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Lignin-carbohydrate complexes of wood and non-wood materials: fractionation and characterisation

Master's Thesis

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Abstract

In lignocelluloses, lignin is currently assumed to be covalently bonded to carbohydrates and these structures are called lignin-carbohydrate complexes. The literature review of this master's thesis handles the structure, fractionation, and analysis of lignin-carbohydrate complexes. Based on current understanding, there are three main lignin-carbohydrate bonds, which are called phenyl glycoside, benzyl ether, and ester bonds. First proper fractionating method for lignin-carbohydrate complexes was established by Björkman and after that many different methods have been presented. The used fractionation method has an impact on the structure of lignin-carbohydrate complexes and modern 2D NMR spectroscopy is one of the most powerful techniques to analyse structures of lignin carbohydrate complexes together with traditional degradation techniques.

In the experimental part, hot water hydrolysates of spruce, birch and wheat straw were fractionated into two fractions containing lignin-carbohydrate complexes, which were ethanol precipitate and ethanol soluble fraction. Lignin and carbohydrates were analysed from fractions using acid hydrolysis, UV/Vis spectroscopy and gas chromatography. In addition, FT-IR spectra of each fraction was measured. Melting points and the amount of extractives were also determined. Ethanol precipitates had less lignin than ethanol soluble fractions and unexpected low melting points of ethanol soluble fractions were observed without depending on used biomass. Birch raw hemi contained significantly more extractives than spruce or wheat straw raw hemi. Wheat straw samples differed slightly from tree samples which were much alike.

Tiivistelmä

Nykytutkimuksen valossa ligniinin oletetaan olevan sitoutunut kovalenttisesti hiilihydraatteihin lignoselluloosapitoisessa biomassassa. Näitä rakenteita kutsutaan ligniini-hiilihydraatti komplekseiksi. Tämän pro gradu- tutkielman kirjallisuuskatsaus käsittelee ligniini-hiilihydraatti kompleksien rakenteita, fraktiointia sekä analysointia. Tämänhetkisen ymmärryksen mukaan ligniini on sitoutunut hiilihydraatteihin pääasiassa kolmentyyppisillä sidoksilla, jotka ovat fenyyliglykosidi-, bentsyylieetteri- ja esterisidoksia. Ensimmäisen kunnollisen menetelmän ligniini-hiilihydraatti kompleksien fraktioimiseksi esitteli Björkman, jonka jälkeen monia erilaisia menetelmiä on julkaistu. Käytetyllä fraktiointimenetelmällä on vaikutusta ligniini-hiilihydraatti kompleksien rakenteisiin. Moderni 2D NMR spektroskopia on tehokkain menetelmä ligniini-hiilihydraatti kompleksien analysoitiin yhdessä perinteisten pilkkomistekniikoiden kanssa.

Kokeellisessa osassa kuusen, koivun ja vehnänoljen kuumavesiuutteita fraktioitiin kahteen ligniini-hiilihydraatti komplekseja sisältävään jakeeseen, jotka olivat etanoliin saostuva ja etanoliin liukeneva jae. Jakeista määritettiin sekä ligniini että hiilihydraattipitoisuudet hyödyntäen happohydrolyysiä, UV/Vis spektroskopiaa sekä kaasukromatografiaa. Lisäksi FT-IR spektri mitattiin jokaisesta jakeesta. Myös sulamispisteet sekä uuteaineiden määrä määritettiin. Etanoliin saostuvat jakeet sisälsivät vähemmän ligniiniä kuin etanoliin liukoiset jakeet ja etanoliin liukoisilla jakeilla huomattiin olevan matala sulamispiste riippumatta käytetystä biomassasta. Koivun raakahemi sisälsi huomattavasti enemmän uuteaineita kuin kuusen tai vehnänoljen raakahemit. Vehnänolkinäytteet erosivat hieman puunäytteistä, jotka olivat keskenään hyvin samankaltaisia.

Preface

This master's thesis was written during spring and summer 2023. The experimental part was performed at the chemistry department of the University of Jyväskylä, and the topic was given by VTT. The literature was searched via JykDok and some were obtained from the supervisors. This project has received funding from the European Union – NextGenerationEU instrument and is funded by the Academy of Finland under grant number 347612, project MIMIC - Microscopy and machine learning in molecular characterization of lignocellulosic materials.

I would like to thank my supervisors Joni Lehto from VTT and Jarmo Louhelainen from University of Jyväskylä for this opportunity, interesting topic, and all the help you gave me. I would also like to thank my family and friends, especially Toni Heikkilä, who has been my IT-support these years I have studied at University of Jyväskylä. Thank you, without you I would have more grey hair than I have today.

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Abbreviations

AcOH Acetic acid

[Amim]Cl 1-Allyl-3-methylimidazolium chloride

Ara Arabinose

ATR Attenuated total reflectance

BE Benzyl ether

CDTA Cyclohexanediaminetetraacetic acid

CEL Cellulolytic enzyme lignin

CIR Chelator-insoluble residue

DDQ 2,3-Dichloro-5,6-dicyano-1,4-benzoquione

DFRC Derivatisation followed by reductive cleavage

DMAc Dimethylacetamide

DMF Dimethylformamide

DMSO Dimethyl sulfoxide

DRI Differential refractive index

DRIFT Diffuse reflectance infrared Fourier-tansform

EHR Enzymatic hydrolysis residue

EtOAc Ethyl Acetate

FID Flame ionization detector

G Guaiacyl unit

Gal Galactose

GC Gas chromatography

GCP Gel permeation chromatography

Glu Glucose

GM Glucomannan

H *p*-Hydroxyphenyl unit

HMBC Heteronuclear multiple bond coherence

HPAEC High performance anion exchange chromatography

HSQC Heteronuclear single quantum coherence

HTAOH Hexadecetyl ammonium hydroxide

IR Infrared

IR-FT Infrared Fourier-transform

KIR KOH-insoluble residue

L Lignin

LC Lignin-carbohydrate

LCC Lignin-carbohydrate complex

LFP Lignin-ferulate-polysaccharide

MAL Mild acidolysis lignin

Man Mannan

MIR Mid-infrared

MS Mass spectrometry

MSD Mass selective detector

MWL Milled wood lignin

NIR Na_2CO_3-insoluble residue

NMR Nuclear magnetic resonance

PhGlc Phenyl glycoside

Py-GC/MS Pyrolysis gas chromatography mass spectrometry

S Syringyl unit

SEC Size exclusion chromatography

TBAF Tetrabutylammonium fluoride

TBAH Tetrabutylammonium hydroxide

TFA Trifluoroacetic acid

WIR Water-insoluble residue

Xyl Xylose

1D One-dimensional

2D Two-dimensional

1 Introduction

Greenhouse gas emissions, global warming and climate change are today's big problems. Due to these environmental problems, finding renewable and sustainable sources to replace fossil raw materials is essential. A lot of research utilising biomass as one solution has been done. Biomass can be divided to plant-based and animal-based. Plant-based biomass is also called lignocellulosic biomass and can be classified in two groups: wood or non-wood. Lignocellulosic biomass consist mainly of three polymers which are cellulose, hemicelluloses, and lignin.[1] In addition, there are some extractives and inorganics.

Cellulose is the most common and abundant organic polymer. It is linear polysaccharide consisting of D-glucose units. The degree of polymerisation varies with the origin but is in plants typically between 800-10 000. Cellulose is extensive and has a lot of hydroxyl groups that form hydrogen bonds. Cellulose has both high order crystalline and low order amorphous areas.[2]

Unlike cellulose, hemicelluloses are not homogenous or necessarily linear. Hemicelluloses are heterogenous polysaccharides, which consist of pentoses like xylose and arabinose, hexoses like mannose, glucose and galactose, and sugar acids. Hemicelluloses' composition varies with species: for example, hardwoods contain mainly xylans and softwoods contain mainly glucomannans. Also, the degree of polymerisation can vary depending on species. Xylans have homopolymeric backbone of β-D-xylopyranose units. In addition to xylose, xylans may contain arabinose, and glucuronic, acetic, ferulic and *p*-coumaric acids as sidegroups.[3] Glucomannans have backbone of β-D-glucopyranose and β-D-mannopyranose units that are partly acetylated and linked to α-D-galactopyranose units.[4]

Lignin is aromatic and amorphous polymer which makes plant cells rigid, impervious, and protects polysaccharides from microbial degradation. Lignin is composed of three hydroxycinnamyl alcohols which are coniferyl alcohol, sinapyl alcohol, and *p*-coumaryl alcohol, which are precursors of lignin biosynthesis.[5] When these three monolignols are part of polymer, they are called guaiacyl (G), syringyl (S) and *p*-hydroxyphenyl (H) units, and are linked by aryl ether and carbon-carbon bonds[6]. Lignin composition varies among taxa and species but also among cell types. In figure 1 are shown some of lignin substructures.[5]

In 1886 Erdman postulated that in wood exist chemical bonding between lignin and carbohydrates forming ‘glycolignose’. After that lot of research has been made and nowadays those structures are called lignin-carbohydrate complexes (LCCs). Interest in LCCs has increased lately because LCCs restrict separation of lignin and carbohydrates. Thus, understanding LCC structures and their chemical behaviour is important for utilizing plant materials in industrial scale. For example, LCCs hinder chemical pulping and bioethanol production.[7,8] LCCs have also displayed pharmacological activities such as anti-viral properties[8] which add interest in LCCs.

Due to complex structure of plant cells, harsh conditions are required for isolating lignin-carbohydrate complexes and question about origin of LCC comes up. Are LCCs native or do they form during isolation or analysis? Lignin-carbohydrate complexes have been studied since 1886 but no direct proof of covalent bonding between lignin and carbohydrates has been found. However, plenty of supporting evidence of covalent bonding has been proposed[7,9,10].

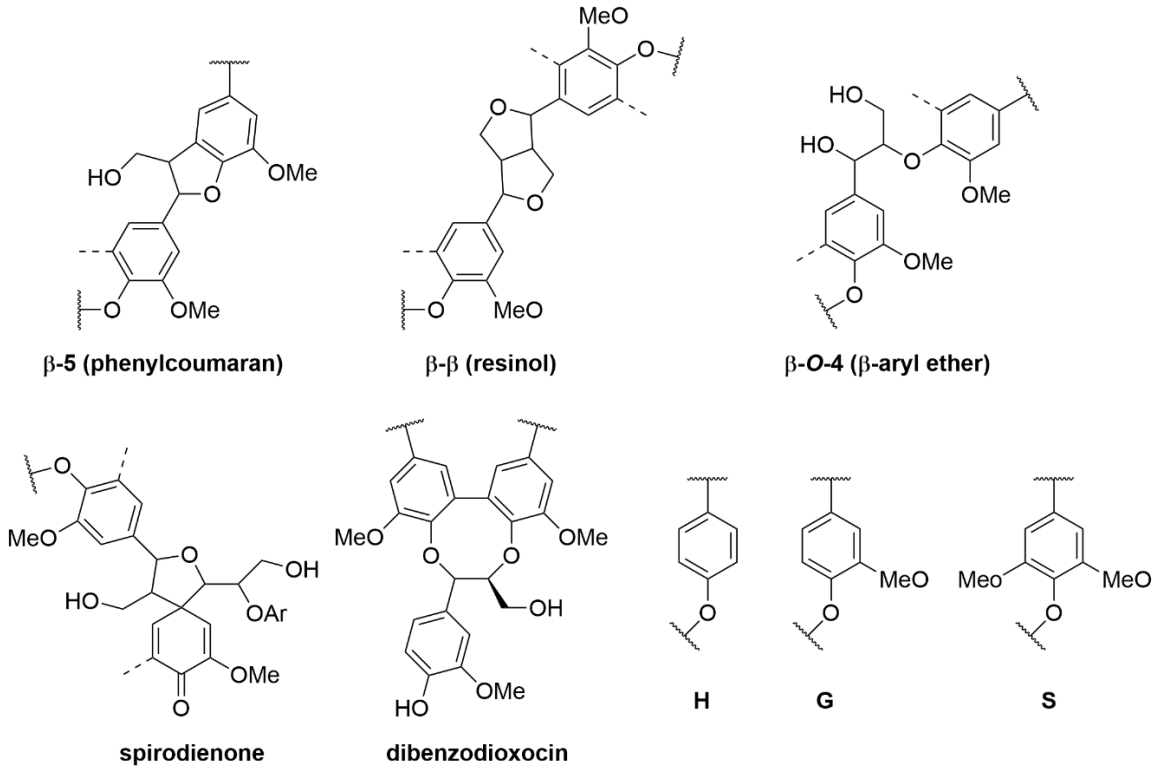

Figure 1. Lignin substructures.[11]

2 Lignin-carbohydrate complexes

As mentioned in the previous chapter, lignin is currently assumed to be covalently bonded to carbohydrates and these structures are called lignin-carbohydrate complexes. These covalent bonds between lignin and carbohydrates are believed to form during the lignin biosynthesis through the addition of nucleophiles to quinone methides, formed as intermediates in the oxidation of *p*-hydroxycinnamyl alcohols.[12] In wood there is relatively low amount of LCC bonds but still, almost all of lignin is bonded to carbohydrates, mainly with hemicelluloses.[11] Level of LCCs is related to the structure of lignin, especially the syringyl to guaiacyl ratio.[8]

In spite of research, ambiguity in types, frequencies and quantity of LCCs exist.[13] Techniques used for analysis of LCC structure are commonly degradation techniques like alkaline hydrolysis, acid hydrolysis, Smith degradation and methylation. Identification of degradation products tells more about carbohydrate parts of LCC and do not provide direct evidence of lignin-carbohydrate bonds.[7] Modern 2D NMR is preferred for structural analysis of LCC though there might be difficulties for dissolving the LCC in common NMR solvents. Difficulties in solubility result mainly from high molecular weight.[14]

2.1 Lignin-carbohydrate bonds

Lignin is mostly covalently bonded to the hemicelluloses through α-carbon and C-4 position in the benzene ring.[15] Despite difficulties in determination, based on current understanding there are three different main types of lignin-carbohydrate linkages in wood, and they are called phenyl glycosides, esters, and benzyl ethers.[8,11,14] In addition to previous three types, acetal linkages can be also counted as one type of lignin-carbohydrate (LC) bond existing in plant materials but acetal linkages are not as widely researched and reported.[15] These main structures are shown in figure 2.

Benzyl ether (BE) bonds exist between α-carbon of the arylpropane lignin unit and the hydroxyl of carbohydrate residue.[15] BE bonds can be divided into two groups: in BE_1 bond is between primary OH-group of carbohydrate and α-carbon of lignin, and in BE_2 bond is between secondary OH-group and α-carbon of lignin.[16] Researchers have proposed as formation mechanism of LC ether and ester bonds nucleophilic addition of hydroxyl groups of carbohydrates or uronic acid to a quinone methide, which is an intermediate during lignin

biosynthesis. If xylan hydroxyl groups are highly substituted by acetyl group, benzyl ether bond formation on xylan decreases. Thus, acetylation of hemicelluloses may function as regulator for the frequency and type of LC bond.[15]

Ester bonds are predominant LC bonds. Ester bond is one important bond that is formed between the β-diaryl lignin unit (α- or γ-carbon) and the side chain of xylan.[15] Thermodynamically less favoured α-ester bond is first formed similarly as benzyl ether bond. Formed ester bond can migrate to the γ-position via a transesterification reaction, forming thermodynamically favoured γ-ester. Migration can happen under neutral or acidic conditions and thus potentially during isolation processes under those conditions.[10]

Phenyl glycosidic bonds are formed between phenolic hydroxyl group of lignin and anomeric hydroxyl group of cellulose or hemicellulose.[15] Mechanism of phenyl glycosidic bond formation is not fully understood but there are two hypotheses. The first hypothesis is that formation of phenyl glycoside is catalysed by transglycosylating enzymes in wood. The second hypothesis is that acid-catalysed hydrolysis leads to acetal formation, which is more likely to happen close to the acidic groups in the cell wall.[10] Phenyl glycosides and benzyl ethers are stable in alkaline pulping conditions while benzyl esters are not.[13]

LCC of herbaceous plants structurally differ from LCC of wood because of the incorporation of hydroxycinnamates into the cell wall.[8] In the cell wall of herbaceous plants ferulic acid is ether linked to lignin and esterified linked to carbohydrates forming a lignin-ferulate-polysaccharide complex (figure 2).[8] Del Rio *et al*.[17] have isolated LCCs from leaf fibers of sisal and abaca. They have isolated two different fractions of LCC containing lignin moieties that structurally differ. The less condensed lignin, enriched in syringyl units, was preferentially associated with xylans. The more condensed lignin, enriched in guaiacyl units, was preferentially associated with glucans.

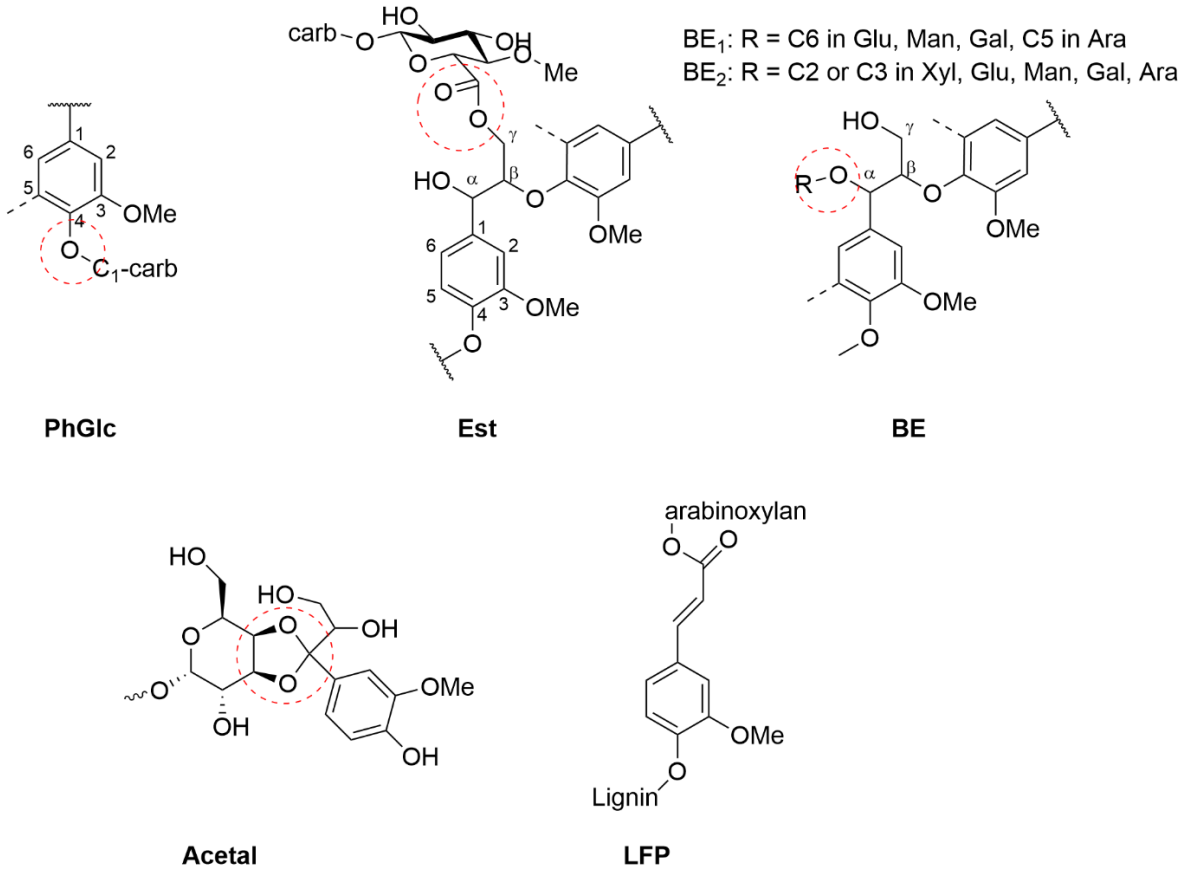

Figure 2. LC bond types: PhGlc[11] = Phenyl glycoside, Est[11] = γ-ester, BE[11] = Benzyl ether, Acetal[18] = Acetal linkage, LFP[19] = lignin-ferulate-polysaccharide.

Balakshin *et al.*[11] have studied LC bonds of pine and birch using 2D NMR spectroscopy. They have reported that pinewood contains more benzyl ether bonds than birch, whereas birch contains more phenyl glycoside and γ-ester bonds. They did not detect any benzyl esters (α-esters) in their studies, and they have explained their observation by migration of α-ester to the γ-position of lignin side chain. In the same study Balakshin *et al.*[11] have noticed that the characteristic signals of non-esterified 4-*O*-methyl-α-D-glucuronic acid are much stronger in samples of birch compared to pine which indicates according to them that the birch LCC preparation still contained significant amount of non-esterified glucuronic acid moieties whereas most of glucuronic acid moieties in the pine LCC preparation are esterified.

In spruce wood benzyl ether and ester bonds exists, and it is proposed that benzyl ether bonds exist between the benzylic α-position in lignin units and primary hydroxyls in hexopyranoses/arabinofuranose or secondary hydroxyls in xylose. Ester bonds are proposed to exist between the 4-*O*-methylglucuronic acid side chains in xylan and the benzylic α-position in lignin units.[14]

Qin *et al.*[20] have isolated LCCs from quince (Chinese fruit) using three different fractionation methods obtaining fractions called milled wood lignin (MWL), LCC-AcOH and Björkman LCC (more about methods is told in the next chapter). Their structural analysis has showed that the main bond types in MWL fraction are benzyl ether and γ-ester, in LCC-AcOH phenyl glycoside and benzyl ether and in Björkman LCC phenyl glycoside and γ-ester. They did also find bonds between lignin and pectin in Björkman LCC fraction.

2.2 Composition

Carbohydrate composition of different LCC fractions in wood and non-wood is shown in table 1. Lawoko *et al.*[13] have studied structure of LCC in spruce wood and in corresponding kraft pulp. In spruce wood, they have found four main types of LCC based on their hemicellulose composition. They were a galactoglucomannan-lignin-pectin complex containing 8 % of the lignin in wood, glucan-lignin complex containing 4 % of the lignin, a glucomannan-lignin-xylan complex containing 48 % of the lignin and xylan-lignin-glucomannan complex containing about 40 % of the lignin. Latter two differ in amount of glucomannan and xylan: the first mentioned was predominant. In corresponding unbleached kraft pulp they have found three major LCC fractions which were the same as in wood but lacking the galactoglucomannan-lignin-pectin complex. The glucomannan-lignin-xylan complex had predominance and after subsequent oxygen delignification it was the only major fraction containing 80 % of the lignin in pulp.

Zhao *et al*.[6] have studied how lignin and LCC varies during growth of eucalyptus using method descripted later in chapter 3.2. They have isolated LCC and lignin fractions from 2-, 3-, and 4-years old eucalyptus trees and analysed the chemical composition and structure of the fractions. They have noticed that the contents of Klason lignin, cellulose and hemicelluloses increased with the growth whereas the ash content slightly decreased. The S/G ratios of three fractions namely MWL, LCC-AcOH and CEL first increased during growth and then decreased but S/G ratio of fraction called EHR only decreased. The amount of phenyl glycoside bonds in LCC-AcOH fraction and ether bonds in MWL fraction varied parallel to the S/G ratio but in CEL fraction amount of the ether bonds were contrary to the S/G ratio.

Table 1. Carbohydrate composition of different LCC fractions in wood and non-wood. Ara = arabinose, Xyl = xylan, Man = mannose, Gal = galactose, Glu = glucose, L=lignin, GM = glucomannan, P = precipitate, S = solution. Fractions are named according to original papers.

LCC fraction	Acid soluble lignin (%)	Total carbohydrates[a] (%)	Relative fraction of total carbohydrates (%)				
			Ara	Xyl	Man	Gal	Glu
Softwood							
(Spruce)[14]							
Glu-L	19.3	80.7	1.9	2.5	8.6	1.2	85.8
GM-L	29.2	70.8	4.7	10.6	30.9	4.4	49.4
Xyl-L	42.7	57.3	13.0	65.3	3.2	3.0	15.6
Hardwood							
(Birch)[21]							
P1	29.2	70.8	0	41.7	11.8	10.0	36.6
P3	15.9	84.1	0.9	16.2	0.5	0.7	81.7
S6[b]	2.7	97.3	0	88.7	3.5	2.8	5.1
S6[c]	35.8	64.2	1.0	75.9	2.2	2.7	18.2
(Eucalypt)[21]							
P1	29.0	71.0	1.2	40.8	10.4	8.0	39.6
P2	53.2	46.8	5.4	19.2	6.7	6.7	62.0
S4	37.5	62.5	1.1	91.3	2.7	1.9	3.0
P3	9.5	90.5	0.8	4.0	0.6	0.5	94.1
S6	13.8	86.2	0	58.7	12.1	2.3	26.9
Non-wood							
(Sisal)[17]							
Glu-L	7.8	92.2	1.5	9.0	0.9	0.2	88.4
Xyl-L	24.1	75.9	0.6	89.4	2.6	0.3	7.1
(Abaca)[17]							
Glu-L	4.4	95.6	0.3	4.1	0.5	0.1	95.0
Xyl-L	29.4	70.6	3.4	75.5	13.0	0.3	7.8

[a] Calculated from relative proportion of lignin.

[b] Solution at pH 7 and 5.

[c] Solution at pH 7, precipitate at pH 5.

2.3 Molecular weight

Molecular weight of LCC depends on the lignocellulosic biomass, but also on the used isolation method. Feng *et al*.[22] have isolated LCC from poplar using hot water extraction pre-treatment and Björkman's fractionation method. According to their study, weight-average molecular weights depended on used extraction temperature and were 19,216 g/mol when the temperature was 140 ℃, 9,688 g/mol when the temperature was 150 ℃ and only 6,197 g/mol when the temperature was 160 ℃. Without the hot water extraction pre-treatment but otherwise in the same way isolated LCC from poplar had weight-average molecular weight of 10,446 g/mol. Based on carbohydrate analysis, depolymerisation during hot water extraction was possibly due to acid hydrolysis of mannose.

In Zhao *et al*.[23] study, six LCC fractions were isolated from eucalyptus. Weight-average molecular weights of these fractions varied from 1,380 g/mol to 14,580 g/mol. The lowest molecular weight was found in the LCC fraction with high carbohydrate content (60.3 %) and the highest molecular weight was found in fraction where carbohydrate content was half lower (30.5 %). In He *et al.*[24] study, three LCC fractions were isolated from sesame hulls. Weight-average molecular weights of those three fractions were 4,600 g/mol to148,600 g/mol. In table 2 is shown weight-average and number-average molecular weights and polydispersity indexes of different LCC fractions. Polydispersity index tells the distribution of the molecular weight within a sample: if the value is one, all molecules have the same weight and greater the value is, more distribution of weights is in the fraction. It can be noticed that molecular weights vary significantly between different fractions but also between the molecules in the same fraction.

Table 2. Weight-average molecular weights (M_w), number-average molecular weights (M_n) and polydispersity indexes (M_w/M_n) of different LCC fractions from different studies and lignocellulosic biomasses.

Study and biomass	LCC	M_w (g/mol)	M_n (g/mol)	M_w/M_n
Feng *et al*.[22]	P-LCC$_{140}$	19,216	3,522	5.46
Poplar[a]	P-LCC$_{150}$	9,688	1,366	7.09
	P-LCC$_{160}$	6,197	1,039	5.96
	B-LCC	10,466	7,635	1.37
Zhao *et al*.[23]	F1	1,380	1,370	1.01
Eucalyptus[b]	F2	14,580	11,060	1.32
	F3	4,590	2,870	1.60
	F1A	1,400	1,380	1.01
	F2A	9,940	8,620	1.15
	F3A	4,780	3,060	1.56
He *et al*.[24]	MWL	4,600	3,700	1.24
Sesame hull[c]	LCC-AcOH	6,100	690	8.84
	Björkman LCC	148,600	129,250	1.15

[a] P-LCC$_x$ is LCC fraction isolated from hot water pre-treatment liquor at temperature x. B-LCC is LCC fraction isolated with Björkman's method. More about fractionation is told in chapter 3.1.

[b] FX is fraction where neutral dioxane was used and FXA is fraction where acidic dioxane where used. More about fractionation is told in chapter 3.7.

[c] MWL (milled wood lignin), LCC-AcOH and Björkman LCC are fractions described in next chapter. More about fractionation is told in chapter 3.2.

3 Isolation of LCC

To investigate structure and properties of LCC, a distinct and complete fractionation is needed. Prerequisite is preserving the covalent bonding between lignin and carbohydrate unaltered. Components in lignocellulosic material are entangled physically with one another, which leads to need for relatively harsh conditions in fractionation. Milling has been commonly used to open the entangled structure. Enzymatic treatment is also commonly used after milling. Due to the complex structure of lignocellulosic biomass isolation of LCC with mild conditions and good yield is challenging. To obtain better recovery, harsher conditions are needed but it might cause damage in LCC structure.[25]

Various methods for isolating LCC have been reported. None of the LCC isolation methods can provide access to all information of LCCs, for example structures and compositions of lignin and carbohydrate parts, bonding types, linking sides, molecular mass, or molecular mass distribution. Thus the best isolation method depends on the purpose of the research.[11] LCC fractions can be divided into lignin-rich and carbohydrate-rich LCC depending on the isolation method. For example Björkman LCC and enzymatic LCC are typically carbohydrate-rich and MWL and CEL are lignin-rich fractions.[6]

3.1 Björkman's method

First proper fractionating method for LCC was established by Björkman in 1954 and it has become a foundation of LCC studies. At first in Björkman's fractionation method (figure 3), lignocellulosic biomass is extracted with toluene and ball milled for 48h. Then milled extractive-free biomass is extracted with 96 % 1,4-dioxane in a wood/solvent ratio of 1/10 wt/wt, stirring at ambient temperature for 24 h under nitrogen atmosphere. After extraction, solution is centrifuged. Solution is evaporated from supernatants, getting fraction called milled wood lignin (MWL). The precipitates are then extracted with dimethylformamide (DMF) or dimethyl sulfoxide (DMSO) and dissolved in 50 % acetic acid. Finally mixture is washed with a dichloromethane/ethanol mixture, getting precipitate considered as Björkman LCC.[26]

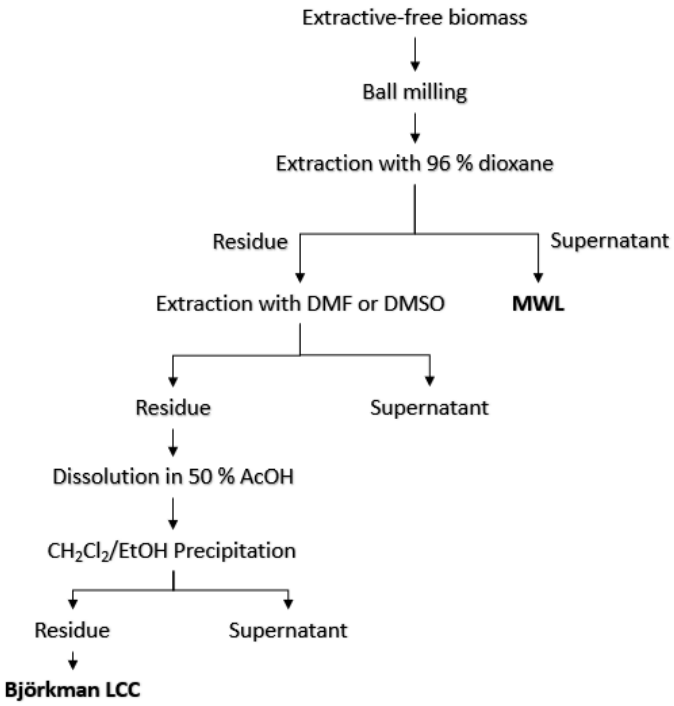

Figure 3. Flowchart adapted from Tarasov *et al.*[26]

Jiang *et al*.[27] have isolated LCC according to Björkman's method from ginkgo shells. Instead of using 50 % acetic acid, they did use 90 % acetic acid for extraction of LCCs and they ball milled samples only for 2 hours. Due to short milling time, cell walls might not be destroyed completely, resulting less lignin being extracted. In their study, yields of fractions were low, under 17 %.

Björkman-LCC from hot water pre-treatment liquor

Feng *et al*.[22] have isolated Björkman-LCC from hot water pre-treatment liquor using Björkman's method. To achieve hot water pre-treatment liquor, they have first extracted poplar meal with benzene and ethanol mixture (2:1) and then they have washed and dried extractive free meal. Next, they have extracted the extractive-free poplar meal with deionised water at different temperatures from 130 ℃ to 190 ℃. Then they have filtered the mixtures and collected solutions. At last, they have dried the solutions and dissolved in DMF and then treated according to Björkman's method to obtain fraction called Björkman LCC.

In Feng *et al*.[22] study, the yield of LCCs depended on hot water pre-treatment temperature. They did divide the process in to two phases, an exponential growth phase at 130-150 ℃ and

an exponential decline phase at 150-190 °C. In the exponential growth phase, the yield increased from 7 % to 83 %. During the exponential decline phase, the yield decreased from 83 % to 6 %. Temperature affected also chemical composition of LCC. The amount of lignin and mannose decreased with increasing temperature as well as the mannose to xylose ratio. Conversely content of xylose increased with increasing temperature. Also, the molecular weights of LCC significantly decreased with increasing temperature.

3.2 Methods with fraction called LCC-AcOH

You *et al*.[8] have isolated LCC from energy crop called A. *donax*, Balakshin *et al.*[11] have isolated LCC from pine and birch, He *et al*.[24] have isolated LCC from sesame hulls and Zhao *et al*.[6] have isolated LCC from eucalyptus, using similar methods which have parts of Björkman's method and have one fraction obtained by using acetic acid extraction. You *et al*.[8], He *et al*.[24] and Zhao *et al.*[6] have used toluene/ethanol mixture and Balakshin *et al*.[11] have used ethanol/benzene mixture to remove extractives from original plant samples. To grind the extractive free samples, You *et al*.[8] have used a planetary ball milling for 12 h, He *et al*.[24] for 4 h and both Zhao *et al*.[6] and Balakshin *et al*.[11] for 5 h. In all these four studies samples were then extracted with dioxane according to Björkman's method.

In Balakshin *et al.*[11] method (figure 4), solvent is evaporated in vacuum and to remove traces of dioxane, a few drops of deionised water is added to solid and evaporated again obtaining crude MWL. Crude MWL is dissolved to 90 % acetic acid (AcOH) (20 ml/g) and precipitated dropwise into water. Solution is collected and evaporated to dryness. Drops of water are added to remove traces of AcOH and evaporated again. The solid is dried in vacuum oven at 35 °C and fraction called LCC-AcOH is obtained. The formed precipitate is washed, freeze dried, and dissolved in dichloroethane-ethanol (2:1) mixture. Then the solution is precipitated dropwise into ether and precipitate is filtered, washed with ether and petroleum ether, and dried. This fraction is called purified MWL. The residue after dioxane extraction is washed with water and then treated with a cellulase in an acetate buffer solution at pH 4.5, at 45 °C for 48 h. Solid matter is separated by centrifugation, washed first with the buffer solution and second with water and freeze dried. The dried sample is extracted with 96 % dioxane and then treated like crude MWL. This fraction is called cellulolytic enzyme lignin (CEL).

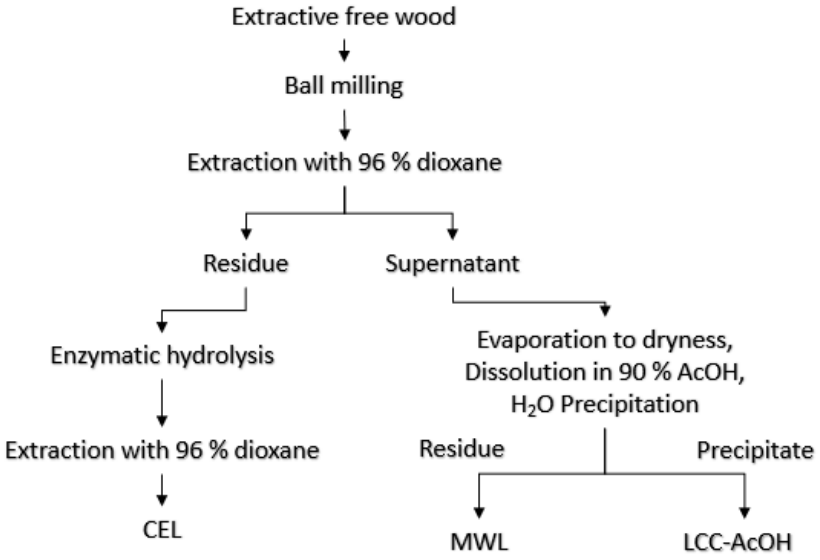

Figure 4. Flowchart adapted from Balakshin *et al.*[11]

In You *et al.*[8] method (figure 5) LCC-AcOH and purified MWL fractions are obtained similarly as in Balakshin *et al.*[11] method descripted above. Residues from the dioxane extraction are dissolved to 50 % AcOH and remaining plant particles are removed by centrifugation. Supernatant is evaporated to dryness, freeze dried and dissolved in DMSO and purified using 1,2-dichloroethane/ethanol solution (2:1), diethyl ether and petroleum ether for several times and fraction called Björkman LCC is obtained. Then Björkman LCC is treated with cellulase in 50 mM sodium acetate buffer for 72 h, washed and freeze dried obtaining fraction called ELCC. The residue is treated with cellulase and hemicellulose in acetate buffer at pH 4.8, at 48 °C for 48 h. After enzymatic treatment the substrate is extracted with 96 % dioxane to obtain fraction called CEL.

Study of Zhao *et al.*[6] used fractionation method from study of Balakshin *et al.*[11] with some changes. Fractions called LCC-AcOH and MWL are obtained as in Balakshin *et al.*[11] study, but the residue from dioxane extract is extracted twice with 80 % aqueous dioxane after enzymatic hydrolysis. All supernatants are collected, concentrated, and regenerated in acidic water at pH 2 and freeze dried to obtain CEL. The residue is treated with cellulase in an acetate buffer solution at 50 °C for 48 h, then centrifuged and the solid is washed and freeze dried to obtain fraction called enzymatic hydrolysis residue (EHR).

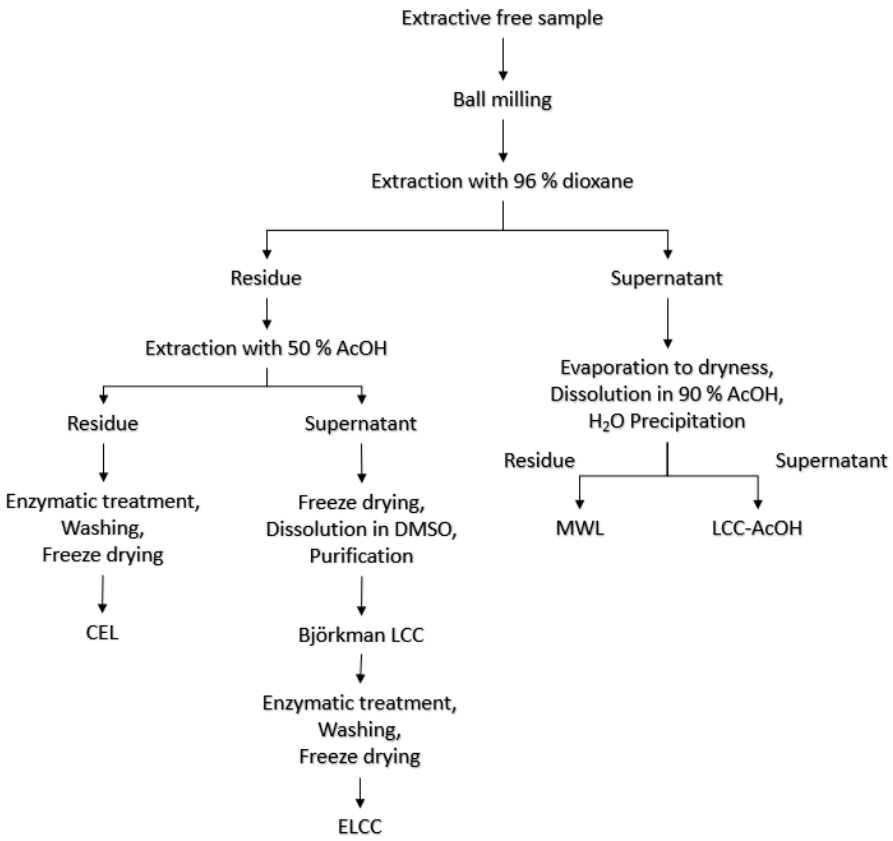

Figure 5. Flowchart adapted from You *et al*.[8]

In study of Balakshin *et al*.[11] total yield of pine and birch LCC fractions were 60-70 % of the total native lignin. Higher yields were obtained from birch. In study of You *et al.*[8] total yield of LCC fractions were 62.1 % of total native lignin and 53.2 % of the lignin was found in the CEL fraction. Only Björkman LCC was carbohydrate-rich having 65.2 % carbohydrates. Other fractions were rich in lignin having 86.5-94.5 % lignin. In study of Zhao *et al*.[6] total yields based on Klason lignin of extractive-free wood were 77.2-84.8 %. According to study of Zhao *et al.*[6] LCC-AcOH fraction is favourable to evaluate phenyl glycoside bonds and CEL is a applicable fraction for calculating benzyl ether bonds.

He *et al*.[24] have isolated three LCC fractions form sesame hulls. Fractions are assigned as MWL, LCC-AcOH and Björkman LCC. The used method (figure 6) is referred to previous studies, including Björkman's method and is similar with methods descripted above. Briefly, in He *et al*.[24] method, extractive free sesame hulls are ball milled and then extracted with 96 % dioxane. The first supernatant is concentrated and dissolved in acetic acid and then added dropwise to deionised water, forming precipitate which is the MWL fraction. The second supernatant is concentrated and freeze dried forming LCC-AcOH. Precipitate from dioxane extraction is extracted with acetic acid and then concentrated. The concentrate is mixed with DMSO, and

the supernatant is added to dichloroethane-ethanol solution and washed with dichloroethane-ethanol, anhydrous diethyl ether, acetone, and petroleum ether. The resulting fraction is dried and called Björkman LCC. Yields in He *et al*.[24] study were very low: MWL was 0.8 %, LCC-AcOH 1.0 % and Björkman LCC 1.3 % of the original sample adding up to total yield of 3.1 %.

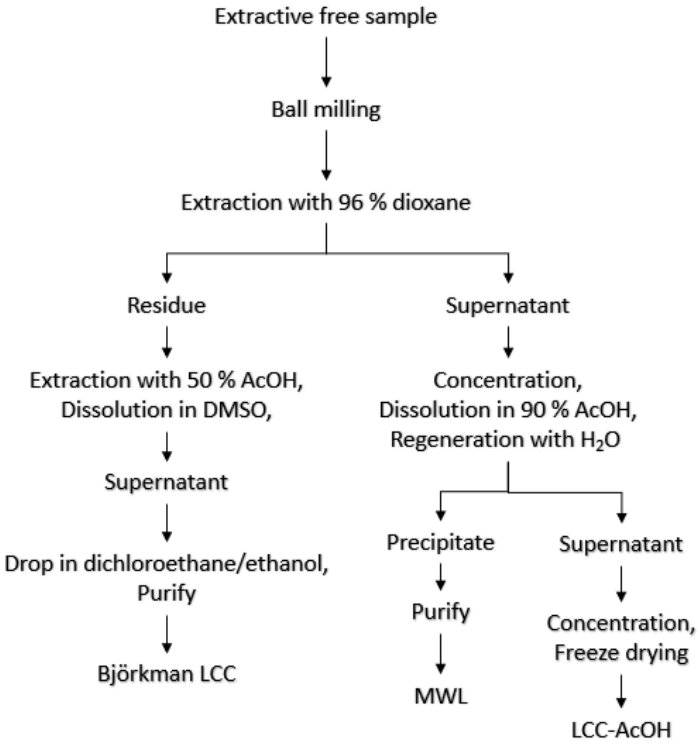

Figure 6. Flowchart adapted from He *et al*.[24]

3.2.1 Method for bamboo

Yue *et al*.[28] have isolated LCC from bamboo using method that is similar with above descripted isolation method of the LCC-AcOH fraction, but lacking other LCC fractions and thus being simpler. It also starts with 96 % dioxane extraction of extractive free ball milled sample according to Björkman's method. The residue is washed with aqueous dioxane and extracted under the same conditions for three times. Extracts and washing solutions are collected and evaporated under vacuum at 50 °C. Few drops of water are added and evaporated again obtaining crude MWL that is dissolved in 90 % acetic acid. Solids are removed by centrifugation and the solution is added dropwise with stirring to the deionised water. Formed precipitate is removed again by centrifugation and the solution is concentrated by rotary vacuum evaporation and freeze dried to obtain LCC fraction.

In Yue *et al*.[28] study, they have isolated LCC from young 2-month-old bamboo and mature 24-month-old bamboo. The yields of LCC were 3.9 % and 1.5 % for young and mature, respectively. According to them, explanation for the yield difference might be that a significant amount of lignin was isolated from the secondary walls of cells and under isolation conditions mature bamboo was not easily extracted by the 96 % dioxane. The weight-average molecular weight of mature bamboo LCC was slightly higher (9,890 g/mol) than young bamboo LCC (8,650 g/mol).

3.2.2 Method used during the removal of pectin and hemicelluloses

Wei *et al*.[29] have isolated Björkman-LCC and LCC-AcOH from Chinese quince fruits during sequential removal of pectic and hemicellulosic polysaccharides. Their polysaccharide removal process includes four steps and is carried out as follows. In the first step extractive free fruit powder is stirred with deionised water at 25 ℃ for 16 hours to extract water-soluble pectic polysaccharides and then remaining solids are separated by filtration and dried at 50 ℃ obtaining first fraction called water-insoluble residue (WIR). In the second step, WIR is mixed with chelator (cyclohexanediaminetetraacetic acid, CDTA) and left to stand for 6 hours in room temperature. Solid residue is separated by centrifugation and dried at 50 ℃ obtaining second fraction called chelator-insoluble residue (CIR). In the third step, CIR is mixed with Na_2CO_3 solution (0.05 mol/l) containing 20 mmol/l $NaBH_4$ and then left to stand at 4 ℃ for 20 h and then at 25 ℃ for 2 h. Again, residue is separated by centrifugation and then dried obtaining the third fraction called Na_2CO_3-insoluble residue (NIR). In the last step, NIR is mixed with KOH solution (4 mol/l) containing 20 mmol/l $NaBH_4$ at 25 ℃ in a N_2 atmosphere for 2 h. Last fraction is called KOH-insoluble residue (KIR) which is obtained as the others by centrifugation and drying.

In Wei *et al*.[29] study Björkman LCC and AcOH-LCC were isolated from extractive free fruit powder, WIR, CIR, NIR and KIR similarly as in studies descripted above. Yields of Björkman LCC were 3.42 %, 5.43 %, 2.89 %, 1.41 % and 0.65 % for extractive free fruit powder, WIR, CIR, NIR and KIR, respectively. Yields of AcOH-LCC were 0.10 %, 0.13 %, 0.08 %, 0.16 % and 0.54 %, respectively. In both fractions, Björkman LCC and AcOH-LCC, highest carbohydrate content was found when they were isolated from KIR. During the sequential

removal of polysaccharides, the S/G ratio of LCC-AcOH fractions increased, referring to that S-type lignins were released from secondary cell walls.

3.3 Method in neutral pH

Giummarella *et al.*[1] have developed pH neutral method for LCC fractionation (figure 7). In their method, milled wood particles are extracted with acetone for 16 h and ball milled for 24 hours. First deionised water is added to extractive free ball milled wood and stirred at 80 ℃ for 4 h. Solution and residue are separated by centrifugation. The solution is passed through polyaromatic resin (Amberlite® XAD-4) twice and the permeate is separated from retentate. Retentate is regenerated from the resin with methanol. This is repeated three times.

Small amount of acetone is added into the residue and dried overnight. Then an ionic liquid, [amim]Cl (1-allyl-3-methylimidazolium chloride) is added (2:1 w/w), and the mixture is stirred at 80 ℃ for 2h. After that DMSO (same weight as [amim]Cl) is added to mixture and is stirred another 2 hours in same conditions. More DMSO (75 ml) is added and left overnight under gently stirring at 70 ℃ achieving completely dissolution. After cooling, deionised water is added and the precipitate 1 is washed with deionized water and freeze dried. Ethanol is added to the remaining solution (3:1) and left to stand overnight at 4 ℃. Formed precipitate 2 is separated by centrifugation, dialysed, and freeze dried. Finally, acetone is added to the remaining solution (2:1) and precipitate 3 is collected like precipitate 2. Giummarella *et al.*[1] reported total recovery of 95 % and lignin recovery of 90 % using their fractionation method for spruce wood.

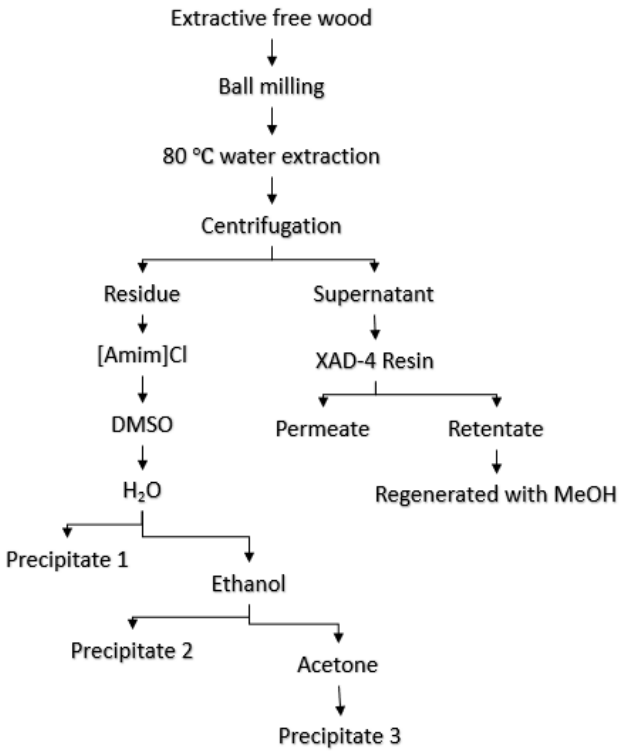

Figure 7. Flowchart adapted from Giummarella *et al.*[1]

Giummarella and Lawoko[30] have done a slight modification on method descripted above. In the last step, instead of using acetone to precipitate the last fraction, they have used three times its volume of water. In their study Giummarella and Lawoko[30] have used birch wood and reported total recovery of 93-97 % and 90 % for lignin. According to them, this protocol is relatively mild and quantitative. The bulk of the hemicelluloses is separated at an earlier stage, which enhances NMR signals of LC bonds when analysing structure. Their observations of acetyl groups partially substituting the hydroxyls in xylan, a high prevalence of aryl ether bonds and the presence of pH-sensitive uronic acid ester linkages indicates that this protocol is not so harsh.

3.4 Universal method

Li *et al.*[31] have developed fractionation method using eucalyptus wood chips and unbleached kraft pulp as samples. In their method wood chips are first milled and then extracted with acetone for 12 h. Sample is air-dried and then ball milled. Enzymatic treatment is conducted with endoglucanase at 50 °C in pH 6 for 48 h. Then sample is dissolved in 1:1 mixture of DMSO and tetrabutylammonium hydroxide (TBAH, 40 % in water). After dissolution at least

6 volumes of deionised water are added to solution dropwise with stirring. Mixture is centrifuged and precipitate is washed with water and freeze dried. The remaining solution is dialysed against running deionised water for 72 h and then freeze dried.

In their study Li *et al*.[31] used also dimethylacetamide (DMAc) containing 8 % LiCl and DMSO containing 16.25 % tetrabutylammonium fluoride (TBAF) as a solvent for extractive free ball milled samples. Eucalyptus wood samples were not soluble in DMAc/LiCl mixture and pulp samples were not totally soluble to DMSO/TBAF mixture. In addition, they have determined the minimal milling time for complete dissolution in DSMO/TBAH mixture as a solvent using eucalypt, spruce, and flax samples. It was 6 h for wood pulps and spruce wood, 12 h for others. According to their study 12 h milling time don't affect to lignin structure.

Du *et al*.[25] have developed method created by Li *et al*. [31] by adding steps (figure 8). In their method an extractive free ball milled sample is dissolved in 1:1 a mixture of DMSO and TBAH (40 % w/w in water). Formed clear solution is dispersed into deionised water and precipitate 1 will be formed. Precipitate 1 is washed with deionised water until the pH is neutral and then freeze dried. Next, saturated $Ba(OH)_2$ solution is added to remaining solution and precipitate 2 is formed. Precipitate 2 is neutralised with HCl and then dialysed and freeze dried. To obtain last LCC fraction, HCl is added to remaining solution until solution is neutralised. Precipitate 3 is dialysed and freeze dried.

Du *et al*.[25] have used spruce as a sample, which is softwood, and reported their method as universal because Li *et al*.[31] had used successfully similar method for eucalyptus which is hardwood, flax which is non-wood and also corresponding pulps. Yields of precipitates 1, 2 and 3 in Du *et al*.[25] study were 49.5 %, 30.9 % and 12.8 %, respectively, adding up to total recovery of 93.2 %. Precipitate 1 was rich in glucan, precipitate 2 was rich in glucomannan and precipitate 3 was rich in xylan.

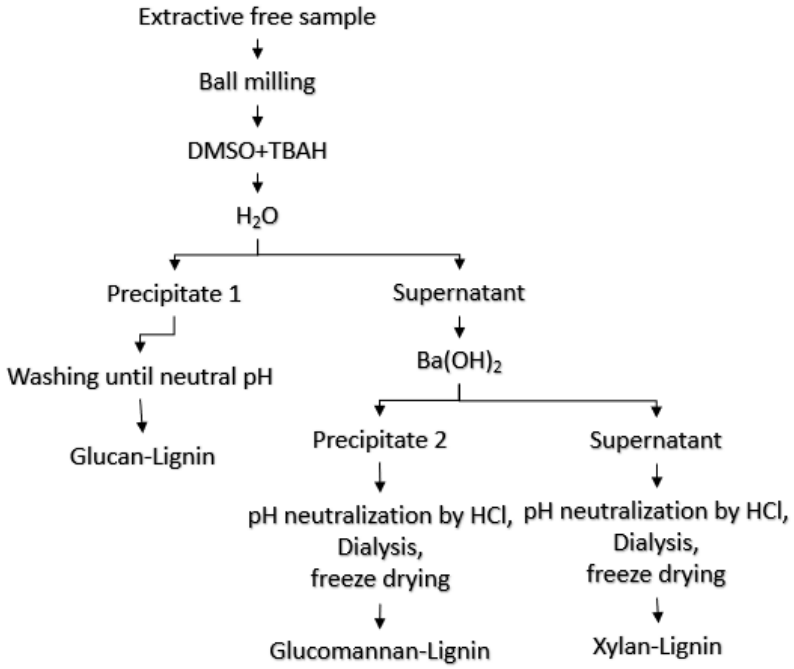

Figure 8. Flowchart adapted from Du *et al*.[25]

Using method described by Li *et al*.[31] and Du *et al*.[25], del Rio *et al*.[17] have isolated LCC from leaf of sisal and abaca plants. They did leave the barium hydroxide step out, because glucomannan is not a major hemicellulose in sisal and abaca. They managed to have quantitative yields: total recovery of 94.0 % and 93.8 % for sisal and abaca, respectively were observed. They have also noticed that despite extensive washing, significant amounts of TBAH remained in the fractions, affecting the yields. In both plants, yield of water precipitate fraction was higher and enriched in glucans. Water soluble fractions were enriched in xylan and had more lignin than glucan-rich fraction. Del Rio *et al*.[17] have pointed out that this fractionation method affects the structure of the carbohydrates and lignin. Their analyses have indicated that groups that are acetylating the γ-carbon of the lignin may be hydrolysed and removed during fractionation. Thus, the fractions would mostly reflect alkali stable LCC fractions.

Monot *et al*.[32] have studied why wood prehydrolysis improves delignification process by isolating LCC from control wood and prehydrolysed wood according to the method by Du *et al*.[25] This wood prehydrolysis is an autohydrolysis, where no external acid is added. Mild acetic conditions where hydrolysis takes place is created by the acetic acid which is released from the hemicelluloses during the heating in water. Monot *et al*.[32] have used a softwood mixture containing pine and spruce species and a hardwood mixture containing mainly beech, poplar, oak and chestnut as samples in their study.

In Monot *et al.*[32] study the total yields of LCC fractions were 78.0 % and 88.5 % without prehydrolysis, and 64.9 % and 53.6 % with prehydrolysis for softwood and hardwood, respectively. According to their study, prehydrolysis reduces the wood components involved in LCC and the effect is depending on wood species. The LCC isolation method was well suited for isolation of glucan-lignin (precipitate 1) and xylan-lignin (precipitate 3), and the results were similar independently of the wood type. For glucomannan-lignin (precipitate 2) the isolation method is less selective. Softwood LCCs contained less lignin and xylans whereas hardwood contained less cellulose, glucomannans and xylans.

3.5 Methods for wheat straw

Zikeli *et al.*[33] have isolated two LCC fractions from wheat straw using the following method (figure 9). Wheat straw is milled and then extracted with acetone for 6 hours. Then extractive free wheat straw is ball-milled using liquid nitrogen cooling for 30 minutes. Cooling the system is done to prevent lignin structure alteration during milling. The straw meal is added to a DMSO/aqueous TBAH (1:1) mixture and stirred for 4 h at room temperature. Solution is slowly added into distilled water and let it stand for 48 hours. Formed precipitate is separated by centrifugation and washed with distilled water. The precipitate is suspended in distilled water and the pH is adjusted to 5.0-5.5 using hydrochloric acid to neutralise residual TBAH and complete precipitation. The precipitate is separated by centrifugation, washed with distilled water, and finally freeze dried. Solutions from centrifugations are subjected to ultrafiltration in order to isolate the second fraction. The retentate is diluted with distilled water to remove dissolved salts and then freeze dried. In their study, Zikeli *et al.*[33] had 94.7 % yield and 49.0 % of it was in first fraction and 45.7 % was in the second fraction.

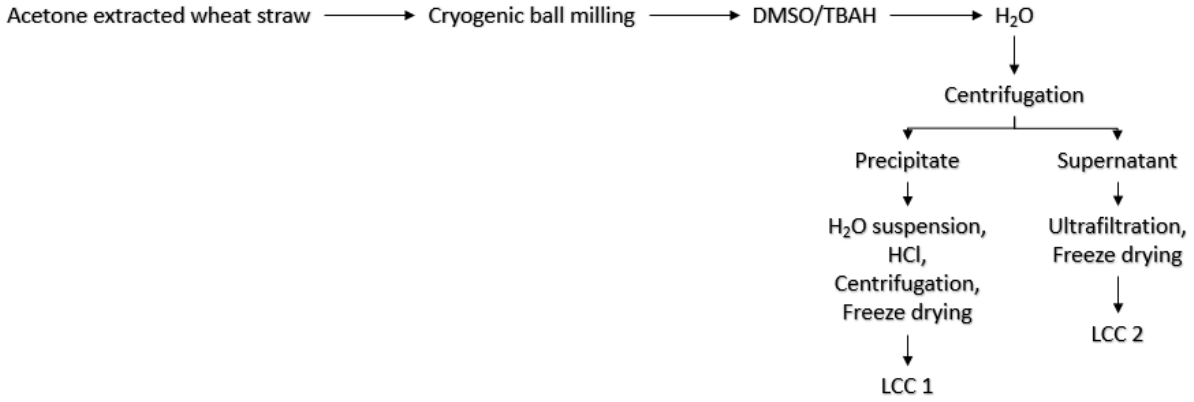

Figure 9. Flowchart adapted from Zikeli *et al.*[33]

Xie *et al*.[34] have isolated LCC from wheat straw stalk, sheath and leaf using a facile method. In their method wheat straw is first extracted with benzene/ethanol solution for 12 hours and after that ball milled for 6 hours. Ball milled material is suspended in 8 % LiCl/DMSO solution and the mixture is stirred at room temperature for 72 h to obtain dissolution. Solution is added dropwise into deionised water and extracted for three days. Solution is centrifuged and liquids are concentrated by rotary evaporation and then precipitated in ethanol. Precipitate is purified with 96 % dioxane, ethanol and ether twice and freeze dried.

The yields in Xie *et al*.[34] study were relative low: 2.1 %, 1.9 % and 2.3 % in case of stalk, sheath and leaf, respectively. According to their study, LCC was hard to isolate due to the degradation. Obtained LCC fractions were carbohydrate-rich and had weight-average molecular weight between 17.5-20.7 g/mol. Main LC bonds were phenyl glycosides and esters.

3.6 Methods for softwoods and corresponding pulps

Lawoko *et al*.[35] have published method for isolating LCC from unbleached softwood kraft pulps. The method starts with enzyme hydrolysis which is conducted with a mono component endoglucanase. Hydrolysis is conducted at pH 6 using a 10 mM bis-tris buffer system at 50 °C for 48 h. Resulting hydrolysate is centrifuged and the residue is washed with 8 M urea solution at pH 8.8 overnight with stirring at room temperature. Mixture is centrifuged again, washed with water properly and the residue is treated with an alkaline borate solution (18 % NaOH with 4 % H_3BO_3) stirring in room temperature for 4 hours. Residue is washed with water until pH is neutral and the procedure is repeated four times. The solution obtained after centrifugation is fractionated by reducing pH. At pH ~ 12 a precipitate 1 is formed, and the solution is reduced to neutral pH. Barium hydroxide (5 % aq.) is added to solution and precipitate 2 is formed, recovered on the centrifuge, dialysed overnight, and freeze dried. The supernatant is shaken with 50 % acetic acid and poured into ethanol forming precipitate 3, which is treated like the precipitate 2. The last supernatant is dialysed and freeze dried. In their study, Lawoko *et al*.[35] have used laboratory prepared unbleached pine kraft pulp and obtained high yield of residual LCC (>90 %) thus making method quantitative.

Lawoko *et al*.[36] have also published similar method for isolating LCC from spruce wood (figure 10). First wood chips are milled to meal and extracted with acetone for 24 hours. Extractive free wood meal is ball-milled for 3 h and then 10 mM bis-tris buffer is added (pH 7)

and stirred for 30 min. Then the endoglucanase is added, and mixture is incubated at 50 °C for 48 h with gently stirring. Mixture is centrifuged and solid part is removed form hydrolysate. An equal amount of 5 % barium hydroxide octahydrate is added dropwise to hydrolysate and stirred for 2 h. Formed precipitate1 is centrifuged and dialysed against deionised water for 12 h. Remaining solution does contain mostly enzyme and degraded cellulose. Solid part from enzyme treatment is washed with water and then added to an 8 M urea solution at pH 8.8 and left stirred at 25 °C for 48 h. Solution is separated and precipitated with barium hydroxide as descripted above. Insoluble part is washed and completely dissolved in 18 % NaOH containing 4 % boric acid at room temperature. HCl is added to solution to reduce pH. At pH 12 precipitate is formed, collected, and freeze dried. At pH 7 solution is concentrated and dialysed against deionised water for 24 h. An equal amount of barium hydroxide is added dropwise to solution and stirred for 2 h. The precipitate is separated, dissolved in 50 % ice cold acetic acid, and re-precipitated in absolute ethanol. Precipitate is separated from ethanol by centrifugation, dialysed against deionised water for 24 h, concentrated by rotary vacuum and freeze dried. The solution from barium hydroxide treatment is concentrated and treated like the precipitate.

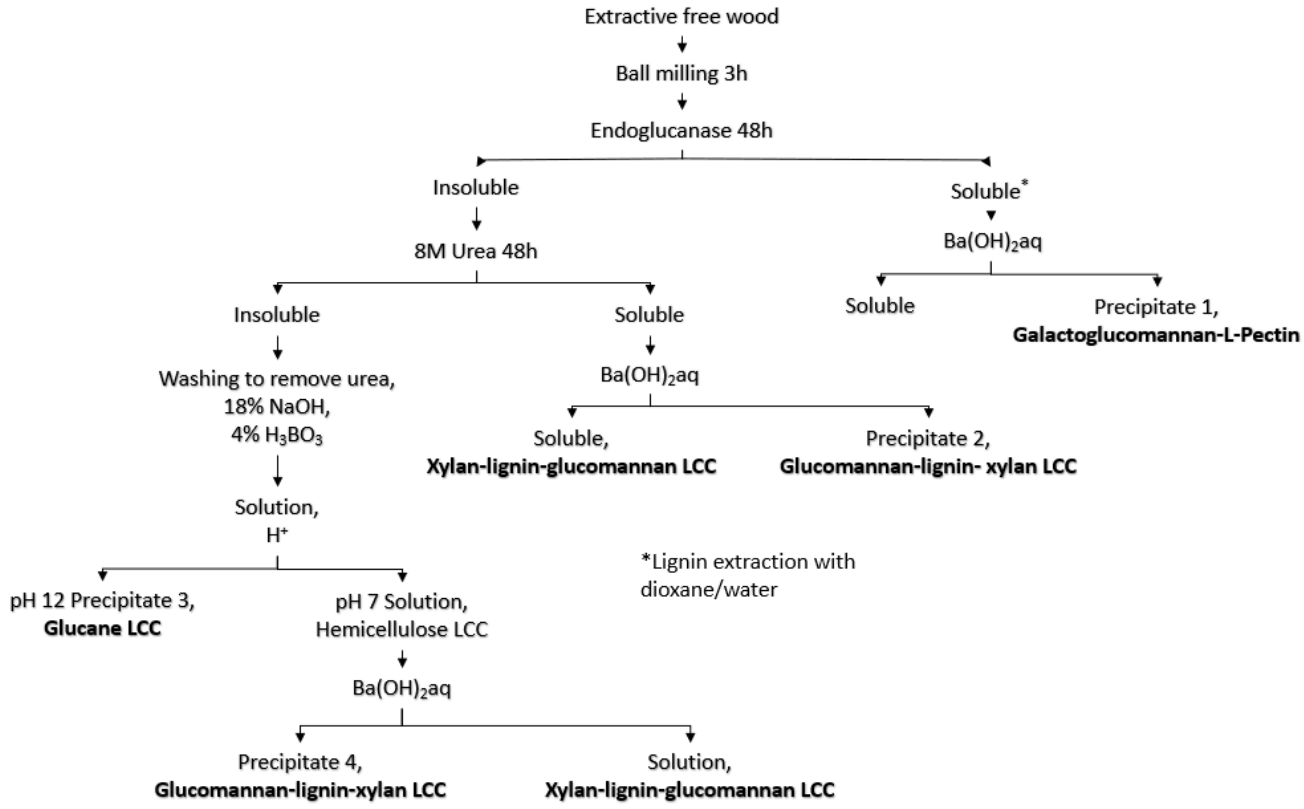

Figure 10. Flowchart adapted from Lawoko *et al.*[36]

Ball milling is needed because cellulases cannot penetrate the cross-linked cell wall and without milling no hydrolysis will happen. Urea treatment has three effects on the insoluble material: first, it swells the fibres forming a gel, second it removes the residual enzyme from the fibres and third it dissolves any fraction with a lignin content ≥ 50 %.[36]

Lawoko[37] has also published milder method (figure 11) for isolating LCC from softwoods. In this method, acetone extracted, and ball milled wood meal is suspended in sodium acetate buffer at pH 5.6 and slowly stirred for an hour. Then mono-component endoglucanase is added, and mixture is incubated at 50 °C for 36 hours. Solution is separated from the residue by centrifugation and concentrated to half and then 5 % saturated barium hydroxide is added slowly, stirred for 2 h and left to stand for 4 h to form a precipitate. The precipitate is dissolved in glacial acetic acid and then re-precipitated in ethanol. The precipitate is separated and dialysed and then concentrated and freeze dried.

In Lawoko's[37] method, residue from the enzymatic treatment is washed with deionised water and then dissolved in DMSO/H_2O (9:1) mixture at 70 °C, slowly stirring for 12 hours. The solution is diluted until the DMSO/H_2O ratio is 1:1, then warmed to 50 °C and added crystalline sodium chloride (1.25 % w/v) and stirred for 2 hours. Formed precipitate is separated by centrifugation and remaining solution can be treated two different ways. The first option is to dilute solution so, that DMSO/H_2O ratio is 1:2 and then a 0.2 M hexadecetyl ammonium hydroxide (HTAOH) solution is added until the pH is between 9 and 10 and left the mixture to stand for 12 hours. The formed precipitate is separated by centrifugation and to the solution left absolute ethanol is added and left to stand for at least 4 hours before separating it by centrifugation. The second option is to add barium hydroxide to form a precipitate and after separating the precipitate, add ethanol to the remaining solution to have the last precipitate. All precipitates are dialysed and freeze dried. In Lawoko's[37] study only about 30 % of the carbohydrates where recovered and about 62 % of the wood lignin was recovered as LCC from the first method option and 72 % from the second method option.

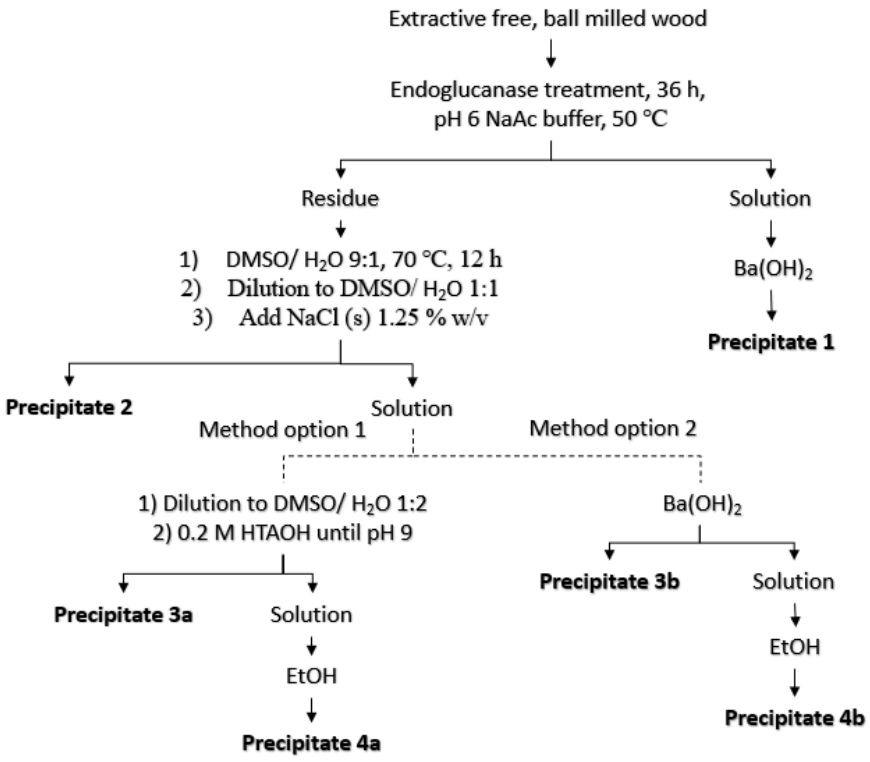

Figure 11. Flowchart adapted from Lawoko[37].

3.7 Method with selective precipitation

Zhao *et al*.[23] have fractionated LCCs from eucalyptus using selective precipitation from neutral and acidic aqueous dioxane. In their method (figure 12), wood sample is first extracted with toluene/ethanol 1:2 solution for 12 h and then ball milled for 5 h. Ball milled sample is stirred in 80 % aqueous dioxane (neutral or acidic containing 0.05 M HCl) with solid to liquid ratio of 1:20 g/ml at 80 °C for six hours and dioxane extraction is repeated twice. Solid residue is washed with dioxane until filtrate is clear. The solution is concentrated using reduced pressure and then three volumes of 70 % ethanol is added, forming precipitate 1, which is collected and freeze dried. The solution is concentrated again and then three volumes of absolute ethanol is added forming precipitate 2. At last, the solution is concentrated, and 10 volumes of acidic water (pH 2) is added to obtain the precipitate 3.

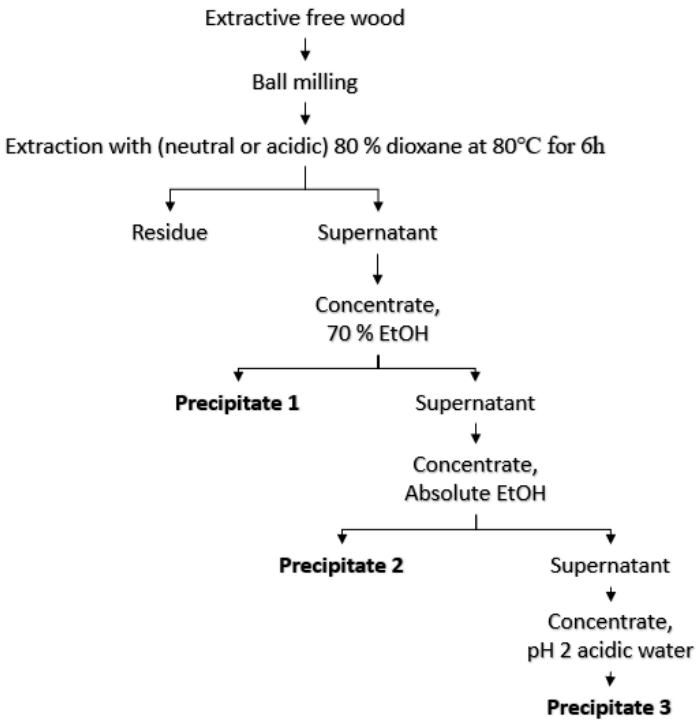

Figure 12. Flowchart adapted from Zhao *et al*.[23]

Zhao *et al*.[23] have reported that 26.1 % LCCs based on Klason lignin was extracted using neutral dioxane and 68.4 % when using acidic dioxane. Sugar content was higher in all acidic dioxane extracted fractions. Fractions that were precipitated in ethanol were carbohydrate-rich but last fraction that was precipitated in acidic water was lignin-rich.

3.8 Method utilising spruce hydrolysate

Carvalho *et al*.[10] have isolated LCC fractions from spruce extract. The spruce extract was obtained using a pressurised hot water flow-through extraction and then recovered from the water extract by spray-drying. Extract was rich in galactoglucomannan. In their fractionation method (figure 13), spray-dried extract is first suspended in water, having 8 % of solids in suspension. The suspension is stirred over night at room temperature. Next the suspension is slowly added to absolute ethanol, stirred for 10 minutes, and then cooled at 4 °C overnight. The ratio of suspension:ethanol is 1:8. Formed precipitate is separated with centrifugation, washed with absolute ethanol, and then dried in a vacuum oven at 40 °C for at least 48 hours. The supernatant is concentrated using a rotary evaporator and then freeze dried. Two fractions of LCC are obtained: ethanol precipitate and ethanol soluble.

In their study Carvalho *et al.*[10] had total yield of 94.9 %, in which yield of ethanol precipitate was 74.1 % and yield of ethanol soluble was 20.8 %. The ethanol precipitate fraction was more pure in relation to the hemicelluloses, and represented 16.0 % of original spruce sawdust. Ethanol soluble fraction was more heterogenous and represented 4.5 % of the original spruce sawdust. Ethanol soluble fraction had a substantially lower molar mass and more homogenous molecular size distribution than ethanol precipitate, which supports assumption that shorter polysaccharide populations are preferentially solubilised in ethanol.

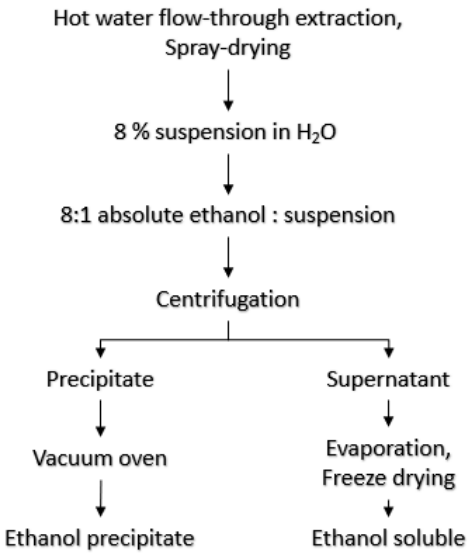

Figure 13. Flowchart adapted from Carvalho *et al.*[10]

To achieve enrichment of LCC bonds and facilitate their identification Carvalho *et al.*[10] have fractionated further ethanol precipitate and ethanol soluble fractions using enzymatic treatment and physical methods (figure 14). Used enzymatic hydrolysis was designed to reduce the molecular size. Their semi-simultaneous enzymatic hydrolysis is performed as follows: sample is suspended in 0.1 M sodium acetate buffer and endomannanase is added. The suspension is incubated at 40 °C for 24 hours. Next, exomannosidase is added and the suspension is incubated at 35 °C for 24 h. Then both enzymes are inactivated by heating the suspension at 100 °C for 10 minutes.

Carvalho *et al.*[10] have fractionated the hydrolysates obtained with enzymatic hydrolysis using ultracentrifugation. Then they have filtered the resulting supernatant and residue through the nanofiltration membrane. Retained fraction was washed properly with water and freeze dried. Fraction poured through the membrane was concentrated using a rotary evaporator and freeze dried.

Carvalho *et al*.[10] have reported that the physical techniques were more efficient in fractionating and enriching the phenyl glycoside bonds than the enzymatic treatment. Phenyl glycoside bonds were identified in all fractions but substantially concentrated in ethanol precipitates which were in residue after ultracentrifugation and retained in nanofiltration. The enzymatic hydrolysis did not concentrate benzyl ether bonds, but physical techniques fractionated benzyl ether bonds in large molecular size fractions. Enzymatic or physical fractionation did not affect on concentration of γ-esters.

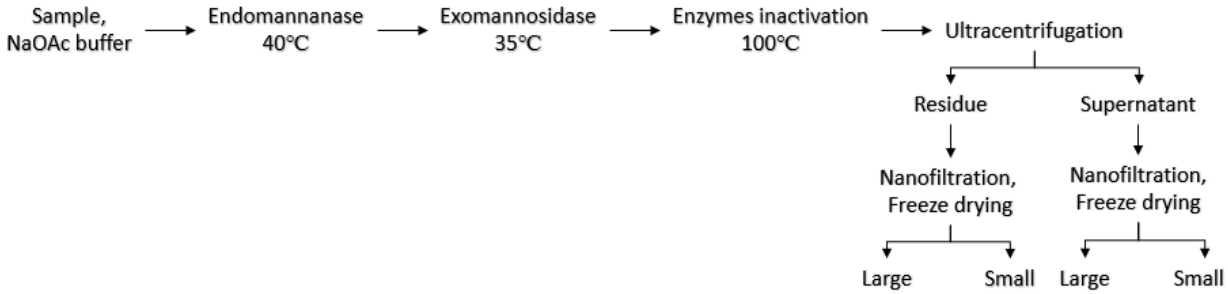

Figure 14. Flowchart adapted from Carvalho *et al*.[10]

3.9 Method with mild acidolysis lignin

To characterise the lignin structures and LCC bonding Yuan *et al*.[16] have isolated two lignin-rich fractions from a fast growing triploid poplar tree. In their method (figure 15), wood sawdust is extracted with toluene/ethanol and then ball milled for 8 hours. During the milling a 10-minute pause is kept after every 10 minutes of milling to prevent overheating. After ball-milling milled wood lignin (MWL) is extracted according to Björkman's method: wood meal is extracted with 96 % dioxane in the dark, under nitrogen atmosphere at room-temperature, for 24 h. Mixture is filtered and washed until filtrate is clear. Extraction and washing are repeated twice. Purification is done according to different method: combined filtrates are concentrated and precipitated in 3 volumes of 95 % ethanol. Precipitates are filtered, washed with 70 % ethanol, and freeze dried. The ethanol is evaporated and the dioxane soluble lignin (MWL) is precipitated at pH 1.5-2.0 using 6 M HCl.

The residual wood meal from dioxane extraction is extracted with 80 % dioxane containing 0.05 M HCl at 85 °C for 5 hours. Mild acidolysis lignin (MAL) is obtained from solution

according the same method as MWL except for washing with acidified water before freeze drying.

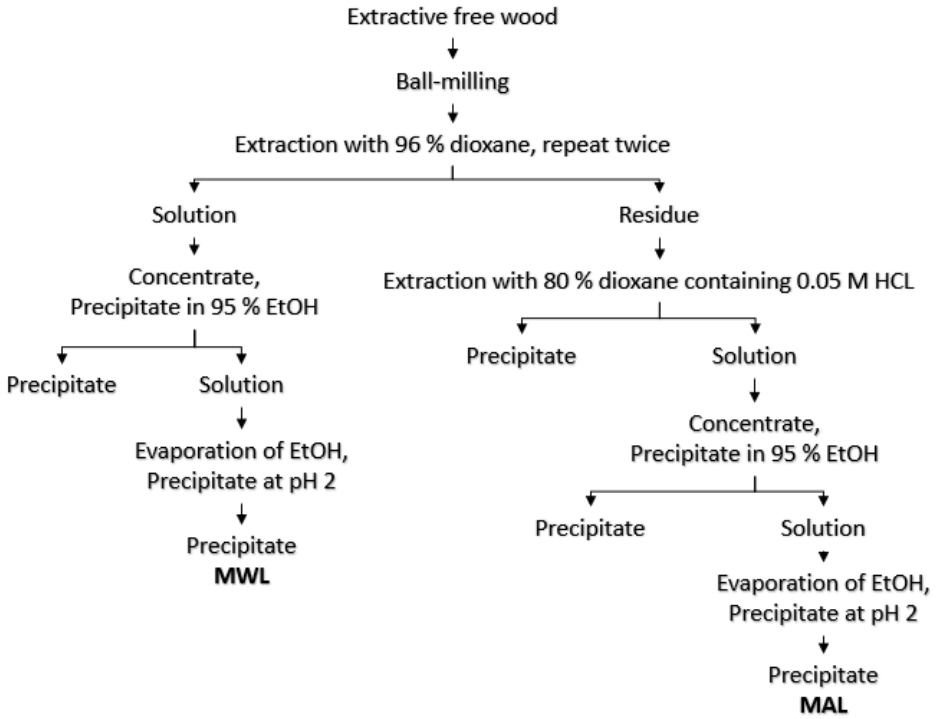

Figure 15. Flowchart adapted from Yuan *et al*.[16]

In the study of Yuan *et al*.[16] the yields of MWL and MAL were 22.1 % and 52.1 %, respectively, adding up to total recovery of 74.2 %. According to their study, some LCC-rich fractions are lost during purification steps. Both MWL and MAL contained relatively high amount of carbohydrates, 15.4 % and 10.4 % respectively. In both fraction lignin-carbohydrate bonds were found. Even though benzyl ethers and phenyl glycoside bonds can be cleaved under mild acidic conditions, both were found in MWL and in MAL. The amount of phenyl glycoside bonds was 4.1 and 4.5 per 100 Ar, amount of benzyl ether bonds was 2.1 and 5.8 per 100 Ar and amount of ester bonds was 3.4 and 1.3 per 100 Ar in MWL and MAL, respectively.

3.10 Microwave acidolysis method

Li *et al*.[38] have developed a method for converting beech wood into LCC exhibiting antiviral properties. In their method (figure 16), wood powder and 0.5 % aq. H_2SO_4 are first loaded into a high-pressure vial for microwave irradiation at 160 °C for 30 minutes with magnetic stirring. Then the mixture is neutralised with sodium bicarbonate ($NaHCO_3$) and filtered. The residue is

washed properly with ultrapure water to completely remove the salt from neutralisation. The solution including water from washing is extracted three times with ethyl acetate (EtOAc). The organic extracts are washed with a saturated NaCl solution, separated, and dried over $MgSO_4$. The residue is extracted with methanol and the residue insoluble in methanol is extracted with DMSO.

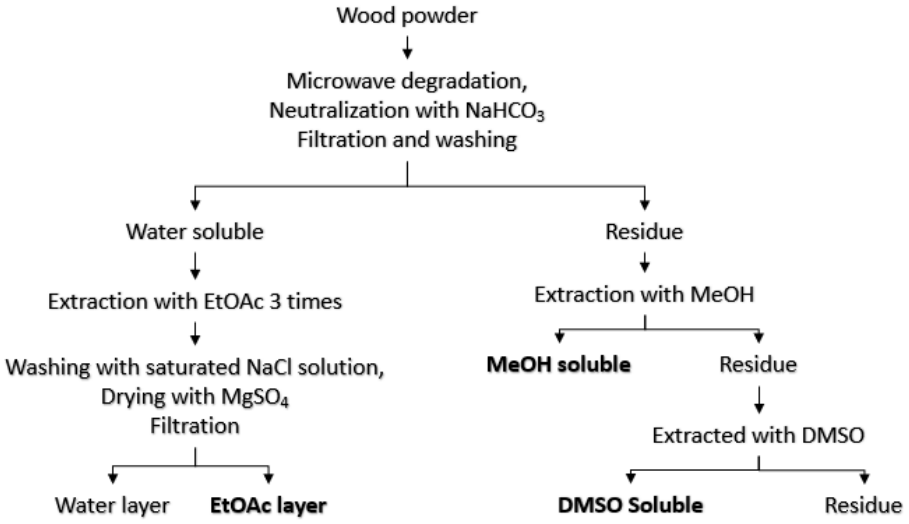

Figure 16. Flowchart adapted from Li *et al*.[38]

Li *et al*.[38] have used silica gel chromatography to separate methanol soluble fraction into four fractions using CH_2Cl_2/MeOH (9:1, 8:2, 7:3 and 100 % MeOH) as the solvent system. They did also use different solvent mixtures and temperatures in microwave acidolysis. For example, 1.0 % aq. H_2SO_4 and 1,4-dioxane (1:1) as a solvent and 140 °C led to better yield than 0.5 % H_2SO_4 and 160 °C, but using organic solvents is not so desirable from the perspective of green chemistry. In their study, the total yield of fractions was low, only about 15 % when using 0.5 % H_2SO_4 as solvent. The highest yield was obtained using 1.0 % H_2SO_4 and acetone (1:1), but it was still low, about 32 %. The structural analyses revealed that fractions were LCC containing 86.8-90.4 % lignin and 3.1-6.1 % carbohydrates.

3.11 Method for ether linkage studies

Nishimura *et al*.[9] have isolated LCC fraction from Japanese red pine for the purpose of determining α-ether linkages between lignin and carbohydrates. In their method (figure 17), wood is extracted with ethanol/benzene mixture and then 0.25 % aqueous potassium acetate solution to remove pectin. Dried wood meal is ball-milled for 48 h under a nitrogen atmosphere

and with external cooling by tap water and then extracted twice with 80 % dioxane at room temperature for 48 h. Remaining solids are extracted with 20 °C water for 12 h and then with 80 °C water for 5 h. Water extracts are combined and concentrated to small volume. Then five volumes of ethanol is added and LCC fraction is precipitated. LCC fraction is fractionated into three subfractions using anion-exchange chromatography. Subfractions are called neutral, acidic and lignin-rich LCC and in their study Nishimura *et al*.[9] have used neutral fraction for determining LCC structures. To have a high purity and concentration of LC bonds, Nishimura *et al*.[9] have used enzymatic treatment on neutral LCC fraction. Hemicellulase treatment was conducted at 45 °C for 24 hours in 0.1 M sodium acetate buffer. After treatment, fractions were separated by using size-exclusion polyvinyl gel with an affinity for lignin.

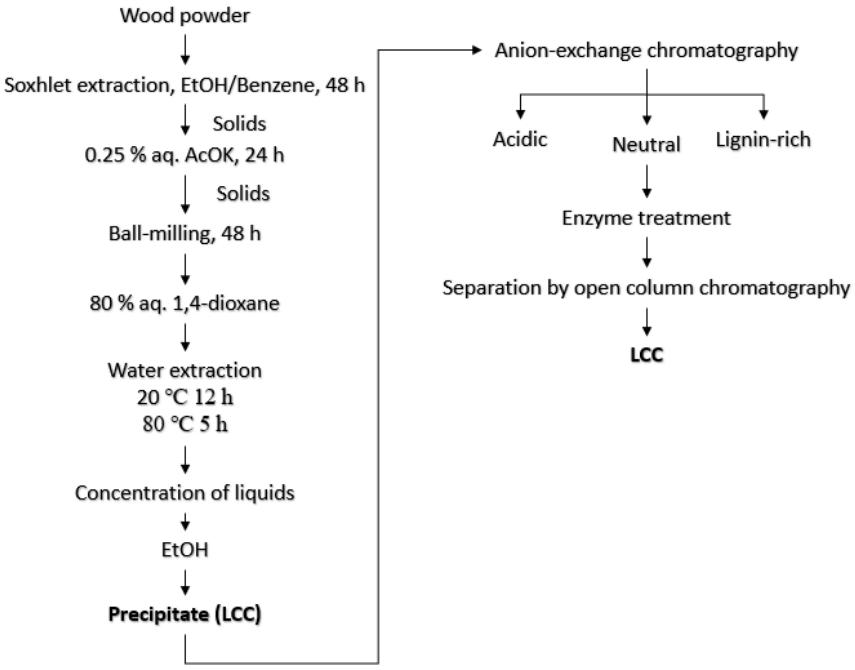

Figure 17. Flowchart adapted from Nishimura *et al.*[9]

4 Characterization of LCC

There are many different analytical techniques that are used in characterisation of LCCs. Typical techniques are different nuclear magnetic resonance (NMR) techniques of which the most important is solution state two-dimensional heteronuclear single quantum coherence nuclear magnetic resonance (2D HSQC NMR) and spectroscopic techniques such as Fourier transform infrared (FT-IR) spectroscopy. In addition, different chromatographic techniques like gel permeation chromatography (GPC), gas chromatography (GC), high performance anion-exchange chromatography (HPAEC) and pyrolysis gas chromatography mass spectrometry (Py-GC/MS) are largely utilised.

Traditionally degradation techniques have been used in analysis of LCC, but degradation products give more information about either carbohydrate part or lignin part of LCC, not about the linkage between them. In latest research 2D HSQC NMR has been the most powerful analytical tool for interunit linkages in LCC. 2D NMR analysis can be complemented with degradation techniques to achieve detection of some linkages that are otherwise hard or impossible to detect.[7,30]

4.1 Degradation techniques

Acid hydrolysis is a process where a protic acid is used to break a chemical bond via a nucleophilic substitution rection. Amount of acid-insoluble lignin, also called Klason lignin, is often determined using sulfuric acid hydrolysis[13,16,17,21], often according to TAPPI method T222. In TAPPI method T222 om-02[39] cold 72 % sulfuric acid is added to the sample and kept in a bath at 20 °C for 2 h, stirring the mixture frequently. After that, the mixture is diluted to 3 % concentration of sulfuric acid and boiled for 4 h under a reflux condenser. Then insoluble lignin is separated with tared filtering crucible, washed with hot water, and dried to constant weight. Instead of boiling step, autoclaving at 125 °C and at 1.4 bar has been used[13,21]. Acid soluble lignin can be measured form hydrolysate by absorbance at 205 nm in UV-vis spectrometer.[34] Also carbohydrate analysis can be done using the hydrolysate from acid hydrolysis and analysing that with chromatographic methods.[8,11]

Smith degradation is a method used to determine the polysaccharides. First in Smith degradation is reaction with sodium periodate followed by reduction with sodium borohydride and at last mild acid hydrolysis that is selective for the oxidized or reduced residues.[40] Nishimura *et al*.[9] have conducted Smith degradation for wood-LCC using reaction with sodium periodate at 4 °C for twenty days, reduction with $NaBH_4$ and hydrolysis with trifluoroacetic acid (TFA) at 100 °C for 3 hours and analysed the products by gas chromatography.

Alkaline hydrolysis, also called saponification, is done under mild conditions. It causes cleavage of ester bonds between benzyl moieties in lignin and glucuronic acid moieties in carbohydrates. Sugar composition and released carboxyl and hydroxyl groups are analysed and the structure of ester bonds in the original LCC is concluded based on the degradation products.[41] Balakshin *et al*.[11] have saponified pine LCC-AcOH using 0.1 M NaOH at room temperature and nitrogen atmosphere overnight. Saponified fraction did not show signals from γ-esters during 2D HSQC NMR analysis but same fraction without saponification did. This indicates that in the pine LCC-AcOH exists γ-ester bonds between lignin and carbohydrates.

Thioacidolysis is a technique that selectively degrades the β-O-4 linkages in lignin to mostly dimeric or monomeric products.[1] Giummarella *et al*.[1] have performed thioacidolysis on LCC as follows. LCC is placed in a vial with 2.5 % BF_3 diethyl etherate with 10 % ethanthiol solution in dioxane and air is evacuated with nitrogen before cap is placed. The vial is heated at 100 °C for 4 hours, gently shaking once in an hour. Vial is placed in ice bath to cool and then neutralised with 0.4 M Na_2CO_3. Deionised water and ethyl acetate are added, and mixture is vortexed. The organic phase contains lignin degradation products, and it is collected. Ethyl acetate extraction is repeated three times. Collected organic fraction is evaporated to dryness. Before analysing degradation products with GC, Giummarella *et al*.[1] have performed desulphuration to form reduced side chain structures and their acetylation for getting quantitative information.

Derivatisation followed by reductive cleavage (DFRC) has been used to monitoring the hydrolysis of acetates attached to the γ-OH of the lignin side chains during the LCC fractionation. A modification of standard DFRC method was done by del Rio *et al*.[17] and performed as follows. Sample is stirred with propionyl bromide in propionic acid at 50 °C for 2 h. Solvents are removed by rotary evaporation and the products are dissolved in dioxane/propionic acid/water mixture (5:4:1) and powdered Zn is added. Mixture is stirred at room temperature for 40 minutes and then transferred into a separatory funnel with dichloromethane and saturated ammonium chloride. The aqueous phase is adjusted to below

pH 3 using HCl. After intense mixing the organic layer is separated, and the water layer is extracted two more times with dichloromethane. Dichloromethane layers are combined and dried over Na_2SO_4, and the filtrate is evaporated using rotary evaporation. Then 1.1 ml of dichloromethane containing 0.2 ml of propionic anhydride and pyridine is added to the residue and let it stay for an hour. The propionylated degradation compounds are analysed by GC/MS after evaporation of solvents.

Nitrobenzene oxidation provides information on the aromatic rings and condensation degree of lignin, thus being great supplement on NMR spectroscopy.[27] Jiang *et al*.[27] have applied nitrobenzene oxidation on LCC structure analysis as follows. Sample, 2 M NaOH and nitrobenzene is added to a stainless-steel reactor and reacted at 170 ℃ for 2 h. Then the reactor is cooled in water and 0.1 M NaOH solution containing 3-ethoxy-4-hydroxybenzaldehyde is added as the internal standard. Mixture is extracted three times with dichloromethane and the aqueous phase is acidified to pH 1 using HCl and then extracted twice with dichloromethane and once with ethyl ether. Organic phases are combined and extracted with deionised water. The organic phase is dried by anhydrous Na_2SO_4 overnight. Insoluble material is removed by filtration and solution evaporated to dryness. Silylation is done using *N,O*-bis(trimethylsilyl) acetamide at 100 ℃ for 10 minutes. The silylated samples are analysed by GC-FID.

2,3-dichloro-5,6-dicyano-1,4-benzoquione (DDQ) oxidation can be used to cleavage the lignin-carbohydrate bonds at the α- and conjugated γ-positions of *p*-etherified phenyl alkane or alkene units.[42] This means only ether and ester bonds between lignin and carbohydrates can be examined by using DDQ oxidation.[7] Watanabe *et al*.[42] have studied the binding side of lignin and carbohydrates and they have developed following method. First LCC fraction is degraded using cellulase and then acetylated with acetic anhydride and pyridine. Then DDQ oxidation is performed by refluxing acetylated LCC samples with DDQ in dichloromethane/water (18:1) mixture for 2 h. After DDQ oxidation LCC samples are methylated with methyl trifluoromethanesulfonate to mark the hydroxyl groups originating from the LC-bonds. At last samples are hydrolysed, reduced, and acetylated before analysing with GC-MS. However, some issues with DDQ oxidation selectivity and completeness have reported.[41]

Acetylation might improve solubility of LCC in NMR solvents.[1,14] Du *et al*.[14] have performed acetylation by dissolving LCC samples in DMSO/*N*-methylimidazole (2:1) and adding acetic anhydride and then stirred mixture at room temperature for 2 h before pouring the mixture into

distilled water. Precipitated acetylated LCC was separated by filtration and washed with ultrapure water.

4.2 Spectroscopic techniques

Spectroscopic techniques are widely used for analysis of lignin and carbohydrates. Strong overlapping of signals is causing difficulties when direct analysis of LCC structures is performed. For example, quantification of LCC bonds is almost impossible with 1D ^{13}C NMR as the signals of the LCC bonds are heavily overlapped with signals from other carbohydrates and lignin. 2D NMR methods overcomes the overlapping problem thus being most important technique when analysing LCC bonding.[11]

4.2.1 NMR spectroscopy

NMR techniques are based on the change of the spin state of NMR active nuclei when sample is placed in a homogenous magnetic field and irritated. NMR active nuclei behave like a magnet and their spins are oriented parallel or antiparallel to the applied magnetic field. Energy difference between two spin states is small and spin states are unequally populated. Samples are irritated with photons that cause nuclei to go transitions between spin states. The exited nuclei emit a magnetic signal that can be detected and converted computationally to a spectrum. Not all atoms emit NMR signal, as atom must have a specific atomic number and isotope. An isotope's NMR activity is a result of the presence of a magnetic moment in its nucleus. NMR-active nuclides are for example, ^{1}H, ^{13}C, ^{15}N, ^{19}F, ^{31}P and ^{2}D (^{2}H).[43]

One-dimensional (1D) NMR spectrum shows amplitude as a function of frequency. Two-dimensional (2D) NMR spectrum is obtained by converting mathematically a matrix of data into spectrum where correlation of axes shows as cross peaks. Typically, both axes show chemical shift which is the resonant frequency of distinctive NMR nuclei relative to a standard in a magnetic field caused by the resistance of the electron cloud to the applied magnetic field.[43]

2D NMR techniques are powerful tools to analyse LCC fractions. Compared to the 1D NMR techniques, 2D NMR allows much better separation of lignin and carbohydrates and thus identification of signals origin is easier. One of the correlation NMR techniques is the hetero

single quantum coherence (HSQC). It shows correlation between ^{1}H and ^{13}C atoms that are directly bonded to each other. Another 2D NMR technique is the heteronuclear multiple bond coherence (HMBC) which gives information of long-range correlations between ^{1}H and ^{13}C atoms.[7]

Dissolving LCC in common NMR solvents can sometimes be difficult due to large molecule size. Degradation and removal of polysaccharides reduces the molecular weight and helps with dissolution. Degradation of polysaccharides also reduces overlapping of NMR signals. Most used solvent in NMR studies of LCC is deuterated DMSO-d_6, but also $DCCl_3$ and other common NMR solvents has been used.[14] Low amount of LC bonds in LCC preparations is a problem and thus enrichment of LC bonds during preparations is often needed when using NMR.[7]

2D-HSQC spectra of lignin samples can be divided into three regions: aliphatic region at δ_C/δ_H 10-40/0.5-2.5, side chain region at δ_C/δ_H 50-95/2.5-6.0 and aromatic region at δ_C/δ_H 95-150/5.5-8.0.[28] When analysing 2D NMR, C2 position on the aromatic ring of a phenyl propane unit can be used as an internal standard for quantification of inter-monolignol units, because in native lignin it is never substituted. Typically, there are few LC linkages compared to abundant inter-monolignol linkages. The signal from BE bond overlaps with signal of spirodienone structure in non-acetylated LCC, but when LCC is acetylated, separation can be achieved.[1] Signal form BE_1 type of LC bond might be overlapped with signal from γ-acylated β-*O*4 substructures linked to a G unit of lignin at δ_C/δ_H 81.0/4.49 ppm and signal from BE_2 type is overlapped with signal from spirodienone β-1 lignin substructures at δ_C/δ_H 81.0/5.01 ppm.[8]

Most used 2D NMR technique in LCC studies is HSQC. In table 3 are shown chemical shifts δ_C/δ_H (ppm) of phenyl glycoside (PhGlc), benzyl ether (BE) and γ-ester bonds in HSQC spectra according to different LCC studies. In these studies, used fractionation method and biomass differs. In figure 18 are shown HSQC spectra from LCC study of Giummarella and Lawoko[30]. However, also HMBC and heteronuclear multiple quantum correlation (HMQC) have been used in LCC studies. Phenyl glycoside LC bonds have been reported in the HMBC spectrum of pine wood LCC at δ_C/δ_H 105.5-99.5/7.69-7.42 ppm.[7] Signals from γ-ester LC bonds at δ_C/δ_H 62-65/4.0-4.5 ppm in the HMQC spectrum and at δ_C/δ_H 166.5-169.4/4.30-4.35 ppm in the HMBC spectrum has been identified from pine LCC.[7]

Table 3. Chemical shifts δ_C/δ_H (ppm) of phenyl glycoside (PhGlc), benzyl ether (BE) and γ-ester bonds in HSQC spectra according to LCC studies.

LCC	PhGlc	BE	γ-Ester	Reference
Bamboo	99.9/4.63	80.9/4.50	62.0-65.0/4.0-4.5	Lv *et al.*[44]
Birch	100.4/5.02	BE1: 80.1-81.2/4.21-4.68 BE2: 82.9/5.23	62-65/4.0-4.5	Giummarella and Lawoko[30]
Bamboo	99-104/4.8-5.2	80.0-82.5/4.3-4.7	62-65/4.0-4.5 α: 75-77/6.0-6.2	Yue *et al.*[28]
Wheat straw	98-104/4.5-5.3	BE1: 80-82/4.5-4.7 BE2: 80-82/4.9-5.1	62-66/4.0-4.5	Xie *et al.*[34]
Ginkgo shell	101-5/4.90	BE1: 81.6/4.64 BE2: 81.4/5.04	62-65/4.0-4.5	Jiang *et al.*[27]
Poplar		BE1: 80.7/4.50 BE2: 81.1/5.06		Feng *et al.*[22]
Spruce	99-102/4.8-5.2	80.2/4.5	62-65/4.0-4.5	Carvalho *et al.*[10]
Quience	100.7-101.6/5.04-4.85	BE1: 85.22/4.69 BE2 :83.65/5.24	62.10/4.24	Qin *et al.*[20]
Pine		80.2/4.50		Nishimura *et al.*[9]
Pine, Birch	104-99/4.8-5.2	80-81/4.5-4.7	65-62/4.0-4.5	Balakshin *et al.*[11]
Arundo donax	105-99.5/5.17-4.28	81.5-80.0/5.3-4.3	65.5-62/4.5-4.0	You *et al.*[8]
Spruce	100.2-101.9/5.03-4.85	80-82/4.4-4.7		Du *et al.*[14]

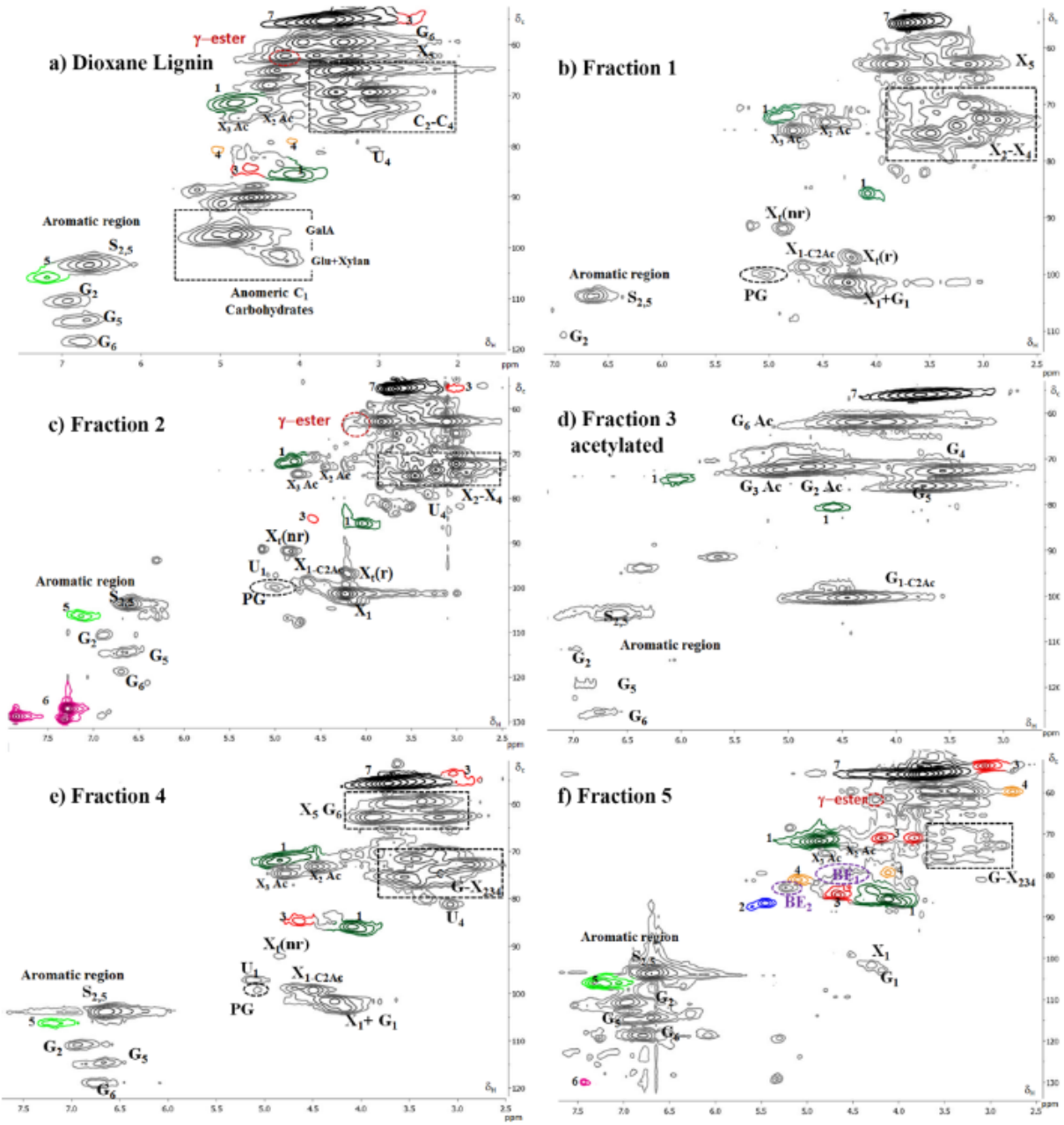

Figure 18. 2D HSQC NMR spectra of birch LCC fractions. LC-bonds: γ-ester, PG = phenyl glycoside and BE_1/BE_2 = benzyl ether. Reprinted with permission from Giummarella, N. and Lawoko, M., Structural Basis for the Formation and Regulation of Lignin–Xylan Bonds in Birch, *ACS Sustain. Chem. Eng.*, **2016**, *4*, 5319–5326. Copyright 2023 American Chemical Society.

4.2.2 IR spectroscopy

In infrared (IR) spectroscopy a sample is placed in the path of an infrared radiation beam. Molecules in the sample can absorb or transmit the radiation depending on its frequency and

the structure of molecules. Atoms in molecules are in continuous motion with respect to each other and have three types of motion which are rotation, vibration, and translation. Molecule absorbs IR radiation when the frequency of radiation is equal to the frequency of a specific vibration of molecule and the associated energy is converted into various types of vibrational or rotational motions. Not all molecules are IR-active: to be IR-active, molecule must undergo a net change in dipole moment in consequence of a vibrational or rotational motion.[45]

Infrared region of electromagnetic spectrum is from 1300 cm^{-1} to 10 cm^{-1}, but mid-IR (MIR) which is from 4000 cm^{-1} to 400 cm^{-1} is usually used for molecular characterisation. Fourier transform IR spectrometers (FT-IR) are often used instead of older dispersive equipment. FT-IR equipment has three main components: the radiation source, the interferometer, and the detector. In dispersive equipment there is monochromator instead of the interferometer. Compared to dispersive equipment, FT-IR is faster and more sensitive.[45]

Three main IR techniques are transmittance FT-IR, diffuse reflectance FT-IR (DRIFT) and attenuated total reflectance (ATR). In transmittance FT-IR, the IR beam is led straight through the sample and the detector is on the other side of the sample. Solid samples are ground with potassium bromide and pressed to form hard, transparent tablet. Wet samples are put on IR-transparent optical window. In DRIFT, the IR beam is projected into the sample. The beam is reflected, scattered, and transmitted from the sample and part of the light is returned to the detector and considered to be diffuse reflection. In ATR, the sample is placed on an optically dense crystal. The IR beam is reflected form the internal surface of the crystal creating an evanescent wave. Some of the evanescent wave is projected into the sample on top of crystal where some of its energy is absorbed into the sample. The radiation reflected from the sample is measured with the detector.[45]

Kostryukov *et al*.[46] have developed FT-IR method for determining cellulose, hemicellulose and lignin by using transmittance technique with potassium bromide tablets as sample preparation. According to them, transmittance gives more representative information than ATR, because plant biomass has a complex 3D structure and ATR method is based on surface reflection which makes ATR more suitable in situations where all components are uniformly distributed in the sample.

Kostryukov *et al*.[46] have determined concentrations of cellulose, hemicellulose and lignin by using calibration equations reflecting the dependence of the intensity of absorption bands in the

model samples. They have determined lignin by using absorption band at 1512 cm^{-1} which is mainly due to the skeletal vibrations of the aromatic ring and cellulose, or hemicellulose do not have absorption band in that area. They have determined cellulose by using absorption band at 1450 cm^{-1} which is mainly due to the scissor vibrations of methylene group and bending in-plane vibrations of the OH-groups. Hemicelluloses they have determined by indirect correlations because the spectra of hemicellulose and cellulose differs a little but the area where it differs clearly was overlapped by the band of crystallisation water.

FT-IR can be used to compare the structure of different LCC fractions. You *et al*.[8] have compared five different LCC fractions isolated from an energy crop plant. In their study, Björkman LCC fraction compared to lignin-rich LCC fractions showed stronger absorbance at 1735 cm^{-1}, which comes from C=O stretching in esterified phenolic acids and acetyls associated with xylose or uronic acid residues of hemicelluloses, at 1630 cm^{-1}, which comes form $-COO^-$ antisymmetric stretching of glucuronic acid or corresponding carboxylate and at 1043 cm^{-1}, which comes from hemicelluloses, but weaker absorbance at 1600 cm^{-1}, 1510 cm^{-1} and 1460 cm^{-1} which are signals from lignin. Phenolic OH region of lignin, 1370 cm^{-1}, had stronger absorbance in Björkman LCC fraction, suggesting that in the fraction, there is higher amount of associated hydroxycinnamic acid in it.

Yue *et al*.[28] and Lv *et al*.[44] have analysed LCC fractions isolated from bamboo by using FT-IR. In figure 19 is shown FT-IR spectrum from study of Yue *et al.*[28] They have identified absorption at 3450 cm^{-1}as O-H stretching vibrations of aromatic and aliphatic OH-groups, 2935 cm^{-1} and 2840 cm^{-1} as C-H stretching vibrations of methylene and methyl groups, 1720 cm^{-1} and 1650 cm^{-1} as conjugated C=O stretching in lignin and unconjugated C=O stretching in carbohydrates. According to them, bands at 1335 cm^{-1}, 1270 cm^{-1} and 1165 cm^{-1} might originate from the S, G and H units of lignin.

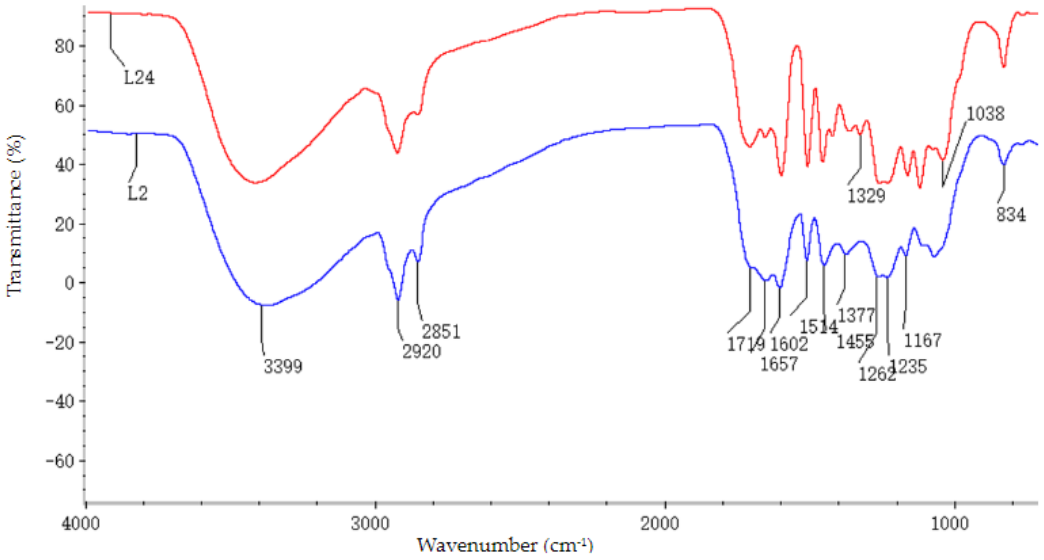

Figure 19. FT-IR spectrum of LCC fractions copied from study of Yue *et al*.[28] The article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

4.3 Chromatographic techniques

Chromatography is an analytical technique where components of a mixture are separated and analysed. In chromatographic techniques a sample is placed in a column with stationary phase inside. The sample travels with the mobile phase, which can be gas or liquid that constantly flows through the column. Different types of molecules have different strengths of intermolecular interactions with the mobile and stationary phases. Molecules that interact more with the stationary phase are retained in column. All molecules spend the same time in the mobile phase but different time in the stationary phase which makes the separation occur. The time it takes for a molecule to travel from end to end of a column is called retention time. At the end of column, there is a detector. There are many different of detectors that can be used depending on the situation and purpose.[47]

In liquid chromatography, the mobile phase is a liquid. Columns used in liquid chromatography are usually packed columns. Packed column is filled with fine particles of the stationary phase. When the stationary phase is more polar than the mobile phase it is called normal phase liquid chromatography and when the mobile phase is more polar than the stationary phase it is called reverse phase liquid chromatography. In normal phase liquid chromatography, polar molecules

interact more with the stationary phase than nonpolar molecules and thus nonpolar molecules come first out from the column. In reverse phase the situation it the opposite.[47]

Size exclusion chromatography (SEC), also known as gel permeation chromatography (GPC), is a type of liquid chromatography. In SEC, separations are based on the size of the molecules. SEC column has particles with different size pores and pore networks inside. As the sample passes thought the column, very large molecules cannot enter many of the pores and come out from column faster than smaller molecules that can fit in the pores. Molecules are separated by the size so that largest comes first out and the smallest as last. SEC is typically used for separating macromolecules and polymers with molecular weight from 1000 Da to over 500 million Da.[47]

SEC with a dual detector system can be used for examining lignin-carbohydrate connectivity. For example, Giummarella and Lawoko[30] have used a dual detector system that consists of a differential refractive index (DRI) detector and a UV detector. DRI detector is a universal concentration detector and UV detector measures the absorbance of lignin. Giummarella and Lawoko[30] have set 280 nm as wavelength for the measurements. When the concentrations of lignin and carbohydrates are known, the elution profiles observed in chromatograms can be used to deduce possible LC bonding by looking possible co-elution. Superimposed DRI and UV signals indicates that there is bonding between lignin and carbohydrates or their hydrodynamic volumes are similar in the unbound state.[30] SEC is used also to determine the molecular weights and the polydispersity indexes of LCC.[13,16,21,44]

Another type of liquid chromatography is ion-exchange chromatography where the stationary phase is charged to provide retention of charged analytes. As the sample moves through the column, the counterions are exchanged with sample ions and analytes are retained. Stationary phase with negatively charged groups is used for exchange cationic species and stationary phase with positively charged groups is for exchanging anionic species.[47] For example, He *et al.*[24] have used high performance anion-exchange chromatography to analyse carbohydrate composition of LCC after hydrolysis with dilute acid.

In gas chromatography the mobile phase is an inert gas, typically nitrogen, helium, hydrogen, or argon. Used columns are capillary columns. Capillary columns are long and there is a thin layer of the stationary phase on the walls of column. Only volatile compounds can be analysed. The mobile phase does not interact with analytes in gas chromatography, only interactions with

the stationary phase and the volatility affects the retention time. Temperature is one of the key values, that controls retention. If the temperature is too high, all molecules are permanently in gas phase and no separation will happen. If the temperature is too low, the molecules will not have enough energy to leave from stationary phase and will not make it to the end of column.[47]

Mass spectrometry (MS) can be combined with both liquid, and gas chromatography by using mass selective detector (MSD). In most common mode of GC/MS, analytes exiting from column are bombarded by high-energy electrons that ionize the molecules and causes bond breaks. The charged fragments are passed through a mass-to-charge analyser and detected as a function of mass and time. Mass spectrometers are universal detectors.[47]

In pyrolysis gas chromatography (Py-GC), temperature is so high that it causes rapid degradation of polymers into smaller fragments. Purpose is to produce smaller volatile molecules from a large molecule like polymer that cannot be analysed by GC without degradation. Used temperature is usually 600-800 °C. When analysing biomass with Py-GC acetic acid, furancarboxaldehyde, and levoglucosan are characteristic of cellulose pyrolysis and phenols of lignin.[48] In figure 20 is shown Py-GC/MS chromatogram of LCC from study of Du *et al*.[25]

According to study of Du *et al*.[14] presence of carbohydrates in LCCs can affect the pyrolytic breakdown pattern. They have compared pyrolytic breakdown patterns of initial LCCs and enzymatically hydrolysed LCCs and have noticed that amount of coniferyl alcohol increased significantly after enzymatic treatment. They have suggested that carbohydrate linkages in the lignin side chains might prevent the alkyl-aryl ether breakdown in lignin units which forms coniferyl alcohol during pyrolytic degradation of lignin.

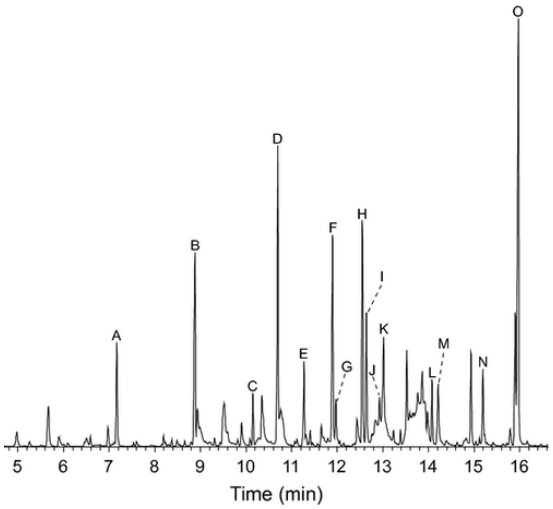

Figure 20. Py-GC/MS chromatogram of LCC copied with permission from Du *et al*.[25]

5 Experimental

Purpose of this experimental work was to find working, relatively simple and mild fractionation method to obtain LCCs and analyse the isolated LCC fractions. Used fractionation method was slightly modified from the study of Carvalho *et al*.[10] Fractionation method was used for one softwood, one hardwood and one non-wood biomass, which were spruce, birch and wheat straw respectively.

5.1 Equipment, reagents, and samples

All used reagents are shown in table 4. Amberlite IRA-67 resin was regenerated by washing it first with UHQ-water, then stirring it overnight in 2 M Na_2CO_3 solution and finally washing with UHQ-water until the pH was neutral. In table 5 are listed all equipment used in this work.

Table 4. Used reagents.

Reagent	Manufacturer
UHQ-water	
Etax, 99.5 % absolute ethanol	Altia Oyj
L-(+)-Arabinose > 99%	Sigma
D-(+)-Galactose ≥ 99 %	Fluka BioChemika
D-(+)-Glucose anhydrous ≥ 99.5 %	Fluka BioChemika
D-(+)-Xylose ≥ 99.5 %	Fluka BioChemika
D-(+)-Mannose ≥ 99.5 %	Fluka BioChemika
Xylitol ≥ 99.5 %	Fluka BioChemika
Pyridine ≥ 99.7 %	AnalaR NORMAPUR
Silylation reagent (BSTFA-TMCS 99:1)	TCI Chemicals
Sulfuric acid 95-97 %	Honeywell fluka
IRA-67 resin	Amberlite
Acetone ≥ 99.8	Fischer Chemical

Table 5. Used equipment.

Equipment	Model	Manufacturer
Centrifuge	Megafuge 1.0	Heraeus Instruments
Scale	Practum 224-1S	Sartorius
Vacuum rotary evaporator	Laborota 400	Heidolph
	Vacuum pump V-700	Büchi
	Vacuum controller V-850	Büchi
Freeze drier	Drywinner	Heto
Autoclave	Autoklav 23	Melag
UV/Vis spectrophotometer	DU 640	Beckman
Shaker	Flask shaker SF1	Stuart Scientific
Gas chromatograph	7820A	Agilent
FT-IR	Nicolet iS50 FT-IR	Thermo Scientific
Melting point apparatus	SMP3	Stuart Scientific
pH meter	320 pH meter	Mettler Toledo
Extraction thimbles	501 22x80	VWR

Raw hemis of spruce, birch and wheat straw were used as samples for fractionation process. Raw hemis were produced by hot water extraction at VTT. Hot water extraction was performed in an 18 l rotating reactor (Haato) where one kg of dry matter was extracted for 2 hours at target temperature 150 °C, prehydrolysis factor being 238. Water/biomass ratio was 5 l/kg, and 5 batches of every material were made. After hot water extraction, the solution was filtered, batches combined, and then spray-dried (Spray dryer Niro P-6.3) to obtain the raw hemi.

5.2 Dry matter content

Dry matter contents of samples were determined by weighing samples before oven and after a night in 105 °C oven. In case of raw hemi, determination was done in three replicates but in case of LCC fractions dry matter content was determined using only one sample or two replicates, because of the low amount of fraction. Dry matter content was calculated according to equation 1:

$$\text{Dry matter content (\%)} = \frac{\text{mass after oven}}{\text{mass before oven}} \cdot 100. \quad (1)$$

5.3 Fractionation

Approximately 8 g of raw hemi was weighted (8.0011 g spruce, 8.0060 g birch, 8.0084 g wheat straw), and 92 g of UHQ-water was added forming an eight weight-% suspension. The suspension was left stirring at room temperature overnight. The suspension was added to 800 ml of absolute ethanol to form a precipitate and stirred 10 minutes. Then the mixture was left to stand in the refrigerator overnight.

The water-ethanol mixture was centrifuged using four 50 ml tubes, 3900 rcf (relative centrifugal force) speed and 10 minutes centrifugation per 40 ml of suspension. Ethanol soluble fraction was removed with pipette and new 40 ml batch of mixture was added on top of solids until all the mixture was centrifuged. To wash the solid, 20 ml of absolute ethanol was added and mixed properly using glass rod and then centrifuged again. This was repeated two times and used washing ethanol was added to ethanol soluble fraction. After washing, the solids were transferred from centrifuge tubes to tared beaker. On the next day, dried solids remaining in centrifuge tubes were scraped and added to beaker in oven. The solid fraction was dried in the oven at 40 °C for 48 hours and weighted.

The ethanol soluble fraction was concentrated using a rotary evaporator and then transferred to tared beaker. The beaker was dipped in liquid nitrogen and held there until the ethanol soluble fraction was completely solid. Then it was dried in freeze dryer for 5 days and then dried at 40 °C until constant weight. Drying until constant weight took so much time that in case of birch and wheat straw, ethanol soluble fraction was dried at 40 °C at least for six days and then the dry matter content was determined. Yields of birch and wheat straw ethanol soluble fractions were calculated by using dry matter content to correct remaining moisture.

In case of birch sample ethanol soluble fraction was centrifuged again in the next day because it was precipitated more during the night. Centrifugation was done as described earlier, but no washing was done. Precipitates were added to the beaker and dried overnight.

5.4 Lignin content

Klason-lignin was determined according to TAPPI T222 om-88. Two replicates of every fraction were analysed. About 250 mg of samples were weighted precisely (appendix 1) into

test tubes and 4 ml cool 72 % sulfuric acid was added. Test tubes were held at 30 °C water bath for one hour, stirring frequently. Mixtures were removed from test tubes to bottles using 112 ml UHQ-water. Bottles were placed into an autoclave for 50 minutes at 1 bar pressure. Precipitated lignin was separated by using tared filtration crucible and dried to constant weight.

Acid soluble lignin was determined from the hydrolysis filtrate using UV/Vis spectrophotometer. Filtrates were diluted to get absorbance between 0.3-0.8. Ethanol precipitates of spruce and birch were diluted first to 250 ml and all other samples were diluted first to 500 ml. All samples needed to be diluted more. Spruce ethanol precipitates were diluted again 10 ml to 20 ml, wheat straw raw hemi and ethanol soluble were diluted 3 ml to 20 ml and all others 5 ml to 20 ml. Ethanol soluble wheat straw fraction needed to be diluted third time, this time 5 ml to 10 ml. Total dilutions are shown in appendix 1. Diluted hydrolysates were measured using UV/Vis spectrophotometer on wavelength of 205 nm. Six measurements per sample were performed (appendix 2) and averages were used to calculate amount of acid soluble lignin.

Amount of the acid soluble lignin was calculated according to equation 2:

$$c = \frac{A}{a \times b}, \tag{2}$$

where c is the concentration of sample (g/L), A is absorbance, a is absorptiveness (120 $Lg^{-1}cm^{-1}$ for spruce and 110 $Lg^{-1}cm^{-1}$ for birch and wheat straw) and b is the length of the light path.

5.5 Carbohydrate content

To determine carbohydrate composition, 5-20 ml hydrolysis filtrate was added to a beaker. The amount of hydrolysis filtrate depended on supposed amount of lignin and carbohydrates and was 5 ml in case of spruce ethanol precipitate, 20 ml in case of spruce and birch ethanol soluble and 10 ml in rest. Filtrates were neutralised to pH 4 using Amberlite IRA 67 ion exchange resin. Then the resin was filtrated by using a filter crucible and washed with UHQ-water. Filtrates were collected in round bottom flasks and 500 µl xylitol standard solution was added as internal standard. Filtrates were evaporated to dryness using a rotary vacuum evaporator. Samples were derivatised by adding 1 ml dry pyridine (dried using KOH-grains) and 0.5 ml silylation reagent and shaking for 30 minutes. Then samples were moved to GC-vials and analysed by GC-FID.

Used GC-method for determining carbohydrates was as follows. Used column was DB-1701 (60 m × 0.32 mm × 0.25 µm) by J&W Scientific, the carrier gas was nitrogen, the flow was 0.9 ml/min, the injection volume was 1 µl and the temperature of inlet was 290 °C. Temperature program of the method is shown in table 6. FID was used as detector and detector heater was set as 300 °C, H_2 flow 40 ml/min, air flow 450 ml/min, make up flow (N_2) 9,1 ml/min and column + make up flow 10 ml/min. Total runtime was 62.25 minutes.

Table 6. Temperature program of GC-method for carbohydrates.

Rate (°C/min)	Temperature (°C)	Hold time (min)
	110	2
5	175	30
5	230	0
40	280	5

To determine response factors for arabinose, galactose, glucose, xylose and mannose, standard solutions of mentioned monosaccharides were made. For this purpose, 50 mg of monosaccharides was weighed precisely (appendix 3) and dissolved to 100 ml of UHQ-water. Standard solution of xylitol was made similarly and used as internal standard. Of each monosaccharide standard, 500 µl was measured to a beaker and 19 ml of diluted sulfuric acid (3 ml 72 % H_2SO_4 to 500 ml) was added. Then ion exchange, addition of internal standard, derivatisation and GC analysis were carried out in the same way as for samples.

Response factors were calculated according to equation 3:

$$\text{Response factor} = \frac{\text{monosaccharide (µg)} \cdot \text{area of internal standard}}{\text{intenal standard (µg)} \cdot \text{area of monosaccharide}}. \quad (3)$$

Response factors were also determined without ion exchange to be sure that resin does not have effect to the results. For this purpose, 500 µl of arabinose, glucose, xylose, galactose, mannose, and xylitol standard solutions were added to a round bottom flask and evaporated to dryness and then derivatised and analysed in the same way as samples. This was done in two replicates.

The monosaccharide contents of samples were calculated according to equation 4:

$$\text{Monosaccharide (\%)} = \frac{\text{response factor} \cdot \text{internal standard (µg)} \cdot \text{area of monosaccharide} \cdot 100}{\text{area of internal standard} \cdot \text{sample (µg)}}. \quad (4)$$

5.6 Milder acid hydrolysis, FT-IR, melting points and extractives

Milder acid hydrolysis was applied to raw hemis but no replicates were made. In this milder acid hydrolysis about 250 mg of sample were weight precisely (appendix 4) and 112 ml UHQ-water and 4 ml 72 % sulfuric acid were added in bottle and autoclaved at 1 bar for 50 minutes. After that lignin and carbohydrate contents were analysed and calculated similar as in harsher acid hydrolysis described before.

To determine functionality of fractions, FT-IR using ATR technique was performed using 32 scans and 4 cm^{-1} as resolution. Samples, that had been in 105 °C oven for dry matter content determination, were used as samples during IR measurements to verify that samples were dry.

Melting points of all raw hemis, ethanol precipitates and ethanol soluble fractions were measured using melting point apparatus with 5 °C/min temperature ramping rate. Melting points of ethanol soluble fractions were measured twice.

To determine amount of extractives, raw hemis were extracted with acetone in two replicates. About 2 g of raw hemis were weighed precisely and extracted with 150 ml acetone in a Soxhlet extractor for 3 hours. Used extraction thimbles and cotton were washed with acetone before use. Extractives were concentrated into small volume by a rotary evaporator, then moved into tared kimax tubes and evaporated to dryness under nitrogen flow. Kimaxes with dry extractives were weighed and the amount of extractives were calculated as difference from tared kimaxes.

6 Results and discussion

6.1 Dry matter content

Dry matter contents were calculated according to equation (1). For example, the dry matter content of spruce raw hemi was calculated as follows:

$$\text{Dry matter} = \left(\frac{45.8058\ \text{g}}{45.7195\ \text{g} + 0.915\ \text{g}} + \frac{50.7896\ \text{g}}{50.6960 + \ 0.991\ \text{g}} + \frac{43.8082\ \text{g}}{43.7115\ \text{g} + 1.031\ \text{g}}\right)/3$$
$$= 0.99988.$$

Dry matter contents were calculated as above and are shown in table 7.

Table 7. Dry matter contents of all samples and LCC fractions.

Sample	Dry matter content
Spruce raw hemi	0.99988
Spruce ethanol precipitate	0.99907
Spruce ethanol soluble	0.99809
Birch raw hemi	0.99925
Birch ethanol precipitate	0.99961
Birch ethanol soluble	0.99851
Wheat raw hemi	0.99903
Wheat straw ethanol precipitate	0.99930
Wheat straw ethanol soluble	0.99730

All ethanol soluble fractions were melted during the night in 105 °C oven (figure 21) which was unexpected. After oven ethanol soluble fractions, especially spruce and birch, were darker than before oven. Ethanol soluble fractions were noticeably bubbling in the oven and formed thin films. Because of this observation, melting points of all fractions were measured and the amount of extractives was determined to explain low melting points.

Figure 21. Wheat straw ethanol soluble fraction before and after a night at 105 °C.

6.2 Fractionation

Precipitation was very fast with all samples, but the slowest reaction was noticed when using birch as a sample. Spruce and birch precipitates were on the bottom of beaker in the morning but some of wheat straw precipitate floated. Wheat straw precipitate looked like it had bigger flakes (figure 22), but when it was mixed again it looked same as others. Birch ethanol soluble fraction precipitated more after separation when it was left to stand overnight. This did not happen with spruce. Wheat straw ethanol soluble was not left to stand overnight before concentrating it.

Figure 22. From left to right spruce, birch, and wheat straw suspensions in absolute ethanol after night in refrigerator.

During separation of ethanol precipitates by centrifugation, birch sample formed darker stripes in the solid layer on the bottom of centrifuge tubes (figure 23). When the solids were washed with pure ethanol, it was observed that solids were glued to the bottom and the glass rod broke when trying to mix solids with ethanol. Finally, when all solids were detached from the bottom and centrifuged again, all stripes were disappeared. These stripes probably contained ethanol soluble components and especially lignin, which could explain toughness.

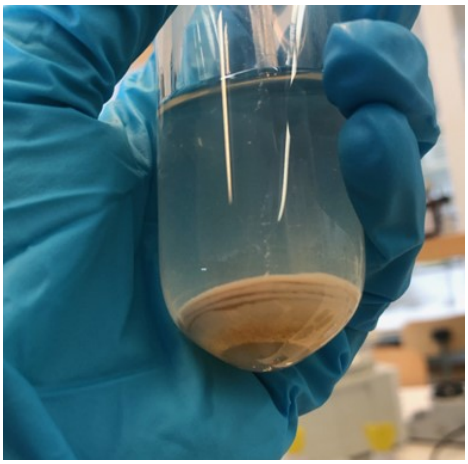

Figure 23. Centrifuge tube having unwashed ethanol precipitates of birch in the bottom. Darker stripes were removed by washing solids with pure absolute ethanol.

Transferring ethanol precipitates from centrifuge tubes to beaker was quite difficult due to the consistency, and some solids were remaining on the edges of tubes. A lot of remaining solids were scraped off the next day and it was easier to remove the rest when solids were dried. However, more loss of yield happened during the concentration of ethanol soluble fraction. Some precipitates were formed in the round bottom flask during concentration and about 0.7 g of birch and 0.3 g of wheat straw fraction remained in the round bottom flask. Some of spruce fraction also remained in the round bottom flaks, but it was never weighed. In table 8 are shown the yields of fractionation processes. In figure 24 are shown spruce raw hemi, ethanol precipitate and ethanol soluble fraction.

Total yield of spruce fractionation (94 %) was similar with the yield of Carvalho *et al.*[10] who have performed similar fractionation. In study of Carvalho *et al.*[10], amount of ethanol precipitate was about 74 % and ethanol soluble only 21 %. In this study, amount of ethanol precipitate was about 62 % and ethanol soluble 32 %. This difference might indicate that separation of ethanol precipitates and ethanol soluble was not complete and some ethanol precipitates remained in the ethanol soluble fraction.

Total yield of birch fractionation was the smallest (88 %), but still good. If the material lost in round bottom flask during concentration is calculated in the yield, then the yield would be about 97 %. Birch was the only sample, where the ethanol soluble fraction (57 %) was larger than the ethanol precipitate (31 %). However, some ethanol precipitates were presumably in the ethanol soluble fraction because dried ethanol soluble fraction had some white areas (looked a lot like ethanol precipitate) in the case of birch.

Table 8. Yields of fractionation.

Sample	Yield	Mass (g)	%
Spruce	**Total**	**7.5559**	**94.42**
	Ethanol precipitate	4.9715	62.13
	Ethanol soluble	2.5844	32.30
Birch	**Total**	**7.0745**	**88.43**
	Ethanol precipitate	2.4761	30.90
	Ethanol soluble	4.5984	57.48
Wheat straw	**Total**	**7.5824**	**94.77**
	Ethanol precipitate	4.1643	52.05
	Ethanol soluble	3.4181	42.72

Wheat straw had total yield (95 %) similar with spruce, but the amount difference between ethanol precipitate (52 %) and ethanol soluble (43 %) was smaller. During freeze drying of wheat straw ethanol soluble fraction, the aluminium foil on top of the beaker flied away and some of ethanol soluble fraction was lost.

Figure 24. From left to right, raw hemi, ethanol precipitate and dried ethanol soluble fraction of spruce.

6.3 Lignin and carbohydrates

Amount of acid soluble lignin was calculated according to equation (2). For example, acid soluble lignin of the spruce raw hemi 1 was calculated as follows:

$$c = \frac{0.4506}{120\ \mathrm{Lg^{-1}cm^{-1}} \cdot 1\ \mathrm{cm}} = 0.003755\ \mathrm{g/l} = 3.755\ \mathrm{mg/l}.$$

Samples weren't in 1 litre volume, so dilutions (appendix 1) have been taken into account. For example, the spruce raw hemi 1 was diluted so that its volume was 2 l so amount of acid soluble lignin was $2\,\mathrm{l} \cdot 3.755\ \mathrm{mg/l} = 7.5103\ \mathrm{mg}$. Klason lignin was determined by weighing and lignin contents are shown together with carbohydrates in tables 10-12.

Ion exchange during carbohydrate analysis did not have a significant impact on response factors so response factors and carbohydrates were determined with the ion exchange. To calculate carbohydrates, response factors were calculated first according to equation (3). For example, the response factor for arabinose was calculated as follows:

$$\text{Response factor} = \frac{255.5\ \mu\mathrm{g} \cdot 3788.95874}{250.5\ \mu\mathrm{g} \cdot 2854.15157} = 1.354022926.$$

Response factors were determined using four replicates and calculated as their average and are shown in table 9.

Table 9. Response factors of monosaccharides.

Monosaccharide	Response factor
Arabinose	1.32271
Glucose	1.20466
Xylose	1.23079
Galactose	1.20884
Mannose	1.19876

Chromatograms of every sample type with labelled peaks are shown in appendixes 5-13. Carbohydrates were calculated according to equation (4). For example, the arabinose content of spruce raw hemi 1 was calculated as follows:

$$\text{Arabinose (\%)} = \frac{1.322712542 \cdot 250.5\ \mu g \cdot 4309.78901 \cdot 100}{3782.92505 \cdot 4990\ \mu g} \approx 7.55\ \%.$$

The carbohydrate content together with lignin content is shown in table 10 for spruce, in table 12 for birch and in table 14 for wheat straw. Same results but scaled to 100 % are shown in tables 11, 13, and 15 for spruce, birch, and wheat straw respectively.

About 62-71 % of spruce fractions could be explained with lignin and analysed carbohydrates. The rest, unrecognised part, may include for example, rhamnose, pectin and extractives. Raw hemi of spruce consisted of about 87 % carbohydrates and 13 % lignin which is in line with the study of Carvalho *et al.*[10] (85 % carbohydrates, 15 % lignin). Ethanol precipitate did not contain much lignin (4 %) and it contained mainly hemicelluloses. However, ethanol soluble fraction had more lignin (24 %) which explains the dark colour compared to almost white ethanol precipitate. Both ethanol precipitate and ethanol soluble fraction had less lignin and more carbohydrates compared to corresponding fractions in the study of Carvalho *et al.*[10].

All spruce samples, especially raw hemi and ethanol precipitate, had a lot of mannose, which is expected because spruce is a softwood and softwood hemicelluloses are mainly glucomannans. Almost all arabinose was found in the ethanol soluble fraction. This may indicate that arabinose is found as monosaccharide in raw hemi because shorter polysaccharides are preferentially solubilised in ethanol[10]. Also, it is probable that arabinose as a side group of hemicelluloses cuts off during hot water extraction and exist as a monosaccharide in raw hemi.

Table 10. The chemical composition of spruce samples as percentages[1)].

Spruce (%)	Raw hemi	Ethanol precipitate	Ethanol soluble
Total carbohydrates	**54.3 ± 1.5**	**64.9 ± 2.4**	**53.8 ± 2.7**
Arabinose	7.1 ± 0.4	0.6 ± 0.0	23.4 ± 0.1
Galactose	5.5 ± 0.0	7.2 ± 0.3	3.9 ± 0.3
Glucose	6.7 ± 0.0	9.1 ± 0.2	3.8 ± 0.4
Mannose	26.5 ± 0.7	38.0 ± 1.6	12.3 ± 1.2
Xylose	8.5 ± 0.4	10.0 ± 0.3	10.4 ± 0.8
Total lignin	**7.8 ± 0.5**	**2.6 ± 0.5**	**17.4 ± 2.2**
Klason-lignin	5.1 ± 0.8	1.4 ± 0.5	12.8 ± 1.8
Acid soluble lignin	2.7 ± 0.3	1.2 ± 0.0	4.6 ± 0.4
Total	**62.2 ± 0.9**	**67.5 ± 2.9**	**71.2 ± 0.5**

1) Average ± standard deviation. If standard deviation <0.05 marked as 0.0.

Table 11. The chemical composition of spruce samples as percentages scaled to 100 %[1)].

Spruce (%)	Raw hemi	Ethanol precipitate	Ethanol soluble
Total carbohydrates	**87.4 ± 1.1**	**96.2 ± 0.6**	**75.6 ± 3.2**
Arabinose	11.5 ± 0.5	0.9 ± 0.0	32.9 ± 0.1
Galactose	8.8 ± 0.2	10.6 ± 0.0	5.4 ± 0.3
Glucose	10.8 ± 0.2	13.5 ± 0.3	5.3 ± 0.5
Mannose	42.7 ± 0.5	56.3 ± 0.0	17.3 ± 1.5
Xylose	13.6 ± 0.4	14.9 ± 0.2	14.7 ± 1.0
Total lignin	**12.6 ± 1.1**	**3.8 ± 0.6**	**24.4 ± 3.2**
Klason-lignin	8.2 ± 1.4	2.1 ± 0.7	18.0 ± 2.6
Acid soluble lignin	4.4 ± 0.4	1.7 ± 0.1	6.4 ± 0.6
Total	**100.0**	**100.0**	**100.0**

1) Average ± standard deviation. If standard deviation <0.05 marked as 0.0.

Only 51-57 % of birch samples could be explained with lignin and analysed carbohydrates. The rest may include same components as spruce (rhamnose, pectin, extractives). The amount of extractives were determined and there was 15 % extractives in birch raw hemi. Birch is a hardwood and hardwood hemicelluloses consist mainly of xylans which explains that in every fraction xylose was the main monosaccharide.

Compared to spruce, all birch fractions had more lignin. This indicates that more lignin is extracted from birch by hot water extraction. This might be the result of different types of lignin. Lignins from gymnosperms like softwoods are composed of G-units of lignin and lignins from angiosperms like hardwoods and grasses are composed of G- and S-units[5].

Table 12. The chemical composition of birch samples as percentages[1)].

Birch (%)	Raw hemi	Ethanol precipitate	Ethanol soluble
Total carbohydrates	**43.0 ± 0.3**	**48.2 ± 0.1**	**37.8 ± 0.1**
Arabinose	1.6 ± 0.0	0.7 ± 0.0	2.0 ± 0.0
Galactose	2.7 ± 0.0	3.3 ± 0.1	1.6 ± 0.1
Glucose	2.0 ± 0.0	1.5 ± 0.1	1.6 ± 0.1
Mannose	1.3 ± 0.0	1.0 ± 0.0	1.1 ± 0.0
Xylose	35.4 ± 0.4	41.8 ± 0.0	31.6 ± 0.0
Total lignin	**14.3 ± 1.2**	**7.7 ± 0.5**	**13.1 ± 0.5**
Klason-lignin	10.0 ± 1.5	5.4 ± 0.5	9.7 ± 0.5
Acid soluble lignin	4.3 ± 0.3	2.3 ± 0.0	3.2 ± 0.0
Total	**57.3 ± 0.8**	**55.9 ± 0.6**	**51.0 ± 0.6**

1) Average ± standard deviation. If standard deviation <0.05 marked as 0.0.

Table 13. The chemical composition of birch samples as percentages scaled to 100 %[1)].

Birch (%)	Raw hemi	Ethanol precipitate	Ethanol soluble
Total carbohydrates	**75.0 ± 1.7**	**86.2 ± 0.7**	**74.2 ± 0.0**
Arabinose	2.8 ± 0.1	1.2 ± 0.1	3.9 ± 0.1
Galactose	4.7 ± 0.1	5.9 ± 0.0	3.2 ± 0.1
Glucose	3.5 ± 0.0	2.7 ± 0.1	3.1 ± 0.0
Mannose	2.2 ± 0.0	1.8 ± 0.0	2.1 ± 0.0
Xylose	61.8 ± 1.7	74.7 ± 0.8	61.9 ± 0.1
Total lignin	**25.0 ± 1.7**	**13.8 ± 0.7**	**25.8 ± 0.0**
Klason-lignin	17.4 ± 2.3	9.7 ± 0.9	19.1 ± 0.6
Acid soluble lignin	7.6 ± 0.6	4.2 ± 0.1	6.3 ± 0.1
Total	**100.0**	**100.0**	**100.0**

1) Average ± standard deviation. If standard deviation <0.05 marked as 0.0.

Wheat straw samples had the highest amount of lignin compared to spruce and birch. Also, wheat straw ethanol soluble fraction was the only fraction that contained more lignin (54 %) than carbohydrates (46 %). Non-woods may have slightly higher amount of H-units in lignin[5], which could be one explanation to the difference between wheat straw and wood samples. Wheat straw samples did also have the strongest smell. Determined lignin and carbohydrates explained only 41-54 % of wheat straw samples. In chromatograms of wheat straw raw hemi and ethanol soluble fraction (appendix 11 and 13) is shown one bigger peak (roughly 1 %) that

was unrecognised but supposed to be some kind of monosaccharide peak. This peak was not found in chromatograms of spruce or birch.

Table 14. The chemical composition of wheat straw samples as percentages[1)].

Wheat straw (%)	Raw hemi	Ethanol precipitate	Ethanol soluble
Total carbohydrates	**33.2 ± 0.5**	**39.4 ± 0.7**	**18.9 ± 0.2**
Arabinose	6.5 ± 0.1	5.0 ± 0.5	5.0 ± 0.1
Galactose	2.7 ± 0.0	4.3 ± 0.0	0.9 ± 0.0
Glucose	5.9 ± 0.1	7.9 ± 0.1	3.1 ± 0.0
Mannose	2.0 ± 0.0	1.5 ± 0.0	1.8 ± 0.3
Xylose	16.0 ± 0.3	20.8 ± 0.0	8.1 ± 0.0
Total lignin	**16.4 ± 0.1**	**14.4 ± 0.4**	**22.1 ± 0.1**
Klason-lignin	9.2 ± 0.1	10.1 ± 0.5	12.5 ± 0.2
Acid soluble lignin	7.2 ± 0.0	4.4 ± 0.1	9.6 ± 0.1
Total	**49.6 ± 0.3**	**53.9 ± 1.1**	**41.0 ± 0.3**

1) Average ± standard deviation. If standard deviation <0.05 marked as 0.0.

Table 15. The chemical composition of wheat straw samples as percentages scaled to 100 %[1)].

Wheat straw (%)	Raw hemi	Ethanol precipitate	Ethanol soluble
Total carbohydrates	**66.9 ± 0.5**	**73.2 ± 0.2**	**46.0 ± 0.1**
Arabinose	13.1 ± 0.1	9.2 ± 0.8	12.3 ± 0.3
Galactose	5.5 ± 0.0	7.9 ± 0.1	2.1 ± 0.1
Glucose	11.9 ± 0.0	14.6 ± 0.1	7.6 ± 0.1
Mannose	4.0 ± 0.0	2.8 ± 0.0	4.4 ± 0.6
Xylose	32.3 ± 0.4	38.7 ± 0.8	19.7 ± 0.2
Total lignin	**33.1 ± 0.5**	**26.8 ± 0.2**	**54.0 ± 0.1**
Klason-lignin	18.6 ± 0.4	18.7 ± 0.5	30.6 ± 0.2
Acid soluble lignin	14.5 ± 0.5	8.1 ± 0.3	23.4 ± 0.3
Total	**100.0**	**100.0**	**100.0**

1) Average ± standard deviation. If standard deviation <0.05 marked as 0.0.

The carbohydrate yields were lower than expected. One reason could be that used acid hydrolysis was too harsh for this kind of highly processed samples and monosaccharides might

decompose during used 2-step acid hydrolysis. To examine this hypothesis milder acid hydrolysis was performed on raw hemis without replicates. Results of milder acid hydrolysis are shown in table 16.

Table 16. The chemical composition of raw hemi samples as percentages determined with milder acid hydrolysis.

Raw hemi (%)	Spruce	Birch	Wheat straw
Total carbohydrates	**58,4**	**53,4**	**37,3**
Arabinose	7,8	2,0	6,8
Galactose	5,3	2,8	2,8
Glucose	6,5	2,0	6,0
Mannose	28,4	1,5	2,4
Xylose	10,3	45,1	19,4
Total lignin	**5,6**	**9,5**	**13,8**
Klason-lignin	-	0,3	3,3
Acid soluble lignin	5,6	9,1	10,5
Total	**64,0**	**62,9**	**51,1**

Determination of Klason-lignin in spruce hemi failed because crucible with Klason lignin was 2.4 mg lighter than tared crucible for some reason. Amount of Klason lignin in spruce were approximately same size with birch, so very low.

With using milder acid hydrolysis higher yields of carbohydrates were obtained. This refers to that two-step acid hydrolysis used before can be a bit too harsh and decompose carbohydrates into furans. However, the yields of lignin were lower when using milder acid hydrolysis. Also, when using milder acid hydrolysis the amount of Klason lignin was lower but the amount of acid soluble lignin was higher. soluble. Strong acid precipitates lignin and removes carbohydrate parts more efficiently. When less lignin is precipitated, more lignin stays as soluble in the acid.

6.4 FT-IR

Measured FT-IR spectra are shown in figures 25-30. Large peak at 3400-3200 cm^{-1} originates from O-H stretching vibrations of aromatic and aliphatic OH-groups. Peaks at about 2930 cm^{-1} and 2850 cm^{-1} are due to symmetric and asymmetric C-H stretch in CH, CH_2 and CH_3 groups. Peak at about 1720 cm^{-1} originates from C=O stretch of unconjugated ketones, carbonyl, and ester groups. Aromatic skeletal vibrations of lignin show as peaks at about 1600 cm^{-1}, 1515 cm^{-1} and 1420 cm^{-1}. However, asymmetric C-H in-plane bending vibrations in OCH_3 groups of lignin and symmetrical bending vibrations in CH_2 groups of hemicellulose shows also at 1450-1400 cm^{-1} and thus might be part of the peak. Peak at about 1370 cm^{-1} might originate from COO-asymmetric and symmetrical vibrations in carboxylate groups or from C-H bending vibrations in CH_3 groups of acetyl fragments. Peak at about 1330 cm^{-1} indicates to syringyl unit and peak at about 1240 cm^{-1} to guaiacyl unit of lignin. C=O in conjugated ester groups of lignin typically causes peak at about 1160 cm^{-1}. Large peak at about 1040 cm^{-1} is combination of aromatic C-H in-plane deformation, C-O deform in primary alcohols and unconjugated C=O stretch.[28,46]

Peak at about 1515 cm^{-1} was found in every ethanol soluble fraction but was significantly smaller in ethanol precipitates and was almost not found in spruce and birch raw hemis. Spruce and birch spectra were very similar, but wheat straw spectra differ slightly. Compared to spruce and birch, wheat straw spectra do not have peak at about 1730 cm^{-1} and comparing ethanol soluble fractions (figure 30) wheat straw has bigger peak at about 1600 cm^{-1}.

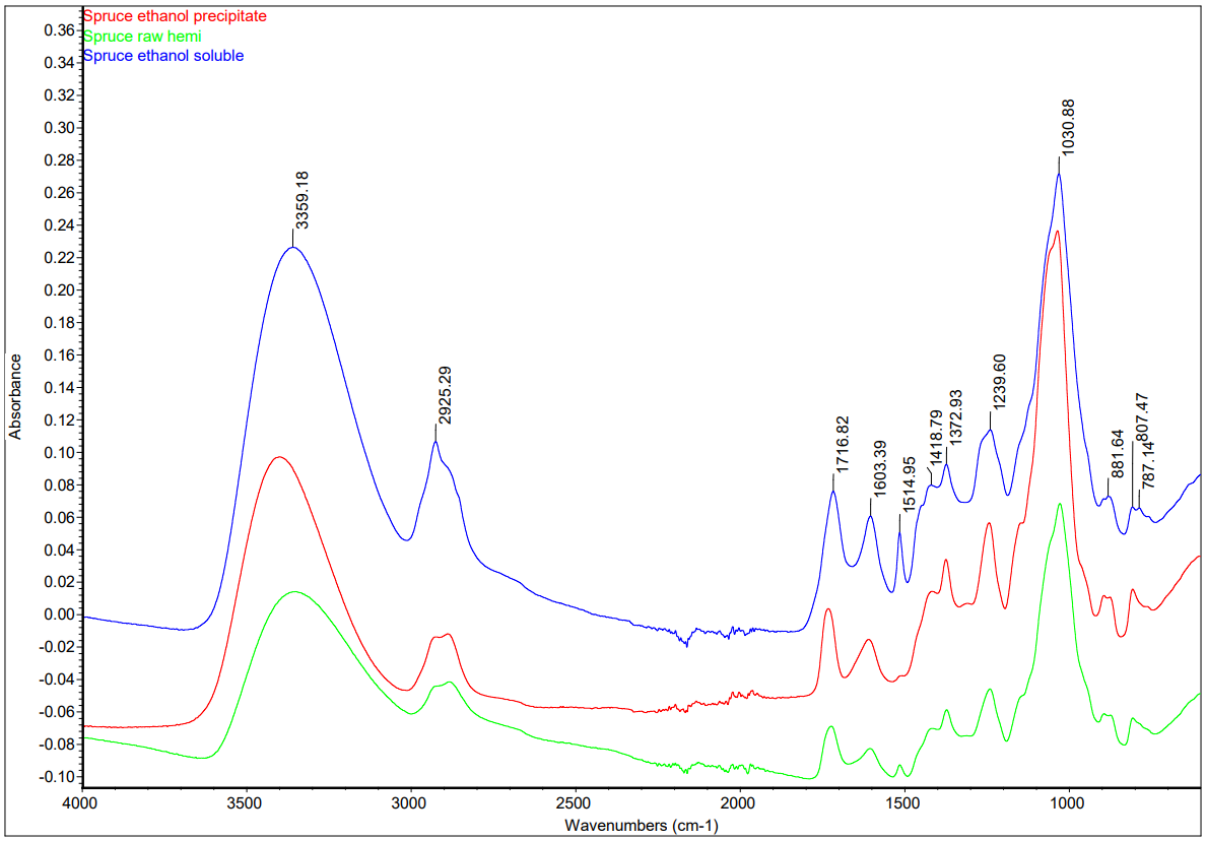

Figure 25. IR-spectra of spruce fractions.

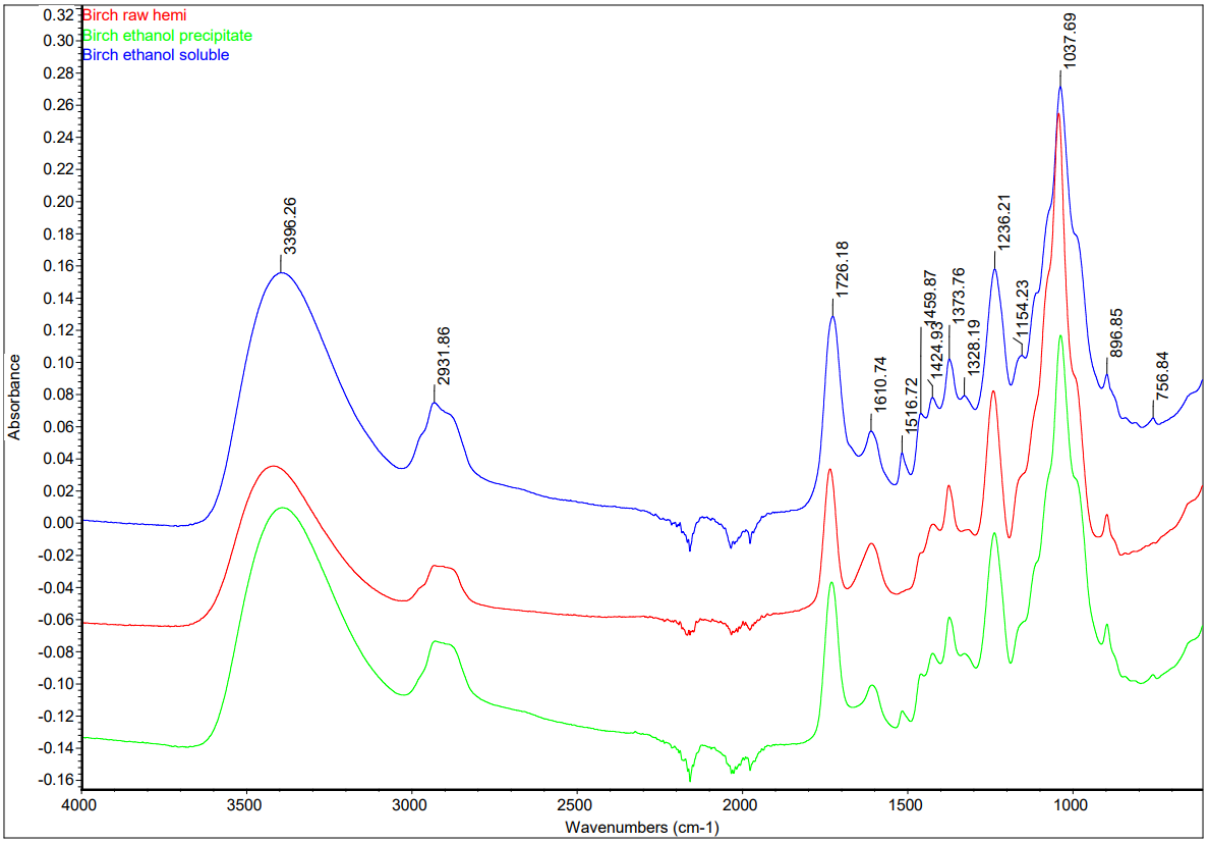

Figure 26. IR-spectra of birch fractions.

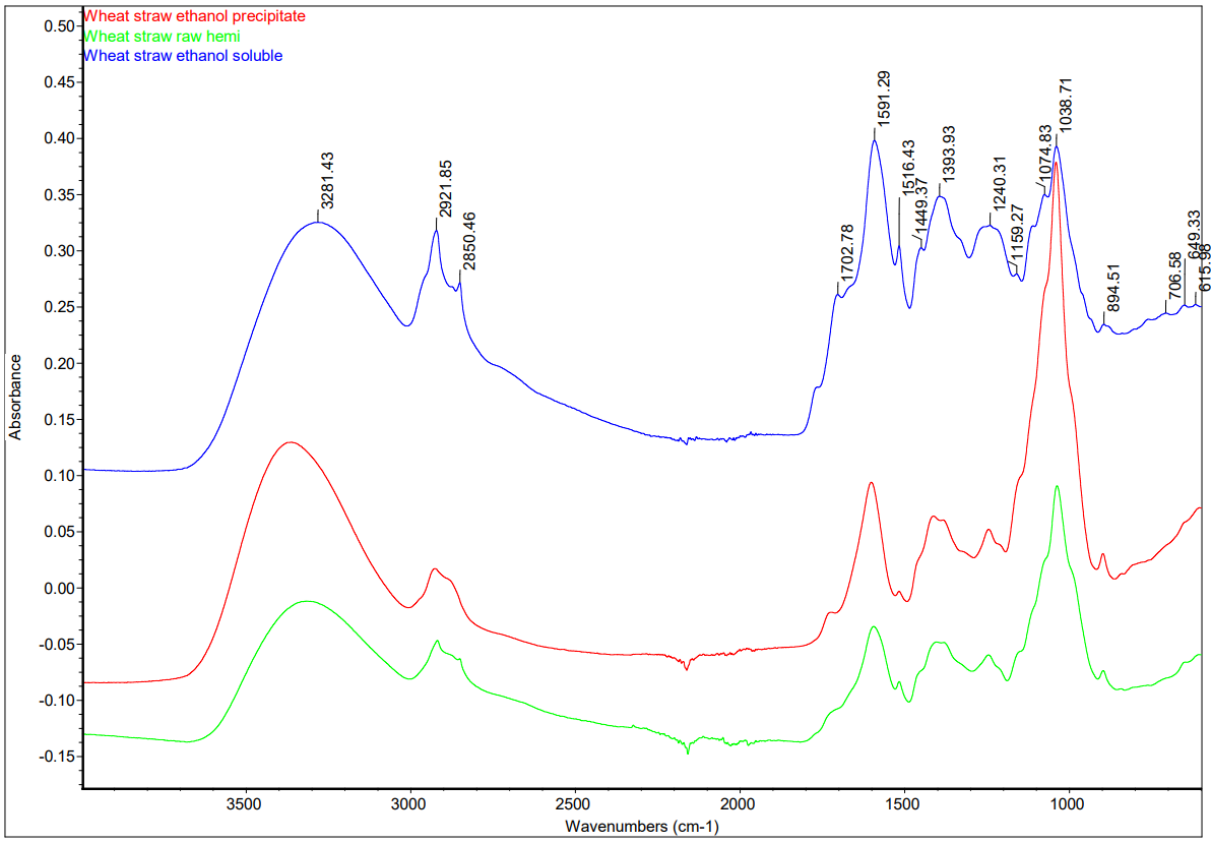

Figure 27. IR-spectra of wheat straw fractions.

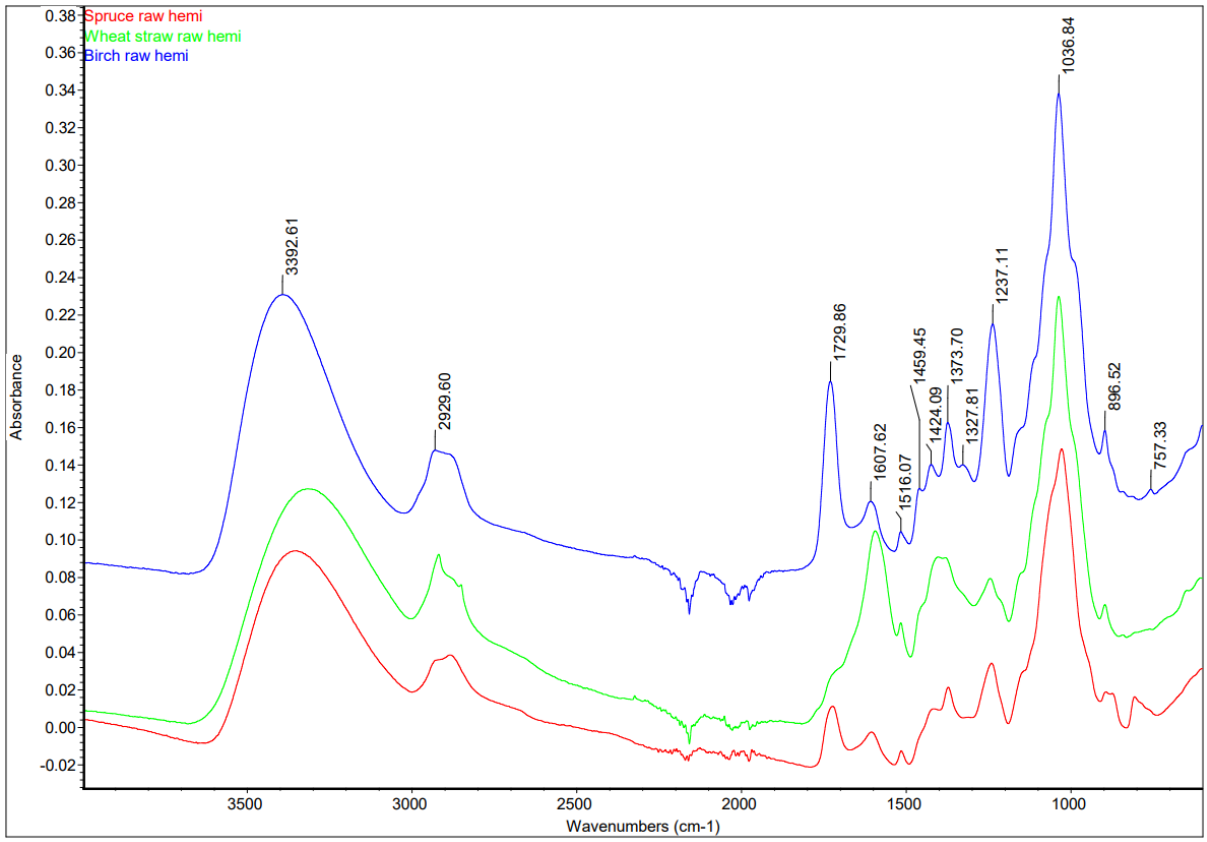

Figure 28. IR-spectra of raw hemis.

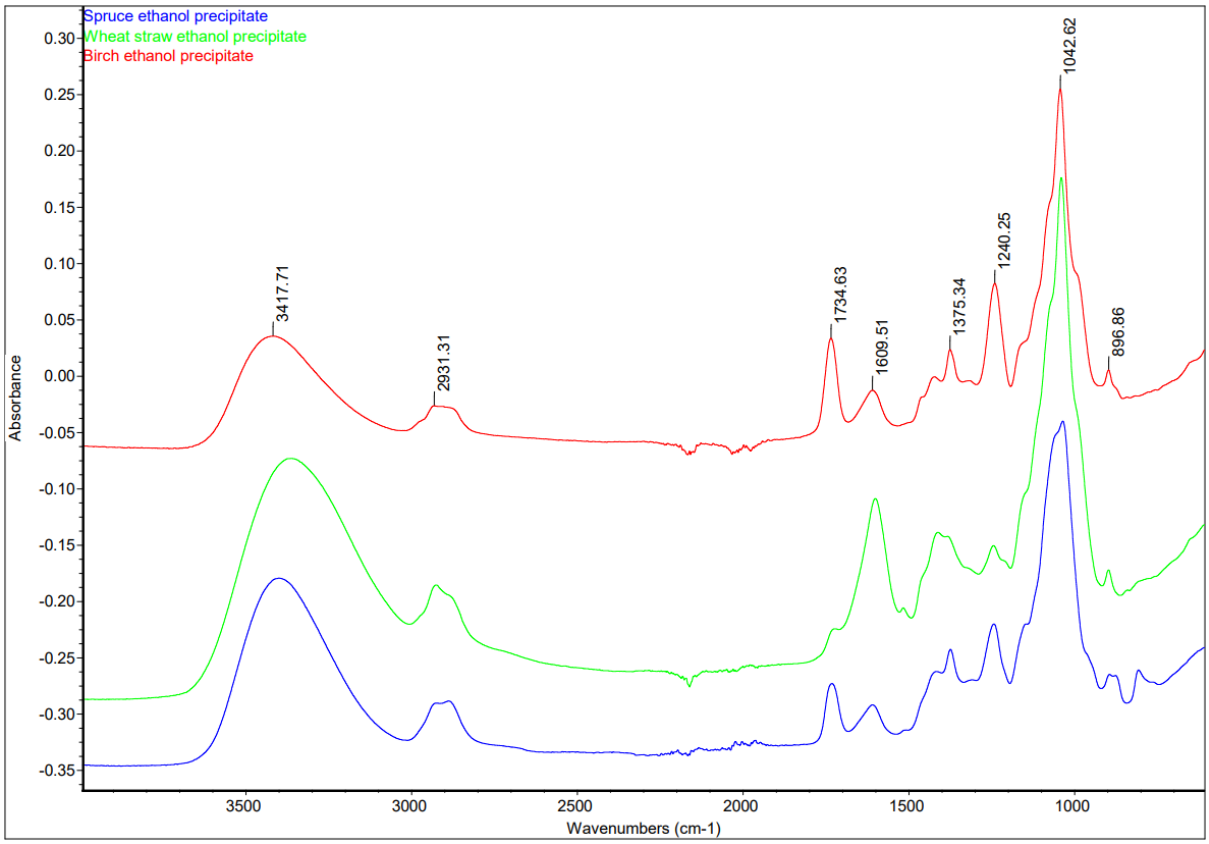

Figure 29. IR-spectra of ethanol precipitates.

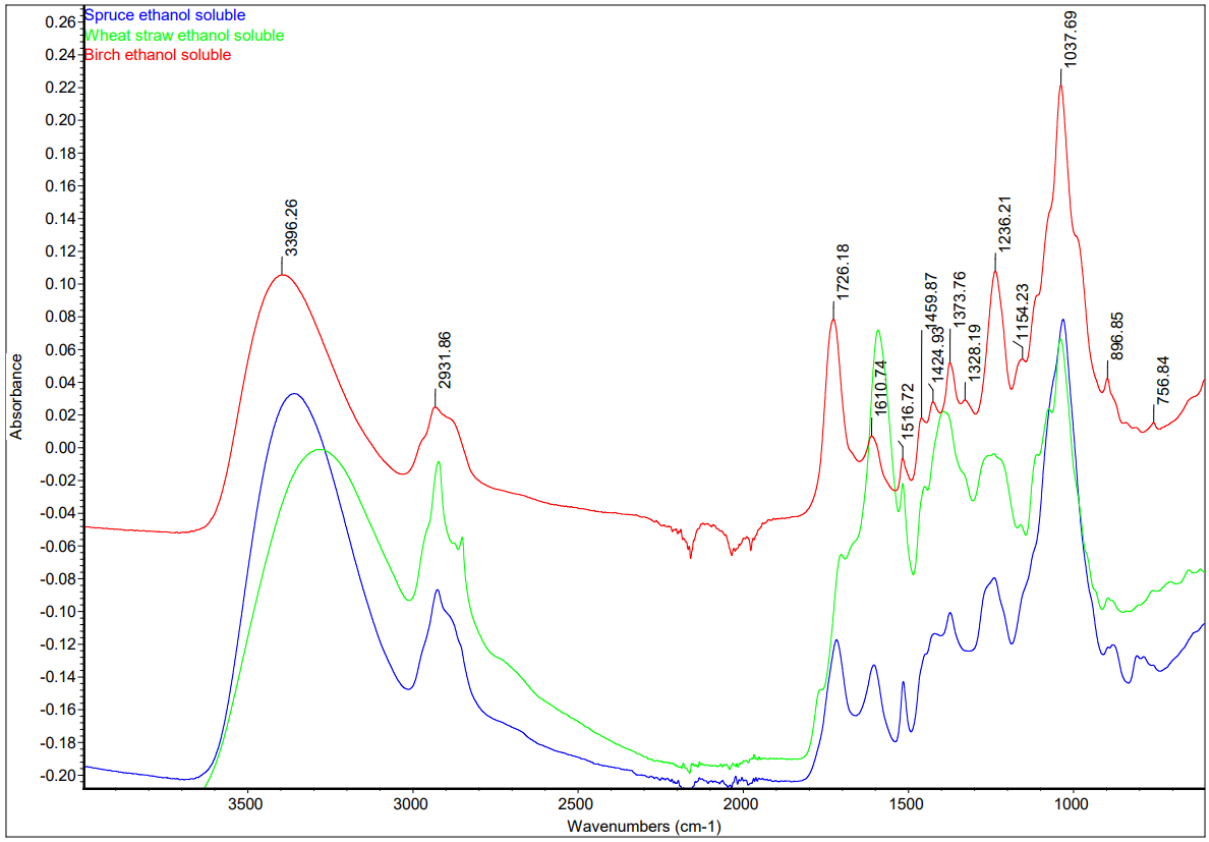

Figure 30. IR-spectra of ethanol solubles.

6.5 Melting points

Melting points of all raw hemis and LCC fractions were determined and are shown in table 17. Determination was challenging because samples did not have clear melting points but changed little by little. The dark colour of ethanol soluble samples did also make determination difficult. Because of these difficulties, melting points are approximates.

Table 17. Determined melting points.

	Spruce	**Birch**	**Wheat straw**
Raw hemi	Burned	150-170 ℃	Partly melted
Ethanol precipitate	Burned	Partly melted	Burned
Ethanol soluble	65-75 ℃	85-95 ℃	75-90 ℃.

Spruce raw hemi did not melt but started to turn darker after 170 ℃ and was all black at 250 ℃ when measurement was ended. Unlike spruce, birch raw hemi did melt. Birch raw hemi started to shrink at 130 ℃ and turn darker at 140 ℃. At 150 ℃ it was most likely melted and at 170 ℃ it started to bubble. Wheat straw raw hemi started to shrink and turn darker at 150 ℃. It did not melt but looked like there was sticky wet parts and solids mixed at 190 ℃. It was completely black at 200 ℃.

Spruce ethanol precipitate did not melt but started to turn darker at 230 ℃ and was all black at 240 ℃. Birch ethanol precipitate started to shrink at 195 ℃ and was completely grey at 200 ℃. At 210 ℃ seemed like there were shiny wet parts and the volume started to increase. At 220 ℃ it was bubbling and looked like dark brow foam. Wheat straw ethanol precipitate started to shrink and turn darker at 195 ℃. At 220 ℃ it was black and dry, so it did not melt.

All ethanol soluble samples had relatively low melting points. Spruce ethanol soluble started to turn darker after 55 ℃, melted between 65-75 ℃ and after 100 ℃ started to bubble. Birch ethanol soluble started to change after 65 ℃, melted between 85-95 ℃ and started to bubble after 105 ℃. Melting point of wheat straw ethanol soluble was determined from sample that was used for dry matter determination because the primary fraction was so sticky that it could not be transferred into glass capillary for melting point apparatus. It was very dark at the beginning what made determination very challenging. Some changes seemed to take place already after 45 ℃ but melting happened probably between 75-90 ℃. At 110 ℃ this fraction was also bubbling.

Melting points this low were not expected. The state of polymer-like lignin and hemicelluloses can be glassy, elastic and melted. The temperature range where the glassy state changes to the elastic state is the glass transition temperature. Properties of lignin such as phenolic side groups, cross-linking, hydrogen bonds, molecular weight, isolation method and thermal prehistory effect on glass transition temperature. According to literature, softwood lignins have glass transition temperatures between 138-160 °C and hardwood lignins between 110-130 °C. The glass transition temperatures of lignin decreases with increasing moisture content until it is saturated with water.[49] Because these lignin melting points were determined by estimating visually, some of these might be actually glass transition temperatures. Presence of extractives in the samples and the hot water extraction might play a part in reasons why melting points were so low.

6.6 Extractives

Analysed carbohydrates and lignin explained about 40-70 % of fractions. Because the raw hemis were obtained by hot water extraction some extractives were included. Amounts of extractives were determined to estimate how much extractives were in the samples. Amounts of extractives are shown in table 18. Spruce and wheat straw raw hemis contained similar, small amounts of extractives, about 1 %. However, birch raw hemi had significantly more extractives, about 15 %. This significant difference indicates that hot water extraction affects differently depending on lignocellulosic biomass used in extraction and probably tells not so much about the original amount of extractives in the raw hemis. It is possible that hot water extraction causes structure changes, and components that are not actually extractives will be able to act like extractives during Soxhlet extraction. Because no other analysis of extractives than the amount was done, it is hard to say why birch had so much more extractives than spruce and wheat straw and what was included in that 15 %.

Table 18. Extractives in raw hemis (average ± standard deviation).

Raw hemi	Extractives (%)
Spruce	1.4 ± 0.1
Birch	15.4 ± 0.2
Wheat straw	1.2 ± 0.0[1)]

1) Standard deviation < 0.05.

7 Conclusions

A lot of research on LCCs has been done but a lot of research remains to be done with these heterogenous materials. Interest in LCCs is increasing and modern 2D NMR techniques are important tools when studying the structure of LCCs. The method used for isolation of LCCs has an essential influence on the fractions and must be chosen for the purpose of the research.

In this experimental work, the used fractionation method was effective for the purpose of this study and the yields were good. Spruce and birch samples were very similar, but the wheat straw fractions differed slightly from the trees. However, birch raw hemi contained significantly more extractives than spruce and wheat straw raw hemis. Ethanol soluble fractions of all samples had unexpected low melting points close to 100 °C thus low melting point did not seem to be depending on the type of lignocellulosic biomass. More experiments would be needed to understand low melting points of ethanol soluble fractions.

Even though used fractionation by ethanol precipitation is very mild treatment, the hot water extraction to obtain raw hemi is quite harsh. Strong acid hydrolysis may cause degradation of monosaccharides when carbohydrates from these kinds of samples are analysed. Amount of rhamnose and pectin could also be determined to give better understanding.

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Appendices

Sample	Weight (mg)	Hydrolysate 1[a)] (ml)	Hydrolysate 2[b)] (l)
Spruce raw hemi 1	249.5	500	2
Spruce raw hemi 2	251.0	500	2
Spruce ethanol precipitate 1	251.2	250	0.5
Spruce ethanol precipitate 2	249.1	250	0.5
Spruce ethanol soluble 1	249.4	500	2
Spruce ethanol soluble 2	253.4	500	2
Birch raw hemi 1	251.8	500	2
Birch raw hemi 2	249.8	500	2
Birch ethanol precipitate 1	249.2	250	1
Birch ethanol precipitate 2	249.0	250	1
Birch ethanol soluble 1	249.5	500	2
Birch ethanol soluble 2	251.0	500	2
Wheat straw raw hemi 1	254.9	500	3.333
Wheat straw raw hemi 2	250.4	500	3.333
Wheat straw ethanol precipitate 1	250.2	500	2
Wheat straw ethanol precipitate 2	251.8	500	2
Wheat straw ethanol soluble 1	254.2	500	6.667
Wheat straw ethanol soluble 2	258.7	500	6.667

a) Hydrolysate which was used to the determination of carbohydrates by using GC.

b) Diluted hydrolysate for determination of acid soluble lignin by using UV/Vis spectrophotometer.

APPENDIX 2

SRH1	SRH2	SEP1	SEP2	SES1	SES2	BRH1	BRH2	BEP1	BEP2	BES1	BES2	WRH1	WRH2	WEP1	WEP2	WES1	WES2
0.4460	0.3724	0.6847	0.6987	0.7409	0.6325	0.6400	0.5590	0.6470	0.6308	0.4594	0.4221	0.6051	0.5963	0.5990	0.5931	0.4072	0.3868
0.4486	0.3724	0.6930	0.7052	0.7487	0.6392	0.6418	0.5615	0.6532	0.6332	0.4586	0.4304	0.6073	0.5919	0.6000	0.5958	0.4037	0.3890
0.4501	0.3731	0.6958	0.7059	0.7495	0.6416	0.6412	0.5625	0.6526	0.6326	0.4570	0.4327	0.6062	0.5940	0.6000	0.6076	0.4045	0.3894
0.4539	0.3686	0.6874	0.7052	0.7370	0.6301	0.6370	0.5503	0.6538	0.6232	0.4435	0.4281	0.6025	0.5935	0.6142	0.5858	0.4003	0.3901
0.4527	0.3690	0.6944	0.7081	0.7479	0.6392	0.6400	0.5556	0.6532	0.6296	0.4538	0.4335	0.6041	0.5956	0.6148	0.5894	0.4053	0.3931
0.4524	0.3639	0.6944	0.7081	0.7487	0.6386	0.6412	0.5561	0.6526	0.6302	0.4538	0.5358	0.6057	0.5077	0.6154	0.5899	0.4064	0.3954
Averages																	
0.4506	**0.3708**	**0.6916**	**0.7033**	**0.7455**	**0.6369**	**0.6402**	**0.5575**	**0.6521**	**0.6299**	**0.4544**	**0.4304**	**0.6052**	**0.5948**	**0.6072**	**0.5936**	**0.4046**	**0.3906**

SRH = Spruce raw hemi, SEP = Spruce ethanol precipitate, SES = Spruce ethanol soluble

BRH = Birch raw hemi, BEP = Birch ethanol precipitate, BES = Birch ethanol soluble

WRH = Wheat straw raw hemi, WEP = Wheat straw ethanol precipitate, WES = Wheat straw ethanol soluble

Monosaccharide	**m(mg)**
Arabinose	51.1
Galactose	51.4
Glucose	50.0
Xylose	49.9
Mannose	49.6
Xylitol	50.1

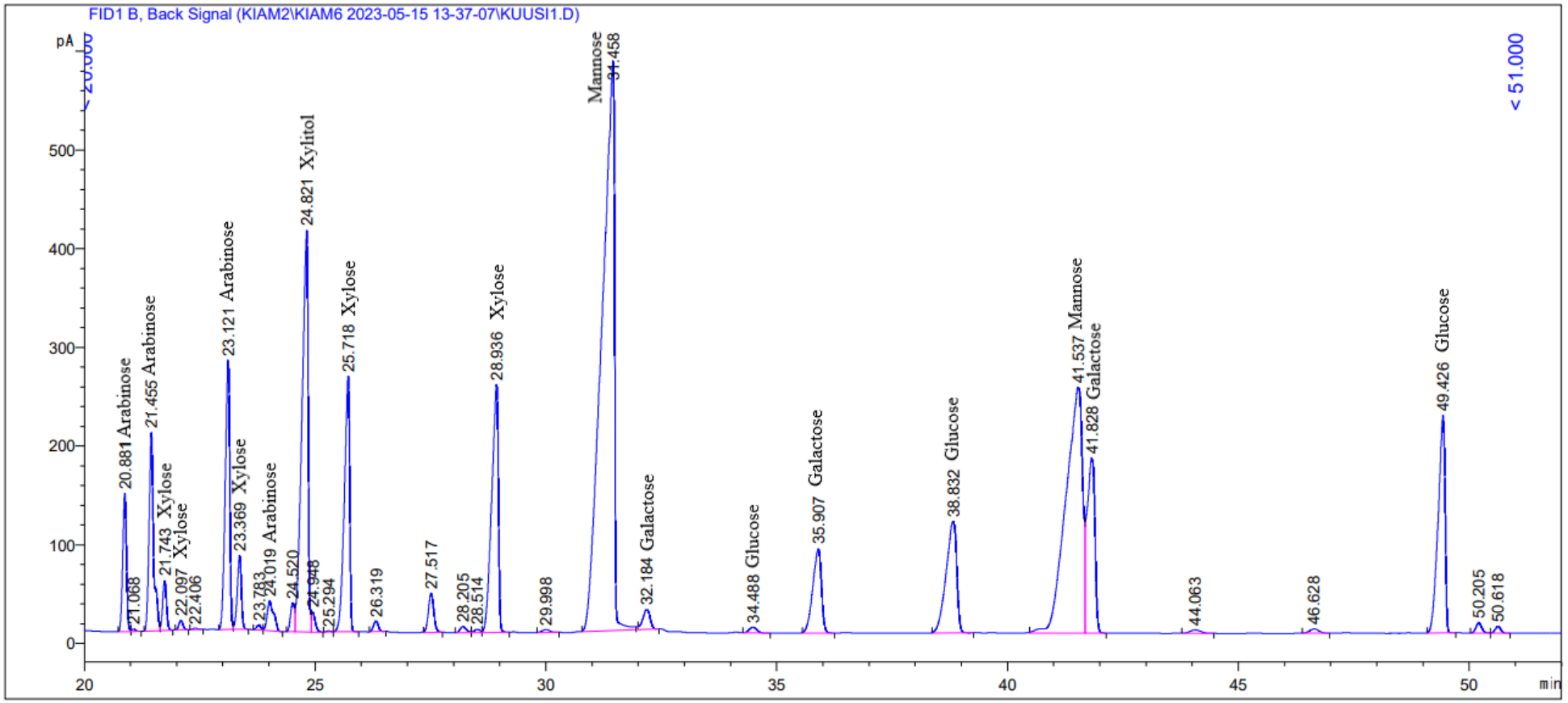
Raw hemi	**m(mg)**	**Hydrolysate 1[a] (ml)**	**Hydrolysate 2[b] (l)**
Spruce	250.4	500	2.5
Birch	250.6	500	5.0
Wheat straw	249.8	500	5.0

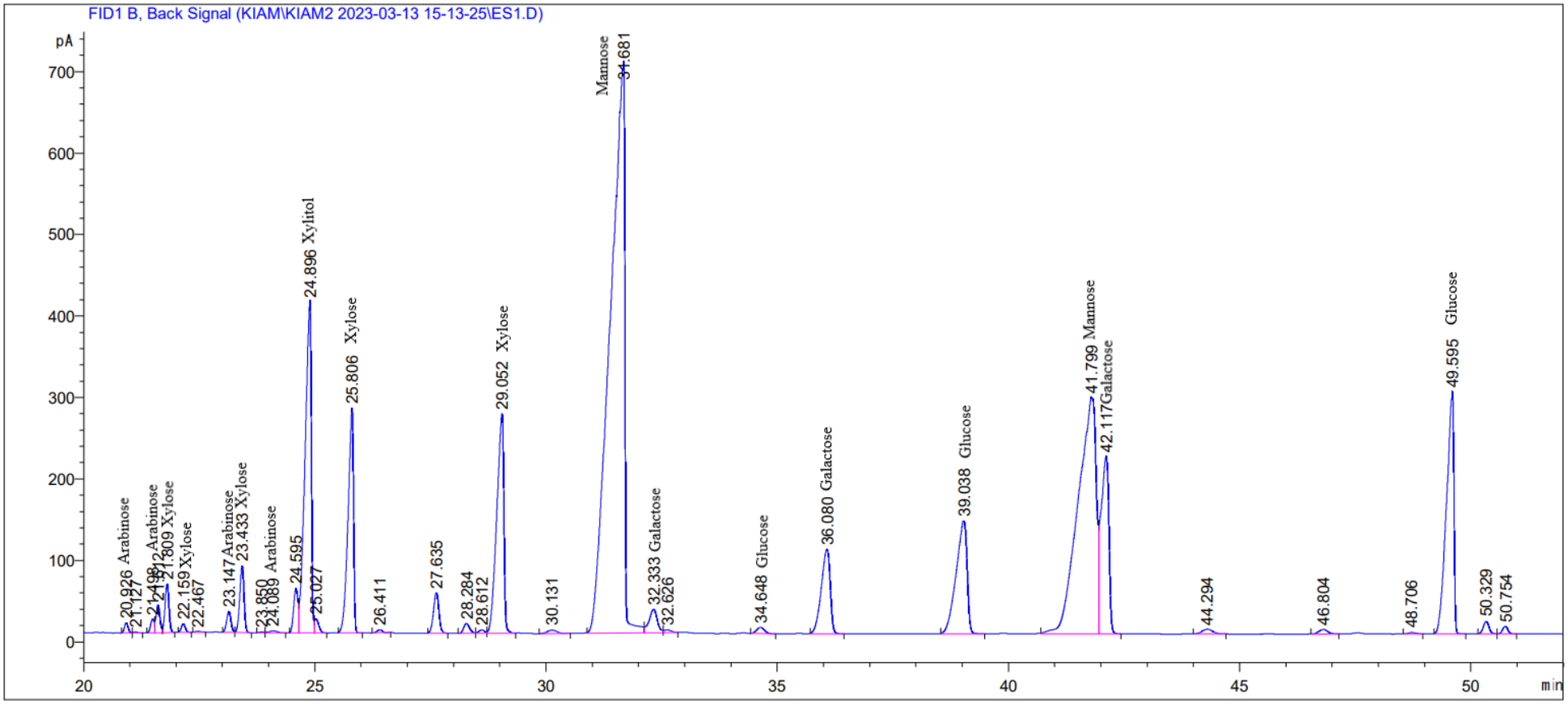
a) Hydrolysate which was used to the determination of carbohydrates by using GC.

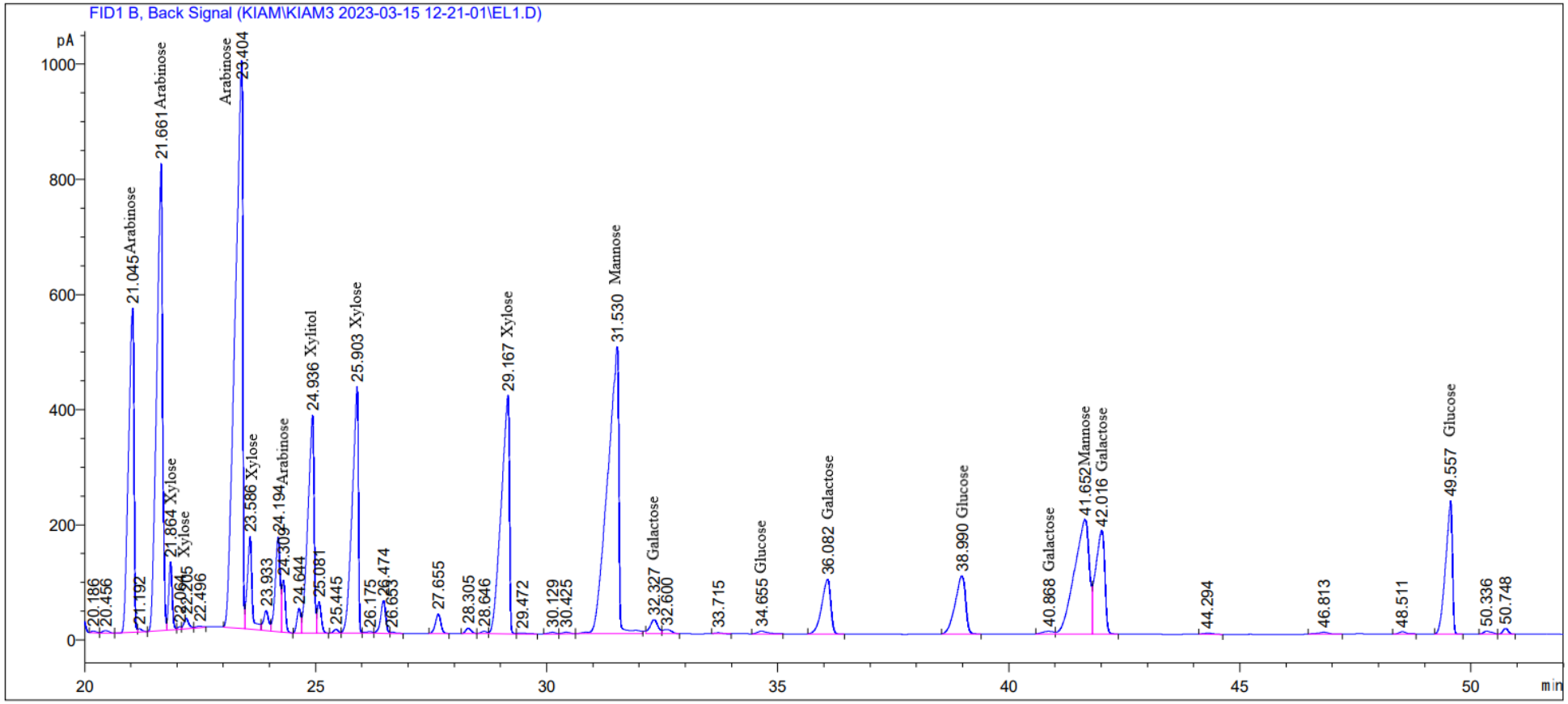
b) Diluted hydrolysate for determination of acid soluble lignin by using UV/Vis spectrophotometer.

Absorption at 205 nm		
Spruce	**Birch**	**Wheat straw**
0.6761	0.5037	0.5734
0.6748	0.5046	0.5743
0.6754	0.5058	0.5777
0.6748	0.5021	0.5748
0.6791	0.5050	0.5753
0.6791	0.5054	0.5767
AVERAGES		
0.6766	**0.5044**	**0.5854**

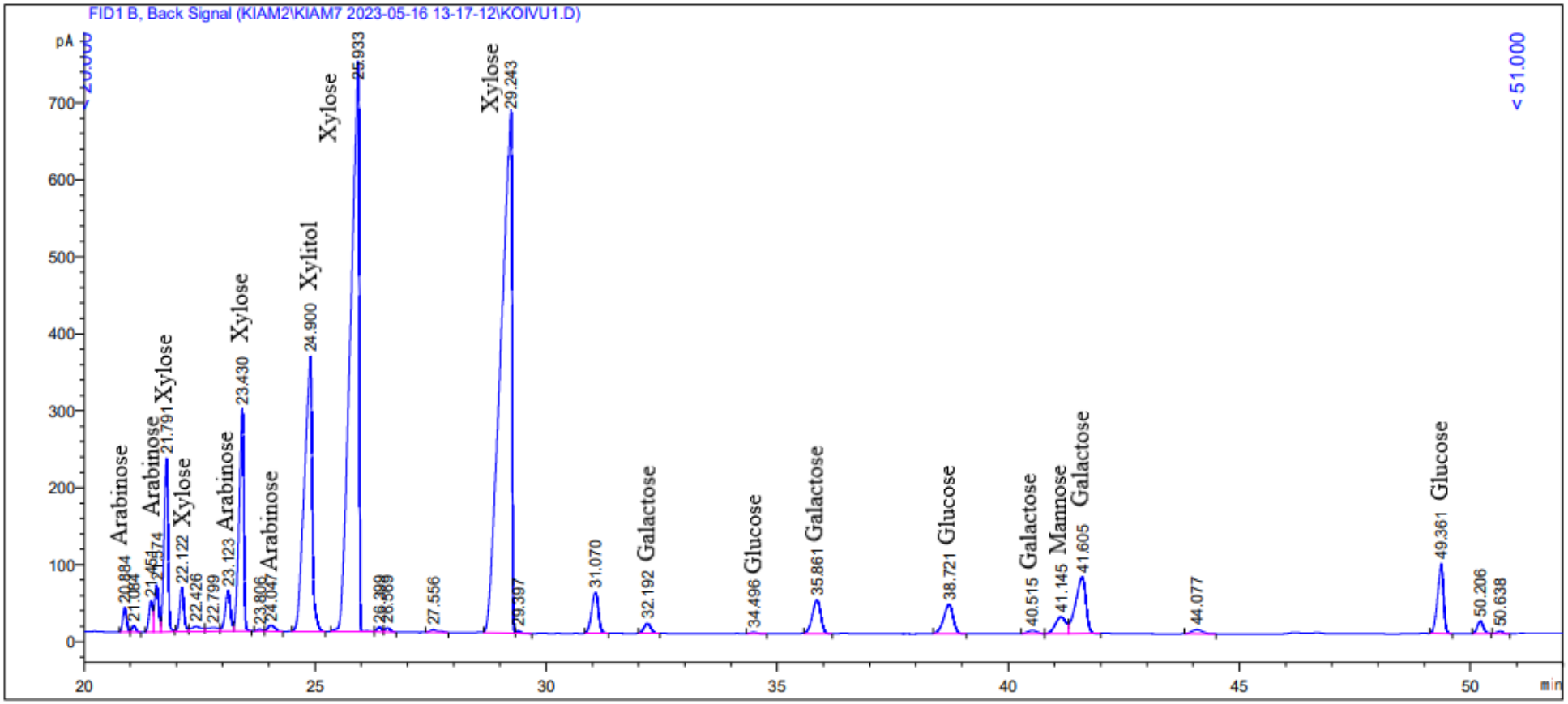
APPENDIX 5


FID1 B, Back Signal (KIAM2\KIAM6 2023-05-15 13-37-07\KUUSI1.D)
pA
< 51.000
20.881 Arabinose
21.068
21.455 Arabinose
21.743 Xylose
22.097 Xylose
22.406
23.121 Arabinose
23.369 Xylose
23.783
24.019 Arabinose
24.520
24.821 Xylitol
24.948
25.294
25.718 Xylose
26.319
27.517
28.205
28.514
28.936 Xylose
29.998
31.458 Mannose
32.184 Galactose
34.488 Glucose
35.907 Galactose
38.832 Glucose
41.537 Mannose
41.828 Galactose
44.063
46.628
49.426 Glucose
50.205
50.618
min

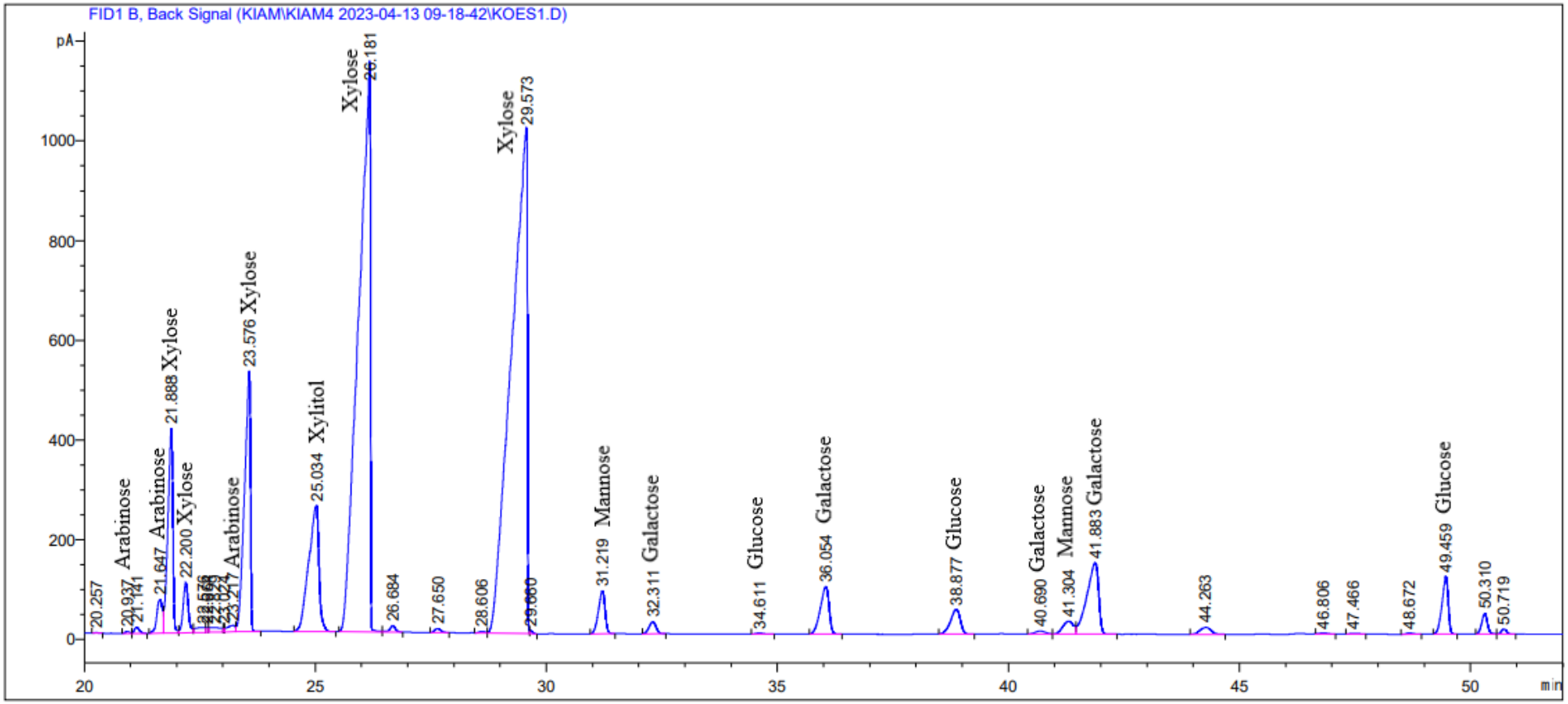

FID1 B, Back Signal (KIAM\KIAM2 2023-03-13 15-13-25\ES1.D)
pA
700
600
500
400
300
200
100
0
20
25
30
35
40
45
50
min
20.926 Arabinose
21.127
21.809 Xylose
22.159 Xylose
22.467
23.147 Arabinose
23.433 Xylose
24.089 Arabinose
24.595
24.896 Xylitol
25.027
25.806 Xylose
26.411
27.635
28.284
28.612
29.052 Xylose
30.131
Mannose
31.681
32.333 Galactose
32.626
34.648 Glucose
36.080 Galactose
39.038 Glucose
41.799 Mannose
42.117Galactose
44.294
46.804
48.706
49.595 Glucose
50.329
50.754

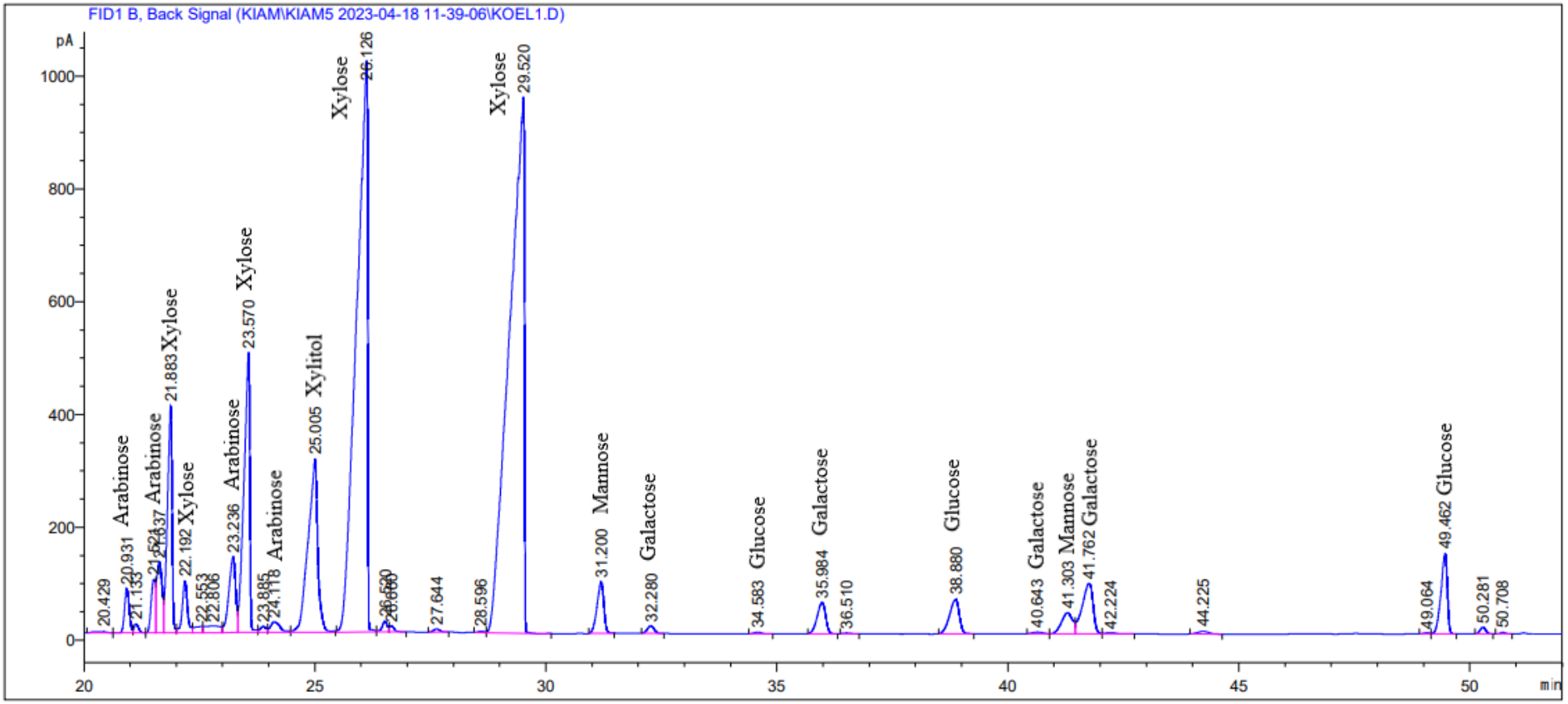

FID1 B, Back Signal (KIAM\KIAM3 2023-03-15 12-21-01\EL1.D)
pA
1000
800
600
400
200
0
20
25
30
35
40
45
50
min
20.186
20.456
21.045 Arabinose
21.192
21.661 Arabinose
21.864 Xylose
22.064 Xylose
22.496
23.404 Arabinose
23.586 Xylose
23.933
24.194 Arabinose
24.309
24.644
24.936 Xylitol
25.081
25.445
25.903 Xylose
26.175
26.474
26.653
27.655
28.305
28.646
29.167 Xylose
29.472
30.129
30.425
31.530 Mannose
32.327 Galactose
32.600
33.715
34.655 Glucose
36.082 Galactose
38.990 Glucose
40.868 Galactose
41.652 Mannose
42.016 Galactose
44.294
46.813
48.511
49.557 Glucose
50.336
50.748

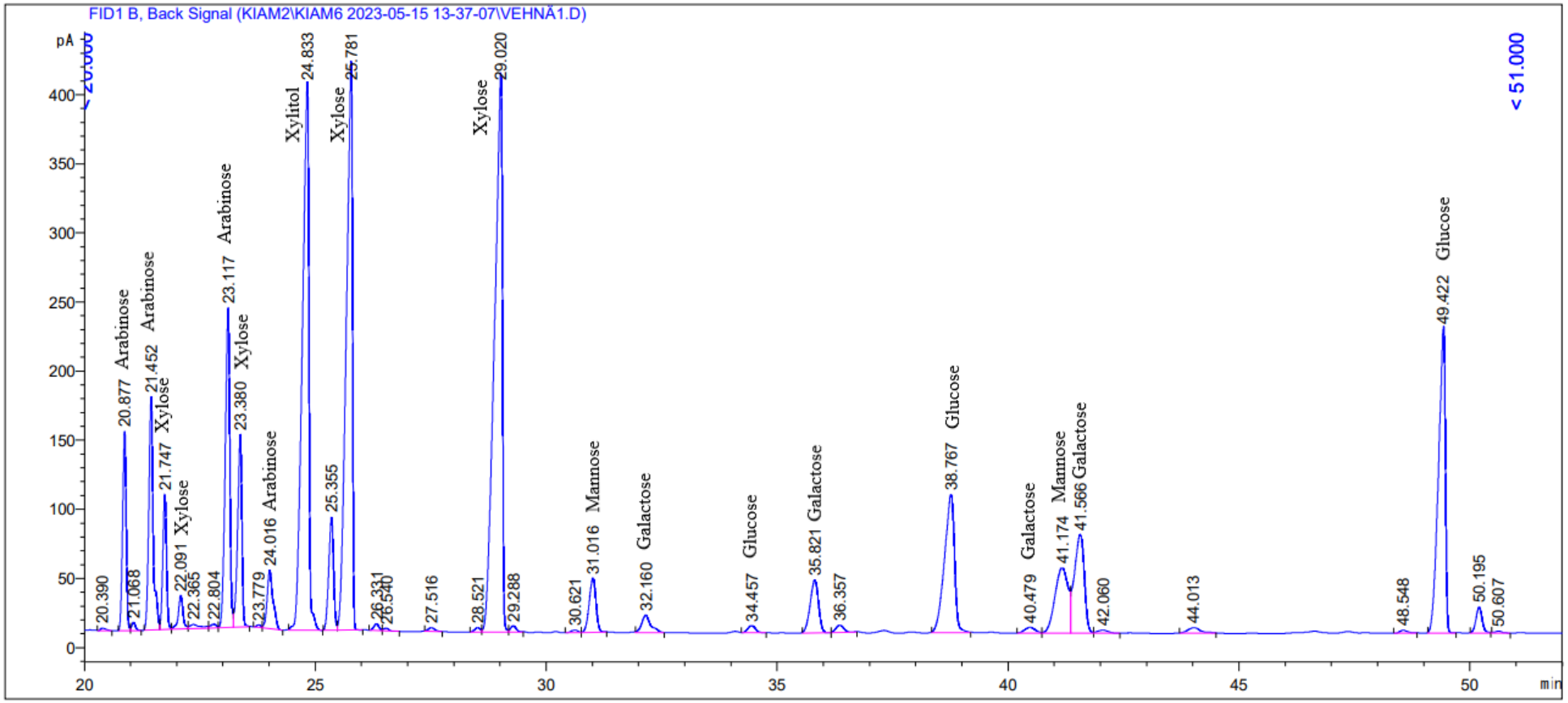
APPENDIX 8

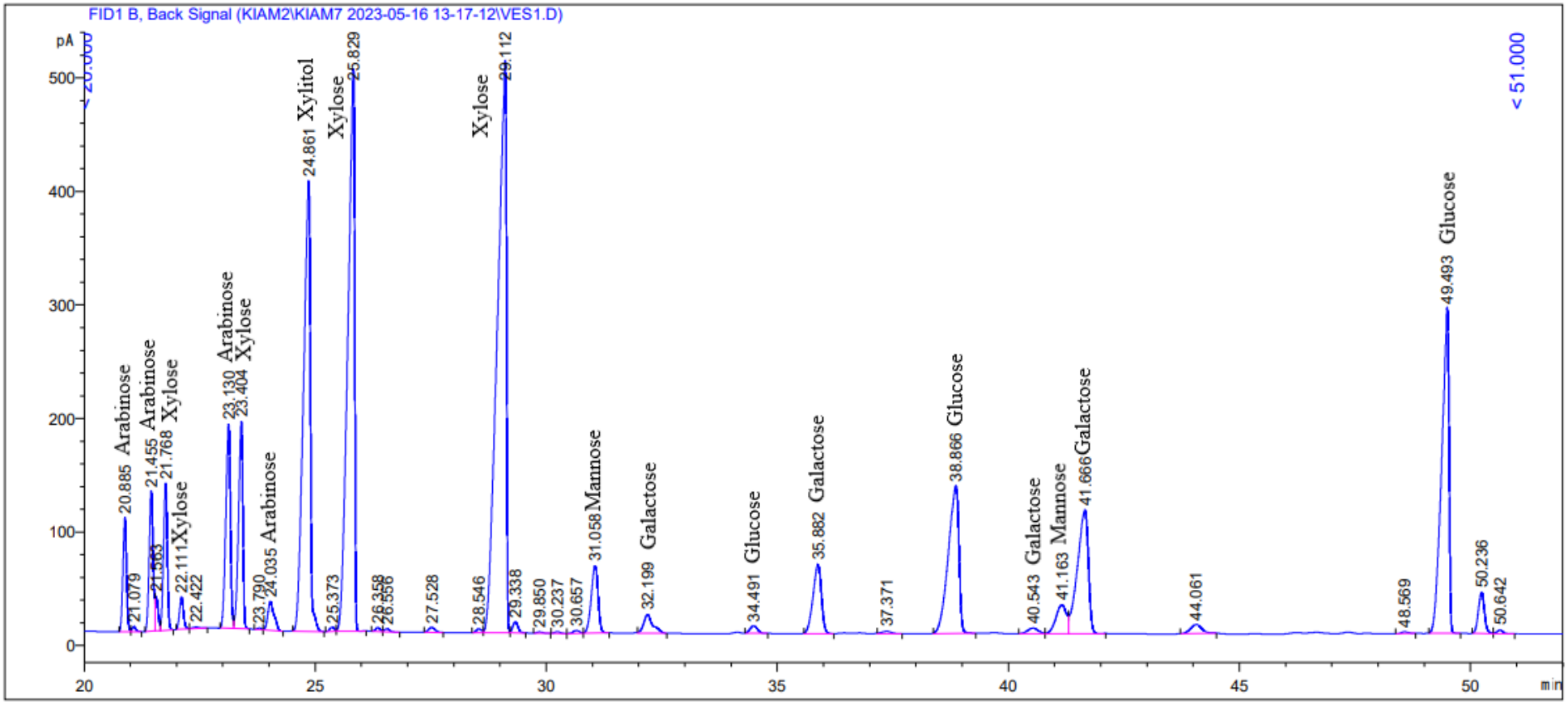

FID1 B, Back Signal (KIAM2\KIAM7 2023-05-16 13-17-12\KOIVU1.D)
pA
< 51.000
Arabinose
Arabinose
Xylose
Xylose
Arabinose
Xylose
Arabinose
Xylitol
Xylose
Xylose
Galactose
Glucose
Galactose
Glucose
Galactose
Mannose
Galactose
Glucose
20.884
21.084
21.374
21.791
22.122
22.426
22.799
23.123
23.430
24.047
24.900
25.933
27.556
29.243
29.397
31.070
32.192
34.496
35.861
38.721
40.515
41.145
41.605
44.077
49.361
50.206
50.638
min

APPENDIX 9

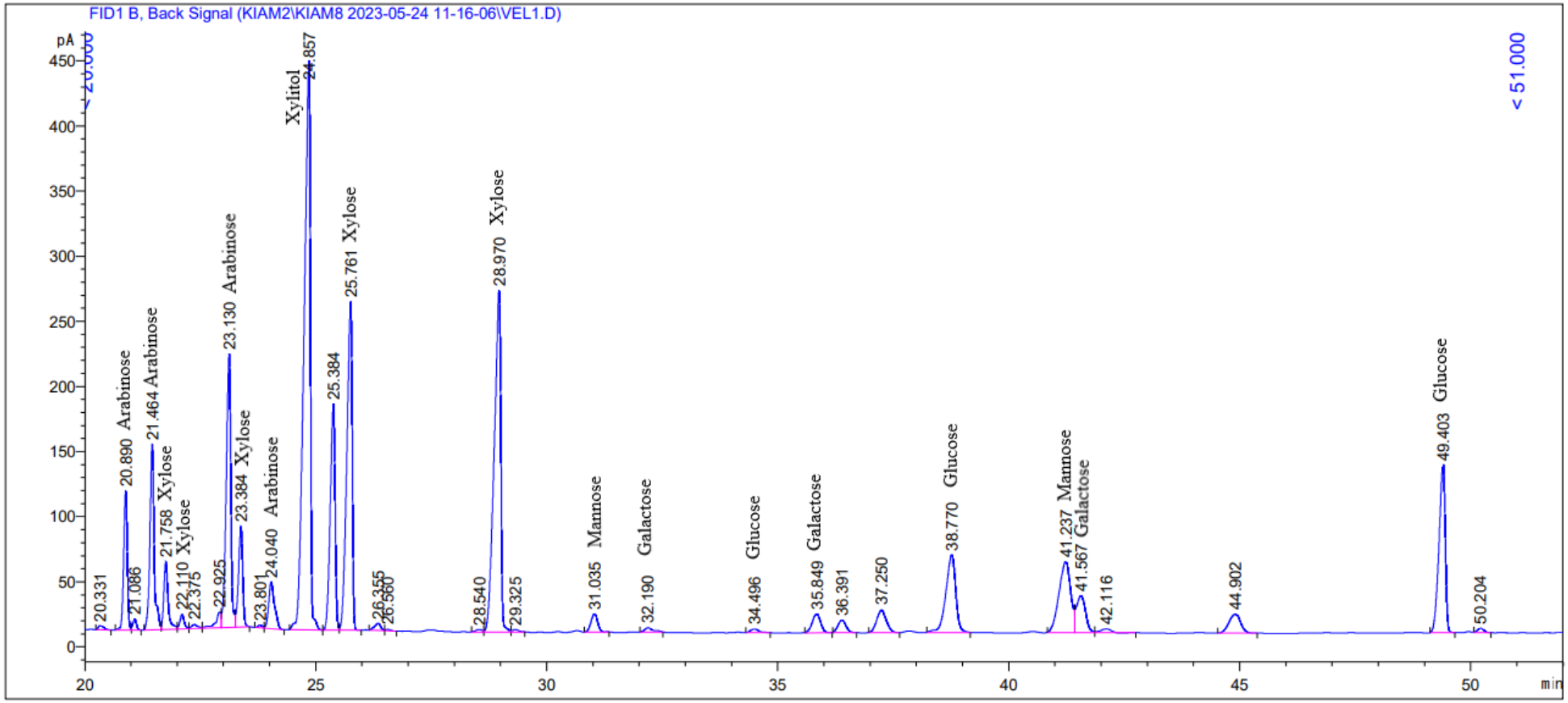

FID1 B, Back Signal (KIAM\KIAM4 2023-04-13 09-18-42\KOES1.D)
pA
1000
800
600
400
200
0
20.257
20.937 Arabinose
21.141
21.647 Arabinose
21.888 Xylose
22.200 Xylose
23.217 Arabinose
23.576 Xylose
25.034 Xylitol
26.181 Xylose
26.684
27.650
28.606
29.573 Xylose
31.219 Mannose
32.311 Galactose
34.611 Glucose
36.054 Galactose
38.877 Glucose
40.690 Galactose
41.304 Mannose
41.883 Galactose
44.263
46.806
47.466
48.672
49.459 Glucose
50.310
50.719
20
25
30
35
40
45
50
min


FID1 B, Back Signal (KIAM\KIAM5 2023-04-18 11-39-06\KOEL1.D)
pA
1000
800
600
400
200
0
20.429
20.931
Arabinose
21.133
21.524
21.637
Arabinose
21.883
Xylose
22.192
Xylose
22.553
22.806
23.236
Arabinose
23.570
Xylose
23.885
24.118
Arabinose
25.005
Xylitol
26.126
Xylose
26.530
26.680
27.644
28.596
Xylose
29.520
31.200
Mannose
32.280
Galactose
34.583
Glucose
35.984
Galactose
36.510
38.880
Glucose
40.643
Galactose
41.303
Mannose
41.762
Galactose
42.224
44.225
49.064
49.462
Glucose
50.281
50.708
20
25
30
35
40
45
50
min


FID1 B, Back Signal (KIAM2\KIAM6 2023-05-15 13-37-07\VEHNA1.D)
pA
< 51.000
20.390
20.877 Arabinose
21.068
21.452 Arabinose
21.747 Xylose
22.091 Xylose
22.365
22.804
23.117 Arabinose
23.380 Xylose
23.779
24.016 Arabinose
24.833 Xylitol
25.355
25.781 Xylose
26.331
26.540
27.516
28.521
29.020 Xylose
29.288
30.621
31.016 Mannose
32.160 Galactose
34.457 Glucose
35.821 Galactose
36.357
38.767 Glucose
40.479 Galactose
41.174 Mannose
41.566 Galactose
42.060
44.013
48.548
49.422 Glucose
50.195
50.607
min


FID1 B, Back Signal (KIAM2\KIAM7 2023-05-16 13-17-12\VES1.D)
pA
< 20.000
< 51.000
20.885 Arabinose
21.079
21.455 Arabinose
21.768 Xylose
22.111 Xylose
22.422
23.130 Arabinose
23.404 Xylose
24.035 Arabinose
23.790
24.861 Xylitol
25.373
25.829 Xylose
26.358
26.556
27.528
28.546
Xylose 29.112
29.338
29.850
30.237
30.657
31.058 Mannose
32.199 Galactose
34.491 Glucose
35.882 Galactose
37.371
38.866 Glucose
40.543 Galactose
41.163 Mannose
41.666 Galactose
44.061
48.569
49.493 Glucose
50.236
50.642
0
100
200
300
400
500
20
25
30
35
40
45
50
min

APPENDIX 13


FID1 B, Back Signal (KIAM2\KIAM8 2023-05-24 11-16-06\VEL1.D)
pA
< 51.000
20.331
20.890 Arabinose
21.086
21.464 Arabinose
21.758 Xylose
22.110 Xylose
22.375
22.925
23.130 Arabinose
23.384 Xylose
23.801
24.040 Arabinose
24.857 Xylitol
25.384
25.761 Xylose
26.355
26.560
28.540
28.970 Xylose
29.325
31.035 Mannose
32.190 Galactose
34.496
35.849 Glucose
36.391 Galactose
37.250
38.770 Glucose
41.237 Mannose
41.567 Galactose
42.116
44.902
49.403 Glucose
50.204
min