dimensional change and lignin content applied to the Amberley data. (D) Linear regression for longitudinal dimensional change and galactosyl residue content applied to the Amberley data. Solid line = model, dashed line = locally weighted regression, compression wood = ●, non-compression wood = O.

Table 2-3 Models describing longitudinal dimensional change as a function of lignin content and galactosyl residue content, applied to the glasshouse data and the Amberley data.

	Glasshouse		Amberley	
Model	R^2	RSE	R^2	RSE
LDC = $e^{0.05lignin}$ - 3.5	0.84	0.28	0.57	0.44
LDC = 0.21 × galactose - 0.05	0.80	0.33	0.50	0.47

LDC = longitudinal dimensional change
RSE = relative standard error

2.4 Discussion

The differences in the cell-wall compositions between compression wood and non-compression wood were consistent with data in the literature for radiata pine (Schwerin, 1958; Nanayakkara *et al.*, 2009; Kibblewhite *et al.*, 2010) and other gymnosperms (Timell, 1986). Although the normal corewood of radiata pine also has high lignin and galactosyl residue contents compared with normal outerwood (Kibblewhite *et al.*, 2010), in the current study the lignin and galactosyl residue contents of compression corewood were significantly higher than the normal corewood. The threshold value of lignin content that distinguishes compression wood in this study, 32%, is similar to the reported value at which the anatomical features typical of compression wood begin to appear in radiata pine, 31% (Nanayakkara *et al.*, 2009). The cell-wall composition of radiata pine wood changes with increasing cambial age (distance from the pith), with the outerwood having a lower lignin and galactosyl residue content and higher cellulose, glucosyl and mannosyl residue content (Harwood, 1971; Uprichard, 1971; Uprichard and Lloyd, 1980; Kibblewhite *et al.*, 2010). There is conflicting data as to whether the xylosyl residue content increases or decreases with distance from the pith (Harwood, 1971; Nanayakkara *et al.*, 2009).

The changes in cell-wall composition with cambial age suggest that the above threshold values may change in accordance with the cambial age of the wood. Although the

anatomical features of the compression corewood samples were not analysed here, it is assumed that these threshold values apply only to severe compression corewood, as this was determined by light microscopy of compression corewood samples corresponding to those examined in the current study (Apiolaza *et al.*, 2011a). These thresholds would certainly also be expected to change among species. For example, the compression corewood of loblolly pine (*Pinus taeda*) has a similar mean lignin content (36.6%) to that measured in the current study for radiata pine, but a much higher mean galactosyl residue content of 21.1% (Yeh *et al.*, 2005). The mean lignin content of compression outerwood (37.4%) from a 35 year old loblolly pine was similar to that of the compression corewood of the same species, but the galactosyl residue content of compression outerwood was much lower (12.8%) compared with the compression corewood (Yeh *et al.*, 2006). However, the ability to quantitatively define radiata pine compression corewood based on lignin and galactosyl residue content could allow the identification of severe compression corewood based on the cell-wall composition, without the need for microscopic analysis of the tracheid morphology.

The flexure wood of Fraser fir (*Abies fraseri*) has been shown to have thick-walled tracheids that are more similar to the tracheids of compression wood than normal wood (Telewski, 1989). Three-point bending tests indicated that Fraser fir flexure wood had significantly increased flexural stiffness and significantly decreased modulus of elasticity of the lower internodes (Telewski and Jaffe, 1986b). The flexure wood of radiata pine corresponding to that used in this study also has a similar tracheid morphology to mild compression wood (Apiolaza *et al.*, 2011a). However, the flexure wood of radiata pine was considered to be "not the same as compression wood", as it had lower density and lower standing tree modulus of elasticity than compression wood (Apiolaza *et al.*, 2011a). Differences between flexure wood and normal wood were also observed by Apiolaza et al.

(2011a), with flexure wood having a higher density and lower standing tree modulus of elasticity than normal wood. However, the modulus of elasticity of dried flexure wood stems was not significantly lower than normal wood (Apiolaza *et al.*, 2011a). Seedlings of loblolly pine grown until 9-months-old under the influence of two oscillating fans, simulating an average wind speed of 1.43 m s^{-1} (about 5.1 km h^{-1}), developed an opposite wood side and a compression wood side, as determined by analysis of the tracheid morphology by light microscopy. The tracheids on the compression wood side had rounded, thick-walled tracheids and intercellular spaces (Yeh *et al.*, 2005). The cell-wall composition of the compression wood side of the seedlings grown under the influence of wind was either the same as compression wood, or intermediate between normal wood and compression wood (Yeh *et al.*, 2005). However, the formation of wood with different tracheid morphologies on each side of the stem is suggestive of the simulated wind causing a preferential lean in one direction, and therefore differences in the cell-wall composition of the wind-affected wood compared with normal wood were more likely to be due to the formation of compression wood, rather than formation of flexure wood with a different cell-wall composition. In the current study, flexion of the radiata pine seedlings was along a uni-directional axis, simulating a wind speed of ~ 10 km h^{-1} and reaching the same degree of lean on each side (Apiolaza *et al.*, 2011a). The cell-wall composition of this radiata pine flexure wood was different from compression wood, supporting the study of Apiolaza et al. (2011a), which indicated that radiata pine flexure wood is not compression wood. Further, the cell-wall composition and moisture-related longitudinal dimensional changes of flexure wood in the current study were not different to normal wood or opposite wood.

The current models of wood longitudinal swelling and shrinkage (longitudinal dimensional change), based on Barber and Meylan's original model (1964), posit that with an increase in moisture content (swelling), the cell-wall matrix provides an expansive force,

which is restrained by the stiff cellulose microfibrils (Yamamoto, 1999; Yamamoto *et al.*, 2001). The microfibril angle of juvenile flexure wood of Fraser fir was reported to be higher than that of normal wood, approaching that of compression wood (Telewski, 1989). However, in the samples examined in the present study, the mean microfibril angle of all of the wood types was high (35.9°), and there was no significant difference in microfibril angle among the wood types (Brennan *et al.*, 2012). However, the longitudinal dimensional change of compression wood was much higher than the longitudinal dimensional change of non-compression wood samples, including flexure wood. This is consistent with the observation of Harris (1977) that the mean microfibril angle of compression wood and opposite wood are not significantly different in radiata pine corewood, although the longitudinal shrinkage of compression wood was significantly higher than opposite wood at the same microfibril angle. The mean microfibril angle of radiata pine compression wood accounts for only one third of the variance in longitudinal shrinkage of this wood type (Harris, 1977). Therefore in accordance with the model of Yamamoto et al. (2001), it is likely that the significantly different cell-wall composition of compression corewood explains a significant proportion of the remaining variance, probably in conjunction with other cell-wall properties such as wood density, which is a measure of the amount of cell-wall per unit volume of the wood.

Consistent with differences in the composition of the cell-wall matrix influencing the longitudinal dimensional change (Yamamoto *et al.*, 2001), the lignin content and galactosyl residue content of the glasshouse wood samples were correlated with the extent of longitudinal dimensional change. That galactosyl residue content is correlated with longitudinal dimensional change is also consistent with the findings of Floyd (2005), who found a correlation between the ratio of galactosyl residue content to glucosyl residue content and longitudinal shrinkage of loblolly pine (*Pinus taeda*). Cell-wall matrix polymers

are known to have different mechanical properties in response to a change in moisture conditions (Cousins, 1976; 1978). The (1→4)-β-D-galactan sidechains of the primary cell-wall pectic polysaccharide rhamnogalacturonan I, which are similar to the (1→4)-β-D-galactans of compresssion wood, have been shown to be highly mobile polymers, acting like "tethered solutes" that can interact with other cell wall polymers (Ha *et al.*, 2005). The mobility of the (1→4)-β-D-galactan sidechains in primary walls, attached only at one end by a glycosidic linkage to rhamnose of the rhamnogalacturonan I backbone, was hypothesised to prevent these sidechains from carrying a mechanical load (Ha *et al.*, 2005). Although it is now known that the (1→4)-β-D-galactan sidechains of sugar beet (*Beta vulgaris*) and potato (*Solanum tuberosum*) can hydrogen bond to cellulose (Zykwinska *et al.*, 2005; Zykwinska *et al.*, 2007). A major role of the (1→4)-β-D-galactans is likely to involve interaction with water. There is also evidence that the (1→4)-β-D-galactans of compression wood are covalently linked to lignin (Mukoyoshi *et al.*, 1981; Minor, 1982), and therefore they may have additional functions to those of primary wall (1→4)-β-D-galactans.

Films of (1→4)-β-D-galactans isolated from lupin seed cell walls have been shown to swell on hydration, and their swelling can be predicted as a function of osmotic pressure, polymer concentration and degree of polymerisation (Ryden *et al.*, 2000). Because the swelling behaviour of (1→4)-β-D-galactans can be predicted (Ryden *et al.*, 2000), the correlation between galactosyl residue content (as an indicator of (1→4)-β-D-galactan content) and longitudinal dimensional change was expected to be stronger. In both data sets, deviation of the residuals from the modelled linear relationship between galactosyl residue content and longitudinal dimensional change were quite large. Part of this variation is probably caused by the galactosyl residue content not being an accurate measure of the (1→4)-β-D-galactan content of the wood samples, as galactosyl residues can also come from other cell-wall polysaccharides such as the galactoglucomannans in the secondary walls, and

xyloglucans in the primary walls. The proportions of the other cell-wall polysaccharides are known to be different in compression wood compared with normal wood (Timell, 1986), and therefore would contribute to variation in the galactosyl residue contents between different wood types. Additionally, the cell-wall composition was measured on only an aliquot of the ground wood of the piece used to measure longitudinal dimensional change, therefore it was not the true composition of that entire sample. The different relationships observed between the glasshouse and Amberley samples when the proportions of cell-wall components were plotted against the extent of longitudinal dimensional change was likely to be influenced by the two different measurements of longitudinal dimensional change used. The longitudinal dimensional change values for the Amberley samples, measured from the green to the dry state, were similar to those in the literature for compression corewood of radiata pine, with longitudinal dimensional changes ranging from 1 to 5 % (Harris, 1977; Xu *et al.*, 2009). The longitudinal dimensional changes measured from wood that had already been oven-dried, as was the case for the glasshouse samples, typically have lower longitudinal dimensional changes, e.g. up to 2% (Floyd, 2005; Leonardon *et al.*, 2009). This is a result of the hysteresis effect of longitudinal dimensional changes of wood, where the length of a sample during drying is shorter than the length of the sample during moisture absorption at the same moisture content (Sadoh and Christensen, 1967; Meylan, 1972), which therefore increased the perceived longitudinal dimensional change measured on drying. The moisture content of the oven-dried Amberley samples was ~4.5% (Apiolaza *et al.*, 2011b), which is within the range where the hysteresis of the longitudinal dimensional change has a measureable effect.

The lack of relationships between cell-wall composition and longitudinal dimensional change in the Amberley data compared with the glasshouse data may be due to the problems cited during growth of the trees, which included "practical difficulties in

maintaining the trees at the desired 25° lean" and "some commingling of compression and opposite wood" (Apiolaza *et al.*, 2011b). These problems appeared to be reflected by a large variation in longitudinal dimensional change of the opposite wood samples in the study of Apiolaza et al. (2011b). The maximum longitudinal dimensional change of the Amberley opposite wood samples exceeded the maximum longitudinal dimensional change of the compression wood samples. The Amberley wood samples investigated in the current study were a subset (100 samples) of the total (492 samples) investigated by Apiolaza et al. (2011b). This subset was carefully chosen by visual inspection of wet samples to include only those samples that contained pure compression wood and opposite wood (wet compression wood is darker than wet normal and opposite wood, Timell, 1986). However, patches of compression wood of intermediate severities that were not visually distinguishable from the surrounding compression wood or opposite wood may still have been present. Compression woods with different severities have different cell-wall compositions (Nanayakkara *et al.*, 2009), and are assumed to have different longitudinal dimensional changes. Indeed, some of the opposite wood samples had greater longitudinal dimensional change than some of the compression wood samples. Therefore, the different relationships observed between the cell-wall components and longitudinal dimensional change within compression wood and non-compression wood in the glasshouse data set, which contained pure corewood types, would not necessarily hold true for the Amberley data if the compression wood and opposite wood samples also contained some mild compression wood. Visual grading of radiata pine compression wood, even by light microscopy, has been shown to be poorly correlated with the degree of longitudinal shrinkage (Harris, 1977). At lower microfibril angles, the influence of the cell-wall matrix on the extent of longitudinal dimensional change is less significant compared with the influence at high microfibril angles (Yamamoto *et al.*, 2001). Therefore, if the Amberley samples have a low microfibril angle, a strong correlation between cell-wall composition and longitudinal dimensional change would not be expected.

Unfortunately the microfibril angle of the Amberley samples is unknown. However, given the prevalence of data in the literature indicating that radiata pine corewood has a high microfibril angle (Harris, 1977; Donaldson, 1992; Downes *et al.*, 2002), it is unlikely that these Amberley samples have low microfibril angles.

The different cell-wall composition of compression wood compared with non-compression wood indicates that detection of compression wood using chemical techniques is possible. The composition of the cell-wall matrix clearly influences the moisture-related longitudinal dimensional changes of wood, but more work needs to be done to determine whether the quality of wood, including compression-free wood can be indicated by the cell-wall composition.

2.5 References

Albersheim, P., D. J. Nevins, P. D. English and A. Karr (1967). "A method for the analysis of sugars in plant cell-wall polysaccharides by gas-liquid chromatography." Carbohydrate Research **5**: 340-345.

Apiolaza, L., A. (2009). "Very early selection for solid wood quality: screening for early winners." Annals of Forest Science **66**: 601.

Apiolaza, L., B. Butterfield, S. Chauhan and J. Walker (2011a). "Characterization of mechanically perturbed young stems: can it be used for wood quality screening?" Annals of Forest Science **68**: 407-414.

Apiolaza, L., S. Chauhan and J. Walker (2011b). "Genetic control of very early compression and opposite wood in *Pinus radiata* and its implications for selection." Tree Genetics & Genomes **7**: 563-571.

Barber, N. F. and B. A. Meylan (1964). "The anisotropic shrinkage of wood - A theoretical model." Holzforschung **18**: 146-156.

Bax, A. (1985). "A spatially selective composite 90° radiofrequency pulse." Journal of Magnetic Resonance **65**: 142-145.

Brendel, O., P. P. M. Iannetta and D. Stewart (2000). "A rapid and simple method to isolate pure alpha-cellulose." Phytochemical Analysis **11**: 7-10.

Brennan, M., J. P. McLean, C. M. Altaner, J. Ralph and P. J. Harris (2012). "Cellulose microfibril angles and cell-wall polymers in different wood types of *Pinus radiata*." Cellulose **Online First**.

Cousins, W. J. (1976). "Elastic modulus of lignin as related to moisture content." Wood Science and Technology **10**: 9-17.

Cousins, W. J. (1978). "Young's modulus of hemicellulose as related to moisture content." Wood Science and Technology **12**: 161-167.

Crowell, E. P. and B. B. Burnett (1967). "Determination of the carbohydrate composition of wood pulps by gas chromatography of the alditol acetates." Analytical Chemistry **39**: 121-124.

Currie, H. A. and C. C. Perry (2006). "Resolution of complex monosaccharide mixtures from plant cell wall isolates by high pH anion exchange chromatography." Journal of Chromatography **1128**: 90-96.

Donaldson, L. A. (1992). "Within- and between-tree variation in microfibril angle in *Pinus radiata* " New Zealand Journal of Forestry Science **22**: 77-86.

Downes, G. M., J. G. Nyakuengama, R. Evans, R. Northway, P. Blakemore, R. L. Dickson and M. Lausberg (2002). "Relationship between wood density, microfibril angle and stiffness in thinned and fertilized Pinus radiata." Iawa Journal **23**: 253-265.

Floyd, S. (2005). Effect of hemicellulose on longitudinal shrinkage in wood. The Hemicelluloses Workshop 2005. K. M. Entwistle and J. C. F. Walker. Christchurch, The Wood Technology Research Centre**:** 115-120.

Ha, M.-A., R. J. Viëtor, G. D. Jardine, D. C. Apperley and M. C. Jarvis (2005). "Conformation and mobility of the arabinan and galactan side-chains of pectin." Phytochemistry **66**: 1817-1824.

Harris, J. M. (1977). "Shrinkage and density of radiata pine compression wood in relation to its anatomy and mode of formation." New Zealand Journal of Forestry Science **7**: 91-106.

Harris, P. J., A. B. Blakeney, R. J. Henry and B. A. Stone (1988). "Gas chromatographic determination of the monosaccharide composition of plant cell wall preparations." Journal of the Association of Official Analytical Chemists **71**: 272-275.

Harwood, V. D. (1971). "Variation in carbohydrate analyses in relation to wood age in *Pinus radiata*." Holzforschung **25**: 73-77.

Iiyama, K., T. B. T. Lam, N. Kasuya and B. A. Stone (1994). "Rapid and simple determination of *O*-acetyl groups bound to plant cell walls by acid hydrolysis and ^{1}H-NMR measurement." Phytochemistry **35**: 959-961.

Kibblewhite, R. P., I. D. Suckling, R. Evans, J. C. Grace and M. J. C. Riddell (2010). "Lignin and carbohydrate variation with earlywood, latewood, and compression wood content of bent and straight ramets of a radiata pine clone." Holzforschung **64**: 101-109.

Lee, Y. C. (1990). "High-performance anion-exchange chromatography for carbohydrate analysis." Analytical Biochemistry **189**: 151-162.

Lee, Y. C. (1996). "Carbohydrate analyses with high-performance anion-exchange chromatography." Journal of Chromatography **720**: 137-149.

Leonardon, M., C. M. Altaner, L. Vihermaa and M. Jarvis (2009). "Wood shrinkage: influence of anatomy, cell wall architecture, chemical composition and cambial age." European Journal of Wood Products **online**

Lina, L., Z. Ping, C. Yaqi and M. Shifen (2006). "High-performance anion exchange chromatography with pulsed amperometric detection for simultaneous determination of monosaccharides and uronic acids." Chinese Journal of Analytical Chemistry **34**: 1371-1374.

Meylan, B. A. (1972). "The influence of microfibril angle on the longitudinal shrinkage-moisture content relationship." Wood Science and Technology **6**: 293-301.

Minor, J. L. (1982). "Chemical linkage of pine polysaccharides to lignin." Journal of Wood Chemistry and Technology **2**: 1-16.

Mukoyoshi, S.-i., J.-i. Azuma and T. Koshijima (1981). "Lignin-carbohydrate complexes from compression wood of *Pinus densiflora* Sieb et. Zucc." Holzforschung **35**: 233-240.

Nanayakkara, B., M. Manley-Harris, I. D. Suckling and L. A. Donaldson (2009). "Quantitative chemical indicators to assess the gradation of compression wood." Holzforschung **63**: 431-439.

R Development Core Team (2011). "R: A language and environment for statistical computing." from http://www.R-project.org.

Ryden, P., A. J. MacDougall, C. W. Tibbits and S. G. Ring (2000). "Hydration of pectic polysaccharides." Biopolymers **54**: 398-405.

Sadoh, T. and G. N. Christensen (1967). "Longitudinal shrinkage of thin sections." Wood Science and Technology **1**: 26-44.

Saeman, J. F., W. E. Moore, R. L. Mitchell and M. A. Millett (1954). "Techniques for the determination of pulp constituents by quantitative paper chromatography." TAPPI Journal **37**: 336-343.

Schwerin, G. (1958). "The chemistry of reaction wood. Part II. The polysaccharides of *Eucalyptus goniocalyx* and *Pinus radiata*." Holzforschung **12**: 43-48.

Smith, B. G. and P. J. Harris (1995). "Polysaccharide composition of unlignified cell walls of pineapple [*Ananas comosus* (L.) Merr.] fruit." Plant Physiology **107**: 1399-1409.

T 249 cm-85 (1985). "Carbohydrate composition of extractive-free wood and wood pulp by gas-liquid chromatography." TAPPI.

Telewski, F. W. (1989). "Structure and function of flexure wood in *Abies fraseri*." Tree Physiology **5**: 113-121.

Telewski, F. W. and M. J. Jaffe (1986). "Thigmorphogenesis: field and laboratory studies of *Abies fraseri* in response to wind or mechanical perturbation." Physiologia Plantarum **66**: 211-218.

Timell, T. E. (1986). Compression wood in gymnosperms. Berlin, Springer-Verlag.

Uprichard, J. M. (1971). "Cellulose and lignin content in *Pinus radiata* D. Don. Within-tree variation in chemical composition, density, and tracheid length." Holzforschung **25**: 97-105.

Uprichard, J. M. and J. A. Lloyd (1980). "Influence of tree age on the chemical composition of radiata pine." New Zealand Journal of Forestry Science **10**: 551-557.

Weitzhandler, M., V. Barreto, C. Pohl, P. Jandik, J. Cheng and N. Avdalovic (2004). "CarboPac™ PA20: a new monosaccharide separator column with electrochemical detection with disposable gold electrodes." Journal of Biochemical and Biophysical Methods **60**: 309-317.

Willför, S., A. Sundberg, J. Hemming and B. Holmbom (2005). "Polysaccharides in some industrially important softwood species." Wood Science and Technology **39**: 245-258.

Xu, P., H. Liu, R. Evans and L. A. Donaldson (2009). "Longitudinal shrinkage behaviour of compression wood in radiata pine." Wood Science and Technology **43**: 423-439.

Yamamoto, H. (1999). "A model of the anisotropic swelling and shrinking process of wood. Part 1. Generalization of Barber's wood fiber model." Wood Science and Technology **33**: 311-325.

Yamamoto, H., F. Sassus, M. Ninomiya and J. Gril (2001). "A model of anisotropic swelling and shrinking process of wood. Part 2. A simulation of shrinking wood." Wood Science and Technology **35**: 167-181.

Yeh, T.-F., J. L. Braun, B. Goldfarb, H.-m. Chang and J. F. Kadla (2006). "Morphological and chemical variations between juvenile wood, mature wood, and compression wood of loblolly pine (*Pinus taeda* L.)." Holzforschung **60**: 1-8.

Yeh, T.-F., B. Goldfarb, H.-m. Chang, I. Peszlen, J. L. Braun and J. F. Kadla (2005). "Comparison of morphological and chemical properties between juvenile wood and compression wood of loblolly pine." Holzforschung **59**: 669-674.

Zykwinska, A., J.-F. Thibault and M.-C. Ralet (2007). "Organization of pectic arabinan and galactan side chains in association with cellulose microfibrils in primary cell walls and related models envisaged." Journal of Experimental Botany **58**: 1795-1802.

Zykwinska, A. W., M.-C. J. Ralet, C. D. Garnier and J.-F. J. Thibault (2005). "Evidence for in vitro binding of pectin side chains to cellulose." Plant Physiology **139**: 397-407.

Chapter 3 - Cell-wall compositions of juvenile radiata pine wood types analysed by 2D NMR spectroscopy

3.1 Introduction

Two-dimensional nuclear magnetic resonance (2D NMR) ^{13}C-^{1}H correlation spectroscopy has been used to examine the structure of cell-wall polymers in their native state within the cell wall, without the need for isolation or fractionation. Early efforts involved dissolution of the cell wall followed by acetylation of cell-wall polymers (Lu and Ralph, 2003). Unfortunately, acetylation of the cell walls meant that information about acetylation of polysaccharide structures could not be determined from the spectra. Subsequently, non-derivatised whole cell walls were dissolved in deuterated di-methyl sulphoxide (DMSO) and N-methylimidazole (NMI) (Yelle *et al.*, 2008), which provided information about acetylation of polysaccharides. Although an improvement on acetylation, this method is not ideal for situations where information about the presence of *p*-hydroxyphenyl units (H-units) of lignin is required, such as the investigation of compression wood lignin in the current study, as the NMI peaks obscure these signals. Swelling the cell walls to a gel-state in DMSO, which did not require complete dissolution of the whole cell wall, produced detailed NMR spectra which gave information about the presence of H-units in lignin (Kim *et al.*, 2008). Most recently, addition of pyridine to the DMSO has been shown to improve swelling of the cell walls, in addition to increasing the resolution and signal intensity of the spectra (Kim and Ralph, 2010).

The use of gel-state 2D NMR spectroscopy of woody plants has been used to examine differences in the cell-wall compositions of both softwoods and hardwoods (Hedenström *et al.*, 2009; Rencoret *et al.*, 2009; Rencoret *et al.*, 2011), and also non-woody

plants (Rencoret *et al.*, 2009; Jensen *et al.*, 2011). It is a powerful tool for detecting changes in the cell-wall compositions of transgenic plants and has been used to detect changes in the lignin composition of radiata pine trees after RNA interference silencing of specific genes in the lignin biosythetic pathway (e.g. Wagner *et al.*, 2007; Wagner *et al.*, 2009). The informative nature of ^{13}C-^{1}H NMR correlation spectroscopy for the investigations of lignin structure has long been exploited (Ralph *et al.*, 1999). However, although the ^{13}C-^{1}H correlations from the anomeric carbon of many of the cell-wall polysaccharides are known for some common NMR solvents (Kim and Ralph, 2010; Rencoret *et al.*, 2011), the literature containing the correlations of the aliphatic carbons has not been compiled in a comprehensive manner. Therefore, the use of 2D NMR for analysis of the polysaccharides in whole cell-wall spectra has not been as widespread as analysis of lignin composition.

In this chapter, oligosaccharide and polysaccharide standards have been analysed by 2D NMR spectroscopy and compared with values in the literature to afford a more comprehensive analysis of wood cell-wall polymers. Gels were prepared from finely milled wood swollen in DMSO-d_6:pyridine-d_5 to compare the composition of the wood cell-wall polymers (lignin and polysaccharides) among four different corewood types of radiata pine: normal wood, opposite wood, flexure wood and compression wood.

3.2 Materials and methods

3.2.1 Wood samples

Wood samples were from the glasshouse-grown trees described in Chapter 2. One sample of each corewood type (normal wood, opposite wood, flexure wood and compression wood) was examined.

3.2.2 Sample preparation

Wood samples were milled to pass a 40-mesh screen (422 µm pore size) in a Wiley® mini-mill (Thomas Scientific, Swedesboro, NJ) before extracting with dichloromethane in a Soxhlet extractor for 8 h at 60°C. A portion of milled, extracted wood was finely milled in a Retsch (Haan, Germany) PM100 planetary ball mill in a zirconium dioxide (ZrO_2) grinding bowl with ZrO_2 ball bearings (10 mm x 10) at 600 rpm following the recommended grinding times of Kim and Ralph (Kim and Ralph, 2010) (1 h 20 min for 100 mg or 2 h 20 min for 200 mg, using grinding intervals of 20 min with 10 min breaks to avoid sample heating). Ball-milled material (70-100 mg) was transferred into a 5 mm NMR tube. Material was dispersed along the side of the tube before addition of a pre-mixed solution containing 4:1 (v/v) DMSO-d_6 (99.9 atom % D, Sigma-Aldrich) and pyridine-d_5 (99.96 atom % D, Sigma-Aldrich) (600 µl). Samples were mixed in the tubes by gently plunging with a thin glass stirring rod until they appeared homogeneous.

3.2.3 Two-dimensional ^{13}C-^{1}H HSQC nuclear magnetic resonance correlation spectroscopy (2D NMR)

Wood gel spectra and most polysaccharide and oligosaccharide gel spectra were acquired on a Bruker 600 MHz UltraShield spectrometer equipped with a 5 mm QNP $^{1}H/^{13}C/^{31}P/^{19}F$ z-gradient cryoprobe. The ^{13}C–^{1}H correlation experiment was an adiabatic HSQC variant (Bruker standard pulse sequence "hsqcetgpsisp2.2") typically with the following parameters: spectra were acquired from -1 to 11 ppm in F2 (^{1}H) using 2048 data

points for an acquisition time of 136 ms, an interscan relaxation delay (D1) of 1 s, and 0 to 210 ppm in F1 (^{13}C) using 512 data points for an F1 acquisition time of 8.08 ms, using 16 scans per increment, with a total acquisition time of 2 h 40 min. Spectra of some oligosaccharides were acquired on a Bruker 400 MHz UltraShield plus spectrometer equipped with a 5 mm BBFO Plus probe using the Bruker standard pulse sequence "hscqetgpsisp.2" typically with the following parameters: spectra were acquired from -1 to 11 ppm in F2 (^{1}H) using 800 data points for an acquisition time of 100 ms, an interscan relaxation delay (D1) of 0.5 s, and 0 to 200 ppm in F1 (^{13}C) using 300 data points for an F1 acquisition time of 7.96 ms, using 16 scans per increment, with a total acquisition time of 10 h. The DMSO solvent peak was used as an internal reference (δ_C 39.5, δ_H 2.49 ppm).

Peaks from glycosyl residues were interpreted by running oligosaccharide and polysaccharide standards in the DMSO-d_6:pyridine-d_5 mixture as described above. Laminaritriose (O-LAM3), (1→4)-β-D-mannotetraose (O-MTE), konjac glucomannan (P-GLCML), 6^3,6^4-di-α-D-galactosyl-β-D-mannopentaose (McCleary *et al.*, 1983) (O-GGM5), (1→4)-β- D-xylotetraose (O-XTE), lupin (1→4)-β- D-galactan (P-GALLU), (1→5)-α-L-arabinotriose (O-ATR), (1→5)-α-L-arabinan (linear) (P-LARB), and sugarbeet arabinan (P-ARAB) were purchased from Megazyme International, Ireland. Cellotetraose (400402) was purchased from Seikagaku Corporation, Tokyo, Japan. 4-*O*-methylglucuronoxylan (M-5144) was purchased from Sigma-Aldrich, St Louis, MO, USA. Lignin aromatic peaks from the lignin units were identified by comparison with values reported in the literature (Kim and Ralph, 2010).

3.3 Results

Peak shifts ($^{13}C/^{1}H$ ppm) of the cell-wall polysaccharides were assigned by comparison with cell-wall, or cell-wall-related, oligosaccharide and polysaccharide standards, and comparison with values in the literature (Table 3-1). Peak shifts of the lignin structures were assigned by comparison with (Kim and Ralph, 2010).

Table 3-1 2D ^{13}C-^{1}H NMR correlation spectroscopy chemical shifts for cell-wall polysaccharide components.

Assignment[a]	C1	H1	C2	H2	C3	H3	C4	H4	C5	H5	C6	H6
Glucosyl residue:[b]												
α-D-Glc*p* (R[c])	92.0	5.03										
β-D-Glc*p* (R)	96.5	4.46										
(1→4)-β-D-Glc*p* (internal)	102.8	4.44	73.7	3.13	74.7	3.46	80.0	3.46	76.8	3.30	60.4	3.71/3.87
(1→4)-β-D-Glc*p* (NR[d])	103.2	4.37										
(1→3)-β-D-Glc*p* (internal)	103.7	4.56										
(1→3)-β-D-Glc*p* (NR)	104.1	4.51										
Mannosyl residue:[e]												
α-D-Man*p* (R)	93.9	5.03										
β-D-Man*p* (R)	94.2	4.67										
(1→4)-β-D-Man*p* (internal)	100.5	4.57	70.0	3.90	71.6	3.56	76.9	3.77	75.7	3.35	60.4-61.4	3.54-3.82
(1→4)-β-D-Man*p* (NR)	100.5	4.62										
2-*O*-Ac-β-D-Man*p*	98.9	4.86	70.6	5.41								
3-*O*-Ac-β-D-Man*p*	99.9	4.80										
(1→4)-β-D-Man*p* with α-D-Gal*p* linked C(O)6	100.6	4.64										
Xylosyl residue:[f]												
α-D-Xyl*p* (R)	92.3	4.99										
β-D-Xyl*p* (R)	97.3	4.36										
(1→4)-β-D-Xyl*p* (internal)	101.8	4.34	72.2	3.18	73.8	3.38	75.3	3.61	62.9	3.25/3.95		
(1→4)-β-D-Xyl*p* (NR)	101.8	4.34										
Galactosyl residue:[g]												
α-D-Gal*p* (R)	93.0	5.05										
β-D-Gal*p* (R)	97.7	4.38										
(1→4)-β-D-Gal*p* (internal)	105.6	4.39	72.6	3.55	73.8	3.52	78.8	3.87	75.5	3.46	60.3	3.61
(1→4)-β-D-Gal*p* (NR)	106.2	4.38										
α-D-Gal*p* linked C(O)6 to (1→4)-β-D-Man*p*	99.1	4.88	68.7	3.74	69.1	3.87	69.6	3.94	71.2	3.80	60.5-61.1	3.56-3.85

Table 3-1 continued 2D ^{13}C-^{1}H NMR correlation spectroscopy chemical shifts for cell wall polysaccharide components.

Assignment[a]	C1	H1	C2	H2	C3	H3	C4	H4	C5	H5	C6	H6
Arabinosyl residue:[h]												
α-L-Ara*f* (R)	102.1	5.08										
β-L-Ara*f* (R)	95.8	5.12										
(1→2)-β-L-Ara*f* (unassigned)	102.9/101.4	5.27/5.25										
(1→5)-α-L-Ara*f* (internal)	108.2	4.88	81.7	3.97	77.4	3.81	84.1	3.88	67.2	3.72/3.60		
(1→5)-α-L-Ara*f* (NR)	108.2	4.88										
α-L-Ara*f* linked C(O)3 to (1→5)-α-L-Ara*f*	107.4	5.06										
Uronosyl residue:[i]												
4-O-Me-α-D-Glc*p*A (NR)	97.5	5.25									60.1	3.42
4-*O*-Me-α-D-Glc*p*A linked C(O)2 to (1→4)-β-D-Xyl*p*	101.4	4.61										

[a]C1/H1 correlations identified and peaks assigned by running oligosaccharide and polysaccharide standards. C2-6/H2-6 correlations identified by running oligosaccharide and polysaccharide standards, and peaks assigned by comparison with values reported in the literature.

[b](Dais and Perlin, 1982; Dudley *et al.*, 1983; Flugge *et al.*, 1999; Moulthrop *et al.*, 2005)

[c]R = reducing termini

[d]NR = non-reducing termini

[e](Davis *et al.*, 1995; Capek *et al.*, 2002; Lundqvist *et al.*, 2002; Teleman *et al.*, 2003; Hannuksela and Hervé du Penhoat, 2004)

[f](van Hazendonk *et al.*, 1996; Vignon and Gey, 1998; Teleman *et al.*, 2000; Habibi and Vignon, 2005)

[g](Davis *et al.*, 1990; Davis *et al.*, 1995; Girault *et al.*, 1997; Habibi *et al.*, 2004; Hannuksela and Hervé du Penhoat, 2004)

[h](Colquhoun *et al.*, 1994; Habibi *et al.*, 2004)

[i](Cavagna *et al.*, 1984; Excoffier *et al.*, 1986; Vignon and Gey, 1998; Teleman *et al.*, 2000; Habibi and Vignon, 2005; Jensen *et al.*, 2011)

3.3.1 Lignin aromatic region

In the aromatic region (100-150/6-8 ppm), guaiacyl (G) unit peaks of lignin were identified in all wood types (Figure 3-1, cyan). The C2/H2 correlation of G units was at ~111.1/7.08 ppm, whereas the C5/H5 and C6/H6 correlations of G units overlapped with each other at ~114.9/6.78 ppm and ~119.0/6.88 ppm (see Kim and Ralph, 2010). A small peak from the C2/H2 correlation of oxidised α-ketone structures from G units was present in all wood types at ~110.6/7.42 ppm. Compression wood was the only wood type to have significant levels of peaks derived from *p*-hydroxyphenyl (H) units, with the C2/H2 and C6/H6 correlations at ~127.7/7.20 ppm (Figure 3-1 C, red). The C3/H3 and C5/H5 correlations of H units overlapped with C5/6 of G units at ~115.3/6.99 ppm (Figure 3-1 C). A peak from the pyridine-d_5 solvent was present at ~123.5/7.30 ppm, and an unassigned peak

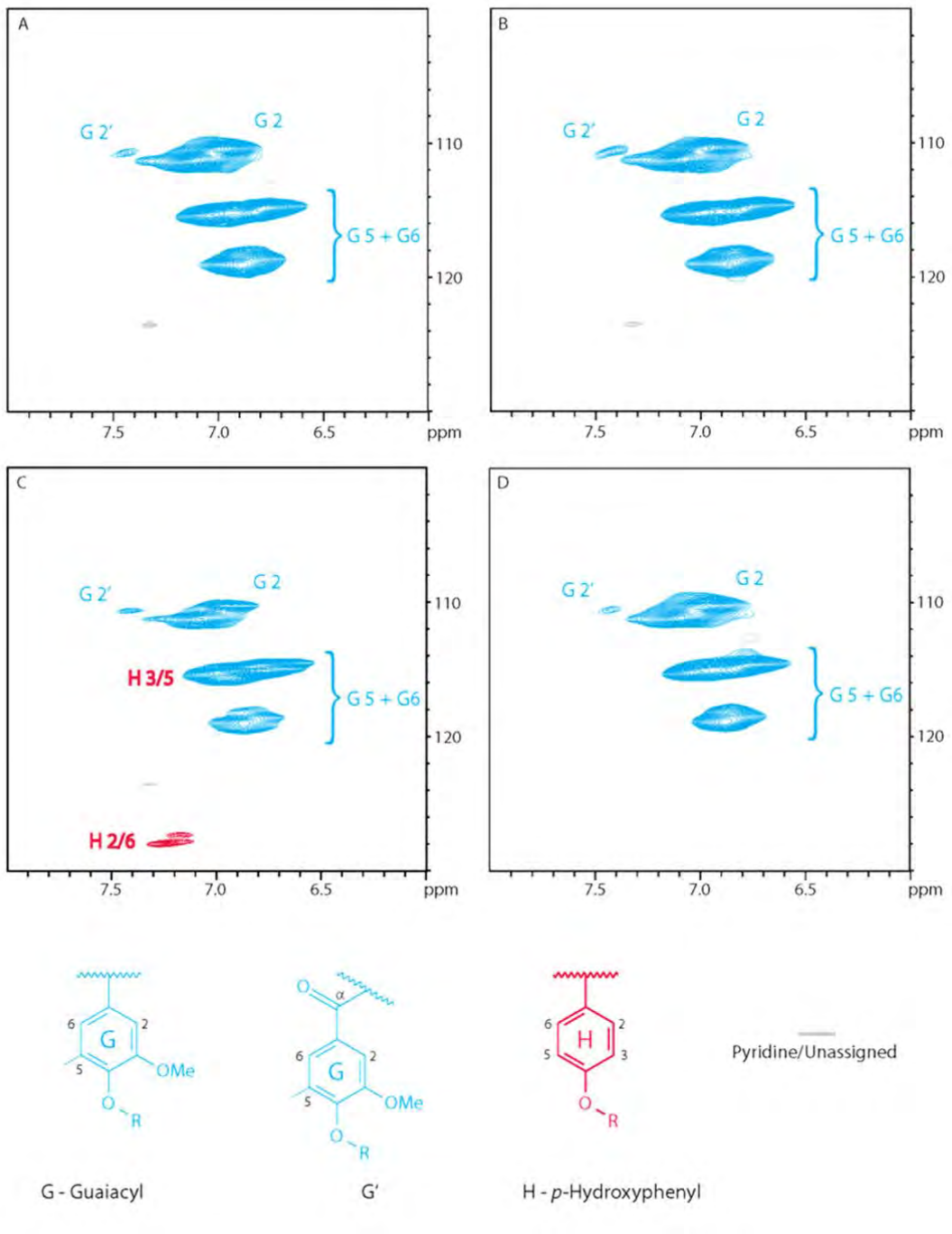

Figure 3-1 Aromatic (lignin) regions of 2D ^{13}C–^{1}H NMR correlation (HSQC) spectra from cell-wall gels of *Pinus radiata* (A) normal wood, (B) opposite wood, (C) compression wood, and (D) flexure wood. The structural units from which the signals are derived are shown below the spectra.

3.3.2 Polysaccharide anomeric region

Differences in the structures of matrix polysaccharides were observed in the polysaccharide anomeric region (90-100/3.5-6 ppm) of the 2D NMR spectra. Differences in the polysaccharides present among the different wood types were found. The spectrum of normal wood is shown in Figure 3-2, opposite wood in Figure 3-3, flexure wood in Figure 3-4 and compression wood in Figure 3-5. Peaks from cellulose, and the most abundant non-cellulosic polysaccharides *O*-acetyl-galactoglucomannans and 4-*O*-methylglucuronoarabinoxylans, were present in all wood types. Note that, as reported previously (Kim *et al.*, 2008; Kim and Ralph, 2010; Rencoret *et al.*, 2011), crystalline cellulose does not swell significantly in these gel-solvents, and is therefore under-represented in the spectra.

The peak from the internal (1→4)-β-D-Glc*p* residues of cellulose was at ~102.8/4.44 ppm. The peak from the internal (1→4)-β-D-Man*p* residues of the galactoglucomannan backbone was at ~100.5/4.57 ppm, but the peak from the internal (1→4)-β-D-Glc*p* residues of *O*–acetyl-galactoglucomannans overlaps with the cellulose peak. A peak from non-reducing terminal α-D-Gal*p*, linked C(O)6 to the internal β-D-Man*p* residues of *O*–acetyl-galactoglucomannans was present at ~99.1/4.88 ppm, with the corresponding signal from β-D-Man*p* substituted with α-D-Gal*p* present at ~100.6/4.64 ppm. A peak from 3-*O*-acetyl-Man*p* was present at ~99.9/4.80 ppm; however, due to overlapping signals it was not obvious whether the peak from 2-*O*-acetyl-Man*p* was present at ~98.9/4.86 ppm. The peak of internal (1→4)-β-D-Xyl*p* from the 4-*O*-methylglucuronoarabinoxylan backbone was at ~101.8/4.34 ppm. The non-reducing terminal 4-*O*-methyl-α-D-Glc*p*A peak was identified at~97.5/5.25 ppm. The peak from non-reducing terminal 4-*O*-methyl-α-D-Glc*p*A linked C(O)2 to the internal β-D-Xyl*p* of 4-*O*-methylglucuronoarabinoxylans was present at ~101.4/4.61 ppm. Non-reducing

terminal Ara*f* peaks likely to be from 4-*O*-methylglucuronoarabinoxylans were also present ~108.2/4.88 ppm. The Ara*f* peak at ~107.4/5.06 was likely to be from arabinan sidechains of rhamnogalacturonan I in the primary walls.

The non-reducing peaks from β-D-Glc*p*, β-D-Man*p* and β-D-Xyl*p* were overlapped by the intense peaks from the internal (main chain) residues of cellulose, *O*-acetyl-galactoglucomannans and 4-*O*-methylglucuronoarabinoxylans, respectively. Small peaks from the reducing ends of polysaccharides were present due to the milling process which physically breaks glycosidic bonds in the polysaccharides and creates reducing (and non-reducing) ends. The peaks from the reducing termini of α-D-Glc*p*, α-D-Gal*p* and α-D-Xyl*p* overlapped with each other at ~92.0-93.0/4.99-5.05 ppm. The peak from reducing terminal β-D-Glc*p* residues was present at ~96.5/4.46 ppm. The reducing terminal β-D-Xyl*p* peak was at ~97.3/4.36 ppm, which overlapped with the reducing terminal β-D-Gal*p* peak at ~97.7/4.38 ppm. A peak from reducing terminal α-D-Man*p* was only present at ~93.9/5.03 ppm; however, the intensity of the signal from reducing terminal β-D-Man*p* was below the detection limit in the spectra. The α-L-Ara*f* reducing peak at ~102.1/5.08 ppm was present, but reducing terminal β-L-Ara*f* was too weak to show above the background noise.

Compression wood was the only wood type with a noticeably different spectrum compared with the normal wood spectrum. Peaks originating from (1→4)-β-D-galactans were present in the spectra of all wood types; however, this peak was proportionally greater in compression wood (Figure 3-5). Both the internal (~105.6/4.39 ppm) and non-reducing (~106.2/4.38 ppm) peaks from (1→4)-β-D-Gal*p* could be distinguished, but in compression wood, the internal (1→4)-β-D-galactan peak was proportionally greater than the non-reducing peak compared with the other wood types. A peak from (1→3)-β-D-Glc*p*, at ~103.7/4.56 ppm, was present in compression wood (Figure 3-5), but was absent from normal wood, opposite wood and flexure wood. The peak at ~101.4/5.25 ppm was present

in compression wood but not in normal wood, opposite wood or flexure wood, and may be from internal and non-reducing terminal (1→2)-linked β-L-Ara*f* residues on extensin sidechains.

The origin of some peaks could not be assigned from the standards or found in the literature. Three of these peaks were present in all four wood types at ~107.8/5.36 ppm, ~107.2/5.54 ppm, and one at ~103.2/4.63 ppm which was larger in compression wood compared with the other wood types. An unassigned peak at ~102.5/5.27 ppm was present in compression wood and normal wood, but not in opposite wood or flexure wood. One unassigned peak was present in opposite wood and flexure wood at ~97.4/4.58 ppm, and another was present only in opposite wood at ~101.8/4.88 ppm.

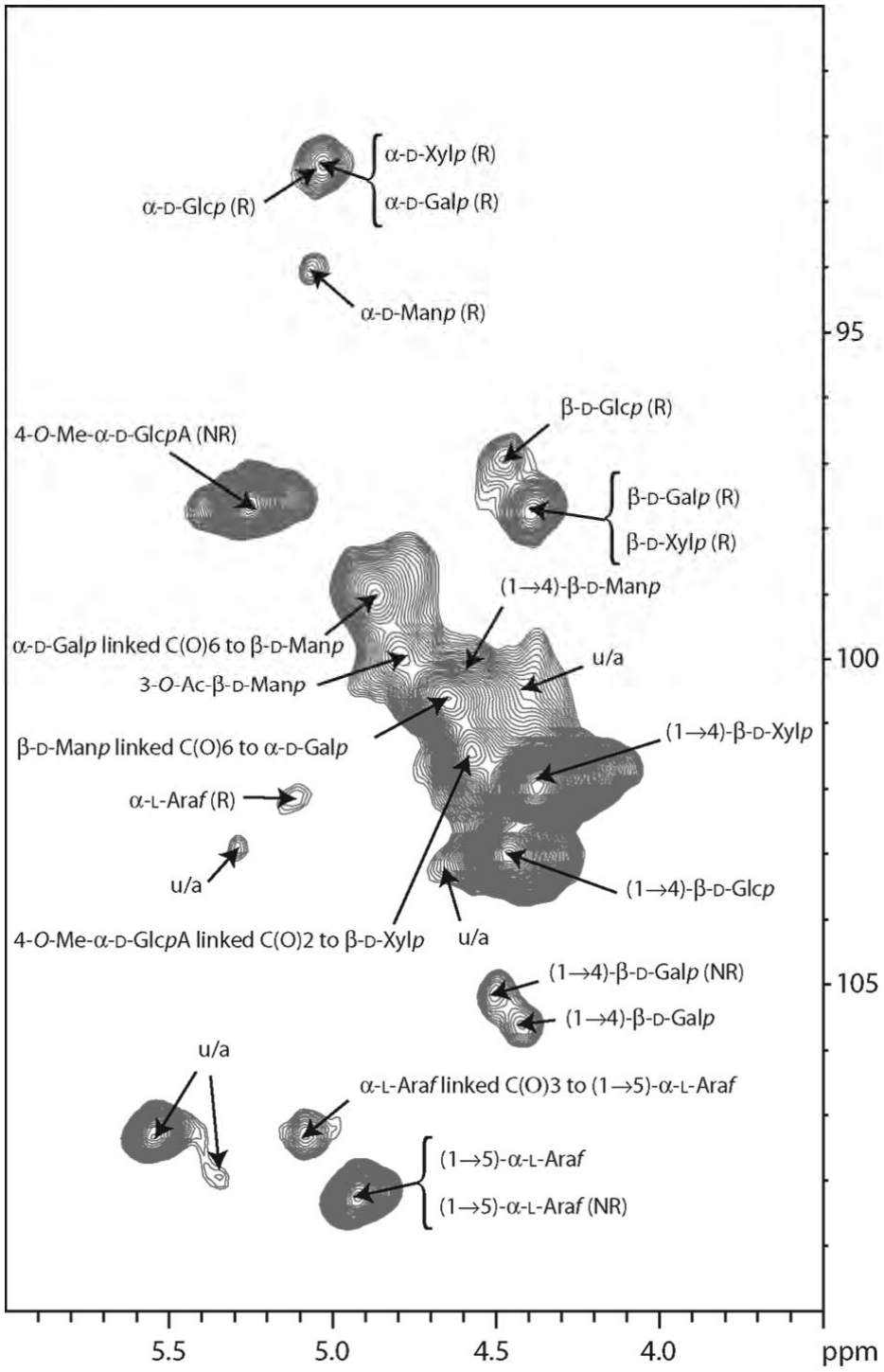

Figure 3-2 Anomeric (polysaccharide) region of the 2D ^{13}C–^{1}H NMR correlation (HSQC) spectrum from a cell-wall gel of *Pinus radiata* normal wood. R = reducing terminus; NR = non-reducing terminus.

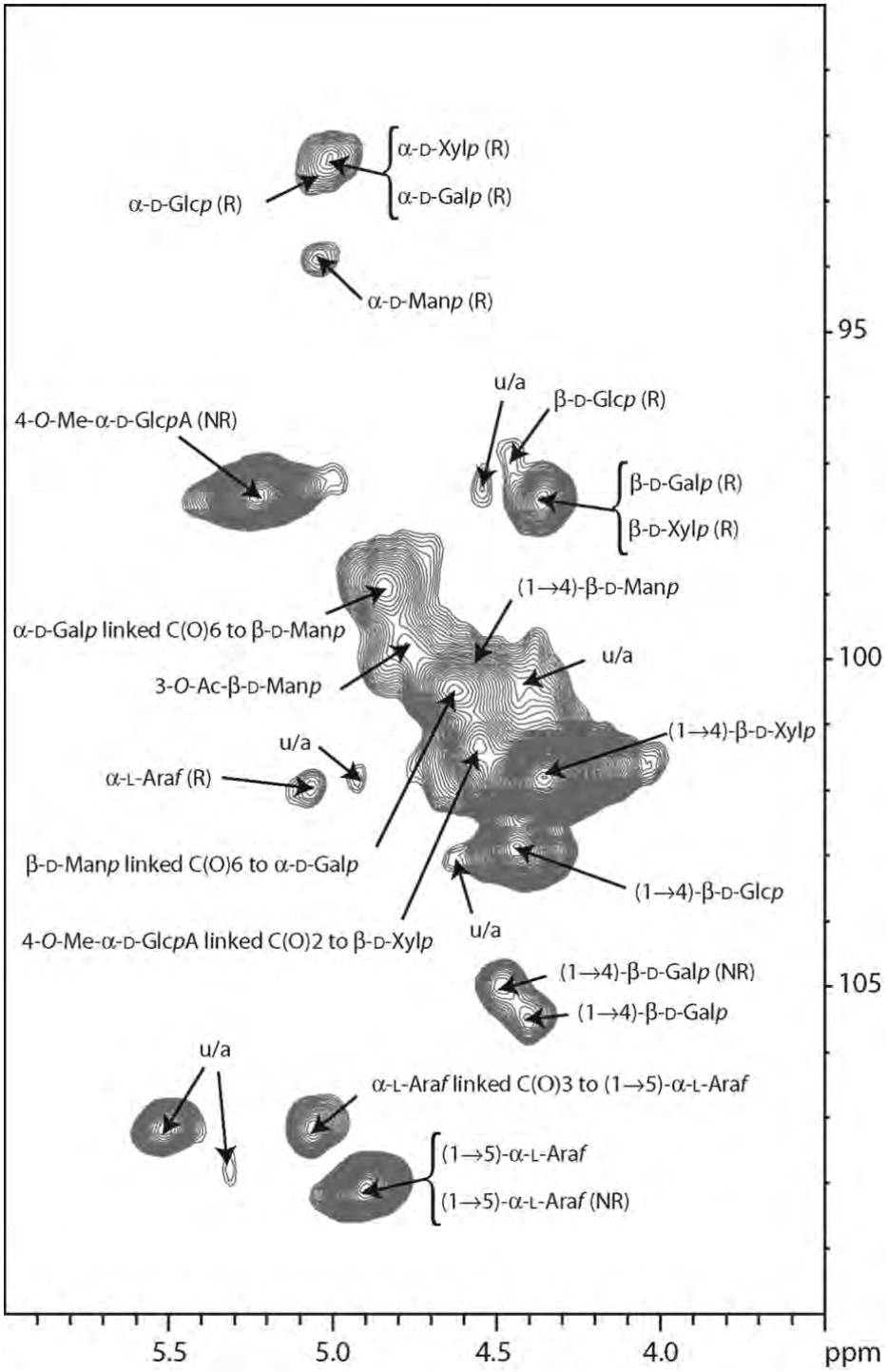

Figure 3-3 Anomeric (polysaccharide) region of the 2D ^{13}C–^{1}H NMR correlation (HSQC) spectrum from a cell-wall gel of *Pinus radiata* opposite wood. R = reducing terminus; NR = non-reducing terminus.

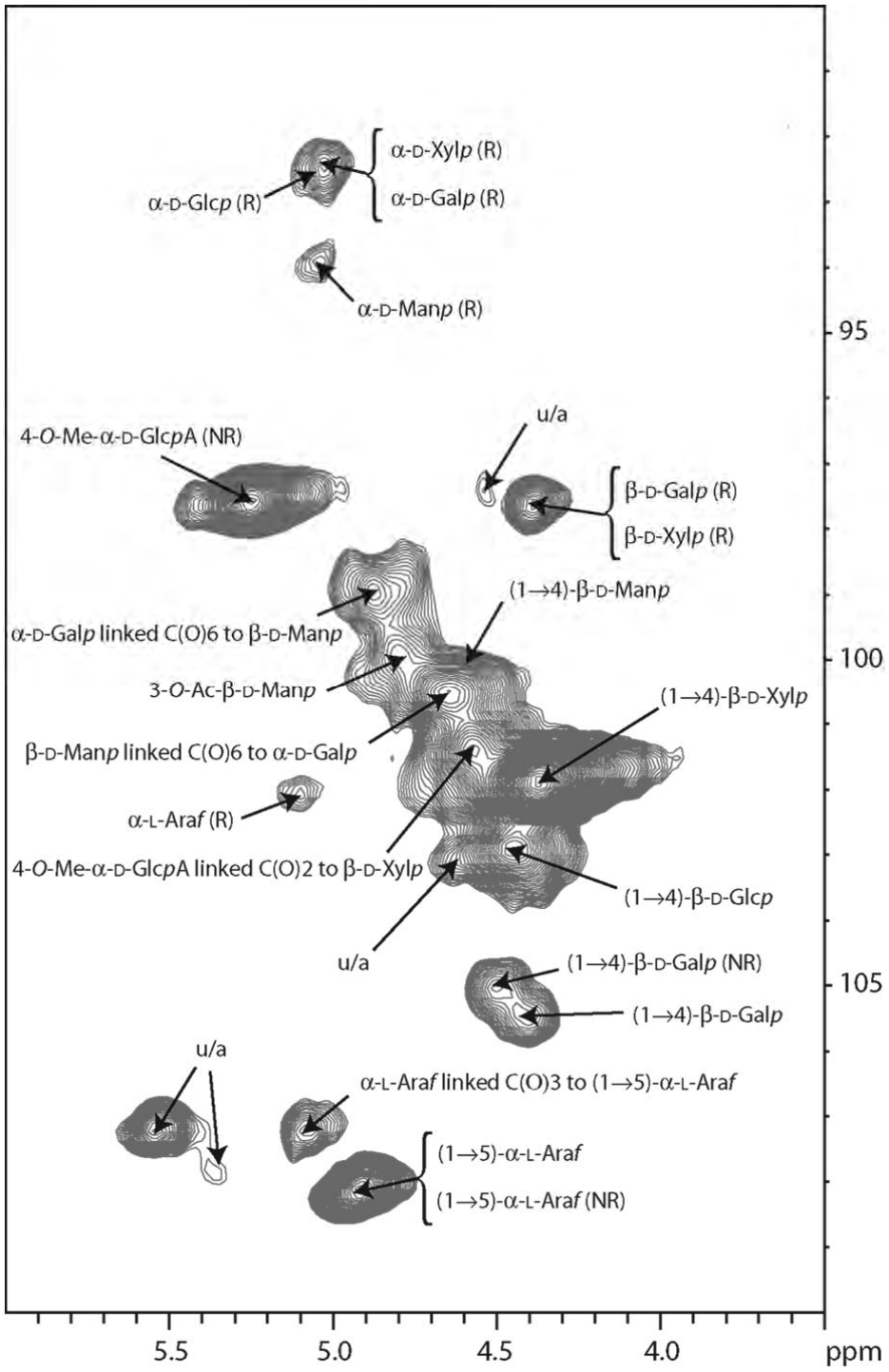

Figure 3-4 Anomeric (polysaccharide) region of the 2D ^{13}C–^{1}H NMR correlation (HSQC) spectrum from a cell-wall gel of *Pinus radiata* flexure wood. R = reducing terminus; NR = non-reducing terminus.

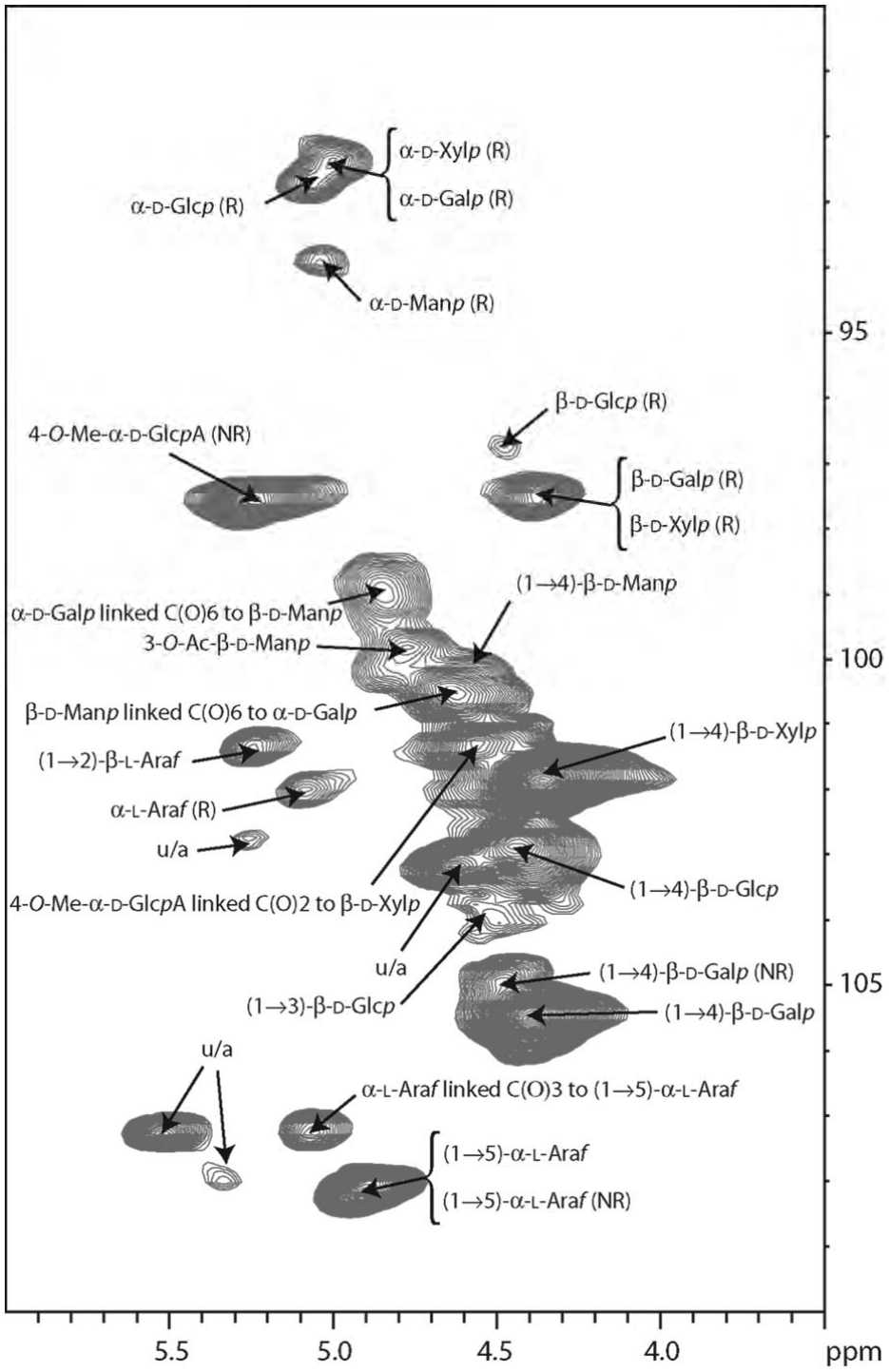

Figure 3-5 Anomeric (polysaccharide) region of the 2D ^{13}C–^{1}H NMR correlation (HSQC) spectrum from a cell-wall gel of *Pinus radiata* compression wood. R = reducing terminus; NR = non-reducing terminus.

3.3.3 Lignin aliphatic and polysaccharide region, and acetyl substitution

The structures of the lignin interunit linkages identified in the lignin aliphatic region (50-90/2-6 ppm) are shown in Figure 3-6. The lignin aliphatic and polysaccharide region spectrum of normal wood is shown in Figure 3-7, opposite wood in Figure 3-8, flexure wood in Figure 3-9 and compression wood in Figure 3-10.

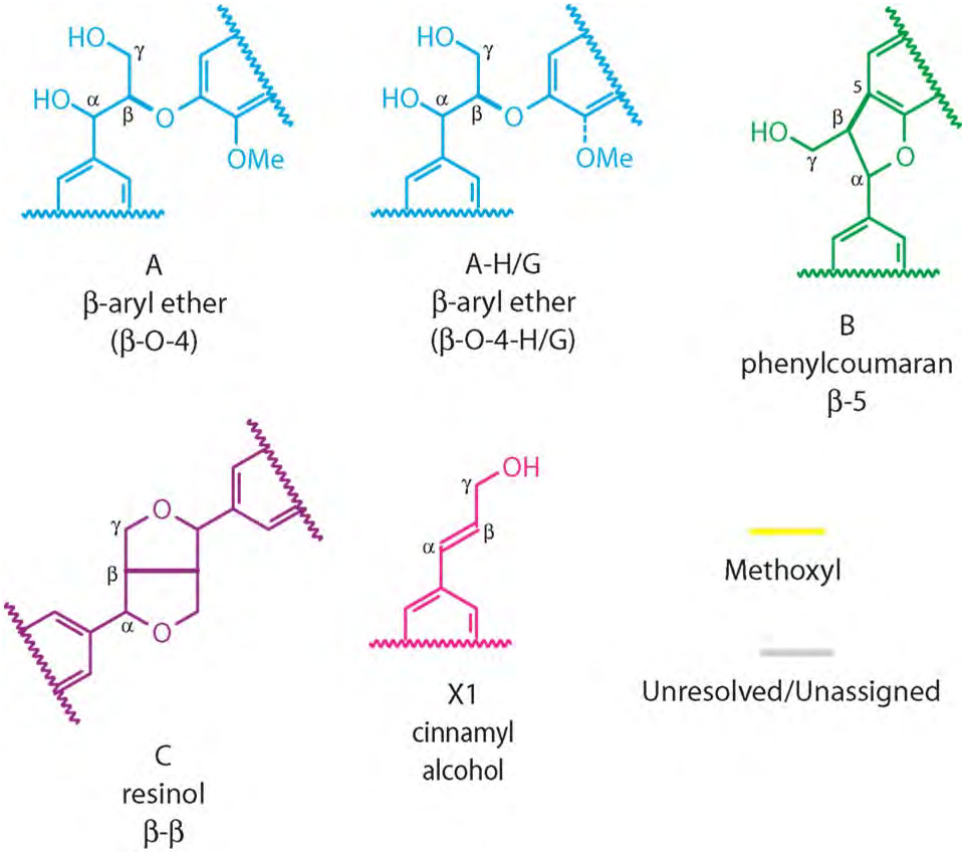

Figure 3-6 Structures of the lignin interunit linkages detected in the aliphatic and lignin side-chain regions of 2D ^{13}C–^{1}H NMR correlation (HSQC) spectra from cell-wall gels of *Pinus radiata* normal wood, opposite wood, flexure wood and compression wood.

Most of the lignin peaks could be distinguished from the polysaccharide peaks. Peaks corresponding to α-, β- and γ-C/H correlations of β-ether units (A) were present in all wood types. The β-ether β-C/H correlation can arise from either G-units or H-units and their origin cannot be distinguished at this resolution. The β-ether γ-C/H correlation overlapped with several polysaccharide signals at ~60-61/3.4-3.7 ppm. Phenylcoumaran (B) α-, β- and γ-C/H correlations were relatively well-resolved from the other lignin signals, and from carbohydrate-derived signals, and were present in all wood types. The resinol (C) α-C/H and β-C/H correlations at ~85.0/4.67 and 53.7/3.06 ppm were present in all wood types; however, a resinol γ-C/H ~71.0/4.14 ppm correlation was only visible in compression wood (Figure 3-10). The cinnamyl-alcohol (X1) γ-C/H correlation was also present in all wood types at ~61.7/4.15 ppm.

Glycosyl residue peaks were often difficult to resolve due to the polysaccharide complexity and the many overlapping signals. Peaks common to those of oligosaccharide and polysaccharide standards are consistent with the presence of cellulose, *O*-acetyl-galactoglucomannans and 4-*O*-methylglucuronoxylans in all wood types. The peak at ~70.6/5.41 ppm from 2-*O*-acetyl-Man*p* was well resolved, and was present in all wood types, but the peak from 3-*O*-acetyl-Man*p* overlapped with that of the β-ether α-C/H correlation. The peak from the acetate methyl group was present in all wood samples at ~20.7/1.97 ppm (region not shown), consistent with the presence of acetylated polysaccharides. A well-resolved peak at ~60.1/3.42 ppm from the C6/H6 correlation of 4-*O*-α-D-methyl-Glc*p*A residues of 4-*O*-methylglucuronoxylans was present in all wood types.

As seen in the anomeric region, peaks from (1→4)-β-D-galactans were more abundant in compression wood than the other wood types, and elevated levels were also discernable in the highly overlapped aliphatic region, most notably from the C2/H2 peak at

~72.6/3.55 ppm (Figure 3-10). A peak which may be derived from internal residues of (1→3)-β-D-glucan at ~86.7/3.55 ppm, evident in the spectrum of the laminaritriose oligosaccharide standard, is present only in compression wood and is absent from the other wood types. Another peak in this region that is unique to compression wood may be from C5/H14 of hydroxyproline residues of extensins, if present, at ~72.9/4.51 ppm (Ulrich *et al.*, 2008). The origin of some peaks could not be assigned, although it is likely that these are from the glycosyl residues of polysaccharides as they were not present in the isolated lignin of loblolly pine reported in (Kim and Ralph, 2010).

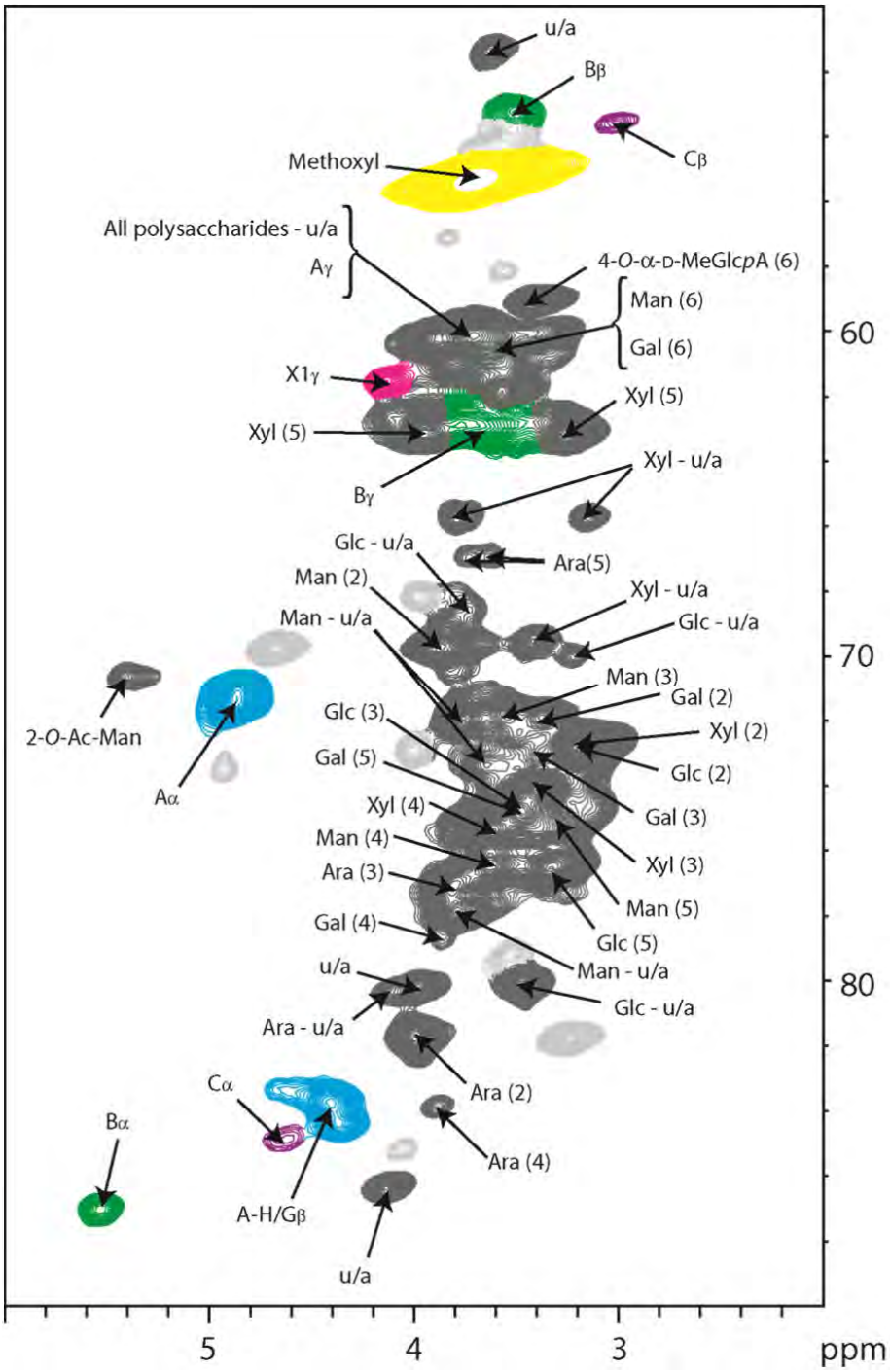

Figure 3-7 Aliphatic and lignin sidechain region of a 2D ^{13}C–^{1}H NMR correlation (HSQC) spectrum from a cell-wall gel of *Pinus radiata* normal wood. The C/H correlations of main chain (internal) residues of Glc*p* of cellulose, Man*p* of *O*-acetyl-galactoglucomannans, Xyl*p* of 4-*O*-methylglucuronoarabinoxylans, Gal*p* of (1→4)-β-D-galactans and Ara*f* of (1→5)-α-L-arabinans are indicated, e.g., Gal(2) = C2/H2 correlation of Gal*p* from (1→4)-β-D-galactan. The α-, β- and γ-C/H correlations of the lignin units are shown. uα = unassigned peak from glycosyl residues of polysaccharides.

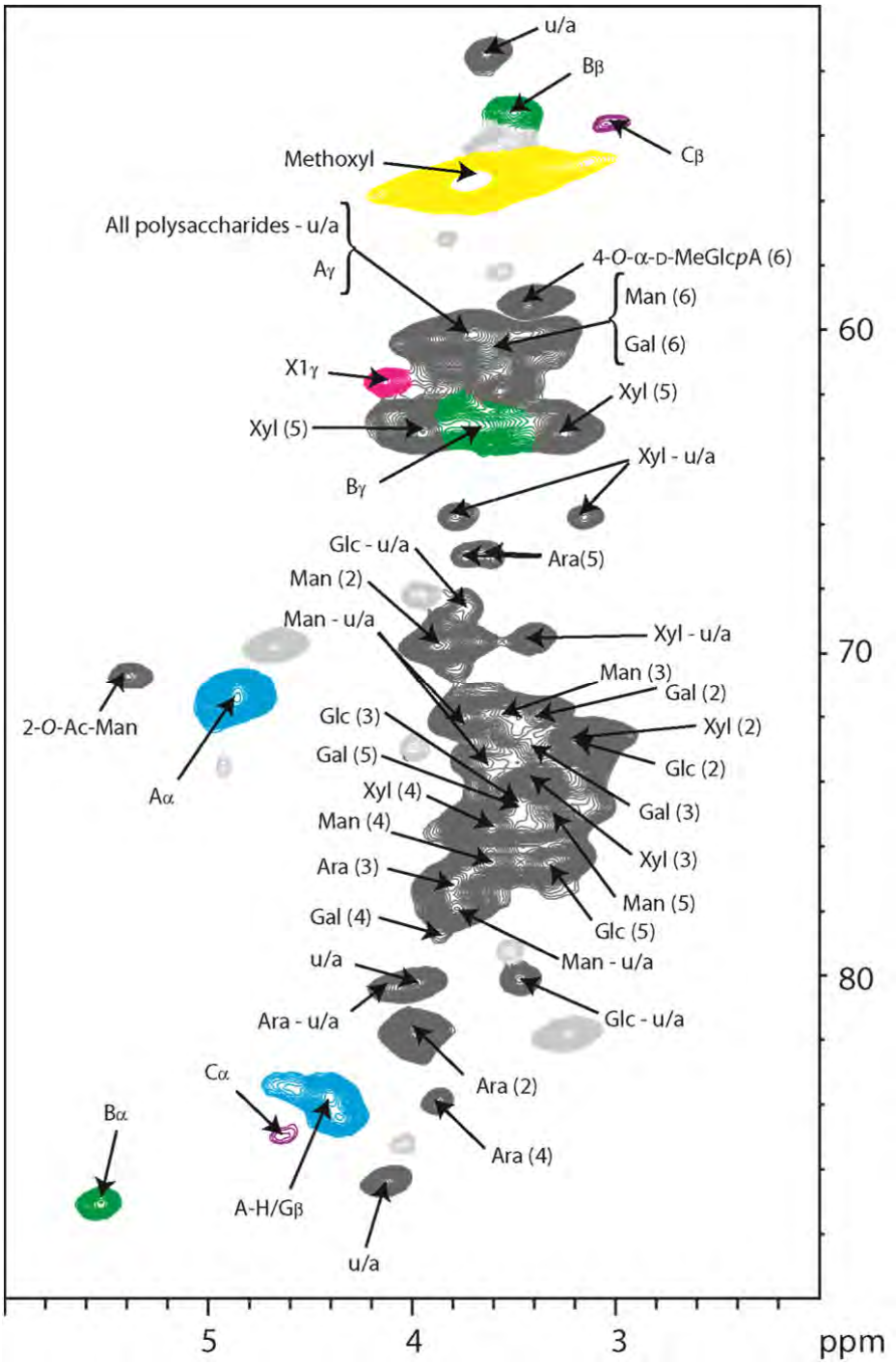

Figure 3-8 Aliphatic and lignin sidechain region of a 2D ^{13}C–^{1}H NMR correlation (HSQC) spectrum from a cell-wall gel of *Pinus radiata* opposite wood. The C/H correlations of main chain (internal) residues of Glc*p* of cellulose, Man*p* of *O*-acetyl-galactoglucomannans, Xyl*p* of 4-*O*-methylglucuronoarabinoxylans, Gal*p* of (1→4)-β-D-galactans and Ara*f* of (1→5)-α-L-arabinans are indicated, e.g., Gal(2) = C2/H2 correlation of Gal*p* from (1→4)-β-D-galactan. The α-, β- and γ-C/H correlations of the lignin units are shown. uα = unassigned peak from glycosyl residues of polysaccharides.

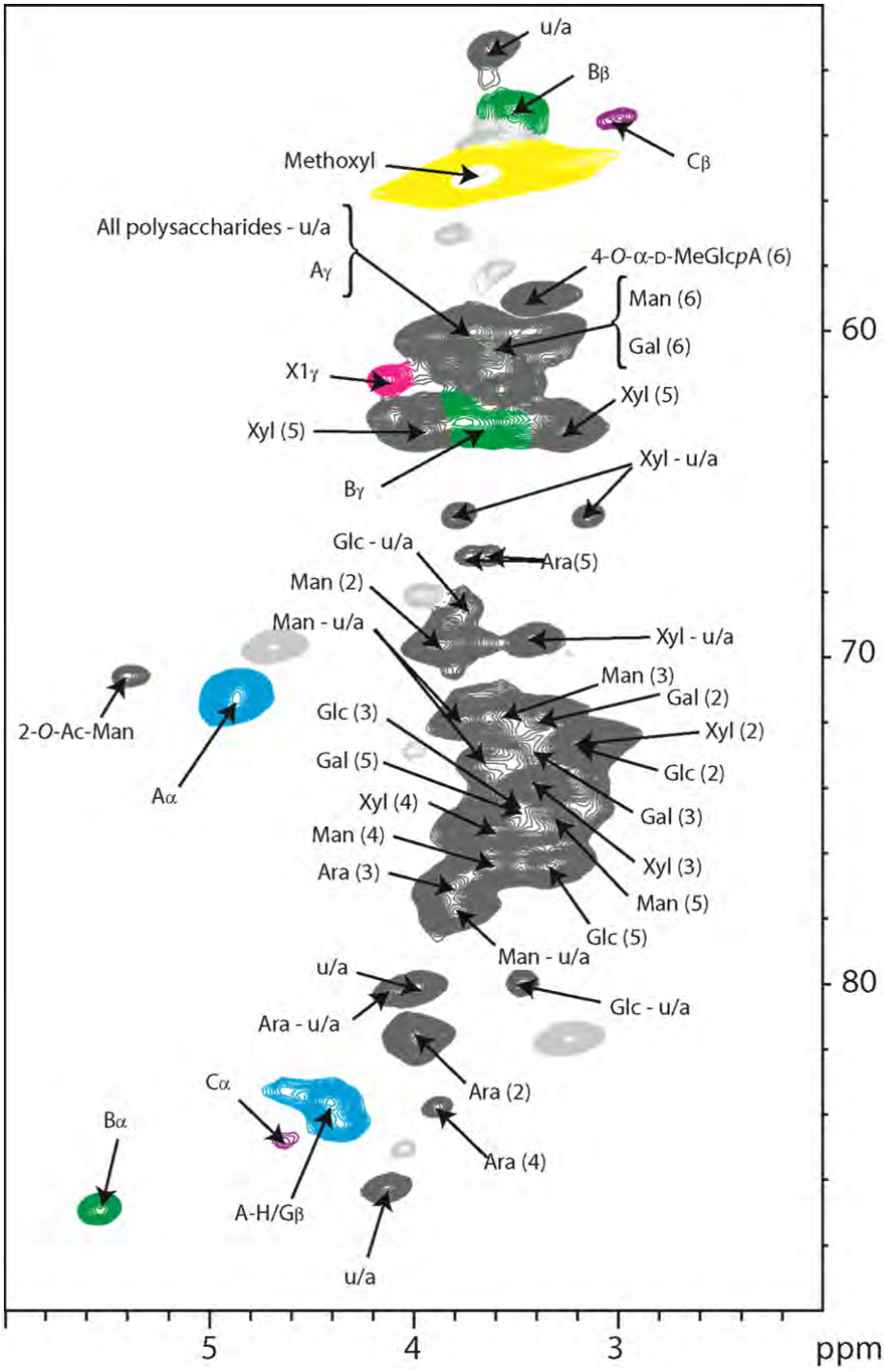

Figure 3-9 Aliphatic and lignin sidechain region of a 2D ^{13}C–^{1}H NMR correlation (HSQC) spectrum from a cell-wall gel of *Pinus radiata* flexure wood. The C/H correlations of main chain (internal) residues of Glc*p* of cellulose, Man*p* of *O*-acetyl-galactoglucomannans, Xyl*p* of 4-*O*-methylglucuronoarabinoxylans, Gal*p* of (1→4)-β-D-galactans and Ara*f* of (1→5)-α-L-arabinans are indicated, e.g., Gal(2) = C2/H2 correlation of Gal*p* from (1→4)-β-D-galactan. The α-, β- and γ-C/H correlations of the lignin units are shown. uα = unassigned peak from glycosyl residues of polysaccharides.

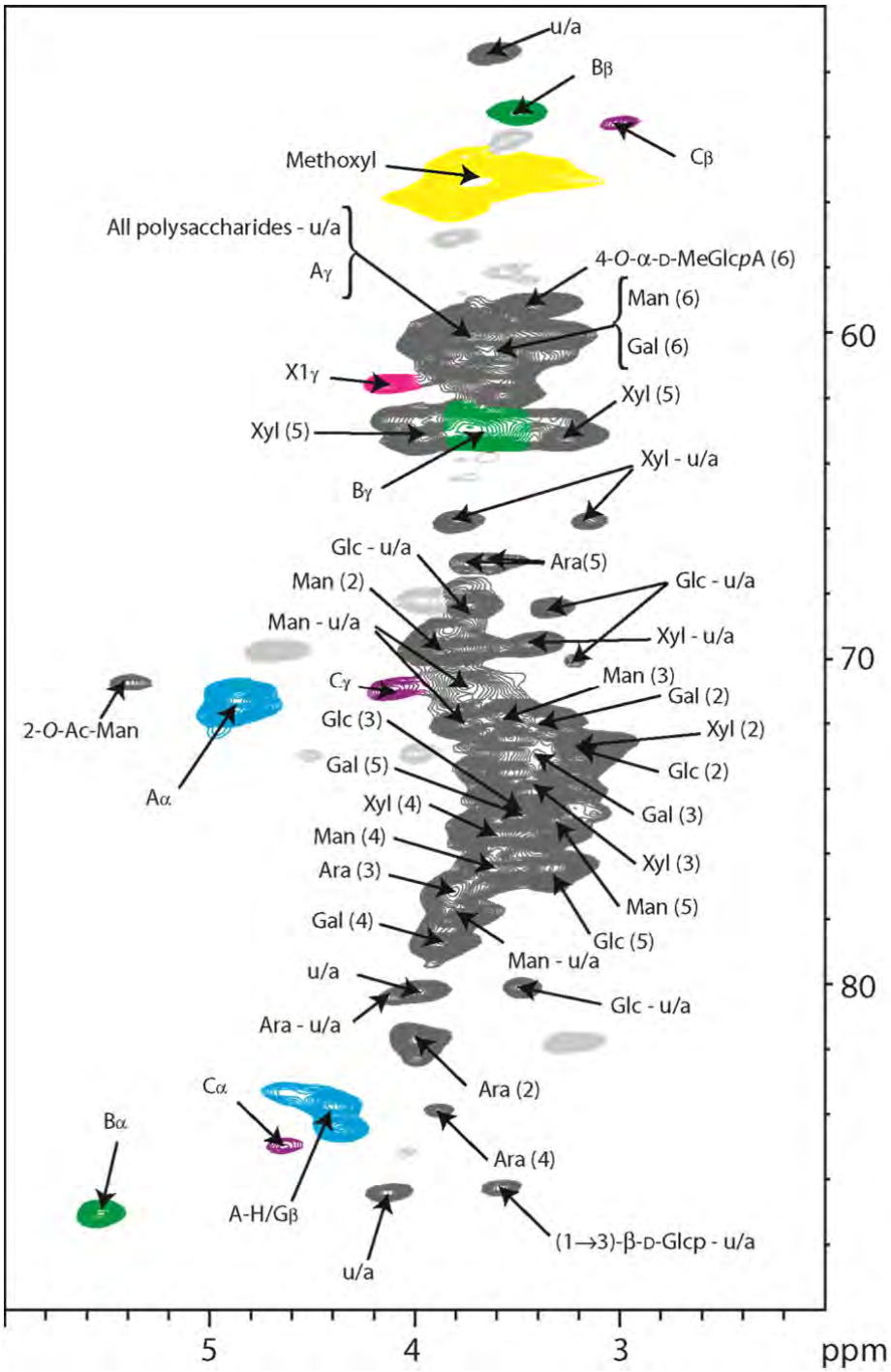

Figure 3-10 Aliphatic and lignin sidechain region of a 2D ^{13}C–^{1}H NMR correlation (HSQC) spectrum from a cell-wall gel of *Pinus radiata* compression wood. The C/H correlations of main chain (internal) residues of Glc*p* of cellulose, Man*p* of *O*-acetyl-galactoglucomannans, Xyl*p* of 4-*O*-methylglucuronoarabinoxylans, Gal*p* of (1→4)-β-D-galactans and Ara*f* of (1→5)-α-L-arabinans are indicated, e.g., Gal(2) = C2/H2 correlation of Gal*p* from (1→4)-β-D-galactan. The α-, β- and γ-C/H correlations of the lignin units are shown. uα = unassigned peak from glycosyl residues of polysaccharides.

3.4 Discussion

Analysis of the cell-wall polymers by 2D NMR spectroscopy supports the data from Chapter 2 and (Brennan *et al.*, 2012), which shows that flexure wood was more similar to normal wood than to severe compression wood. Flexure wood did not have differences in lignin composition, or structures of the cell-wall polysaccharides compared with normal wood and opposite wood. Compression wood however, had a different lignin composition compared with the non-compression wood samples, contained a higher proportion of (1→4)-β-D-galactans and also showed evidence of the presence of callose and extensins, consistent with this wood type having a significantly different cell-wall composition (Chapter 2, Brennan *et al.*, 2012).

That the lignin in compression wood of radiata pine contains H-units is well known (Bland, 1958; Nanayakkara *et al.*, 2009). Peaks from the H-units of lignin were found in detectable amounts in only the 2D NMR spectrum of compression wood. As the H-unit peaks were not evident in opposite wood, normal wood or flexure wood, either the proportions of H-units are too small to be detected using this method, or they are not present. Small proportions of H-units have been found in normal wood (Nanayakkara *et al.*, 2009; Kim and Ralph, 2010) and also in opposite wood (Whiting and Goring, 1982; Terashima and Fukushima, 1988; Yeh *et al.*, 2005; Yeh *et al.*, 2006; Tokareva *et al.*, 2007; Nanayakkara *et al.*, 2009). Although it has been suggested that the small proportions of H-units in the middle lamella lignin of normal wood of coniferous gymnosperms observed by Whiting and Goring (1982) was due to the presence of small amounts of compression wood (Westermark, 1985), it is possible that small proportions of H-units are present in the normal wood, opposite wood and flexure wood produced in this study, but are undetectable in these spectra using the parameters described here.

Compression wood lignin typically has fewer releasable β-ether interunit bonds than normal wood, and more phenylcoumaran (β-5) structures (Timell, 1982). The lignin of radiata pine compression wood has been shown by thioacidolysis to contain more β-5 and resinol (β-β) structures than the lignin of normal wood (Nanayakkara *et al.*, 2009). The spectrum of the aliphatic and lignin sidechain region for the compression wood sample in the current study shows that compression wood has a higher proportion of β-β structures, but a lower proportion of β-5 structures compared with the spectra of normal wood, opposite wood and flexure wood. Dibenzodioxocin and spirodienone structures were not detected in any of the wood types, despite being commonly found in isolated softwood lignins (Wagner *et al.*, 2007; Kim *et al.*, 2008; Wagner *et al.*, 2009; Kim and Ralph, 2010).

The peak from (1→4)-β-D-galactans in the anomeric region of the 2D NMR spectrum was more intense for compression wood than the non-compression wood samples, confirming that the higher proportions of galactosyl residues released from compression wood walls (Chapter 2) was due to a higher proportion of (1→4)-β-D-galactans, and not from a higher degree of substitution of galactoglucomannans with α-D-galactosyl residues, or from the presence of arabino-3,6-galactans such as those found in larch (*Larix laricina*) (Haq and Adams, 1961; Goellner *et al.*, 2011). The peak from the internal residues of (1→4)-β-D-galactans of compression wood was larger than the peak from the non-reducing residues, whereas in the normal wood, opposite wood, and flexure wood, the peaks from the internal residues and the non-reducing residues were a similar size. This suggests that the (1→4)-β-D-galactans present in compression wood have a higher degree of polymerisation than those of the (1→4)-β-D-galactans of non-compression wood. A lower degree of polymerisation could be indicative of rhamnogalacturonan I in the primary walls as suggested by Altaner et al. (2010). The degree of polymerisation of radiata pine compression wood (1→4)-β-D-galactans have been shown to be ~ 390 (Nanayakkara, 2007),

similar to that of tension wood galactan from beech (*Fagus sylvatica*) (Meier, 1962a) although significantly higher than the orignally reported compression wood galactan which had a degree of polymerisation of 52 (Bouveng and Meier, 1959a). It is certainly higher than the degrees of polymerisation reported for the (1→4)-β-D-galactans of rhamnogalacturonans of flax (*Linum usitatissimum*) bast fibres which consisted of short and long sidechains with degrees of polymerisation of 2 to 3 and ~28, respectively (Gur'janov *et al.*, 2006).

The polymers associated with compression wood as identified by 2D NMR spectroscopy may be synthesised in response to, or are present in, walls that are subjected to mechanical (abiotic) or biotic stress. One of the most well-known plant cell-wall polysaccharides synthesised in response to mechanical stress (wounding) or pathogen attack is (1→3)-β-D-glucan (callose or laricinan in compression wood) (Bacic *et al.*, 2009). The 2D NMR spectra in the current study show that (1→3)-β-D-glucans were present only in compression wood and not normal wood, opposite wood or flexure wood. This is consistent with the immunolabelling results of Altaner et al. (2010) which show that (1→3)-β-D-glucans are localised to the helical grooves of the S2 layer of severe compression wood, and are not present in normal wood. The (1→2)-linked β-L-Ara*f* residues in the 2D NMR spectrum of compression wood may be from the side-chains of extensins which are hydroxyproline-rich proteins glycosylated with short chains of (1→2)-linked β-L-Ara*f* residues (Bacic *et al.*, 1988). A peak in the aliphatic region of the compression wood spectrum was identified as being likely to originate from hydroxyproline residues of extensin, supporting the possibility that the walls of compression wood have more extensins than normal wood. Extensins have been reported in differentiating wood cell walls of *Pinus taeda* (Bao *et al.*, 1992). They are upregulated in response to mechanical wounding (Chen and Varner, 1985), and have been found complexed to pathogen-elicited extracellular lignin of suspension cell cultures of spruce (*P. abies*) (Lange *et al.*, 1995). This pathogen-elicited spruce lignin is similar to

compression wood lignin in that it contains a higher proportion of *p*-hydroxyphenyl (H) units (19%), and β–β interunit bonds than normal wood lignin (Lange *et al.*, 1995). Pathogen-elicited changes in the anatomy and chemical composition of wood of *Abies* spp. were also similar to those in compression wood (Timell, 1986). Infestation of *Abies* spp. with the woolly aphid *Adelges piceae* results in production of wood with rounded, thick-walled tracheids that produce intercellular spaces (Timell, 1986). Aphid-induced wood of fir (*Abies fraseri*) also contained H-units, and had a high galactosyl residue content compared with wood from un-infested fir (Balakshin *et al.*, 2005). Biotic stresses and abiotic stresses can thus induce similar changes to tracheid wall composition.

Comparisons of the 2D NMR spectra of the different wood types examined in the present study did not indicate that there were any differences in the structures or proportions of either the *O*–acetyl-galactoglucomannans or 4-*O*-methylglucuronoarabinoxylans among the wood types. Peaks from *O*-acetyl-Glc*p* were not evident in the 2D-NMR spectra, which is consistent with the finding of Willför et al. (2003a) for Norway spruce (*Picea abies*) that the glucosyl residues of galactoglucomannans are not acetylated, although acetylated glucosyl residues have been reported in the galactoglucomannans of Paraná pine (*Araucaria angustifolia*) (Katz, 1965). No peaks from acetylated xylosyl residues were evident in the 2D-NMR spectra, consistent with the current understanding that the 4-*O*-methylglucuronoarabinoxylans of softwoods are not acetylated (Whistler and Chen, 1991). However, the small proportions of water-soluble 4-*O*-methylglucuronoarabinoxylans of Norway spruce have been reported to contain *O*-acetyl-xyl*p* residues (Willför *et al.*, 2003a).

Further analysis of whole cell-wall gels of radiata pine by 2D NMR spectroscopy will be aided by compilation of the polysaccharide assignments compiled in this chapter, which have been published in Brennan *et al.* (2012). The cell-wall composition of radiata pine

compression wood is different from that of normal, opposite and flexure wood. Two-dimensional NMR spectroscopy could be used to examine differentiating compression wood tracheids to determine at which stage during tracheid differentiation the changes to cell-wall polymer structures occurs.

3.5 References

Altaner, C. M., E. N. Tokareva, M. C. Jarvis and P. J. Harris (2010). "Distribution of (1→4)-β-galactans, arabinogalactan proteins, xylans and (1→3)-β-glucans in tracheid cell walls of softwoods." Tree Physiology **30**: 782-793.

Bacic, A., G. B. Fincher and B. Stone, Eds. (2009). Chemistry, biochemistry, and biology of (1→3)-β-glucans and related polysaccharides, Academic Press.

Bacic, A., P. J. Harris and B. A. Stone (1988). Structure and function of plant cell walls. The biochemistry of plants. J. Preiss, Academic Press, San Diego, California. **14:** 297-371.

Balakshin, M. Y., E. A. Capanema, B. Goldfarb, J. Frampton and J. F. Kadla (2005). "NMR studies on Fraser fir *Abies fraseri* (Pursh) Poir. lignins." Holzforschung **59**: 488-496.

Bao, W., D. M. O'Malley and R. R. Sederoff (1992). "Wood contains a cell-wall structural protein." Proceedings of the National Academy of Sciences USA **89**: 6604-6608.

Bland, D. E. (1958). "The chemistry of reaction wood. Part I. The lignins of *Eucalyptus goniocalyx* and *Pinus radiata*." Holzforschung **12**: 36-43.

Bouveng, H. O. and H. Meier (1959). "Studies on a galactan from Norwegian spruce compression wood (*Picea abies* Karst.)." Acta Chemica Scandinavica **13**: 1884-1889.

Brennan, M., J. P. McLean, C. M. Altaner, J. Ralph and P. J. Harris (2012). "Cellulose microfibril angles and cell-wall polymers in different wood types of *Pinus radiata*." Cellulose **Online First**.

Capek, P., J. Alföldi and D. Lisková (2002). "An acetylated galactoglucomannan from *Picea abies* L. Karst." Carbohydrate Research **337**: 1033-1037.

Cavagna, F., H. Deger and J. Puls (1984). "2D-N.M.R. analysis of the structure of an aldotriouronic acid obtained from birch wood." Carbohydrate Research **129**: 1-8.

Chen, J. and J. E. Varner (1985). "An extracellular matrix protein in plants: characterization of a genomic clone for carrot extensin." The EMBO Journal **4**: 2145-2151.

Colquhoun, I. J., M.-C. Ralet, J.-F. Thibault, C. B. Faulds and G. Williamson (1994). "Structure identification of feruloylated oligosaccharides from sugar-beet pulp by NMR spectroscopy." Carbohydrate Research **263**: 243-256.

Dais, P. and A. S. Perlin (1982). "High-field, ^{13}C-N.M.R. spectroscopy of β-D-glucans, amylopectin, and glycogen." Carbohydrate Research **100**: 103-116.

Davis, A. L., R. A. Hoffmann, A. L. Russell and M. Debet (1995). "^{1}H- and ^{13}C-NMR characterization of the digalactosylmannopentaose liberated from legume seed galactomannan by β-mannanase action." Carbohydrate Research **271**: 43-54.

Davis, E. A., C. Derouet, C. Herve Du Penhoat and C. Morvan (1990). "Isolation and an N.M.R. study of pectins from flax (*Linum usitatissimum* L.)." Carbohydrate Research **197**: 205-215.

Dudley, R. L., C. A. Fyfe, P. J. Stephenson, Y. Deslandes, G. K. Hamer and R. H. Marchessault (1983). "High-resolution carbon-13 CP/MAS NMR spectra of solid cellulose oligomers and the structure of cellulose II." Journal of the American Chemical Society **105**: 2469-2472.

Excoffier, G., R. Nardin and M. R. Vignon (1986). "Détermination de la structure primaire d'un acide tri-d-xylo-4-*O*-méthyl-D-glucuronique par R.M.N. à deux dimensions." Carbohydrate Research **149**: 319-328.

Flugge, L. A., J. T. Blank and P. A. Petillo (1999). "Isolation, modification, and NMR assignments of a series of cellulose oligomers." Journal of the American Chemical Society **121**: 7228-7238.

Girault, R., F. Bert, C. Rihouey, A. Jauneau, C. Morvan and M. Jarvis (1997). "Galactans and cellulose in flax fibres: Putative contributions to the tensile strength." International Journal of Biological Macromolecules **21**: 179-188.

Goellner, E. M., J. Utermoehlen, R. Kramer and B. Classen (2011). "Structure of arabinogalactan from *Larix laricina* and its reactivity with antibodies directed against type-II-arabinogalactans." Carbohydrate Polymers **86**: 1739-1744.

Gur'janov, O. P., T. Gorshkova, M. A. Kabel, H. A. Schols and v. J. E. G. Dam (2006). "Structural characterization of tissue-specific galactan from flax fibers by 1H NMR and MALDI TOF mass spectrosmetry." Russian Journal of Bioorganic Chemistry **32**: 558 - 567.

Habibi, Y., M. Mahrouz, M.-F. Marais and M. R. Vignon (2004). "An arabinogalactan from the skin of *Opuntia ficus-indica* prickly pear fruits." Carbohydrate Research **339**: 1201-1205.

Habibi, Y. and M. R. Vignon (2005). "Isolation and characterization of xylans from seed pericarp of *Argania spinosa* fruit." Carbohydrate Research **340**: 1431-1436.

Hannuksela, T. and C. Hervé du Penhoat (2004). "NMR structural determination of dissolved *O*-acetylated galactoglucomannan isolated from spruce thermomechanical pulp." Carbohydrate Research **339**: 301-312.

Haq, S. and G. A. Adams (1961). "Structure of an arabinogalactan from tamarack (*Larix laricina*)." Canadian Journal of Chemistry **30**: 1563-1573.

Hedenström, M., S. Wiklund-Lindström, T. Öman, F. Lu, L. Gerber, P. F. Schatz, B. Sundberg and J. Ralph (2009). "Identification of lignin and polysaccharide modifications in *Populus* wood by chemometric analysis of 2D NMR spectra from dissolved cell walls." Molecular Plant **2**: 933-942.

Jensen, J. K., H. Kim, J.-C. Cocuron, R. Orler, J. Ralph and C. G. Wilkerson (2011). "The DUF579 domain containing proteins IRX15 and IRX15-L affect xylan synthesis in Arabidopsis." The Plant Journal **66**: 387-400.

Katz, G. (1965). "The location and significance of the *O*-acetyl groups in a glucomannan from Paraña pine." TAPPI Journal **48**: 34-41.

Kim, H. and J. Ralph (2010). "Solution-state 2D NMR of ball-milled plant cell wall gels in DMSO-d_6/pyridine-d_5." Organic and Biomolecular Chemistry **8**: 576-591.

Kim, H., J. Ralph and T. Akiyama (2008). "Solution-state 2D NMR of ball-milled plant cell wall gels in DMSO-d_6." Bioenergy Research **1**: 65-66.

Lange, B. M., C. Lapierre and H. Sandermann Jr (1995). "Elicitor-induced spruce stress lignin (structural similarity to early developmental lignins)." Plant Physiology **108**: 1277-1287.

Lu, F. and J. Ralph (2003). "Non-degradative dissolution and acetylation of ball-milled plant cell walls: high-resolution solution-state NMR." The Plant Journal **35**: 535-544.

Lundqvist, J., A. Teleman, L. Junel, G. Zacchi, O. Dahlman, F. Tjerneld and H. Stålbrand (2002). "Isolation and characterization of galactoglucomannan from spruce (*Picea abies*)." Carbohydrate Research **48**: 29-39.

McCleary, B. V., E. Nurthen, F. R. Taravel and J.-P. Joseleau (1983). "Characterisation of the oligosaccharides produced on hydrolysis of galactomannan with β-D-mannanase." Carbohydrate Research **118**: 91-109.

Meier, H. (1962). "Studies on a galactan from tension wood of beech (Fagus-Silvatica L.)." Acta Chemica Scandinavica **16**: 2275-2283.

Moulthrop, J. S., R. P. Swatloski, G. Moyna and R. D. Rogers (2005). "High-resolution ^{13}C NMR studies of cellulose and cellulose oligomers in ionic liquid solutions." Chemical Communications **12**: 1557-1559.

Nanayakkara, B. (2007). Chemical characterisation of compression wood in plantation grown *Pinus radiata*. Doctoral thesis Doctoral thesis, The University of Waikato.

Nanayakkara, B., M. Manley-Harris, I. D. Suckling and L. A. Donaldson (2009). "Quantitative chemical indicators to assess the gradation of compression wood." Holzforschung **63**: 431-439.

Ralph, J., J. Marita, S. A. Ralph, R. D. Hatfield, F. Lu, R. M. Ede, J. Peng, S. Quideau, R. F. Helm, J. H. Grabber, H. Kim, G. Jimenez-Monteon, Y. Zhang, H. J. G. Jung, L. L. Landucci, J. J. MacKay, R. R. Sederoff, C. Chapple and A.-M. Boudet (1999). Solution-state NMR of lignins. Advances in lignocellulosics characterization. D. S. Argyropoulos. Atlanta, GA., TAPPI Press**:** 55-108.

Rencoret, J., A. Gutiérrez, L. Nieto, J. Jiménez-Barbero, C. B. Faulds, H. Kim, J. Ralph, Á. T. Martínez and J. C. del Río (2011). "Lignin composition and structure in young versus adult *Eucalyptus globulus* plants." Plant Physiology **155**: 667-682.

Rencoret, J., G. Marques, A. Gutiérrez, L. Nieto, J. I. Santos, J. Jiménez-Barbero, Á. T. Martínes Ferrer and J. C. d. Río Andrade (2009). "HSQC-NMR analysis of lignin in woody (*Eucalyptus globulus* and *Picea abies*) and non-woody (*Agave sisalana*) ball-milled plant materials at the gel state." Holzforschung **63**: 691-698.

Teleman, A., J. Lundqvist, F. Tjerneld, H. Stålbrand and O. Dahlman (2000). "Characterization of acetylated 4-*O*-methylglucuronoxylan isolated from aspen employing ^{1}H and ^{13}C NMR spectroscopy." Carbohydrate Research **329**: 807-815.

Teleman, A., M. Nordström, M. Tenkanan, A. Jacobs and O. Dahlman (2003). "Isolation and characterization of *O*-acetylated glucomannans from aspen and birch wood." Carbohydrate Research **338**: 525-534.

Terashima, N. and K. Fukushima (1988). "Heterogeneity in formation of lignin - XI: An autoradiographic study of the heterogeneous formation and structure of pine lignin." Wood Science and Technology **22**: 259-270.

Timell, T. E. (1982). "Recent progress in the chemistry and topochemistry of compression wood." Wood Science and Technology **16**: 83-122.

Timell, T. E. (1986). Compression wood in gymnosperms. Berlin, Springer-Verlag.

Tokareva, E. N., A. V. Pranovich, P. Fardim, G. Daniel and B. Holmbom (2007). "Analysis of wood tissues by time-of-flight secondary ion mass spectrometry." Holzforschung **61**: 647-655.

Ulrich, E. L., H. Akutsu, J. F. Doreleijers, Y. Harano, Y. E. Ioannidis, J. Lin, M. Livny, S. Mading, D. Maziuk, Z. Miller, E. Nakatani, C. F. Schulte, D. E. Tolmie, R. Kent Wenger, H. Yao and J. L. Markley (2008). "BioMagResBank." Nucleic Acids Research **36**: D402-D408.

van Hazendonk, J. M., E. J. M. Reinerik, P. de Waard and J. E. G. van Dam (1996). "Structural analysis of acetylated hemicellulose polysaccharides from fibre flax (*Linum usitatissimum* L.)." Carbohydrate Research **291**: 141-154.

Vignon, M. R. and C. Gey (1998). "Isolation, ^{1}H and ^{13}C NMR studies of (4-*O*-methyl-glucurono)-xylans from luffa fruit fibres, jute bast fibres and mucilage of quince tree seeds." Carbohydrate Research **307**: 107-111.

Wagner, A., L. Donaldson, H. Kim, L. Phillips, H. Flint, D. Steward, K. Torr, G. Koch, U. Schmitt and J. Ralph (2009). "Suppression of 4-coumarate-CoA ligase in the coniferous gymnosperm *Pinus radiata*." Plant Physiology **149**: 370-383.

Wagner, A., J. Ralph, T. Akiyama, H. Flint, L. Phillips, K. Torr, B. Nanayakkara and L. Te Kiri (2007). "Exploring lignification in conifers by silencing hydroxycinnamoyl-CoA:shikimate hydroxycinnamoyltransferase in *Pinus radiata*." Proceedings of the National Academy of Sciences **104**: 11856-11861.

Westermark, U. (1985). "The occurrence of *p*-hydroxyphenylpropane units in the middle-lamella lignin of spruce (*Picea abies*)." Wood Science and Technology **19**: 223-232.

Whistler, R. L. and C.-C. Chen (1991). Hemicellulose. Wood structure and composition. M. Lewin and I. S. Goldstein. New York, Marcel Dekker**:** 287-319.

Whiting, P. and D. Goring (1982). "Chemical characterization of tissue fractions from the middle lamella and secondary wall of black spruce tracheids." Wood Science and Technology **16**: 261-267.

Willför, S., R. Sjöholm, C. Laine, M. Roslund, J. Hemming and B. Holmbom (2003). "Characterisation of water-soluble galactoglucomannans from Norway spruce wood and thermomechanical pulp." Carbohydrate Polymers **52**: 175-187.

Yeh, T.-F., J. L. Braun, B. Goldfarb, H.-m. Chang and J. F. Kadla (2006). "Morphological and chemical variations between juvenile wood, mature wood, and compression wood of loblolly pine (*Pinus taeda* L.)." Holzforschung **60**: 1-8.

Yeh, T.-F., B. Goldfarb, H.-m. Chang, I. Peszlen, J. L. Braun and J. F. Kadla (2005). "Comparison of morphological and chemical properties between juvenile wood and compression wood of loblolly pine." Holzforschung **59**: 669-674.

Yelle, D. J., J. Ralph and C. R. Frihart (2008). "Characterization of nonderivatized plant cell walls using high-resolution solution-state NMR spectroscopy." Magnetic Resonance in Chemistry **46**: 508-517.

Chapter 4 - Pyrolysis gas-chromatography/mass-spectrometry of compression wood and opposite wood

4.1 Introduction

Analytical pyrolysis methods such as pyrolysis followed by combined capillary gas-chromatography mass spectrometry (pyrolysis-GC/MS) yields detailed information about the composition of cell walls, and have been used in research on wood, forages and biomass for biofuel production (Mohan *et al.*, 2006; Torri *et al.*, 2010). Pyrolysis of plant cell walls produces volatile degradation products from both lignin and polysaccharides. These products can be separated by gas chromatography and identified by their retention times and by their mass spectra. Quantification of lignin in softwoods by pyrolysis-GC/MS has been investigated, and found to have an accuracy similar to that of Klason lignin measurement, even when compression wood samples were included (Alves *et al.*, 2006; Alves *et al.*, 2008). Not only can pyrolysis-GC/MS be used to determine the lignin content of wood, but the characteristic pyrolysis degradation products of lignin make it a useful tool for the structural analysis of ligin (Möller *et al.*, 2003; Rencoret *et al.*, 2011). For example, structural differences between compression wood and normal wood lignin of radiata pine have been determined using analytical pyrolysis (Nanayakkara, 2007; Mast *et al.*, 2009). Differences in the pyrolysis degradation products have allowed pyrolysis-GC/MS to be used, in conjunction with multivariate statistical analysis, to distinguish different species of oak (*Quercus*) from each other (Nonier *et al.*, 2006), and to discriminate genetically modified poplar (*Populus*) clones from wild-type clones (Meier *et al.*, 2005).

The pyrolysis products of lignin include phenolic compounds that may be substituted with a methoxyl group, or groups, on the benzene ring. Having no methoxyl

groups indicates that they originate from the H-units of lignin (Meier and Faix, 1992; Serban, 1998). A methoxyl group at C3 indicates that they are derived from the G-units of lignin and a methoxyl on C3 and on C5 indicates that they are from S-units (Figure 4-1). These products can have a short side chain of one to three carbons. For H-unit derived products, a sidechain of two or three carbons identifies them as being derived from lignin. This is an important distinguishing feature as the pyrolysis products of polysaccharides can also produce phenolic compounds with a single carbon sidechain (Serban, 1998), e.g. 4-methyl-phenol (Figure 4-1). The structures of some of the compounds that can be produced by pyrolysis of both polysaccharides and H-units of lignin, and those that can only be produced only by pyrolysis of the H-units of lignin are shown in Figure 4-1.

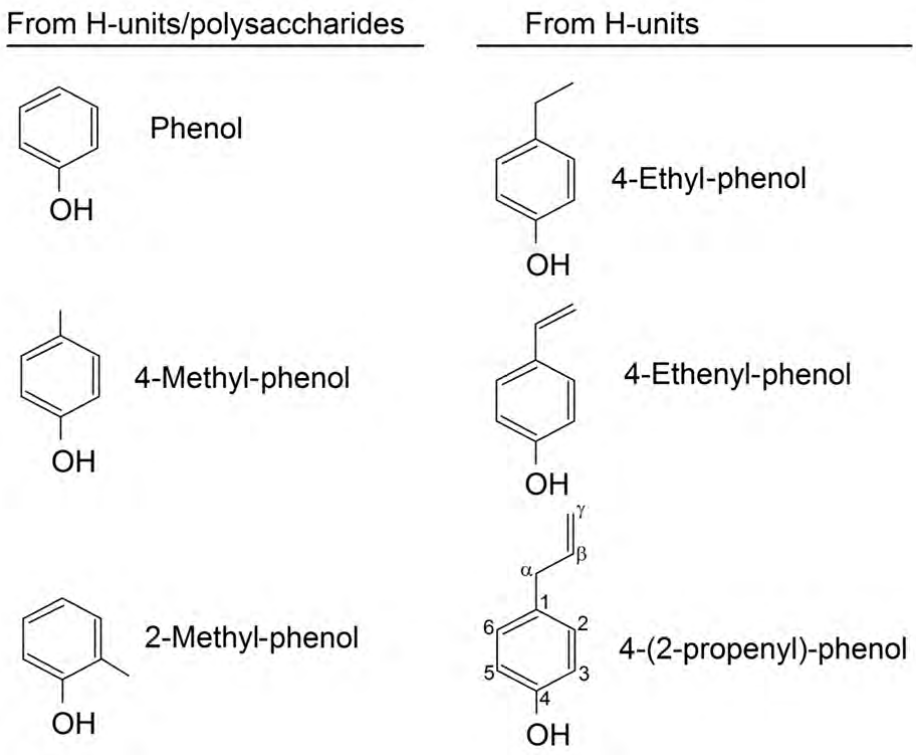

Figure 4-1 Phenolic compounds that are produced by pyrolysis of the H-units of lignin and also by pyrolysis of the polysaccharides have either no sidechain, or a single-carbon sidechain. Those that are produced by pyrolysis of the H-units of lignin have longer sidechains of two or three carbons. Note that ethenyl is often called "vinyl", and propenyl is often called "allyl".

Other pyrolysis products of polysaccharides result from cleavage of the glycosidic bonds in the polysaccharides and the formation of anhydrosugars from the monosaccharides. The production of the stable compounds (1,6)-anhydrohexopyranose and (1,6)-anhydrohexofuranose from (1→4)-linked hexosans is well characterised (Serban, 1998). For example, the dominant pyrolysis products of cellulose are 1,6-anhydro-β-D-glucopyranose, followed by 1,6-anhydro-β-D-glucofuranose (Shafizadeh and Fu, 1973; Ponder *et al.*, 1992). Analytical pyrolysis is not as efficient for polysaccharides as it is for lignin, due to a high proportion of the polysaccharides degrading to mostly low molecular weight compounds (e.g. furfurals). Arabinose however, does pyrolyse relatively efficiently to an anhydrosugar (Ralph and Hatfield, 1991).

Some of the inorganic cations that are naturally present in wood, such as sodium and potassium, are known to act as catalysts in pyrolytic reactions, particularly those of polysaccharide pyrolysis, resulting in increased production of low molecular weight compounds such as carbon dioxide, carbon monoxide, acetic acid and furfurals which are not diagnostic of the polymer from which they are derived (Pan and Richards, 1989; Kleen and Gellerstedt, 1995). Calcium cations on the other hand, do not act as catalysts in pyrolytic reactions, and exchange of sodium cations for calcium cations by washing samples with a solution of calcium chloride has been shown to increase the yields of anhydrosugars (Kleen and Gellerstedt, 1995). As the pyrolysis products of compression wood (1→4)-β-D-galactans were of potential interest in this study, pre-treatment of the samples by washing with a solution of calcium-chloride was investigated.

The aim of this chapter was to identify pyrolysis products that could be used to indicate the presence of compression wood. The relative proportions of the pyrolysis products of compression wood and opposite wood were calculated. Products were identified that were unique to compression wood and were therefore considered to be indicators of compression wood. Products that differed significantly in their relative proportions between compression wood and opposite wood were also identified. Pyrolysis products that had different relative proportions among compression wood samples were also identified. The proportions of pyrolysis products were then related to the cell-wall compositions (presented in Chapter 2) of the compression wood and opposite wood samples examined.

4.2 Materials and methods

4.2.1 Samples

Wood samples were from the Amberley field trial described in Chapter 2 and Apiolaza et al. (2011b). Twelve representative samples (6 × compression wood and 6 × opposite wood) were chosen for pyrolysis. A lupin seed (1→4)-β-D-galactan preparation, treated with α-L-arabinofuranosidase to remove any attached arabinosyl residues, (P-GALLU, Megazyme International Ireland Ltd.) was used as a reference standard for (1→4)-β-D-galactans.

4.2.2 Sample preparation

Samples were prepared for pyrolysis in three ways. "Coarse" samples were prepared by milling wood in a Wiley® mini-mill (Thomas Scientific, Swedesboro, NJ) to pass a 40-mesh screen (422 µm pore size). "Fine" samples were prepared from a fraction (~50 mg) of each of the coarse samples, which was cooled in liquid nitrogen for 15 min before milling in liquid nitrogen using a SPEX CertiPrep 6750 Freezer/Mill (Spex, Metuchen, NJ, USA). Total milling time was 3 min (3 cycles of milling for 1 min each time followed by a 2 min cooling period). "Calcium-treated" samples were prepared by washing portions (~25 mg) of the fine samples in 0.1 M $CaCl_2$ (500 µl, 30 min), followed by washing twice in double-distilled water (500 µl, 10 min each time). Samples were dried in a vacuum oven (35°C) containing phosphorous pentoxide (18 h), and weighed (~65-85 µg) into a pyrolysis cup. Three aliquots of each sample were analysed.

4.2.3 Pyrolysis gas-chromatography/mass-spectrometry (pyrolysis GC/MS)

The pyrolysis interface was set at 360°C, and pyrolysis was done at 500°C with a Py-2020iD micro-furnace pyrolyser (Frontier Laboratories Ltd., Fukushima, Japan). Combined gas-chromatography/mass-spectrometry was done on an Agilent 6890 gas chromatograph fitted with a flame ionisation detector (FID) and an Agilent 5973 mass selective detector. Split injection (1:30) was done onto a DB-1701 fused silica column (60 m x 0.25 mm x 0.25 µm film thickness, Agilent) with helium (flow rate 1 ml min^{-1}) as the carrier gas. The oven programme was 4 min at 45°C followed by ramping at 3°C min^{-1} to 280°C and held for 20 min. The GC/MS interface and FID were at 280°C. The ionisation energy of the mass selective detector was 70 eV, with a scan range of 15-600 m/z. Samples were analysed in random order. Product identification was done at the Center of Wood Science and Technology, University of Hamburg, Germany, using an in-house-library of mass spectra (Faix *et al.*, 1990; Faix *et al.*, 1991) and the NIST 02 library (NIST/EPA/NIH Mass Spectral Database., 2002). Pyrolysis of the lupin seed (1→4)-β-D-galactan preparation was done in the same way. A blank run, containing no sample, was done after every four samples.

4.2.3.1 Processing of chromatograms

The total ion current chromatograms were baseline corrected using metAlign software, version 080311 (Lommen, 2009). Integration of peaks was done using Wsearch32 Mass Spectrometry software, version 1.6.2005 (http://www.wsearch.com.au), using a minimum threshold for peak area of 0.05%. Reported values were normalised by dividing the peak

area of each product by the sum of all integratable peak areas, and are expressed as a percentage. Statistical analysis was done using the statistical analysis software R (R Development Core Team, 2011).

4.3 Results

4.3.1 Identification of pyrolysis products from (1→4)-β-D-galactans

The pyrolysis products of lupin seed (1→4)-β-D-galactan were used to identify (1→4)-β-D-galactan pyrolysis products in the chromatograms of the wood samples. The pyrolysis chromatogram of lupin seed (1→4)-β-D-galactan yielded three major pyrolysis products at retention times 53.83, 58.92 and 65.28 min (Figure 4-2, A). The mass spectrum of each of the pyrolysis products is shown in Figure 4-2, B-D. Comparison of the retention order of the products, and their mass spectra, with those in the literature (Budgell *et al.*, 1987; Faix *et al.*, 1991) indicated that the first product (Figure 4-2, B) was (1,6)-anhydrogalactopyranose, the second product (Figure 4-2, C) was (1,4)-anhydrogalactopyranose and the third product (Figure 4-2, D) was (1,6)-anhydrogalactofuranose. Identification of (1,4)-anhydrogalactopyranose is tentative as there is no mass spectrum of this product in the available literature, however the retention order is identical to that of Budgell et al. (1987), who analysed pure samples of the above anhydrogalactosyl compounds on the same type of DB-1701 column.

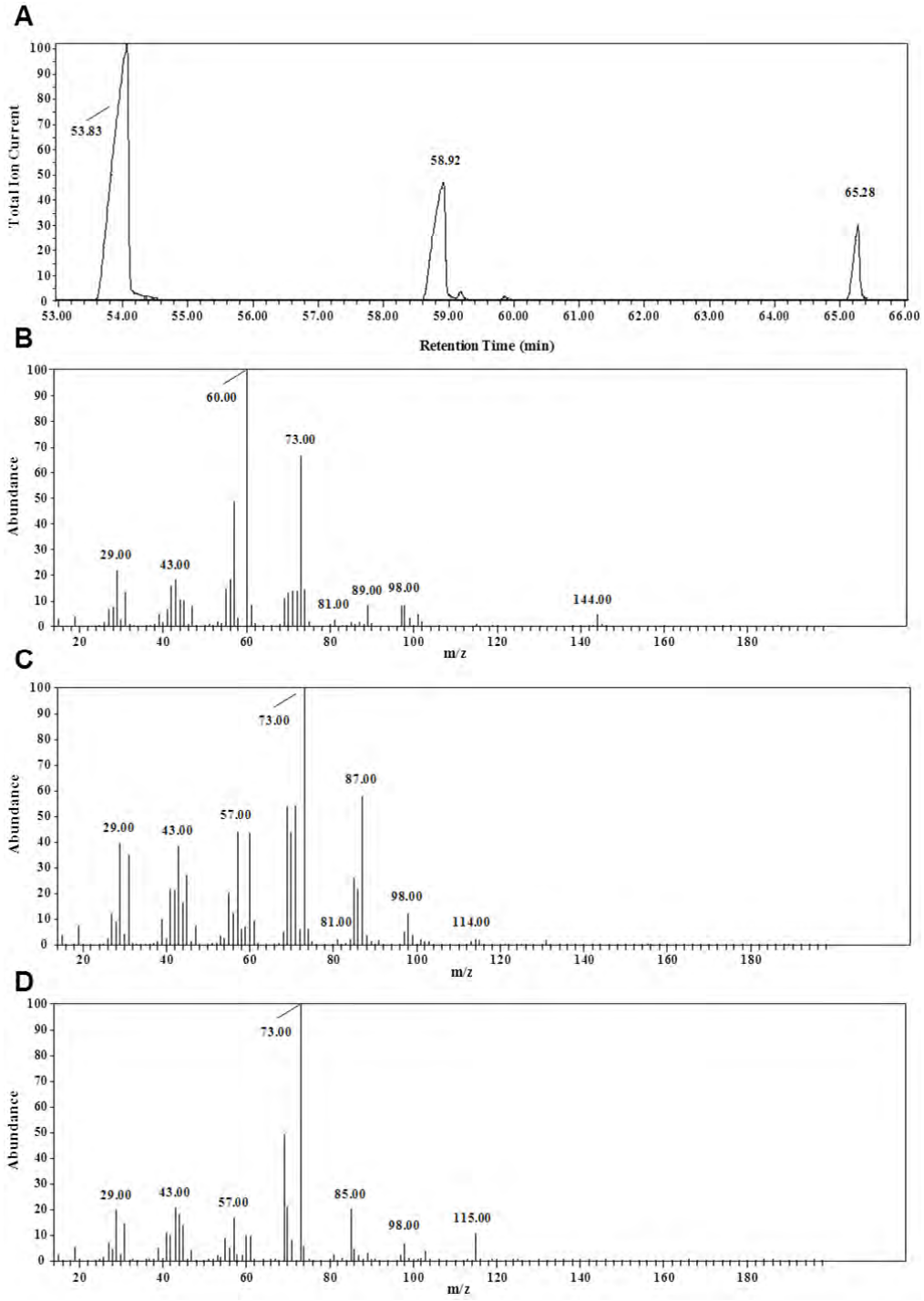

Figure 4-2 Pyrolysis products of lupin seed (1→4)-β-D-galactan measured on the DB-1701 column. (A) Total ion current chromatogram showing the three major pyrolysis products of (1→4)-β-D-galactan, the mass spectra of these products with retention times of (B) 53.83 min, (C) 58.92 min and (D) 65.28 min are shown.

The lupin seed (1→4)-β-D-galactan was analysed on a newer DB-1701 column (with the same dimensions) than the wood samples, which were analysed on an older DB-1701 column. There was a shift in retention time of the products, which eluted ~ 2.2 minutes faster on the older column. The shift in retention time was determined by analysing the same compression wood sample using the newer and older columns (Figure 4-3, A,B), and comparing the retention times of the pyrolysis products. The retention time of (1,6)-anhydrogalactopyranose (peak 131) was 53.83 min on the newer column and 51.57 min on the older column. The retention time of (1,4)-anhydrogalactopyranose (peak 145) was 58.92 min on the newer column and 56.67 min on the older column.

(1,6)-Anhydrogalactofuranose was not present in sufficient amounts in the wood samples to be detected on either the newer or the older column. The mass spectrum of (1,6)-anhydrogalactopyranose from the compression wood sample obtained using the newer column (Figure 4-3, C) and the older column (Figure 4-3, D) was the same. The mass spectra of (1,4)-anhydrogalactopyranose were also the same between the newer and older column.

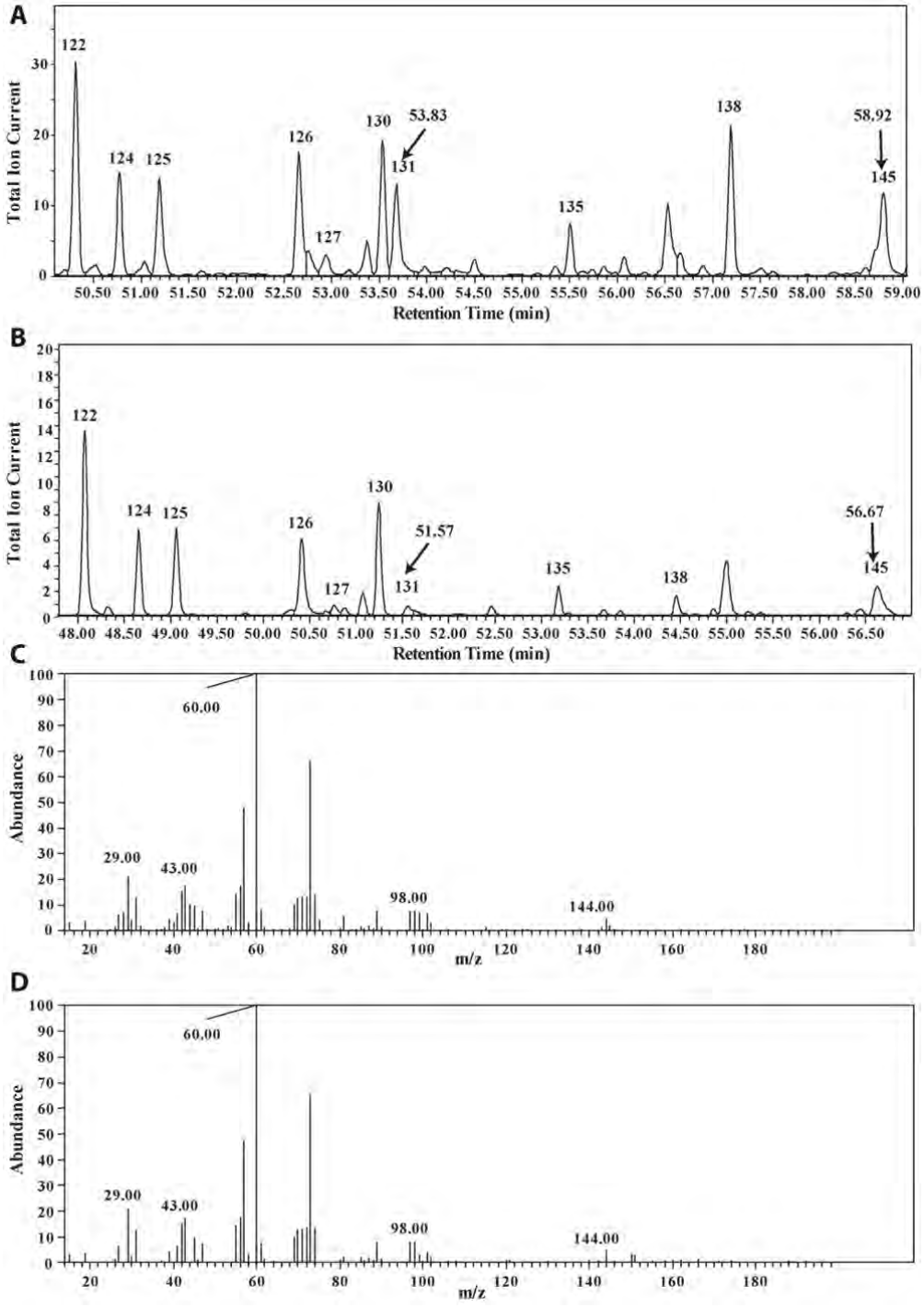

Figure 4-3 The retention times of (1,6)-anhydrogalactopyranose (peak 131, arrows) and (1,4)-anhydrogalactopyranose (peak 145, arrows) in a compression wood sample obtained using (A) the newer DB-1701 column and (B) the older DB-1701 column. The mass spectra of (1,6)-anhydrogalactopyranose are shown for the sample obtained using (C) the newer column and (D) the older column, which were used to confirm the identity of the peak.

4.3.2 Pyrolysis products of opposite and compression woods

To compare differences in proportions of pyrolysis products between compression wood and opposite wood, a retention-time-range of interest was selected (29.0 to 64.0 min), which corresponds to the phenolic compounds and anhydrosugar pyrolysis products of lignin and polysaccharides. This region was chosen because these compounds give structural information about the composition of the lignin or the polysaccharides from which they are derived. The retention times, identification and mass spectra of the pyrolysis products are shown in Table 4-1. These products were derived from G-units (G) or H-units (H) of lignin, from polysaccharides (CHO), or were phenolic compounds with no methoxyl groups and a single carbon sidechain which consequently could have been derived from either H-units of lignin or polysaccharides (H/CHO) Table 4-1. No products were identified in any of the chromatograms other than those that would be expected from the pyrolysis of wood. For a full list of identified pyrolysis products please see the appendix. All of the results presented in the following sections are from the wood samples measured on the older DB-1701 column.

There were six pyrolysis products that were unique to compression wood, all of which were H-unit derived products, or polysaccharide-derived products. The following products did not occur in any opposite wood samples: 4-(2-propenyl)-phenol, (1,6)-anhydrogalactopyranose and an unknown product at retention time 63.89 min (peak 153). Other products were only present in small proportions in one aliquot of one opposite wood sample: 4-ethyl-phenol, or they were present in small proportions in one aliquot of two opposite wood samples: 4-propyl-phenol and 4-hydroxy-benzaldehyde, and

their occurrence in opposite wood can most likely be considered to be experimental artefacts, possibly due to contamination.

Table 4-1 Pyrolysis products identified in the retention-time-range of interest in opposite wood and compression wood samples

Peak	RT	Identification	H/G/CHO	CW/OW	Base ion (100)	Ion m/z (relative intensity)
65	29.75	Phenol	H/CHO	Both	94 (100)	66 (27), 65 (21), 39 (13), 95 (7), 40 (6), 55 (6), 63 (5), 38 (4), 51 (4)
66	30.38	Guaiacol (phenol, 2-methoxy-)	G	Both	109 (100)	124 (87), 81 (54), 53 (13), 52 (7), 110 (7), 125 (7), 51 (6), 39 (5), 65 (5)
69	32.09	Phenol, 2-methyl-	H/CHO	Both	108 (100)	107 (90), 79 (35), 77 (33), 90 (22), 89 (14), 80 (13), 51 (11), 53 (9), 39 (9)
77	33.81	Phenol, 4-methyl-	H/CHO	Both	107 (100)	108 (83), 77 (27), 123 (19), 79 (18), 138 (14), 51 (9), 90 (8), 53 (7), 80 (7)
78	33.90	Phenol, 3-methyl-	H/CHO	Both	108 (100)	107 (96), 79 (29), 77 (28), 53 (9), 90 (9), 51 (9), 80 (8), 39 (8), 109 (8)
83	35.25	Guaiacol, 4-methyl	G	Both	138 (100)	123 (92), 95 (28), 67 (16), 77 (15), 55 (11), 139 (9), 65 (8), 39 (8), 51 (7)
86	35.96	Phenol, 3,4-dimethyl-	H/CHO	Both	122 (100)	107 (99), 121 (51), 56 (31), 77 (25), 91 (25), 79 (15), 51 (11), 26 (11), 108 (11)
88	37.84	Phenol, 4-ethyl-	H	CW	107 (100)	122 (32), 77 (15), 108 (8), 39 (5), 91 (4), 51 (4), 78 (4), 79 (3), 103 (3)
89	38.20	Guaiacol, 3-ethyl-	G	Both	152 (100)	137 (95), 109 (23), 79 (17), 91 (12), 77 (12), 151 (9), 107 (8), 51 (7), 108 (7)
91	39.01	Guaiacol, 4-ethyl-	G	Both	137 (100)	152 (40), 122 (11), 138 (9), 91 (9), 77 (7), 94 (6), 79 (5), 65 (5), 148 (4)
99	41.12	(1,5)-Anhydroarabinofuranose	CHO	Both	57 (100)	73 (53), 29 (30), 43 (25), 60 (22), 55 (19), 86 (17), 58 (17), 45 (15), 42 (15)
100	41.62	Guaiacol, 4-ethenyl-	G	Both	150 (100)	135 (80), 107 (30), 77 (29), 151 (12), 79 (9), 136 (8), 78 (7), 51 (7), 53 (6)
101	41.76	Phenol, 4-ethenyl- (t)	H	Both	120 (100)	91 (42), 119 (26), 65 (13), 121 (9), 39 (7), 94 (7), 63 (7), 51 (5), 92 (5)
102	42.04	Phenol, 4-(2-propenyl)-	H	Both	134 (100)	133 (81), 107 (50), 77 (28), 105 (24), 71 (17), 69 (17), 135 (15), 97 (15), 91 (15)
104	42.59	Eugenol	G	Both	164 (100)	149 (33), 103 (26), 131 (25), 77 (24), 91 (21), 137 (19), 133 (17), 104 (16), 121 (14)
105	42.71	Guaiacol, 4-propyl-	G	Both	137 (100)	166 (25), 122 (9), 138 (8), 77 (5), 94 (5), 91 (4), 51 (3), 164 (3), 66 (3)
113	44.97	Isoeugenol (*cis*), 2-Methoxy-4-(1-propenyl)-phenol	G	Both	164 (100)	149 (33), 77 (24), 103 (23), 91 (21), 131 (21), 44 (17), 133 (14), 55 (14), 121 (12)
116	46.50	Phenol, 4-propenyl- (*trans*)	CW	Both	134 (100)	133 (79), 107 (28), 105 (25), 77 (19), 115 (13), 91 (12), 79 (10), 135 (10), 119 (9)
119	47.23	Isoeugenol (*trans*), 2-Methoxy-4-(1-propenyl)-phenol	G	Both	164 (100)	149 (32), 77 (21), 103 (21), 131 (20), 91 (20), 133 (13), 55 (12), 121 (12), 165 (11)
122	48.07	Vanillin	G	Both	151 (100)	152 (91), 81 (20), 109 (15), 123 (15), 53 (8), 153 (8), 51 (8), 52 (8), 108 (6)
126	50.41	Homovanillin	G	Both	137 (100)	166 (26), 122 (19), 94 (10), 138 (9), 51 (5), 77 (5), 116 (5), 66 (5), 65 (4)
127	50.76	Phenol, 4-propyl-	H	CW	107 (100)	136 (22), 77 (21), 108 (9), 78 (7), 51 (7), 164 (6), 45 (6), 79 (6), 73 (5)
130	51.24	Acetoguaiacone	G	Both	151 (100)	166 (50), 123 (21), 152 (9), 108 (9), 43 (7), 52 (6), 65 (6), 77 (5), 167 (5)
131	51.57	(1,6)-Anhydrogalactopyranose	CHO	CW	60 (100)	73 (67), 57 (49), 29 (22), 56 (18), 43 (18), 42 (16), 55 (15), 74 (14), 71 (14)
134	52.46	Benzaldehyde, 4-hydroxy-	H/CHO	CW	121 (100)	122 (84), 93 (34), 65 (28), 39 (14), 63 (9), 123 (7), 66 (6), 62 (5), 38 (5)
135	53.18	Guaiacylacetone	G	Both	137 (100)	180 (22), 122 (15), 138 (10), 94 (7), 43 (7), 77 (3), 51 (3), 66 (3), 65 (3)

Table 4-1 continued Pyrolysis products identified in the retention-time-range of interest in opposite wood and compression wood samples

Peak	RT	Identification	H/G/CHO	CW/OW	Base ion (100)	Ion m/z (relative intensity)
138	54.46	Propioguaiacone	G	Both	151 (100)	180 (26), 107 (15), 123 (14), 152 (9), 108 (8), 77 (8), 51 (5), 52 (5), 135 (5)
145	56.67	(1,4)-Anhydrogalactopyranose (t)	CHO	Both	73 (100)	87 (58), 71 (55), 69 (54), 70 (44), 57 (44), 60 (43), 29 (39), 43 (38), 31 (35)
146	57.27	(1,6)-Anhydroglucopyranose (levoglucosan)	CHO	Both	60 (100)	57 (43), 73 (38), 29 (18), 70 (16), 56 (16), 43 (15), 42 (14), 55 (13), 31 (11)
147	57.92	Dihydroconiferyl alcohol	G	Both	137 (100)	182 (41), 138 (32), 122 (13), 77 (9), 123 (8), 91 (8), 106 (7), 79 (5), 107 (5)
148	58.93	Coniferyl alcohol (*cis*)	G	Both	137 (100)	180 (61), 124 (47), 91 (32), 119 (22), 77 (16), 103 (15), 147 (13), 109 (13), 65 (11)
150	60.71	Unknown anhydrosugar	CHO	Both	73 (100)	45 (92), 69 (85), 41 (71), 91 (62), 115 (30), 42 (23), 114 (21), 164 (18), 27 (17)
151	61.89	Coniferyl alcohol (*trans*)	G	Both	137 (100)	180 (72), 124 (50), 91 (34), 119 (23), 77 (17), 103 (15), 147 (15), 131 (14), 65 (12)
152	62.54	Coniferaldehyde	G	Both	178 (100)	135 (39), 147 (36), 107 (28), 77 (28), 177 (25), 161 (19), 89 (15), 118 (14), 109 (14)
153	63.89	Unknown	L	CW	147 (100)	148 (95), 91 (48), 119 (38), 120 (31), 131 (24), 89 (15), 39 (14), 51 (10), 92 (10)

Peak refers to the peak number as displayed in the chromatogram figures

RT = retention time (minutes)

(t) = tentative assignment

G = G-unit pyrolysis product

H = H-unit pyrolysis product

CHO = polysaccharide pyrolysis product

H/CHO = pyrolysis products of either H-units or polysaccharides which are phenolic compounds with no methoxyl group and no, or a single carbon, sidechain

L = unknown lignin pyrolysis product

CW/OW = indicates whether products were from compression wood (CW), opposite wood (OW) or both wood types.

Base ion is the most abundant ion (relative intensity = 100%), the m/z of the next nine most abundant ions are shown, with their relative intensities in brackets

4.3.3 Comparison of sample pre-treatments

Three different sample pre-treatments, “coarse”, “fine” and “calcium-treated”, were compared to determine which one maximised differences in the relative proportions of pyrolysis products from compression wood and opposite wood. Chromatograms obtained using the three different pre-treatments for the same sample of compression wood are shown in Figure 4-4. The proportions of polysaccharide derived products (anhydrosugars) were smaller for the coarse (Figure 4-4, A, arrows) and fine samples (Figure 4-4, B, arrows) compared with the calcium-treated samples (Figure 4-4, C, arrows). This is most easily observed by comparing the relative proportions of (1,6)-anhydroglucopyranose (levoglucosan, peak 146), which is the primary pyrolysis product of cellulose. Treatment had no effect on the retention times of the pyrolysis products.

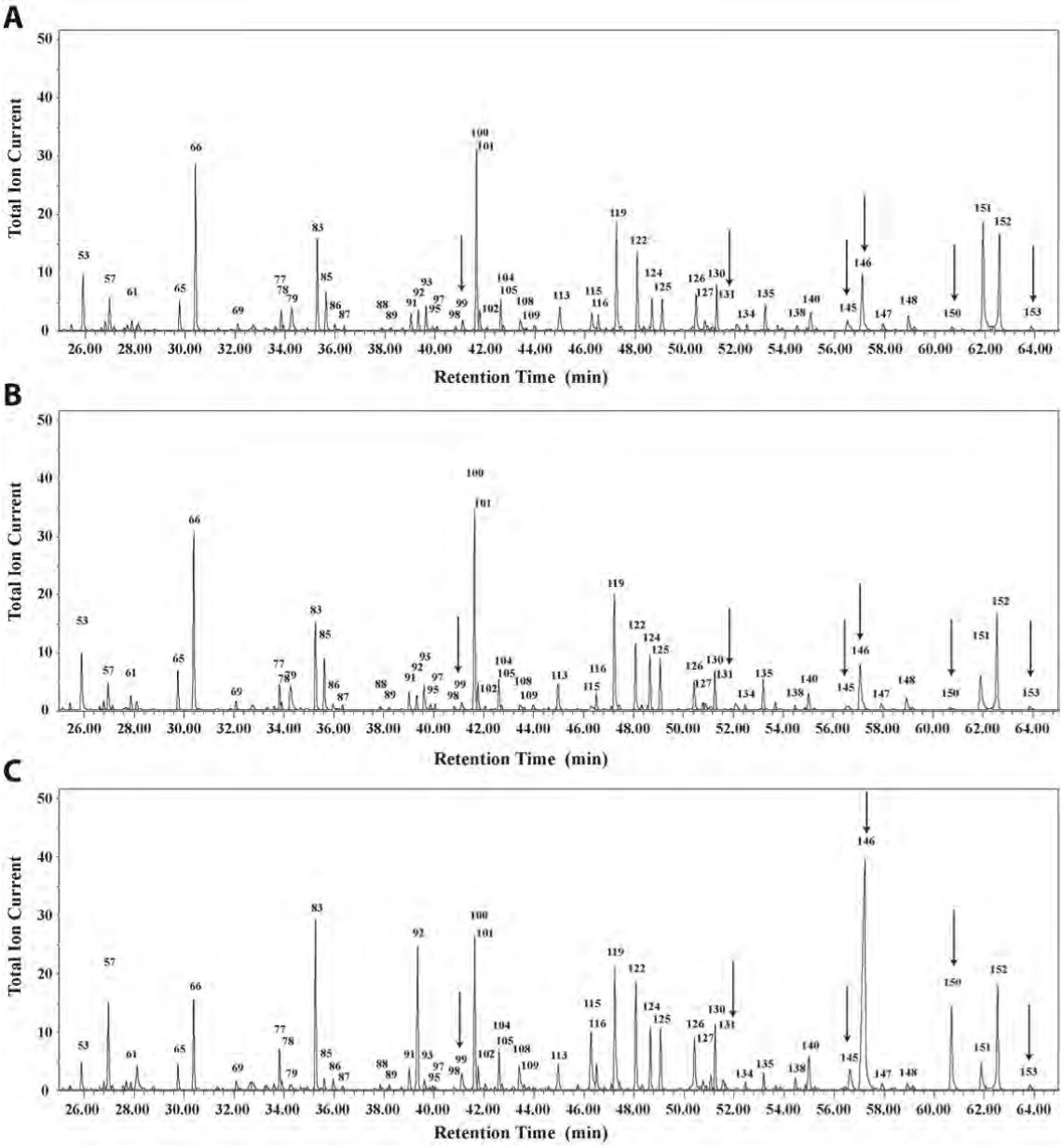

Figure 4-4 Comparison of the pyrolysis products in the retention time range of interest of a compression wood sample after three different treatments: (A) coarse, (B) fine and (C) calcium-treated. The numbers above the peaks are the 'peak numbers', and the products that they refer to are given in Table 4-1 and in the appendix. Arrows indicate the peaks of anhydrosugars.

The peak areas of each of the the pyrolysis products in the retention time range of interest (Table 4-1) were integrated and were normalised as a percentage of the total integratable area of the total ion chromatogram. The three different sample pre-treatments were

compared separately for compression wood and opposite wood (Table 4-2). To examine the statistical significance of the pre-treatments on the proportions of these products, generalised linear models were built following the form:

$y_i = Treatment + \varepsilon$

Where "y_i"= the proportion of the product of interest, "*Treatment*" is the treatment type and ε is the error. The generalised linear models were tested using an *F*-test in ANOVA ($a = 0.05$). Those products that had significantly different proportions between treatments as determined by ANOVA of the linear models were then tested by post-hoc Tukey's HSD tests to determine which sample pre-treatments were significantly different (Table 4-2).

Milling the coarse samples to a smaller particle size ("fine" pre-treatment) had an effect on the proportions of a greater number of products among opposite wood samples compared with compression wood samples. In opposite wood samples, the proportions of all products that were significantly different between coarse and fine sample pre-treatments were lower after milling more finely, except for phenol, which was present in significantly higher proportions in the fine samples. Fine-milling of the opposite wood samples did not have a significant effect on the proportions of anhydrosugars. Fine-milling of the compression wood samples significantly reduced the proportions of only 4-ethenyl-guaiacol, and the unknown anhydrosugar at retention time 60.71 min (peak 150). Fine-milling did not have a significant effect on the proportions of any of the other pyrolysis products of compression wood. In the opposite wood samples, calcium-treatment of the fine samples did not have a consistent effect on the proportions of lignin derived products. The proportions of some lignin-derived products were increased (e.g. 4-methyl-guaiacol and vanillin), whereas the proportions of other lignin-derived products were decreased (e.g. phenol and 4-ethenyl-guaiacol). Calcium-treatment of the fine, opposite wood samples

increased the proportions of the anhydrosugars (1,5)-anhydroarabinofuranose, (1,6)-anhydroglucopyranose and the unknown anhydrosugar at retention time 60.71 min (peak 150). Proportions of lignin-derived products after calcium-treatment of the fine, compression wood samples were either increased (e.g. 4-methyl-guaiacol and vanillin) or decreased (e.g. phenol and 4-ethenyl-guaiacol). The proportions of the anhydrosugars (1,5)-anhydroarabinofuranose, (1,6)-anhydrogalactopyranose, (1,4)-anhydrogalactopyranose, (1,6)-anhydroglucopyranose and the unknown anhydrosugar at retention time 60.71 min (peak 150) were increased after calcium-treatment of the fine, compression wood samples. The pre-treatments had the same effect on the proportions of pyrolysis products across both wood types. That is to say, if fine-milling reduced the proportions of a product detected in the opposite wood samples, it also reduced the proportions of that product in the compression wood sample.

The total proportion of lignin products was highest in the coarse samples, and lowest in the calcium-treated samples. However, the difference in proportions of total lignin products between compression wood and opposite wood was the lowest in the coarse samples, and the highest in the calcium-treated samples. The same was true of the total proportion of G-unit derived products. Although the coarse samples gave the highest proportion of G-unit products, the largest difference between compression wood and opposite wood was in the calcium-treated samples. The total proportion of H-units was highest from the coarse, opposite wood samples, but highest from the fine, compression wood samples. The biggest difference in the proportion of H-units between the two wood types was in the fine samples, followed by the coarse samples, and lastly the calcium-treated samples. The difference in the ratio of H-units to G-units between compression wood and opposite wood however, decreased from fine samples to calcium-treated samples and finally to coarse samples, although the values were all quite similar. The greatest differences in

proportions of products between compression wood and opposite wood samples were observed in the pyrolysis products of the polysaccharides. Here, the calcium-treated samples had a difference in the total amounts of anhydrosugars produced between compression wood and opposite wood that was seven times larger than the difference observed for the coarse samples, and nearly nine times that of the fine samples. The differences in proportions of (1→4)-β-D-galactan pyrolysis products between compression wood and opposite wood calcium-treated samples was nearly eight times that of the coarse samples. (1→4)-β-D-Galactan pyrolysis products in the opposite wood fine samples were not detectable. As the differences between the relative proportions of total lignin products and anhydrosugars were typically greatest in the calcium-treated samples, differences in the proportions of pyrolysis products in the calcium-treated samples were analysed further.

Table 4-2 Differences in proportions of pyrolysis products among different pre-treatments of compression wood and opposite wood samples

			OW			CW			CW-OW		
Peak	**Identification**	**H/G/CHO**	**Coarse**	**Fine**	**Calcium**	**Coarse**	**Fine**	**Calcium**	**Coarse**	**Fine**	**Calcium**
65	Phenol	H/CHO	0.17 a	0.23 b	0.10 c	0.59 df	0.87 ef	0.32 d	0.42	0.64	0.23
66	Guaiacol (phenol, 2-methoxy-)	G	2.41 ac	2.24 c	0.99 b	2.99 df	2.73 f	1.27 e	0.59	0.50	0.27
69	Phenol, 2-methyl-	H/CHO	0.17 a	0.10 b	0.12 ab	0.17	0.16	0.16	0.00	0.06	0.03
77	Phenol, 4-methyl-	H/CHO	0.12 a	0.11 ac	0.09 bc	0.37	0.34	0.45	0.25	0.24	0.36
78	Phenol, 3-methyl-	H/CHO	0.08 a	0.08 ac	0.06 bc	0.13	0.13	0.14	0.05	0.05	0.07
83	Guaiacol, 4-methyl	G	1.27 a	1.09 b	1.65 c	1.62 df	1.43 f	2.14 e	0.35	0.33	0.49
86	Phenol, 3,4-dimethyl-	H/CHO	0.09 ac	0.09 c	0.15 b	0.13 df	0.12 f	0.21 e	0.04	0.03	0.06
88	Phenol, 4-ethyl-	H	NA	NA	NA	0.07	0.03	0.07	0.07	0.03	0.06
89	Guaiacol, 3-ethyl-	G	0.02 ac	0.01 c	0.07 b	0.06	0.04	0.09	0.04	0.03	0.02
91	Guaiacol, 4-ethyl-	G	0.34 a	0.25 b	0.26 b	0.37	0.31	0.33	0.03	0.06	0.07
99	(1,5)-Anhydroarabinofuranose	CHO	0.47 ac	0.39 c	0.68 b	0.29 df	0.25 f	0.44 e	-0.18	-0.14	-0.24
100	Guaiacol, 4-ethenyl-	G	3.58 a	3.14 b	1.88 c	3.47 d	3.18 e	2.18 f	-0.11	0.04	0.31
101	Phenol, 4-ethenyl- (t)	H	0.09 ac	0.09 c	0.06 b	0.47	0.47	0.35	0.38	0.38	0.30
102	Phenol, 4-(2-propenyl)-	H	NA	NA	NA	0.10	0.09	0.11	0.09	0.09	0.11
104	Eugenol	G	0.54 a	0.46 b	0.50 b	0.58	0.52	0.57	0.03	0.06	0.07
105	Guaiacol, 4-propyl-	G	0.12 a	0.10 b	0.10 b	0.13 d	0.11 de	0.11 e	0.01	0.01	0.01
113	Isoeugenol (*cis*), 2-Methoxy-4-(1-propenyl)-phenol	G	0.54 a	0.48 b	0.46 b	0.62 d	0.57 de	0.51 e	0.07	0.09	0.06
116	Phenol, 4-propenyl- (*trans*)	H	0.01 ac	0.00 c	0.06 b	0.31	0.28	0.37	0.30	0.28	0.31
119	Isoeugenol (*trans*), 2-Methoxy-4-(1-propenyl)-phenol	G	2.18 ac	1.81 c	1.93 b	2.28	2.10	2.07	0.10	0.28	0.14
122	Vanillin	G	0.99 ac	0.91 c	1.22 b	1.23 df	1.14 f	1.54 e	0.24	0.23	0.32
126	Homovanillin	G	0.40 ac	0.36 c	0.55 b	0.59 df	0.55 f	0.93 e	0.19	0.18	0.38
127	Phenol, 4-propyl-	H	NA	NA	NA	0.23 df	0.18 ef	0.14 e	0.13	0.18	0.11
130	Acetoguaiacone	G	0.60 a	0.51 b	0.69 c	0.72 df	0.65 f	0.92 e	0.13	0.14	0.23
131	(1,6)-Anhydrogalactopyranose	CHO	NA	NA	NA	0.00 df	0.01 f	0.16 e	0.00	0.01	0.16
134	Benzaldehyde, 4-hydroxy-	H/CHO	NA	NA	NA	0.13	0.11	0.12	0.11	0.11	0.11

Table 4-2 continued Differences in proportions of pyrolysis products among different pre-treatments of compression wood and opposite wood samples

			OW			CW			CW-OW		
Peak	**Identification**	**H/G/CHO**	**Coarse**	**Fine**	**Calcium**	**Coarse**	**Fine**	**Calcium**	**Coarse**	**Fine**	**Calcium**
135	Guaiacylacetone	G	0.47 ac	0.44 c	0.22 b	0.50 df	0.50 f	0.26 e	0.03	0.06	0.04
138	Propioguaiacone	G	0.07 ac	0.06 c	0.19 b	0.11 df	0.10 f	0.19 e	0.03	0.04	0.01
145	(1,4)-Anhydrogalactopyranose (t)	CHO	0.14	0.00	0.05	0.04 df	0.05 f	0.59 e	-0.09	0.05	0.54
146	(1,6)-Anhydroglucopyranose (levoglucosan)	CHO	1.41 ac	1.39 c	9.56 b	1.51 df	1.26 f	8.01 e	0.11	-0.13	-1.55
147	Dihydroconiferyl alcohol	G	0.40 a	0.36 ab	0.32 b	0.27	0.23	0.22	-0.13	-0.12	-0.09
148	Coniferyl alcohol (*cis*)	G	0.27 ac	0.27 c	0.13 b	0.38 df	0.33 f	0.20 e	0.11	0.06	0.07
150	Unknown anhydrosugar	CHO	0.24 ac	0.12 c	1.93 b	0.11 d	0.10 e	1.86 f	-0.13	-0.02	-0.08
151	Coniferyl alcohol (*trans*)	G	1.91 a	1.52 b	0.33 c	2.64 df	1.93 f	0.64 e	0.73	0.41	0.31
152	Coniferaldehyde	G	1.63	1.68	1.58	1.95	2.00	1.97	0.33	0.33	0.38
153	Unknown	L	NA	NA	NA	0.14	0.11	0.12	0.14	0.11	0.12
	Total lignin		18.56	16.38	13.66	23.21	21.21	18.59	4.65	4.82	4.93
	Sum "G"		17.73	15.70	13.05	20.51	18.43	16.15	2.78	2.73	3.10
	Sum"H"		0.20	0.09	0.08	1.18	1.05	1.04	0.98	0.96	0.96
	Sum "H" + " H/CHO"		0.83	0.69	0.61	2.71	2.78	2.44	1.88	2.10	1.83
	Ratio of "H" to "G"		0.01	0.01	0.01	0.06	0.06	0.06	0.35	0.35	0.31
	Ratio of "H" + "H/CHO" to "G"		0.05	0.04	0.05	0.13	0.15	0.15	0.68	0.77	0.59
	Total anhydrosugars		2.25	1.90	12.23	2.10	1.78	11.18	-0.15	-0.12	-1.05
	Total anhydrogalactose		0.14	0.00	0.05	0.05	0.06	0.75	-0.09	0.06	0.70

Peak refers to the peak number as displayed in the chromatogram figures
(t) = tentative assignment
G = G-unit pyrolysis product
H = H-unit pyrolysis product
CHO = polysaccharide pyrolysis product
H/CHO = pyrolysis products of either H-units or polysaccharides which are phenolic compounds with no methoxyl group and no, or a single carbon, sidechain
L = unknown lignin pyrolysis product
Values are the mean proportion of the total integratable area (%) of 6 samples of each wood type. Significant differences between treatments are denoted by lower case letters after the proportion of the product (tested by post-hoc Tukey's HSD tests).

4.3.4 Differences in proportions of pyrolysis products that were unique to compression wood

The six pyrolysis products that were unique to compression wood were the H-unit derived products 4-(2-propenyl)-phenol, 4-ethyl-phenol and 4-propyl-phenol; the anhydrosugar (1,6)-anhydrogalactopyranose; the unknown product at retention time 63.89 min (peak 153) and the phenolic compound 4-hydroxy-benzaldehyde which may be derived from either the H-units of lignin or from polysaccharides. To examine the statistical significance of the observed differences in the proportions of these products between samples of compression wood, generalised linear models were built following the form:

$y_i = Sample + \varepsilon$

Where "y_i"= the proportion of the product of interest, "*Sample*" is the sample measured and ε is the error. These generalised linear models were tested using an *F*-test in ANOVA ($a = 0.05$), and the proportions of each product and the ANOVA results are shown in Table 4-3.

Table 4-3 Differences in proportions of pyrolysis products that were unique to compression wood

Peak	Identification	H/CHO	CW 1	CW 2	CW 3	CW 4	CW 5	CW 6	*F*	*p*-value	HSD
88	Phenol, 4-ethyl-	H	0.056 (0.002)	0.078 (0.002)	0.069 (0.002)	0.040 (0.035)	0.082 (0.009)	0.074 (0.003)	3.3	0.0418	1
102	Phenol, 4-(2-propenyl)-	H	0.097 (0.021)	0.125 (0.010)	0.109 (0.014)	0.095 (0.005)	0.133 (0.007)	0.130 (0.010)	5.6	0.007	3
127	Phenol, 4-propyl-	H	0.118 (0.007)	0.182 (0.016)	0.115 (0.018)	0.109 (0.008)	0.164 (0.026)	0.143 (0.013)	10.2	0.0005	6
131	(1,6)-Anhydrogalactopyranose	CHO	0.035 (0.061)	0.206 (0.023)	0.138 (0.021)	0.045 (0.041)	0.269 (0.125)	0.270 (0.085)	6.9	0.003	4
134	Benzaldehyde, 4-hydroxy-	H/CHO	0.089 (0.004)	0.144 (0.005)	0.114 (0.006)	0.075 (0.006)	0.173 (0.013)	0.133 (0.011)	59.1	<0.0001	12
153	Unknown	L	0.072 (0.014)	0.143 (0.020)	0.119 (0.004)	0.085 (0.003)	0.169 (0.021)	0.132 (0.010)	20.1	<0.0001	8

Peak refers to the peak number as displayed in the chromatogram figures

Values are the mean proportion of the total integratable area (%) of 6 samples of compression wood, standard deviation is given in brackets.

The *F*-value and *p*-value are given for ANOVA of the generalised linear model for the effect of sample on the proportion of each product.

Degrees of freedom are the following: sample = 5, residuals = 12

HSD = the number of post-hoc Tukey's HSD pairwise comparisons (out of a possible 15 comparisons) that have significantly different proportions of each product between samples.

H = H-unit pyrolysis product

CHO = polysaccharide pyrolysis product

H/CHO = pyrolysis products of either H-units or polysaccharides which are phenolic compounds with no methoxyl group and no, or a single carbon, sidechain

L = unknown lignin pyrolysis product

The proportions of all of these products were significantly different among compression wood samples ($p < 0.04$). Post-hoc Tukey's HSD pairwise comparisons of the samples were done to determine the number of sample comparisons (out of a total of 15 possible comparisons) that had significantly different proportions of each product. The product 4-hydroxy-benzaldehyde, derived from either H-units of lignin or polysaccharides, had significantly different proportions between pairwise comparisons of the greatest number of samples (12 out of 15 pairwise comparisons). Proportions of 4-hydroxy-benzaldehyde from replicate aliquots of each sample are shown in Figure 4-5.

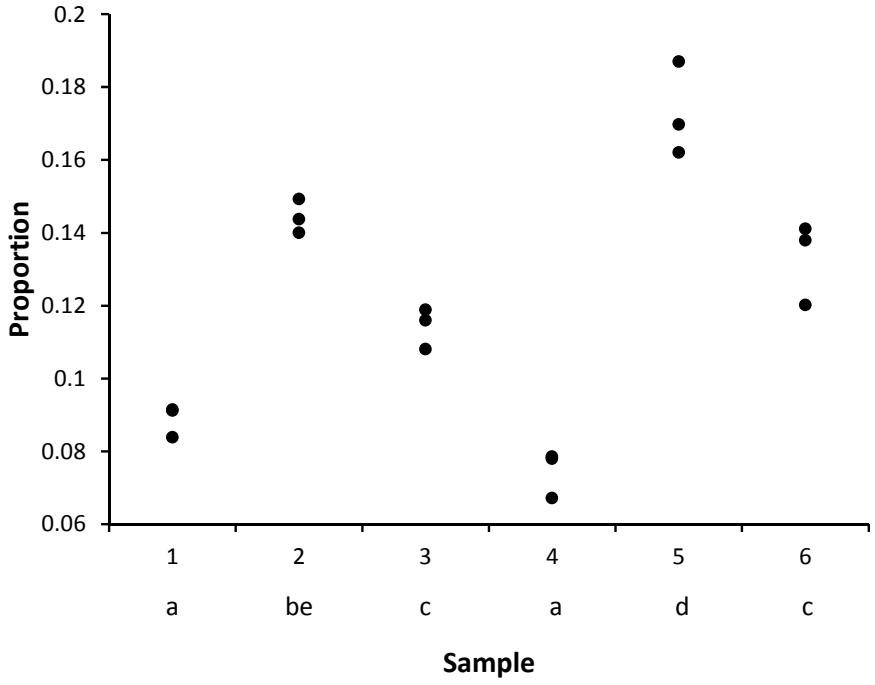

Figure 4-5 Differences in proportions of 4-hydroxy-benzaldehyde between aliquots of each compression wood sample. Significant differences between samples (post-hoc Tukey's HSD) are indicated by letters beneath the sample numbers.

4.3.5 Differences in proportions of the pyrolysis products present in both opposite wood and compression wood

To determine the statistical significance of the effect of wood type and wood sample on the relative proportions of each of the compounds that were produced from pyrolysis of both wood types, generalised linear models were built following the form:

$y_i = Type + Sample + \varepsilon$

Where "y_i"= the proportion of the product of interest, "*Type*"is the wood type, "*Sample*" is the sample measured and ε is the error. These generalised linear models were tested using an *F*-test in analysis of variance (ANOVA) ($a = 0.05$) (Table 4-4).

4.3.5.1 Differences in proportions between wood types

Wood type had a significant effect on the proportions of pyrolysis products in the retention time range of interest, with the exception of the G-unit product propioguaiacone, and the unknown anhydrosugar at retention time 60.71 min (peak 150), the proportions of which were not significantly different between compression wood and opposite wood ($p = 0.35$ and 0.05, respectively) (Table 4-4). Wood type had a significant effect on the proportions of products that could have been derived from either H-units or polysaccharides or both. These included phenol ($F_{1\,34} = 348.5$, $p < 0.0001$), 2-methyl-phenol ($F_{1\,34} = 47.2$, $p < 0.0001$), 4-methyl-phenol ($F_{1\,34} = 435.9$, $p < 0.0001$) and 3,4-dimethyl-phenol ($F_{1\,34} = 54.5$, $p < 0.0001$). Wood type also had a significant effect on the proportions of guaiacol ($F_{1\,34} = 80.21$, $p < 0.0001$) and all of the other products derived from the G-units of lignin except for propioguaiacone as previously mentioned. The proportion of dihydroconiferyl alcohol was

greater from opposite wood compared with compression wood ($F_{1\ 34}$ = 79.6, p < 0.0001), which was unusual for a lignin-derived product. Among polysaccharide derived products, wood type had a significant effect on the proportions of (1,5)-anhydroarabinofuranose ($F_{1\ 34}$ = 89.2, p < 0.0001), (1,4)-anhydrogalactopyranose ($F_{1\ 34}$ = 287.9, p < 0.0001) and (1,6)-anhydroglucopyranose (levoglucosan) ($F_{1\ 34}$ = 46.3, p < 0.0001).

Table 4-4 The effect of wood type and sample on differences in the proportions of pyrolysis products

					Wood type		Sample		HSD	
Peak	**Identification**	**H/G/CHO**	**CW (SD)**	**OW (SD)**	***F***	***p*-value**	***F***	***p*-value**	**CW**	**OW**
65	Phenol	H/CHO	0.32 (0.07)	0.10 (0.01)	348.5	<0.0001	6	<0.0001	4	NA
66	Guaiacol (phenol, 2-methoxy-)	G	1.27 (0.12)	0.99 (0.04)	80.2	<0.0001	4.5	<0.0001	2	NA
69	Phenol, 2-methyl-	H/CHO	0.16 (0.01)	0.12 (0.01)	47.2	<0.0001	1.4	0.2600	NA	NA
77	Phenol, 4-methyl-	H/CHO	0.45 (0.10)	0.09 (0.01)	435.9	<0.0001	5.7	<0.0001	3	NA
78	Phenol, 3-methyl-	H/CHO	0.14 (0.02)	0.06 (0.01)	124.8	<0.0001	1.1	0.4000	NA	NA
83	Guaiacol, 4-methyl	G	2.14 (0.11)	1.65 (0.09)	227.6	<0.0001	2.2	0.0700	NA	NA
86	Phenol, 3,4-dimethyl-	H/CHO	0.21 (0.02)	0.15 (0.01)	54.5	<0.0001	1.2	0.3500	NA	NA
89	Guaiacol, 3-ethyl-	G	0.09 (0.01)	0.07 (0.01)	22.5	<0.0001	1.5	0.2100	NA	NA
91	Guaiacol, 4-ethyl-	G	0.33 (0.02)	0.26 (0.02)	132.4	<0.0001	2	0.1100	NA	NA
99	(1,5)-Anhydroarabinofuranose	CHO	0.44 (0.10)	0.68 (0.10)	89.2	<0.0001	6.4	<0.0001	NA	2
100	Guaiacol, 4-ethenyl-	G	2.18 (0.12)	1.88 (0.07)	63.7	<0.0001	3.1	0.0200	2	NA
101	Phenol, 4-ethenyl- (t)	H	0.35 (0.08)	0.06 (0.02)	477	<0.0001	6	<0.0001	4	NA
104	Eugenol	G	0.57 (0.02)	0.5 (0.02)	50.9	<0.0001	1.9	0.1100	NA	NA
105	Guaiacol, 4-propyl-	G	0.11 (0.01)	0.1 (0.01)	22.8	<0.0001	2.2	0.0700	NA	NA
113	Isoeugenol (*cis*), 2-Methoxy-4-(1-propenyl)-phenol	G	0.51 (0.01)	0.46 (0.03)	32.3	<0.0001	0.5	0.7900	NA	NA
116	Phenol, 4-propenyl- (*trans*)	H	0.37 (0.08)	0.06 (0.03)	295.6	<0.0001	3.1	0.0200	1	NA
119	Isoeugenol (*trans*), 2-Methoxy-4-(1-propenyl)-phenol	G	2.07 (0.05)	1.93 (0.13)	15.8	<0.0001	3.9	0.0100	NA	2
122	Vanillin	G	1.54 (0.07)	1.22 (0.05)	219.5	<0.0001	2.5	0.0500	NA	NA
126	Homovanillin	G	0.93 (0.13)	0.55 (0.05)	104.8	<0.0001	0.4	0.8500	NA	NA
130	Acetoguaiacone	G	0.92 (0.05)	0.69 (0.03)	223.2	<0.0001	2.3	0.0600	NA	NA
135	Guaiacylacetone	G	0.26 (0.02)	0.22 (0.01)	40.2	<0.0001	1.9	0.1100	NA	NA
138	Propioguaiacone	G	0.19 (0.01)	0.19 (0.01)	0.9	0.3500	NA	NA	NA	NA
145	(1,4)-Anhydrogalactopyranose (t)	CHO	0.59 (0.13)	0.05 (0.03)	287.9	<0.0001	2.5	0.0500	NA	NA
146	(1,6)-Anhydroglucopyranose (levoglucosan)	CHO	8.01 (0.96)	9.56 (0.64)	46.3	<0.0001	5.7	<0.0001	2	NA
147	Dihydroconiferyl alcohol	G	0.22 (0.07)	0.32 (0.05)	79.6	<0.0001	16.7	<0.0001	4	1

Table 4-4 continued The effect of wood type and sample on differences in the proportions of pyrolysis products

					Wood type		Sample		HSD	
Peak	**Identification**	**H/G/CHO**	**CW (SD)**	**OW (SD)**	***F***	***p*-value**	***F***	***p*-value**	**CW**	**OW**
148	Coniferyl alcohol (*cis*)	G	0.20 (0.02)	0.13 (0.01)	86.2	<0.0001	1.2	0.3500	NA	NA
150	Unknown anhydrosugar	CHO	1.86 (0.08)	1.93 (0.06)	4.2	0.0500	NA	NA	NA	NA
151	Coniferyl alcohol (*trans*)	G	0.64 (0.13)	0.33 (0.04)	27.6	<0.0001	1.1	0.3800	NA	NA
152	Coniferaldehyde	G	1.97 (0.09)	1.58 (0.08)	195	<0.0001	4.1	<0.0001	2	NA

Peak refers to the peak number as displayed in the chromatogram figures

Values are the mean proportion of the total integratable area (%) of 6 samples of each wood type, standard deviation (SD) is given in brackets.

The *F*-value and *p*-value are given for ANOVA of the generalised linear model for the effect of wood type and sample on the proportion of each product.

Degrees of freedom are the following: type = 1, residuals = 34; sample = 6, residuals = 28.

HSD = the number of post-hoc Tukey's HSD pairwise comparisons (out of a possible 15 comparisons) that have significantly different proportions of each product between samples of each wood type.

(t) = tentative assignment

G = G-unit pyrolysis product

H = H-unit pyrolysis product

CHO = polysaccharide pyrolysis product

H/CHO = pyrolysis products of either H-units or polysaccharides which are phenolic compounds with no methoxyl group and no, or a single carbon, sidechain

NA = not tested as either wood type or sample did not have a significant effect on the proportions of this product

4.3.5.2 Differences in proportions among samples

There was a significant difference in the proportions of the following products which may be derived from H-units of lignin and/or polysaccharides: phenol and 4-methyl-phenol, and the H-unit products 4-ethenyl-phenol and 4-propenyl-phenol, among samples of compression wood ($p < 0.02$). There were also significant differences in the proportions of the following G-unit products, guaiacol, 4-ethenyl-guaiacol, dihydroconiferyl alcohol and coniferaldehyde, and also the the polysaccharide-derived anhydrosugar (1,6)-anhydroglucopyranose ($p < 0.02$). For those products that had significantly different proportions in ANOVA ($a = 0.05$) of the model described above, post-hoc Tukey's HSD pairwise comparisons of the samples were done to determine the number of sample comparisons (out of a total of 15 possible comparisons for each wood type) that had significantly different proportions of each product. Among compression wood samples the *F*-values for the effect of wood type (Table 4-4) were much smaller compared with the *F*-values for the effect of sample, indicating that the effect of sample on the proportions of these products is much less than the effect of wood type.

The effect of sample on the proportions of each product was even smaller for opposite wood. In this wood type, only (1,5)-anhydroarabinofuranose and the G-unit derived products isoeugenol (*trans*) and dihydroconiferyl alcohol had significantly different proportions among samples ($p < 0.01$). Proportions of phenol from aliquots of each compression wood sample and opposite wood sample are shown in Figure 4-6. It can be seen that differences in the proportions among samples (in compression wood) are much smaller than differences in the proportions between wood types.

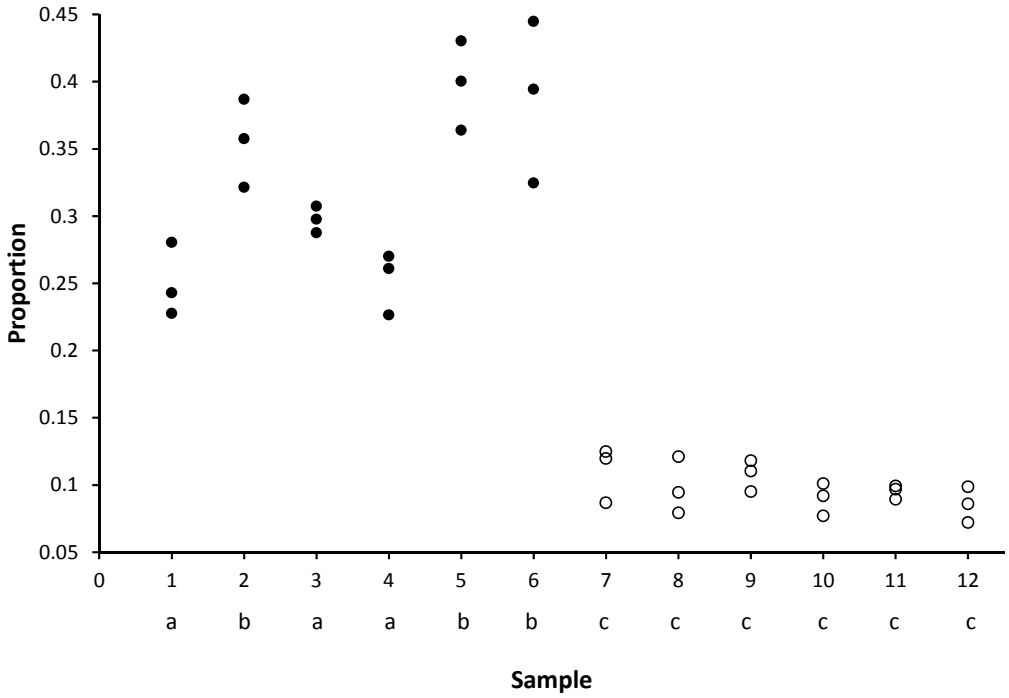

Figure 4-6 Proportions of phenol for three aliquots of each of the compression wood samples (samples 1 to 6, ●) and opposite wood samples (samples 7 to 12, ○). Significant differences between samples (post-hoc Tukey's HSD) are indicated by letters beneath the sample numbers.

1.1.1 Relating the proportions of pyrolysis products to the Klason lignin, galactosyl residue and glucosyl residue content

To determine whether the proportions of pyrolysis products derived from lignin and polysaccharides were correlated with the proportions of lignin and the glycosyl residue contents of the two wood types (Chapter 2), the proportions of pyrolysis products derived from cellulose and (1→4)-β-D-galactans were plotted against the glucosyl residue contents and galacotosyl residue contents of the wood samples determined after acid hydrolysis (Chapter 2). The proportions of lignin-derived pyrolysis products, and H-unit-derived pyrolysis products were plottled against the Klason lignin content indicated by the weight

of the hydrolysis residue (Chapter 2). The Klason lignin content, glucosyl and galactosyl residue contents, and the proportions of the different pyrolysis products were clustered into two groups, compression wood and opposite wood, due to the differences in their cell-wall compositions. Therefore relationships were plotted separately and compared for each wood type. Differing slopes of regression between the two wood types indicated that there was a lack of coherent relationships between compression wood and opposite wood. In many cases there was no relationship within the wood types.

There was no correlation between the proportions of (1,6)-anhydroglucose and the glucosyl residue content of compression wood samples (Figure 4-7, A), or opposite wood samples (Figure 4-7, B). A positive relationship was observed when the proportions of the total (1,4)-anhydrogalactopyranose and (1,6)-anhydrogalactopyranose were plotted against the galactosyl residue content of both compression wood (Figure 4-7, C) and opposite wood (Figure 4-7, D), although the relationships were not coherent between the two wood types as can be observed by the different slopes of the regression lines. The slope of regression is more similar between compression wood and opposite wood when (1,4)-anhydrogalactopyranose is plotted against the galactosyl residue content, but the relationships are still not coherent.

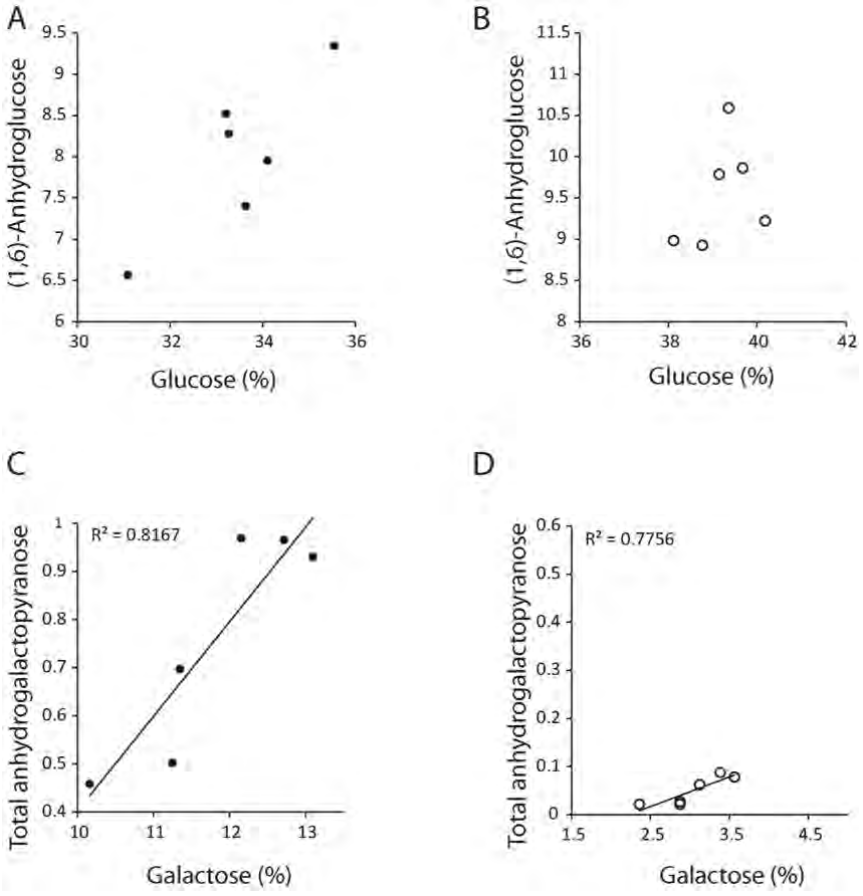

Figure 4-7 Proportions of (1,6)-anhydroglucose plotted against the proportions of glucosyl residues measured in Chapter 2 for (A) compression wood and (B) opposite wood. Proportions of total anhydrogalactopyranose products are plotted against the galactosyl residue content measured in Chapter 2 for (C) compression wood and (D) opposite wood. Compression wood = ●, opposite wood = ○.

Lignin content and the proportion of H-units in the lignin have been related to compression wood severity (Nanayakkara *et al.*, 2009). The proportions of H-unit derived products were compared with the Klason lignin content (Chapter 2) of the compression wood and opposite wood samples. Again, the data were clustered into groups of compression wood and opposite wood, with compression wood having the higher Klason lignin content. The total proportion of H-units was strongly correlated with the proportions of products that may be derived from either the H-units of lignin, from polysaccharides, or both (H-units+H/CHO) (Figure 4-8, A), suggesting that pyrolysis of carbohydrates did not yield significant

proportions of phenolic compounds. The total proportion of H-units did not have an obvious relationship to the Klason lignin content of either compression wood (Figure 4-8, B) or opposite wood (Figure 4-8, C). The total proportion of H-units products that may be derived from either the H-units of lignin, from polysaccharides, or both (H-units+H/CHO) had the same relationship with Klason lignin (Figure 4-8, D) in the compression wood samples as was observed for the total proportion of H-units (Figure 4-8, B). The unknown product at retention time 63.89 min (peak 153) was strongly correlated with the total proportion of H-units (Figure 4-8, E).

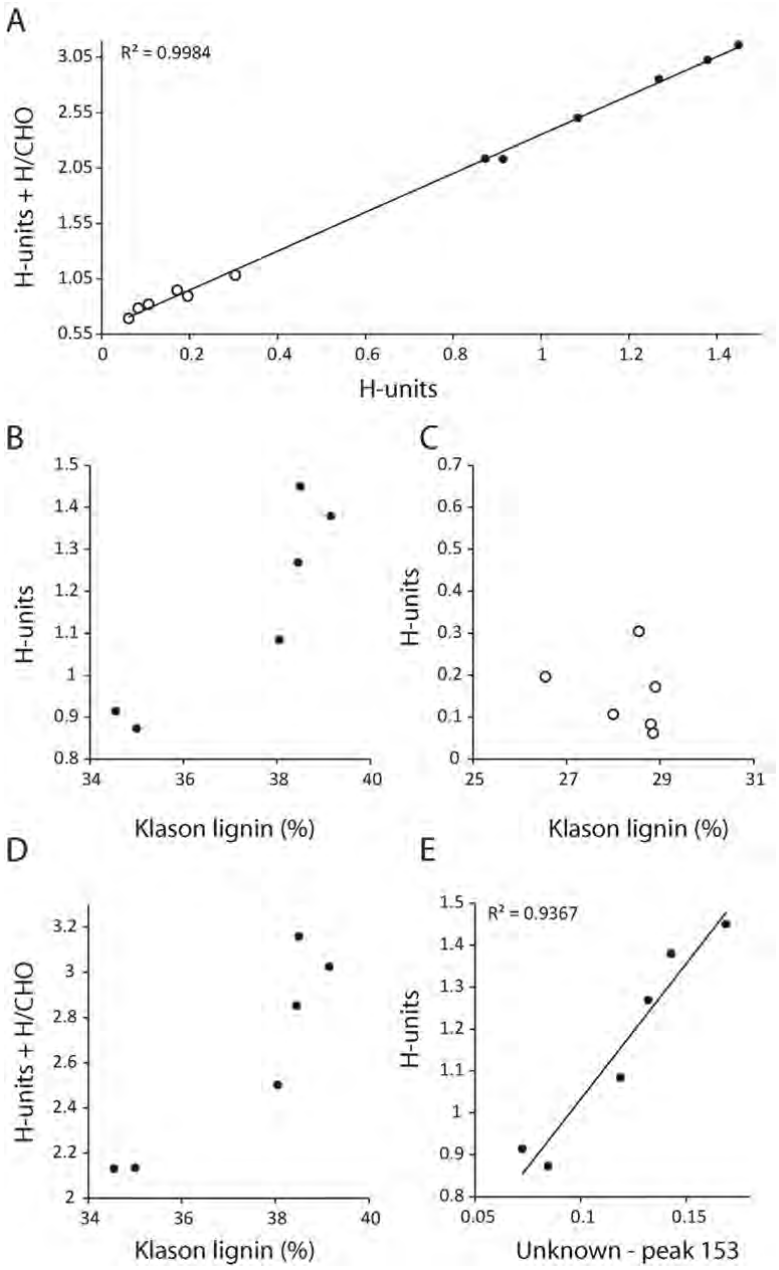

Figure 4-8 (A) The total proportion of H-units is plotted against the total proportion of H-units and phenolic compounds that do not contain methoxyl groups and have no, or a single carbon, sidechain (H/CHO). The total proportion of H-units is plotted against the Klason lignin content (measured in Chapter 2) of (B) compression wood and (C) opposite wood. The proportions of H-units and H/CHO products are plotted against the Klason lignin content of compression wood samples. (D) The proportions of the unknown product at retention time 63.89 (peak 153) is plotted against the total proportion of H-units. Compression wood = ●, opposite wood = ○.

The ratio of the summed proportions of (1,4)-anhydrogalactopyranose and (1,6)-anhydrogalactopyranose to (1,6)-anhydroglucopyranose was plotted against the summed proportion of products from the H-units of lignin and those products that may be derived from either the H-units of lignin, from polysaccharides, or both (H-units+H/CHO), in compression wood (Figure 4-9, A) and opposite wood (Figure 4-9, B). There was a relationship between the ratio of (1,4)-anhydrogalactopyranose and (1,6)-anhydrogalactopyranose to (1,6)-anhydroglucopyranose and the proportions of H-units+H/CHO-derived products within compression wood, but there was no relationship within the opposite wood samples. There was no relationship between the ratio of (1,4)-anhydrogalactopyranose and (1,6)-anhydrogalactopyranose to (1,6)-anhydroglucopyranose and the Klason lignin content of compression wood (Figure 4-9 C) or opposite wood (Figure 4-9, D).

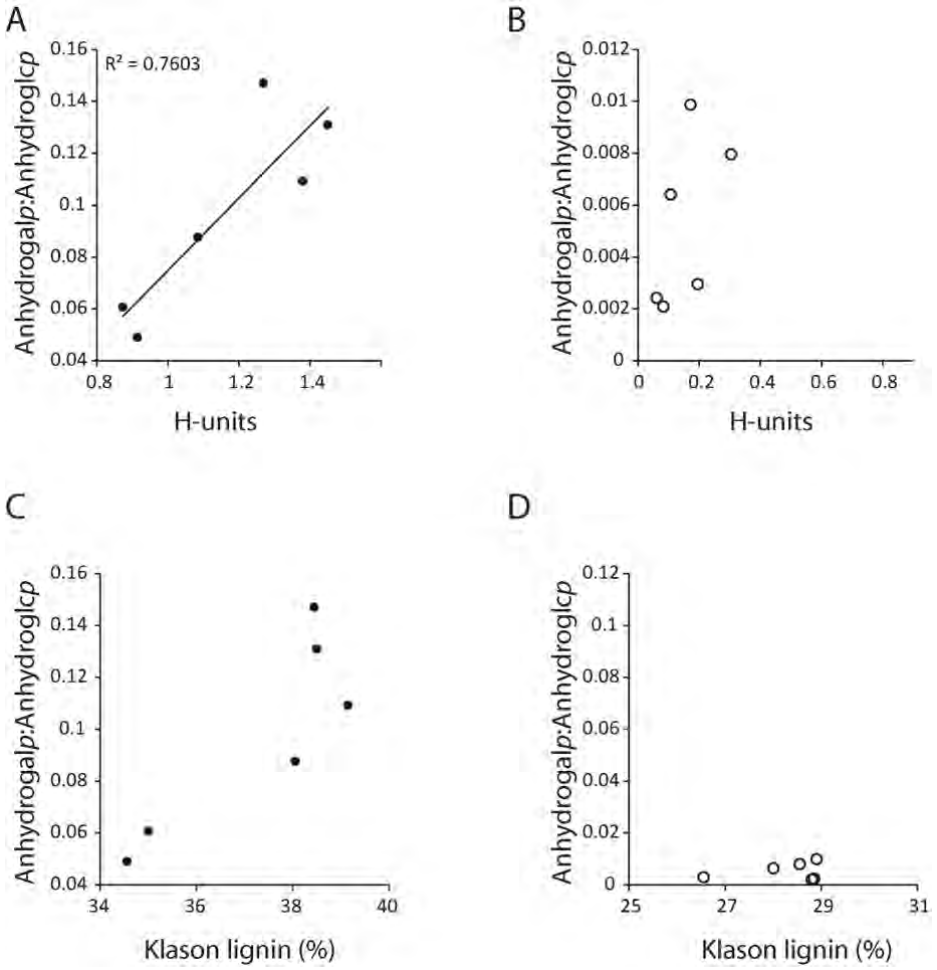

Figure 4-9 Ratio of total anhydrogalactose to anhydroglucose plotted against the total proportion of H-units for (A) compression wood and (B) opposite wood, and against the Klason lignin content measured in Chapter 2 for (C) compression wood and (D) opposite wood. Compression wood = ●, opposite wood = ○.

4.4 Discussion

As expected, pyrolysis of lupin seed (1→4)-β-D-galactan produced large proportions of (1,6)-anhydrogalactopyranose, and smaller proportions of (1,6)-anhydrogalactofuranose. Production of a third compound (peak 145) in large quantities was unexpected. Pyrolysis of galactose, and galactose-containing polysaccharides is known to yield both (1,6)-anhydrogalactopyranose and (1,6)-anhydrogalactofuranose, and additionally small proportions of (1,4)-anhydrogalactopyranose (Serban, 1998). The retention order of peak 145,

relative to (1,6)-anhydrogalactopyranose and (1,6)-anhydrogalactofuranose and analysed on the same type of column (DB-1701), is consistent with the product being (1,4)-anhydrogalactopyranose (Budgell *et al.*, 1987). Therefore, peak 145 was identified as (1,4)-anhydrogalactopyranose, although as no mass spectrum of a (1,4)-anhydrohexose could be found in the literature to confirm this. It is possible that this compound could be another product derived from pyrolysis of (1→4)-β-D-galactans.

On pyrolysis of compression wood, the proportion of (1,4)-anhydrogalactopyranose was unexpectedly greater than that of (1,6)-anhydrogalactopyranose. Pyrolysis of apple and citrus pectins, which have high proportions of (1→4)-β-D-galactan sidechains of rhamnogalacturonan I (RG I) (Renard *et al.*, 1995), yielded (1,4)-anhydrogalactopyranose, but (1,6)-anhydrogalactopyranose and (1,6)-anhydrogalactofuranose were not detected (Serban, 1998). As pyrolysis of the preparation of lupin seed (1→4)-β-D-galactan did not yield (1,4)-anhydrogalactopyranose as the primary product, this suggests that (1→4)-β-D-galactans in the wood cell-wall matrix may have some property that influences the products formed during pyrolysis. The (1→4)-β-D-galactans of compression wood are found in the highly lignified $S2_{(L)}$ layer of the secondary wall (Altaner *et al.*, 2010; Kim *et al.*, 2010). It is possible that covalent bonds between (1→4)-β-D-galactans and lignin (Mukoyoshi *et al.*, 1981; Minor, 1982) in the cell walls may preferentially cause the formation of higher proportions of (1,4)-anhydrogalactopyranose. In opposite wood, (1→4)-β-D-galactans are present in the primary walls (Altaner *et al.*, 2010; Kim *et al.*, 2010), and hydrogen bonds between the (1→4)-β-D-galactans and other polysaccharides such as cellulose (Zykwinska *et al.*, 2005; Zykwinska *et al.*, 2007; Gur'janov *et al.*, 2008), may also influence the formation of higher proportions of (1,4)-anhydrogalactopyranose. Galactose is also a component of other polysaccharides, for example as sidechains of the pectic polysaccharides of RG I (as mentioned above) and xyloglucans in the primary walls and galactoglucomannans in the

secondary walls (Harris, 2005). Therefore some of the anhydrogalactose products detected may also be derived from these polysaccharides. However, compression wood of radiata pine corewood has a larger proportion of (1→4)-β-D-galactans compared with galactoglucomannans, as determined by two-dimensional ^{13}C-^{1}H nuclear magnetic resonance correlation spectroscopy (Chapter 3; Brennan *et al.*, 2012), and the primary walls are only a small proportion of the cell walls of wood. Therefore it is likely that the increased proportions of (1,4)-anhydrogalactopyranose and (1,6)-anhydrogalactopyranose in compression wood compared with opposite wood is due to increased proportions of (1→4)-β-D-galactans.

The fine-milled wood samples had a lower proportion of phenolic compounds and anhydrosugar pyrolysis products compared with the coarse samples, suggesting that the milling process did have an effect on the structure of the polymers in the wood. Room-temperature milling of both softwood and hardwood pulps has been shown to decrease the proportions of anhydrosugars detected using pyrolysis gas-chromatography/mass-spectrometry (Syverud *et al.*, 2003). In the current study, the samples were milled under liquid nitrogen to prevent damage to the polymers by heating from friction during milling, but Ikeda et al. (2001) have shown that dry-milling (under liquid nitrogen) of loblolly pine (*Pinus taeda*) can still alter the structure of lignin, causing depolymerisation and formation of a higher proportion of low molecular weight structures. It is likely that in the current study the fine-milled samples, having a higher surface area to volume ratio, were pyrolysed more rapidly and completely compared to the coarse samples, decreasing the total proportion of phenolic compounds and anhydrosugars. However, it is possible that polymer cleavage during fine-milling contributed to the production of lower proportions of phenolic compounds and anhydrosugars.

As the pyrolysis of arabinose is efficient relative to pyrolysis of the other monosaccharides found in plant cell walls (Ponder *et al.*, 1990; Ralph and Hatfield, 1991), it is not surprising that the proportions of (1,5)-anhydroarabinofuranose were significantly different between wood types, and even among some of the opposite wood samples, despite arabinosyl residues being present in only small amounts in these wood samples (Chapter 2). Washing the samples with calcium-chloride, as reported by Kleen and Gellerstedt (1995), increased the total proportion of the other anhydrosugars detected (ie, those not from arabinose), including those derived from (1→4)-β-D-galactans, compared with the fine samples that were not calcium-treated. This is consistent with the results of Syverud et al. (2003), who showed that calcium-treatment improved the yields of galactose-, xylose-, mannose- and arabinose-derived pyrolysis products of pulps. Although the proportions of galactose-derived pyrolysis products were not investigated in the study of Syverud et al. (2003), it is likely that if they had been investigated, calcium-treatment would have had a similar effect. Therefore in future investigations of compression wood cell-wall composition using pyrolysis gas-chromatography with mass spectrometry, calcium-chloride washing is recommended if information about the pyrolysis products of the (1→4)-β-D-galactans of compression wood is desired. Although (1→4)-β-D-galactans are known to be present in the primary walls of normal wood (Altaner *et al.*, 2010; Kim *et al.*, 2010), and are present in the cell walls of opposite wood in comparable proportions to normal wood (Chapter 3), the proportions of (1→4)-β-D-galactans in these opposite wood samples are probably too small for the production of detectable proportions of (1,6)-anhydrogalactopyranose, even after treatment with calcium-chloride.

(1,6)-Anhydrogalactopyranose was one of six pyrolysis products identified in this investigation as being good markers for the presence of compression wood. The other markers were either phenolic compounds with no methoxyl groups that had carbonyl side

chains of two or three carbons, and thus could not have been produced by pyrolysis of the cell-wall polysaccharides, and 4-hydroxy-benzaldehyde which may be derived from pyrolysis of either the H-units of lignin, polysaccharides or both. An unknown product (peak 153) that was strongly correlated with the total proportions of H-units was also unique to compression wood. As treating the fine-milled samples with calcium-chloride did not significantly increase the proportions of phenol, or phenolic compounds with no methoxyl groups and only a single carbon sidechain, it is likely that the pyrolysis of polysaccharides only produces small proportions of these compounds, which would explain the small proportions observed in the opposite wood samples. As the compound 4-hydroxy-benzaldehyde was unique to compression wood, it is unlikely that the pyrolysis of polysaccharides forms this product. Multivariate analyses (principal component analysis) have successfully been used to distinguish between compression wood samples and normal wood from different softwood species based on the products derived from H-units and G-units of lignin (Alves *et al.*, 2009). The proportions of the six unique products identified in the current study were significantly different among some of the compression wood samples. Further investigation of differences in the proportions of these products in different compression wood samples would indicate whether they could be useful in distinguishing between samples with differing degrees of compression wood severity.

Using a different polarity gas-chromatography column, Mast et al. (2009) examined a sample of compression wood and a sample of opposite wood from a 6-year-old radiata pine by pyrolysis gas-chromatography/mass-spectrometry. Their results indicated that the proportions of the six unique products identified here were increased in compression wood compared with opposite wood, but that they were not unique to compression wood. However, the peaks in their chromatograms were not well resolved from each other, with the exception of 4-ethenyl-phenol (4-vinyl-phenol) which was also present in opposite wood

in the present study; therefore, it is not obvious to what extent other pyrolysis products contributed to those peaks. Pyrolysis gas-chromatography/mass-spectrometry was also used to examine compression wood and opposite wood of radiata pine to determine whether this technique could be useful to determine the H/G ratio of wood as a measure of compression wood severity (Nanayakkara, 2007). In the study of Nanayakkara (2007), the same column as Mast et al. (2009) was used and it was not clear from examining the chromatograms whether the products identified as unique to compression wood in the current study were present in the opposite wood sample examined by Nanayakkara (2007). The peaks in the chromatograms of Nanayakkara (2007) were not well resolved and additionally the baseline was not flat, therefore quantification of these products by integrating the peak areas would not have been precise and it is not surprising that the H/G ratio calculated from these chromatograms was not related to compression wood severity.

Whiting and Goring (1982) used infrared spectroscopy and also hydriodic acid hydrolysis followed by gas chromatography to determine the methyl iodide released, to compare the relative proportions of methoxyl groups in ground wood from the middle lamella, secondary wall and whole wood in the normal wood of black spruce (*Picea mariana*). The lower methoxyl content of wood from the middle lamella compared to wood from the secondary walls was considered indicative of a higher proportion of H-units in the middle lamella lignin (Whiting and Goring, 1982). Westermark (1985) suggested that the small proportions of H-units in the middle lamella lignin of normal wood observed by Whiting and Goring (1982) was due to the presence of small amounts of compression wood. Pyrolysis products from lignin H-units, albeit in small proportions, were present in the opposite wood of the samples examined in the current study. It is possible that there was some mild compression wood present in the opposite wood of these samples, due to the practical difficulties cited during their growth (Apiolaza *et al.*, 2011b), which resulted in

some "commingling of compression wood and opposite wood". The samples chosen for investigation in the current study were visually assessed for the presence of compression wood as explained in Chapter 2, but mild compression wood may not have been visibly detectable. The pyrolysis products found in opposite wood that are apparently from the H-units of lignin could alternatively be from pyrolysis of *p*-coumaric acid esters. These are found in the primary walls of radiata pine, where they are likely to be attached to pectic polysaccharides (Carnachan and Harris, 2000), and they are known to pyrolyse to 4-ethenyl-phenol (Serban, 1998). However, other studies have indicated that there are H-units found in the 'normal' lignin of both straight-grown trees and opposite wood (Terashima and Fukushima, 1988; Yeh *et al.*, 2005; Yeh *et al.*, 2006; Tokareva *et al.*, 2007; Nanayakkara *et al.*, 2009). A microautoradiographic study of lignin deposition in black pine (*Pinus thunbergii*) fed tritiated monolignol glucosides showed that, in normal wood, H-units are deposited before G-units, and are deposited in the compound middle lamella; whereas in compression wood, H-units are likewise deposited first, but their deposition is more active in the outer S2 layer (Terashima and Fukushima, 1988).

The apparent spatial regulation of H-unit deposition in normal wood and compression wood, and the significance of the proportion of H-units in compression wood, suggest that the composition of lignin may have some functional significance in the wall. A modelling study by Besombes et al. (2003) suggests that the lack of a methoxyl oxygen in H-units confers higher conformational flexibility to lignins rich in H-units, thus the different lignin composition in compression wood may have functional significance in the compression wood tracheid walls. However, it has been suggested that plants produce lignin because they require a polymer with specific properties, but that the composition of the lignin is not significant (Ralph, 1997). The total proportion of H-units is strongly correlated with lignin content (Wadenback *et al.*, 2004; Nanayakkara *et al.*, 2009). It may be

that the observed relationships between H-units and compression wood severity in radiata pine (Nanayakkara *et al.*, 2009) is due to the increase in total lignin content rather than indicating a functional role of the H-units of compression wood lignin.

A number of pyrolysis degradation products that could be used to detect compression wood have been identified in the current study. Those products that are derived from the H-units of lignin are typically more diagnostic of the presence of compression wood than those derived from the galactosyl residues detected in compression wood, probably due to the lower efficiency of pyrolysis of carbohydrates, and that the anhydrogalactose products can be derived from the galactosyl residues present in polysaccharides other than the (1→4)-β-D-galactans of compression wood. Products derived from the H-units of lignin which have sidechains of two or three carbons should be further investigated as indicators of compression wood severity.

4.5 References

Altaner, C. M., E. N. Tokareva, M. C. Jarvis and P. J. Harris (2010). "Distribution of (1→4)-β-galactans, arabinogalactan proteins, xylans and (1→3)-β-glucans in tracheid cell walls of softwoods." Tree Physiology **30**: 782-793.

Alves, A., N. Gierlinger, M. Schwanninger and J. Rodrigues (2009). "Analytical pyrolysis as a direct method to determine the lignin content in wood: Part 3. Evaluation of species-specific and tissue-specific differences in softwood lignin composition using principal component analysis." Journal of Analytical and Applied Pyrolysis **85**: 30-37.

Alves, A., J. Rodrigues, R. Wimmer and M. Schwanninger (2008). "Analytical pyrolysis as a direct method to determine the lignin content in wood. Part 2: Evaluation of the common model and the influence of compression wood." Journal of Analytical and Applied Pyrolysis **81**: 167-172.

Alves, A., M. Schwanninger, H. Pereira and J. Rodrigues (2006). "Analytical pyrolysis as a direct method to determine the lignin content in wood. Part 1: Comparison of pyrolysis lignin with Klason lignin." Journal of Analytical and Applied Pyrolysis **76**: 209-213.

Apiolaza, L., S. Chauhan and J. Walker (2011). "Genetic control of very early compression and opposite wood in *Pinus radiata* and its implications for selection." Tree Genetics & Genomes **7**: 563-571.

Besombes, S., D. Robert, J.-P. Utille, F. R. Taravel and K. Mazeau (2003). "Molecular modeling of syringyl and *p*-hydroxyphenyl β-O-4 dimers. Comparative study of the computed and experimental conformational properties of lignin β-O-4 model compounds." Journal of Agricultural and Food Chemistry **51**: 34-42.

Brennan, M., J. P. McLean, C. M. Altaner, J. Ralph and P. J. Harris (2012). "Cellulose microfibril angles and cell-wall polymers in different wood types of *Pinus radiata*." Cellulose **Online First**.

Budgell, D. R., E. R. Hayes and R. J. Helleur (1987). "Direct identification of pentoses and hexoses by pyrolysis/capillary gas chromatography." Analytica Chimica Acta **192**: 243-253.

Carnachan, S. M. and P. J. Harris (2000). "Polysaccharide compositions of primary cell walls of the palms *Phoenix canariensis* and *Rhopalostylis sapida*." Plant Physiology and Biochemistry **38**: 699-708.

Faix, O., I. Fortmann, J. Bremer and D. Meier (1991). "Thermal degradation products of wood. Gas chromatographic separation and mass spectrometric characterization of polysaccharide derived products." Holz als Roh- und Werkstoff **49**: 213-219.

Faix, O., D. Meier and I. Fortmann (1990). "Thermal degradation products of wood. Gas chromatographic separation and mass spectrometric characterization of monomeric lignin derived products." Holz als Roh- und Werkstoff **48**: 281-285.

Gur'janov, O. P., N. N. Ibragimova, O. I. Gnezdilov and T. A. Gorshkova (2008). "Polysaccharides, tightly bound to cellulose in cell wall of flax bast fibre: Isolation and identification." Carbohydrate Polymers **72**: 719-729.

Harris, P. J. (2005). Diversity in plant cell walls. Plant diversity and evolution: genotypic and phenotypic variation in higher plants. R. J. Henry. Wallingford, Oxon, UK, CAB International Publishing**:** 201-227.

Ikeda, T., K. Holtman, J. F. Kadla, H.-m. Chang and H. Jameel (2001). "Studies on the effect of ball milling on lignin structure using a modified DFRC method." Journal of Agricultural and Food Chemistry **50**: 129-135.

Kim, J. S., T. Awano, A. Yoshinaga and K. Takabe (2010). "Immunolocalization of β-1-4-galactan and its relationship with lignin distribution in developing compression wood of *Cryptomeria japonica*." Planta **232**: 109-119.

Kleen, M. and G. Gellerstedt (1995). "Influence of inorganic species on the formation of polysaccharide and lignin degradation products in the analytical pyrolysis of pulps." Journal of Analytical and Applied Pyrolysis **35**: 15-41.

Lommen, A. (2009). "MetAlign: interface-driven, versatile metabolomics tool for hyphenated full-scan mass spectrometry data preprocessing." Analytical Chemistry **81**: 3079-3086.

Mast, S. W., L. A. Donaldson, K. Torr, L. Phillips, H. Flint, M. West, T. J. Strabala and A. Wagner (2009). "Exploring the ultrastructural localization and biosynthesis of β(1,4)-galactan in *Pinus radiata* compression wood." Plant Physiology **150**: 573-583.

Meier, D. and O. Faix (1992). Pyrolysis-gas chromtography-mass spectrometry. Methods in lignin chemistry. S. Y. Lin and C. W. Dence. Berlin, Springer.

Meier, D., I. Fortmann, J. Odermatt and O. Faix (2005). "Discrimination of genetically modified poplar clones by analytical pyrolysis-gas chromatography and principal component analysis." Journal of Analytical and Applied Pyrolysis **74**: 129-137.

Minor, J. L. (1982). "Chemical linkage of pine polysaccharides to lignin." Journal of Wood Chemistry and Technology **2**: 1-16.

Mohan, D., C. U. Pittman and P. H. Steele (2006). "Pyrolysis of wood/biomass for bio-oil: a critical review." Energy & Fuels **20**: 848-889.

Möller, R., A. McDonald, C. Walter and P. J. Harris (2003). "Cell differentiation, secondary cell-wall formation and transformation of callus tissue of *Pinus radiata* D. Don." Planta **217**: 736-747.

Mukoyoshi, S.-i., J.-i. Azuma and T. Koshijima (1981). "Lignin-carbohydrate complexes from compression wood of *Pinus densiflora* Sieb et. Zucc." Holzforschung **35**: 233-240.

Nanayakkara, B. (2007). Chemical characterisation of compression wood in plantation grown *Pinus radiata*. Doctoral thesis Doctoral thesis, The University of Waikato.

Nanayakkara, B., M. Manley-Harris, I. D. Suckling and L. A. Donaldson (2009). "Quantitative chemical indicators to assess the gradation of compression wood." Holzforschung **63**: 431-439.

NIST/EPA/NIH Mass Spectral Database. (2002). National institute of standards and technology.

Nonier, M. F., N. Vivas, N. Vivas de Gaulejac, C. Absalon, P. Soulié and E. Fouquet (2006). "Pyrolysis-gas chromatography/mass spectrometry of *Quercus* sp. wood. Application to structural elucidation of macromolecules and aromatic profiles of different species." Journal of Analytical and Applied Pyrolysis **75**: 181-193.

Pan, W.-P. and G. N. Richards (1989). "Influence of metal ions on volatile products of pyrolysis of wood." Journal of Analytical and Applied Pyrolysis **16**: 117-126.

Ponder, G. R., H.-X. Qiu and G. N. Richards (1990). "Pyrolytic conversion of biomass to anhydrosugars." Applied Biochemistry and Biotechnology **24/25**: 41-47.

Ponder, G. R., G. N. Richards and T. T. Stevenson (1992). "Influence of linkage position and orientation in pyrolysis of polysaccharides: A study of several glucans." Journal of Analytical and Applied Pyrolysis **22**: 217-229.

R Development Core Team (2011). "R: A language and environment for statistical computing." from http://www.R-project.org.

Ralph, J. (1997). Recent advances in characterizing "non-traditional" lignins. 9th International symposium on wood and pulping chemistry.

Ralph, J. and R. D. Hatfield (1991). "Pyrolysis-GC-MS characterization of forage materials." Journal of Agricultural and Food Chemistry **39**: 1426-1437.

Renard, C. M. G. C., M.-J. Crépeau and J.-F. Thibault (1995). "Structure of the repeating units in the rhamnogalacturonic backbone of apple, beet and citrus pectins." Carbohydrate Research **275**: 155-165.

Rencoret, J., A. Gutiérrez, L. Nieto, J. Jiménez-Barbero, C. B. Faulds, H. Kim, J. Ralph, Á. T. Martínez and J. C. del Río (2011). "Lignin composition and structure in young versus adult *Eucalyptus globulus* plants." Plant Physiology **155**: 667-682.

Serban, C. M. (1998). Analytical pyrolysis of natural organic polymers. Amsterdam, Elsevier. **20:** 496.

Shafizadeh, F. and Y. L. Fu (1973). "Pyrolysis of cellulose." Carbohydrate Research **29**: 113-122.

Syverud, K., I. Leirset and D. Vaaler (2003). "Characterization of carbohydrates in chemical pulps by pyrolysis gas chromatography/mass spectrometry." Journal of Analytical and Applied Pyrolysis **67**: 381-391.

Terashima, N. and K. Fukushima (1988). "Heterogeneity in formation of lignin - XI: An autoradiographic study of the heterogeneous formation and structure of pine lignin." Wood Science and Technology **22**: 259-270.

Tokareva, E. N., A. V. Pranovich, P. Fardim, G. Daniel and B. Holmbom (2007). "Analysis of wood tissues by time-of-flight secondary ion mass spectrometry." Holzforschung **61**: 647-655.

Torri, C., A. Adamiano, D. Fabbri, C. Lindfors, A. Monti and A. Oasmaa (2010). "Comparative analysis of pyrolysate from herbaceous and woody energy crops by Py-GC with atomic emission and mass spectrometric detection." Journal of Analytical and Applied Pyrolysis **88**: 175-180.

Wadenback, J., D. Clapham, G. Gellerstedt and S. von Arnold (2004). "Variation in content and composition of lignin in young wood of Norway spruce." Holzforschung **58**: 107-115.

Westermark, U. (1985). "The occurrence of *p*-hydroxyphenylpropane units in the middle-lamella lignin of spruce (*Picea abies*)." Wood Science and Technology **19**: 223-232.

Whiting, P. and D. Goring (1982). "Chemical characterization of tissue fractions from the middle lamella and secondary wall of black spruce tracheids." Wood Science and Technology **16**: 261-267.

Yeh, T.-F., J. L. Braun, B. Goldfarb, H.-m. Chang and J. F. Kadla (2006). "Morphological and chemical variations between juvenile wood, mature wood, and compression wood of loblolly pine (*Pinus taeda* L.)." Holzforschung **60**: 1-8.

Yeh, T.-F., B. Goldfarb, H.-m. Chang, I. Peszlen, J. L. Braun and J. F. Kadla (2005). "Comparison of morphological and chemical properties between juvenile wood and compression wood of loblolly pine." Holzforschung **59**: 669-674.

Zykwinska, A., J.-F. Thibault and M.-C. Ralet (2007). "Organization of pectic arabinan and galactan side chains in association with cellulose microfibrils in primary cell walls and related models envisaged." Journal of Experimental Botany **58**: 1795-1802.

Zykwinska, A. W., M.-C. J. Ralet, C. D. Garnier and J.-F. J. Thibault (2005). "Evidence for in vitro binding of pectin side chains to cellulose." Plant Physiology **139**: 397-407.

Chapter 5 - Using *endo*-(1→4)-β-D-galactanases to detect (1→4)-β-D-galactans in compression wood

5.1 Introduction

Purified preparations of enzymes that cleave cell-wall polysaccharides at specific glycosidic linkages are useful for the structural assessment and determination of cell-wall polysaccharides present in isolated cell walls, or cell-wall fractions. Pure enzymes can be acquired by purification of enzyme preparations (Huber and Nevins, 1977; Andersson *et al.*, 1978) and by generation of recombinant enzymes which may be followed by purification (Mort *et al.*, 2005) and are now commercially available (Biosupplies Australia Pty Ltd.; Megazyme International Ltd.). The purity and high specificity of the enzymes means that the released products are unambiguously known to be from a particular polysaccharide and their structures can be further analysed. For example, xyloglucan-specific *endo*-(1→4)-β-glucanases have been used to hydrolyse xyloglucans, and the released oligosaccharides were identified using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and matrix-assisted laser-desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) (Lerouxel *et al.*, 2002; Hsieh and Harris, 2009). In addition to structural analysis, the products of specific enzyme hydrolysis can be quantified, for example glucose can be quantified colorimetrically by reactions with glucose-oxidase/peroxidase (McCleary and Codd, 1991) or *p*-hydroxybenzoic acid hydrazide (Blakeney and Mutton, 1980), by HPAEC-PAD (Kazmaier *et al.*, 1998) or by MALDI-TOF MS (Kazmaier *et al.*, 1998; Grant *et al.*, 2003; Bungert *et al.*, 2004). Analysis of oligosaccharides by MALDI-TOF

MS is attractive because it is a rapid, sensitive technique that does not require derivitisation of the oligosaccharides for detection (Cohen and Gusev, 2002; Günl *et al.*, 2011). The incorporation of a matrix in the samples allows a "soft" ionisation of the molecules present without fragmentation (breaking bonds).

Cell-wall fractions enriched for the polysaccharide of interest can be obtained by treating isolated cell walls with solutions of alkali such as sodium hydroxide or potassium hydroxide containing sodium borohydride to prevent alkaline peeling (Browning, 1967; Fengel and Wegener, 1989). Quantitative isolation of the polysaccharide of interest by extraction with alkali is not possible from lignified walls with currently available methods. In particular, a large proportion of the polysaccharides that are tightly associated with lignin, for example the (1→4)-β-D-galactans of compression wood (Bouveng and Meier, 1959a; Mukoyoshi *et al.*, 1981; Minor, 1982), can remain with the lignin. However, complete dissolution of whole wood cell-walls in ionic liquids, which interrupt the hydrogen bonds between cell-wall polymers, followed by regeneration (precipitation) of the polysaccharide-containing fraction, has been used to fractionate the cell-wall polysaccharides, making them more accessible for enzyme hydrolysis (Kilpeläinen *et al.*, 2007). Complete dissolution of the cell walls and regeneration of the polysaccharide-containing fraction could possibly isolate a greater proportion of the polysaccharides from highly lignified materials such as wood, compared with alkali extracts.

In this chapter I aimed to investigate the feasibility of using *endo*-(1→4)-β-D-galactanases to release (1→4)-β-D-galactan oligosaccharides from wood samples, which could be quantified and used to determine the severity of compression wood as a measure of wood quality. A commercial preparation of lupin seed (1→4)-β-D-galactan was hydrolysed with *endo*-(1→4)-β-D-galactanase as a reference

standard, and the sodium adduct ions of released galactosyl oligosaccharides were detected by MALDI-TOF MS. The products from the lupin seed (1→4)-β-D-galactan hydrolysates were then compared with the products yielded by *endo*-(1→4)-β-galactanase hydrolysis of wood extracts (sodium hydroxide extracts and ionic liquid extracts), and of ball-milled wood. Standard solutions of different concentrations of lupin seed (1→4)-β-D-galactan were hydrolysed with two different *endo*-(1→4)-β-galactanases: one isolated from *Aspergillus niger*, and a recombinant, thermophilic enzyme from *Clostridium thermocellum*. The precision of quantification using the ratio of different galactosyl oligosaccharide sodium adduct ions to an internal standard was compared for each of the different enzymes in order to determine how the future quantification of (1→4)-β-D-galactans in wood might best be achieved.

5.2 Materials and methods

5.2.1 Enzymes

The following highly purified enzymes were used: an *endo*-(1→4)-β-galactanase from *Aspergillus niger* (catalogue number: E-EGALN) and a recombinant *endo*-(1→4)-β-galactanase from *Clostridium thermocellum* (catalogue number: E-GALCT), from Megazyme International Ireland Ltd. These enzyme preparations have only small proportions of other enzyme activities, e.g. the cellulase activity is 0.0006% of the activity of the *A. niger endo*-(1→4)-β-galactanase activity, and 0.003% of the activity of the *C. thermocellum endo*-(1→4)-β-galactanase activity on potato (1→4)-β-D-galactan (Megazyme International Ltd.). No other enzyme activities in these preparations would yield hexosyl oligosaccharides.

5.2.2 Wood materials and standards

A (1→4)-β-D-galactan preparation from lupin seeds (P-GALLU, Gal:Ara:Rha:Xyl:GalUA = 91:2:1.8:0.2:5.0, Megazyme International Ireland Ltd.) was used as the reference standard for developing a method of estimating (1→4)-β-D-galactans. The xyloglucan heptasaccharide XXXG (using the nomenclature of Fry *et al.*, 1993) (X_3GLC_4, Megazyme International Ireland Ltd.) was used as an internal standard for MALDI-TOF MS quantification of oligosaccharides derived from (1→4)-β-D-galactans. Wood samples were from the Amberley field trial described in Chapter 2 and by Apiolaza et al. (2011b). For sodium hydroxide extracts of wood, the wood samples were ground in a Wiley® mini-mill (Thomas Scientific, Swedesboro, NJ) to pass a 40-mesh screen (422 µm pore size). For ionic liquid dissolution of wood and enzyme hydrolysis of ball-milled (untreated) wood, the 40-mesh material (300 mg) was finely milled in a Retsch PM100 (Haan, Germany) planetary ball mill in a zirconium dioxide (ZrO_2) grinding bowl with 10 zirconium dioxide ball bearings (10 mm diameter) at 600 rpm for 3 h 20 min (with 10 min cooling breaks every 20 min) following the recommended grinding times of Kim and Ralph (2010) to avoid polymer degradation.

5.2.2.1 NaOH extracts of wood

Wood ground to 40-mesh particle size (~300 mg) was treated with 8 M NaOH (15 ml) containing 1% (w/v) $NaBH_4$ to prevent oxidative degradation (alkaline peeling) (Whistler and BeMiller, 1958) on an orbital shaker at 37°C for 24 h. The extract was filtered through Miracloth, cooled on ice, and then brought to neutral pH by dropwise addition of 17.4 M acetic acid. A drop of toluene was added to prevent microbial growth. The filtrate was

dialysed (molecular weight cut-off 12 to 14 kDa) against tap water for 24 h, followed by Milli-Q® water for 1 h. The dialysate was freeze-dried and stored in a desiccator over indicating silica gel.

5.2.2.2 Ionic liquid dissolution of wood

Ball-milled wood (8 mg) was added to the ionic liquid 1-ethyl-3-methylimidazolium acetate (often abbreviated to 'EMIMAc' or '[C_2mim]OAc') (200 mg, 0.195 ml) (Sigma-Aldrich, St Louis, MO, USA) in a sealed Pierce reaction vial and placed in an oven at 80°C for 4 h (Kilpeläinen *et al.*, 2007), after which time the wood was fully dissolved. Regeneration (precipitation) of the polysaccharide-rich fraction was based on Sun et al. (2009) as follows. The solution was quantitatively transferred to a beaker with acetone:water (1:1, v/v) (20 ml), covered with parafilm and stirred constantly at room temperature for 1 h. The beaker contents were transferred onto a glass-fibre filter (Whatman-GC, pre-fired at 500°C for 1 h) and washed with acetone:water (1:1, v/v) (10 ml), followed by 100% acetone (5 ml). The residue was air-dried on the filter overnight at room temperature. Enzyme hydrolysis was done by submerging the ionic liquid residue, while still on the filter, in the enzyme solution (0.5 ml 50 mM ammonium formate buffer, pH 4.0, and 0.5 units of enzyme as detailed below).

5.2.2.3 Enzymic hydrolysis and MALDI-TOF MS sample preparation

5.2.2.3.1 (1→4)-β-D-Galactan detection

To detect (1→4)-β-D-galactans, 2 mg of the lupin seed (1→4)-β-D-galactan reference standard, NaOH extract, ball-milled wood or the ionic liquid residue was suspended in 50 mM ammonium formate buffer (pH 4.0) (0.5 ml). To these mixtures 0.5 U of enzyme (0.5 µl from *A. niger endo*-(1→4)-β-galactanase or 4 µl from *C. thermocellum endo*-(1→4)-β-galactanase) was added. Hydrolysis with *A. niger endo*-(1→4)-β-galactanase was done at 37°C, whereas hydrolysis with *C. thermocellum endo*-(1→4)-β-galactanase was done at 60°C. Hydrolysis was done for 30 min unless otherwise stated. Hydrolysis was stopped by boiling at 100°C for 2 min. Aliquots (10 µl) of enzyme hydrolysate were added to matrix (2,5-dihydroxybenzoic acid, 10 mg ml^{-1} in water, 10 µl) and of NaCl (10 mM, 6 µl). An aliquot (1 µl) of this mixture was spotted on a MALDI sample plate, air-dried at room temperature and loaded into the MALDI-TOF mass spectrometer.

The following controls were done (incubated for 30 min as described above) to ensure that the ions detected in the mass spectra were derived from cleavage of (1→4)-β-D-galactans by *endo*-(1→4)-β-galactanase:

1. Buffer only (50 mM ammonium formate buffer, pH 4.0).
2. Buffer and substrate (lupin seed (1→4)-β-D-galactan, NaOH extract, ionic liquid extract or ball-milled wood) only.
3. Buffer and enzyme (*A. niger endo*-(1→4)-β-galactanase or *C. thermocellum endo*-(1→4)-β-galactanase) only (no substrate).

5.2.2.3.2 Quantification of lupin seed (1→4)-β-D-galactan

A 20 mg ml^{-1} stock solution of lupin galactan in 50 mM ammonium formate buffer (pH 4.0) was prepared. Aliquots of this were diluted in 50 mM ammonium formate buffer (pH 4.0) to give final concentrations of 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg ml^{-1}. To each standard, 0.5 U of enzyme was added before hydrolysis for 30 min at 50°C (*A. niger endo*-(1→4)-β-galactanase) or 60°C (*C. thermocellum endo*-(1→4)-β-galactanase) as above. Hydrolysis was stopped by boiling at 100°C for 2 min. When cool, 125 µg of xyloglucan heptasaccharide (XXXG) internal standard (50 µl of 2.5 mg ml^{-1} in 50 mM ammonium formate buffer, pH 4.0) was added. The sodium adduct ion of XXXG has an m/z of 1085, which is sufficiently different from the m/z of any hexosyl oligosaccharide sodium adduct ions that they are clearly separated. An aliquot (50 µl) of the enzyme hydrolysate and internal standard mixture was added to 50 µl of a 1:6 (v/v) mixture of matrix (2,5-dihydroxybenzoic acid, 10 mg ml^{-1} in ammonium formate buffer) and NaCl (10 mM). An aliquot (1 µl) of this mixture was spotted onto a MALDI sample plate, air-dried at room temperature and loaded into the MALDI-TOF mass spectrometer.

5.2.3 MALDI-TOF MS

Spectra were acquired on a Voyager-DE™ PRO MALDI-TOF workstation (Applied Biosystems, Foster City, CA, USA) operated in reflectron mode with an acceleration voltage of 20 kV and a delay time of 200 ns. Compilation of each spectrum was of accumulated data from 100 laser shots. Calibration of the mass spectrometer was done using a peptide mixture (Cal Mix 1) containing three external standards: des-Arg1-bradykinin (m/z = 904.5), angiotensin I (m/z = 1296.7) and [Glu1]-fibrinopeptide B (m/z = 1570.6).

5.2.4 Analysis of the spectra

Spectra were analysed in the Voyager Data Explorer™ version 4.0. For quantification of lupin seed (1→4)-β-D-galactans, the heights of the peaks of each of the sodium adduct galactosyl oligosaccharide ions, or the summed peak heights of all sodium adduct galactosyl oligosaccharide ions, were divided by the height of the internal standard sodium adduct ion. Linear models were built using the statistical analysis software R (R Development Core Team, 2011) to compare quantification using *A. niger endo*-(1→4)-β-galactanase with quantification using *C. thermocellum endo*-(1→4)-β-galactanase.

5.3 Results

5.3.1 Detection of (1→4)-β-D-galactan oligosaccharides released from enzyme hydrolysis of lupin seed (1→4)-β-D-galactan

Hydrolysis of lupin galactan with *A. niger endo*-(1→4)-β-galactanase released galactosyl oligosaccharides which can be detected by the presence of the sodium-adduct ions $[M+Na]^+$ of galactosyl oligosaccharides in matrix-assisted laser-desorption ionisation time-of-flight (MALDI-TOF) mass spectra. After a hydrolysis time of 24 h, the sodium adduct ion of only galactobiose (m/z = 365) (two (1→4)-linked β-D-galactosyl residues) was detected (Figure 5-1, A). As the hydrolysis time was decreased, the degree of polymerisation of the galactosyl oligosaccharides increased. After a four hour hydrolysis,

galactosyl oligosaccharide sodium adduct ions with a degree of polymerisation of two, three (m/z = 527) and four (m/z = 689) were detected (Figure 5-1, B). After a 30 min hydrolysis, galactosyl oligosaccharide sodium adduct ions with degrees of polymerisation of five (m/z = 851), six (m/z = 1014) and seven (m/z = 1176) were detected (Figure 5-1, C) in addition to the galactosyl oligosaccharide sodium adduct ions with degrees of polymerisation of two, three and four. The 30 minute hydrolysis time was chosen as the shortest sensible hydrolysis time as the degree of polymerisation of the oligosaccharides released were in the molecular weight range of the molecular weight standard used for calibration of the instrument, discounting the need for a second calibrant for oligosaccharides of a higher molecular weight.

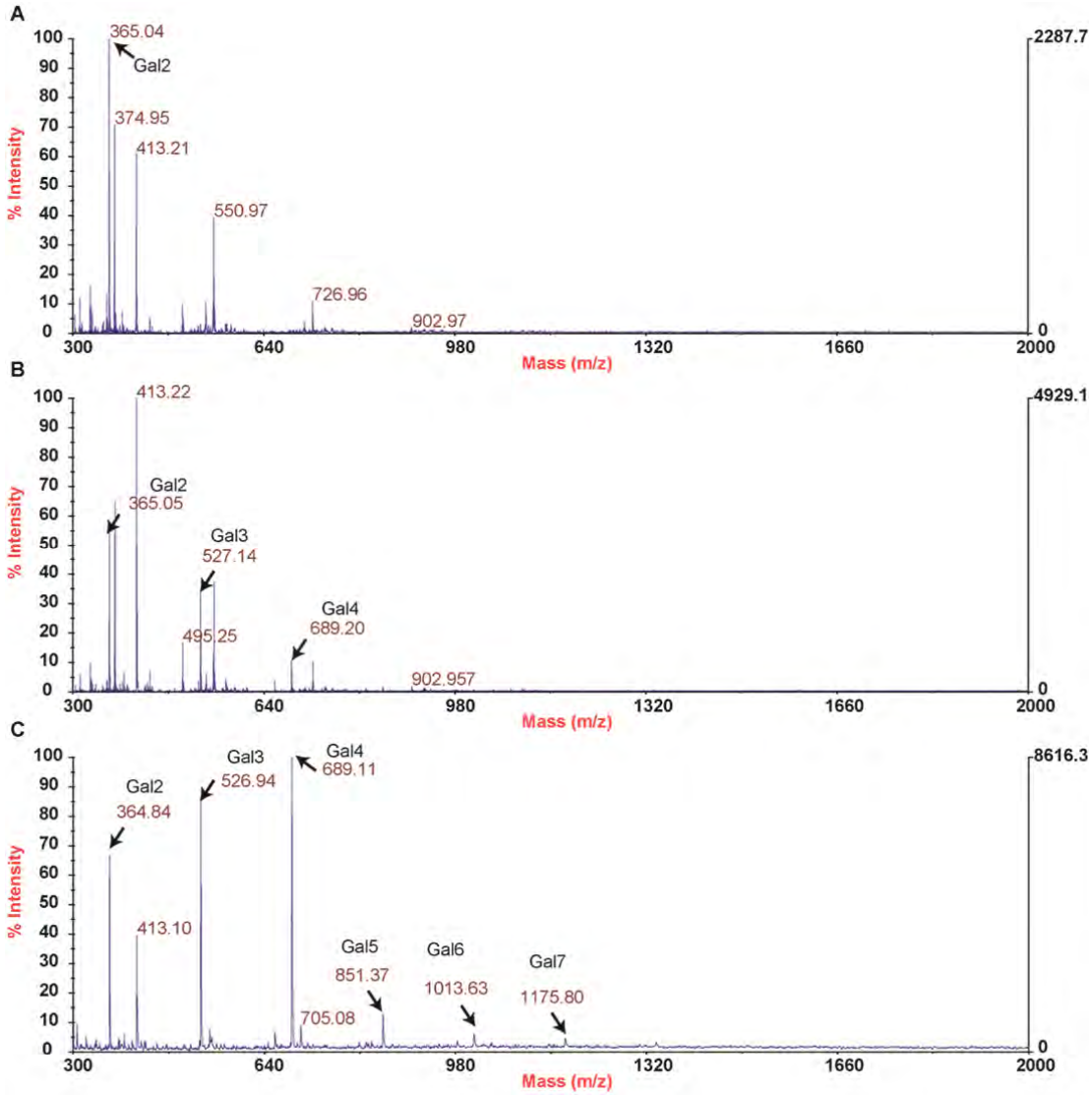

Figure 5-1 MALDI-TOF mass spectra of galactosyl oligosaccharides released from lupin seed (1→4)-β-D-galactan after hydrolysis with *A. niger endo*-(1→4)-β-galactanase for (A) 24 h, (B) 4h and (C) 30 min. The degree of polymerisation is indicated by Gal2, Gal3 etc.

No sodium-adduct hexosyl oligosaccharide ions were detected in the spectra of the buffer only, enzyme only (Figure 5-2, A, *A. niger endo*-(1→4)-β-galactanase) or galactan only (Figure 5-2, B) controls. This showed that the products in the hydrolysate were galactosyl oligosaccharides released by cleavage of the glycosidic bonds between the galactosyl residues by the *endo*-(1→4)-β-galactanase. The m/z = 413 ion, shown in the controls

(Figure 5-2) is from the matrix (2,5-dihydroxybenzoic acid). Sodiated monohexosyl ions were not detected in any of the hydrolysates. Thus MALDI-TOF MS can be used for the detection of (1→4)-β-D-galactan oligosaccharides released by *endo*-(1→4)-β-galactanase hydrolysis.

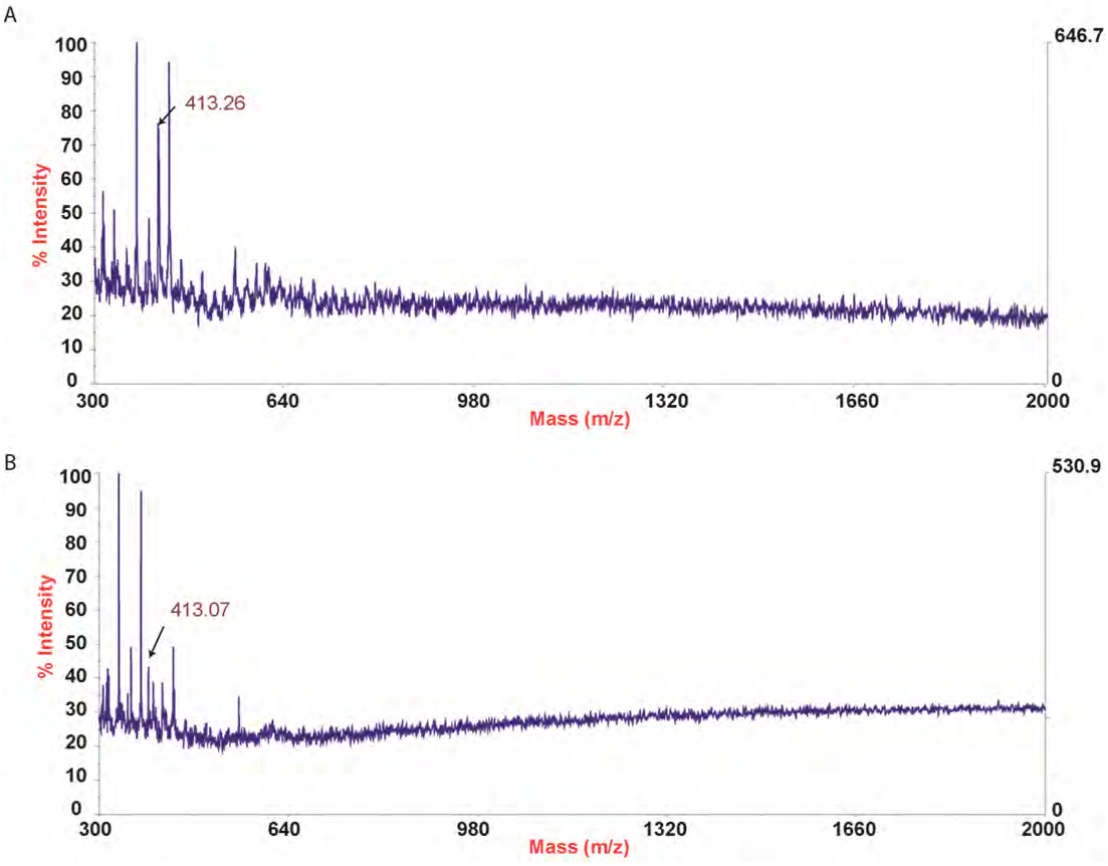

Figure 5-2 MALDI-TOF mass spectra of (A) the *A. niger endo*-(1→4)-β-galactanase (buffer and enzyme only) control and (B) the lupin seed (1→4)-β-D-galactan (buffer and substrate only) control.

5.3.2 Detection of (1→4)-β-D-galactan oligosaccharides released from enzyme hydrolysis of sodium hydroxide extracts from wood

Sodium hydroxide (NaOH) extracts of wood were hydrolysed with *A. niger* *endo*-(1→4)-β-galactanase. The sodium adduct ion of galactobiose was detected in the hydrolysate of the NaOH extract of compression wood (Figure 5-3, A). No sodium adduct ions of galactosyl oligosaccharides were detected in the hydrolysates of NaOH extracts of opposite wood (Figure 5-3, B). Sodium adduct ions of oligosaccharides with higher degrees of polymerisation were not detected. No sodium adduct ions of galactosyl oligosaccharides were detected in the controls.

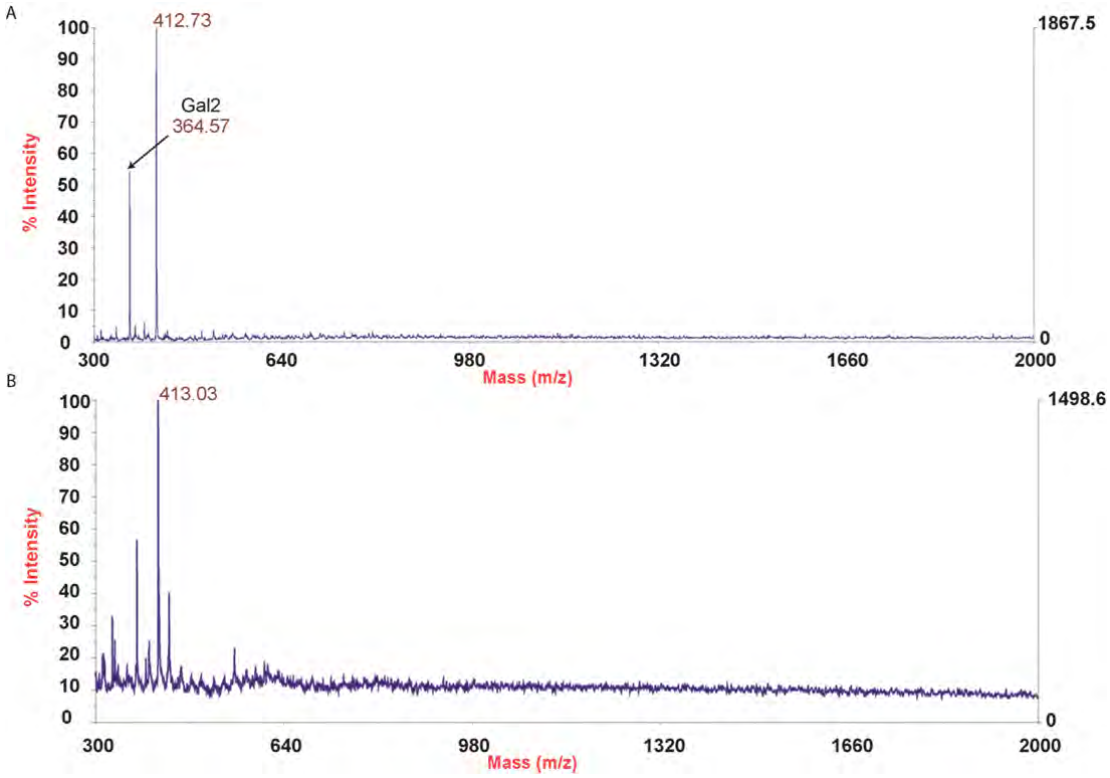

Figure 5-3 MALDI-TOF mass spectra of the products of *A. niger endo*-(1→4)-β-galactanase hydrolysis of NaOH extracts of (A) compression wood and (B) opposite wood. Only di-galactosyl sodium adduct ions (Gal2) were present in the hydrolysate of the compression wood NaOH extract.

5.3.3 Detection of (1→4)-β-D-galactan oligosaccharides released from enzyme hydrolysis of ionic liquid extracts from wood

Hydrolysis of the ionic liquid extracts of both opposite wood and compression wood with *A. niger endo*-(1→4)-β-galactanase yielded an unusual series of ions. The products differed by 162 mass units which corresponds to the molecular weight of an anhydrohexosyl residue, although the m/zs of the products did not correspond to a mono-sodiated galactosyl series. One series was composed of ions of m/z = 713, 875 and 1037, having the expected molecular weights of di-sodium adducts of galactosyl oligosaccharides with the

degree of polymerisation of four, five and six, respectively (Figure 5-4, arrows). The second ion series was composed of ions with m/z = 375, 537 and 699, which correspond to galactosyl oligosaccharides with degrees of polymerisation of two, three and four respectively, with an additional mass of 33 from an unknown adduct (Figure 5-4, arrows). Comparison of the relative abundances of the ions with the relative abundance of the matrix ion with m/z = 413 indicates that the galactosyl oligosaccharide ions are more abundant in the hydrolysate of the compression wood extract than they are in the opposite wood extract. These ions were not present in the controls.

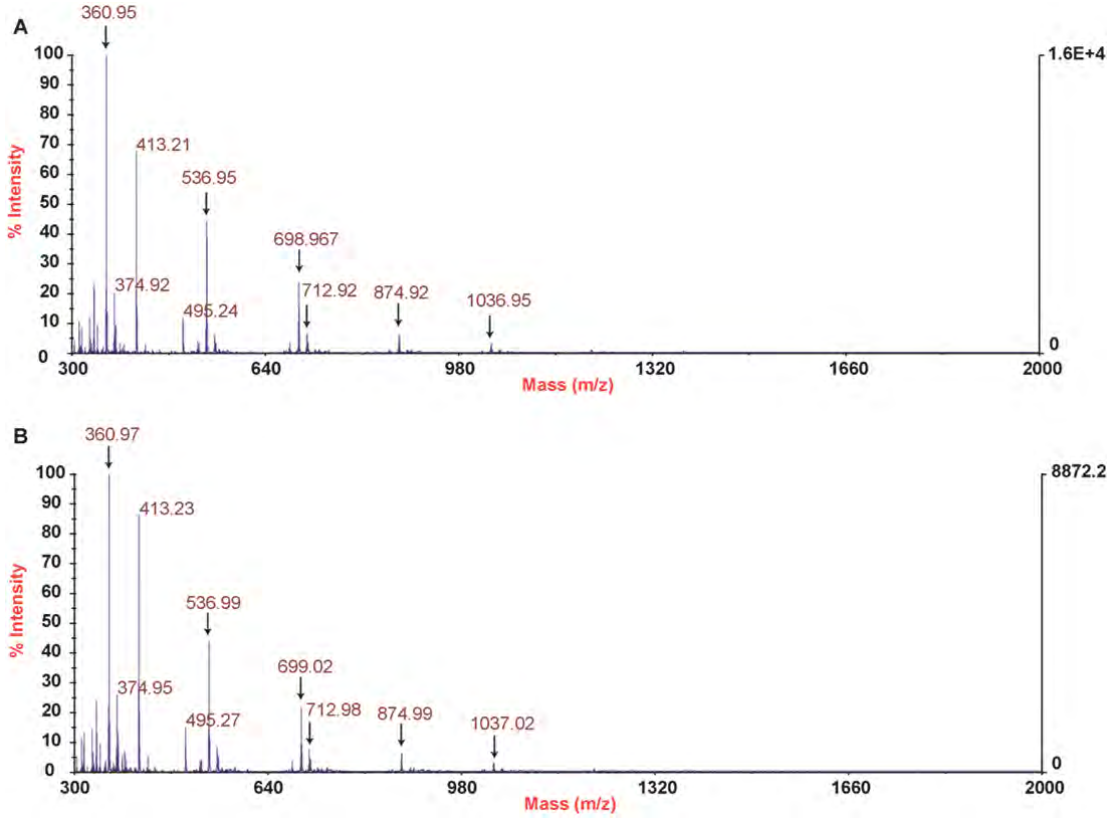

Figure 5-4 MALDI-TOF mass spectra of the products of *A. niger endo*-(1→4)-β-galactanase hydrolysis of ionic liquid extracts from (A) compression wood and (B) opposite wood. Arrows indicate the ions present in the two hexosyl series detected.

5.3.4 Detection of (1→4)-β-D-galactan oligosaccharides released from enzyme hydrolysis of ball-milled wood

Ball-milled wood, without fractionation or delignification, was directly hydrolysed with *A. niger endo*-(1→4)-β-galactanase. Sodium adduct ions of galactosyl oligosaccharides with degrees of polymerisation of two, three and four were detected in the compression wood hydrolysates (Figure 5-5, A). No sodium adduct ions of galactosyl oligosaccharides were detected in the opposite wood hydrolysates (Figure 5-5, B). No sodium adduct ions of galactosyl oligosaccharides were detected in the enzyme only, buffer only or galactan only controls.

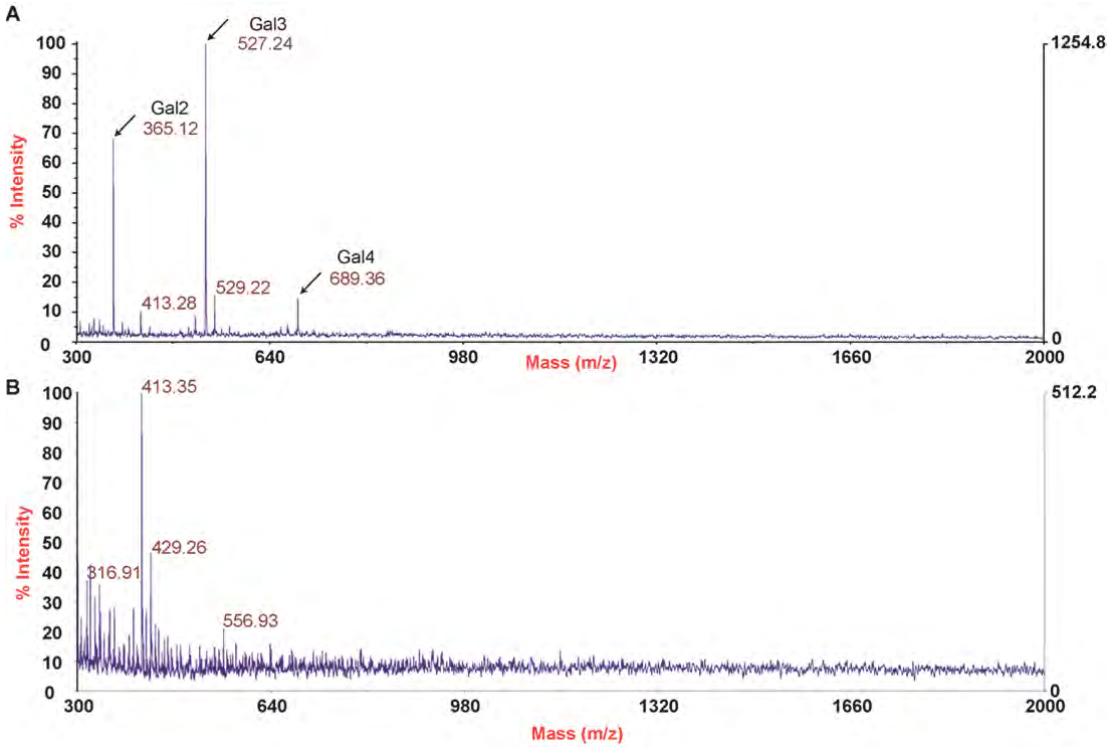

Figure 5-5 MALDI-TOF mass spectra of the products of *A. niger endo*-(1→4)-β-galactanase hydrolysis of (A) ball-milled compression wood and (B) ball-milled opposite wood. The degree of polymerisation is indicated by Gal2, Gal3 etc.

5.3.5 Comparison of the two *endo*-(1→4)-β-galactanases

After confirmation that (1→4)-β-D-galactans from compression wood extracts and ball-milled compression wood can be specifically hydrolysed by *A. niger endo*-(1→4)-β-galactanase, the different *endo*-(1→4)-β-galactanases available were considered for investigation. A recombinant, thermophilic galactanase from *Clostridium thermocellum* was available, and was here compared with the *A. niger endo*-(1→4)-β-galactanase for quantification of (1→4)-β-D-galactans. Solutions of different lupin seed (1→4)-β-D-galactan concentrations were used to compare the precision of quantification of (1→4)-β-D-galactan oligosaccharides by *A. niger*

endo-(1→4)-β-galactanase and *C. thermocellum endo*-(1→4)-β-galactanase. For each concentration of lupin seed (1→4)-β-D-galactan, triplicate hydrolysates were prepared for MALDI-TOF MS. Three spectra were acquired for each hydrolysate. The concentration of lupin seed (1→4)-β-D-galactan was plotted against the ratio of the ion strength of the sodium adduct galactosyl oligosaccharide ions to the internal standard, XXXG, to determine whether there was a linear relationship using both enzymes. The strength (peak heights) of the sodium adduct ions of the galactosyl oligosaccharides were summed and divided by the strength (peak height) of the sodium adduct ion of the internal standard. The mean ratio calculated for each concentration of lupin seed (1→4)-β-D-galactan is plotted, with the standard deviation (error bars) in Figure 5-6. Hydrolysis of the lupin seed (1→4)-β-D-galactan standards by both enzymes resulted in release of sodium adduct ions of galactosyl oligosaccharides with a total abundance (relative to the internal standard) that was linearly correlated with the concentration of (1→4)-β-D-galactan.

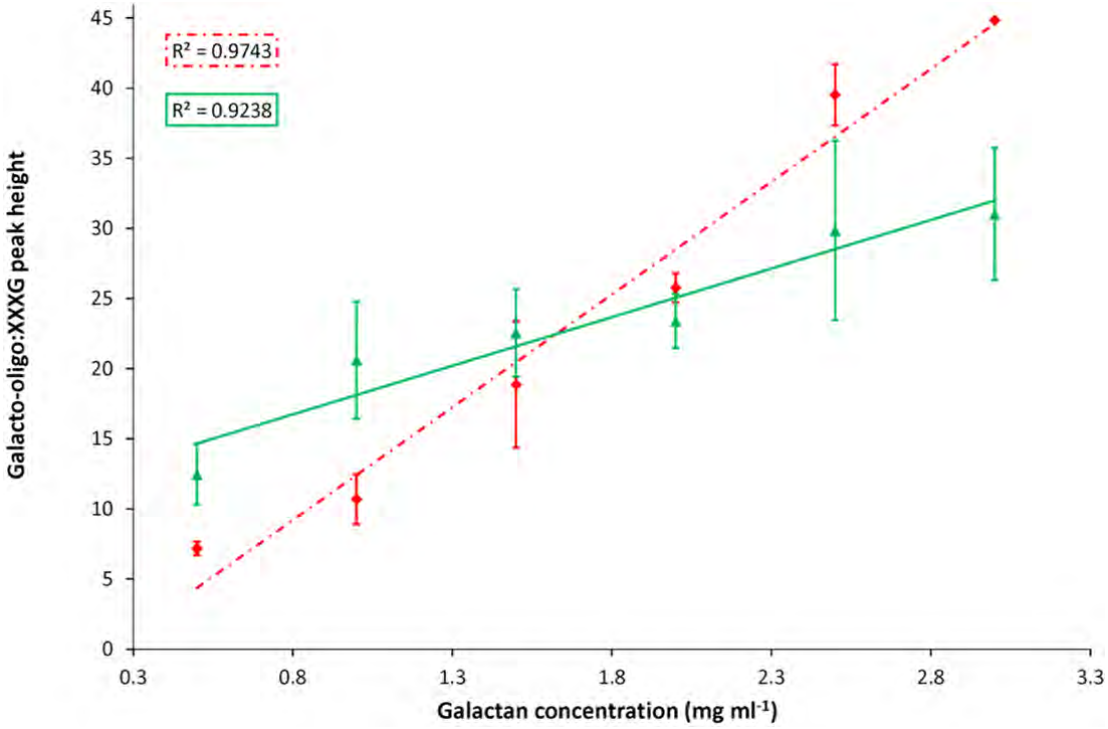

Figure 5-6 Comparison of two *A. niger* (green, solid line) and *C. thermocellum* (red, dashed line) *endo*-(1→4)-β-galactanases used to quantify different concentrations of lupin seed (1→4)-β-D-galactan. The mean ratio of the summed galactosyl oligosaccharide sodium adduct ion strengths relative to the strength of the internal standard sodium adduct ion is plotted ± standard deviation.

From the mass spectra of each of the different lupin seed (1→4)-β-D-galactan standards, it can be seen that as the concentration of lupin seed (1→4)-β-D-galactan is increased, galactosyl oligosaccharides with a higher degree of polymerisation are released (Figure 5-7). The concentration of the standard solutions of lupin seed (1→4)-β-D-galactan was increased from 0.5 mg ml^{-1} to 3.0 mg ml^{-1} by 0.5 mg ml^{-1} increments. Hydrolysis of the 0.5 mg ml^{-1} solution of lupin seed (1→4)-β-D-galactan with *C. thermocellum* *endo*-(1→4)-β-galactanase released galactosyl oligosaccharides with degrees of polymerisation from two to six (Figure 5-7, A). With each increase in lupin seed (1→4)-β-D-galactan concentration, the degree of polymerisation of galactosyl oligosaccharides was increased by one anhydrosugar unit. For example, hydrolysis of the

1.0 mg ml^{-1} solution of lupin seed (1→4)-β-D-galactan with *C. thermocellum endo*-(1→4)-β-galactanase released galactosyl oligosaccharides with degrees of polymerisation from two to seven (Figure 5-7, B) and hydrolysis of the 1.5 mg ml^{-1} solution of lupin seed (1→4)-β-D-galactan with *C. thermocellum endo*-(1→4)-β-galactanase released galactosyl oligosaccharides with degrees of polymerisation from two to eight (Figure 5-7, C). Small proportions of galactosyl oligosaccharides with degrees of polymerisation higher than those shown in the spectra in Figure 5-7 were detected, but were not present in high enough proportions to be labelled in the spectra at the resolution shown here. The same trend was observed when the lupin seed (1→4)-β-D-galactan standard solutions were hydrolysed with *A. niger endo*-(1→4)-β-galactanase, although at the same concentration of lupin seed (1→4)-β-D-galactan, galactosyl oligosaccharides released typically had a lower degree of polymerisation, by one anhydrosugar unit, compared with hydrolysis with *C. thermocellum endo*-(1→4)-β-galactanase.

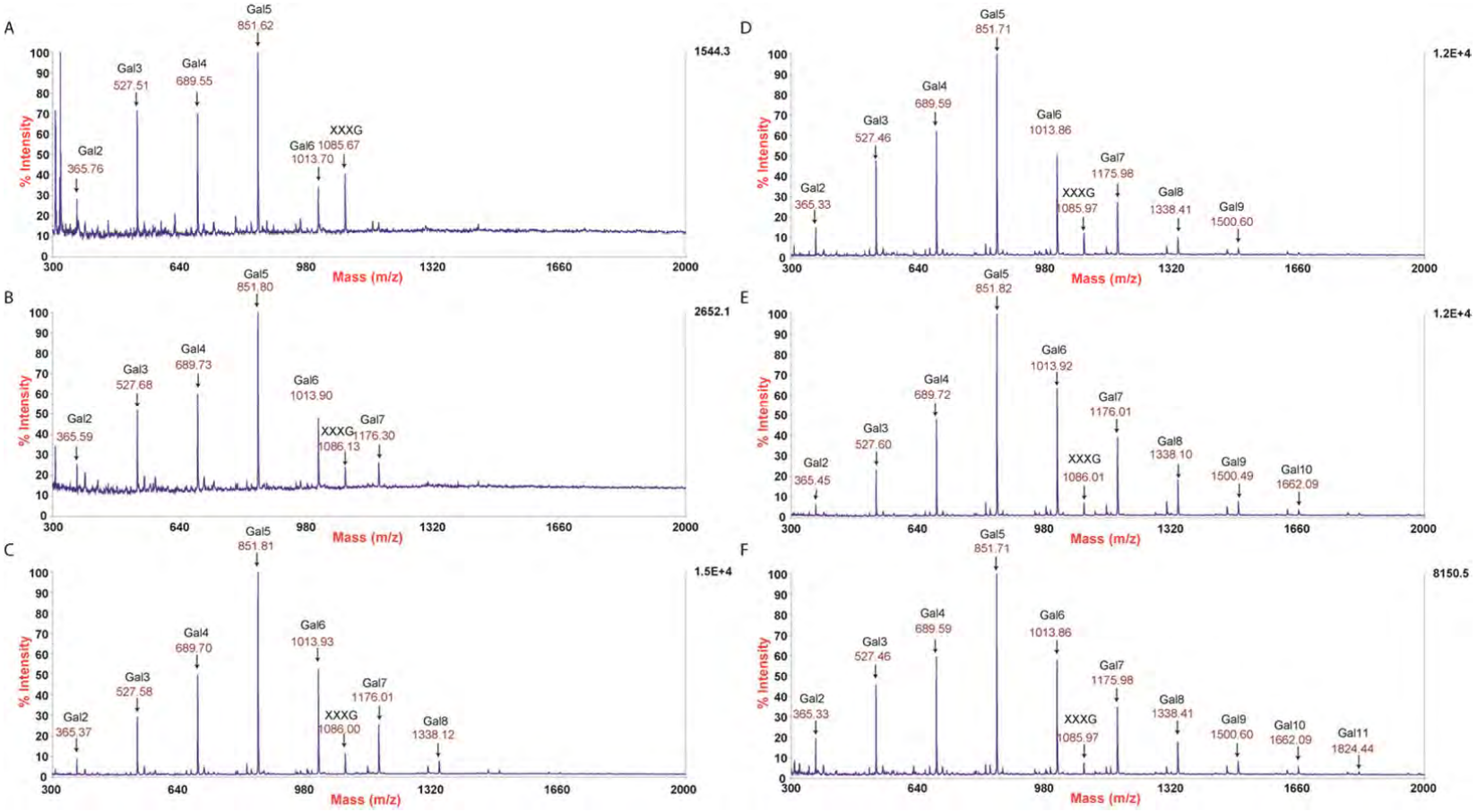

Figure 5-7 Galactosyl oligosaccharides released by hydrolysis of increasing concentrations of lupin (1→4)-β-D-galactan with *C. thermocellum endo*-(1→4)-β-galactanase. Concentrations of galactan were: (A) 0.5 mg ml^{-1}, (B) 1.0 mg ml^{-1}, (C) 1.5 mg ml^{-1}, (D) 2.0 mg ml^{-1}, (E) 2.5 mg ml^{-1} and (F) 3.0 mg ml^{-1}.The degree of polymerisation is indicated by Gal2, Gal3 etc.

To determine whether the strength of one particular galactosyl oligosaccharide sodium adduct ion (e.g. Gal2 or Gal3 etc) relative to the internal standard was more highly correlated with the concentration of (1→4)-β-D-galactan, linear models were built following the form:

$$y = Ratio + \varepsilon$$

Where "*y*" is the concentration of lupin seed (1→4)-β-D-galactan, "*Ratio*" is the ratio of the strength of each ion to the sodium adduct ion of the internal standard and "*ε*" is the error. This was done for each of the enzymes to compare their precision of quantification (Table 5-1). Linear models were not built using oligosaccharides with a degree of polymerisation higher than nine, as these were not present in all spectra. The coefficient of determination (R^2) of each oligosaccharide to the internal standard using the enzyme *C. thermocellum endo*-(1→4)-β-galactanase was higher compared with *A. niger endo*-(1→4)-β-galactanase. The relative standard error (RSE) was smaller for quantification using *C. thermocellum endo*-(1→4)-β-galactanase compared with *A. niger endo*-(1→4)-β-galactanase. Therefore quantification of the (1→4)-β-D-galactans using *endo*-(1→4)-β-galactanase-mediated hydrolysis is likely to be more precise using *C. thermocellum endo*-(1→4)-β-galactanase compared with *A. niger endo*-(1→4)-β-galactanase.

Table 5-1 Comparison of the linear models of the ratio of each of the galactosyl oligosaccharide sodium adducts to the internal standard sodium adduct ion strength using *A. niger endo*-(1→4)-β-galactanase and *C. thermocellum endo*-(1→4)-β-galactanase.

Ion:XXXG	*C. thermocellum*[a]		*A. niger*[a]	
	R^2	RSE	R^2	RSE
Gal2	0.744	0.440	0.004	0.869
Gal3	0.854	0.333	0.022	0.861
Gal4	0.944	0.207	0.280	0.738
Gal5	0.889	0.290	0.711	0.468
Gal6	0.835	0.353	0.762	0.425
Gal7	0.750	0.435	0.717	0.463
Gal8	0.811	0.378	0.735	0.448
Gal9	0.799	0.390	0.569	0.571
All ions[b]	0.939	0.214	0.481	0.627

[a]The R^2 value for correlations between the ratio of each sodium adduct ion to the internal standard (XXXG) sodium adduct ion and the concentration of lupin (1→4)-β-D-galactan is given, followed by the relative standard error in brackets.
[b]The summed total of all galactosyl oligosaccharides released. In some cases these include galactosyl oligosaccharides with a degree of polymerisation higher than nine.

The strengths of galactotetraose (Gal4) sodium adduct ions and the summed strengths of all galactosyl oligosaccharide sodium adduct ions relative to the internal standard using *C. thermocellum endo*-(1→4)-β-galactanase had the greatest coefficients of determination and smallest relative standard error within the linear models built for *C. thermocellum endo*-(1→4)-β-galactanase. Therefore the ratio of galactotetraose or all galactosyl oligosaccharide sodium adduct ions to the internal standard were plotted against the concentration of the (1→4)-β-D-galactan standards (Figure 5-8, A and B, respectively). Replicate spectra of each standard are shown by points with the same shape. It can be seen that the stronger correlation and lower RSE observed using the sodium adduct ion of galactotetraose is due to lower variation, not among replicate spectra, but among the different aliquots of the same standard.

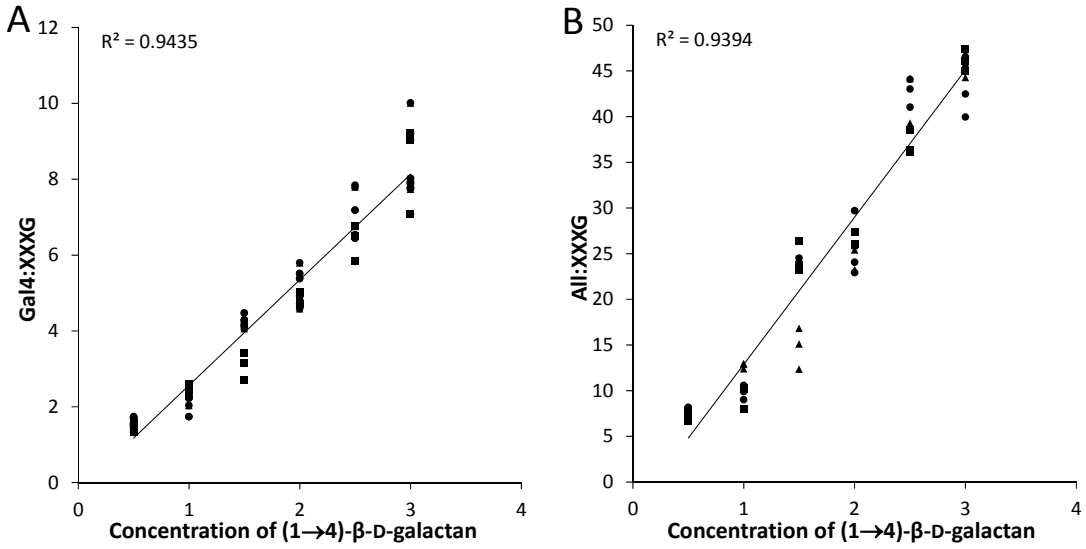

Figure 5-8 Proportions of (A) Gal4, and (B) all galactosyl oligosaccharides (relative to the internal standard) from hydrolysis with *C. thermocellum endo*-(1→4)-β-galactanase.

5.4 Discussion

Small proportions of (1→4)-β-D-galactans are known to be present in opposite wood (Chapter 3), and have been shown to be located in the primary walls (Altaner *et al.*, 2010; Kim *et al.*, 2010) where they are likely to be side chains on the pectic polysaccharide rhamnogalacturonan I (RG I). Despite this, sodium adduct galactosyl oligosaccharide ions were not detected in the enzyme hydrolysates of the NaOH extracts from opposite wood, indicating that they were not present in sufficient concentration to be detected with the conditions and *A. niger endo*-(1→4)-β-galactanase used in this study. Sodium adducts of galactosyl oligosaccharide ions were detected in the mass spectra of hydrolysates of ionic liquid extracts; therefore ionic liquid extraction is likely to be a more efficient method of making the (1→4)-β-D-galactans of wood available for enzyme hydrolysis. Although quantification of galactosyl oligosaccharides from the ionic liquid extracts was not attempted, the total signal intensity of ions from the compression wood extract was

higher compared with those from opposite wood, and the ion strengths were comparatively greater than that of the strongest intensity matrix ion at m/z = 413 which was present in all samples, suggesting that higher proportions of galactans may have been present in the ionic liquid extracts of compression wood. Preparation of ball-milled wood is less time-consuming than preparing wood extracts, therefore direct hydrolysis of ball-milled compression wood and opposite wood was investigated. Although direct hydrolysis of ball-milled opposite wood did not yield galactosyl oligosaccharides, hydrolysis of ball-milled compression wood yielded galactosyl oligosaccharides with similar degrees of polymerisation as the lupin seed (1→4)-β-D-galactan. This is encouraging for future investigation of the use of *endo*-(1→4)-β-D-galactanases to determine the abundance of (1→4)-β-D-galactans in compression wood, requiring minimal sample preparation.

Although MALDI-TOF MS has often been used to structurally characterise cell-wall polysaccharides (Lerouxel *et al.*, 2002; Hsieh and Harris, 2009; Obel *et al.*, 2009; Günl *et al.*, 2011), historically it has been regarded as an unsuitable tool for quantification. Heterogeneous concentrations of analyte and the crystalline nature of most MALDI-TOF matrices within the sample spot cause "hot-spot" effects, regions of particularly intense signals (Kang *et al.*, 2001), resulting in spectra which are not reproducible, therefore hindering quantification. Despite these inherent problems, there are several examples where the implementation of internal standards has made successful quantification of carbohydrates using MALDI-TOF MS possible. Quantification by MALDI-TOF MS of cell-wall polysaccharides that have been hydrolysed with purified, highly specific enzymes has not been demonstrated. However, quantification of malto-oligosaccharides using MALDI-TOF MS with an *N*-acetylglucosamine internal standard was shown to be suitable for quantification of oligosaccharides with a degree of polymerisation up to 15

compared with quantification using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (Kazmaier *et al.*, 1998). Monosaccharides can also be quantified by MALDI-TOF MS as demonstrated by Grant et al. (2003), although the high variability compared with quantification using a standard enzyme colorimetry method was only partially compensated for by increasing the number of spectra acquired.

Bungert et al. (2004) has proposed a set of pre-requisites for the quantification of low molecular weight compounds using MALDI-TOF MS. Firstly, that the choice of matrix should avoid ions with the same m/z as the analyte. Secondly, it was proposed that the molar ratio of matrix to analyte should be between 10:1 and 100:1. Thirdly, an internal standard must be used, that has the best possible molecular identity with the analyte. The fourth pre-requisite concerns automated spectral acquisition and integration, which is not necessarily a fundamental requirement for quantification, but would certainly save time and possibly minimise experimental error.

In the current study, the chosen matrix does not have ions with the same m/z as the analyte, and the internal standard used is an oligosaccharide and therefore is molecularly similar to the galactosyl oligosaccharides of interest. The ratio of matrix to analyte in this study falls between 100:1 and 1000:1; however, this pre-requisite is likely to depend on the analyte and the matrix used. As a good correlation between the relative galactosyl oligosaccharide content of the enzyme hydrolysates of lupin galactan (calculated from MALDI-TOF mass spectra) and the known concentration of lupin seed (1→4)-β-D-galactan in standard solutions was achieved in this study, the ratio of matrix to analyte used was suitable for quantification. If further investigation of using this method to quantify changes in the abundance of (1→4)-β-D-galactans in compression wood are successful, future applications could be teamed with software such as Bruker's

AutoExecute, as used by Kang et al. (2001), to automate the spectral acquisition and integration processes.

The (1→4)-β-D-galactans of compression wood are present in the highly lignified $S2_{(L)}$ layer of the secondary wall (Altaner *et al.*, 2010; Kim *et al.*, 2010), and are tightly associated with the lignin (Bouveng and Meier, 1959a; Mukoyoshi *et al.*, 1981; Minor, 1982). Lignin has been shown to be a physical barrier to enzyme hydrolysis of cell-wall polysaccharides (Akin, 2007). As the proportion of galactosyl residues increases in compression wood, the proportion of lignin also increases (Nanayakkara *et al.*, 2009). Therefore as the (1→4)-β-D-galactan content increases, it is likely that access to the (1→4)-β-D-galactans by the enzymes will be increasingly hindered by the lignin. Further work on quantification of (1→4)-β-D-galactans in compression wood by *endo*-(1→4)-β-galactanase hydrolysis and MALDI-TOF MS of the hydrolysis products may require delignification or extraction of the polysaccharides if a quantitative relationship is not found using ball-milled compression wood. However, due to the strong association between compression wood (1→4)-β-D-galactans and lignin, delignification by conventional methods such treatment with acid-chlorite (Wise *et al.*, 1946) may result in the loss of a significant proportion of the (1→4)-β-D-galactans. Dissolution with ionic liquids, and regeneration of the polysaccharide-containing fraction may overcome much of this loss.

As previously noted, ionic liquid extracts are likely to be more efficient as far as making the (1→4)-β-D-galactans of compression wood available for hydrolysis with *endo*-(1→4)-β-galactanases. Further investigation is required before quantification of (1→4)-β-D-galactans from ionic liquid extracts of compression wood can be examined. Two unusual ion series were present in the spectra from hydrolysates of ionic liquid (1-ethyl-3-methylimidazolium acetate, often abbreviated to 'EMIMAc' or '$[C_2mim]OAc$')

extracts of opposite wood and compression wood. The *endo*-(1→4)-β-galactanases specifically cleave the glycosidic bonds of (1→4)-β-D-galactans, therefore it is likely that in both series the hexosyl oligosaccharide products were due to the presence of (1→4)-β-D-galactans in the ionic liquid extract. Each series however, was composed of products with an unexpected adduct. The m/z of the ions 713, 875 and 1037 correspond to the expected m/z of di-sodium adducts of galactosyl oligosaccharides. Di-sodium adducts of xyloglucan oligosaccharides were found to be present in similar proportions to the mono-sodium adducts of xyloglucan oligosaccharides in the study of Peña et al. (2008). Di-sodium adduct ions were detected in large proportions in preparations of human milk oligosaccharides using MALDI MS with an ionic liquid matrix (2,5-dihydroxybenzoic acid butylamine) (Mank *et al.*, 2004), but these were not detected in greater proportions than the mono-sodium adducts, which was the case in this current study. Ionic liquids are salts that are liquids at room temperature and in this study consist of a 1-ethyl-3-methylimidazolium cation and an acetate anion. The complexity of the charged molecules in the preparations in the current study may have induced the preferential formation of di-sodium adducts.

The second series had ions with m/z = 375, 537 and 699. Conversion of cello-oligosaccharides by the ionic liquid EMIMAc to a semi-acetal-like structure has been reported (Handy and Okello, 2005; Liebert and Heinze, 2008) (Figure 5-9).

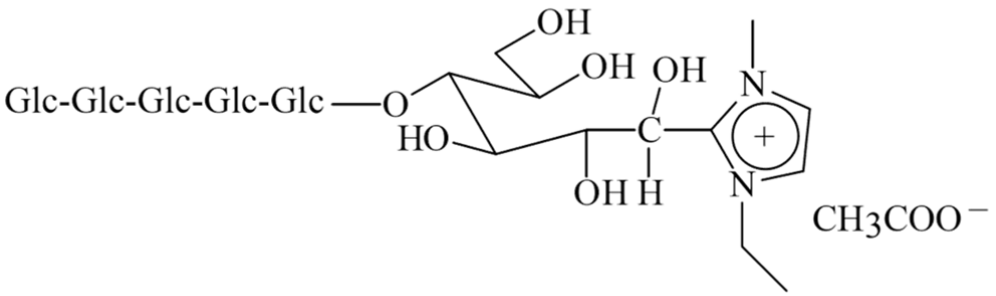

Figure 5-9 Semi-acetal-like structure proposed by Liebert and Heinze (2008) for conversion of cello-oligosaccharides by EMIMAc. Figure from Liebert and Heinze (2008).

A sodium adduct of this compound (adducted with the acetate moiety) would still not account for the molecular weights of the ions observed, as these would have m/z = 373, 535 and 697, two mass units lower than the ions observed. Additionally, the ion with m/z = 375 would be due to the presence of a mono-galactosyl residue adducts, which were not detected in the mass spectra of hydrolysates of lupin-seed galactan, NaOH extracts or ball-milled wood, although it is possible that they are present in the hydrolysates of ionic liquid extracts. MALDI MS/MS of the ions in this series would help to elucidate their elemental composition and structure.

In addition to dissolution of whole cell-walls, ionic liquids are increasingly being used in MALDI MS as matrices to prevent the "hot-spot" effect (Laremore *et al.*, 2006; Tholey and Heinzle, 2006; Fukuyama *et al.*, 2008; Crank and Armstrong, 2009), but formation of these unusual ions have not been reported. However, if the composition of the products detected can be determined, the use of ionic liquids both for extracting the (1→4)-β-D-galactans of compression wood, and as a MALDI matrix with more homogeneous properties than the crystalline matrix used in this study, would be

advantageous, particularly as a large proportion of the ionic liquid would be recoverable for re-use (Zhao, 2006).

5.5 References

Akin, D. E. (2007). "Grass lignocellulose - strategies to overcome recalcitrance." Applied Biochemistry and Biotechnology **137**: 3-15.

Altaner, C. M., E. N. Tokareva, M. C. Jarvis and P. J. Harris (2010). "Distribution of (1→4)-β-galactans, arabinogalactan proteins, xylans and (1→3)-β-glucans in tracheid cell walls of softwoods." Tree Physiology **30**: 782-793.

Andersson, M. A., J. A. Cook and B. A. Stone (1978). "Enzymatic determination of 1,3;1,4-β-glucans in barley grain and other cereals." Journal of the Institute of Brewing **84**: 233-239.

Apiolaza, L., S. Chauhan and J. Walker (2011). "Genetic control of very early compression and opposite wood in *Pinus radiata* and its implications for selection." Tree Genetics & Genomes **7**: 563-571.

Biosupplies Australia Pty Ltd. from http://www.biosupplies.com.au/.

Blakeney, A. B. and L. L. Mutton (1980). "A simple colorimetric method for the determination of sugars in fruits and vegetables." Journal of the Science of Food and Agriculture **31**: 889-897.

Bouveng, H. O. and H. Meier (1959). "Studies on a galactan from Norwegian spruce compression wood (*Picea abies* Karst.)." Acta Chemica Scandinavica **13**: 1884-1889.

Browning, B. L. (1967). Hemicellulose isolation and separation. Methods of wood chemistry. New York, Interscience Publishers. **2**.

Bungert, D., S. Bastian, D. M. Heckmann-Pohl, F. Giffhorn, E. Heinzle and A. Tholey (2004). "Screening of sugar converting enzymes using quantitative MALDI-ToF mass spectrometry." Biotechnology Letters **26**: 1025-1030.

Cohen, L. and A. Gusev (2002). "Small molecule analysis by MALDI mass spectrometry." Analytical and Bioanalytical Chemistry **373**: 571-586.

Crank, J. A. and D. W. Armstrong (2009). "Towards a second generation of ionic liquid matrices (ILMs) for MALDI-MS of peptides, proteins, and carbohydrates." Journal of the American Society for Mass Spectrometry **20**: 1790-1800.

Fengel, D. and G. Wegener (1989). Wood chemistry, ultrastructure, reactions. Berlin, Walter de Gruyter.

Fry, S. C., W. S. York, P. Albersheim, A. G. Darvill, T. Hayashi, J.-P. Joseleau, Y. Kato, E. P. Lorences, G. A. Maclachlan, M. McNeil, J. S. G. Reid, H. U. Seitz, R. F. Selvendran, A. G. J. Voragen and A. R. White (1993). "An unambiguous nomenclature for xyloglucan-derived oligosaccharides." Physiologia Plantarum **89**: 1-3.

Fukuyama, Y., S. Nakaya, Y. Yamazaki and K. Tanaka (2008). "Ionic liquid matrixes optimized for MALDI-MS of sulfated/sialylated/neutral oligosaccharides and glycopeptides." Analytical Chemistry **80**: 2171-2179.

Grant, G. A., S. L. Frison, J. Yeung, T. Vasanthan and P. Sporns (2003). "Comparison of MALDI-TOF mass spectrometric to enzyme colorimetric quantification of glucose from enzyme-hydrolyzed starch." Journal of Agricultural and Food Chemistry **51**: 6137-6144.

Günl, M., F. Kraemer and M. Pauly (2011). Oligosaccharide mass profiling (OLIMP) of cell wall polysaccharides by MALDI-TOF/MS. The plant cell wall. Methods in molecular biology. Z. A. Popper. New York, Springer.

Handy, S. T. and M. Okello (2005). "The 2-Position of imidazolium ionic liquids: substitution and exchange." The Journal of Organic Chemistry **70**: 1915-1918.

Hsieh, Y. S.-Y. and P. J. Harris (2009). "Xyloglucans of monocotyledons have diverse structures." Molecular Plant **2**: 943-965.

Huber, D. J. and D. J. Nevins (1977). "Preparation and properties of a β-D-glucanase for the specific hydrolsis of β-D-glucans." Plant Physiology **60**: 300-304.

Kang, M.-J., A. Tholey and E. Heinzle (2001). "Application of automated matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for the measurement of enzyme activities." Rapid Communications in Mass Spectrometry **15**: 1327-1333.

Kazmaier, T., S. Roth, J. Zapp, M. Harding and R. Kuhn (1998). "Quantitative analysis of malto-oligosaccharides by MALDI-TOF mass spectrometry, capillary electrophoresis and anion exchange chromatography." Fresenius' Journal of Analytical Chemistry **361**: 473-478.

Kilpeläinen, I., H. Xie, A. King, M. Granstrom, S. Heikkinen and D. S. Argyropoulos (2007). "Dissolution of wood in ionic liquids." Journal of Agricultural and Food Chemistry **55**: 9142-9148.

Kim, H. and J. Ralph (2010). "Solution-state 2D NMR of ball-milled plant cell wall gels in DMSO-d_6/pyridine-d_5." Organic and Biomolecular Chemistry **8**: 576-591.

Kim, J. S., T. Awano, A. Yoshinaga and K. Takabe (2010). "Immunolocalization of β-1-4-galactan and its relationship with lignin distribution in developing compression wood of *Cryptomeria japonica*." Planta **232**: 109-119.

Laremore, T. N., F. Zhang and R. J. Linhardt (2006). "Ionic Liquid Matrix for Direct UV-MALDI-TOF-MS Analysis of Dermatan Sulfate and Chondroitin Sulfate Oligosaccharides." Analytical Chemistry **79**: 1604-1610.

Lerouxel, O., T. S. Choo, M. Seveno, B. Usadel, L. Faye, P. Lerouge and M. Pauly (2002). "Rapid structural phenotyping of plant cell wall mutants by enzymatic oligosaccharide fingerprinting." Plant Physiology **130**: 1754-1763.

Liebert, T. and T. Heinze (2008). "Interaction of ionic liquids with polysaccharides. 5. Solvents and reaction media for the modification of cellulose." BioResources **3**: 576-601.

Mank, M., B. Stahl and G. Boehm (2004). "2,5-Dihydroxybenzoic acid butylamine and other ionic liquid matrixes for enhanced MALDI-MS analysis of biomolecules." Analytical Chemistry **76**: 2938-2950.

McCleary, B. V. and R. Codd (1991). "Measurement of (1 → 3),(1 → 4)-β-D-glucan in barley and oats: A streamlined enzymic procedure." Journal of the Science of Food and Agriculture **55**: 303-312.

Megazyme International Ltd. from http://www.megazyme.com.

Minor, J. L. (1982). "Chemical linkage of pine polysaccharides to lignin." Journal of Wood Chemistry and Technology **2**: 1-16.

Mort, A. J., S. Gao, X. Wu and R. Prade (2005). "The need for pure enzymes that degrade cell wall polysaccharides and how to get them." Plant Biosystems - An International Journal Dealing with all Aspects of Plant Biology **139**: 84-87.

Mukoyoshi, S.-i., J.-i. Azuma and T. Koshijima (1981). "Lignin-carbohydrate complexes from compression wood of *Pinus densiflora* Sieb et. Zucc." Holzforschung **35**: 233-240.

Nanayakkara, B., M. Manley-Harris, I. D. Suckling and L. A. Donaldson (2009). "Quantitative chemical indicators to assess the gradation of compression wood." Holzforschung **63**: 431-439.

Obel, N., V. Erben, T. Schwarz, S. Kühnel, A. Fodor and M. Pauly (2009). "Microanalysis of plant cell wall polysaccharides." Molecular Plant **2**: 922-932.

Peña, M. J., A. G. Darvill, S. Eberhard, W. S. York and M. A. O'Neill (2008). "Moss and liverwort xyloglucans contain galacturonic acid and are structurally distinct from the xyloglucans synthesized by hornworts and vascular plants*." Glycobiology **18**: 891-904.

R Development Core Team (2011). "R: A language and environment for statistical computing." from http://www.R-project.org.

Sun, N., M. Rahman, Y. Qin, M. L. Maxim, H. Rodriguez and R. D. Rogers (2009). "Complete dissolution and partial delignification of wood in the ionic liquid 1-ethyl-3-methylimidazolium acetate." Green Chemistry **11**: 646-655.

Tholey, A. and E. Heinzle (2006). "Ionic (liquid) matrices for matrix-assisted laser desorption/ionization mass spectrometry—applications and perspectives." Analytical and Bioanalytical Chemistry **386**: 24-37.

Whistler, R. L. and J. N. BeMiller (1958). Alkaline degradation of polysaccharides. Advances in Carbohydrate Chemistry. L. W. Melville, Academic Press. **Volume 13:** 289-329.

Wise, L. F., M. Murphy and A. A. D'Addieco (1946). "Chlorite holocellulose, its fractionation and bearing on summative wood analysis and on studies on the hemicelluloses." Technical Association of the Pulp and Paper Industry **122**: 35-43.

Zhao, H. (2006). "Innovative applications of ionic liquids as green engineering liquids." Chemical Engineering Communications **193**: 1660-1677.

Chapter 6 - General discussion

How the different cell-wall polymers that are characteristic of compression wood function to return leaning stems to their vertical growth orientation is still unknown. Although the microfibril angle in the cell walls of wood has been shown to determine the extent of moisture-related longitudinal dimensional changes (swelling and shrinkage) (Barber and Meylan, 1964; Yamamoto, 1999; Yamamoto *et al.*, 2001), the ways in which the matrix polymers interact and contribute to the physical properties of wood is still to be determined. From the current investigation however, it is known that the presence of compression wood has a significant effect on the longitudinal dimensional changes of radiata pine corewood, and that the characteristic chemical composition of compression wood compared with normal wood can potentially be exploited to detect the presence of compression wood.

6.1 Radiata pine corewood "types"

Corewood is the earliest formed wood at any given height in the stem, therefore it is subjected to a number of forces that would be resisted by a stem with a large diameter, as is the case for outerwood. "Normal" wood is difficult to define. All wood types produced by trees, including compression, opposite and flexure wood, can be considered normal. However, the term "normal" is used here to describe wood produced by trees that are not subjected to obvious external forces (i.e. they are grown in a vertical posture), for comparison with wood produced by trees grown under mechanical stresses. Normal wood and opposite wood examined in the current study were shown by the *O*-acetyl and cellulose content, monosaccharide composition and their two-dimensional ^{13}C-^{1}H nuclear

magnetic resonance correlation (2D NMR) spectra, to have the same cell-wall composition, except that opposite wood had a significantly lower Klason lignin content. These data support the idea that there is a spectrum ranging from pure opposite wood, through normal wood to pure compression wood (Timell, 1973), although the differences in cell-wall composition between opposite and normal wood are much smaller compared with the differences between normal and compression wood. It is likely that all normal wood produced from trees with vertical stems contains some compression wood which is responsible for the straight appearance of these stems (Timell, 1986), and normal wood could therefore be considered the mildest form of compression wood. The cell-wall compositions of radiata pine corewoods in the current study indicate that corewood types examined here could be segregated into "compression corewood" and "non-compression corewood". The cell-wall composition between different samples of compression wood (from different trees) was here shown by the Klason lignin content, monosaccharide composition and pyrolysis products to be more variable compared with that of opposite wood, normal wood and flexure wood.

Flexure corewood is produced by wind-affected trees subjected to a higher degree of deformation under mechanical stress (wind). It is not certain whether mature wind-affected wood would exist, as mature trees have larger diameter stem which resist bending. The mature flexure wood described by Telewski and Jaffe (1986b) is more likely to be mild compression wood as the trees were grown on a site with prevailing wind and had flag-formed crowns which result from uni-directional wind. Additionally, the formation of compression wood and opposite wood "sides" on seedlings of loblolly pine (*Pinus taeda*) subjected to growth under the influence of oscillating fans which simulated a windy environment (Yeh *et al.*, 2005) suggests that the wind in their study caused a lean on one axis preferentially, and the wood produced was more correctly termed

compression wood and opposite wood rather than flexure wood. The study of Yeh et al. (2005) therefore provides evidence that trees can form compression wood as a result of wind. The Klason lignin content, *O*-acetyl content, monosaccharide composition and 2D NMR spectrum of radiata pine flexure wood examined in the current study were not different from those of the normal wood examined. Seedlings grown under more random (multi-directional) and sustained flexure events compared with those of the current study may produce wood with an altered cell-wall composition; however, the high microfibril angle of radiata pine corewood (Barnett and Bonham, 2004; Donaldson *et al.*, 2004; Brennan *et al.*, 2012) may provide the necessary flexibility to withstand bending in the wind without failure, negating the need for altered cell-wall composition.

6.2 Chemical indicators of compression wood

6.2.1 Lignin

Both the lignin content and the lignin composition of radiata pine corewood were found to be indicators of the presence of compression wood. In the current study, the lignin content was measured as Klason lignin (the weight of the residue after sulphuric acid hydrolysis), which takes days to measure due to the need to dry to constant weight. However, lignin content can also be predicted using pyrolysis gas-chromatography/mass-spectrometry (pyrolysis-GC/MS) (Alves *et al.*, 2006; Alves *et al.*, 2008) or Fourier-transform infrared spectroscopy (FTIR) (Allison *et al.*, 2009). The presence of compression wood does not alter the statistical model used to predict lignin content using pyrolysis-GC/MS (Alves *et al.*, 2008). Therefore pyrolysis-GC/MS could be used to segregate compression wood from non-compression wood by predicted lignin content. The current study further indicated that some of the pyrolysis products of

compression wood differed among samples. Thus pyrolysis-GC/MS could be useful to both detect the presence of compression wood and determine its severity, although further investigation of a larger set of compression wood samples of known severity is required to test this hypothesis.

Two commonly used methods of analysing the composition of lignin in wood are thioacidolysis (Lapierre *et al.*, 1985) and derivitisation followed by reductive cleavage (DFRC) (Lu and Ralph, 1997), both of which only release monomers joined by β-O-4- linkages which can then be quantified. However, these methods cannot be used to quantify the total amount of each of the different units (e.g. H-units and G-units in softwoods) that are present in the lignin. Pyrolysis gas-chromatography followed by mass-spectrometry could be used, as demonstrated in the current study, to calculate the proportions of H-units and G-units in the isolated lignin, giving a more complete analysis of the lignin composition.

Using 2D NMR spectroscopy, the lignin of compression wood was shown to contain H-units, whereas H-unit signals were not detected in the spectra of the non-compression wood samples examined by 2D NMR. Similar results were obtained using pyrolysis-GC/MS, where most of the pyrolysis products that were known to be derived from H-units (phenolic compounds with no methoxyl group and a carbon sidechain of two or three carbons) were unique to compression wood, or were only present in small proportions in the opposite wood samples examined. The presence of H-units in opposite wood is still under debate. As previously mentioned, compression wood is at the extreme end of a spectrum ranging from opposite wood, through normal wood to compression wood. Therefore, the presence of H-units in normal wood cannot be ruled out as being due to the formation of mild compression wood because trees that appear straight are only likely to be so due to formation of compression wood. Small

proportions of H-units have been reported from the opposite corewood (Yeh *et al.*, 2005) and mature wood (Yeh *et al.*, 2006) of loblolly pine (*Pinus taeda*), radiata pine (Nanayakkara *et al.*, 2009) and in the cell corners of opposite wood of Norway spruce (*Picea abies*) (Tokareva *et al.*, 2007). The possible presence of some mild compression wood in the opposite wood samples examined by pyrolysis-GC/MS in the present study is a likely source of the H-units detected using pyrolysis-GC/MS. The samples examined by 2D NMR on the other hand, were grown under more tightly controlled conditions (Apiolaza *et al.*, 2011a) compared with the samples examined by pyrolysis-GC/MS (Apiolaza *et al.*, 2011b), and the absence of signals from H-units in the non-compression wood samples suggests that their presence in radiata pine corewood may be indicative of the formation of mild compression wood. Examination of pure opposite wood, such as that from the glasshouse trees in the present study, by pyrolysis-GC/MS, would indicate whether there really are H-units in the opposite wood of radiata pine corewood. Altaner et al. (2007) suggested that the synthesis of (1→4)-β-D-galactans was one of the first physiological processes in the formation of compression wood, before the tracheid morphology becomes visibly altered. The synthesis of *p*-hydroxyphenyl alcohol and its incorporation into the lignin macromolecule as H-units of lignin, may also be one of the earliest physiological responses of the tree to displacement from the vertical.

6.2.2 (1→4)-β-D-Galactans

In the current study, pure compression wood from radiata pine corewood has been shown to be distinguishable from non-compression wood by having a galactosyl residue content of greater than 6% of the dry weight of the wood. What is not known, is how the galactosyl residue content of compression wood relates empirically to the proportion of

(1→4)-β-D-galactans in the wood. This is because in softwoods, galactosyl residues are also present in xyloglucans (Acebes *et al.*, 1993; Andrew and Little, 1997; Kakegawa *et al.*, 1998) and rhamnogalacturonan I (Thomas *et al.*, 1987; Edashige and Ishii, 1997) in the primary walls, and in galactoglucomannans (Harwood, 1973; Lundqvist *et al.*, 2002; Willför *et al.*, 2003b) in the secondary walls. Additionally, (1→4)-β-D-galactans are not specific to compression wood, but are found in small proportions in the primary walls of normal and opposite wood (Altaner *et al.*, 2010; Kim *et al.*, 2010), where they are probably present as sidechains on rhamnogalacturonan I. They are also found in the water-soluble type II arabinogalactans that have been reported to be present in small proportions in Norway spruce and Scots pine (*Pinus sylvestris*) (Willför and Holmbom, 2004). Therefore, quantification of the galactosyl residues in acid-hydrolysates of wood is not a true measure of the (1→4)-β-D-galactan content. The galactoglucomannans of radiata pine only contribute a small proportion of galactosyl residues to acid hydrolysate of wood. Galactoglucomannans make up ~ 12 to 15% of the dry weight of the normal wood of softwoods (Whistler and Chen, 1991), but compression wood contains only ~ 8 to 9% galactoglucomannan (Timell, 1986), which otherwise have the same structure as normal wood galactoglucomannans (Côté *et al.*, 1967; Hoffmann and Timell, 1972). As primary walls are only a small proportion of the wood (~ 1 % of the wood area, Goldstein, 1991), changes in the galactosyl residue content of compression wood are likely to be a reasonable indicator of changes in the proportion of (1→4)-β-D-galactans, although it is important to distinguish between measurement of the galactosyl residue content and the (1→4)-β-D-galactan content.

Comparison of 2D NMR spectrum of compression wood with the spectra obtained from non-compression wood samples, showed that compression wood did have a higher proportion of (1→4)-β-D-galactans, indicating that the elevated galactosyl residue content

in acid-hydrolysates of compression wood compared with non-compression wood samples was related to an elevated proportion of (1→4)-β-D-galactans. This finding was further supported by the use of *endo*-(1→4)-β-galactanases, which specifically cleave glycosidic linkages between galactosyl residues of (1→4)-β-D-galactans, to hydrolyse compression wood, and extracts of compression wood, which released galactosyl oligosaccharides. These results are in agreement with characterisation of a galactose-containing polysaccharide that was isolated from the chlorite liquor (lignin-containing fraction) of delignified radiata pine compression wood (Nanayakkara, 2007). Methylation (linkage) analysis of this polysaccharide showed that it was composed primarily of an unbranched (1→4)-linked polymer of galactosyl residues (Nanayakkara, 2007).

Although methylation analysis can give quantitative information about the polysaccharides present in cell walls, precise, routine methods that can quantify the polysaccharides in cell walls are not yet available. Highly specific *endo*-(1→4)-β-galactanases were shown in this work to release galactosyl oligosaccharides that were proportionally related to the content of (1→4)-β-D-galactans in the substrate. Further investigation is reqiured to determine whether specific *endo*-(1→4)-β-galactanase hydrolysis of compression wood samples containing different proportions of (1→4)-β-D-galactans releases galactosyl oligosaccharides that are related to the (1→4)-β-D-galactan content of the wood. However, to use differences in the relative proportions of (1→4)-β-D-galactans that are specific to the secondary cell walls of compression wood tracheids as an indicator of compression wood severity, one must either assume that the proportions of (1→4)-β-D-galactans found in the primary walls do not change significantly among compression wood samples of differing severity and can

therefore be disregarded, or somehow distinguish between the (1→4)-β-D-galactans of primary and secondary walls.

The 2D NMR spectrum of compression wood cell walls indicated that the degree of polymerisation of compression wood (1→4)-β-D-galactans is higher than the degree of polymerisation of non-compression wood (1→4)-β-D-galactans. Therefore, the (1→4)-β-D-galactans of compression wood may be distinguishable from the (1→4)-β-D-galactans in the primary walls of wood by their degree of polymerisation. Although 2D NMR spectra can not yet provide quantitative information between different samples, chemometric techniques (e.g. multivariate regression) can be used to quantify within- and between-spectra variation (Hedenström *et al.*, 2008; Hedenström *et al.*, 2009). Chemometrics may therefore be used to quantify changes in the ratio of the internal (1→4)-β-D-galactan signal to the non-reducing (1→4)-β-D-galactan signal, perhaps in relation to signals from other primary cell-wall polysaccharide signals when these are better characterised (Free *et al.*, in preparation) to quantify the (1→4)-β-D-galactan content of different compression wood samples. This would still not be a truly quantitative measure of the proportion of (1→4)-β-D-galactans specifically present in the secondary walls of compression wood tracheids. Ball-milling cleaves the polysaccharides, indicated by the production of reducing and non-reducing ends of polysaccharides as detected in the 2D NMR spectra and also by decreased proportions of phenolic compounds and anhydrosugars detected in the pyrolysis-GC/MS chromatograms of fine-milled wood samples in the current study. Therefore the ball-milling process will both decrease the degree of polymerisation and create more non-reducing ends. A better method for quantitative analysis of (1→4)-β-D-galactans with differing degrees of polymerisation might be to de-lignify the wood without using an extensive milling step, for example using ionic liquds on wood chips (Kilpeläinen *et al.*,

2007), followed by separation of the regenerated polysaccharide fractions using size-exclusion chromatography. The relative proportions of (1→4)-β-D-galactans with low and high degrees of polymerisation could then be characterised, for example using specific hydrolysis with *endo*-(1→4)-β-galactanase, followed by quantification of the oligosaccharides released by matrix-assisted laser-desorption ionisation time-of-flight mass spectrometry, as demonstrated for soluble lupin seed (1→4)-β-D-galactans in the current study.

The genes for the galactosyltransferases that synthesise (1→4)-β-D-galactans have not yet been identified, although an enzyme with (1→4)-β-D-galactosyltransferase activity has been isolated from a microsome preparation from developing tracheids of radiata pine compression wood (Mast *et al.*, 2009). A number of *endo*-(1→4)-β-galactanase genes have been identified (e.g. those described in Christgau *et al.*, 1995; Yang *et al.*, 2006) which, by transformation of radiata pine to constitutively express such a gene, could help to elucidate the mechanical function, if any, that the (1→4)-β-D-galactans have in compression wood. A similar study has already been done using potato (*Solanum tuberosum*) which was modified to express an *endo*-(1→4)-β-galactanase from *Aspergillus aculeatus* (Sørensen *et al.*, 2000); the modified potato tuber did not contain (1→4)-β-D-galactans and was found to be more brittle compared with wild type tuber material (Ulvskov *et al.*, 2005).

6.3 Quantitative analysis of all polymers of whole cell walls

The cell-wall polymers (lignin and polysaccharides) are polydispersed throughout the walls and routine methods of quantitative isolation followed by characterisation of each of the cell wall polymers are currently unavailable. That the ionic liquid extracts of both compression wood and opposite wood yielded galactosyl oligosaccharides after hydrolysis with an *endo*-(1→4)-β-galactanase is encouraging for further investigation of using ionic liquids to quantify families of polysaccharides and different lignin units from highly-lignified materials such as wood. Sun et al. (2009) used ^{13}C NMR spectroscopy to show that the regenerated polysaccharide-containing fraction of wood dissolved in the ionic liquid 1-ethyl-3-methylimidazolium acetate contained some residual lignin, but that the lignin-containing fraction did not contain residual polysaccharides. Loss of ~40% of the weight of the polysaccharides of the wood was attributed to the washing steps during regeneration (Sun *et al.*, 2009). It is therefore likely that the regeneration process could be optimised, and a more complete recovery of the polysaccharides achieved, without substantial proportions of the polysaccharide remaining in the lignin. Characterisation of both the regenerated polysaccharide-containing fraction and the lignin-containing fraction could then be achieved using, for example, a number of methods to quantify the polymers that are of interest in quantifying degrees of compression wood severity (H-units of lignin and (1→4)-β-D-galactans), including pyrolysis-GC/MS, HPAEC-PAD and MALDI-TOF MS as demonstrated in the present study.

6.4 Limitations associated with this type of study

A constraint inherent to the study of wood quality is the time it takes to grow the trees to produce wood in sufficient quantities to analyse. This PhD project was part of a larger, longer-term, collaborative study with the School of Forestry at the University of Canterbury (Apiolaza *et al.*, 2011a; Apiolaza *et al.*, 2011b), funded by the New Zealand Foundation for Research, Science and Technology. As part of this project, the trees were grown by colleagues at the School of Forestry and mechanically tested by them as part of their own research before the wood was made available to Auckland University for chemical analyses, some of which are described in this PhD thesis. That the trees had previously been grown by the Forestry Department made this PhD possible, as otherwise there would not have been time for seedlings to be selected and grown to produce sufficient wood within the time-frame of a PhD project.

A drawback of this collaborative research was that I was unable to choose the trees (e.g. specific clones/families) or their growing conditions. In retrospect, it would have been preferable to examine the wood from a greater number of clones grown only in the glasshouse, rather than including the Amberley field-grown samples. Inclusion of the Amberley samples in the analysis added further variables that affect wood quality, specifically variation in heritability of traits (these samples were from different families) and also variation in the growth conditions across the site (Apiolaza *et al.*, 2011b). However, the aim of the study was to investigate whether there were correlations between the compositions of the cell walls and the quality of wood produced, regardless of the biological causation of the wood quality.

A more significant limitation of the study was the time-consuming methods used for chemical analysis, and also the limited availability of the analytical instruments. These limitations meant that only a few samples of each wood type were examined using pyrolysis-GC/MS. The limited number of samples that were able to be analysed using pyrolysis-GC/MS does mean that these results should be interpreted with some caution. Measurement of the pyrolysis products of a larger number of samples of compression wood and opposite wood would provide greater confidence that the differences between the two wood types described here applies generally to these wood types, and is not only representative of the samples analysed here.

The limitation of the availability of the analytical instruments applied also to the use of 2D NMR spectroscopy. The aim of examining the different wood types using 2D NMR spectroscopy was to determine whether there were clear differences in the polymers present between different wood types, rather than to quantify small changes in the proportions of the polymers present. The 2D NMR spectra showed differences in the presence (the H-units of lignin) and proportion (the (1→4)-β-D-galactans) of polymers in the compression wood spectrum compared to the non-compression wood spectra are likely to be representative of that wood type. As discussed previously, small amounts of H-units of lignin have been reported in non-compression wood (Yeh *et al.*, 2005; Yeh *et al.*, 2006; Tokareva *et al.*, 2007; Nanayakkara *et al.*, 2009). The present study indicates that if H-units of lignin are present in proportions large enough to be detected using 2D NMR spectroscopy of the whole cell walls of wood, that compression wood is present, although it does not rule out the possibility that non-compression wood contains smaller proportions of H-units in the lignin.

6.5 Concluding remarks

The current study has shown that radiata pine compression corewood has a significantly altered cell-wall composition compared with non-compression corewood. This could be exploited by the timber industry to improve the average quality of structural timber, by using chemical methods to detect, and therefore discard, timber with unacceptable proportions of compression wood. For this to be feasible, faster methods of cell-wall compositional analysis are required. There is evidence that infrared radiation techniques could be used for this purpose (Åkerholm and Salmén, 2002; Kelley *et al.*, 2004; Jones *et al.*, 2006), and other members of the collaborative research project, of which the current study was a part, are currently investigating this possibility.

6.6 References

Acebes, J. L., E. P. Lorences, G. Revilla and I. Zarra (1993). "Pine xyloglucan. Occurrence, localization and interaction with cellulose." Physiologia Plantarum **89**: 417-422.

Åkerholm, M. and L. Salmén (2001). "Interactions between wood polymers studied by dynamic FT-IR spectroscopy." Polymer **42**: 963-969.

Åkerholm, M. and L. Salmén (2002). "Dynamic FTIR spectroscopy for carbohydrate analysis of wood pulps." Journal of Pulp and Paper Science **28**: 245-249.

Åkerholm, M. and L. Salmén (2003). "The oriented structure of lignin and its viscoelastic properties studied by static and dynamic FT-IR spectroscopy." Holzforschung **57**: 459-465.

Akin, D. E. (2007). "Grass lignocellulose - strategies to overcome recalcitrance." Applied Biochemistry and Biotechnology **137**: 3-15.

Albersheim, P., D. J. Nevins, P. D. English and A. Karr (1967). "A method for the analysis of sugars in plant cell-wall polysaccharides by gas-liquid chromatography." Carbohydrate Research **5**: 340-345.

Allison, G. G., S. C. Thain, P. Morris, C. Morris, S. Hawkins, B. Hauck, T. Barraclough, N. Yates, I. Shield, A. V. Bridgwater and I. S. Donnison (2009). "Quantification of hydroxycinnamic acids and lignin in perennial forage and energy grasses by Fourier-transform infrared spectroscopy and partial least squares regression." Bioresource Technology **100**: 1252-1261.

Altaner, C. M., A. I. Hapca, J. P. Knox and M. C. Jarvis (2007). "Detection of β-1-4-galactan in compression wood of Sitka spruce [*Picea sitchensis* (Bong.) Carriére] by immunofluorescence." Holzforschung **61**: 311-316.

Altaner, C. M., E. N. Tokareva, M. C. Jarvis and P. J. Harris (2010). "Distribution of (1→4)-β-galactans, arabinogalactan proteins, xylans and (1→3)-β-glucans in tracheid cell walls of softwoods." Tree Physiology **30**: 782-793.

Altaner, C. M., E. N. Tokareva, J. Wong, A. I. Hapca, J. P. McLean and M. C. Jarvis (2009). "Measuring compression wood severity in spruce " Wood Science and Technology **43**: 279-290.

Alves, A., N. Gierlinger, M. Schwanninger and J. Rodrigues (2009). "Analytical pyrolysis as a direct method to determine the lignin content in wood: Part 3. Evaluation of species-specific and tissue-specific differences in softwood lignin composition using principal component analysis." Journal of Analytical and Applied Pyrolysis **85**: 30-37.

Alves, A., J. Rodrigues, R. Wimmer and M. Schwanninger (2008). "Analytical pyrolysis as a direct method to determine the lignin content in wood. Part 2: Evaluation of the common model and the influence of compression wood." Journal of Analytical and Applied Pyrolysis **81**: 167-172.

Alves, A., M. Schwanninger, H. Pereira and J. Rodrigues (2006). "Analytical pyrolysis as a direct method to determine the lignin content in wood. Part 1: Comparison of pyrolysis lignin with Klason lignin." Journal of Analytical and Applied Pyrolysis **76**: 209-213.

Andersson, M. A., J. A. Cook and B. A. Stone (1978). "Enzymatic determination of 1,3;1,4-β-glucans in barley grain and other cereals." Journal of the Institute of Brewing **84**: 233-239.

Andrew, I. G. and J. W. Little (1997). "A xyloglucan in etiolated seedlings of *Pinus radiata*." Phytochemistry **46**: 203-207.

Apiolaza, L., A. (2009). "Very early selection for solid wood quality: screening for early winners." Annals of Forest Science **66**: 601.

Apiolaza, L., B. Butterfield, S. Chauhan and J. Walker (2011a). "Characterization of mechanically perturbed young stems: can it be used for wood quality screening?" Annals of Forest Science **68**: 407-414.

Apiolaza, L., S. Chauhan and J. Walker (2011b). "Genetic control of very early compression and opposite wood in *Pinus radiata* and its implications for selection." Tree Genetics & Genomes **7**: 563-571.

Bacic, A., G. B. Fincher and B. Stone, Eds. (2009). Chemistry, biochemistry, and biology of (1→3)-β-glucans and related polysaccharides, Academic Press.

Bacic, A., P. J. Harris and B. A. Stone (1988). Structure and function of plant cell walls. The biochemistry of plants. J. Preiss, Academic Press, San Diego, California. **14:** 297-371.

Balakshin, M. Y., E. A. Capanema, B. Goldfarb, J. Frampton and J. F. Kadla (2005). "NMR studies on Fraser fir *Abies fraseri* (Pursh) Poir. lignins." Holzforschung **59**: 488-496.

Bao, W., D. M. O'Malley and R. R. Sederoff (1992). "Wood contains a cell-wall structural protein." Proceedings of the National Academy of Sciences USA **89**: 6604-6608.

Barber, N. F. and B. A. Meylan (1964). "The anisotropic shrinkage of wood - A theoretical model." Holzforschung **18**: 146-156.

Barnett, J. R. and V. A. Bonham (2004). "Cellulose microfibril angle in the cell wall of wood fibres." Biological Reviews **79**: 461-472.

Bax, A. (1985). "A spatially selective composite 90° radiofrequency pulse." Journal of Magnetic Resonance **65**: 142-145.

Besombes, S., D. Robert, J.-P. Utille, F. R. Taravel and K. Mazeau (2003). "Molecular modeling of syringyl and *p*-hydroxyphenyl β-O-4 dimers. Comparative study of the computed and experimental conformational properties of lignin β-O-4 model compounds." Journal of Agricultural and Food Chemistry **51**: 34-42.

Biosupplies Australia Pty Ltd. from http://www.biosupplies.com.au/.

Björkman, A. (1956). "Studies on finely divided wood. Part 1. Extraction of lignin with neutral solvents." Svensk Papperstidning **59**: 477-485.

Blakeney, A. B. and L. L. Mutton (1980). "A simple colorimetric method for the determination of sugars in fruits and vegetables." Journal of the Science of Food and Agriculture **31**: 889-897.

Bland, D. E. (1958). "The chemistry of reaction wood. Part I. The lignins of *Eucalyptus goniocalyx* and *Pinus radiata*." Holzforschung **12**: 36-43.

Blumenkrantz, N. and G. Asboe-Hansen (1973). "New method for quantitative determination of uronic acids." Analytical Biochemistry **54**: 484-489.

Boerjan, W., J. Ralph and M. Baucher (2003). "Lignin biosynthesis." Annual Review of Plant Biology **54**: 519-546.

Bouveng, H. O., P. J. Garegg and B. Lindberg (1960). "Position of the *O*-acetyl groups in birch xylan." Acta Chemica Scandinavica **14**: 742-748.

Bouveng, H. O. and H. Meier (1959a). "Studies on a galactan from Norwegian spruce compression wood (*Picea abies* Karst.)." Acta Chemica Scandinavica **13**: 1884-1889.

Bouveng, H. O. and H. Meier (1959b). "Studies on a galactan from Norwegian spruce compression wood (*Picea abies* Karst.)." Acta Chemica Scandinavica **13**: 1884-1889.

Brendel, O., P. P. M. Iannetta and D. Stewart (2000). "A rapid and simple method to isolate pure alpha-cellulose." Phytochemical Analysis **11**: 7-10.

Brennan, M. and P. J. Harris (2011). "Distribution of fucosylated xyloglucans among the walls of different cell types in monocotyledons determined by immunofluorescence microscopy." Molecular Plant **4**: 144-156.

Brennan, M., J. P. McLean, C. M. Altaner, J. Ralph and P. J. Harris (2012). "Cellulose microfibril angles and cell-wall polymers in different wood types of *Pinus radiata*." Cellulose **Online First**.

Browning, B. L. (1967). Hemicellulose isolation and separation. Methods of wood chemistry. New York, Interscience Publishers. **2**.

Budgell, D. R., E. R. Hayes and R. J. Helleur (1987). "Direct identification of pentoses and hexoses by pyrolysis/capillary gas chromatography." Analytica Chimica Acta **192**: 243-253.

Bungert, D., S. Bastian, D. M. Heckmann-Pohl, F. Giffhorn, E. Heinzle and A. Tholey (2004). "Screening of sugar converting enzymes using quantitative MALDI-ToF mass spectrometry." Biotechnology Letters **26**: 1025-1030.

Burdon, R. D., R. P. Kibblewhite, J. C. F. Walker, R. A. Megraw, R. Evans and D. J. Cown (2004). "Juvenile versus mature wood: a new concept, orthogonal to corewood versus outerwood with special reference to *Pinus radiata* and *P. taeda*." Forest Science **50**: 399-415.

Butterfield, B. G. and B. A. Meylan (1980). Three-dimensional structure of wood: an ultrastructural approach. London, Chapman and Hall.

Capek, P., J. Alföldi and D. Lisková (2002). "An acetylated galactoglucomannan from *Picea abies* L. Karst." Carbohydrate Research **337**: 1033-1037.

Carnachan, S. M. and P. J. Harris (2000). "Polysaccharide compositions of primary cell walls of the palms *Phoenix canariensis* and *Rhopalostylis sapida*." Plant Physiology and Biochemistry **38**: 699-708.

Cavagna, F., H. Deger and J. Puls (1984). "2D-N.M.R. analysis of the structure of an aldotriouronic acid obtained from birch wood." Carbohydrate Research **129**: 1-8.

Chen, J. and J. E. Varner (1985). "An extracellular matrix protein in plants: characterization of a genomic clone for carrot extensin." The EMBO Journal **4**: 2145-2151.

Christgau, S., T. Sandal, L. V. Kofod and H. Dalbøge (1995). "Expression cloning, purification and characterization of a β-1,4-glactanase from *Aspergillus aculeatus*." Current Genetics **27**: 135-141.

Clarke, A. E., R. L. Anderson and B. A. Stone (1979). "Form and function of arabinogalactans and arabinogalactan-proteins." Phytochemistry **18**: 521-540.

Cohen, L. and A. Gusev (2002). "Small molecule analysis by MALDI mass spectrometry." Analytical and Bioanalytical Chemistry **373**: 571-586.

Colquhoun, I. J., M.-C. Ralet, J.-F. Thibault, C. B. Faulds and G. Williamson (1994). "Structure identification of feruloylated oligosaccharides from sugar-beet pulp by NMR spectroscopy." Carbohydrate Research **263**: 243-256.

Çöpür, Y., D. J. Kiemle, A. J. Stipanovic, J. Koskinen and H. Makkonen (2003). "^{1}H-NMR spectroscopic determination of carbohydrates and yield in pine and maple pulps." Paperi ja Puu - Paper and Timber **85**: 158-162.

Côté, W. A., A. C. Day, B. W. Simson and T. E. Timell (1966). "Studies on larch arabinogalactan I. The distribution of arabinogalactan in larch wood." Holzforschung **20**: 178-192.

Côté, W. A., P. A. Pickard and T. E. Timell (1967). "Studies on compression wood. IV. Fractional extraction and preliminary characterization of polysaccharides in normal and compression wood of balsam fir." Tappi **50**: 350-356.

Cousins, W. J. (1976). "Elastic modulus of lignin as related to moisture content." Wood Science and Technology **10**: 9-17.

Cousins, W. J. (1978). "Young's modulus of hemicellulose as related to moisture content." Wood Science and Technology **12**: 161-167.

Cown, D. J. (1992). "Corewood (juvenile wood) in *Pinus radiata* - should we be concerned?" New Zealand Journal of Forestry Science **22**: 87-95.

Crank, J. A. and D. W. Armstrong (2009). "Towards a second generation of ionic liquid matrices (ILMs) for MALDI-MS of peptides, proteins, and carbohydrates." Journal of the American Society for Mass Spectrometry **20**: 1790-1800.

Crowell, E. P. and B. B. Burnett (1967). "Determination of the carbohydrate composition of wood pulps by gas chromatography of the alditol acetates." Analytical Chemistry **39**: 121-124.

Currie, H. A. and C. C. Perry (2006). "Resolution of complex monosaccharide mixtures from plant cell wall isolates by high pH anion exchange chromatography." Journal of Chromatography **1128**: 90-96.

Dais, P. and A. S. Perlin (1982). "High-field, ^{13}C-N.M.R. spectroscopy of β-D-glucans, amylopectin, and glycogen." Carbohydrate Research **100**: 103-116.

Davis, A. L., R. A. Hoffmann, A. L. Russell and M. Debet (1995). "^{1}H- and ^{13}C-NMR characterization of the digalactosylmannopentaose liberated from legume seed galactomannan by β-mannanase action." Carbohydrate Research **271**: 43-54.

Davis, E. A., C. Derouet, C. Herve Du Penhoat and C. Morvan (1990). "Isolation and an N.M.R. study of pectins from flax (*Linum usitatissimum* L.)." Carbohydrate Research **197**: 205-215.

Donaldson, L., K. Radotić, A. Kalauzi, D. Djikanović and M. Jeremić (2010). "Quantification of compression wood severity in tracheids of *Pinus radiata* D. Don

using confocal fluorescence imaging and spectral deconvolution." Journal of Structural Biology **169**: 106-115.

Donaldson, L. A. (1992). "Within- and between-tree variation in microfibril angle in *Pinus radiata* " New Zealand Journal of Forestry Science **22**: 77-86.

Donaldson, L. A. (2001). "Lignification and lignin topochemistry - an ultrastructural view." Phytochemistry **57**: 859-873.

Donaldson, L. A., J. Grace and G. M. Downes (2004). "Within-tree variation in anatomical properties of compression wood in radiata pine." IAWA Journal **25**: 253-271.

Downes, G. M., J. G. Nyakuengama, R. Evans, R. Northway, P. Blakemore, R. L. Dickson and M. Lausberg (2002). "Relationship between wood density, microfibril angle and stiffness in thinned and fertilized Pinus radiata." Iawa Journal **23**: 253-265.

Driouich, A. and T. I. Baskin (2008). "Intercourse between cell wall and cytoplasm exemplified by arabinogalactan proteins and cortical microtubules." American Journal of Botany **95**: 1491-1497.

Dudley, R. L., C. A. Fyfe, P. J. Stephenson, Y. Deslandes, G. K. Hamer and R. H. Marchessault (1983). "High-resolution carbon-13 CP/MAS NMR spectra of solid cellulose oligomers and the structure of cellulose II." Journal of the American Chemical Society **105**: 2469-2472.

Eastin, I. L., S. R. Shook and S. J. Fleishman (2001). "Material substitution in the US residential construction industry, 1994 versus 1998." Forest Products Journal **51**: 30-37.

Easty, D. B. and N. A. Thompson (1991). Wood analysis. Wood structure and composition. M. Lewin and I. S. Goldstein. New York, Marcel Dekker**:** 49-138.

Edashige, Y. and T. Ishii (1997). "Rhamnogalacturonan I from xylem differentiating zones of Cryptomeria japonica." Carbohydrate Research **304**: 357-365.

Ellis, M., J. Egelund, C. J. Schultz and A. Bacic (2010). "Arabinogalactan-proteins: key regulators at the cell surface?" Plant Physiology **153**: 403-419.

Excoffier, G., R. Nardin and M. R. Vignon (1986). "Détermination de la structure primaire d'un acide tri-d-xylo-4-*O*-méthyl-D-glucuronique par R.M.N. à deux dimensions." Carbohydrate Research **149**: 319-328.

Fahlén, J. and L. Salmén (2005). "Pore and matrix distribution in the fiber wall revealed by atomic force microscopy and image analysis." Biomacromolecules **6**: 433-438.

Faix, O., I. Fortmann, J. Bremer and D. Meier (1991). "Thermal degradation products of wood. Gas chromatographic separation and mass spectrometric characterization of polysaccharide derived products." Holz als Roh- und Werkstoff **49**: 213-219.

Faix, O., D. Meier and I. Fortmann (1990). "Thermal degradation products of wood. Gas chromatographic separation and mass spectrometric characterization of monomeric lignin derived products." Holz als Roh- und Werkstoff **48**: 281-285.

Fengel, D. and G. Wegener (1989). Wood chemistry, ultrastructure, reactions. Berlin, Walter de Gruyter.

Floyd, S. (2005). Effect of hemicellulose on longitudinal shrinkage in wood. The Hemicelluloses Workshop 2005. K. M. Entwistle and J. C. F. Walker. Christchurch, The Wood Technology Research Centre**:** 115-120.

Flugge, L. A., J. T. Blank and P. A. Petillo (1999). "Isolation, modification, and NMR assignments of a series of cellulose oligomers." Journal of the American Chemical Society **121**: 7228-7238.

Free, H. C. A., M. Brennan, P. J. Harris, J. Ralph and B. G. Smith 2D-NMR spectroscopy of oligosaccharides and polysaccharides to facilitate plant cell wall research. In preparation.

Fry, S. C., W. S. York, P. Albersheim, A. G. Darvill, T. Hayashi, J.-P. Joseleau, Y. Kato, E. P. Lorences, G. A. Maclachlan, M. McNeil, J. S. G. Reid, H. U. Seitz, R. F. Selvendran, A. G. J. Voragen and A. R. White (1993). "An unambiguous nomenclature for xyloglucan-derived oligosaccharides." Physiologia Plantarum **89**: 1-3.

Fu, Y.-L. and T. E. Timell (1972). "Polysaccharides in the secondary phloem of scots pine *Pinus sylvestris* L). III. The constitution of a galactoglucomannan." Cellulose Chemistry and Technology **4**: 517-519.

Fukuyama, Y., S. Nakaya, Y. Yamazaki and K. Tanaka (2008). "Ionic liquid matrixes optimized for MALDI-MS of sulfated/sialylated/neutral oligosaccharides and glycopeptides." Analytical Chemistry **80**: 2171-2179.

Girault, R., F. Bert, C. Rihouey, A. Jauneau, C. Morvan and M. Jarvis (1997). "Galactans and cellulose in flax fibres: Putative contributions to the tensile strength." International Journal of Biological Macromolecules **21**: 179-188.

Goellner, E. M., J. Utermoehlen, R. Kramer and B. Classen (2011). "Structure of arabinogalactan from *Larix laricina* and its reactivity with antibodies directed against type-II-arabinogalactans." Carbohydrate Polymers **86**: 1739-1744.

Goldstein, I. S. (1991). Overview of the chemical composition of wood. Wood structure and composition. M. Lewin and I. S. Goldstein. New York, Marcel Dekker.

Grant, G. A., S. L. Frison, J. Yeung, T. Vasanthan and P. Sporns (2003). "Comparison of MALDI-TOF mass spectrometric to enzyme colorimetric quantification of glucose from enzyme-hydrolyzed starch." Journal of Agricultural and Food Chemistry **51**: 6137-6144.

Günl, M., F. Kraemer and M. Pauly (2011). Oligosaccharide mass profiling (OLIMP) of cell wall polysaccharides by MALDI-TOF/MS. The plant cell wall. Methods in molecular biology. Z. A. Popper. New York, Springer.

Gur'janov, O. P., T. Gorshkova, M. A. Kabel, H. A. Schols and v. J. E. G. Dam (2006). "Structural characterization of tissue-specific galactan from flax fibers by 1H NMR and MALDI TOF mass spectrosmetry." Russian Journal of Bioorganic Chemistry **32**: 558 - 567.

Gur'janov, O. P., N. N. Ibragimova, O. I. Gnezdilov and T. A. Gorshkova (2008). "Polysaccharides, tightly bound to cellulose in cell wall of flax bast fibre: Isolation and identification." Carbohydrate Polymers **72**: 719-729.

Ha, M.-A., R. J. Viëtor, G. D. Jardine, D. C. Apperley and M. C. Jarvis (2005). "Conformation and mobility of the arabinan and galactan side-chains of pectin." Phytochemistry **66**: 1817-1824.

Habibi, Y., M. Mahrouz, M.-F. Marais and M. R. Vignon (2004). "An arabinogalactan from the skin of *Opuntia ficus-indica* prickly pear fruits." Carbohydrate Research **339**: 1201-1205.

Habibi, Y. and M. R. Vignon (2005). "Isolation and characterization of xylans from seed pericarp of *Argania spinosa* fruit." Carbohydrate Research **340**: 1431-1436.

Handy, S. T. and M. Okello (2005). "The 2-Position of imidazolium ionic liquids: substitution and exchange." The Journal of Organic Chemistry **70**: 1915-1918.

Hannuksela, T. and C. Hervé du Penhoat (2004). "NMR structural determination of dissolved *O*-acetylated galactoglucomannan isolated from spruce thermomechanical pulp." Carbohydrate Research **339**: 301-312.

Haq, S. and G. A. Adams (1961). "Structure of an arabinogalactan from tamarack (*Larix laricina*)." Canadian Journal of Chemistry **30**: 1563-1573.

Harris, J. M. (1977). "Shrinkage and density of radiata pine compression wood in relation to its anatomy and mode of formation." New Zealand Journal of Forestry Science **7**: 91-106.

Harris, J. M. and B. A. Meylan (1965). "The influence of microfibril angle on longitudinal and tangential shrinkage in *Pinus radiata*." Holzforschung **19**: 144-153.

Harris, P. J. (2005). Diversity in plant cell walls. Plant diversity and evolution: genotypic and phenotypic variation in higher plants. R. J. Henry. Wallingford, Oxon, UK, CAB International Publishing**:** 201-227.

Harris, P. J., A. B. Blakeney, R. J. Henry and B. A. Stone (1988). "Gas chromatographic determination of the monosaccharide composition of plant cell wall preparations." Journal of the Association of Official Analytical Chemists **71**: 272-275.

Harris, P. J., R. J. Henry, A. B. Blakeney and B. A. Stone (1984). "An improved procedure for the methylation analysis of oligosaccharides and polysaccharides." Carbohydrate Research **127**: 59-73.

Harris, P. J. and B. A. Stone (2008). Chemistry and molecular organization of plant cell walls. Biomass recalcitrance: deconstructing the plant cell wall for bioenergy. M. E. Himmel. Oxford, Blackwell**:** 61-93.

Harwood, V. D. (1971). "Variation in carbohydrate analyses in relation to wood age in *Pinus radiata*." Holzforschung **25**: 73-77.

Harwood, V. D. (1973). "Studies on the cell wall polysaccharides of *Pinus Radiata*. II. Structure of a glucomannan." Svensk Papperstidning **10**: 377-379.

Hatfield, R. D. and R. S. Fukushima (2005). "Can lignin be accurately measured?" Crop Science **45**: 832-839.

Hedenström, M., S. Wiklund-Lindström, T. Öman, F. Lu, L. Gerber, P. F. Schatz, B. Sundberg and J. Ralph (2009). "Identification of lignin and polysaccharide modifications in *Populus* wood by chemometric analysis of 2D NMR spectra from dissolved cell walls." Molecular Plant **2**: 933-942.

Hedenström, M., S. Wiklund, B. Sundberg and U. Edlund (2008). "Visualization and interpretation of OPLS models based on 2D NMR data." Chemometrics and intelligent laboratory systems **92**: 110-117.

Hillis, W. E., Ed. (1987). Heartwood and tree exudates. Springer series in wood science. Berlin, Springer-Verlag.

Hoffman, G. C. and T. E. Timell (1970). "Isolation of a β-1,3-glucan (laricinan) from compression wood of *Larix laricina*." Wood Science and Technology **4**: 159-162.

Hoffmann, G. C. and T. E. Timell (1972). "Polysaccharides in compression wood of tamarack (*Larix laricina*) III. Constitution of a galactoglucomannan " Svensk Papperstidning **75**: 297-298.

Hsieh, Y. S.-Y. and P. J. Harris (2009). "Xyloglucans of monocotyledons have diverse structures." Molecular Plant **2**: 943-965.

Hsieh, Y. S.-Y., M. Paxton, C. P. Ade and P. J. Harris (2009). "Structural diversity, functions and biosynthesis of xyloglucans in angiosperm cell walls." New Zealand Journal of Forestry Science **39**: 187-196.

Huber, D. J. and D. J. Nevins (1977). "Preparation and properties of a β-D-glucanase for the specific hydrolsis of β-D-glucans." Plant Physiology **60**: 300-304.

Iiyama, K., T. B. T. Lam, N. Kasuya and B. A. Stone (1994). "Rapid and simple determination of *O*-acetyl groups bound to plant cell walls by acid hydrolysis and ^{1}H-NMR measurement." Phytochemistry **35**: 959-961.

Ikeda, T., K. Holtman, J. F. Kadla, H.-m. Chang and H. Jameel (2001). "Studies on the effect of ball milling on lignin structure using a modified DFRC method." Journal of Agricultural and Food Chemistry **50**: 129-135.

Jensen, J. K., H. Kim, J.-C. Cocuron, R. Orler, J. Ralph and C. G. Wilkerson (2011). "The DUF579 domain containing proteins IRX15 and IRX15-L affect xylan synthesis in Arabidopsis." The Plant Journal **66**: 387-400.

Johansson, M. (2003). "Prediction of bow and crook in timber studs based on variation in longitudinal shrinkage." Wood and Fiber Science **35**: 445-455.

Johansson, M. and R. Kliger (2002). "Influence of material characteristics on warp in Norway spruce studs." Wood and Fiber Science **34**: 325-336.

Jones, P. D., L. R. Schimleck, G. F. Peter, R. F. Daniels and A. Clark (2006). "Nondestructive estimation of wood chemical composition of sections of radial wood strips by diffuse reflectance near infrared spectroscopy." Wood Science and Technology **40**: 709-720.

Kakegawa, K., Y. Edashige and T. Ishii (1998). "Xyloglucan from xylem-differentiating zones of *Cryptomeria japonica*." Phytochemistry **47**: 767-771.

Kang, M.-J., A. Tholey and E. Heinzle (2001). "Application of automated matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for the measurement of enzyme activities." Rapid Communications in Mass Spectrometry **15**: 1327-1333.

Katz, G. (1965). "The location and significance of the *O*-acetyl groups in a glucomannan from Paraña pine." TAPPI Journal **48**: 34-41.

Kazmaier, T., S. Roth, J. Zapp, M. Harding and R. Kuhn (1998). "Quantitative analysis of malto-oligosaccharides by MALDI-TOF mass spectrometry, capillary electrophoresis and anion exchange chromatography." Fresenius' Journal of Analytical Chemistry **361**: 473-478.

Kelley, S. S., T. G. Rials, R. Snell, L. H. Groom and A. Sluiter (2004). "Use of near infrared spectroscopy to measure the chemical and mechanical properties of solid wood." Wood Science and Technology **38**: 257-276.

Kenne, L., K.-G. Rosell and S. Svensson (1975). "Studies on the distribution of the *O*-acetyl groups in pine glucomannan." **44**: 69-76.

Kibblewhite, R. P., I. D. Suckling, R. Evans, J. C. Grace and M. J. C. Riddell (2010). "Lignin and carbohydrate variation with earlywood, latewood, and compression wood content of bent and straight ramets of a radiata pine clone." Holzforschung **64**: 101-109.

Kiemle, D. J., A. J. Stipanovic and K. E. Mayo (2004). Proton NMR methods in the compositional characterization of polysaccharides. 1st International symposium on xylans, mannans and other hemicelluloses, Orlando, Florida, Amer Chemical Soc.

Kilpeläinen, I., H. Xie, A. King, M. Granstrom, S. Heikkinen and D. S. Argyropoulos (2007). "Dissolution of wood in ionic liquids." Journal of Agricultural and Food Chemistry **55**: 9142-9148.

Kim, H. and J. Ralph (2010). "Solution-state 2D NMR of ball-milled plant cell wall gels in DMSO-d_6/pyridine-d_5." Organic and Biomolecular Chemistry **8**: 576-591.

Kim, H., J. Ralph and T. Akiyama (2008). "Solution-state 2D NMR of ball-milled plant cell wall gels in DMSO-d_6." Bioenergy Research **1**: 65-66.

Kim, J. S., T. Awano, A. Yoshinaga and K. Takabe (2010). "Immunolocalization of β-1-4-galactan and its relationship with lignin distribution in developing compression wood of *Cryptomeria japonica*." Planta **232**: 109-119.

Kleen, M. and G. Gellerstedt (1995). "Influence of inorganic species on the formation of polysaccharide and lignin degradation products in the analytical pyrolysis of pulps." Journal of Analytical and Applied Pyrolysis **35**: 15-41.

Knox, J. P. (2006). "Arabinogalactan-proteins (AGPs) and plant cell development." Foods and Food Ingredients Journal **211**: 1-6.

Lamport, D. T. A., M. J. Kieliszewski, Y. Chen and M. C. Cannon (2011). "Role of the extensin superfamily in primary cell wall architecture." Plant Physiology **156**: 11-19.

Lange, B. M., C. Lapierre and H. Sandermann Jr (1995). "Elicitor-induced spruce stress lignin (structural similarity to early developmental lignins)." Plant Physiology **108**: 1277-1287.

Lapierre, C., B. Monties, C. Rolando and L. d. Chirale (1985). "Thioacidolysis of lignin: comparison with acidolysis." Journal of Wood Chemistry and Technology **5**: 277-292.

Laremore, T. N., F. Zhang and R. J. Linhardt (2006). "Ionic Liquid Matrix for Direct UV-MALDI-TOF-MS Analysis of Dermatan Sulfate and Chondroitin Sulfate Oligosaccharides." Analytical Chemistry **79**: 1604-1610.

Larson, P. R. (1994). The vascular cambium: development and structure. Berlin, Springer-Verlag.

Lee, Y. C. (1990). "High-performance anion-exchange chromatography for carbohydrate analysis." Analytical Biochemistry **189**: 151-162.

Lee, Y. C. (1996). "Carbohydrate analyses with high-performance anion-exchange chromatography." Journal of Chromatography **720**: 137-149.

Leonardon, M., C. M. Altaner, L. Vihermaa and M. Jarvis (2009). "Wood shrinkage: influence of anatomy, cell wall architecture, chemical composition and cambial age." European Journal of Wood Products **online**

Lerouxel, O., T. S. Choo, M. Seveno, B. Usadel, L. Faye, P. Lerouge and M. Pauly (2002). "Rapid structural phenotyping of plant cell wall mutants by enzymatic oligosaccharide fingerprinting." Plant Physiology **130**: 1754-1763.

Lewis, N. G. and E. Yamamoto (1990). "Lignin: occurrence, biogenesis and biodegradation." Annual Review of Plant Physiology and Plant Molecular Biology **41**: 455-496.

Liebert, T. and T. Heinze (2008). "Interaction of ionic liquids with polysaccharides. 5. Solvents and reaction media for the modification of cellulose." BioResources **3**: 576-601.

Lina, L., Z. Ping, C. Yaqi and M. Shifen (2006). "High-performance anion exchange chromatography with pulsed amperometric detection for simultaneous determination of monosaccharides and uronic acids." Chinese Journal of Analytical Chemistry **34**: 1371-1374.

Lommen, A. (2009). "MetAlign: interface-driven, versatile metabolomics tool for hyphenated full-scan mass spectrometry data preprocessing." Analytical Chemistry **81**: 3079-3086.

Lu, F. and J. Ralph (1997). "Derivatization followed by reductive cleavage (DFRC method), a new method for lignin analysis: protocol for analysis of DFRC monomers." Journal of Agricultural and Food Chemistry **45**: 2590-2592.

Lu, F. and J. Ralph (2003). "Non-degradative dissolution and acetylation of ball-milled plant cell walls: high-resolution solution-state NMR." The Plant Journal **35**: 535-544.

Lundqvist, J., A. Teleman, L. Junel, G. Zacchi, O. Dahlman, F. Tjerneld and H. Stålbrand (2002). "Isolation and characterization of galactoglucomannan from spruce (*Picea abies*)." Carbohydrate Research **48**: 29-39.

Mank, M., B. Stahl and G. Boehm (2004). "2,5-Dihydroxybenzoic acid butylamine and other ionic liquid matrixes for enhanced MALDI-MS analysis of biomolecules." Analytical Chemistry **76**: 2938-2950.

Mast, S. W., L. A. Donaldson, K. Torr, L. Phillips, H. Flint, M. West, T. J. Strabala and A. Wagner (2009). "Exploring the ultrastructural localization and biosynthesis of β(1,4)-galactan in *Pinus radiata* compression wood." Plant Physiology **150**: 573-583.

McCleary, B. V. and R. Codd (1991). "Measurement of (1 → 3),(1 → 4)-β-D-glucan in barley and oats: A streamlined enzymic procedure." Journal of the Science of Food and Agriculture **55**: 303-312.

McCleary, B. V., E. Nurthen, F. R. Taravel and J.-P. Joseleau (1983). "Characterisation of the oligosaccharides produced on hydrolysis of galactomannan with β-D-mannanase." Carbohydrate Research **118**: 91-109.

Megazyme International Ltd. from http://www.megazyme.com.

Meier, D. and O. Faix (1992). Pyrolysis-gas chromtography-mass spectrometry. Methods in lignin chemistry. S. Y. Lin and C. W. Dence. Berlin, Springer.

Meier, D., I. Fortmann, J. Odermatt and O. Faix (2005). "Discrimination of genetically modified poplar clones by analytical pyrolysis-gas chromatography and principal component analysis." Journal of Analytical and Applied Pyrolysis **74**: 129-137.

Meier, H. (1961). "Isolation and characterisation of an acetylated glucomannan from pine (*Pinus silvestris* L.)." Acta Chemica Scandinavica **15**: 1381-1385.

Meier, H. (1962a). "Studies on a galactan from tension wood of beech (Fagus-Silvatica L.)." Acta Chemica Scandinavica **16**: 2275-2283.

Meier, H. (1962b). "Studies on a galactan from tension wood of beech (*Fagus silvatica* L.)." Acta Chemica Scandinavica **16**: 2275-2283.

Meylan, B. A. (1968). "Cause of high longitudinal shrinkage in wood." Forest Products Journal **18**: 75-78.

Meylan, B. A. (1972). "The influence of microfibril angle on the longitudinal shrinkage-moisture content relationship." Wood Science and Technology **6**: 293-301.

Ministry of Agriculture and Forestry (2007). "New Zealand forest industry facts and figures 2006/2007." from http://www.maf.govt.nz/statistics/.

Minor, J. L. (1982). "Chemical linkage of pine polysaccharides to lignin." Journal of Wood Chemistry and Technology **2**: 1-16.

Mohan, D., C. U. Pittman and P. H. Steele (2006). "Pyrolysis of wood/biomass for bio-oil: a critical review." Energy & Fuels **20**: 848-889.

Möller, R., A. McDonald, C. Walter and P. J. Harris (2003). "Cell differentiation, secondary cell-wall formation and transformation of callus tissue of *Pinus radiata* D. Don." Planta **217**: 736-747.

Moore, J. R., J. D. Tombleson, J. A. Turner and M. van der Colff (2008). "Wind effects on juvenile trees: a review with special reference to toppling of radiata pine growing in New Zealand." Forestry **81**: 377-387.

Mort, A. J., S. Gao, X. Wu and R. Prade (2005). "The need for pure enzymes that degrade cell wall polysaccharides and how to get them." Plant Biosystems - An International Journal Dealing with all Aspects of Plant Biology **139**: 84-87.

Moulthrop, J. S., R. P. Swatloski, G. Moyna and R. D. Rogers (2005). "High-resolution ^{13}C NMR studies of cellulose and cellulose oligomers in ionic liquid solutions." Chemical Communications **12**: 1557-1559.

Mukoyoshi, S.-i., J.-i. Azuma and T. Koshijima (1981). "Lignin-carbohydrate complexes from compression wood of *Pinus densiflora* Sieb et. Zucc." Holzforschung **35**: 233-240.

Nanayakkara, B. (2007). Chemical characterisation of compression wood in plantation grown *Pinus radiata*. Doctoral thesis Doctoral thesis, The University of Waikato.

Nanayakkara, B., M. Manley-Harris, I. D. Suckling and L. A. Donaldson (2009). "Quantitative chemical indicators to assess the gradation of compression wood." Holzforschung **63**: 431-439.

Nicholls, J. W. P. (1982). "Wind action, leaning trees and compression wood in *Pinus radiata* D. Don." Australian Forest Research **12**: 75-91.

NIST/EPA/NIH Mass Spectral Database. (2002). National institute of standards and technology.

Nonier, M. F., N. Vivas, N. Vivas de Gaulejac, C. Absalon, P. Soulié and E. Fouquet (2006). "Pyrolysis-gas chromatography/mass spectrometry of *Quercus* sp. wood. Application to structural elucidation of macromolecules and aromatic profiles of different species." Journal of Analytical and Applied Pyrolysis **75**: 181-193.

Norman, A. G. and S. H. Jenkins (1934). "The determination of lignin. I. Errors introduced by the presence of certain carbohydrates." Biochemical Journal **28**: 2147-2160.

O'Neill, M. A., T. Ishii, P. Albersheim and A. G. Darvill (2004). "Rhamnogalacturonan II: structure and function of a borate cross-linked cell wall pectic polysaccharide." Annual Review of Plant Biology **55**: 109-139.

Obel, N., V. Erben, T. Schwarz, S. Kühnel, A. Fodor and M. Pauly (2009). "Microanalysis of plant cell wall polysaccharides." Molecular Plant **2**: 922-932.

Pan, W.-P. and G. N. Richards (1989). "Influence of metal ions on volatile products of pyrolysis of wood." Journal of Analytical and Applied Pyrolysis **16**: 117-126.

Peña, M. J., A. G. Darvill, S. Eberhard, W. S. York and M. A. O'Neill (2008). "Moss and liverwort xyloglucans contain galacturonic acid and are structurally distinct from the xyloglucans synthesized by hornworts and vascular plants*." Glycobiology **18**: 891-904.

Ponder, G. R., H.-X. Qiu and G. N. Richards (1990). "Pyrolytic conversion of biomass to anhydrosugars." Applied Biochemistry and Biotechnology **24/25**: 41-47.

Ponder, G. R. and G. N. Richards (1997). "Arabinogalactan from Western larch, Part III: alkaline degradation revisited, with novel conclusions on molecular structure." Carbohydrate Polymers **34**: 251-261.

Ponder, G. R., G. N. Richards and T. T. Stevenson (1992). "Influence of linkage position and orientation in pyrolysis of polysaccharides: A study of several glucans." Journal of Analytical and Applied Pyrolysis **22**: 217-229.

R Development Core Team (2011). "R: A language and environment for statistical computing." from http://www.R-project.org.

Ralph, J. (1997). Recent advances in characterizing "non-traditional" lignins. 9th International symposium on wood and pulping chemistry.

Ralph, J., G. Brunow and W. Boerjan (2007). Lignins. eLS, John Wiley & Sons, Ltd.

Ralph, J., G. Brunow, P. J. Harris, R. A. Dixon and W. Boerjan (2009). Lignification: are lignins biosynthesized via simple combinatorial chemistry or via proteinaceous control and template reaction? Recent advances in polyphenol research. F. Daayf and V. Lattanzio, Wiley-Blackwell**:** 36-66.

Ralph, J. and R. D. Hatfield (1991). "Pyrolysis-GC-MS characterization of forage materials." Journal of Agricultural and Food Chemistry **39**: 1426-1437.

Ralph, J., K. Lundquist, G. Brunow, F. Lu, H. Kim, P. F. Schatz, J. M. Marita, R. D. Hatfield, S. A. Ralph, J. H. Christensen and W. Boerjan (2004). "Lignins: natural polymers from oxidative coupling of 4-hydroxyphenyl-propanoids." Phytochemistry Reviews **3**: 29-60.

Ralph, J., J. Marita, S. A. Ralph, R. D. Hatfield, F. Lu, R. M. Ede, J. Peng, S. Quideau, R. F. Helm, J. H. Grabber, H. Kim, G. Jimenez-Monteon, Y. Zhang, H. J. G. Jung, L. L. Landucci, J. J. MacKay, R. R. Sederoff, C. Chapple and A.-M. Boudet (1999). Solution-state NMR of lignins. Advances in lignocellulosics characterization. D. S. Argyropoulos. Atlanta, GA., TAPPI Press**:** 55-108.

Renard, C. M. G. C., M.-J. Crépeau and J.-F. Thibault (1995). "Structure of the repeating units in the rhamnogalacturonic backbone of apple, beet and citrus pectins." Carbohydrate Research **275**: 155-165.

Rencoret, J., A. Gutiérrez, L. Nieto, J. Jiménez-Barbero, C. B. Faulds, H. Kim, J. Ralph, Á. T. Martínez and J. C. del Río (2011). "Lignin composition and structure in young versus adult *Eucalyptus globulus* plants." Plant Physiology **155**: 667-682.

Rencoret, J., G. Marques, A. Gutiérrez, L. Nieto, J. I. Santos, J. Jiménez-Barbero, Á. T. Martínes Ferrer and J. C. d. Río Andrade (2009). "HSQC-NMR analysis of lignin in woody (*Eucalyptus globulus* and *Picea abies*) and non-woody (*Agave sisalana*) ball-milled plant materials at the gel state." Holzforschung **63**: 691-698.

Ryden, P., A. J. MacDougall, C. W. Tibbits and S. G. Ring (2000). "Hydration of pectic polysaccharides." Biopolymers **54**: 398-405.

Sadoh, T. and G. N. Christensen (1967). "Longitudinal shrinkage of thin sections." Wood Science and Technology **1**: 26-44.

Saeman, J. F., W. E. Moore, R. L. Mitchell and M. A. Millett (1954). "Techniques for the determination of pulp constituents by quantitative paper chromatography." TAPPI Journal **37**: 336-343.

Salmén, L. and A.-M. Olsson (1998). "Interaction between hemicelluloses, lignin and cellulose: structure-property relationships." Journal of Pulp and Paper Science **24**: 99-103.

Schwerin, G. (1958). "The chemistry of reaction wood. Part II. The polysaccharides of *Eucalyptus goniocalyx* and *Pinus radiata*." Holzforschung **12**: 43-48.

Scurfield, G. (1973). "Reaction wood: its structure and function." Science **179**: 647-655.

Seifert, G. J. and K. Roberts (2007). "The biology of arabinogalactan proteins." Annual Review of Plant Biology **58**: 137-161.

Serban, C. M. (1998). Analytical pyrolysis of natural organic polymers. Amsterdam, Elsevier. **20:** 496.

Shafizadeh, F. and Y. L. Fu (1973). "Pyrolysis of cellulose." Carbohydrate Research **29**: 113-122.

Shimizu, K., M. Hashi and K. Sakurai (1978). "Isolation from a softwood xylan of oligosaccharides containing two 4-*O*-methylglucuronic acid residues." Carbohydrate Research **62**: 117-126.

Shin, S.-J. (2008). "Conversion factors for carbohydrate analysis by hydrolysis and ^{1}H-NMR spectroscopy." Cellulose **15**: 255-260.

Smith, B. G. and P. J. Harris (1995). "Polysaccharide composition of unlignified cell walls of pineapple [*Ananas comosus* (L.) Merr.] fruit." Plant Physiology **107**: 1399-1409.

Solar, R., F. Kačik and I. Melcer (1987). "Simple semimicro method for the determination of O-acetyl groups in wood and related materials." Nordic Pulp and Paper Research **2**: 139-141.

Sørensen, S., M. Pauly, M. Bush, M. Skjøt, M. C. McCann, B. Borkhardt and P. Ulvskov (2000). "Pectin engineering: modification of potato pectin by *in vivo* expression of an endo-1,4-β-D-galactanase." Proceedings of the National Academy of Sciences **97**: 7639-7644.

Stevanic, J. S. and L. Salmén (2009). "Orientation of the wood polymers in the cell wall of spruce wood fibres." Holzforschung **63**: 497-503.

Sun, N., M. Rahman, Y. Qin, M. L. Maxim, H. Rodriguez and R. D. Rogers (2009). "Complete dissolution and partial delignification of wood in the ionic liquid 1-ethyl-3-methylimidazolium acetate." Green Chemistry **11**: 646-655.

Sutton, W. R. J. (1999). "The need for planted forests and the example of radiata pine." New Forests **17**: 95-110.

Syverud, K., I. Leirset and D. Vaaler (2003). "Characterization of carbohydrates in chemical pulps by pyrolysis gas chromatography/mass spectrometry." Journal of Analytical and Applied Pyrolysis **67**: 381-391.

T 249 cm-85 (1985). "Carbohydrate composition of extractive-free wood and wood pulp by gas-liquid chromatography." TAPPI.

TAPPI standard (1997). "T 204 cm-97. Solvent extractives of wood and pulp."

TAPPI standard (1998). "T-222 om-98. Acid-insoluble lignin in wood and pulp."

Teleman, A., J. Lundqvist, F. Tjerneld, H. Stålbrand and O. Dahlman (2000). "Characterization of acetylated 4-*O*-methylglucuronoxylan isolated from aspen employing ^{1}H and ^{13}C NMR spectroscopy." Carbohydrate Research **329**: 807-815.

Teleman, A., M. Nordström, M. Tenkanan, A. Jacobs and O. Dahlman (2003). "Isolation and characterization of *O*-acetylated glucomannans from aspen and birch wood." Carbohydrate Research **338**: 525-534.

Telewski, F. W. (1989). "Structure and function of flexure wood in *Abies fraseri*." Tree Physiology **5**: 113-121.

Telewski, F. W. and M. J. Jaffe (1981). "Thigmorphogenesis: changes in the morphology and chemical composition induced by mechanical perturbation in 6-month-old *Pinus taeda* seedlings." Canadian Journal of Forest Research **11**: 380-387.

Telewski, F. W. and M. J. Jaffe (1986a). "Thigmorphogenesis: anatomical, morphological and mechanical analysis of genetically different sibs of *Pinus taeda* in response to mechanical perturbation." Physiologia Plantarum **66**: 219-226.

Telewski, F. W. and M. J. Jaffe (1986b). "Thigmorphogenesis: field and laboratory studies of *Abies fraseri* in response to wind or mechanical perturbation." Physiologia Plantarum **66**: 211-218.

Terashima, N., T. Akiyama, S. Ralph, D. Evtuguin, C. P. Neto, J. Parkås, M. Paulsson, U. Westermark and J. Ralph (2009). "2D-NMR (HSQC) difference spectra between specifically ^{13}C-enriched and unenriched protolignin of *Ginkgo biloba* obtained in the solution state of whole cell wall material." Holzforschung **63**: 379-384.

Terashima, N. and K. Fukushima (1988). "Heterogeneity in formation of lignin - XI: An autoradiographic study of the heterogeneous formation and structure of pine lignin." Wood Science and Technology **22**: 259-270.

Tholey, A. and E. Heinzle (2006). "Ionic (liquid) matrices for matrix-assisted laser desorption/ionization mass spectrometry—applications and perspectives." Analytical and Bioanalytical Chemistry **386**: 24-37.

Thomas, J. R., M. McNeil, A. G. Darvill and P. Albersheim (1987). "Structure of plant cell walls. XIX. Isolation and characterization of wall polysaccharides from suspension-cultured Douglas fir cells." Plant Physiology **83**: 659-671.

Thomas, R. J. (1991). Wood: formation and morphology. Wood structure and composition. M. Lewin and I. S. Goldstein. New York, Marcel Dekker**:** 7-47.

Timell, T. E. (1964). Wood hemicelluloses: Part I. Advances in carbohydrate chemistry. M. L. Wolfrom and S. R. Tipson. New York, Academic Press. **19:** 247-302.

Timell, T. E. (1965). Wood hemicelluloses: Part II. Advances in carbohydrate chemistry. M. L. Wolfrom and S. R. Tipson. New York, Academic Press. **20:** 410-483.

Timell, T. E. (1973). "Studies on opposite wood in conifers part II: Histology and ultrastructure." Wood Science and Technology **7**: 79-91.

Timell, T. E. (1982). "Recent progress in the chemistry and topochemistry of compression wood." Wood Science and Technology **16**: 83-122.

Timell, T. E. (1986). Compression wood in gymnosperms. Berlin, Springer-Verlag.

Tokareva, E. N., A. V. Pranovich, P. Fardim, G. Daniel and B. Holmbom (2007). "Analysis of wood tissues by time-of-flight secondary ion mass spectrometry." Holzforschung **61**: 647-655.

Torri, C., A. Adamiano, D. Fabbri, C. Lindfors, A. Monti and A. Oasmaa (2010). "Comparative analysis of pyrolysate from herbaceous and woody energy crops by

Py-GC with atomic emission and mass spectrometric detection." Journal of Analytical and Applied Pyrolysis **88**: 175-180.

Ulrich, E. L., H. Akutsu, J. F. Doreleijers, Y. Harano, Y. E. Ioannidis, J. Lin, M. Livny, S. Mading, D. Maziuk, Z. Miller, E. Nakatani, C. F. Schulte, D. E. Tolmie, R. Kent Wenger, H. Yao and J. L. Markley (2008). "BioMagResBank." Nucleic Acids Research **36**: D402-D408.

Ulvskov, P., H. Wium, D. Bruce, B. Jørgensen, K. B. Qvist, M. Skjøt, D. Hepworth, B. Borkhardt and S. O. Sørensen (2005). "Biophysical consequences of remodeling the neutral side chains of rhamnogalacturonan I in tubers of transgenic potatoes." Planta **220**: 609-620.

Updegraff, D. M. (1969). "Semimicro determination of cellulose in biological materials." Analytical Biochemistry **32**: 420-424.

Uprichard, J. M. (1971). "Cellulose and lignin content in *Pinus radiata* D. Don. Within-tree variation in chemical composition, density, and tracheid length." Holzforschung **25**: 97-105.

Uprichard, J. M. and J. A. Lloyd (1980). "Influence of tree age on the chemical composition of radiata pine." New Zealand Journal of Forestry Science **10**: 551-557.

van Hazendonk, J. M., E. J. M. Reinerik, P. de Waard and J. E. G. van Dam (1996). "Structural analysis of acetylated hemicellulose polysaccharides from fibre flax (*Linum usitatissimum* L.)." Carbohydrate Research **291**: 141-154.

Vignon, M. R. and C. Gey (1998). "Isolation, ^{1}H and ^{13}C NMR studies of (4-*O*-methyl-glucurono)-xylans from luffa fruit fibres, jute bast fibres and mucilage of quince tree seeds." Carbohydrate Research **307**: 107-111.

Wadenback, J., D. Clapham, G. Gellerstedt and S. von Arnold (2004). "Variation in content and composition of lignin in young wood of Norway spruce." Holzforschung **58**: 107-115.

Wagner, A., L. Donaldson, H. Kim, L. Phillips, H. Flint, D. Steward, K. Torr, G. Koch, U. Schmitt and J. Ralph (2009). "Suppression of 4-coumarate-CoA ligase in the coniferous gymnosperm *Pinus radiata*." Plant Physiology **149**: 370-383.

Wagner, A., J. Ralph, T. Akiyama, H. Flint, L. Phillips, K. Torr, B. Nanayakkara and L. Te Kiri (2007). "Exploring lignification in conifers by silencing hydroxycinnamoyl-CoA:shikimate hydroxycinnamoyltransferase in *Pinus radiata*." Proceedings of the National Academy of Sciences **104**: 11856-11861.

Walker, J. (2006). Primary wood processing. Principles and practice. Dordrecht, Springer.

Wardrop, A. B. and D. E. Bland (1959). Biochemistry of wood. London, Pergamon Press.

Weitzhandler, M., V. Barreto, C. Pohl, P. Jandik, J. Cheng and N. Avdalovic (2004). "CarboPac™ PA20: a new monosaccharide separator column with electrochemical detection with disposable gold electrodes." Journal of Biochemical and Biophysical Methods **60**: 309-317.

Westermark, U. (1985). "The occurrence of *p*-hydroxyphenylpropane units in the middle-lamella lignin of spruce (*Picea abies*)." Wood Science and Technology **19**: 223-232.

Whistler, R. L. and J. N. BeMiller (1958). Alkaline degradation of polysaccharides. Advances in Carbohydrate Chemistry. L. W. Melville, Academic Press. **Volume 13:** 289-329.

Whistler, R. L. and C.-C. Chen (1991). Hemicellulose. Wood structure and composition. M. Lewin and I. S. Goldstein. New York, Marcel Dekker**:** 287-319.

Whiting, P. and D. Goring (1982). "Chemical characterization of tissue fractions from the middle lamella and secondary wall of black spruce tracheids." Wood Science and Technology **16**: 261-267.

Willför, S. and B. Holmbom (2004). "Isolation and characterisation of water soluble polysaccharides from Norway spruce and Scots pine." Wood Science and Technology **38**: 173-179.

Willför, S., R. Sjöholm, C. Laine, M. Roslund, J. Hemming and B. Holmbom (2003a). "Characterisation of water-soluble galactoglucomannans from Norway spruce wood and thermomechanical pulp." Carbohydrate Polymers **52**: 175-187.

Willför, S., R. Sjöholm, C. Laine, M. Roslund, J. Hemming and B. Holmbom (2003b). "Characterisation of water-soluble galactoglucomannans from Norway spruce wood and thermomechanical pulp." Carbohydrate Polymers **52**: 175-187.

Willför, S., A. Sundberg, J. Hemming and B. Holmbom (2005). "Polysaccharides in some industrially important softwood species." Wood Science and Technology **39**: 245-258.

Wise, L. F., M. Murphy and A. A. D'Addieco (1946). "Chlorite holocellulose, its fractionation and bearing on summative wood analysis and on studies on the hemicelluloses." Technical Association of the Pulp and Paper Industry **122**: 35-43.

Xu, P., H. Liu, R. Evans and L. A. Donaldson (2009). "Longitudinal shrinkage behaviour of compression wood in radiata pine." Wood Science and Technology **43**: 423-439.

Yamamoto, H. (1999). "A model of the anisotropic swelling and shrinking process of wood. Part 1. Generalization of Barber's wood fiber model." Wood Science and Technology **33**: 311-325.

Yamamoto, H., F. Sassus, M. Ninomiya and J. Gril (2001). "A model of anisotropic swelling and shrinking process of wood. Part 2. A simulation of shrinking wood." Wood Science and Technology **35**: 167-181.

Yang, H., H. Ichinose, M. Yoshida, M. Nakajima, H. Kobayashi and S. Kaneko (2006). "Characterization of a thermostable *endo*-β-D-galactanase from the hyperthermophile *Thermotoga maritima*." Bioscience, Biotechnology, and Biochemistry **70**: 538-541.

Yeh, T.-F., J. L. Braun, B. Goldfarb, H.-m. Chang and J. F. Kadla (2006). "Morphological and chemical variations between juvenile wood, mature wood, and compression wood of loblolly pine (*Pinus taeda* L.)." Holzforschung **60**: 1-8.

Yeh, T.-F., B. Goldfarb, H.-m. Chang, I. Peszlen, J. L. Braun and J. F. Kadla (2005). "Comparison of morphological and chemical properties between juvenile wood and compression wood of loblolly pine." Holzforschung **59**: 669-674.

Yelle, D. J., J. Ralph and C. R. Frihart (2008). "Characterization of nonderivatized plant cell walls using high-resolution solution-state NMR spectroscopy." Magnetic Resonance in Chemistry **46**: 508-517.

Zanuttini, M., M. Citroni and M. J. Martínez (1998). "Application of diffuse reflectance infrared fourier transform spectroscopy to the quantitative determination of acetyl groups in wood." Holzforschung **52**: 263-267.

Zhao, H. (2006). "Innovative applications of ionic liquids as green engineering liquids." Chemical Engineering Communications **193**: 1660-1677.

Zykwinska, A., J.-F. Thibault and M.-C. Ralet (2007). "Organization of pectic arabinan and galactan side chains in association with cellulose microfibrils in primary cell walls and related models envisaged." Journal of Experimental Botany **58**: 1795-1802.

Zykwinska, A. W., M.-C. J. Ralet, C. D. Garnier and J.-F. J. Thibault (2005). "Evidence for in vitro binding of pectin side chains to cellulose." Plant Physiology **139**: 397-407.

Appendix

Pyrolysis product identification table

Peak	RT	Compound
1	4.05	Ethanedial, monohydrate, dimer
2	4.50	2-Propenal
3	4.70	Propanal-2-one
4	5.32	Methacrolein
5	5.62	(1,5)-Hexadien-3-ol (t)
6	5.78	Unknown
7	5.94	Unknown/Ethylvinylketone
8	6.39	Unknown/3-Pentanone
9	7.07	Hydroxyacetaldehyde
10	7.90	2-Butenal/Crotonaldehyde/(2,3)-Dihydrofuran
11	8.16	Acetic acid
12	8.89	Unknown/(2,3)-Pentanedione
13	9.63	2-Propanone, 1-hydroxy- (Acetol)
14	9.97	Toluene
15	10.86	1,2-Dihydroxyethene
16	11.07	Unknown
17	11.60	Methyl pyruvate
18	12.32	3-Methyl-furan
19	13.15	2-Propenoic acid, methyl ester
20	13.70	(1,2)-Ethanediol
21	14.34	3-Hydroxypropanal
22	14.90	3-Butenal-2-one/Furan-(3H)-2-one
23	15.02	3-Butenal-2-one/(S)-5-Hydroxymethyl-2[5H]-furanone
24	15.67	Furan-(2H)-3-one
25	15.93	3-Furaldehyde
26	16.54	2-Hydroxy-3-oxo-butanal/(Methyl pyruvate?)
27	16.79	Unknown
28	17.19	2-Cyclopenten-1-one
29	17.33	2-Furaldehyde
30	19.39	2(3H)-Furanone, 5-methyl-
31	19.50	2-Furfuryl alcohol
32	19.92	Acetol acetate
33	20.10	2-Methyl-2-cyclopenten-1-one
34	20.39	2-Ethylbutanal/2-Butanone
35	20.83	Dihydro-methyl-furanone or Dimethyl-dihydro-furan
36	20.92	2-Acetylfuran
37	21.44	Unknown
38	21.47	Methoxy-dihydrofuran or 2-Butenoic acid methyl ester
39	21.67	Unknown
40	21.81	2-Cyclopentene-1,4-dione
41	22.29	5-Methyl-2,3-dihydro-1,4-dioxine (t)
42	22.58	Similar to 2-Hydroxy-butanedial or 4-Hydroxydihydro-2(3H)-furanone
43	22.75	Unknown
44	22.96	2-Hydroxycyclopent-2-en-1-one
45	23.23	Benzofuran
46	23.36	Benzaldehyde
47	24.00	Dihydro-methyl-furanone
48	24.17	4-Methoxytoluene
49	24.40	5-Methyl-2-furancarboxaldehyde
50	24.51	2-hydroxymethylene-tetrahydrofuran-3-one or 4-Hydroxy-(5,6)-dihydro-(2H)-pyran-2-one
51	25.14	3-Methyl-2-cyclopenten-1-one
52	25.42	Butyrolactone
53	25.88	2(5H)-Furanone

54	26.26	3-Pentanone
55	26.61	5-Methyl-2(5H)-furanone
56	26.78	Unknown
57	26.97	4-Hydroxy-(5,6)-dihydro-pyran-(2H)-2-one
58	27.13	3-Methyl-(2,5)-furandione
59	27.55	Unknown
60	27.67	2H-Pyran-2-one
61	27.86	2-Hydroxy-3-methyl-2-cyclopenten-1-one
62	28.07	Methyl-dihydro-(2H)-pyran-2-one
63	28.11	Unknown
64	28.75	Unknown
65	29.75	Phenol
66	30.38	Guaiacol, 2-Methoxy-phenol
67	31.32	Difurfuryl ether
68	31.55	Unknown/(1,3)-Dioxolan-2-one (t)
69	32.09	Phenol, 2-methyl-
70	32.26	Unknown/Propane-(1,1)-diol diacetate (t)
71	32.57	Unknown/5-Hydroxy-2-methyl-4H-pyran-4-one (t)
72	32.64	Unknown
73	32.72	Unknown/similarities to 3-Hydroxydihydro-2(3H)-furanone
74	33.23	(2,5)-Furandicarboxaldehyde
75	33.31	2-Hydroxy-5-methylbenzaldehyde
76	33.59	4-Methyl-5H-furan-2-one
77	33.81	Phenol, 4-methyl-
78	33.90	Phenol, 3-methyl-
79	34.25	5-Hydroxymethyldihydrofuran-2-one
80	34.66	Unknown/ similarities to (2,5)-Furandione, dihydro-3-methyl-
81	34.78	4H-Pyran-4-one, (2,3)-dihydro-(3,5)-dihydroxy-6-methyl-
82	34.90	Unknown
83	35.25	4-Methyl guaiacol
84	35.48	Unknown
85	35.60	Unknown
86	35.96	Phenol, (3,4)-dimethyl-
87	36.33	unknown G-lignin/ similarities to (3,4)-Dimethoxytoluene
88	37.84	4-Ethyl phenol
89	38.20	3-Ethyl guaiacol
90	38.69	unknown/lactone derivative
91	39.01	4-Ethylguaiacol
92	39.35	Unknown
93	39.61	4-Hydroxy-3-methyl-(5H)-furanone or 3-Methyl-(2,4)-furandione
94	39.70	Unknown H-lignin
95	39.82	(3,4)-Anhydro-D-galactosan or (2,3)-Anhydro- D-galactosan
96	39.94	Unknown
97	40.05	Anhydro- D-mannosan or (3,4)-Anhydro- D-galactosan
98	40.80	(1,4:3,6)-Dihydro-α- D-glucopyranose
99	41.11	(1,5)-Anhydroarabinofuranose
100	41.62	Vinyl guaiacol
101	41.76	Phenol, -4-ethenyl (t)
102	42.04	4-(2-propenyl)-Phenol (4 allylphenol)
103	42.43	Unknown lignin
104	42.59	Eugenol
105	42.71	4-Propyl guaiacol
106	42.88	5-Acetoxymethyl-2-furaldehyde
107	43.17	Unknown
108	43.40	5-Hydroxymethyl-2-furaldehyde
109	43.61	Unknown H-lignin (4-propenylphenol (*cis*))
110	43.79	Unknown
111	43.96	5-Hydroxymethyldihydrofuran-2-one
112	44.59	Unknown G lignin (similar to Isoeugenol)
113	44.97	Isoeugenol (*cis*), 2-Methoxy-4-(1-propenyl)-phenol
114	45.75	3-Methoxy-5-methylphenol (t)
115	46.28	2-Hydroxymethyl-5-hydroxy-2,3-dihydro-(4H)-pyran-4-one

116	46.50	4-propenylphenol (*trans*)
117	46.81	Unknown
118	47.09	1H-Indenol
119	47.23	Isoeugenol (trans), 2-Methoxy-4-(1-propenyl)-phenol
120	47.41	Unknown lignin
121	47.72	Unknown
122	48.07	Vanillin
123	48.32	Unknown lignin
124	48.65	Unknown, similarity to 6-Methoxy-3-methylbenzofuran
125	49.06	Unknown, similarity to 6-Methoxy-3-methylbenzofuran
126	50.41	Homovanillin
127	50.76	Phenol, 4-propyl-
128	50.88	Unknown lignin compound
129	51.08	Unknown
130	51.24	Acetoguaiacone
131	51.57	(1,6)-Anhydrogalactopyranose
132	51.65	Unknown phenol
133	52.08	Unknown
134	52.46	Benzaldehyde, 4-hydroxy-
135	53.18	Guaiacylacetone
136	53.67	Unknown H-lignin compound
137	53.85	Unknown
138	54.46	Propioguaiacone
139	54.86	Acetophenone, 4'-hydroxy-
140	55.00	Unknown G-lignin
141	55.23	Unknown lignin compound
142	55.37	(1,6)-Anhydromannopyranose (t)
143	56.30	Unknown
144	56.43	Unknown
145	56.67	(1,4)-Anhydrogalactopyranose (t)
146	57.27	(1,6)-Anhydroglucopyranose (levoglucosan)
147	57.92	Dihydroconiferyl alcohol
148	58.93	Coniferyl alcohol (*cis*)
149	59.16	Unknown lignin compound
150	60.71	Anhydrosugar (unknown)
151	61.89	Coniferyl alcohol (*trans*)
152	62.54	Coniferyl aldehyde
153	63.89	Unknown