Imperial College London

DESIGN OF A HYBRID ORGANOSOLV-IONOSOLV LIGNIN FRACTIONATION METHOD AND LIGNIN-LIKE POLYMER SYNTHESIS FOR VALUE-ADDED APPLICATIONS

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Thesis submitted for the degree of Doctor of Philosophy PhD Department of Chemical Engineering Imperial College London

October 2019

Declaration of Originality

The research work conducted and presented in this thesis was carried out at Imperial College London from November 2015 to October 2019. The work was conducted by myself, unless otherwise stated, and has not been submitted for a postgraduate doctoral degree at this or any other university.

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Publications

The research work related to lignin fractionation has been presented in the peer-reviewed literature.

- C. L. Chambon[1†], M. Chen[1†], P. S. Fennell[1] and J. P. Hallett[1]* Efficient Fractionation of Lignin- and Ash-rich Agricultural Residues Following Treatment with a Low-Cost Protic Ionic Liquid, *Frontiers in Chemistry*, 2019 † Clementine L. Chambon and Meng Chen are joint first authors and contributed equally to this work. (published)
- M. Chen , F. Malaret, A. E. J. Firth, A. R. Abouelela, Y. Chen, J. P. Hallett* Design of an Organosolv-ionoSolv biomass fractionation process for biofuel production and high value-added lignin valorisation, *Green Chemistry*, 2019 (ready for submission)
- M. Chen , A. E. J. Firth, Y. Chen, J. P. Hallett* Integrated fractionation of lignin- and ash-rich agricultural residues by a hybrid pretreatment method, *Bioresource technology*, 2020 (manuscript prepared)

Acknowledgements

I would like to thank Jason for all the guidance and help. Without you, I will not be able to start and finish the PhD. Thank you for being on my side all the time. Thank you for giving me the freedom to do what I want. Thank you for always being there for me when I am in trouble. I am so grateful for you to accept the fact that I am always the NAUGHT one. It is my honour to be one of your students. Also, I would like to thank Agi, you were like mum to me when I was in the first year, you were always so kind, patient and caring. I would like to thank all the people I worked with: Clem, Francisco, Adam, Yiyan and Anton. I have learnt so much from you guys. My research work would not be completed without your effort. Big thanks to Anton. I am so appreciated that you could proofread my staff whenever I need you to. I think we have made a good team and it was so fun having my seat next to yours in the office.

I would like to thank Weichien, Aida and the Bings. In the past four years, my time has been almost used up by numerous rounds of experiments, endless pretreatments. Because you guys, I managed to have some quality time outside the Bone building. I will miss the tea time with you, Weichiem. The healing trip to Switzerland was remarkable, Aida. Thank you, Bing and Lisa. Thank you for always having me when you guys are having home-made hotpot and thank you for being not angry when I could not meet you for lunch so last minute. Thank you for being there for me when I was so down. It was my honour to have you as my friend. At last, I want to say, no matter where I go, the friendship will last forever, I will chase you down!

In the end, I want to thank MYSELF. Good job for not quitting at the half way. I know you have suffered a lot, but YOU NAILED IT NOW!

Glossary

AFEX	ammonia fibre expansion
AIL	acid insoluble lignin (Klason lignin)
APIL	aprotic ionic liquid
ASL	acid soluble lignin
BTX	benzene, toluene, xylene
[Bmim]	1-butyl-3-methylimidazolium
[BF_4]	tetrafluoroborate anion
CEL	cellulolytic enzyme lignin
C	carbon
CO_2	carbon dioxide
[C_2C_1im]	1-ethyl-3-methylimidazolium
[C_4C_1im]	1-butyl-3-methylimidazolium
[C_4Him]	3-butylimidazolium
[DMBA]	*N,N*-dimethylbutylammonium
DMF	*N,N*-dimethylformamide
DMSO	dimethyl sulfoxide
EtOH	ethanol
[Emim]	1-ethyl-3-methylimidazolium
EMAL	enzymatic mild acidolysis lignin
FA	ferulic acid
G	guaiacyl
GHG	greenhouse gases
GPC	gel permeation chromatography

g	gram
H	*p*-hydroxyphenyl
[HC_4im]	3-butylimidazolium cation
[HSO_4]	hydrogen sulphate anion
H_2SO_4	sulphuric acid
HMF	5-hydroxymethylfurfural
HPLC	high performance liquid chromatography
HSQC	heteronuclear single quantum correlation NMR spectroscopy
HCl	hydrochloric acid
H_2O_2	hydrogen peroxide
HRP	horseradish peroxide
IL(s)	ionic liquid(s)
L	litre
LCC	lignin carbohydrate complex
[$MeCO_2$]	acetate
MeOH	methanol
$MgCl_2$	magnesium chloride
mg	milligram
mL	millilitre
mmol	millimole
mol	mole
M_n	number average molecular weight
M_w	weight average molecular weight
NMR	nuclear magnetic resonance spectroscopy
NaOH	sodium hydroxide

NAD(P)H	Nicotinamide Adenine Dinucleotide Phosphate oxidase
NREL	National Renewable Energy Laboratory
$[OAc]^-$	acetate
PCA	p-coumaric acid
PDI	polydispersity index
PIL	protic ionic liquid
Ppm	parts per million
S	syringyl
SE	steam explosion
SRS	sugar recovery standard [TEA] triethylammonium
TGA	thermogravimetric analysis
UV	ultraviolet spectroscopy
ZL	Zulaufverfahren
ZT	Zutrophverfahren
wt%	weight percent
α	Kamlet-Taft acidity
β	Kamlet-Taft basicity
δ	chemical shift (ppm)
ε	molar extinction coefficient
π*	Kamlet-Taft polarizability
°C	degree Celsius
a/b	acid/base ratio of protic ionic liquid

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Abstract

Biofuel technology has been introduced to reduce the reliance on fossil fuels. It is also superior for addressing environmental issues, such as greenhouse gas (GHG) emissions. Biofuel production from lignocellulosic biomass has been attracting attention due to the high abundance of the feedstock and the incredible GHG emission reduction associated with the production process. Ionic liquid pretreament, the ionoSolv process, and organosolv pretreatment are well-known for their selective fractionation performance. The ionoSolv process is able to generate a highly digestible cellulose fraction and the organosolv process is famous for producing high quality lignin as the side product, where the lignin generated is suitable for value-added applications. Here, a hybrid pretreament process has been developed based on these two processes, where two protic ionic liquids and three organic solvents were the selected solvents. The new process has been tested on three classes of feedstocks, miscanthus, pine and agricultural residues. The pretreatment effectiveness was determined by enzymatic saccharification and compositional analysis. The isolated lignin fraction was subjected to HSQC and GPC analysis.

For miscanthus, ethanol/butanol-IL process was able to produce a highly digestible pulp with a glucose yield of up to 85%, 10% higher than the standard ionoSolv pulp, due to more profound lignin removal for the hybrid process. The process maintained its functionality with a range of IL acidities, 1.00 to 1.02 (acid/base) and up to 50% wt biomass loading. Similar glucose yield increases were observed for pine, rice husk and bagasse. For the process of two straws, additional hemicellulose releases were detected in enzymatic hydrolysis while the level of glucose yields remained the same as for the ionoSolv ones. HSQC NMR of the lignin indicated that α-alkoxylation took place during ethanol/butanol-IL fractionation, inhibiting lignin condensation. Three major monolignols were synthesised and radical polymerisation induced by horseradish peroxidases was conducted for the monolignols synthesised. The lignin-like polymer was analysed by GPC.

1 Introduction

In 1700s, the invention of steam engines along with the discovery of vast fossilised-plant reserves including coal, oil and natural gas, results in replacing water power with engine power in conventional manufacturing. For a long period of time, these engines had been purely relied on fossil fuels due to its vast scale of reserve and low extraction capital cost. In recent decades, the drastically rising energy demand in both industrial activities and households raised the gasoline shortage crisis. Besides the insecurity in the energy supply, the overuse of petroleum-based fuels has also brought several environmental issues, like greenhouse gas emissions (GHG).[1] This drives the society to revalue the importance of fossil fuels as an energy resource and the discovery and implementation of sustainable energy resources are encouraged, in order to reduce our reliance on fossil fuels. Among the various renewable energy production technologies, "integrated biorefinery" is believed to be one of the most promising technologies.[2,3]

Biorefinery is the term to describe a multistep biochemical process generating fuels from biomass, in order to replace petroleum-based fuels for all type of engines. The fuels derived from biomass, also referred as biofuels, are mainly used in powering vehicles[4] The first generation of biofuels, mainly bioethanol and biobutanol, have been studied in depth, subsequently their commercial-scale production have been achieved in Europe, North and South America. These biofuels can be derived from various food crops depending on the production location, e.g. corn for the US and Canada, sugarcane for Brazil, wheat for Europe and Canada.[2,5,6] Biodiesel has been commercially produced in Germany using animal fats and vegetable oils including soy oil and rapeseed oil, and also in south pacific area using palm oil.[5,7,8] Hydrous bioethanol, ethanol mixing with 5% water, has been used for powering internal combustion engines in modified and unmodified (depending on ratio of bioethanol to petrol) petrol engines.

Compared to petroleum-powered engines, the engines powered by bioethanol could function with a higher thermal efficiency, thanks to the higher octane number of the ethanol relative to gasoline, thus the bioethanol engines have better fuel economies.[4] Biomass-derived ethanol has replaced methyl tertiary butyl ether as a gasoline additive.[5] The presence of the gasoline additive is significantly reduce the carbon monoxide and hydrocarbon emissions of the engines.[9] It is reported that the US produced 15.2 billion litres of ethanol from corn merely in 2005, which corresponds to over 10% of its annual transport fuel consumption.[4] The bioethanol product in Brazil is reported in a similar scale to the US, which could supply 30% of its annual energy demand for transportation. [10 11 12] It is worth mentioning that these production scale have kept rising with an incredible annual increase rate, ≥10%, ever since. Biodiesels are thought to be non-toxic and biodegradable, and are suitable alternative to petroleum-based diesel. The commercialisation of biodiesels is even earlier than bioethanol and the first commercial-scale plant was put to use in 1989.[13]

Despite the upsides coming along with the utilisation of the first (1st) generation biofuels, it is still not the perfect solution to replace fossil fuel for power. Compared to fossil fuels, 1st generation biofuels could only effectively reduce greenhouse gas emission up to 50% due to high energy input, not enough to eliminate the environmental problems associated with the usage of fossil fuels, e.g. climate change. [1,4,9,10] Moreover, the production process of biofuels raised many other issues, e.g. high feedstock capital cost, inevitable high labour requirement, water and soil pollution for the local area, insecurity of the local food supply for selective regions. [8,14,15,16,17] This makes 1st generation biofuels less environmentally-friendly and cost-effective, consequently driving the introduction and integration of 2nd generation biofuel technology, in which the biofuels are no longer derived from food crops but cellulosic biomass.[3]

The first (1st) generation biofuels are usually converted from food crops, e.g. corn, sugarcane, wheat, while 2nd generation biofuel production uses inedible plants, lignocellulosic biomass, which consists of various feedstocks with incredible availability wordwide.[3,18] Feedstocks commonly studied for 2nd generation biofuel technology are fast growing-energy crops, agricultural residues, municipal wastes and algae.[5,8,19,20,21] The production of these feedstocks generally require low capital costs, and some are referred to as waste, currently not being used for value added applications. Europe's biomass production capacity reached 190 million tons of oil equivalents in 2010, and is expected to double by 2030, which will be sufficient to replace all the fossil fuel usage in the chemical industry.[22] Replacing food crops with lignocellulosic biomass in biofuel production not only relief the issue of low insecurity for food supply, but also can reduce the greenhouse gas (GHG) emission to a further extent, i.e. cellulosic ethanol could displace 90% fuel-based CO_2 emissions, while the emission reduction achieved by corn ethanol is less than 20%.[4,8,22]

Although a massive amount of work has been done to develop lignocellulosic biofuel technology, the technology has not been mature enough for industrialisation. The bottleneck for commercialisation is to convert cellulosic biomass into biofuels, meanwhile generating high quality side products (hemicellulose and lignin) at an industrial-scale, with reasonable capital and operating costs. Compared to food crops, cellulosic biomass has increased complexity in term of structure and composition, which subsequently requires an additional step to pretreat (deconstruct) the feedstock before being subjected to hydrolysis and fermentation processes.[3,8] This deconstruction process is named pretreatment, where the process cost occupies a significant portion, ca 20%, in the overall capital and operating expense for the biorefinery. Reasons for this relative high cost are higher energy consumption, increased process complexity as well as higher enzyme consumption, relative to 1st generation biofuel production.[3,23] Therefore, integration of the pretreatment is crucial to

commercialise cost-effective 2nd generation biofuel technology. Fig 1.1 described the major steps involved in both 1st and 2nd biofuel generation process.

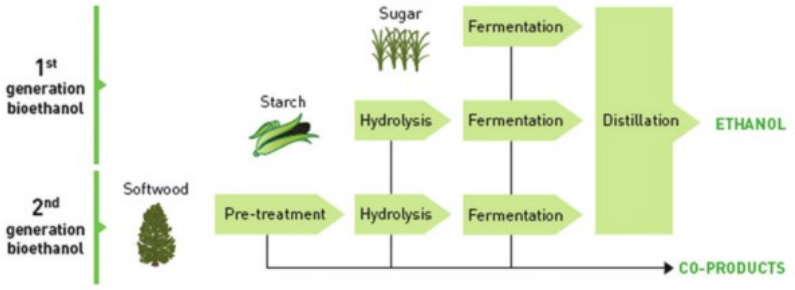


Figure 1. 1 Biorefinery processes for 1st and 2nd generation biofuels[3]

Lignocelluloses biomass mainly consists of cellulose, hemicellulose and lignin, where the sum of these three components' dry weight accounting for more than 90% of the biomass weight.[3,8,23] Cellulose and hemicelluloses' valorisation are developed in depth, and several decent reviews have been published for this topic.[24,25] The fermentation and hydrogenation products of glucose, xylose originated from cellulose and hemicellulose, have been reviewed in details, listed in Figure 1.2.[24] Some of these chemical applications have been commercialised and are becoming popular in both chemical and material industries. However, this is not the case for lignin.

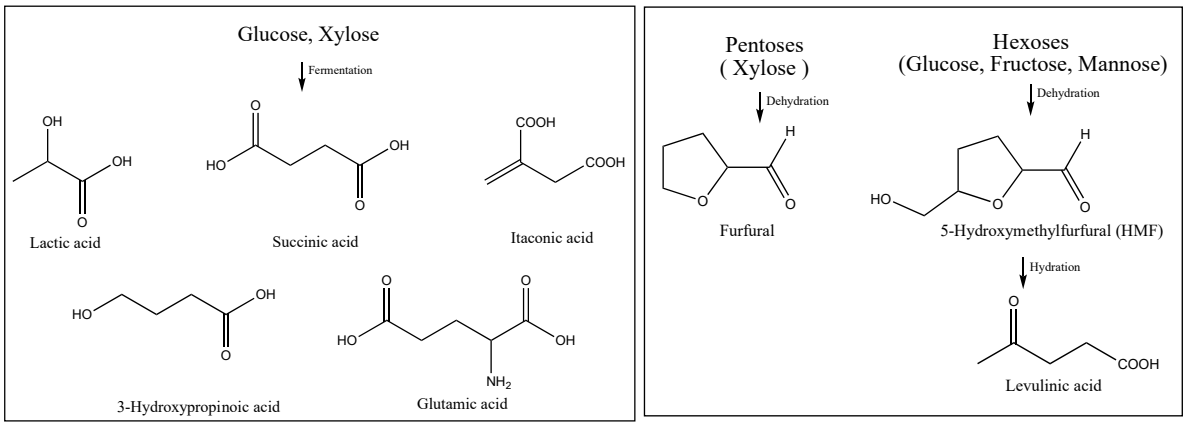


Figure 1. 2 Major platform chemicals produced from cellulose and hemicellulose (Left: the chemicals produced by sugar fermentation; Right: chemicals produced by sugar hydrogenation)[26]

Although lignin is the naturally occurring aromatic polymeric resource with the highest abundance worldwide, little attention has been drawn to its value-added applications. In 2004, lignin production reached 50 million tons merely in the pulp and paper industry, where the production increased to 70 million tons by 2010.[27,28] Only 2% was developed for low value-added applications, such as vanillin where its market is quite narrow. The most common utilisation for lignin is directly subjected to open field burning for heat and electricity production.[26] This is largely due to the isolated lignin structure not being ideal for high value applications, and further modification is needed. Among the lignins generated from different biomass fractionation processes, only Kraft lignin and lignosulphonates are commercially produced.[29] The world's largest Kraft lignin producer is MeadWestvaco, owned by Metso Corporation, able to produce Kraft lignin from black liquor on a commercial scale, the socalled Lignoboost process.[30,31] There is a rising need for generating aromatic-based chemicals or material from renewable resource, instead of unsustainable resources, such as petroleum. Resultantly, the development of lignin extraction technologies are urgently needed, as the overall pretreatment cost could effective reduced if the lignin fraction's economical value was increased.

As lignin has a more complex structure than carbohydrates and it has more diverse connections with other molecular species in the cell wall, lignin removal in the pretreatment is rather complex. Insufficient lignin removal will hinder the carbohydrate hydrolysis.[26,32] Therefore, effective lignin removal without chemically altering the lignin structure in a undesired way is necessary for an ideal pretreatment technology. Apart from improving current lignin isolation technologies, solving this issue in a genetic approach has also been suggested.[33] Specifically, by understanding the natural lignin biosynthesis pathway, the biomass gene is altered in order to produce modified biomass with lower lignin content. Lignin-like polymers can also be synthesized according to the biosynthesis pathway, which can have a great potential in industrial valorization.[34,35,36]

This thesis was separated into two parts. First part was integrating current pretreatment technologies. A new hybrid process was developed by incorporating the idea of an organosolv pretreatment with a ionic liquid pretreatment, named organosolv-ionoSolv pretreatment. A better lignin extraction was expected to be achieved, relative to current level of pretreatment technology, such as ionoSolv process, and the lignin extracted from this hybrid process was expected to have a higher quality, with a less condensed polymeric structure which can be benefit for its development in high value-added valorizations. The second part was: current level of technical lignins are not ideal for high value added applications, and cannot be utilized without any chemical modifications, but these lignins could be easily depolymerized into small aromatic fragments. A questions is raised at this point and yet to be answered: could we reproduce a polymeric material in a controllable manner using these aromatic fragments? A test was conducted to produce lignin-like polymers from small aromatic molecules, more specifically, lignin model compounds. The major lignin monomers, three phenylpropanoid alcohols, were synthesized from the commercial-available cinnamic acids. Enzymatic polymerizations were conducted and the polymers synthesized were characterized, to confirm whether we could produce some lignin-like polymers with desired physical/chemical properties by manipulating the polymerization conditions.

1.1 Literature background

1.1.1 Lignocellulosic feedstocks

The three predominate components of lignocellulosic biomass are two carbohydrate polymers (cellulose and hemicellulose) and one random aromatic polymer (lignin), detailed in Figure 1.3.[8] The homogenous polymer, cellulose, which is made up by glucose only, packs into microfibrils; the branched polymers, hemicellulose, which is made of pentoses, hexoses and sugar acids, cover the surface of microfibrils though non-covalent bonds; after decoration with hemicellulose, these

microfibrils are then assembled together via crosslinking with lignin, a random amorphous polymer which consists of several aromatic monomers. [25,37,38,39]

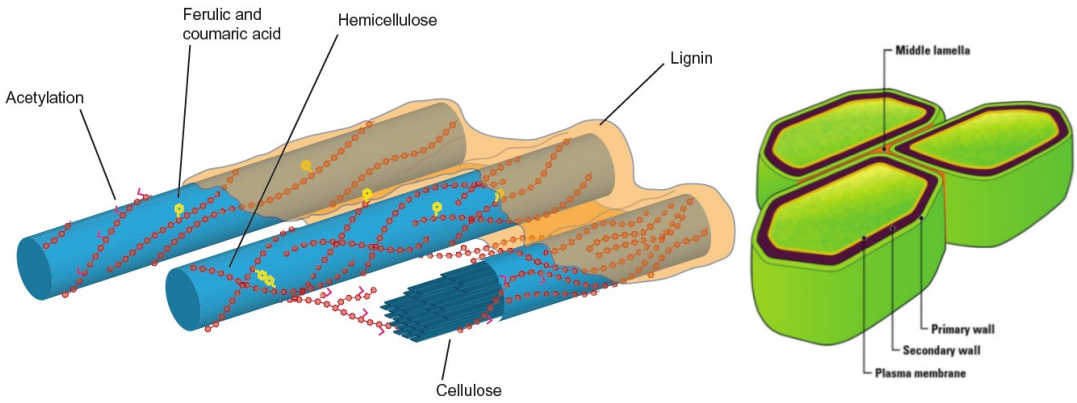


Figure 1. 3 Microstructure and ultrastructure of a lignocellulosic cell wall3 (Left: the 3-D structure of cellulose, hemicellulose and lignin inside the cell wall; Right: the arrangement of cells, cell wall and middle lamella inside a plant) [40]

The exact composition of the lignocellulosic biomass differs from type to type, and also varies with the growth conditions and locations. [40] Any changes in the biomass's composition could directly affect the overall results of biorefinery, which subsequently changes the type of platform chemicals and biofuels derived from the process. Lignocellulose can be grouped in many ways. They can be classified as virgin biomass (trees), industrial and agricultural waste (corn stover, saw mill, rice husk, rice straw, wheat straw), or dedicated energy crops (switch grass). [8,41,42,43,44] However, lignocellulose can also be catalogued into softwoods (pine, fir, spruce), hardwoods (polar, willows) and grasses (perennial ones: miscanthus, switchgrass; non-perennial ones: corn stalks, sugar cane bagasse, straw), by judging their actual compositions.[3,5,45,46]

Plant cell wall is composed of lignocellulosic tissues, which are light and porous.[47] These tissues enhance the cell wall structure in terms of stiffness.[47] Within the cell wall, elongated cells create channels to deliver water and nutrients throughout the plant. These channels determine the speed at

which the water and nutrients are diffused within the lignocellulosic tissues during the biomass fractionation process, detailed in Figure 1.3.[3] Two adjacent cell walls are isolated from each other by a lumen, named the middle lamella. This middle lamella mainly consists of cellulose and hemicellulose. With the increasing maturity of the lignocellulosic tissue, the lignin content of this lumen rises as well.[3]

1.1.1.1 Cellulosic biomass component: cellulose

Cellulose is the most important biomass component, as it could be used to generate bioethanol and other biofuels via a biorefinery. Among all the components in the cell wall, cellulose is the one with highest weight percentage, 35% to 50 % (depends on the biomass type).[23] For grassy feedstock, miscanthus, cellulose content was 37-45%, 25-42% for pine, and 36-53% for agricultural waste like rice husk.[40,48] Cellulose has a linear homomeric-polymer structure and mainly facilitates the frame work of the cell wall. Its monomers, glucose units, are linked together via 1-4 β glycosidic linkages. These glycosidic linkages result in a planar stretched chain conformation for all cellulosic polymer chains. The polymer chains have a degree of polymerization ranging from 15000 (cotton) to 36000 (line).[8,49]

Covalent bonds, hydrogen bonds and the Van der Waals interactions within or between cellulosic polymer chains, make cellulosic microfibrils crystalline and insoluble in water and many other solvents. For each of the glucose monomers, it forms intra-molecular hydrogen bonding with the two adjacent monomers on the same polymer chain, and also bonds with the adjacent polymer chain which is in the same plane, via hydrogen bonding. All the polymeric planes are connected to each other via van der Waals interactions. In the perspective of cell wall ultrastructure, cellulose fibres are formed by microfibrils assembled by 20 to 300 cellulose polymer chains.[3] The hydrogen bonding within a microfibril contributes into the straightness of the cellulose fibre, while the same type of interactions in between microfibrils determine the crystalline and amorphous areas of the cellulose. Cellulose

fibres are separated out in the deconstruction step (pretreatment), and subsequently hydrolyzed into glucose, which are subsequently fermented into biofuels, which are currently the most important products of the biorefinery.

The primary structure (monomers and linkages between monomers) of the cellulose fibers are the same, but the hydrogen bonding and the van der Waals interactions between cellulosic polymers chains defines the secondary structure of the cellulose fibers, where can be different and allow the fibers to have 6 different secondary structures.[3,8,50] Cellulose I is the natural occurring form of cellulose, which could be converted into a more thermally stable form, Cellulose II, via mercerization or dissolution (followed with regeneration), detailed in Figure 1.4. Cellulose III_I and Cellulose III_{II} could be generated from Cellulose I and II, via chemical treatments. The last two types, IV_I and IV_{II}, of cellulose are formed from Cellulose III_I , III_{II} , by thermal treatment in the solvent medium, glycerol.

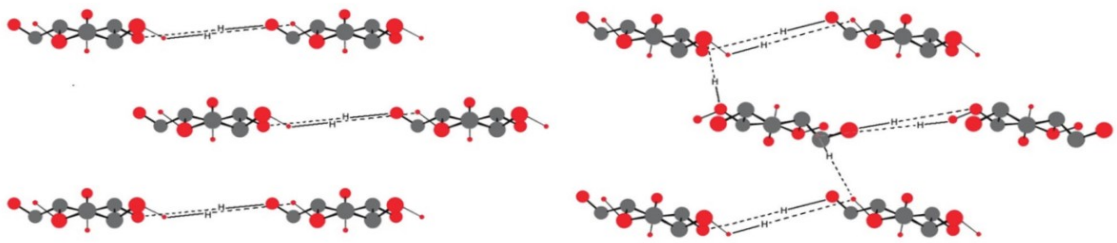


Figure 1. 4 molecular arrangements of Cellulose I (right)and Cellulose II (left)[3]

1.1.1.2 Cellulosic biomass component: hemicellulose

Hemicellulose, the second largest single component of cell walls, generally occupies 20 to 25 wt% of dry biomass. It connects to the cellulosic microfibrils via non-covalent interactions, and acts as a structural matrix providing stiffness to cellulose fibrils. It is an amorphous heterogeneous polymer,

and is linear and branched in the same time, with a lower degree of polymerization , typically ranging from 100 to 200, relative to cellulosic polymers.[3,8]

Hemicellulose often consists of a range of pentose (xylose, arabinose) and hexose (mannose, glucose, galactose) sugar monomers. Some of these monomers are coated with acetyl and methyl groups, and these acetylated monomers are named as glucuronic, galacturonic and cinnamic acids.[3] The appearance of these acetylated sugar units strengthens the hemicellulose's affinity towards lignin, binding cellulose and hemicellulose with lignin more tightly.

The actual monomer compositions vary with biomass feedstocks, which directly leads to require different conditions for the effective hemicellulose fractionation. Generally, hemicellulose contains two or more of those pentose and hexose sugar units, listed in Figure 1.5. In woody feedstocks (softwood and hardwood), xylans as well as glucomannans made up the hemicellulose polymer chains. Xylan is the second most abundant carbohydrate units within biomass (glucose is the first abundant), where it occupies up to 30% of dry mass for hardwood. For softwood, two forms of xylan appear as :1) arabinoglucuronoxylans, named as arabino-4-O-methylglucuronoxylans with a degree of polymerization, 70 to 130, and this xylans usually take up 5 to 10% of dry mass; 2) galactoglucomannans, also named as O-acetyl-galactoglucomannans, which is the dominant xylan type and occupies up to 25% of dry mass.[51,52] For grasses and hardwood, arabino-4-O-methylglucuronoxylans was the predominant type of xylan. For grassy xylan, arabinofuranosyl side chains are crosslinked with the main xylan chains. These differences in the hemicellulose structure result in different level of ease to fractionation (extract) hemicellulose during pretreatment, softwood appears to be the most difficult feedstock type to achieved effective hemicellulose fractionation.[37]

For grasses, hemicellulose is composed of xylose, arabinose and glucuronic acid, whereas sorftwood hemicellulos is made of mannose (80%) and gluclose (20%).[37] Rice-related hemicellulose usually consists of 46% xylose, 45% arabinose, 6% galactose, 2% glucose and its anhydrouronic acid content is around 1%.[37] For wheat, 66% xylose, 34% arabinose made up the hemicellulose of the biomass, accompanied with traces of mannose, galactose and glucose.[37]

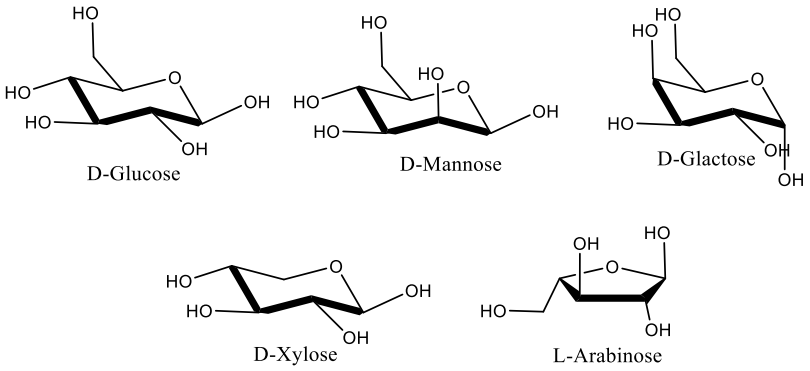


Figure 1. 5 Typical sugar monomers found in hemicellulose (Top: hexose monomers; Bottom: pentose monomers) [3]

Due to its amorphous nature, hemicellulose can easily undergo hydrolysis even under mild conditions, but the process is significantly disrupted by lignin carbohydrate complexes (LLC), which also hinder the cellulose hydrolysis.[53,54,51] These complexes are made up by binding lignin fragments to the hemicellulose polymers though various bonds, detailed in lignin section. Apart from this, hemicellulose also degrades while being fractionated under harsh conditions, forming furfurals and hydroxyl methyl furfurals (HMF).[26] Thus, the conditions of the biorefinery, especially pretreatment, need to be carefully designed in order to avoid these issues.

It is important to note that hemicellulose recovered from the pretreatment could be hydrolyzed and fermented to produce bioethanol and other biofuels, or modified to yield value added chemicals, such as polyols, xylitol, yeast extract, levulinic acid, furfural, and some other aromatic platform chemicals.[55,56,57,58]

1.1.1.3 Cellulosic biomass component: lignin

1.1.1.3.1 Lignin structure and composition

The third abundant component within the lignocellulosic biomass, lignin's composition varies with feedstock: a typical lignin content of softwood is 30 wt%, while hardwood has a lower lignin content, 20 - 25 wt%; lignin content is generally even lower for grasses, 10- 15 % of dry mass, but there is some exceptions, such as miscanthus, which has a lignin content of more than 20wt%; for those rice/wheat/bagasses related agricultural residues, their lignin contents range from 8 to 23.[26,32,43,59,60] Lignin's polymeric structure is built up by a few aromatic monomers, also named as monolignols, and is highly crosslinked and hence amorphous. It enhances the cell wall structure in a few aspects:1) it acts as a "glue", sticking the carbohydrate and non-carbohydrate components (of the biomass) together and provide an additional stiffness to the cell wall; 2) it is water insoluble in nature, therefore its hydrophobicity promotes an efficient transportation of water and nutrients via cell wall within the plant; 3) It also acts as a call-wall protector, largely reducing the damage of the cell wall due to the attack from pathogens and insects.[26]

Compared to cellulose and hemicellulose, lignin has not been studied in depth due to its more complex nature. Until now, a full image of lignin's internal structure and composition cannot be drawn. However, according to the investigations done in the past, it is certain that lignin is mainly assembled by the three phenylpropanoid alcohols via an enzymatic random radical polymerization. [3,26,59] These alcohols are named as sinapyl (S), coniferyl (G) and *p*-coumaryl alcohol (H), in which their subsequent

monolignols are named as syringyl (S), guaiacyl (G) and *p*-hydroxyphenyl (H) lignin subunits, presented in Figure 1.6. These monolignols only differ with each other in the aspect of the degree of methoxylation; the S monolignol has two methoxy groups located at the C3 and C5 positions of the aromatic ring; the G unit was decorated with one methoxy group located at C3; the H lignin subunit has no attached methoxy groups on the aromatic ring. The monolignols' composition varies with feedstock. Commonly, softwood lignins are made up by G units with traces of S and H, hardwood lignins are built up by a combination of S and G units, where S dominates, while grassy lignins have a good combination of S, G, and H units.[61] Several study suggested that S/G ratio is correlated with the enzymatic digestibility of the pretreated biomass. Whether it is positively related or negatively related to the biomass hydrolysis has not reached an agreement yet. [62,63,64] The lignin composition for different feedstocks is detailed in Table 1.1. Softwood is mainly built up by G units and the aromatic C5 position of G units are free to undergo chemical modification during pretreatment, including forming new carbon bonds (condensation reaction); However, hardwood has a high content of S units on which the C5 position are sterically hindered and cannot forming new carbon-carbon bonds before cleaving the methoxyl group first.[26]

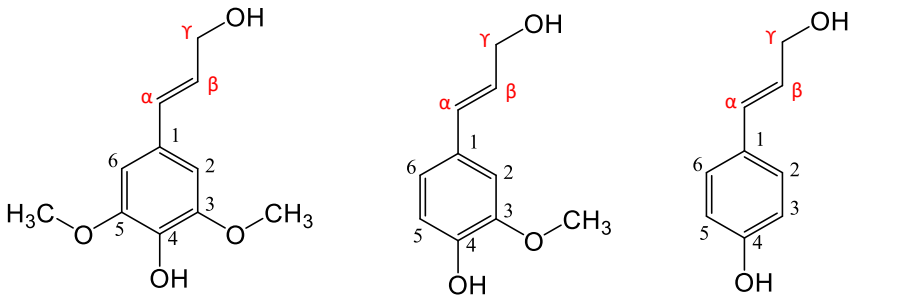
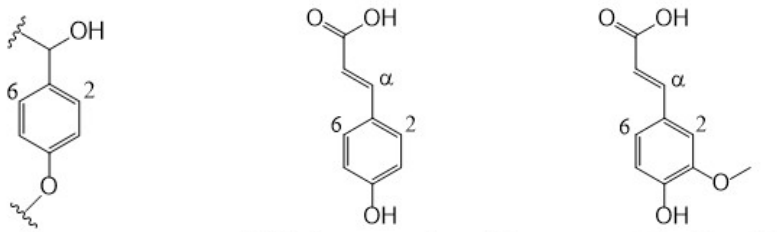


Figure 1. 6 Lignin's primary building blocks: Phenylpropaniod alcohols[32]

Table 1. 1 Lignin monomer composition for all biomass types[32]

Monolignol	Grass (wt%)	Sorftwood (wt%)	Hardwood (wt%)
Syringyl unit	20-50	0-1	50-75
Guaiacyl unit	25-50	90-95	25-50
***p*-hydroxyphenyl unit**	10-25	0.5-3.4	Trace

Apart from the phenylpropanoid building blocks, other molecular species were detected also, such as ferulates (FA), coniferaldehyde, sinapaldehyde, *p*-coumarate units (PCA) and hydroxyphenyl units, listed in Figure 1.7. FA was detected in the softwood lignins by ^{1}H-^{13}C two-dimension (HSQC) NMR.[65] Grasses have a relatively different lignin composition, relative to softwood and hardwood and, where its FA and PCA content is much higher.[32] FA is involved in the formation of LLC (Lignin-polysaccharide cross-Linking Complex), bridging the carbohydrates to other lignin fragments via crosslinking.[53,66] Monolignols are covalently connected with carbohydrates units though benzyl esters, benzyl ethers and glycosidic linkages (mainly via arabinose).[66] These bonds are labile under many pretreatment conditions, and therefore increase the difficulty for the final stage of delignification, breaking the lignin polymer chains into lignin fragments, shorter chain or even lignin oligomers.[53,67] A few studies suggested that these complex has a direct effect on the lignin and carbohydrate fractionation, therefore could negatively influence the enzyme digestibility of the pretreated biomass.[68,69] Other studies also suggested that the low residual lignin reactivity may be responsible for the presence of lignin condensation reactions during the pretreatment, which is one of the major obstacles to developing an efficient fractioning process for the biorefinery and lignin valorization.[53,67,70] Hydroxylphenyl species is detected in lignin extracted by ionic liquid, which was converted from PCA units.[71]



H (*p*-hydroxyphenyl) **PCA** (p-coumaric acid) **FA** (ferulic acid)

Figure 1. 7 Lignin's minor building blocks[32,67]

The most common linkages detected within the lignin structure are ether C-O and carbon-carbon C-C bonds (the C-C bonds formed during biomass fractionation are referred as condensed bonds), where the different types of ether linkages share more than half of the total linkages.[32,26] During biomass fractionation, ether bonds are relatively easily to cleave, whereas C-C bonds are more chemically stable and require more energy input and harsh conditions to break. Ether bonds detected in the lignin are: β aryl ethers (β-*O*-4) (major), diaryl ethers (4-*O*-5) (minor) and aliphatic ethers (α-*O*-ϒ) bonds (minor), while carbon single linkages discovered are resinol (β-β) (major), phenylcoumaran (β-5) (major), 5-5 (minor) and spirodienone (β-1) bonds (minor). The proportion of each linkages depends on the enzymatic polymerisation of the lignin biosynthesis process and differs by feedstocks.[72] The lignin linkage composition leads to different lignin structure, therefore influence the effectiveness of biomass fractionation in terms of delignification: softwood has a higher proportion of carbon single bonds but lower proportion of ether bonds, relative to hardwood, whereas the grasses has the highest ether content. During lignin fractionation, the process is believed to kick off with the cleavage of the ether bonds, where carbon single bonds are never cleaved but chemical modified. Hence, the lignin fractionation is expected to happen faster and more easily for grasses, slowest for softwood, i.e. hardest to delignify (fractionate). Typical compositions of the ether and carbon linkages are listed in Table 1.2, for grass, softwood and hardwood.

Table 1. 2 Compositions of lignin linkages for different biomass [32,73,71]

Number/100 ppu	β-O-4	β-5	α-O-4	β-β	5-5	4-O-5	β-1
Softwood	**43-50**	**9-12**	**6-8**	**2-4**	**12-25**	**4**	**3-7**
Hardwood	**50-65**	**4-6**	**4-8**	**3-7**	**4-10**	**6-7**	**5-7**
Grass[a]	**80**	**10**	**n/a**	**10**	**n/a**	**n/a**	**n/a**

a: Linkages composition for grassy lignin is obtained from HSQC NMR.

The literature has suggested the different functional groups, especially hydroxyl groups contribute to the overall lignin reactivity. [32,53] Three types of hydroxyl groups were discovered in lignin, phenolic, aliphatic and carboxylic OH. The hydroxyl group contents for softwood ranks as carboxylic < guaiacyl phenolic < aliphatic; that for hardwood is carboxylic ≈ p-hydroxyphenyl phenolic < syringyl phenolic ≈ guaiacyl phenolic < aliphatic; and the trend for grasses is carboxylic < p-hydroxyphenyl phenolic < syringyl phenolic ≈ guaiacyl phenolic < aliphatic. The exact hydroxyl group compositions of softwood, hardwood and grassy lignin are listed in Table 1.3. It is important to note that different lignin extraction technologies will lead to a different hydroxyl group composition of the lignin extracted, as not all of the hydroxyl groups remain chemically stable during the lignin fractionation.

Table 1. 3 Compositions of lignin hydroxyl groups for various feedstock.[74,75,76,77,78]

Number[a]/mmol g^{-1}	Phenolic[b]	Aliphatic	Caboxyl
Softwood	**0.77-3.1**	**3.4-4.7**	**0.02**
Hardwood	**0.21-1.21**	**0.92-4.57**	**0.02-0.22**
Grass	**3.48-5.54**	**1.24-1.53**	**0.12-0.18**

a: The actual hydroxyl contents vary with feedstocks within the same catalogue

b : the phenolic hydroxyl groups detected locate at four different location, G, S, and H monolignols, and aromatic carbon 5 position

Lignin has been identified as one of the biggest obstacles for efficient biorefining, as it exerts a fairly large negative impact on the pretreatment process regardless of the process type. Lignin restrains the biomass fractionation via two ways: 1) it prevents hydrolases' access to their substrates, i.e. enzymes cannot reach the target carbohydrate polymer chains that easily; 2) after the preteatment, modified (condensed) lignin sticks (redeposits) to the pulp and hence induces ineffective hydrolase binding. More specifically, after biomass fractionation, lignin adhering to the pulp can either form a physical barrier preventing the pulp being attacked by enzymes (steam explosion pretreatment), or makes the pulp-enzyme binding to be non-productive via enhancing lignin-enzyme interactions (organosolv pretreatment).[79,80] Therefore, enzyme loadings for the current polysaccharide hydrolysis technology are too high to meet industrial requirements (high enzyme loading makes the expense of the hydrolysis process too high and the process is not cost-effective enough for industrial biorefinery), and enzyme recycling techniques for the hydrolysis are yet to develop. For the benefit of hydrolysis and fermentation steps, developing a pretreatment process with effective lignin removal is the key to the development of cost-effective biorefinery.

1.1.1.4 lignin-like polymer synthesis

The lignin fraction of biomass is negatively correlated to the overall efficiency of the biorefinery process, therefore inversely related to the process capital expense. In order to make biorefining more cost-effective, work has been done to reduce the lignin content of the raw biomass via a genetic engineering approach. In order to get more control on the biomass's lignin content, the biosynthesis pathway of the lignin polymer have been intensively studied recently. [33,72,81,82] A fairly detailed phenylpropanoid biosynthesis route has been introduced, but some information about this route are still missing.[33,83] The discovery of this synthesis route may offer an opportunity to produce lignin-like polymers with desired chemical and physical properties for industrial valorization. The required properties can be achieved by carefully selecting the building blocks of these polymers, monolignols. Moreover, the biosynthesis route only requires mild conditions and does not use hazardous chemicals, thus is a 'green' synthetic route for the formation of lignin-like polymers. A typical example is the industrial production of phenol-formaldehyde resin.[84] Using a biosynthesis pathway can limit the utilization of toxic formaldehyde, which is strongly desired.

1.1.1.4.1 An overview of the lignin biosynthesis pathway

The lignin biosynthesis pathway is a multiple-step reaction.[33,72,81,82,85] The suggested synthesis pathway is presented in Figure 1.8. It begins with a deamination in which phenylalanine is converted into cinnamic acid by phenylalanine ammonia-lyase (PAL). *p*-Coumaric acid is generated from enzymatic hydroxylation of cinnamic acid, catalyzed by a monooxygenase, named cinnamic acid 4-hydroxylase (C4H). Caffeic acid is the hydroxylation product of P-coumaric acid and the catalyst of the hydroxylation is coumaric acid 3-hydroxylase (C3H), where the process has not been fully understood till now. The hydroxylation reaction at cinnamoyl-CoA level (from p-coumaroyl-CoA to caffeoyl-CoA) is suggested to be catalyzed by *p*-coumaroyl-CoA 3-hydroxylase (CCoA3H). The lignin methylation steps take place at both the cinnamic acid level and hydroxycinnamoyl-CoA level, but different

enzymes are involved in these processes: for the cinnamic acid level, 5-hydroxylferulic acid *O*-methyltransferase (COMT) is used: for hydroxycinnamoyl-CoA level, both caffeoyl-CoA O-metyltransferase (CCoAOMT) and hydroxycinnamoyl-CoA ester *O*-methyltransferase (AEOMT) are utilized. Methylations catalyzed by COMT and AEOMT do not form products with significantly different specificity, while CCoAOMT does have a specificity preference. The hydroxycinnamoyl-CoA esters can be esterified from its acid, where the esterification is facilitated by 4-coumarate-CoA ligase (4CL). However, it seems to be a different case for sinapic acid. As it was suggested that sinapic acid is not a substrate of 4CL, whether sinapic acid is syringyl lignin's precursor is still under debate.[82,83] Finally, all the CoA esters undergo a two-step reduction process, generating three dominant monolignols. Two reduction steps are catalyzed by cinnamoyl-CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD), respectively.

The final lignin polymers are formed via dehydropolymerizations catalyzed by peroxidases, laccases or phenol oxidases, where the process is named as lignification.[33,36,86] This lignification in the cell wall is composed of several subprocesses: activated oxygen species generation, oxidizing enzyme synthesis and lignin monomer coupling. Firstly, the lignin monomers are oxidized in to phenoxy radicals by enzymes. Depending on the choice of enzyme, different activated oxygen species, H_2O_2 or O_2, are involved in the oxidation reaction. For laccases, O_2 is utilized, while the peroxidases use H_2O_2. The exact H_2O_2 formation procedure within the cell wall is still under debate.[36,86] Back in 1978, Halliwell *et al.* found out peroxidase has multiple functions in lignification, H_2O_2 producer and as the catalyst for monomer oxidation.[87] In 1997, Ogawa *et al.* reported NAD(P)H is able to form H_2O_2 and lignin in spinach hypocotyls, where NAD(P)H is a superoxide-generating plasma membrane oxidases.[88] In 1998, Moller *et al.* suggested that a copper amine oxidase (CuAO) can also produce H_2O_2.[33] They suggested that: in tracheary elements of Arabidopsis, lignin staining and peroxidase activity are colocalized with the promoter activity of the CuAO gene.

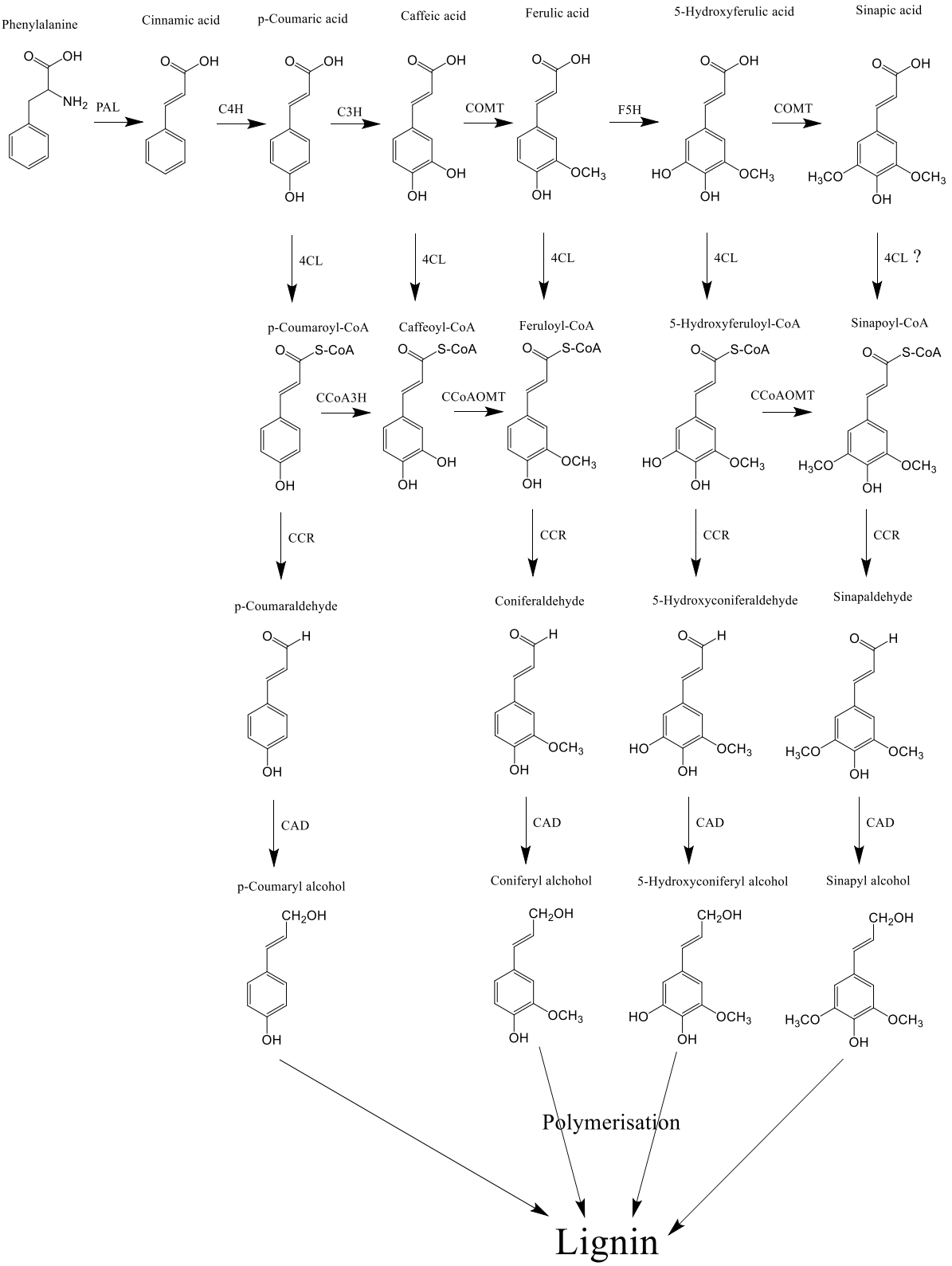


Figure 1. 8 Lignin biosynthesis pathway[85]

With the presence of the activated oxygen species, peroxidases and laccases can readily produce phenoxy radicals. Peroxidases are monomeric glycoproteins, which dehydrogenate lignin precursors into cinnamyl alcohol radicals.[33] The common peroxidases used are horseradish peroxidase (HRP), manganese peroxidase (MnP), and soy bean peroxidase (SBP).[36,89,90,91] In this project, HRP was chosen. Laccases are one type of glycoenzymes binding with copper, and this facilitates the phenoxy radicals' formation via single electron oxidations.[92] How laccases facilitate the radical formation is still under investigation.[72] Furthermore, peroxidases oxidize the lignin monomer at a higher speed but with a higher consumption of activated oxygen species, compared to laccases (for producing every four radicals from monolignols, laccase uses up one oxygen molecule while peroxidase consumes two H_2O_2; laccase performs coniferyl alcohol oxidation with a maximum speed of 6.7 nmol/s/mg, while the speed of lignifying peroxidase for the same reaction is 359000 nmol/s/mg).[33,72,93]

For the final step of the polymerization, radicals generated by activated oxygen species are stabilized by delocalized electron density, which provides β carbon on the aliphatic side chain with a single electron density. They are cross-coupled together to give an overall three-dimension polymeric lignin structure.[33]

1.1.1.4.2 Historical view of hydrogenative monolignol polymerisation using HRP

Dehydrogenation (polymerisation)of lignin-like polymers using HRP was first introduced in 1956 by *Freudenber*.[34,94] This enzymatic polymerisation has been attractive ever since and much research has been done in this area, in order to understand the underlying chemistry and to integrate the process for industrial interests. In 1990, a mechanism for this catalytic reaction was introduced by Sakurada *et al*, shown in Figure 1.9:[88]

$$Enzyme + H_2O_2 \rightarrow Enzyme_{compound\ 1}$$

$$Enzyme_{compound\ 1} + AH_2 \rightarrow Enzyme_{compound\ 2} + AH\cdot$$

$$Enzyme_{compound\ 2} + AH_2 \rightarrow Enzyme + AH\cdot$$

$$AH\cdot + AH\cdot \rightarrow A_2H_2$$

or $AH\cdot + AH\cdot \rightarrow A + AH_2$

Figure 1. 9 Mechanism for dehydrogenation polymerisation catalysed by HRP[88]

According to Freudenberg's discovery, two polymerisation methods have been introduced, named "Zulaufverfahren" (ZL) and "Zutrophverfahren" (ZT): the ZL method describes a continuous addition process of the monolignols and HRP within a reactor, which results in a bulk polymerization; the ZT process alters the way in which monolignols are introduced into the reactor, dropwise addition instead of direct dissolution, which leads to an end-wise polymerisation.[34]

Several studies have shown that the choice of solvent has a large influence on lignin- like polymer's yield and quality, and some also proved evidence that the solvent can negatively affect the enzyme's catalytic activity.[90,95,96,97] It is well known that aqueous conditions are not ideal for polymerisation as the enzyme will entrap into polymer and its catalytic activity is then reduced. A group of Japanese scientists have investigated the relationship between the HRP catalytic activity, polymer yield and the choice of organic solvent used.[91] In their work, pure water, 20 to 80% of aqueous 1,4-dioxane, 20 to 100% of aqueous DMF and 20 to 100% of the aqueous method were tested in the same phenol polymerisation. The results of the test showed: water significantly reduced enzyme's activity and therefor has the lowest yield; the polymeric reaction only preforms well at low 1,4 dioxane concentration and the impurity in the organic solvent can decompose the apo-protein in HRP, consequently the lignin yield is reduced. This work also suggested the both the nature of the organic solvent and the aqueous organic solvent composition can result in a deactivation of HRP.

Some have suggested using reverse micelles instead as this water-in-oil microstructured media has a limited effect on the enzyme activity.[24,97,98] Back in 1993, Rao *et al.* tested reverse micelles for the HRP catalysed polymerisation.[99] They found out the reaction is as feasible as the process using monophasic organic solvents, and they also suggested it is possible to control the molecular weight of lignin via varying the surfactant concentration, the highest molecular weight recorded in their test was 400 kDa. Kobayashi *et al.* also reported a work which compares the HRP enzymatic Cresol polymerisation in aqueous 1,4 dioxane with the same process in a reverse micellar system.[97] Their results show that the polymer's average molecular weight is the same for two solvent systems but the reverse micellar system has a lower lignin yield. Furthermore, a newly developed solvent, ionic liquid is also believed to have limited impact on HRP's catalytic activity. Zaragoza-Gasca *et al.* reported a polymeric process using a phosphate buffer with [BMIM][BF_4] instead of a conventional solvent system.[100] The integrated process achieved a 100% polymer yield and with high molecular weights. The ability of reusing the solvent system makes this process even more remarkable.

1.1.1.5 Softwood, Grass, Agricultural residues

Lignocellulosic feedstocks are abundant and sustainable; Therefore, the biorefinery process has been investigated extensively recently to reduce the usage of fossil fuels. Feedstocks like dedicated energy crops, Miscanthus (*Miscanthus* x *giganteus*) are particularly good for biofuel generation. Miscanthus is one type of perennial grasses and is cheap to grow and easy to maintain. It can even grow on degraded land with a low water requirement.[8] Its growth requires little fertilizer, and is not invasive, is very fast, which leads to a potential annual production of 20 tons per hectare.[101] Softwood feedstocks such as pine (*Pinus sylvestris*) are the dominant biomass sources in the Northern hemisphere, growing in America, Asia and the Europe continent.[36] Pine is often referred as a forest residue or waste produced by pulping and timber industry, which is significantly underused at this point.[102] It grows rapidly with increased annual production for every year. It is estimated a 1.3 million

tons of production for the UK alone, the cost of its harvest and transportation is estimated at rate of 18-50 British sterling per ton, comparing to 40-70 British sterling per ton for miscanthus.[103] The large abundance and the low capital cost makes pine very promising as a lignocellulosic feedstock for a commercial-scale biorefinery process.[104]

Besides miscanthus and pine, the other class of feedstocks, agricultural residues, including rice husk, rice straw, wheat straw and sugarcane bagasse, are studied in depth for biorefining, but the current pretreatment technologies are not advance enough to overcome their low digestibilities.[60] It was reported in 2004, the potential bioethanol production rate from agricultural waste is 491 billion liters.[105] These feedstocks are available in high quantities worldwide, especially in those countries with massive crop productions, e.g. China, Japan, Brazil and Canada.[106] Among crop wastes, rice straw, is highly abundant, with an annual production rate of 731 million tons, corresponding a 282 billion liters bioethanol production if suitable biorefinery technology is developed.[105] Comparatively, the annual production rate for rice husk is smaller, but is still large, 137 million tons.[107] Wheat straw is commonly generated in Asia, America and Europe. In 2007, an estimation for its annual production rate, 850 million tons, was made, corresponding a 120 billion liters bioethanol production.[108] Sugarcane is an important agricultural residue for countries like China and Brazil, where its production rate in 2010 was 1.69 billion tons worldwide.[109,110,111,112] The feedstock itself is heterogenous, containing fractions, fiber and pith, where the pith occupied around 5% of the dry mass.[112] The pith fraction is problematic for paper industry and often removed by depithing process prior to use.[113] The ash fraction of the feedstock also is removed by industrial depithing processes. These crop residues have high lignin- and ash-contents, especially rice husk and rice straw, and mostly are subject to direct burning for power supply.[44] Due to the large inorganic matter contained by the feedstocks, burning these residues has produced a significant amount of air pollution, making climate change even more severe. Therefore,

turning these feedstocks into useful products like bioethanol will be very beneficial in both economic and environmental aspects.[109]

1.1.2 Biorefinery, pretreatment and lignin fractionation

1.1.2.1 Definition of the biorefinery

For the needs of industry, an ideal biorefinery process should be able to produce high quality sugar solutions with low capital costs; meanwhile by-products of the process can be easily modified into value-added applications. Generally, a biorefinery can be separated into four major steps, pretreatment, hydrolysis, fermentation and distillation or regeneration.[23] The aim of pretreatment is to change or eliminate the structural as well as composition barriers of hydrolysis, i.e. the crystalline regions of cellulose are disrupted and the lignin-polysaccharide linkages are partially broken during this step. Resultantly, cellulose can achieve a higher accessibility towards enzymes and more of it can be converted into fermentable sugars and the lignin extracted out from this step can potentially be used for a range of applications. Additionally, the formation of sugar degradation products and lignin inhibitors of the subsequent hydrolysis and fermentation need to be eliminated in pretreatment.[8 23] The following hydrolysis step involves the conversion of holocellulose (cellulose and hemicellulos) into hexoses and pentoses, which are then fermented into organic alcohols, using either chemicals or enzymes. High purity biofuels are generated via product purification process, which removes all the undesired residuals via distillation.

1.1.2.2 An overview of pretreatment methods to date

Pretreatment can be classified into four categories: physical, biological, chemical and combined processes. Physical pretreatments include chipping, grinding, and milling.[8,114,115] These mechanical size reduction processes increase the cellulose' enzyme accessibility though increasing its specific surface area as well as though decreasing cellulose's crystallinity content and degree of

polymerization.[66] The energy requirement is defined by the nature of the feedstock and the size of the final product. For milling, it is possible to reduce the particle sizes down to 0.2mm, but the energy cost is enormously high.[116] Even though several studies suggest that milling increases final biogas (such as hydrogen) yield up to 30% via increasing the amount of soluble substrates for fermentation, it is still not economically effective for industrial scale biofuel production.[116,117]

Biological pretreatments use fungi's ability to selectively degrade biomass components (mostly lignin) to achieve the separation between lignin, hemicellulose and cellulose.[118] Back in 1993, Hatakka *et al* already reported that white and soft-rot fungi such as phanerochaete chrysosporium are able to perform selective delignification.[93] The main obstacles of scaling up biological processes are their long processing time, the strict requirement of maintaining suitable growth conditions and the high space requirement. What makes it even worse: fungi sometimes not only can degrade lignin, but also can consume cellulose.[119]

Steam pretreatment (SP), Liquid Hot Water pretreatment (LHW), Wet Oxidation pretreatment and Ammonia Fibre Expansion (AFEX) are typical examples of physiochemical pretreatments.[80,120,121,122,123,124] SP is using high pressure saturated steam to dissolve hemicellulose and transform lignin, leaving cellulose with higher accessibility. A typical process temperature range from 230 to 240.[125,126] The term "autohydrolysis" is introduced to describe the process in which acetyl groups built into the hemicellulose release during the pretreatment and hydrolyse hemicellulose.[127] SP has low energy requirements and limited chemical usage.[126] However, incomplete lignin-carbohydrate linkage separation results in lignin condensation and the subsequent hydrolysis and fermentation may be disrupted by the inhibitors generated from holocellulose degradation. LHW is similar to SP but replace pressurised steam with high temperature liquid-state water (180-190°C).[125,128] Comparing to SP, although it eliminates the holocellulose degradation by reducing

reaction temperature, the energy cost for down-stream processing is increased as more water is used.[129] AFEX uses pressurised ammonia to reduce the crystallinity of cellulose as well as to remove lignin.[130] Some studies suggested that this method cannot achieve effective lignin removal for biomass with high lignin content, such as softwoods.

Acid, alkali, ionic liquid (IL) and organic solvents are the common solvent choices for chemical pretreatment.[131,132,133,134] Dilute acid pretreatment (DA) has received intensive attention due its potential of replacing enzymatic hydrolysis in biorefinery.[135] Diluted aqueous mineral acid, e.g. sulfuric acid, is commonly used to dissolve hemicellulose and remove lignin, which thus increases pretreated biomass' digestibility. Several studies has confirmed that after fractionated by dilute acid, the carbohydrate hydrolysis rate of the pretreated biomass is increased significantly, while other groups reported that the degradation products generated from carbohydrates (especially hemicellulose), such as HMF and FA, significantly inhibit the subsequent hydrolysis and fermentation, therefore, reduce the biofuel production yield.[8,23,133] Currently, Organosolv and ionic liquid pretreatments have been attracting a lot of attentions.[3,45,136,137] The solvents used for both pretreatments can be recycled after the process, increasing the "greenness" of these processes, which is referred as one of the upsides about these two pretreatment technology. Selective lignin and hemicellulose fractionation and enhanced biomass digestibility make them very promising.[45,47] These two pretreatment process are discussed in more details in Section 1.1.2.4 and 1.1.2.5.

1.1.2.3 Lignin extraction for varies pretreatments

Due to complex linkages between lignin and carbohydrates within the cell wall, the lignin isolation process has remained relatively challenging, comparing to holocellulose fractionation during pretreatment. Non ideal lignin removal potentially inhibit the enzyme digestibility of the pretreated biomass, where enzymatic hydrolysis for the feedstock would have low efficiency, consequently

increasing the overall capital cost of the biorefinery process. Since lignin fractionation plays such a crucial role in the biorefinery process, many lignin fractionation technologies have been continuously introduced in the last century, and integration of these technologies is thought to be one of the latest hot topics. Generally, lignin fractionation technologies function *via* two mechanisms: 1) the first route is to remove the carbohydrates of the feedstock by solubilisation, while the lignin fraction is left as a solid residue (all the enzymatic lignin extraction processes and the Klason method work in this way); [138,139] 2) the second route is to remove lignin by dissolution, leaving cellulose with or without hemicellulose as a solid residue, and the dissolved lignin is then recovered from the pretreatment liquor (Kraft method, lignosulfonate process, Björkman process, organosolv, ionic liquid and alkaline wet oxidation pretreatments function *via* this mechanism).[32,92,122] Each of these lignin isolation processes is different to each other in some aspects, therefore they have their unique strengths and weaknesses. Lignin fractionation technologies applied in some typical pretreatment processes are listed in Table 1.4, including the pretreatment conditions, key features of the lignin fractionation and the biomass components dissolved by the solvent during pretreatment.

The Klason method is to dissolve carbohydrate by acidic solvent medium for isolating the lignin fraction of biomass.[140] This method can achieve high lignin yields, but the lignins isolated are usually highly degraded, and it does not work well with all feedstocks: the extraction of hardwood might suffer from partial lignin dissolution due to strong acidic hydrolysis. [47,137] klason lignin is also referred as acid-insoluble lignin.[139] The klason method have been widely used for determine the lignin content of the untreated and treated biomass.[141] The Kraft process is one of the process which could achieve an industrial scale of lignin production, often involved in pulping and paper-making industry.[54,142] Kraft lignin is often produced from the black liquor via a pH-induced precipitation, where the black liquor is generated as a waste with a massive quantity during pulp and paper production process.[92] Due to its drastic conditions, the lignin produced does not have a particular high quality. Kraft lignin generally

has a highly modified structure and is soluble in highly polar organic/inorganic solvent, such as alkali solutions, i.e. more hydrophobic compared to native lignin. Over 80% of lignin's hydroxyl groups are sulfonated during Kraft pulping process. Due to the high degree of lignin modifications, kraft lignins usually have a low molecular weight Mn, below 3000 Da, and are not sulphur-free, which common sulphur content of commercial available kraft lignin is around 1% to 3%.[143,144] Lignosulfonates is another type of lignin having industrial scale production, often produced from pulping process. This type of lignins are also produced from waste liquor generated by the pulping process, and has a similar degree of lignin modification, therefore similar solubility in polar solvents, relative to Kraft lignin. However, it has a higher molecular weight (lignosulfonate is up to 140,000 Da).[92] The 'greenness' of the lignin generating process is not ideal due to the large amount of waste water produced.[86,145,29]

Relatively new isolation techniques, such as organosolv and ionic liquid (IL) pretreaments have received more attention due to their 'greenness' and their good ability to isolate lignin.[29,31,41,47,146,147] High lignin solubility and easy lignin recovery were reported for organosolv processes using methanol and ethanol.[137] Organic acids like formic, acetic acid were suggested to be good pretreatment solvents due to their little impact on the environment and excellent lignin fractionation ability. Moreover, IL pretreament has been suggested as a promising technique due to its mild operational conditions.[8] The chemical and physical properties of ILs can be tuned to achieve a higher lignin extraction efficiency. The lignins produced often have a higher average molecular weight, narrower molar mass distribution and limited structural change, compared to Kraft lignin.[146] Nevertheless, the high IL cost and IL's recyclability need to be investigated before developing this process up to industrial scale.[3]

More recent studies have suggested a new lignin fractionation approach, lignin-first fractionation.[148] The new approach sets the lignin valorisation as the main goal of biomass fractionation rather than turning cellulosic pulp into useful fuels. The lignin-first fractionation aims to avoid lignin condensation

and to deliver lignin-derived aromatic products, such as fuels under moderate conditions.[149] This type of processes usually prevent the lignin degradation via two strategies: 1) tandem depolymerisation-stabilisation, e.g. Reductive Catalytic Fractionation (RCF); 2) aryl β-O-4 ether linkage preservation, e.g. formaldehyde-assisted stablisation.[148,150] RCF actually is one type of lignin hydrogenolysis processes, and also has other names, e.g. Early-Stage Catalytic Conversion of Lignin (ECCL), Catalytic Upstream Biorefining (CUB). Rinaldi *et al* tested an ECCL process for Polar lignin using Raney Ni in aqueous 2-propanol at various temperatures, 160°C to 220°C.[151] They reported that the delignification of the ECCL process could reach 87% at 220°C. Another work of Ferrini and Rinaldi suggested that lignin-first biorefinery using Raney Ni in aqueous 2-propanol could yield a high-quality lignin-oil but the pulp produced suffered from a relative lower enzyme accessibility, comparing to organosolv process.[152] However, ECCL process is particular promising for lignin-to-fuel conversion. Cao *et al* successfully demonstrated a two-step process, ECCL followed by hydrodeoxygenation process, turning native hardwood lignin into high purity gasoline and diesel.[153]

It is important for an ideal lignin fractionation process to consider technological issues, economic and environmental impacts. Lately, the idea of developing a fractionation process having the key advantages from multiple isolations has been put into practice. Back in 2008, *Zoia* introduced a modified enzymatic mild acidolysis process (EMAL), where the oil-based heating was replaced with microwaves, and this method showed a higher lignin yield with a better purity comparing to conventional EMAL processing.[154] In 2012, *Wang* reported the purity of lignin extracted can be largely improved by using a process combining CEL with alkaline organosolv process. With the support from these successful examples, the ideal of a combined lignin fractionation process is also applied in this project.[32]

Lignin isolation process	Lignin name	Process conditions	Lignin characteristics	Dissolved species
Kraft process	Kraft lignin	Na_2S, NaOH	Highly modified, partially fragmented	lignin
Lignosulfonate process	Lignosulfonate	Extract lignin from the black liquor generated from softwood sulfate pulping	Highly modified, high average molecular weights, the ether linkages is cleavage, methoxyl groups are lost and new C-C bond is forming.	Lignin
Organosolv process	Organosolv lignin	Using organic solvents to dissolve lignin and extract it	Mild process conditions, extracted lignin has minor modifications, solvent is recycled via distillation	lignin
Ionic liquid process	Ionic liquid lignin	Stepwise precipitation (Dissolution process) or selective extraction (ionoSolv process)	Higher yield and purity than lignin generated in enzymatic or mechanical pretreatment	Cellulose, hemicellulose /hemicellulose, lignin
Enzyme process	Cellulolytic enzyme lignin (CEL)	Hydrolysis cellulose and leave lignin as a solid residue	Low structure change	Hemicellulose, Cellulose
Björkman process	Milled wood lignin	Ball milling followed with extraction using aqueous dioxane	Close to the natural lignin structure, extensive milling might cause depolymerization	Lignin
Lignin-first process	Reductive Catalytic Fractionation (RCF)	Redox catalyst, e.g. Pd/C Reducing agent, e.g. pressurized H_2 Hydrogen donor, e.g. iPrOH, formic acid	Depolymerized into lignin oil accompanied with some monomers with high yields,	Lignin

Table 1. 4 A list of lignin isolation methods for biomass fractionation [29,32 92, 148]

1.1.2.4 Ionic liquids and ionoSolv pretreatment

1.1.2.4.1 An overview of ionic liquids

Ionic liquids(ILs) are also called molten salts. Their ions are poorly coordinated, leading them to be in a liquid form at room temperature. Their low vapour pressure, good thermal stability and tuneable chemical and physical properties make this class of solvents so "green" that they are considered as a replacement for conventional industrially-used organic solvents. [3,47,136,155] The discovery of water and air stable room temperature ILs started around the 1990s.[155] The very first IL synthesised was ethanolammonium nitrate by Gabriel *et al*.[156] Intensive research has been done for understanding this type of solvents and for their valorisations covering the area of chemical synthesis, electrochemical devices, chemical and biological catalysis.[157] Lately, it has been proven to show a great potential for being used as a solvent in biomass deconstruction processes based on the discovery of IL's ability of dissolving cellulose.[29,47,157]

ILs, often referred as a "solvent of the future", are well known for their designable properties. We can carefully manage the cation and anion type, in order for the subsequent IL to have desired chemical and physical properties fulfilling the industrial interests.[157,158] The choice of cation is usually bulky organic cation, typically ammonium ions, occasionally alkylated phosphonium or sulfonium cations.[159] The anion is normally inorganic or organic anions. Figure. 1.10 lists a range of commonly picked anions and cations for ILs. The polarity of the solvent defines its solubility towards solutes. The polarity is defined by both the specific and the non-specific interactions between solute and solvent.[160] The polarity of the solvent is depending on the coulombic, dipole-dipole, hydrogen-bonding interactions between the solvent and solute molecules, where solute exerts high solubilities with solvents having a similar polarity as the solute.[161] Therefore, understanding the polarity of the IL is crucial for developing its usages as a solvent in all areas. There are several polarity scales existing, which define IL's polarity with different aspects.[162] The most commonly used scale is dielectric constant, ε_r, where

ε_r< 9 are classified as non-polar ε_r > 15 are referred as polar.[161] A range of imidazolium-based IL was reported to have a dielectric constant value between 8.9 to 27.9, e.g. [C_4C_1im][BF_4] has the ε_r value of 11.7.[163] Multiparameter scales such as Kamlet-Taft scales could provide a more sophisticated description about the solvent behaviour towards different solutes. [159] Kamlet-Taft scales contain three parameters, hydrogen bond acidity (α) and basicity (β), dipolarity or polarizability effects (π*).[164] The IL's polarity (solubility) largely depends on its ability being a hydrogen bonding donor or acceptor. For [C_4C_1im][BF_4], its Kamlet-Taft parameters are reported as 1.05(π*), 0.63(α), 0.38(β).[165] For IL pretreatments, anions with high hydrogen basicity are generally preferred, such as hydrogen sulfate, methyl sulfate and halides.[146] IL's viscosity is influenced by temperature, any impurities associated with the IL and the choice of the anion.[166] It is believed that anion's ability to form hydrogen bonding with the cation could affect the viscosity, rather than the size of the anion. For imidazolium-based ILs (same anion choice), the cation with alkyl substituents with longer carbon chain length would results in a lower IL viscosity.[167] ILs with high viscosity would restrain their dissolving ability towards many solutes, requiring the addition of less viscous co-solvent, such as water. A typical example is the ionoSolv pretreatment process (detailed below), which is using an aqueous IL to pretreatment biomass rather than anhydrous IL.[3] Different to IL's viscosity, IL's density is not significantly correlated to temperature and IL's impurities. For non-haloaluminate ILs, the cation size is inversely related to the density.[168] The acidity of the IL is beyond the scale of pH, and is defined by Hammett acidity, expressed as H_0.[169] This Hammett acid function defines the degree of IL protonation induced by a UV dye.[169] The Hammett acidity offers the possibility to measure the acidities of those solutions, where their pH values are between 0 to -12. The UV dye is required to be a weak base, can only partially deprotonate the IL. A typical example of the dyes is 4-chloro-2-nitroaniline.[170] In 2014, a proton NMR approached was introduced for determining the acidities for a group of hydrogen sulphate ILs, e.g. [Bmim][HSO_4], and this approach was suggested to be easier to handle.[171]

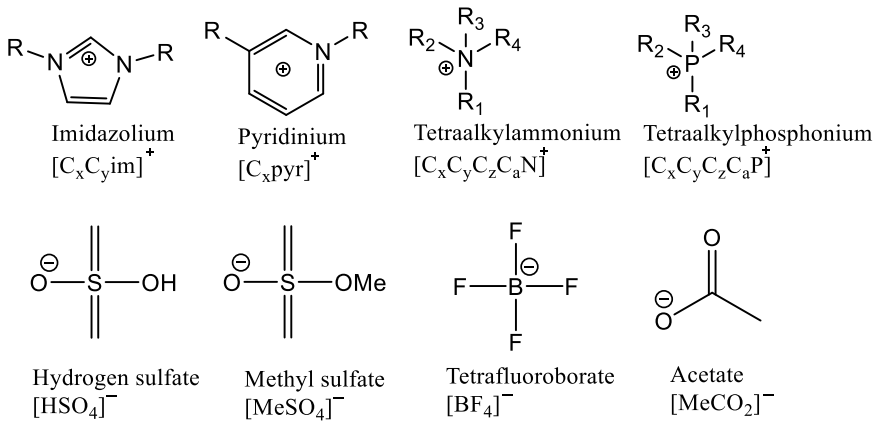


Figure 1. 10 Common choices of cation and anion for ionic liquid[3]

The "greenness" of the IL as a solvent is tightly correlated to its toxicity. Up till now, the study about IL's toxicity has not completed.[161] A estimation of the toxicity could be obtained by investigating the hazards of the alkali metal (sodium cation with IL anion)as well as the chloride-based salt (IL cation with chloride anion) related to the IL.[166] Due to the excellent stability and low biodegradability, the ILs tend to accumulate in the nature environment, potentially causing non-degradable waste if being used in industrial scales. This is the driving force for tuning the IL properties in order to be biodegradable or biocompatible.[172] One solution suggested is to synthesis ILs using bio-resources, such as amino acid, lignin.[173] Alkylammonium-based ILs, which has been widely used in our research group, are catalogued as protic ILs, processing excellent fractionation ability, and also is believed to be less toxic then imidazolium-based ILs.[174] The "greenness" of the IL usage is also linked to the recycling of this type of solvents. As IL is fairly hard to decompose naturally, the solvent recovery could not only eliminate the non-degradable wastes, but also reduce the capital costs for industrial-scale processes. Hydrophobic ILs can easily regenerated via liquid-liquid extraction, while hydrophobic ILs requires an energy intensive distillation process due to their low vapor pressure. However, this energy capital cost for the IL recovery could trade off by the reduced solvent capital cost(the IL recovered could be used again, one batch of the IL could fractionate several batches of the biomass). Brandt *et al* reported that the [TEA][HSO_4] could maintain the same biomass fractionation ability even after being used and recovered for four cycles.[175]

1.1.2.4.2 Protic ionic liquid

With increased concern about the production cost and environmental concerns, a group of cheap "protic IL" were introduced as a solvent in biomass pretreatment.[176,177] They can be produced via simple acid-base neutralization, which is also referred as a proton transfer process from a Brønsted acid (electron acceptor) or a Brønsted base (electron donor). Typical examples are triethylammonium hydrogen sulfate, which only costs $ 1.24 per kilogram, and N,N-dimethylbutylammonium hydrogen sulfate.[104,177] Apart from being used as a ionic liquid pretreatment (ionoSolv process) solvent, protic ILs also found its use as fuel cell electrolytes, catalysts for microwave-facilitated chemical reactions, such as glucose dehydration.[178,179]

Protic ILs has relative high conductivity, high thermal stability, and its viscosity is highly temperature-dependent as well as anion-dependent.[159] They can be synthesised via a one-step addition reaction without any side products. This proton transfer reaction could be incomplete due to ion aggregation, limiting the ionicity of the IL.[180] IL thermal stability is analysed by thermogravimetry analysis (TGA), where the sample mass loss is detected as a function of temperature. It is reported that ILs with an imidazolium cation process a high decomposition temperature of above 200°C.[169] George *et al* reported the decomposition temperature for a group of low cost ammonium-based protic ILs.[181] They suggested that the benchmark dissolution IL $[C_2C_1im][OAc]$ has a decomposition temperature of 215°C, which can be problematic for pretreatment at high operational temperature. Cough *et al* also suggested a similar decomposition behaviour for this benchmark IL.[182] However, George *et al* also suggested that the group of ionoSolv ILs decompose at 270°C to 300°C, more stable than $[C_2C_1im][OAc]$. More alkyl group substituted on the ammonium cation ion would lead to a drop at the decomposition temperature. Viscosity is also an important feature of IL needed to take into consideration when selecting IL for pretreatment.[159 169] Low viscosity would lead to a low mass transfer of the IL during fractionation even at high temperatures, resulting in an incomplete mixing between the biomass and

the IL solvent and the fractionation effectiveness is hence hindered. The viscosity of IL positively depends on the degree of intermolecular van der Waals and hydrogen bonding interactions between the cations and anions, where anions has a stronger influence on the viscosity.[183] Stacking of the cation aromatic rings and cation-anion interactions are also reported to influence the viscosity.[178,169,183]

1.1.2.4.3 Deconstruction of biomass: Dissolution process

Two distinct approaches of IL pretreatment have emerged to date, namely the dissolution process and the ionoSolv process. [104,146,176,184,185,] The former refers to a two-stage process: after the complete solvation of the biomass into IL, cellulose was regenerated in the first stage, and the second stage is the recovery of lignin using anti-solvents like water or acetone[30]; the regenerated cellulose has a much higher digestibility toward enzymatic hydrolysis and a better cellulose separation can be accomplished by precipitating cellulose with organic water solvent systems or solvent systems having a protic component. Typical dissolution processes studied in the past used $[C_2C_1im][MeCO_2]$. Biomass feedstocks tested ranged from grasses (miscanthus, switchgrass) to softwoods (pine).[3,29,47] Reasonable lignin removal, 17% - 65%, and excellent hemicellulose removal, 0% - 83%, were achieved.[3] Better lignin and hemicellulose fractioning were performed for hardwoods than that for softwoods which are more recalcitrant in nature and therefore harder to pretreat. The dissolution process is reported to have low tolerant towards water moisture. Generally, water contents less than 1% are required for this type of IL pretreatment as cellulose's hydroxyl groups might bond to water molecules instead of solvent molecules resulting in a reduced cellulose solubility.[146] Another benchmark IL for this dissolution solvent was reported, [Emim][OAc] due the decent hydrogen bonding basicity of the acetate anion.[136] The cellulose fraction went through a dissolution and regeneration process during pretreatment, yielding a fraction of cellulose with a high enzyme digestibility up to 90%. However, the

low thermal stability of the IL, high water sensitivity and hence high IL regeneration cost of the corresponding pretreatment has significantly reduced the potential of this dissolution process being used in an commercial-scale biorefinery.[181,186,187] The residual [Emim][OAc] in the pretreated biomass is considered as toxic towards conventional saccharification enzymes and microbial process host, in order to solve this issue, a group of Joint BioEnergy Institute scientists has introduced a one-pot biorefinery process for swichgrass using this IL , instead of multi-step process, and the pulp produced in this process was hydrolysed by a IL tolerant cellulase, *E. coli* strain. This process is believed to have less water usage and generate less wastes.[171]

1.1.2.4.4 Deconstruction of biomass: ionoSolv process

The ionoSolv process has much less literature than the previous pretreatment approaches but its popularity has been rising lately. [104,176,184] In ionoSolv process, lignin and hemicellulose are dissolved into the IL solvent medium, while cellulose is kept as a solid residue throughout the process. A flow diagram of the ionoSolv process in an industrial perspective is presented in Figure 1.11. One good thing about this type of pretreatment is: it can tolerant water content up to 40% without inhibit the overall fractionation performance, resultantly eliminating the energy-intensive IL drying process when developing the process into a commercial-scale.[51] The increased saccharification yield reported indicates that a certain level of structural modifications have happened to cellulose (cellulose crystallinity) while it almost remained untouched during the fractionation.[47,184]

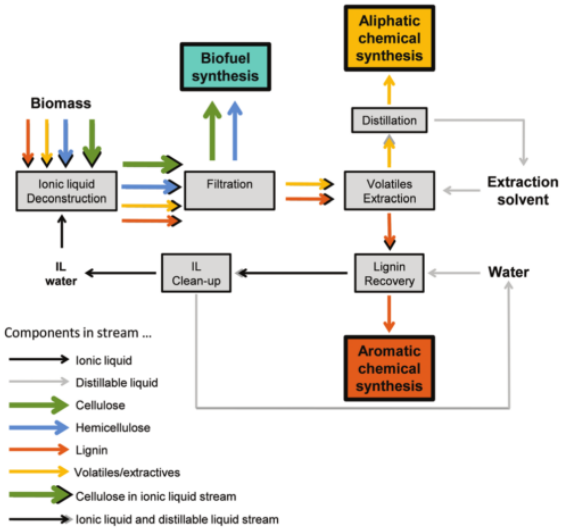


Figure 1. 11 A flow diagram of ionosolv pretreatment process[3]

During ionoSolv process, the lignin fraction is dissociated by the IL from the biomass by cleaving the ether bonds like β aryl ethers (β-*O*-4) and breaking/ modifying carbon-carbon linkages, such as resinol (β-β) and phenylcoumaran (β-5). Hydrogen bond basic anions were reported to be suitable for lignin dissolution as they are able to break the most abundant ether linkages in the lignin.[51] A recent paper published by Brandt *et al.* mentioned that over 60% of lignin removal yield for untreated miscanthus was obtained after an aqueous $[C_4C_1im][HSO_4]$ pretreatment.[176] They believed that a slightly more acidic $[C_4Him][HSO_4]$ is the most promising solvent among all the IL they tested, as its subsequent lignin recovery yield is reaching 100% , in which an extra addition of 1 mol% sulfuric acid was involved the IL pretreatment. They also noticed that the acidity of the IL might cause lignin degradation during the pretreatment, which is one of the obstacles to scaling up the $[C_4Him][HSO_4]$ pretreatment process that needs to be resolved urgently.

As the bulk production of the protic IL for ionoSolv process heavily relies on the price of the amine, the cost of synthesising imidazolium-based ILs is more expensive than other IL such as $[HNEt_3][HSO_4]$, triethylammonium hydrogen sulfate (named as $[TEA][HSO_4]$ in this thesis), $[DMBA][HSO_4]$ N,N-dimethylbutylammonium hydrogen sulfate, where these ILs bulk price ranges from $1.24 -5.88 per

kilogram.[188] Additional to the low production cost, the relative high thermal stability also make these two ILs very promising as the biomass fractionation solvent, their decomposition temperature is reported to around 280°C, 215°C for the benchmark dissolution solvent [EMIM][OAc].[181] Consequently, this cheap and highly thermal stable ILs have been involved in several ionoSolv process studies for a wide range of feedstocks in recent years, including miscanthus, pine, willow, sugarcane bagasse and other agricultural residues. [46,60,67,70,189,190] [TEA][HSO_4] has proved its excellent fractionation ability towards all the feedstocks, by selective removing lignin and hemicellulose and modifying cellulose fraction in to an amorphous and highly digestible form. Weigand *et al* reported that aqueous [TEA][HSO_4] (20% wt. moisture) with acid to base ratio of 1.02 and 0.98 effectively pretreated hardwood willow in an ionoSolv fashion and achieved a glucose releasing yield for the pretreated willow up to 82% (peaked at 150°C, 1 hour).[70] According to this study, the IL with excess acid achieved a faster hemicellulose and lignin removal with more severe lignin condensation. Brandt *et al* conducted an ionoSolv process for grassy feedstock, miscanthus using [TEA][HSO_4] with 20% wt. water content.[175] A maximum saccharification yield of pretreated miscathus was reported to be 77% at 120°C for 8 hours. Excellent lignin removal (up to 90%) and quantitative hemicellulose removal were achieved by this ionoSolv process, and the trend of delignification as a function of time is in line with the saccharification yields, suggesting lignin is one of the biggest obstacles for the pretreatment. Brandt *et al* also suggested that increasing the acidity of the IL could speed up the fractionation. In another miscanthus study conducted by Gschwend *et al*, the pulps produced by the ionoSolv process preformed with a biomass loading of 1:5 g g^{-1} at 170°C, 30 minutes or 180°C, 15 minutes could obtain a glucose releasing yield of 76%, almost identical to the yield for 120°C, 8 hours with a biomass loading of 1:10 g g^{-1}.[67] However, Gschwend *et al* suggested that using higher operational temperature with shorter pretreatment duration at higher biomass loading could reduce the reactor volume requirement up to 64-fold, leading a significant reduce in the capital cost of the pretreatment due to a 95% reduction in reactor expense reduction.[67] Chambon *et al* reported an ionoSolv process study for sugarcane bagasse, a 69% saccharification accompanied with a 90% lignin removal was achieved

by the bagasse pretreatment at 120°C for 4 hours.[46] Chambon *et al* also tested the same fractionation process at higher temperature 170°C for shorter duration (45minutes for rice husk and bagasse, 30minutes for straws) with a group of high lignin- and ash-content agricultural residues, where rice straw, wheat straw and bagasse obtained a glucose yield of 90% approximately and more recalcitrant rice husk obtained a 73% yield.[60] Hornification induced air-drying the pretreated biomass was reported repeatedly by Chambon *et al* and Gschwend *et al* to have a negative impact on the enzymatic saccharification. The lignin condensation, coupling of small lignin fragment to yield large water insoluble lignin fragments, and the formation of pseudo-lignin, the product of the degraded sugar fragments crosslinked to lignin, are detected in all ionoSolv pretreament using [TEA][HSO_4]. The degree of the lignin degradation is proportional to the severity of the pretreatment conditions.

[TEA][HSO_4] possesses excellent fractionation ability towards a wide range of feedstocks, but its ability is less powerful towards highly recalcitrant feedstocks, such as softwood, compared to [DMBA][HSO_4] and [HBim][HSO_4].[71] Gschwend *et al* compared the fractionation effectiveness of the ionoSolv process for pine. [DMBA][HSO_4] and [HBim][HSO_4] were reported to be superior to [TEA][HSO_4] in terms of sugar releasing yield.[190] The former achieved a saccharification yield above 70%, while the later only obtained a yield just below 40%, at 170°C for 30 minutes. Despite that, [TEA][HSO_4] is still an excellent IL candidate for industrial biorefinery due to its low cost and decent fractionation performance towards various biomass.

1.1.2.5 Organosolv pretreatments

1.1.2.5.1 Overview

Organosolv pretreatments have been intensively developed in the pulp and paper industries, e.g. ALCELL process. [41,101,137,191,192,193] Because of this, this type of pretreatment processes have drawn a lot of attention lately and is believed to be one of the emerging “greener” biomass deconstruction

processes compared to the ones discussed earlier, e.g. AFEX, dilute acid process. It is defined as a physicochemical pretreatment method, in which the biomass is deconstructed with a hot aqueous organic solvent, sometimes in the present of a catalyst (usually acid), and then the biomass particle size is physically reduced.[29] The signature process during the organosolv pretreament is lignin fractionation, in which the lignin's ether linkages are cleaved, producing low molecular weight lignin species and phenolics, and the lignin fragments generated is dissolved into the aqueous organic solvent medium.[41] Organosolv is well known for its high selectivity towards lignin and the major biomass components are separated in to three fractions: solid lignin (dissolved by the solvent and then recovered from the solvent), a solid fraction with high cellulose content and a liquid fraction mostly including hemicellulose. [137,193] The isolated lignin is considered to have a relative high purity (little carbohydrate impurity), low molecular weight, a less condensed structure and most importantly zero sulfur content, which can be further developed into highly value added biorefinery side products.[29]

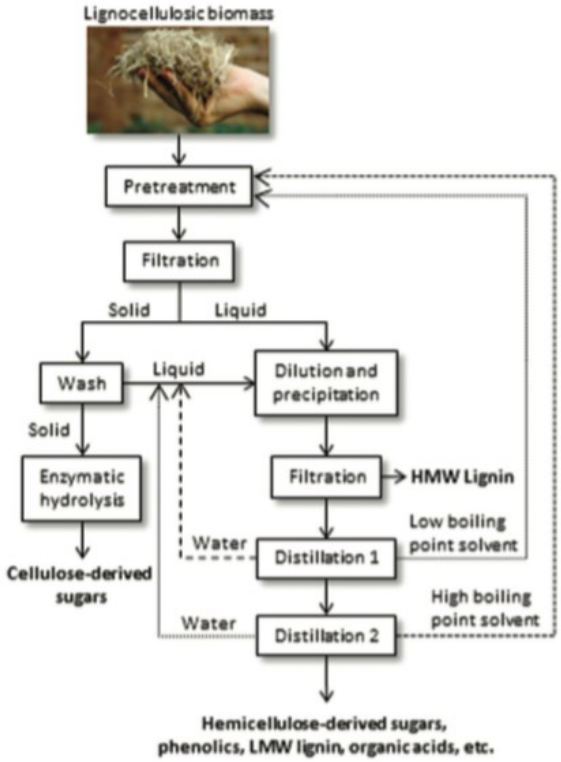


Figure 1. 12 A flow diagram of organosolv pretreatment process[137]

Several classes of solvents can be utilized in Organosolv pretreatment: aliphatic alcohols, e.g., ethanol, butanol, polyols, e.g., glycerol, organic acid, e.g., acetic acid, and some other organic solvents, e.g. acetone or phenols.[193,194,195] Organic solvents improve the biomass fractionation *via* promoting solvent penetration into the biomass matrix as well as improving lignin and hemicellulose dissolution. Solvents' performances are related to a solubility parameter, named the Hildebrand's solubility parameter δ.[146,137,196] This parameter is experimentally derived, and is used to describe the solvent dissolution ability. Generally, good dissolution occurs when the δ value of the solvent is close to that for solute, lignin (this suggests solvent with low polarity tend to be suitable co-solvent as the IL during fractionation). As only lignin's δ value is available, 22.4 $MPa^{1/2}$ or 13.7 cal cm^{-1} (depending on the unit), among the three major biomass components, we can only use δ to interpret the solvent behavior in the aspect of the delignification process.[137,197] Furthermore, depending on the solvent (with high boiling point or low boiling point) used, the solvent recovery process is modified slightly.[198] In general, the pretreatment process undergoes a solid-liquid separation despite the type of solvent used: the solid residue is separated to be ready for the subsequent hydrolysis process; the lignin is recovered from the liquid fraction by adding antisolvent. Two distillation steps are preformed after the separation to recover the water and solvent used in the fractionation process: if a low boiling point solvent is utilized, then it will be recycled in the first distillation and water is recovered in the second one; however, if a high boiling point solvent is used, the distillation steps are reversed.[198] A flow diagram of an Organosolv process is presented in Figure 1.12. The main weakness of using low boiling point solvent is the environmental issues related to their volatility and flammability, whereas high boiling point solvents requires a much higher capital cost and a higher energy requirement for solvent recovery.[198] Organosolv processes may require relative expensive high-pressure reactors, compared with other fractionation processes, but this is considered trade-off with the cheaper solvent recovery and high-quality lignin fraction which can be used for a wide range of applications.

1.1.2.5.2 Choices of organic solvent and their related pretreatment processes

1.1.2.5.2.1 Ethanol, butanol and acetone

Low boiling point solvents have been widely used in organosolv fractionation, and ethanol is one of the earliest developed solvents.[193] Some are renewable, low cost and easy to be recovered due to their low boiling points. Both acids and bases can be used as catalysts for the fractionation and when the operational temperature is low, below 200°C, an acid is preferred as acid catalysts can increase cellulose digestibility and reduce reaction temperature and time, compared to ethanol alone or base catalysed ethanol pretreatment.[198] Several studies have suggested that organic acids and inorganic salts have better delignification ability than mineral acids. Park *et al.* compared three pitch pine pretreatment process using 1% sulfuric acid H_2SO_4, 1% magnesium chloride $MgCl_2$ and 2% sodium hydroxide NaOH.[199] Their results shows that both $MgCl_2$ and NaOH have a 10% increase in the cellulose yield and NaOH's delignification yield is 7 times higher than that for the H_2SO_4 catalysed process. Several studies suggested that the delignification yield is positively linked to ethanol concentration in acid catalysed processes, due to the positive correlation between lignin solubility and ethanol concentration; according to Ni *et al,* lignin solubility reaches a maximum at 70% aqueous ethanol solution.[192] It was also mentioned that ether linkages generate lignin species with lower molecular weights, which are water soluble, at lower ethanol contents while higher ethanol content leads to better lignin dissolution; thus the fragmentation is no longer needed. Hage *et al* suggested that cleavage of β-*O*-4 linkages are the major cause of delignification.[101] This has been confirmed by many other studies using different biomass feedstocks, including Kanlow switchgrass, Buddleja, and loblolly pine.[137]

The low boiling point solvents, including short-chain aliphatic alcohols (ethanol, butanol) and ketones (acetone) can fractionate various feedstocks, including highly recalcitrant softwoods. The lignol process developed by Pan *et al* is an aqueous ethanol pretreatment for mixed

softwood (pine, spruce and Douglas fir), performed with a 40% wt. ethanol content at 185°C to 198°C for 0.5 to 1 hour, catalysed by sulfuric acid.[200] The pretreated softwood was highly digestible and a quantitative cellulose conversion of the pulp was reported after being hydrolysed for merely 24 hours. Sidiras *et al* compared the fractionation ability of four organic solvents, methanol, ethanol, butanol and acetone towards wheat straw.[201] The pretreatments conducted by Sidiras *et al* were identical apart from the organic solvent choice: 50% wt organic solvent concentration, 160°C, 20 minutes, catalysed by 0.045N sulfuric acid, biomass loading 1:20 g g^{-1}. Butanol gave the highest lignin removal, 63%, similar yield (59%) was achieved by acetone, while ethanol was less powerful, only achieving a delignification around 50%. Quantitative hemicellulose dissolution into the solvent medium was achieved by butanol and acetone. The separated hemicellulose fraction (xylan) could easily be hydrolysed into xylose. Several studies have suggested that the acetone is very promising as a delignifying agent and can hydrolysis hemicellulose easily. A wheat straw organosolv process using aqueous acetone (50% wt.) was reported by Huijgen *et al*, and the lignin and hemicellulose removals reported were around 80%.[202] A similar fractionation performance was reported for sweet sorghum bagasse by Jafari *et al*.[203] The process used aqueous acetone (50% wt.) with 0.1% wt. sulfuric acid, and operated at 180°C for 1 hour. The enzymatic saccharification yield reported was 94%.

The organosolv process could generatw a fairly high quality lignin fraction. The quality of the lignin isolated from the fractionation largely depends on the chemical reactions taking place during the pretreatment. Several studies have taken a close look at the signature chemical reactions happening during lignin fractionation for organosolv pretreatments using ethanol and butanol. Bauer *et al*. investigated a series of organosolv lignins derived from miscanthus, while Lancefield studied the lignin isolated from beech, Douglas fir and walnut.[204,205] Their observations were in line with each other. They both suggested that an α-alkoxylation was taking place at side chain regions for the major lignin

subunits, S,G,H monolignols, where the reaction was competing with lignin condensation during fractionation. They also suggested that the degree of lignin degradation is inversely proportional to the ethanol/butanol content of the pretreatment. A series of chemical reactions which could potentially take place at the side chain of the monolignols are detailed in Figure. 1.13, including the α-alkoxylation (ethoxylation or butoxylation) and the lignin condensation.[204,205] After losing a proton at the hydroxyl group on the α carbon position, monolignols turn into benzylic cations, which can undergo different chemical reaction and yield different end products:1) preform a condensation coupling with themselves or the adjacent monolignols, it could also be followed with a hydrolysis to produce Hibbert Ketones, shown as route 1,2,3 in Figure 1.13; 2) trapped by ethanol/butanol via α-alkoxylation to produce α-ethoxylated/butoxylated β-*O*-4 linkages, which stop the cations from undergoing condensation coupling. The α-ethoxylated/butoxylated β-*O*-4 linkages formed might tune the lignin solubility for a more profound lignin dissolution. According to Bauer's work, traces of hemicellulose was detected for organosolv lignins and believed to linked to other lignin monolignols via *p*-coumarate acid.

In recent years, a ketone based solvent, methyl isobutyl ketone (MIBK), combined with alcohol and water has been used in the biomass deconstruction process.[206] This biphasic fractionation system was claimed to provide finer control of biomass fractioning process and is suggested to produce "cleaner" lignin than conventional organosolv processing. Brudecks *et al*. reported an 87% lignin removal yield for prairie cord grass using this type of pretreatment process.[206] Ethyl acetate has been proposed as an alternative of MIBK as it has a lower toxicity and is cheaper.[137]

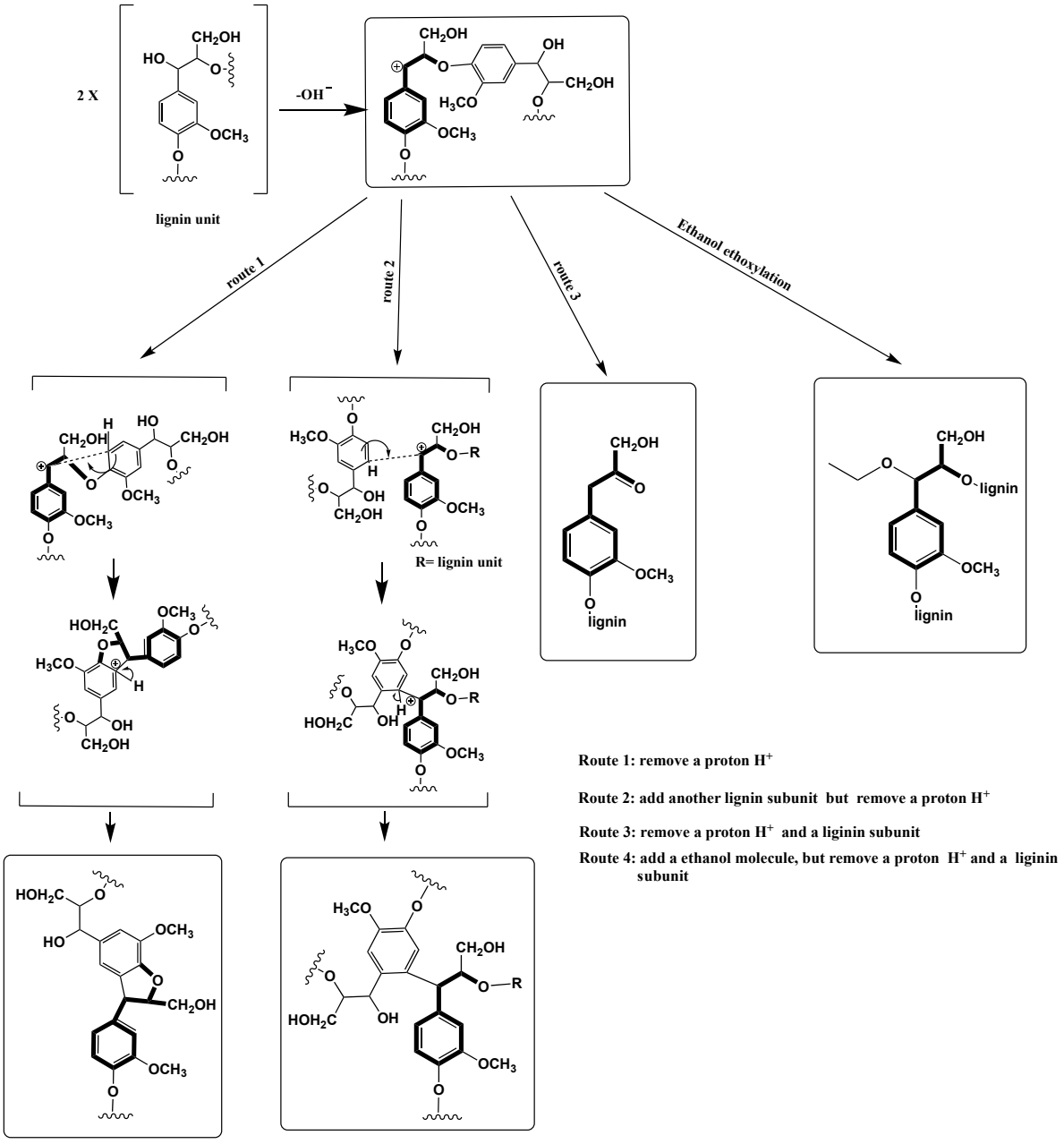


Figure 1. 13 Proposed chemical reaction happening at side chain β carbon of monolignols under organosolv-ionoSolv pretreatment conditions.[204,205]

1.1.2.5.2.2 Glycerol

Glycerol, a polyhydric alcohol, is derived from propylene or triglycerides in fats and oils. It can be used along with or combined with basic catalysts in pretreatment processes. Chen *et al* reported a wheat straw pretreatment using solely glycerol at 220 °C for 3 hours can achieve a hemicellulose removal yield of 70%, a lignin removal yield of 65% and the cellulose digestibility yield was recorded as 90%.[207] A similar result was reported for Eucalyptus wood after treated with glycerol only at 200°C for 70 minutes.[137] The main cause of delignification for the process using glycerol is also the cleavage of ether bonds. Zhang *et al* suggested that the glycerol (more than 5%) and other polyhydric alcohols left in the cellulose fraction will become inhibitors for the following enzymatic hydrolysis as well as for fermentation.[208]

1.1.2.5.2.3 Organic acids

Organic acids, such as acetic acid or formic acid can react with hydrogen peroxide to generate powerful delignification agents, peroxyformic acid and peroxyacetic acid.[137,198] These acids combined with hydrogen peroxide have been studied for pretreament and pulping.[209] Previous studies have performed pretreatments with formic acid or acetic acid for many feedstocks, including corn cob, miscanthus, sugarcane bagasse and wheat straw.[77,137,210,211,212] All of these studies shows decent hemicellulose (around 80%) and lignin removal (above 80%), Compared to the other organosolv process mentioned above, the higher delignification yield may be explained by the small difference in the Hildebrand solubility parameters between lignin and organic acids, 24.9 $MPa^{1/2}$ for formic acid and 21.4 $MPa^{1/2}$ for acetic acid.[137]

1.1.2.6 Hybrid pretreatments

Until now, there is no single pretreatment technology which could fulfil all the requirements for a cost effective industrial biorefinery process, producing bioethanol alone with other value-added chemical

products derived from hemicellulose and lignin fractions. Therefore, hybrid pretreatments, combining the basic concepts of two or more fractionation processes might be the future development of current pretreament technologies. There have been some investigations of hybrid fractionation processes, but the processes developed so far are mainly based on an alkaline or acid pretreatment and a physical process, e.g. acid-microwave process, photo-induced alkaline process.[213,214] Organosolv pretreatment is also reported to co-operate with other pretreatment processes to give a more effective and cleaner biomass fractionation. This strategy was first used by *Rughani et al*. in 1980 in a RASH organosolv process, which is a combination of organosolv pretreatment with a rapid stream hydrolysis.[8] In 2003, a group of Japanese scientists reported a bio-organosolv process for beech wood chips; this bio-organosolv process involves a white rot fungi pretreatment process and an ethanolysis.[8] Moreover, in 2007, a steam explosion with an ethanol extraction process was performed by *Hongzhang et al.* They reported that hemicellulose and lignin removal yields are 80% and 75%, respectively, and 85% of the ethanol used can be recovered.[215]

No work has been done to developing a hybrid process based on the organosolv and ionoSolv processes. However, there are potential advantages to this approach, including: 1) both processes are considered as 'green' fractionation, as the pretreatment solvent could be recycled after the fractionation; 2) the two methods function in the same way (selectively dissolving lignin and hemicellulose into the solvent medium with the leftover cellulose-rich residue modified to be more digestible), therefore the two methods could be combined into a one-step pretreatment process (less complex and lower cost), while many of the current hybrid process require a two-step process; 3) the ionoSolv process could process provides extremely high enzyme-accessible cellulose for biofuel production, but its lignin fraction has limited usage due to its condensed structure, while the organosolv process generates a high quality lignin fraction for value-added applications.[29] Furthermore, from an industrial perspective, the solvent regeneration process (generation of IL from

the aqueous solution) for ionoSolv pretreatments is fairly energy intensive and hence expensive. Partially replacing the IL with solvents with a lower boiling point and/or heat of vaporization, e.g. ethanol, butanol, could reduce the energy requirement for the solvent recovery after the pretreatment. As ethanol/butanol are the final products of a biorefinery process, the ethanol/butanol recovery for pretreatments could be conducted in a distillation column which is also used to purify the cruel ethanol/butanol produced during the fermentation process. This could further reduce the solvent cost for the overall biorefinery and makes the process more industrially feasible, as the ethanol/butanol used in the pretreatment process is produced by the biorefinery and no extra distillation column built only for recovering the organic solvent of the pretreatment is needed for separating the organic solvent from the organic-IL solvent medium after the pretreaetment.

1.1.3 After the lignin fractionation: lignin depolymerisation and valorisations

1.1.3.1 Lignin depolymerisation

Lignin extracted from biomass usually needs to be depolymerised before being used as an aromatic-based feedstock for the chemical industry. Depolymerisation processes can be classified into acid/ base catalysed depolymerisation, pyrolysis, hydrogenation, chemical oxidation, gasification as well as biodegradation.[26,32,59,216,217] Figure 1.14 presents a list of lignin depolymerisation processes their corresponding process conditions, including the operational temperature and the oxidising/ reducing agents if it is applicable.

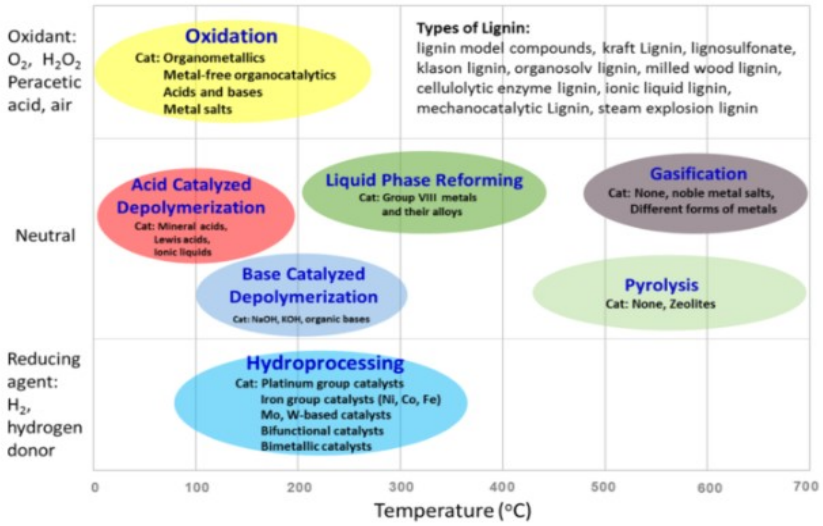


Figure 1. 14 A summary of lignin depolymerisation processes[32]

Base/acid catalysed processes are mainly utilized for effective lignin fractioning rather than lignin industrial valorisation.[218,219] Base catalysed processes perform at a temperature ranging from 270°C to 330 °C, while the operational temperature of acid catalysed process is up to 380°C .[219,220] The common commercially available base catalysts are lithium hydroxide, sodium hydroxide, and potassium hydroxide, where sodium hydroxide has been used most frequently.[221] The predominant chemical reaction occurring in this type of processes is the cleavage of aryl-alkyl ether linkages as they are the weakest linkages within the lignin structural. Similarly, acid catalysed processes disrupt the lignin structure *via* breaking α and β- aryl ether linkages. It has a relative long history, in which the very first process was introduced in 1924 by *Hägglund al et.*[222] They reported to depolymerise lignin using two solvent mixture, hydrogen chloride/ ethanol and formic acid/ ethylene glycol, where the depolymerised lignin was seperated into water soluble and insoluble fractions, and the end products reported were thiobarbituric acid and phloroglucinol.

Different from the two processes described above, hydrogenation and oxidation processes are mainly designed to produce lignin-based aromatic materials for the chemical industry. Hydrogenation is

actually a pyrolysis process with the presence of hydrogen. It has an operational temperature between 200 and 350 °C.[223,224] Hydrogen-donating solvents such as formic acid may be added to accelerate the process and maximise the product yield.[87] Commercial-scale phenol production using lignin hydrogenation was firstly built in 1952 in Japan, named as the "Noguchi Process".[24] The production plant treated desulfonated lignin in a two-step process catalysed by sulfated metal. The monophenol yield recorded was 44%.[24] Since then, several large-scale pilot plants have been designed, but most of them have been suffered from low phenol yields and process control difficulties.[32] Oxidation processes involve a chemical cracking reaction which breaks all kinds of lignin linkages including ether bonds, aromatic rings and resistant carbon-carbon linkages.[225] Typical oxidants used are nitrobenzene, molecular oxygen, and hydrogen peroxide. The oxidant products range from carboxylic acids to aromatic aldehydes, for example, vanillin and syringaldehyde.[32] This process is thought to be promising for replacing petroleum-based chemical production.

1.1.3.2 Applications for current technical lignins

The annual lignin production of current biorefinery processes is predicted to be around 200,000 tons, the majority is subjected to direct burning for powering and only a minor proportion of the lignin generated is used for other value-added utilisations.[226] Back in 2004, Pacific Northwest National Laboratory announced 12 value-added chemicals generated in biorefineries which are compatible with conventional petroleum-based platform chemicals, but none of them are derived from lignin, only from carbohydrates.[217] This was improved in 2007. Three lignin-based chemicals are commercially available, vanillin, dimethyl sulfide as well as dimethyl sulfoxide.[26,227] Vanillin is produced from lignin alkaline oxidation, while dimethyl sulfide is produced from treating kraft lignin with molten sulfur under basic conditions. Compared to vanillin and its chemical derivatives, dimethyl sulphide and dimethyl sulfoxide has a relatively smaller industrial potential. However, vanillin (used as a flavouring compound in food and pharmaceutical products) and its derivatives, such as vanillic acid

and vanillic alcohol, have narrow market segments, which does not allow lignin to be used to its full potential. Other processes, for example, phenol production via alkaline hydrolysis, aromatic chemical production by the Noguchi Process, were mentioned in some literature.[24,217] The high capital cost makes these processes less compatible than petroleum-based processes. Integrations are desirable, for the purpose of reducing our reliance on petroleum resources.

1.1.3.3 Potential valorisations

Apart from the three commercially available platform chemicals mentioned above, lignin can theoretically derive a range of useful chemicals and polymeric materials, which could potentially benefit the biorefinery economically via improving lignin fraction's value-added valorisations.[228,26] Typical examples of these potential applications are lignin-based carbon fibers, oil drilling fluids, plasters and dust suppressants.[79,227,229,230] Back in 1997, Baumberger *et al* discovered that kraft lignin could act as a plasticizer and incorporate with starch films to improve the films' hydrophobicity.[231] In 2013, Berghel et al also suggested that kraft lignin could be used as an additive in pellet press for improving pellet's mechanical durability and length.[232] A list of lignin potential value-added applications are presented in Figure 1.15.

Figure 1. 15 A summary of lignin based aromatic chemicals and macromolecular materials (reproduced from Ayyachamy *et al*) [228]

Lignin is believed to be a good starting material for macromolecular materials, such as carbon fibers, adhesives and resins.[217,233,84] Carbon fiber is an essential material for daily applications like light-weight vehicles and computers.[233,234] Carbon fiber needs to be light, fatigue resistant and possesses high strength as well as hardness. Natural or industrial extracted lignin is thought to be a "greener" alternative to synthetic materials, polyacrylonitrile (PAN) in carbon fiber production.[233] Generally, lignin is melted and spun into fiber at a high speed. The obstacle for this technology is that: native or industrial lignins are different in molecular weight distributions, melting points and other polyelectrolyte properties, and not all the lignins are suitable for this high-speed melt spinning process; only the lignins with high purities and limited molecular weight distributions are able to generate high quality carbon fibers. Moreover, lignin can take a part in polymer synthesis to improve the polymer properties, such as in polyesters. Cetin *et al.* reported the organosolv lignin can replace phenol in phenol formaldehyde resin production, and the subsequent resin produced possesses better properties than conventional ones.[84] Kosbar *et al.* reported that lignin based epoxy resins can be used in the production of printed circuit boards.[235]

Lignin also can potentially be converted into many high-volume aromatic chemicals, typically benzene, toluene, xylenes (BTX) and phenolic compounds.[216] BTX can be derived via a defunctionalisation of the lignin subunits followed with a hydrodeoxygenation. Phenolic compounds can be produced via selective catalytic hydrolysis, which is hard to generate using conventional petroleum-based synthesis routes. Phenolic compounds can also be derived from a direct lignin pyrolysis. The process normally requires quite harsh conditions, >600°C, and solid char and condensed hydrocarbons are the side products.[236,237] The phenolic chemical produced can be further updated into liquid fuels with high energy density.

Research objectives

Part 1: Some work has been done to understand the protic ionic liquid pretreatment processes.[47,104,176,184,177] The main focus was to optimise the glucose release of the pretreated biomass, and the recorded glucose release for this type of pretreatment is superior, up to 100%. However, the lignin degradation of the process was observed repeatedly but little attention has been paid to address this issue. Organosolv pretreatment is well known for its selective lignin fractionation and high-quality lignin recovery. Combing two or more pretreatment technology into one process is the future of the current pretreatment process development. However, no work has been done to develop a new pretreatment process based on the ionoSolv and organosolv pretreatments, but it is worth looking into this due to the excellent fractionation performances delivered by both pretreatment processes. The reasons for choosing [DMBA][HSO_4] for pine and [TEA][HSO_4] for the rest of feedstocks over other protic ILs are: 1) [TEA][HSO_4] preformed the best at increasing cellulose digestibility for miscanthus among all the protic IL tested in previous study, 75% as efficient as the benchmark IL [C_2C_1im][OAC]; 2) [DMBA][HSO_4] fractionate softwood feedstocks more efficiently. The reasons for choosing ethanol and butanol are: 1) As in the later stage of the biorefinery, fermentation mainly produces bioethanol/butanol; the use of this solvent in the pretreatment step simplifies the solvent

recovery process, as the solvents can be recycled using the purification apparatus which is also for purifying the bioethanol/butanol produced during fermentation step; 2) no extra cost needed to purchase ethanol/butanol as the biorefinery is already producing them. Acetone was picked as it is a low boiling point solvent, easy to recover, and it is an excellent lignin solvent due to solubility parameter really close to lignin.

A hybrid process named organosolv-ionoSolv is originated from ionoSolv process using aqueous protic IL and the organosolv process using low boiling point solvents. This process has been developed in this project for achieving a better lignin fractionation including the upgraded quality for the isolated lignin fraction, without inhibiting the fractionation effectiveness for the sugar fractions of the biomass. The fractionation performance of the process was tested with a range of feedstocks, including miscanthus, pine, rice husk, rice straw, wheat straw and sugarcane bagasse, where the last four feedstocks listed are classified as agricultural wastes. [TEA][HSO_4] and ethanol, butanol, acetone were the protic IL and organic solvents for miscanthus pretreatments. [DMBA][HSO_4] and ethanol were the solvents selected for pine pretreatments. Ethanol, butanol and [TEA][HSO_4] were selected for agricultural residue pretreatments.

Miscanthus

- The organosolv-ionoSolv pretreatment process was carried out using different [TEA][HSO_4] and organic solvent mixtures to evaluate their fractionation effectiveness.
- Selected [TEA][HSO_4]-ethanol process was conducted for a second time in order to understand to what extent the pulp hornification could influence the pulp digestibility.
- Three ionoSolv processes using aqueous [TEA][HSO_4] were performed and their fractionation effectiveness was compared to the corresponding hybrid process.

- The hybrid process was conducted with using different [TEA][HSO_4] and ethanol mixtures, where IL acid to base ratio was 1.02 and 0.98. Their fractionation effectiveness was studied to investigate the impact of IL acidity on the performance of the newly developed process.
- Two ionoSolv processeses using aqueous [TEA][HSO_4] (the acid to base ratio was 1.02 and 0.98) were performed, and their fractionation effectiveness was compared to the hybrid processes.
- The hybrid process was conducted as a function of biomass loading (1:10 to 5:10 g g^{-1}) to examine if the process could function efficiently at high loadings.
- All pretreatmemt effectiveness were described by the saccharification assay and compositional analysis of the pretreated biomass, in terms of pulp's enzyme digestibility (sugar releasing yield), hemicellulose and lignin removal, cellulose recovery and lignin recovery yield.
- The HSQC NMR and GPC analysis were used to study the isolated lignin. The information about the internal structure and average molecular weight of the isolated lignin was revealed, giving more details about the major lignin modification during fractionation as well as the degree of undesired lignin condensation.

Pine

- Three hybrid processes with different [DMBA][HSO_4]-ethanol mixtures and a ionoSolv process using aqueous [DMBA][HSO_4] were conducted, to compare the pretreatment effectiveness between these two types of pretreatments.
- A series of biomass loading experiments (1:10 to 5:10 g g^{-1}) were conducted to test if the hybrid process could fractionate recalcitrant softwood feedstocks at high loadings.
- The isolated lignin fractions were also studied by HSQC NMR and GPC, to determine the quality of the lignin generated from the new pretreatment, and to compare with the conventional ionoSolv lignins.

Agricultural residues

- The ionoSolv process using aqueous [TEA][HSO_4] were conducted for rice husk at 150°C, 170°C with various durations, to determine the optimal ionoSolv process conditions for this feedstock.
- The processes were repeated, where the repeated pretreatment did not conduct a drying process for the pretreated biomass, to investigate the impact of pulp hornification on the pulp digestibility.
- Based on the time course experiment of the rice husk, rice straw, wheat straw and sugarcane bagasse were fractionated by aqueous [TEA][HSO_4] at the predicted optimal conditions.
- The ethanol/butanol-IL process were preformed for all feedstocks to investigate if the hybrid process could effectively fractionate this type of high ash- and lignin- content feedstocks.
- The optimal conditions for the organic-IL process was identified for agricultural residues
- The isolated lignin was studied for any structural changes happening to it during the pretreatment.

Part 2: According to a previous work conducted by our research group, the lignin-like polymers for sinapyl alcohol and coniferyl alcohol were successfully syntheses, but the polymers produced had low molecular weight.[83] So, the polymerisation protocol needs to be altered, in order to produce large uniform polymers for applications such as carbon fiber production.

A synthetic route was designed and carried out for selected monolignols, sinapyl alcohol, coniferyl alcohol, *p*-coumaryl alcohol. Random radical polymerisation was performed with these selected monolignols.

- An intermediate ester was synthesized from sinapic aicd, ferulic acid and *p*-coumaric acid via an esterification.
- The reduction reaction was conducted to generate monoligols from intermediate esters.

- Homogenous polymerisation will be carried out for selected monoligols using phosphate buffer, 1,4-dioxane, H_2O_2 and HRP. Compared to previous work done by our group, the way monolignols are introduced into the polymerisation is altered in order to achieve a higher molecular weight polymer
- Using GPC analysis to investigate the molecular weight and polydispersity of the lignin like polymer.

2 Experimental

2.1 Biorefinery

2.1.1 General materials and equipment

All chemicals were purchased from VWR international or Sigma Aldrich, and used as received, unless stated otherwise. Here, the Karl-Fisher titrator used was a V20 volumetric Titrator (Mttler-Toledo), and the analytical balance used was a Sartorius CPA 1003 S balance (±0.001 g).

Pretreatments were conducted in either Ace Pressure Tubes or Hydrothermal Autoclave Reactors with a Teflon Chamber, purchased from Amazon.com, Inc., depending on the pretreatment solvent composition and the operational temperature. For miscanthus, both the ionoSolv and organosolv-ionoSolv pretreatments were carried out in Ace Pressure Tubes. For pine, Hydrothermal Autoclave Reactors were used for the ionoSolv and organosolv-ionoSolv pretreatments. For rice husk, one type of agricultural residues studied here, Ace Pressure Tubes were used to investigate the optimal reaction time for the ionoSolv process. For all agricultural residues investigated, including rice straw, wheat straw, depithed sugarcane bagasse, and rice husk, the ionoSolv pretreatments with an optimised reaction duration were conducted in Ace Pressure Tubes, and the processes were repeated once using Hydrothermal Autoclave Reactors. Organosolv-ionoSolv fractionation processes for the four agricultural residues were conducted in Hydrothermal Autoclave Reactors.

2.1.2 Ionic liquid synthesis

Two amines and one type of acid purchased for IL synthesis were triethylamine, *N,N*-dimethylbutylamine, and sulfuric acid. The minimum purity of the amines was listed as ≥99 % for triethylamine, 99 % for *N,N*-dimethylbutylamine. The concentration of sulfuric acid in the aqueous

solution used in the synthesis was 49 wt% or 72 wt%. The structures of ILs synthesised were further confirmed by ^{1}H NMR, ^{13}C NMR, and mass spectrometry. ^{1}H NMR and ^{13}C NMR spectra were both recorded on a Bruker 400 MHz spectrometer. Chemical shifts are reported in ppm and the solvent (DMSO) signal is located at 2.500 (^{1}H spectrum) and 39.520 (^{13}C spectrum). Electrospray mass spectrometry experiments were conducted on a Micromass Premier spectrometer by Dr Lisa Haigh (Imperial College London, Chemistry department).

A detailed protic IL synthesis standard operating protocol was established earlier by our research group.[176] A typical protic IL synthesis procedure involves three steps: 1) amine with required amount is pre-cooled in a round bottom flask; 2) a 49 wt% or 72 wt%. aqueous solution of the acid, typically sulfuric acid, with an equimolar amount as the pre-cooled amine is dropwise-added into the round bottom flask with stirring; 3) an excess amount of water is then removed by a rotary evaporator (Büchi) in order to keep ILs moisture concentration at 20 wt%. In this study, as ILs with moisture content ≤1% were required, an extra drying step was conducted for the ILs mentioned below. After drying the ILs water content down to 5 wt%, using the rotary evaporator, the ILs were transferred into round bottom flasks and further dried using a Schlenk line at 40 °C for 24 hours to 72 hours.

2.1.2.1 Triethylammonium hydrogensulfate [TEA][HSO_4]

Sulfuric acid (72 wt%, 252g, 2500 mmol) was dropwise added into anhydrous triethylamine (341g, 2500 mmol) which was pre-cooled in an ice bath. Distilled water (29.2 g, 1620 mmol) was added into the amine-acid mixture after the addition of the acid. Stirring was on throughout the acid and water addition. The mixture was stirred for another 3 hours before the ice bath was removed. A colourless viscous liquid was synthesised. (499g, 2500 mmol, 100%)

IL produced was subjected to proton NMR spectrometry for characterisation and the assigned spectra is detailed here: ^{1}H NMR, 400 MHz, DMSO-d6, δH 8.98 (1 H, s, N-H^{+}), 7.03 (1 H, br s, HSO_4^-), 3.09 (6 H, qd, J=7.3, 4.9 Hz, N-CH_2), 1.18 (9 H, t, J=7.3 Hz, N-CH_2CH_3).

IL produced was subjected to mass spectrometry for characterisation and the assigned spectra is detailed here: MS (Magnet FB^{+}) m/z: 102.13 ([TEA]$^{+}$, 100%), (Magnet FB^{-}) m/z: 96.96 ($[HSO_4]^-$, 100%).

2.1.2.2 N,N-dimethylbutylammonium hydrogensulfate [DMBA][HSO_4]

5M sulfuric acid (500mL, 2.5mol) was added dropwise into N,N-dimethylbutylamine (253g, 2500 mmol) which was placed in an ice bath, under stirring. The acid-amine mixture was stirred for another 3 hours. The excess amount of water was removed, and a transparent viscous liquid was synthesised. (499g, 2500 mmol, 100%)

IL produced was subjected to proton NMR spectrometry for characterisation and the assigned spectra is detailed here: ^{1}H NMR , 400 MHz, DMSO-d6, δH 9.44 (2 H, br, s, N-H^{+},HSO_4^-), 3.04-2.99 (2 H, m, N-CH_2), 2.76 (6 H, s, N-$(CH_3)_2$), 1.64-1.50 (2 H, m, N-CH_2CH_2), 1.30 (2 H, h, J=7.4 Hz, N-$CH_2CH_2CH_2$), 0.90 (3 H, t, J=7.4 Hz, N-$CH_2CH_2CH_2CH_3$).

IL produced was subjected to mass spectrometry for characterisation and the assigned spectra is detailed here: MS (Magnet FB^{+}) m/z: 102.13 ([DMBA]$^{+}$, 100%), (Magnet FB^{-}) m/z: 96.96 ($[HSO_4]^-$, 100%)

2.1.2.3 Adjusting the water contents of the ionic liquids

Moisture contents of [TEA][HSO_4] and [DMBA][HSO_4] were altered to 20% (±0.05wt%, w/w IL). After the moisture content adjustment, ILs were kept in glass bottles, labelled as stock solution A ([TEA][HSO_4]) and B ([DMBA][HSO_4]). Stock solutions were used directly for all ionoSolv pretreatments. For the organosolv-ionoSolv pretreatments, aqueous ILs were further dried under vacuum at 40°C with stirring. The drying duration varied with different ILs, 24 hours for [DMBA][HSO_4] and 72 hours

for [TEA][HSO_4]. The final moisture contents of [TEA][HSO_4] and [DMBA][HSO_4] were 1% and 0.02% (w/w IL). ILs with a water content ≤1% needed to be used in less than three days; otherwise, a repeated drying process might be needed, as the glass bottles were not completely isolated from the open air.

2.1.3 Feedstocks

In this study, six different lignocellulosic biomasses were studied. A dedicated energy crop, *Miscanthus* x *giganteus* (described as miscanthus below) was harvested from Silwood Park campus Imperial College London, the UK in 2016. One common type of softwood, *Pinus sylvestris* (described as pine below) were originated from Bedfordshire, the UK, supplied by Bark UK Online and received as chips in 2017. *Oryza sativa* (described as rice husk in the later section) was originated from Bahraich district, Uttar Pradesh, India in May 2015. *Oryza sativa* (described as rice straw) was supplied by the Institute of Chemical Technology, Mumbai, which was harvested in Tirunelveli district, Tamil Nadu, India in 2015. *Triticum aestivum* (referred as wheat straw) was originated from Glasgow, the UK in 2015. *Saccharum officinarum* (described as bagasse in the later section) was obtained from a South African pulp mill in Kwazulu-Natal province. This sugarcane bagasse was subjected to a depithing process before being packed and delivered. Before shipping, all feedstocks were washed to remove adhering inorganic debris to prevent microbial degradation. Once received, the feedstocks were air-dried, ground and sieved to 180-850 µm (20 + 80 US mesh scale). The sieved feedstocks were contained in sealed plastic bags, stored in cupboards, kept away from sunlight.

2.1.4 Biomass fractionation

The procedure of pretreatments, determination of IL moisture content and oven-dried weight of biomass (ODW) were exactly following the published standard operating procedure.[189] The pretreatment conditions varied from process to process. The operational temperature varied from 120°C to 170°C; pretreatment duration ranged from 0.5 hours to 8 hours; biomass to liquid loading

varied from 1:10 g g^{-1} (10 wt%) to 5:10 g g^{-1} (50 wt%); the organic solvents used for pretreating biomass were ethanol, butanol and acetone; the organic solvent to IL ratio ranged from 1:9 g g^{-1} to 8:2 g g^{-1}, also described as the organic solvent concentration in the pretreatment solvent ranged from 10 wt% to 80 wt%. When a comparison was made between ionoSolv and organosolv-ionoSolv pretreatments, the ionoSolv pretreatment was named as the control process. The minimum purity of ethanol, butanol and acetone used in the pretreatment was ≥99 %. A complete pretreatment condition description for each pretreatment process was detailed below.

The pretreatments were all conducted in triplicate, regardless of the conditions and feedstock types. For each biomass sample, 1 g of biomass on the oven-dried basis, which was equivalent to 1.07 g for air-dried miscanthus and agricultural residues or 1.05 g for air-dried pine, was added into a pressure tube or an autoclave reactor, followed with adding 10 g of the stock solution or a mixture of the organic solvent and the IL with a water content ≤1%. The actual weight of biomass added was recorded, and its corresponding weight on the oven-dried basis was obtained from the calculation using the untreated biomass moisture content obtained on the same day as the pretreatment process was performed. After the solid and liquid additions, the pressure tube or the reactor was sealed and subjected to an oven treatment. The oven treatment was carefully timed to prevent overcooking the feedstock. The cooked biomass (also named as the pulp) was cooled in the apparatus to the ambient temperature before the pulp washing step.

The pulp washing step was performed in the same fashion for all feedstocks but only varies with different organic solvents involved in the pulp cooking step. For biomass pretreated by aqueous IL or ethanol-IL mixtures, the pulps were washed by absolute ethanol. For biomass pretreated by butanol-IL mixtures, the pulp washing solvent was butanol, and likewise, pulps cooked by acetone-IL mixtures were washed with acetone

The pulp was washed with 40 mL of washing solvent, and the dark brown suspension was then transferred into a 50 mL Falcon centrifuge tube. For each tube, the suspension was completely vortexed and left to settle for 1 hour or more before being vortexed for a second time. The Falcon tube containing the pulp mixture was subjected to a centrifugation for 50 minutes at 3000 rpm. The supernatant was decanted into a labelled round bottom flask. This washing process was repeated for four times. All the supernatants were collected and combined into the round bottom flask, and the washed pulp was transferred into a cellulose thimble and subjected to a Soxhlet extraction process. More specifically, the thimble with the pulp was repeatedly flashed with 150mL of boiling ethanol for at least 24 hours. After that, the thimble was left in the fume hood to dry overnight. The dried pulp was recovered from the cellulose thimble, weighed, and its moisture content was determined for obtaining the oven-dried weight of the cooked biomass.

The light brown pulp wash generated from the Soxhlet extraction was combined with the previous washes and subjected to evaporation. A dark brown solid containing dried IL and extracted lignin was left in the round bottom flask after removing the washing organic solvent. 30 mL of distilled water (lignin anti-solvent) was added into the round bottom flask in order to precipitate the extracted lignin. The suspension containing precipitated lignin was then transferred into another 50 mL Falcon tube, completely vortexed, left for at least one hour before being vortexed again. The suspension was then subjected to centrifugation, and the supernatant was decanted. The lignin washing process was repeated for four times. The washed lignin was freeze dried for at least three days and then weighed to obtain the lignin recovery yield.

2.1.4.1 Wet pulp preparation

For pretreatments that did not air-dry the pulp to prevent hornification, the procedure was conducted in the same way as the other pretreatments until the Soxhlet extraction process. After the Soxhlet

extraction, the pulp was transferred from the Soxhlet adapters to 50 mL Falcon tube, and each sample was washed with 50 mL DI water, vortexed, and left to settle for an hour or more. The water-pulp suspensions were centrifuged for 30 min at 2000g and the water wash decanted. The washing step was repeated for one more time. All washed pulps were store at 4 °C. Their water content measurements and saccharification analysis were conducted within the next 3 days, otherwise the growing fungi might significantly reduce the amount of sugar releasing during enzymatic hydrolysis.

2.1.4.2 Pretreatment conditions for miscanthus pretreatments

All miscanthus pretreatments were carried out in Ace Pressure Tubes 35mL.

- **For miscanthus fractionation process using different organic solvents**

  The operational temperature: 120°C

  The pretreatment duration: 8 hours

  The biomass to liquid loading: 1:10 g g^{-1}

  The organic solvents used for pretreatment: ethanol, butanol and acetone

  The ionic liquid used: [TEA][HSO_4] with an acid to base ratio of 1

  The organic solvent to ionic liquid ratios: Table 2.1 and Table 2.2

Table 2. 1 Solvent compositions for miscanthus pretreatments with ethanol and [TEA][HSO4], [TEA][HSO4] had three different IL acid to base ratios, 1.00:1.00, 1.02:1.00 and 0.98:1.00, at 120°C at a 1:10 biomass loading

Sample ID	Absolute Ethanol (g)	Ionic liquid [TEA][HSO_4] (g)	Water(g)
Control (ionoSolv)	**0**	**8**	**2**
Ratio 1	**1**	**8**	**1**
Ratio 2	**2**	**8**	**0**
Ratio 3	**3**	**7**	**0**
Ratio 4	**4**	**6**	**0**
Ratio 5	**5**	**5**	**0**
Ratio 6	**6**	**4**	**0**
Ratio 7	**7**	**3**	**0**
Ratio 8	**8**	**2**	**0**

Table 2. 2 Solvent compositions for miscanthus pretreatments with butanol/ acetone and [TEA][HSO_4], [TEA][HSO_4] acid to base ratio was 1.00:1.00, at 120°C at a 1:10 biomass loading

Sample ID	Absolute butanol/acetone (g)	Ionic liquid [TEA][HSO_4] (g)	Water(g)
Control (ionoSolv)	**0**	**8**	**2**
Ratio 1	**2**	**8**	**0**
Ratio 2	**4**	**6**	**0**
Ratio 3	**6**	**4**	**0**
Ratio 4	**8**	**2**	**0**

- **For miscanthus fractionation process using different IL acidities**

  The operational temperature: 120°C

The pretreatment duration: 8 hours

The biomass to liquid loading: 1:10 g g^{-1}

The organic solvents used for pretreatment: ethanol

The ionic liquid used: [TEA][HSO_4] with acid to base ratios of a/b=1, a/b=1.02, a/b=0.98

The organic solvent to ionic liquid ratios: detailed in Table 2.1

- **For miscanthus fractionation process using different biomass loadings**

  The operational temperature: 120°C

  The pretreatment duration: 8 hours

  The biomass to liquid loadings: 1:10 g g^{-1}, 2:10 g g^{-1}, 3:10 g g^{-1}, 4:10 g g^{-1}, 5:10 g g^{-1}

  The organic solvents used for pretreatment: ethanol

  The ionic liquid used: [TEA][HSO_4] with an acid to base ratio of 1

  The organic solvent to ionic liquid ratio: ethanol: IL = 4: 6 g g^{-1}

2.1.4.3 Pretreatment conditions for pine pretreatments

All pine pretreatments were carried out in Hydrothermal Autoclave Reactors 100mL.

- **For pine fractionation process using different organic solvent-ionic liquid mixtures**

  The operational temperature: 170°C

  The pretreatment duration: 80 minutes

  The biomass to liquid loading: 1:10 g g^{-1}

  The organic solvents used for pretreatment: ethanol

  The ionic liquid used: [DMBA][HSO_4] with an acid to base ratio of 1

  The organic solvent to ionic liquid ratios: Table 2.3

Table 2. 3 Solvent compositions for pine pretreatments with [DMBA][HSO_4] and ethanol, at 170°C at a 1:10 biomass loading

Sample ID	Absolute Ethanol (g)	Ionic liquid [DMBA][HSO_4] (g)	Water(g)
Control (ionoSolv)	**0**	**8**	**2**
Ratio 1	**2**	**8**	**0**
Ratio 2	**4**	**6**	**0**
Ratio 3	**8**	**2**	**0**

- **For pine fractionation process using different biomass loadings**

  The operational temperature: 170°C

  The pretreatment duration: 80 minutes

  The biomass to liquid loadings: 1:10 g g^{-1}, 3:10 g g^{-1} , 5:10 g g^{-1}

  The organic solvents used for pretreatment: ethanol

  The ionic liquid used: [DMBA][HSO_4] with an acid to base ratio of 1

  The organic solvent to ionic liquid ratio: ethanol : IL = 4 : 6 g g^{-1}

2.1.4.4 Pretreatment conditions for agriculture residues

- **For rice husk fractionation process with different pretreatment durations and temperatures**

  The operational temperature: 150°C, 170°C

  The pretreatment duration: Table 2.4

Table 2. 4 A list of selected pretreatment durations and temperatures for optimising the rice husk ionoSolv process

Pretreatment temperature (°C)	Pretreatment time (min)							
	15	30	45	60	90	120	150	180
150	✔	✔	✔	✔				
170		✔		✔	✔	✔	✔	✔

The biomass to liquid loadings: 1:10 g g^{-1}

The organic solvents used for pretreatment: none

The ionic liquid used: stock solution A, [TEA][HSO_4] with an acid to base ratio of 1

The pretreatments were carried out in Ace Pressure Tubes.

- **For agricultural residue ionoSolv fractionation**

The pretreatments for four agricultural wastes, bagasse, rice husk, rice straw and wheat straw, were conducted in Ace Pressure Tubes with an estimated optimal pretreatment conditions, Table 2.5.

Table 2. 5 The predicated optimal conditions of the ionoSolv pretreatments for bagasse, rice husk, rice straw and wheat straw

Pretreatment conditions	Bagasse	Rice husk	Rice straw	Wheat straw
Operational temperature (°C)	170	170	170	170
Duration (min)	45	45	30	30
Biomass loading (g g^{-1})	1:10	1:10	1:10	1:10
Ionic liquid[a]	[TEA][HSO_4]	[TEA][HSO_4]	[TEA][HSO_4]	[TEA][HSO_4]
[a] The acid to base ratio of the ionic liquid was 1				

- **For agricultural residue organoSolv-ionoSolv pretreatments**

For each feedstock, an ionoSolv process (also named control pretreatment was conducted in parallel for comparison. All pretreatments were carried out in Hydrothermal Autoclave Reactors. The estimated optimal conditions for organosolv-ionoSolv process were listed in Table 2.6.

Table 2. 6 The predicated optimal conditions of the organosolv-ionoSolv pretreatments for bagasse, rice husk, rice straw and wheat straw

Pretreatment conditions	Bagasse	Rice husk	Rice straw	Wheat straw
Operational temperature (°C)	170	170	170	170
Duration (min)	100	100	80	80
Biomass loading (g g^{-1})	1:10	1:10	1:10	1:10
Organic solvent to ionic liquid ratio [a, b] (g g^{-1})	4:6	4:6	4:6	4:6
[a] The ionic liquid was [TEA][HSO_4] and the acid to base ratio of it was 1.				
[b] Two organic solvents used were ethanol, butanol.				

2.1.5 Pulp analysis

2.1.5.1 Moisture content

For both untreated biomass and pulps (including air-dried and wet pulps), the water content measurements were conducted following the National Renewable Energy Laboratory Analytical Procedure (also known as NREL protocol) "Determination of Total Solids in Biomass and Total Dissolved Solids in Liquid Process Samples".[238] Approximately 100 mg of air-dried biomass/pulp or 1 g of water-washed pulp was weighed out and transferred onto a piece of aluminium foil. The biomass/pulp with the foil was weighed again, folded and oven dried at 105 °C for at least 12 hours. The oven dried aluminium packet containing biomass/pulp was cooled to the room temperature in a desiccator before its weight was measured again. Water content of the measured sample was determined by the weight difference of the metal packet before and after drying. This procedure was carried out in triplicated for biomass and once per sample for both dry and wet pulp.

2.1.5.2 Compositional analysis

The compositions of raw biomass and pulps are determined by following the NREL protocol "Determination of Structural Carbohydrates and Lignin in Biomass".[239] The analysis of raw biomass was conducted in triplicate, but one sample was analysed for each pulp. For each compositional sample, the acid soluble and insoluble lignin contents, ash content, glucose contents and

hemicellulose compositions were determined. The analysis equipment consisted of a 105°C oven (VWR Venti-Line 115), a muffle oven (Nabertherm with Controller P330), an analytical balance (Sartorius CPA 1003 S balance), an autoclave (Sanyo Labo ML5 3020U), a pH meter (VWR SB70P), and a UV-Vis spectrometer (Perkin Elmer Lambda 650 with STD detector module).

300 mg of air-dried pulp or raw biomass (on an oven-dried basis) was weighed and transferred into a 100 mL Ace pressure tube, followed with an addition of 72 vol% sulfuric acid (3 mL). The pressure tube was kept in a 30°C water bath, the mixture was stirred with a Teflon rod and left for 15 minutes. The stirring was repeated for four times with a total duration of 1 hour, making sure the sulfuric acid was well mixed with the sample. The pressure tube was taken out from the water bath, before the solid sample was diluted with 84mL of distilled water, sealed, autoclaved at 120°C for 1 hour, and cooled to 80°C for another hour. The solid sample was then subjected to a fast filtration though a pre-weighted ceramic crucible in order to separate the aqueous filtrate containing acid soluble lignin and sugar from the solid residues made up of acid insoluble lignin and ash. 70 mL filtrate was collected and transferred into two individual Falcon tubes for HPLC (50 mL) and UV (20 mL) analysis.

The black solid residues were washed thoroughly using hot distilled water and contained in the crucible, which was placed in the 105°C oven and dried overnight. The crucible with the residues was cooled in a desiccator for 20 minutes, then weighed on the second day, before being ashed to a constant weight in the muffle oven with a maximum ashing temperature of 575°C. After the ashing process, the crucible which was only left with white ash was cooled for 20 minutes and weighted again. Three crucible weight readings were recorded: the empty crucible, the crucible with acid insoluble lignin and ash and the crucible with ash. The acid insoluble lignin and ash contents were calculated from equations 1 and 2:

$$AIL(\%) = \frac{(m_{AIL+ASH} - m_0) - (m_{ASH} - m_0)}{m_{oven\ dried\ pulp}} \times 100\% \qquad \text{(eq. 1)}$$

$$ASH(\%) = \frac{m_{ASH} - m_0}{m_{oven\ dried\ pulp}} \times 100\% \quad \text{(eq. 2)}$$

where m_0 is the recorded weight of the empty crucible, $m_{AIL+ASH}$ is the recorded weight of the crucible with acid insoluble lignin and ash, m_{ASH} is the recorded weight of the crucible with ash, $m_{oven\ dried\ pulp}$ is the recorded weight on the oven dry basis of the compositional sample (300 mg).

The collected filtrate was partially (20 mL) subjected to a UV analysis for determining the acid soluble lignin content of the measured biomass/pulp. The filtrate was always subjected to a dilution with water, prior to measuring its UV absorbance. Up to three UV absorbance readings at a single wavelength 240 nm were recorded. The absorbance readings were required to range between 0.7 to 1.0. The average absorbance reading was used in equation 3 for determining the acid soluble lignin content:

$$ASL(\%) = \frac{UV_{average} \cdot Volume_{filtrate}}{m_{oven\ dried\ pulp} \cdot \varepsilon \cdot l} \times 100\% \quad \text{(eq. 3)}$$

where $UV_{average}$ is the average reading of the UV absorbance, $Volume_{filtrate}$ is 86.73 mL, $m_{oven\ dried\ pulp}$ is the recorded weight on the oven dry basis of the compositional sample (300 mg), ε is the absorptivity, 12 L/g cm for miscanthus and agricultural residues, 25 L/g cm for pine, l is the path length of the cuvette in cm (1 cm).

The hexose and pentose compositions of the sample was obtained by the HPLC analysis. This analysis was conducted on a Shimadzu HPLC system with an AMINEX HPX-87P Column (BioRad, 300 x 7.8 mm) and a refractive index detection. The mobile phase (0.6 mL/min) was purified water with a resistivity of 15.6 MΩ, and the column was set to 85°C with an acquisition time of 40 min. 20 mL of the collected filtrate was neutralized with calcium carbonate until its pH value reached around 5. The mixture was left to settle for 5 minutes, and 1 mL of the liquid phase was collected using a syringe and filtered

through a 0.2 µm PTEE syringe filter into a HPLC vial and submitted for analysis. Sugar calibration standards were also prepared and run by the HPLC prior to the compositional samples: the standards with concentrations of 0.1, 1, 2 and 4 mg mL^{-1} consisted of glucose, xylose, mannose, arabinose and galactose; the 8 mg mL^{-1} standard only contained glucose. Sugar recovery standards were prepared as following: a 10 mL aqueous solution close to the expected sugar concentration of the sample, transferred to pressure tube, followed with an addition of 72 wt% sulfuric acid (278 µL); the sugar-acid mixture was then autoclaved. The sugar recovery coefficient was determined using equation 4 and two series of sugar recovery coefficients used in the later calculations are listed in Table 2.7. The sugar compositions of each sample were obtained according to equation 5:

$$SRC = \frac{C_{HPLC} \cdot V}{m_{initial\ weight}} \quad \text{(eq. 4)}$$

$$Sugar(\%) = \frac{C_{HPLC} \cdot V \cdot corr_{anhydro}}{SRC \cdot m_{oven\ dried\ pulp}} \cdot 100\% \quad \text{(eq. 5)}$$

where SRC is the Sugar recovery standards, C_{HPLC} is the sugar concentration recorded by HPLC, V is the initial solution volume in mL (10.00 mL for the sugar recovery standards and 86.73 mL for the samples), $m_{initial\ weight}$ is the recorded weight of the sugars used for sugar recovery standards, $corr_{anhydro}$ is the correction of the mass increase during polymeric sugars hydrolysis and $m_{oven\ dried\ pulp}$ is the recorded weight on the oven dry basis of the compositional sample (300 mg).

Table 2. 7 A list of recovery sugar standards and anhydrous correction values used in miscanthus and pine compositional analysis

Sugar	Sugar recovery standards (miscanthus, agricultural residues/pine)	Anhydrous correction
Glucose	0.949/0.98	0.9
Xylose	0.878/1.08	0.88
Galactose	0.878/1.29	0.9
Arabinose	0.878/0.79	0.88
Mannose	0.878/0.86	0.9

For the pulps, three key indicators of the pretreatment effectiveness, hemicellulose removal, delignification and glucan recovery in percentage yields were calculated according to equations 6, 7 and 8, respectively:

$$\text{Hemi. removal } \% = \frac{\text{Hemi.}_{\text{untreated}} - (\text{Yield}_{\text{pulp}} \cdot \text{Hemi.}_{\text{pulp}})}{\text{Hemi.}_{\text{untreated}}} \cdot 100\% \quad \text{(eq. 6)}$$

$$\text{Deligninfication } \% = \frac{\text{Lignin}_{\text{untreated}} - (\text{Yield}_{\text{pulp}} \cdot \text{Lignin}_{\text{pulp}})}{\text{Lignin}_{\text{untreated}}} \cdot 100\% \quad \text{(eq. 7)}$$

$$\text{Gluc. recovery } \% = \frac{\text{Gluc.}_{\text{untreated}} - (\text{Yield}_{\text{pulp}} \cdot \text{Gluc.}_{\text{pulp}})}{\text{Gluc.}_{\text{untreated}}} \cdot 100\% \quad \text{(eq. 8)}$$

where Hemi. is short for hemicellulose, Gluc. is short for glucose, $\text{Hemi.}_{\text{untreated}}$, $\text{Lignin}_{\text{untreated}}$ and $\text{Gluc.}_{\text{untreated}}$ stand for hemicellulose , lignin and glucose contents of the raw biomass, respectively; $\text{Hemi.}_{\text{pulp}}$, $\text{Lignin}_{\text{pulp}}$ and $\text{Gluc.}_{\text{pulp}}$ stand for hemicellulose, lignin and glucose contents of the pulp based on composition analysis, respectively; $\text{Yield}_{\text{pulp}}$ is the pulp yield on an oven dry basis.

2.1.5.3 Saccharification assays

Enzymatic hydrolysis experiments were conducted in triplicate, according to the National Renewable Energy Laboratory Analytical Procedure "Enzymatic Saccharification of Lignocellulosic Biomass".[240] Novozymes experimental enzyme mixture, Cellic® CTec 2, was used. The enzyme loadings were 20 µL(miscanthus, bagasse, rice straw, wheat straw) and 50 µL (pine, rice husk). Three untreated biomass samples for each feedstock were prepared and analysed in parallel to the pulp samples.

For each pulp sample, one saccharification sample was prepared and analysed. 100±10 mg of air-dried pulp was weighed and contained into a 25 mL Sterilin tube. The exact weight of the pulp sample was recorded, and the corresponding oven-dried weight was calculated. Three enzyme-only samples were run with an additional of 100 µL distilled water to the buffer-enzyme stock solution for correcting any

sugar residues appearing in the stock solution. A buffer-enzyme mixture containing two antibiotics was made and labelled as the saccharification stock solution. Each saccharification sample was mixed with 9.9 mL stock solution consisting of 5 mL sodium citrate buffer (100 mM, pH 4.8), 30 µL cycloheximide solution (10 mg mL^{-1} in 100% distilled water), 40 µL tetracycline solution (10 mg mL^{-1} in 70% v:v ethanol and 30% v:v distilled water), distilled water (4.81 mL for miscanthus, bagasse, rice straw, wheat straw, 4.78 mL for pine, rice husk) and Novozymes experimental enzyme mixture Cellic® CTec 2. All samples were sealed, incubated in a Stuart Orbital Incubator (S1500) at 50°C for 7 days at 250 rpm. After that, 1 mL of the saccharification mixture was collected, filtered, contained in a HPLC vial after the mixture was cooled to the room temperature. The filtered samples were analysed on a Shimadzu HPLC system with RI detector and an Aminex HPX-87P column (BioRad, 300 x 7.8 mm) with purified water (resistivity 15.6 MΩ) as mobile phase (0.6 mL/min). The column temperature was set to 85 °C and acquisition time of the column system was 40 min. Five sugar calibration standards were also prepared and run: the standards with concentrations of 0.1,1,2 and 4 mg mL^{-1} consisted of glucose, xylose, mannose, arabinose and galactose; the 8 mg mL^{-1} standard contained merely glucose.

Between wet and air-dried pulps, saccharification hydrolysis differed by the amount of water added during sample preparation. Moisture contents of the wet pulps were determined prior to assay. Each wet sample was weighed, recorded. The water content of the saccharification sample was determined using the recorded sample weight and its moisture content. Distilled water was then added where the total volume of water contained in the sample was kept at 1.5 mL, and the sample moisture was subtracted from 1.5 mL prior to the water addition. The sample was then mixed with 8.4 mL stock solution containing enzyme, buffer, and antibiotics before being incubated.

2.1.6 Lignin analysis

2.1.6.1 Enzymatic mild acidolysis lignin for agriculture residues (EMAL)

Enzymatic mild acid hydrolysis lignins were extracted from four agriculture residues following the protocol established by Wu *et al*.[241] (Only bagasse EMAL sample was produced successfully) For each EMAL sample, the raw biomass was ground to a particle size ≤180 µm using a SM200 cutting mill. The biomass powder was subjected to an acetone extraction for 2 days, following by a drying process at 45 °C under vacuum. Around 60 g extracted biomass powder was separated into four ball milling jars, immersed in toluene, and subjected to a planetary ball milling process for 14 days at 150 rpm. After that, the toluene-biomass mixtures were left in the fume hood to dry for 3 days.

A two-stage enzymatic acid hydrolysis was conducted for the ball-milled biomass. In the first stage of the hydrolysis, the ball-milled biomass was treated with cellulase enzymes Cellic® CTec 2 with an enzyme to biomass concentration of 190 mg g^{-1}. This stage of the hydrolysis was conducted in a citrate buffer (0.1 M, pH of 4.75) which contains 2% Tween 20 for 2 days. The buffer-biomass slurry was stirred at 120 rpm in a 2 L jacketed borosilicate glass pressure vessel with an anchor impeller.

The slurry temperature was monitored and maintained at 50 °C by an oil recirculator equipped with a thermostat. After the hydrolysis, the suspension was centrifuged at 3000 rpm for 30 min and supernatant was decanted. The remaining solid residues were mixed with a new batch of enzyme-buffer mixture and subjected to another acid hydrolysis for 2 days at 50 °C. After completing the two-stage hydrolysis, the solid residues were washed with acidified deionized water (pH 2.0) twice, for removing any soluble sugars, and then washed with 6 M guanidine hydrochloride to remove any protein residues. The washed grey residues were freeze dried for three days to yield a crude lignin sample. (rice husk, rice straw, wheat straw failed to generate the crude lignin samples.)

The crude lignin was ground using a pestle and mortar. The ground lignin was mixed with azeotrope of dioxane-water (96: 4 v/v, lignin to liquid ratio of 1:20 g mL^{-1}), hydrochloric acid (0.01 M) and butyl hydroxytoluene acting as a radical scavenger and stabilizer during hydrolysis. The lignin-acid slurry was then subjected to an acid hydrolysis, refluxed at 87°C under a nitrogen atmosphere. The slurry was centrifuged and supernatant decanted. The lignin residues were washed with fresh aqueous dioxane until the supernatant was colourless. The supernatants were collected, combined, and then neutralized using sodium bicarbonate. 8 L acidified water (pH 2.0) was dropwise added into the neutralised supernatant to precipitate EMAL lignin. The aqueous lignin mixture was left to equilibration for 12 hours. The precipitated EMAL was isolated from the aqueous phase by centrifugation, washed twice with distilled water, washed once with hexane, and finally subjected to freeze dry for 3 days. The yield of the EMAL sample extracted from bagasse was 3 wt% relative to the weight of air-dried biomass.

2.1.6.2 ^{1}H-^{13}C HSQC NMR spectroscopy

For each lignin sample, 40 ± 0.1 mg of the measured sample was dissolved in 0.5 mL DMSO-d_6 with stirring for at least 12 hours. The well stirred lignin solution was then transferred to an NMR tube. HSQC NMR spectra were recorded on a Bruker 600 MHz spectrometer (pulse sequence hsqcetgpsi2, spectral width of 10 ppm in F2 (^{1}H) with 2048 data points and 160 ppm in F1 (^{13}C) with 256 data points, 16 scans and 1 s interscan delay). MestReNova 8.0.0 was used to assign and integrate the spectra. The solvent peaks at 2.500 ppm (^{1}H) and 39.520 ppm (^{13}C) was referenced prior to peak integration. Integral areas were kept the same for all spectra. Integration areas were located according to peak assignments in the literature[71,205] and normalised to (G_2 + $G_{2,cond}$) signals. All spectra can be found in the appendix.

2.1.6.3 Gel permeation chromatography（GPC）

All GPC measurements were carried out in an Agilent 1260 Infinity instrument equipped with a Viscotek column set (AGuard, A6000 M and A3000 M) and an Agilent 1260 Infinity RID detector. The instrument calibrations used were ten pullulan standards (Agilent calibration kit, 180 < Mp < 780 000). The column set was eluted with a GPC grade DMSO and LiBr mixture (1 g x L^{-1}) at a flow rate of 0.4 mL min^{-1}at 60 °C. Each lignin sample, 20± 0.1 mg, was weight and dissolved in 1 mL eluent mixture for at least 12 hours before being filtered.

2.2 Artificial lignin polymer synthesis

2.2.1 Monolignol synthesis

2.1.1.1 *Synthesis of ethyl sinapate*

Sinapic acid (4.83 g, 21.5 mmol) was dissolved in absolute ethanol (88.0 mL, 1500 mol), and kept in an ice bath until the complete dissolution was achieved. Acetyl chloride (8.79 mL, 123 mmol) was added over a period of 10 minutes. The mixture was kept stirring for 24 hours and then partially evaporated at 30°C. 1:1 aqueous solution of ethyl acetate (v/v) (134 mL) was added into the remaining reaction mixture. The organic fraction was washed with distilled water (134 mL, 2 times) and saturated aqueous sodium bicarbonate (134 mL, 1 time), before being washed with distilled water (134 mL). The upper organic fraction was then washed with saturated aqueous sodium chloride (134 mL) before being dried over magnesium sulphate. The organic layer was collected and evaporated to dryness. The final product, a pale yellow crystalline solid, was obtained by a crystallisation using ethyl acetate/hexane (3.14 g, 12.5 mmol, 57.8 %).

Compound produced was subjected to proton NMR spectrometry for characterisation and the assigned spectra is detailed here: ^{1}H NMR, 400 MHz, DMSO-d_6, δ_H 8.96 (1 H, s, OH), 7.56 (1H, d, J=15.9

Hz), 7.04 (2H, s, H(2)), 6.54 (1H, d, J=15.9 Hz), 4.17 (2H, q, J=7.1 Hz), 3.81 (6H, s), 1.25 (3H, t, J=7.1 Hz) Compound produced was subjected to Carbon-13 NMR spectrometry for characterisation and the assigned spectra is detailed here: ^{13}C NMR, DMSO-d_6, δ_c 167.06, 148.48, 145.69, 138.73, 124.87, 115.46, 106.67, 60.16, 56.54, 14.71.

2.1.1.2 *Synthesis of methyl sinapate*

Sinapic acid (2.00 g, 9.30 mmol) was fully dissolved in pre-heated anhydrous methanol (20 mL) at 87°C. 6 drops of aqueous sulphuric acid (acid to water ratio was 72:28 w/w) were added into the reaction mixture, with stirring. The acid-organic mixture was kept refluxing for one day, before being cooled to room temperature. The creamy suspension was then concentrated before dissolving into ethyl acetate (50 mL). The organic mixture was then washed with brine (20 mL, 2 times), distilled water (20mL), dried over magnesium sulphate. The final product, a pale yellow solid, was obtained after evaporation. (1.93 g, 8.08 mmol, 86.8 %).

Compound produced was subjected to proton NMR spectrometry for characterisation and the assigned spectra is detailed here: ^{1}H NMR, 400 MHz, DMSO-d_6, δ_H 8.97 (1H, s, OH), 7.58 (1H, d, *J* = 15.9 Hz), 7.04 (2H, s), 6.55 (1H, d, *J* = 15.9 Hz), 3.81 (6H, s), 3.72 (3H, s)

Compound produced was subjected to Carbon-13 NMR spectrometry for characterisation and the assigned spectra is detailed here: ^{13}C NMR, DMSO-d_6, δ_c 167.52, 148.49, 145.87, 138.81, 124.83, 115.09, 106.76, 56.57, 51.69.

2.1.1.3 *Synthesis of sinapyl alcohol*

Ethyl sinapate (2.49 g, 9.58 mmol) was fully dissolved in fresh distilled toluene (110 mL), kept in an ice bath under nitrogen. Diisobutylaluminium hydride (28.2 mL, 41.58 mmol) was slowly added into over

a period of 20 minutes, with stirring. The reaction mixture was stirred for another hour before dropwise adding distilled water (1.76 mL), 15 wt% aqueous solution of sodium hydroxide (1.76 mL). Distilled water (4.40mL) was again added into the mixture. The reaction was kept stirring for another 15 minutes, before being cooled to room temperature. Another water addition was performed (57.20 mL). As the aqueous layer of the mixture containing gelatinous aluminum salts precipitates, it was extracted using ethyl acetate (176 mL, 4 times). All the organic layers were collected, combined and dried over magnesium sulfate. The solid- liquid solution was stirred for 15 minutes. The filtrate was evaporated into solid. The final product, a yellow-orange oil, was purified from dichloromethane/petroleum benzene (1.73 g, 8.33 mmol, 86.2%).

Compound produced was subjected to proton NMR spectrometry for characterisation and the assigned spectra is detailed here: ^{1}H NMR, 400 MHz, DMSO-d_6 δ_H 8.36(1H, s), 6.64(2H,d, J=32.2 Hz), 6.48 (1H, m), 6.23 (1H, dt, J = 15.9, 5.3 Hz), 4.09 (2H, td, J = 5.4 ,1.5 Hz), 3.75 (6 H, m), 3.33 (1 H, S, OH)

Compound produced was subjected to Carbon-13 NMR spectrometry for characterisation and the assigned spectra is detailed here: ^{13}C NMR, DMSO-d_6, δc 148.53, 135.65, 129.72, 128.43, 127.92, 104.23, 62.20, 56.43.

Methyl sinapate (1.00 g, 4.18 mmol) was added into pre-cooled anhydrous toluene (50 mL) at 0°C under vacuum. Diisobutylaluminium hydride (17 mL, 25.1 mmol) was added dropwise, and the mixture was kept stirring for 2 hours at 0°C and continued to be stirred for 1 day at room temperature. The mixture was quenched by absolute ethanol (10 mL), concentrated. The remaining organic mixture was mixed with distilled water (20 mL), forming yellow solid. The solid-liquid mixture was extracted with ethyl acetate (50 mL, 5 times). The ethyl acetated mixture was washed with brine (50 mL, 1 time), distilled water (50 mL, 1 time), dried with magnesium sulfate. The final, product, a yellow-orange oil,

was obtained after evaporation. The purification with dichloromethane/ hexane was preformed for the crude product (0.68 g, 3.27 mmol, 78.2%).

Compound produced was subjected to proton NMR spectrometry for characterisation and the assigned spectra is detailed here: ^{1}H NMR, 400 MHz, DMSO-d_6, δ_H 8.35 (1H, s, OH), 6.69 (2H, s), 6.43 (1H, d, *J* = 15.9 Hz), 6.23 (1H, d, *J* = 15.7 Hz), 4.77 (2H, t, *J* = 5.5 Hz), 4.09 (6H, td, *J* = 5.4, 1.5 Hz)

Compound produced was subjected to Carbon-13 NMR spectrometry for characterisation and the assigned spectra is detailed here: ^{13}C NMR, DMSO-d_6 δ_c 148.50, 135.67, 129.67, 128.39, 127.89, 104.27, 62.14, 56.41.

2.1.1.4 *Synthesis of ethyl ferulate*

Ferulic acid (10.0 g, 51.5 mmol) was dissolved in absolute ethanol (180 mL), followed by an addition of acetyl chloride (12 mL, 168.17 mmol). The mixture was under stirring for two nights. The remaining ethanol and acetyl chloride were evaporated at 30°C. The final product, a creamy crystalline solid, was crystallised from viscous oil, using ethyl acetate/hexane. (9.28 g, 41.8 mmol, 81.1%). (More details in **2.1.1.1 *synthesis of ethyl sinapate***)

Compound produced was subjected to proton NMR spectrometry for characterisation and the assigned spectra is detailed here: ^{1}H NMR, 400 MHz, DMSO-d_6, δ_H 9.60 (1H, s, OH), 7.55 (1H, d, J=15.9 Hz), 7.33 (1H, d, J=2.0 Hz), 7.12 (1H, dd, J=8.2 Hz), 6.79 (1H, d, J=8.1 Hz), 6.48 (1H, d, J=15.9 Hz), 4.17 (2H, q, J=7.1 Hz), 3.82 (3H, s), 1.25 (3H, t, J=7.1 Hz)

Compound produced was subjected to Carbon-13 NMR spectrometry for characterisation and the assigned spectra is detailed here: ^{13}C NMR, DMSO-d_6, δ_C 167.08, 149.27, 148.38, 145.37, 126.04, 123.56, 115.94, 115.02, 111.65, 60.15, 56.32, 14.73.

2.1.1.5 *Synthesis of coniferyl alcohol*

Ethyl ferulate (2.20 g, 11.3 mmol) was dissolved in fresh distilled toluene (110 mL), followed by addition of diisobutylaluminium hydride (28.2 mL, 41.6 mmol), kept in an ice bath under nitrogen. The final product, a pale yellow crystalline solid, was crystallized from dichloromethane/petroleum benzene (2.30 g, 113%). (see more details in **2.1.1.*3 synthesis of sinapyl alcohol***)

Compound produced was subjected to proton NMR spectrometry for characterisation and the assigned spectra is detailed here: ^{1}H NMR, 400 MHz, DMSO-d_6, δ_H 8.99 (1H, s , OH), 7.00(1H, d, J=2.0 Hz), 6.80 (1H, dd, J=8.1, 1.9 Hz), 6.71 (1H, d, J =8.1 Hz), 6.42 (1H, dt, J= 15.9, 1.7 Hz), 6.18 (1H, dt, J= 15.9, 5.4 Hz), 4.76 (2H, t， J= 5.4 Hz), 3.33 (1H, s, OH), 4.08 (3H, s)

Compound produced was subjected to Carbon-13 NMR spectrometry for characterisation and the assigned spectra is detailed here: ^{13}C NMR, DMSO-d_6, δ_C 148.14, 146.59, 129.41, 127.93, 119.86, 115.89, 110.12, 62.17, 56.00.

2.1.1.6 *Synthesis of methyl p-coumarate*

P-coumaric acid (9.85 g, 60 mmol) was fully dissolved in pre-heated anhydrous methanol (400 mL) at 87°C. aqueous sulphuric acid (3 mL, acid to water ratio was 72: 28 w/w) were added into the reaction mixture, with stirring. The final product, a creamy solid, was obtained after evaporation. (8.17 g, 0.046 mmol, 76.4 %). (more details in **2.1.1.2 *synthesis of methyl sinapate***)

Compound produced was subjected to proton NMR spectrometry for characterisation and the assigned spectra is detailed here: ^{1}H NMR, 400 MHz, DMSO-d_6, δ_H 10.00 (1H, s,OH), 7.58 (1H, d, J = 12.2 Hz), 7.55 (2H, d, J = 4.8 Hz), 6.80 (2H, dd, J = 8.7 Hz), 6.40 (1H, d, J = 16.0 Hz), 2.54 – 2.48 (3H, m)

Compound produced was subjected to Carbon-13 NMR spectrometry for characterisation and the assigned spectra is detailed here: ^{13}C NMR, DMSO-d_6, δ_c 167.48, 160.31, 145.19, 130.74, 125.54, 116.23, 114.39, 51.65.

2.1.1.7 *Synthesis of p-coumaryl alcohol*

Methyl coumarate (2.01 g, 11.3 mmol) was added into pre-cooled anhydrous toluene (50 mL) at 0°C under vaccumn. Diisobutylaluminium hydride (33.2 mL, 49.0 mmol) was added dropwise, and the mixture was kept stirring for 2 hours at 0°C and continued to be stirred for 1 day at room temperature. The final, product, a yellow-orange oil, was obtained after evaporation. The purification with dichloromethane/ hexane was performed for the crude product (1.25 g, 8.41 mmol, 74.5%). (see more details in **2.1.1.*3 synthesis of sinapyl alcohol***)

Compound produced was subjected to proton NMR spectrometry for characterisation and the assigned spectra is detailed here: ^1H NMR, 400 MHz, DMSO-d_6, δ_H 9.40 (1H, s, OH), 7.19 (1H, d, J = 8.6, 2.0 Hz), 7.07 – 6.82 (2H, m), 6.75 – 6.59 (2H, m), 6.38 (1H, dd, J = 15.9, 1.9 Hz), 6.18 – 5.92 (2H, m), 4.02 (2H, td, J = 5.4, 4.5, 2.7 Hz)

Compound produced was subjected to Carbon-13 NMR spectrometry for characterisation and the assigned spectra is detailed here: ^{13}C NMR, DMSO-d_6, δ_c 157.33, 129.19, 128.41, 127.91, 127.66, 115.89, 62.25.

2.1.2 Enzymatic homogeneous polymerization

Monolignols (e.g for coniferyl alcohol, 1.12g monomer, 0.62 mmol) were dissolved in a mixture of 40mM phosphate buffer, pH of 6, and aqueous 1,4-dioxane (40% v/v), labelled mixture 1. H_2O_2 (e.g for coniferyl alcohol, 0.22 g H_2O_2, 0.62 mmol) was dissolved in a mixture of 40mM phosphate buffer and a aqueous 1,4-dioxane (20% v/v), labelled mixture 2. Horseradish peroxidases HRP (e.g for

coniferyl alcohol, 2 mg HRP) was dissolved in 40mM phosphate, labelled mixture 3. Mixtures 1,2, and 3 were dropwise added into a mixture of phosphate buffer with 1,4-dioxane (20% v/v). After the addition, the reaction mixture was kept on stirring for 1 day before filtering the precipitate. The precipitated polymer mixture was washed with distilled water (100mL, 5 times). The washed polymer mixture underwent fractionation: polymer mixture firstly dissolved in methanol, and the methanol insoluble fraction was filtered, dried in the desiccator, and its weight was recorded; the methanol insoluble fraction was then dissolved in DMF, the insoluble fraction (if applicable) was filtered, dried, weighted. The buffer soluble fraction was isolated out by dropwise adding water into the solution, following with centrifuging at 3000 rpm for 50 minutes. The isolation process was repeated for three times. The precipitate isolated out was dried in vacuum oven at 40°C overnight. The methanol soluble and methanol insoluble polymers were subjected to GPC and HSQC analysis.

3 Results: Miscanthus organosolv-ionoSolv fractionation

Chapter summary

The focus of this chapter is to investigate the optimal fractionation conditions for pretreating a grassy feedstock, miscanthus, using the IL [TEA][HSO_4] mixed with an organic co-solvent, e.g. ethanol. butanol, acetone. By varying different characteristics of the fractionation process, e.g. pretreatment solvent composition, IL acidity, biomass to solvent loading, the organosolv-ionoSolv process has been studied in depth. The effect of hornification due to air-drying pulp on saccharification yields has also been investigated. The relationship between lignin removal and the sugar releasing yield of the pulp for this newly-developed fractionation process has been understood and the lignins isolated have been characterised by HSQC NMR and GPC analysis. The chapter finishes out with an economical evaluation of the organosolv-ionoSolv process, in order to examine if the process is cost-effective for industrialisation. The feedstock loading pretreatments were conducted by master student, Adam Raif and the economical evaluation was conducted by a PhD student, Francisco Malaret. The full content of the chapter was also included in the written paper 'Design of an organosolv-ionoSolv biomass fractionation process for biofuel production and high value-added lignin valorisation' which is ready for submission, as detailed in **Publications**.

3.1 Optimisation of combined organosolv-ionoSolv pretreatments

Based on the main concepts of ionoSolv and organosolv processes, a hybrid biomass fractionation process was developed here, using anhydrous [TEA][HSO_4] with an organic co-solvent to pretreat Miscanthus. The effectiveness of the pretreatment was determined by the saccharification yield and

the pulp composition. The co-solvents chosen were ethanol, butanol, and acetone, and the concentration of each co-solvent was varied from 0 to 80 wt%. For this hybrid process, the pretreated biomass (pulp), in this case, miscanthus, was typically subjected to an oven cooking process at 120°C for 8 hours, then washed with the organic solvent repeatedly to remove dissolved lignin, hemicellulose and IL, and finally air-dried before subjected to compositional analysis and saccharification assays. The pulp washing solvents were decanted and collected. The organic solvents were subsequently separated from the pulp washings by rotary evaporation, leaving a solid mixture of lignin and IL. Water was added into the solid mixture as an anti-solvent for separating the lignin from the IL, as well as washing the lignin. The washed lignin was then freeze-dried to remove any remaining moisture. For ethanol-IL pretreatment, the cooked biomass was washed by ethanol, while for butanol-IL pretreament, the pulp was washed by butanol, likewise for acetone-IL pretreament. The reason for using different pulp washing solvents was for the simplicity of recycling solvent in the later stage of the pretreatment. IonoSolv processes using aqueous [TEA][HSO_4] (with 20 wt% moisture) were conducted for comparison. Three ionoSolv pretreatments were performed, and each with a different pulp washing solvent. The ionoSolv process using ethanol to wash the pulp is compared to the ethanol-IL fractionation process, likewise for the ionoSolv process using butanol or acetone as the pulp washing solvent.

3.1.1 Effect of organic solvent choice and their concentrations on fractionation

All the air-dried pulps were characterised by saccharification assay and compositional analysis. Figure 3.1 presents the pulp composition for organosolv-ionoSolv pretreatments with 10 different ratios of ethanol and IL at 120°C for 8 hours, with a 1:10 g g^{-1} biomass loading.

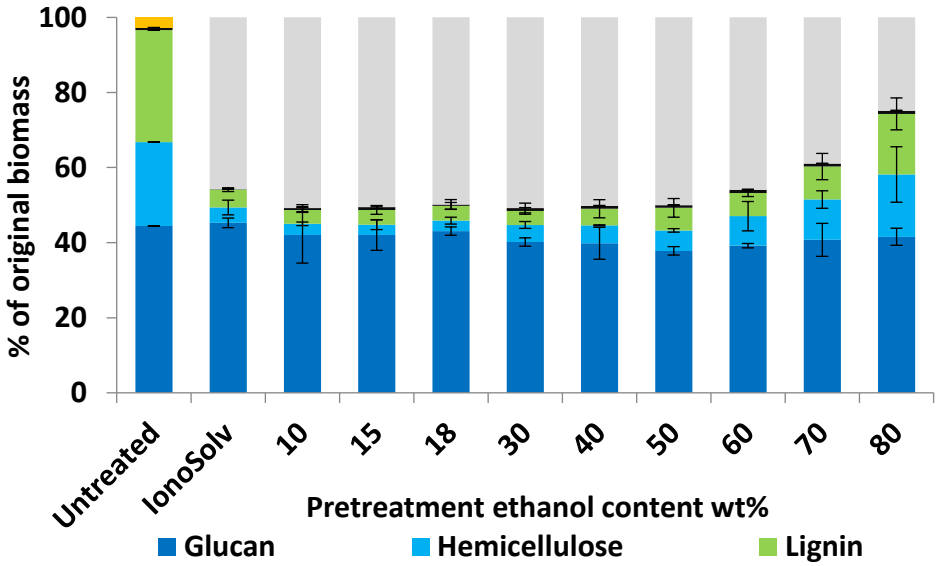


Figure 3. 1 Compositional analysis of Miscanthus pulp recovered from ethanol-[TEA][HSO4] pretreatment at 120°C for varying ethanol content in pretreatment solvent at 1:10 g g^{-1} biomass to solvent loading. IonoSolv (second from left) represents the pulp composition for pretreatment with 80wt% [TEA][HSO4] and 20wt% water for comparison. Error bar is included.

3.1.1.1 Saccharification yields

The saccharification yields for the ethanol-IL processes kept constant around 85% from 20 wt% to 60 wt% ethanol but the yield was halved at 80 wt%. ethanol. This suggests there is a minimum IL concentration required in order to keep an overall pretreatment effectiveness of >80%, i.g ethanol-IL mixture with 20% IL is not powerful enough to fractionate the feedstock. Figure 3.1 presents the trend of the saccharification yields, glucose recovery, hemicellulose removal, lignin removal and lignin recovery yields for the pulps pretreated with different ethanol-[TEA][HSO_4] mixtures. All the yields were listed in Table 3.1. The sugar (glucose) releasing yields are presented as percentages relative to the glucose content of untreated *Miscanthus*. The ionoSolv process, using IL with 20 wt% water, achieved a saccharification yield of 75%. This yield was increased to 85% when the fractionation process was using anhydrous IL with 40% ethanol. This 10% glucose-yield increase is important for any potential commercialisation of this modified ionoSolv pretreatment, which is often hindered by the

energy intensive and low cost-effective IL regeneration process.[175] Organosolv process using aqueous ethanol could achieve a similar glucose conversion, 78%, similar to the ionoSolv process, but the process operated at a higher temperature, 170°C.[242]

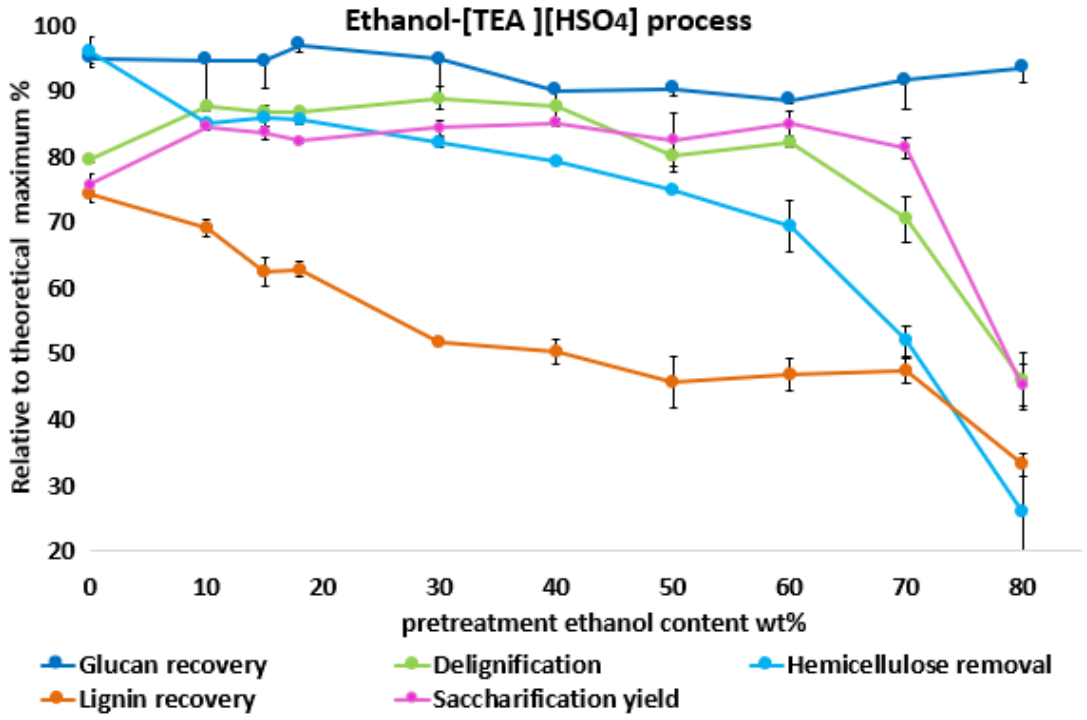


Figure 3. 2 Key indicators of fractionation effectiveness for ethanol -[TEA][HSO_4] pretreatment at 120°C for 8 hours with a varied organic solvent content in pretreatment solvent with 1:10 g g-1 biomass loading, all data points with 0% organic solvent represent corresponding ionoSolv processes, using [TEA][HSO_4] containing 20 wt% water. 0% ethanol data points represent the ionoSolv process where the pulp was washed by ethanol. Yields are relative to the glucose, hemicellulose and lignin content in the untreated Miscanthus. Error bar is included.

Table 3. 1 A list of compositional and saccharification key indicators for miscanthus fractionation process using a mixture of ethanol and [TEA][HSO_4]

wt% of ethanol in pretreatment solvent (wt% IL+water)	Glucan recovery[a]	hemicellulose removal[a]	lignin recovery yield[a]	Delignification[a]	Saccharification yield[a]
0[b] (100)	95	96	65	79	76
10[c](90)	95	85	56	88	85
15 (85)	95	86	54	87	84
18 (82)	97	85	54	87	82
30 (70)	95	82	55	89	85
40 (60)	90	79	53	88	85
50 (50)	91	75	48	80	83
60 (40)	89	69	43	82	85
70 (30)	92	52	48	70	81
80 (20)	94	26	33	46	45

[a] The yield is presented in precentages of the theoretical maximum, relative to untreated biomass

[b] The pretreatment with 0% ethanol content represents the ionoSolv process where the pretreated biomass is subjected to the ethanol pulp washing process.

[C] The pretreatment solvent composition for 10% ethanol was 80 wt% IL, 10 wt% water and 10 wt% ethanol.

For an organosolv pretreatment to achieve a competitive performance, comparing to ionoSolv and organosolv-ionoSolv preteatments, it often requires a high operational temperature (>200°C), otherwise a catalyst is needed, e.g. mineral acids (commonly sulfuric acid).[243,203] In this organosolv-ionoSolv pretreatment process, the acidic IL, in this case, [TEA][HSO_4], plays a dual role: 1) removing hemicellulose and lignin by breaking down their linkages with cellulose 2) activating the co-solvent, ethanol, allowing it to fractionate biomass at a comparatively lower temperature, 120°C. When IL concentration is high enough (≥20 wt%.), both IL and ethanol could act as biomass fractionation solvents at the same time without any inhibiting

interactions. Thus, this hybrid (organosolv-ionoSolv) process could be seen as more effective than the process using ethanol or IL alone. If the IL concentration is below 20 wt% the amount of IL presented in the pretreatment is not enough to remove hemicellulose and lignin quantitatively, and the large amount of ethanol (≥80 wt%) is not activated as a pretreating solvent, i.e. the ethanol only appeared in the pretreatment as a co-solvent for IL, not a fractionation solvent for biomass. Brandt-Talbot *et al*'s study has reported a strong positive correlation between saccharification yield and lignin removal, also referred as delignification.[71] Therefore, the enzymatic saccharification of those biomass pretreated with low IL-content organic mixtures will be hindered by the large amount lignin preserved in the pulps.

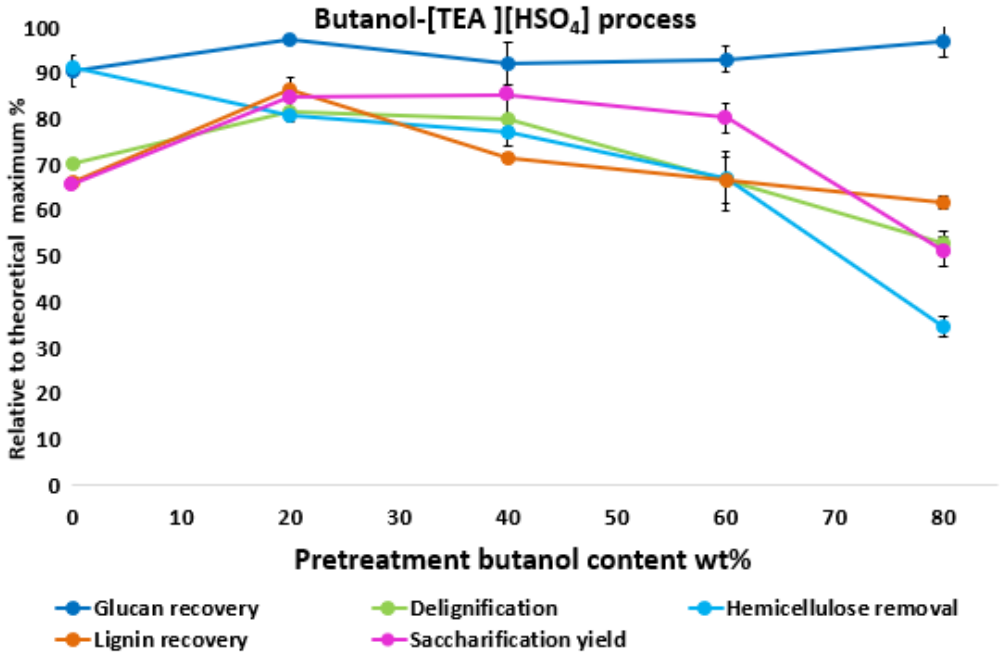


Figure 3. 3 Key indicators of fractionation effectiveness for butanol -[TEA][HSO_4] pretreatment, all data points with 0% organic solvent represent corresponding ionoSolv processes, using [TEA][HSO_4] containing 20 wt%. water. 0% butanol data points represent the ionoSolv process where the pulp was washed by butanol. Yields are relative to the glucose, hemicellulose and lignin content in the untreated Miscanthus. Error bar is included.

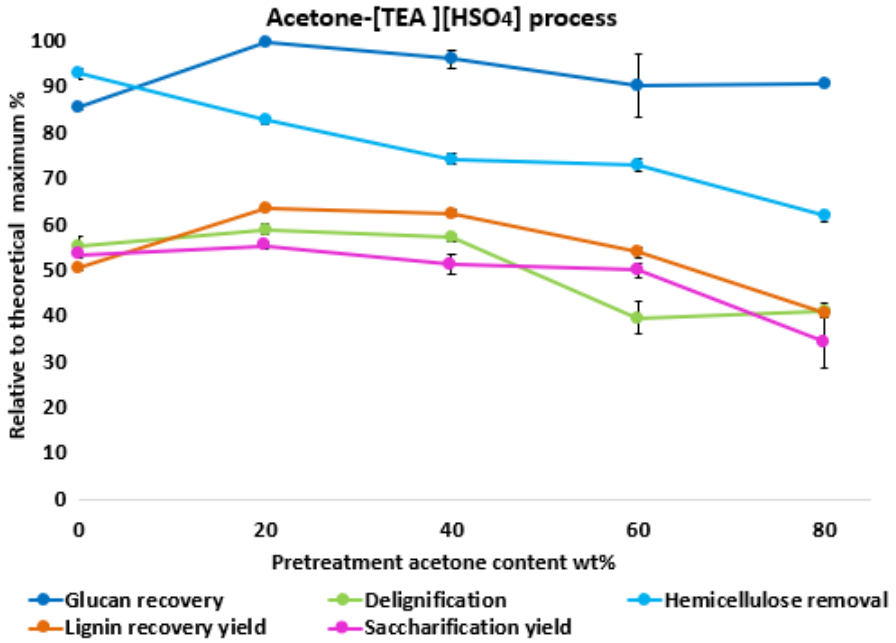


Figure 3. 4 Key indicators of fractionation effectiveness for acetone -[TEA][HSO_4] pretreatment, all data points with 0% organic solvent represent corresponding ionoSolv processes, using [TEA][HSO_4] containing 20 wt%. water. 0% acetone data points represent the ionoSolv process where the pulp was washed by acetone. Yields are relative to the glucose, hemicellulose and lignin content in the untreated Miscanthus. Error bar is included.

Figure 3.3 and 3.4 display the trends of glucose releasing yields and other key fractionation indicators, e.g. delignification, for miscanthus fractionated by butanol-IL and acetone-IL mixtures with various organic solvent contents. According to Table 3.2, for pretreatment using butanol-IL mixtures, the glucose releasing yield peaked at 40 wt% butanol with a value of 85% and maintained above 80% between 20 wt% and 60 wt% butanol. Comparing to ethanol-IL, butanol-IL achieved a similar maximum saccharification yield, indication that changing the aliphatic carbon chain length for the organic alcohol does not affect the sugar yield or have a significant impact of the overall pretreatment effectiveness.

Table 3. 2 A list of compositional and saccharification key indicators for miscanthus fractionation process using a mixture of butanol and [TEA][HSO_4]

wt% of butanol in pretreatment solvent (wt% IL+water)	Glucan recovery[a]	hemicellulose removal[a]	lignin recovery yield[a]	Delignification[a]	Saccharification yield[a]
0[b] (100)	90	91	66	70	66
20 (80)	98	81	87	82	85
40 (60)	92	77	72	80	85
60 (40)	93	67	67	67	80
80 (20)	97	35	62	53	51

a the yield is presented in precentages of the theoretical maximum, relative to untreated biomass

b The pretreatment with 0% butanol content represents the ionoSolv process where the pretreated biomass is subjected to the butanol pulp washing process.

Table 3. 3 A list of compositional and saccharification key indicators for miscanthus fractionation process using a mixture of acetone and [TEA][HSO_4]

wt% of acetone in pretreatment solvent	Glucan recovery[a]	hemicellulose removal[a]	lignin recovery yield[a]	Delignification[a]	Saccharification yield[a]
0[b] (100)	86	93	50	56	54
20 (80)	99	83	64	59	56
40 (60)	96	74	63	57	51
60 (40)	90	73	54	40	50
80 (20)	91	62	41	42	34

[a] the yield is presented in percentages of the theoretical maximum, relative to untreated biomass

[b] the pretreatment with 0% acetone content represents the ionoSolv process where the pretreated biomass is subjected to the acetone pulp washing process.

As listed in Table 3.3, glucose yields for acetone-IL processes peaked at 55%. This peak sugar yield was significantly lower than butanol and ethanol, 30% lower, but appear in an agreement with the literature. The organosolv process using aqueous acetone for bagasse was conducted by Jafari *et al*. Jafari reported that the peak saccharification achieved by aqueous acetone pretreatment was 55% at 150°C and 94% at 180°C.[203] The other study using aqueous acetone to pretreat wheat straw demonstrated that the organosolv process could achieve a saccharification yield of 87%, while operating at a comparatively high temperature, 205°C, relative to the acetone-IL processes developed here. [243] This study also demonstrated that sugar yields were below 45% when aqueous acetone process was operated at 160°C and 175°C, suggesting the wheat straw fractionation was not effective at these temperatures. The temperature that the pretreatments were operating at (120°C) could be blamed for the low sugar yield achieved by acetone-IL pretreatment in our study.

Three ionoSolv processes using aqueous IL were performed, where the only differences came from the pulp washing solvent. The pulps washed by ethanol achieved the best saccharification yield, 10% higher the pulps washed by butanol and 22% higher than the acetone-washed pulps. This suggested that compared to ethanol, using butanol and acetone to wash pulp could negatively affect lignin removal. Subsequently, non-ideal pulp washing solvent could be blamed for the lowered saccharification yields for all acetone-IL processes.

3.1.1.2 Delignification and lignin recovery

Delignification is the term to describe the degree of lignin dissolution in to the solvent during fractionation, while lignin yield, also known as lignin recovery yield, quantifies the amount of lignin precipitated from the pulp washing due to the addition of anti-solvent, water. Delignification and lignin yields for organosolv-ionoSolv pretreatments using three different

organic solvents were listed in Table 3.1, 3.2 and 3.3, while their trend was presented in Figure 3.2, 3.3 and 3.4.

The trend of delignification was in line with the saccharification yield. This could be explained as lignin is the major recalcitrance towards effective enzymatic saccharification for all lignocellulosic biomass, regardless the feedstock type.[71] For ethanol-IL fractionation process, a delignification, up to 89% was achieved, which was 10% higher than the ionoSolv process (using ethanol to wash the pulp), and it remained stable between 10 wt% to 60 wt% ethanol, detailed in Table 3.1 and Figure 3.2. A similar case was observed for butanol-IL pretreatments, where the higher delignification achieved was 82% at 20 wt%. butanol, 11% higher than the ionoSolv process (using butanol to wash the pulp), detailed in Table 3.2 and Figure 3.3. However, acetone-IL processes was a different case. A significant difference in lignin removal between acetone-IL and ionoSolv process was not observed, detailed in Table 3.3 and Figure 3.4. The ionoSolv process (using acetone to wash the pulp) obtained a 55% lignin removal. When the pretreating solvent switched from aqueous IL to anhydrous IL with 20 wt% acetone, the lignin removal was only increased to 59%. When the acetone content in the hybrid (acetone-IL) process increased to 60%, the delignification was dramatically dropped ≤40%. The comparatively little difference in the lignin removal, ≤ 4%, between ionoSolv and the hybrid processes could be explained: 1) the lignin solubility in acetone is smaller than in ethanol or butanol 2) the operational temperature for effective acetone-IL fractionation needs to be higher than 120°C. According to the literature, delignifications of 42%, 63%, 58% were reported by organosolv pretreatment using aqueous ethanol, butanol and acetone, respectively.[201]

For three ionoSolv processes, where the only differences was the pulp washing solvent choice, ethanol, butanol and acetone, the order of lignin removal observed was acetone < butanol <

ethanol, with values of 56%, 70%, 80%, respectively. The order of lignin removal could be explained by the lignin solubilities in different organic solvents. Theoretically, the highest lignin dissolution happens when the lignin's solubility parameter (δ) is equal, or close to the δ value of the solvent.[197] According to Ni *et al.*'s estimation, lignin has a δ value of 13.7 $cal^{1/2}$ $cm^{-3/2}$.[192] Yagi *et al* reported the δ values for ethanol, butanol and acetone are 12.7 $cal^{1/2}$ $cm^{-3/2}$,11.4 $cal^{1/2}$ $cm^{-3/2}$ and 9.9 $cal^{1/2}$ $cm^{-3/2}$, respectively.[244] Along these three organic solvents, ethanol and butanol have two δ values which are similar to each other, fairly close to the value of lignin (>2 $cal^{1/2}$ $cm^{-3/2}$), while the difference between lignin and acetone's solubility parameter is around 4 $cal^{1/2}$ $cm^{-3/2}$. Hence the order of lignin solubility in the three organic solvents is expected to be ethanol ≥ butanol > acetone. Different lignin solubility did not have a significant effect on the lignin dissolution in the organic-IL mixture during cooking step of the pretreatment, but did have a noticeable influence on the pulp washing process. When washing the pulps, the washing solvent with lower solubility could potentially result in an incomplete dissolution of the lignin which is dissolved by the IL during cooking process of the pretreatment, leaving a small proportion of lignin (possibly with some residual IL) not being dissolved into organic washing solvent but stuck on the surface of the cellulose-rich pulp. The current compositional analysis used in this study is based on an acid hydrolysis. This method is not able to distinguish the redeposited lignin from the residual lignin, which is a limitation and need a protocol integration to analysis the lignin composition more precisely.

For ethanol-IL processes, increasing ethanol content led to a drop in the lignin recovery yield, detailed in Table 3.1 and Figure 3.2. For the process containing 10 to 60 wt% ethanol, their lignin recovery were > 30% lower than the corresponding delignifications. The lignin yield was only 14% lower than for the ionoSolv process (using ethanol to wash pulp). The comparatively lower lignin recovery for ethanol-IL process could be explained by the improved lignin solubility

in water, which was in line with experimental observation. A yellow colloidal suspension was formed during lignin water washing step, where the colloidal lignin was not able to be isolated from water washings even after being centrifuged repeatedly. A similar experimental observation was reported by Bauer *et al*.[204]

For butanol-IL pretreatments, the lignin yield peaked at 20 wt% butanol, and the value was 20% higher than ionoSolv process (using butanol to wash pulp), detailed in Table 3.2 and Figure 3.3. It could be attributed to the presence of sugar impurities, mainly arabinofuranose and the increased lignin molecular weight due to lignin modification (α-butoxylation, more details in lignin section) during fractionation. When lignin was fractionated by butanol-IL mixtures, it is subjected to α-butoxylation and the hydroxyl group of the α carbon on the lignin side chain is replaced by a butoxy group. Therefore, the lignin molecular weight is increased.[205] Different to the ethanol-IL process, lignin yields of butanol-IL pretreatment having 20, 60 and 80 wt% butanol-content exceeded their delignification. The reason for this unexpected high lignin yields is: compared to α-ethoxylated lignin, α-butoxy lignin is relatively larger in molecular weight, hence less polar and less water-soluble; consequently no colloidal suspension formed in the lignin washing step, and the lignin dissolved during previous cooking process could be precipitated easily by adding anti-solvent into the lignin-IL solid mixture.

For acetone-IL processes, all lignin yields exceeded the deligninfications, detailed in Table 3.3 and Figure 3.4. The lignin recoveries for acetone-IL pretreatments were 63% for 20 wt% acetone (delignification 59%), 62% for 40 wt% acetone and 54% for 60 wt% acetone. These values were all higher than that of ionoSolv process (using acetone to wash pulp), 50%. The reason for this high lignin yield could be attributed to the formation of condensed lignin units and pseudo-lignin, which is formed by sugar degradation units and lignin units. Condensed

lignin oligomers are large in size and insoluble in water, which is formed by aggregation of small lignin oligomer. Carbohydrate degradation also took place during the lignin fractionation, forming sugar degradation products like 5-HMF and furfural. The degradation product is incorporated with lignin oligomer during the lignin fractionation, forming lignin-like polymers, also named pseudo-lignin. These polymers could not be distinguished by the compositional analysis protocol we currently use, and is detected as acid-insoluble lignin. Brandt *et al* reported the presence of pseudo-lignin units and the lignin condensation for ionoSolv lignin using severe conditions (long pretreatment time and high temperature).[71]

3.1.1.3 glucan recovery and hemicellulose

Figure 3.2, 3.3 and 3.4 also show the trend of glucose recovery and hemicellulose removal for all organic-IL pretreatments. The actual values were expressed in percentages of theoretical maximum, relative to the glucose and hemicellulose content of untreated miscanthus, listed in Table 3.1, 3.2 and 3.3. Glucan recoveries were kept above 90% for all pretreatments, suggesting little glucan degradation took place in this type of pretreatments. This is in line with the ionoSolv process and organosolv process reported in the literature, suggesting the organic-IL process fractionate biomass in the same way as the two individual pretreatment methods, dissolving hemicellulose, lignin and leaving cellulose as a solid residue.[71 201] Regardless of the organic solvent type, increasing the organic content of the pretreatment led to a reduced hemicellulose removal. The best hemicellulose removals, 85%, 80% and 82% were achieved by the organic-IL processes with 20 wt% ethanol, butanol, and acetone, respectively, which were 12%, 11%,11% lower than the corresponding ionoSolv processes. Organosolv pretreatments reported were able to remove up to 88%, 93%, and 94% of hemicellulose using ethanol, butanol, and acetone; but these pretreatments required a higher

operational temperature, 160°C, and the fractionation processes were also facilitated by sulfuric acid.[201]

3.1.1.4 Summary

After analysing the saccharification yield and pulp compositions for all pretreatments, an optimal pretreatment condition could be concluded here. The most effective pretreatment had a solvent composition of 40 wt% ethanol and 60 wt% [TEA][HSO_4], as it achieved the highest lignin removal, 88%, and subsequently the highest saccharification yield, 85%.

3.1.2 Effect of ionic liquid acidity on fractionation

The acidity of protic hydrogen sulfate ILs could be easily alternated via changing the amount of acid/amine added during their synthesis. [TEA][HSO_4] with a small excess of acid has been reported to speed up the pretreatment.[176] This favours the process at an industrial scale as the IL cost is reduced, and the optimal residence time is reduced. However, one of the main drawbacks about using acidic ILs is the increased degree of lignin condensation and formation of pseudo-lignin.[176] The potential of extracted lignin for high value-added applications would consequently be substantially lower.

[TEA][HSO_4] with two acid-base ratios (a/b =1.02, 0.98) were prepared here to study whether changing the IL acidity would improve the performance of the organosolv-ionoSolv process. Ethanol was the organic solvent chosen as it performed the best among the three-solvent tested. Parallel to the organosolv-ionoSolv pretreatments, two ionoSolv pretreatments were also conducted using IL with two acidities, a/b =1.02, 0.98, for comparison. Key indicators of pretreatment effectiveness including saccharification yields, pulp composition, and lignin recovery yields are presented in Figure 3.5 and 3.6, and the actual value of these key indicators are listed in Table 3.4 and 3.5.

Increasing IL acidity (and thus pretreatment severity) maintained the excellent fractionation performance and any form of overtreatment was not observed. A significant reduction of fractionation effectiveness was noticed when less acidic IL was used in pretreatment.

Saccharification yield (up to 89%) and delignification (up to 83%) for the IL with a/b =1.02 displayed the same trend as that for 1:1 IL mixtures, detailed in Figure 3.5 and Table 3.4. Saccharification yield and delignification for the IL with a/b =0.98 continuously dropped as the ethanol concentration increased, detailed in Figure 3.6 and Table 3.5. Glucan degradation decreased with increased ethanol content for both ILs, indicating that ethanol could remove hemicellulose and lignin more selectively compared to ILs and therefore ethanol is better in preserving more glucan. For Hemicellulose removal, a 40% decrease was observed at 40 wt% ethanol for the IL with a/b =0.98 (39%) compared to 1:1 IL mixtures (79%). This is attributed to the decreased pretreatment severity. The lignin yield for IL with a/b =1.02 was up to 20% lower than delignification. This large discrepancy is likely due to the increased water solubility of the lignin.

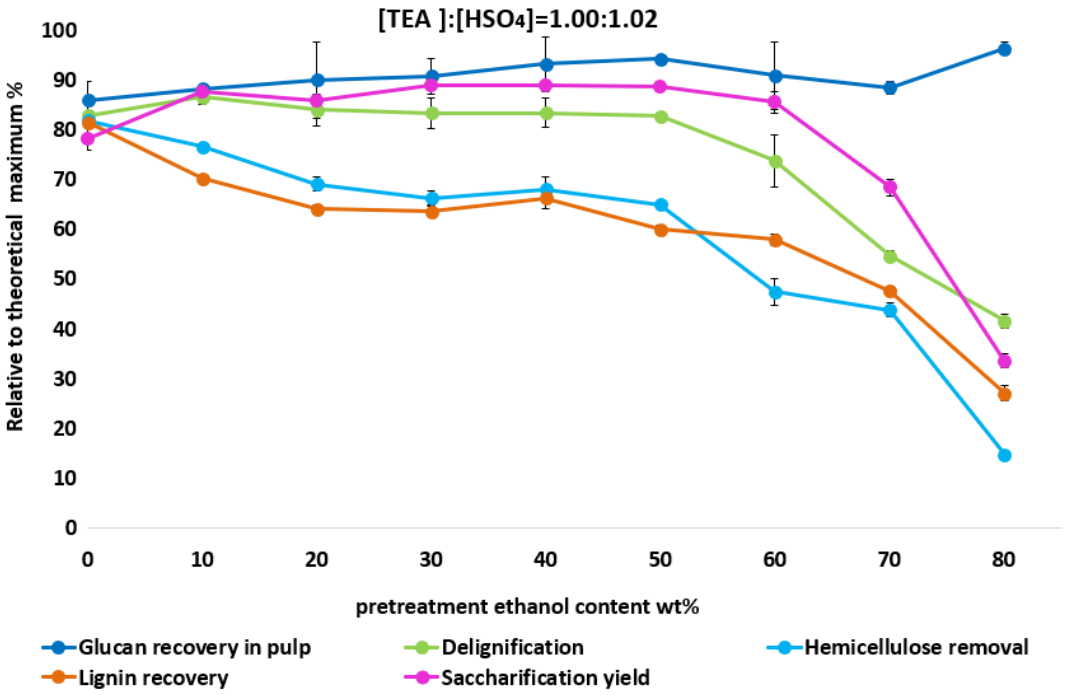


Figure 3. 5 Key indicators of fractionation effectiveness for ethanol-[TEA][HSO4] pretreatment using ionic liquid with an acid to base ratio of 1.02 at 120°C and 1:10 g g-1 biomass loading, 0% organic solvent data points represent the ionoSolv process using aqueous ionic liquid with 2% acid excess. Error bar is included.

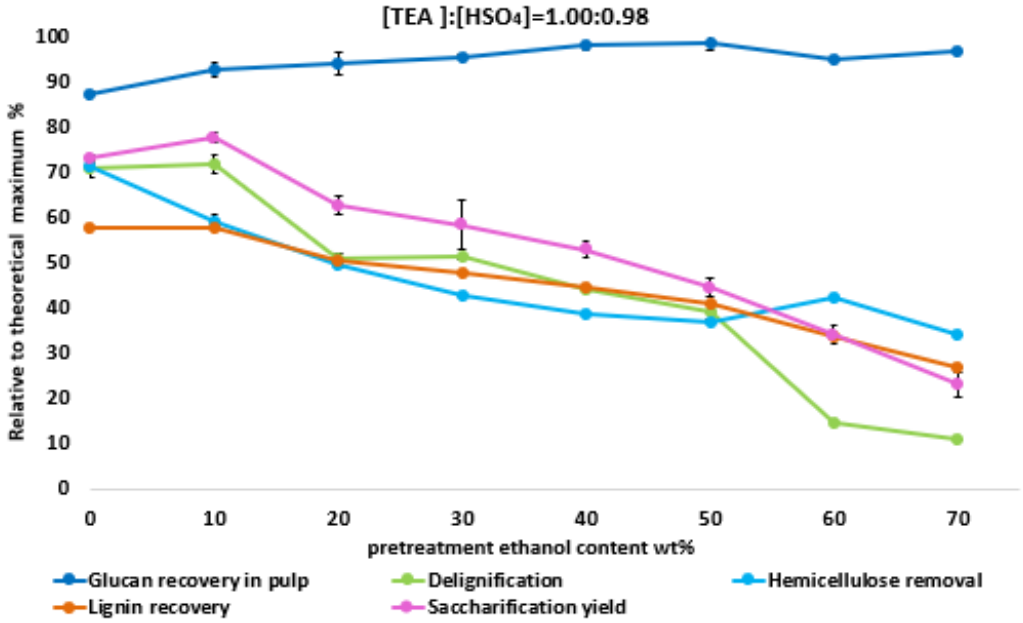


Figure 3. 6 Key indicators of fractionation effectiveness for ethanol-[TEA][HSO4] pretreatment using ionic liquid with an acid to base ratio of 0.98 at 120°C and 1:10 g g-1 biomass loading, 0% organic solvent data points represent the ionoSolv process using aqueous ionic liquid with 2% base excess. Error bar is included.

Table 3. 4 A list of compositional and saccharification key indicators for miscanthus fractionation process using a mixture of ethanol and [TEA][HSO_4] with an acid to base ratio of 1.02

wt% of ethanol in pretreatment solvent(wt% IL+water)	Glucan recovery[a]	Hemicellulose removal[a]	Lignin recovery yield[a]	Delignification[a]	Saccharification yield[a]
0[b] (100)	86	82	82	83	78
10 (90)	88	77	70	87	88
20 (80)	90	69	64	84	86
30 (70)	91	66	64	83	89
40 (60)	93	68	66	83	89
50 (50)	94	65	60	83	89
60 (60)	91	47	58	74	86
70 (30)	88	44	48	55	68
80 (20)	96	15	27	42	34

[a] the yield is presented in precentages of the theoretical maximum, relative to untreated biomass

[b] the pretreatment with 0% ethanol content represents the ionoSolv process where the biomass is pretreated with IL with an acid to base ratio of 1.02.

Table 3. 5 A list of compositional and saccharification key indicators for miscanthus fractionation process using a mixture of ethanol and [TEA][HSO_4] with an acid to base ratio of 0.98

wt% of ethanol in pretreatment solvent (wt% IL+water)	Glucan recovery[a]	Hemicellulose removal[a]	Lignin recovery yield[a]	Delignification[a]	Saccharification yield[a]
0[b] (100)	88	72	58	71	73
10 (90)	93	59	58	72	78
20 (80)	94	50	51	51	63
30 (70)	96	43	48	51	63
40 (60)	98	39	45	44	54
50 (50)	99	37	41	39	45
60 (60)	95	42	34	15	34
70 (30)	97	34	27	11	23

[a] the yield is presented in precentages of the theoretical maximum, relative to untreated biomass

[b] the pretreatment with 0% ethanol content represents the ionoSolv process where the biomass is pretreated with IL with an acid to base ratio of 0.98.

A similar study, pretreating willow using [TEA][HSO_4] with two acidities (a/b =1.02, 0.98), was conducted by Weigand *et* al.[70] Their work clearly showed that at 120°C, more acidic ILs could achieve better hemicellulose and lignin removal. Severe glucan degradation and lignin condensation were observed at 170°C. Brandt *et al* demonstrated that by using IL with 9 mol% excess acid, the saccharification yield peaked at 2h at 120°C, whereas the peak postponed to 8h for 1:1 IL.[71] Peak hemicellulose and lignin removal also shifted to 4h (24h for 1:1 IL) and 2h (4h for 1:1 IL) by increasing the severity of the pretreatment conditions.

Different from ionoSolv process, increasing IL acidity for organosolv-ionoSolv process did not fundamentally change the fractionation ability of the organic-IL mixture, this opens up an opportunity

to reduce the solvent cost for the process when scaling up to industrial sizes as acidic IL could replace stoichiometric 1:1 IL.

3.1.3 Effect of feedstock loading on fractionation

The pretreatment processes used in current lignocellulosic ethanol plants often require high capital costs, in which the cost for this particular step is usually accounting for 20% of the overall cost for the biorefinery process.[245] By increasing the biomass loading, the reduction in reactor size as well as the solvent capital cost can potentially be achieved, which subsequently reduce the capital expenditure (CAPEX) of the biorefinery plant up to 40%.[190] For industrially-viable pretreatment process, it is important for it to operate at a high biomass loading. Therefore, we tested the newly developed hybrid (organosolv-ionoSolv) pretreatment process with 5 different biomass to liquid loadings, 10 wt% (1 :10 g g^{-1}), 20 wt% (2 :10 g g^{-1}), 30 wt% (3 :10 g g^{-1}), 40 wt%(4 :10 g g^{-1}), 50 wt% (5 :10 g g^{-1}), in order to evaluate the fractionation performance of the organic-IL process at high solids loadings.

All pretreatments were performed using miscanthus at 120°C for 8h with an IL: ethanol mass ratio of 60:40, which is the optimal condition identified earlier. Figure 3.7 presents the overall tendencies of the performance indicators for the organosolv-ionoSolv processes with different biomass loadings. All the important fractionation indicators including saccharification yield and delignification are listed as actual numbers in Table 3.6.

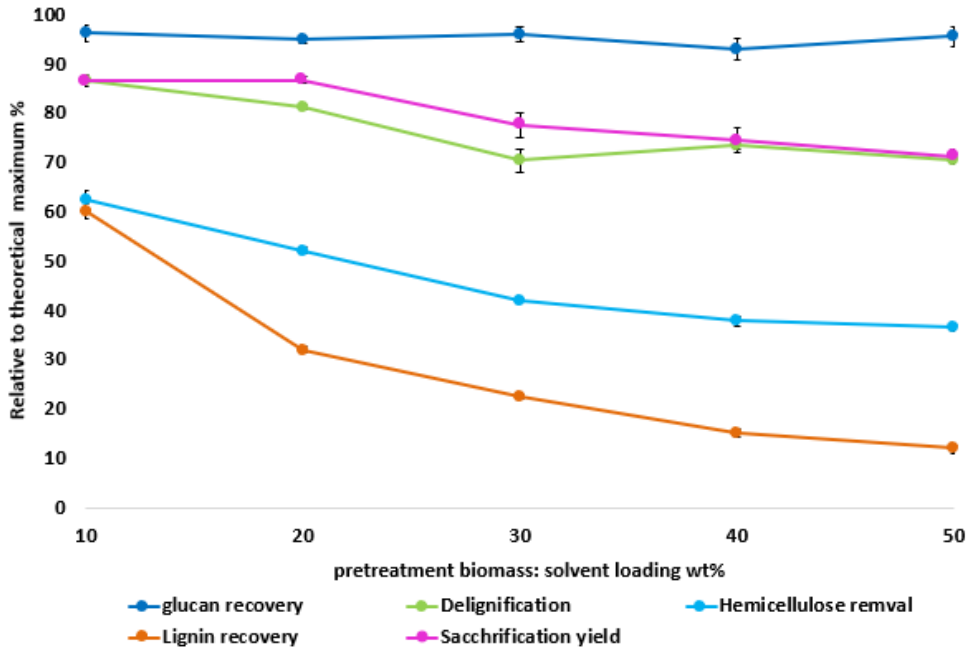


Figure 3. 7 Effect of biomass loading on fractionation effectiveness for miscanthus ethanol-[TEA][HSO_4] pretreatment with 60 wt% IL and 40 wt% ethanol at 120°C. Error bar is included.

Table 3. 6 A list of compositional and saccharification key indicators for miscanthus fractionation process using an ethanol-[TEA][HSO_4] mixture with 40 wt% ethanol content with five different biomass loadings

% wt biomass to solvent loading	Glucan recovery[a]	Hemicellulose removal[a]	Lignin recovery yield[a]	Delignification[a]	Saccharification yield[a]
10	96	63	60	87	87
20	95	52	32	81	87
30	96	42	23	71	78
40	93	38	15	74	75
50	96	37	12	71	71

[a] the yield is presented in percentages of the theoretical maximum, relative to untreated biomass

Increasing biomass loading has a stronger effect on the overall fractionation effectiveness of the ionoSolv process, compared to the ethanol-IL process. When the process was performed at 10 wt% loading, 87% of glucose was released in enzymatic saccharification, the amount of glucose released was maintained at 71% as the loading increased 5 fold, detailed in Table 3.6 and Figure 3.7. This result is very promising and is not in line with the literature: the ionoSolv processes reported a significantly reduced delignification and hence largely reduced saccharification yield (usually around half compared to 10% loading) at higher loadings. The reason behind this drop is believed to be the limited mass transfer: ionoSolv process at high solid loadings, IL is highly viscous, hence it forms lump with the biomass even after mixing; high portion of raw biomass remain untreated during the pretreatment, resulting in poor delignification. However, it is not the same case for ethanol-IL process, suggesting the mass transfer was not significantly limited in this hybrid organic-ionic pretreatment medium, as much less viscous pretreatment solvent was used during the fractionation.[246] More specifically, the decent performance of this hybrid pretreatment at high loadings, presented in Figure 3.7, could be explained by a dual effect of: 1) compared to ionoSolov pretreatment, the ethanol-IL pretreatment uses a less viscous medium as 40 wt% ethanol is incorporated with the IL, instead of 20 wt% water; and 2) compared to organosolv processes, the hybrid process replaces water with IL (60 wt% in this case), where IL is not only a co-solvent to the ethanol but also acting as a fractionation solvents; water could not achieve this. The protic hydrogen sulfate IL used in this study has been shown effective delignification performance at high solid loading with challenging feedstocks such as pine wood.[190] A saccharification yield decrease of 52% was reported for the ionoSolv process using [TEA][HSO_4], 78% for 10 wt% loading, but 26% for 50 wt% loading.[247]

For all solid loadings, glucose recovery remained stable. Hemicellulose removal for 10% loading was 62% and 37% for 50% loading, a 25% drop when the loading increased 5 times. Although larger amount of hemicellulose residues was detected at higher loadings, the saccharification yields were not largely affected. As mentioned in the earlier section, lignin is the biggest recalcitrance hindering the pulp enzymatic hydrolysis. The delignification was observed to be in line with the sugar releasing yield, and a 16% drop in lignin removal was observed as the loading increased 5-fold. The delignification recorded for ionoSolv process were 80% for 10 wt% loading and 47% for 50 wt% loading.[247] The lignin removal was higher at both loadings for ethanol-IL processes, it might be due to more lignin was hydrolysed in the ethanol-IL mixture regardless the loadings.

Lignin recovery yield decreased from 60% for 10 wt% loading to 12% at 50 wt% loading. Two reasons could explain this substantial drop: 1) the amount of water-soluble lignin fraction forming colloidal suspensions was increased, and hence less amount of lignin was precipitated out; 2) when the loading was increased by 5 times, increased amount of lignin dissolved in ethanol-IL mixtures during the cooking process led to an incomplete dissolution of the lignin into the organic washings during the pulp washing step, substantially the undissolved lignin fraction was trapped with the pulp. Compositional analysis could not distinguish this from the residual lignin fraction of the pulp.

In summary, the organosolv-ionoSolv pretreatment could maintain a similar fractionation effectiveness when the biomass loading increased from 10 to 50 wt% This is due to little impact on the mass transfer of the process, which is reported to be the limiting effect for ionoSolv and organosolv pretreatments to achieve a compatible fractionation performance in the hybrid process.

3.1.4 Effect of air-drying pretreated biomass on enzymatic saccharification

In industrial pretreatments, the pulp is subjected to enzymatic hydrolysis without being air-dried after going through the washing step. However, the air-drying step was not eliminated in our bench scale procedure for the ease of handling, and to improve the accuracy of pulp yields by using pulps with low moisture contents. Air-drying of the pulp causing hornification (collapse of the biomass pores) has been reported by several studies. [60,190] It is believed that hornification could cause a decrease of sugar releasing yield, but how much the yield is quantitively affected has not been fully understood. Therefore, the sugar releasing yield for an industrial organosolv-ionoSolv process using wet pulps could not be estimated from the results obtained using air-dried pulp when the process turns from a bench scale into an industrial size. Three ethanol-IL pretreatments with ethanol concentrations of 0 wt%, 50 wt%, 80 wt% were repeated for miscanthus to get a better understanding of the correlation between hornification and saccharification yield. After being washed with ethanol, pulps were washed with water instead of being air-dried. Here, we refer the water-washed pulp as wet pulp and dried pulp as dry pulp. Table 3.7 lists the saccharification yields for both the dry and wet pulps.

Table 3. 7 Saccharification yield obtained from air-dried and wet miscanthus pulp, pretreated with [TEA][HSO4] and ethanol at at 120°C and 1:10 g g^{-1} biomass loading.

	Saccharification yield	
Ethanol content wt%	**Dry pulp**	**Wet pulp**
0	75±1.7%	78±1.2%
50	82±4.0%	89±3.9%
80	45±3.1%	50±7.5%

The saccharification yield differed from 2.9% for 0 wt% ethanol, to 7.2% for 50 wt% ethanol. In the case of pine pulp, Gschwend reported a 37% glucose yield drop due to hornification.[190] Chambon also reported that the saccharification yields differed by 5.6% (wheat straw), 3.4%(rice straw), 8.1% (sugarcane bagasse), 30.4%(rice husk) between wet and dry pulps.[60] The four agricultural residues studied were thought to be similar to a grassy biomass such as Miscanthus. The degree of saccharification yield decrease due to hornification varies with feedstocks. The enzymatic hydrolysis for grassy biomass tends to be impacted less.

Here, we could conclude that for grassy feedstock, the negative impact on biomass enzymatic hydrolysis due to hornification is insignificant. The batch-scale pretreatment with the pulp air drying process could provide a reliable predication of the actual industrial process.

3.2 Lignin characterisation

For organosolv-ionoSolv and ionoSolv processes, an effective biomass fractionation always requires a decent lignin removal. During the lignin fractionation, the structure of the native lignin is often altered though various chemical reactions which usually take place at the intrinsic functional groups of the lignin, e.g. aliphatic hydroxyl group. The degree of lignin structural modification varies with the types and the severities of the pretreament process, which has a critical impact on the isolated lignin structure. The lignin structure determines the suitable applications for the lignin isolated. Selective pretreatment being studied, e.g. organosolv lignin, could generate a high-quality lignin fraction which could be used in high value-added applications, such as lignin-based carbon fiber and aromatic platform chemicals.

Process integration for most of the current pretreatment process is needed for generating better quality lignin side products. Therefore, a comprehensive understand of the lignin structure would be essential for suggesting suitable integration for a pretreatment process

Here, a detailed structural characterisation of the lignin generated from various organosolv-ionoSolv pretreatments gives more insights of how the lignin modification was influenced by different pretreatment conditions, including organic solvent choices, organic solvent-contents and IL acidities. ^{1}H-^{13}C heteronuclear quantum coherence (HSQC) NMR spectroscopy was used to monitor changes in the lignin's key functionalities or major subunits. Gel Permeation Chromatography (GPC) was also carried out to investigate lignin's molecular weight changes with various pretreatment conditions.

3.2.1 HSQC NMR analysis

HSQC NMR analysis were conducted for the ionoSolv lignin, 6 organic-IL lignins (ethanol 40 wt%, 60 wt%, butanol 40 wt%, 60 wt%, acetone 40 wt%, 60 wt%), three lignins extracted by an ethanol-IL mixture with an IL acidity of 1.02, (ethanol 0 wt%, 40 wt%, 80 wt%), and another three lignins extracted by an ethanol-IL mixture with an IL acidity of 0.98 (ethanol 0 wt%, 40 wt%, 80 wt%).

The major subunits detected in HSQC were presented in Fig 3.8, which are β-*O*-4 ether (A), β-β resinol (B), β-5 phenylcoumaran (C), α-alkoxy ether (A'), Lignin-carbohydrate linkages (lignin with arabinose (Ara) or xylose (Xyl)), uncondensed and condensed guaiacyl (G_2, G_5, G_6, $G_{2cond.}$), uncondensed and condensed syringyl ($S_{2,6}$, $S_{2,6\ cond.}$), *p*-coumaric acid (PCA), and *p*-hydroxyphenyl (H).

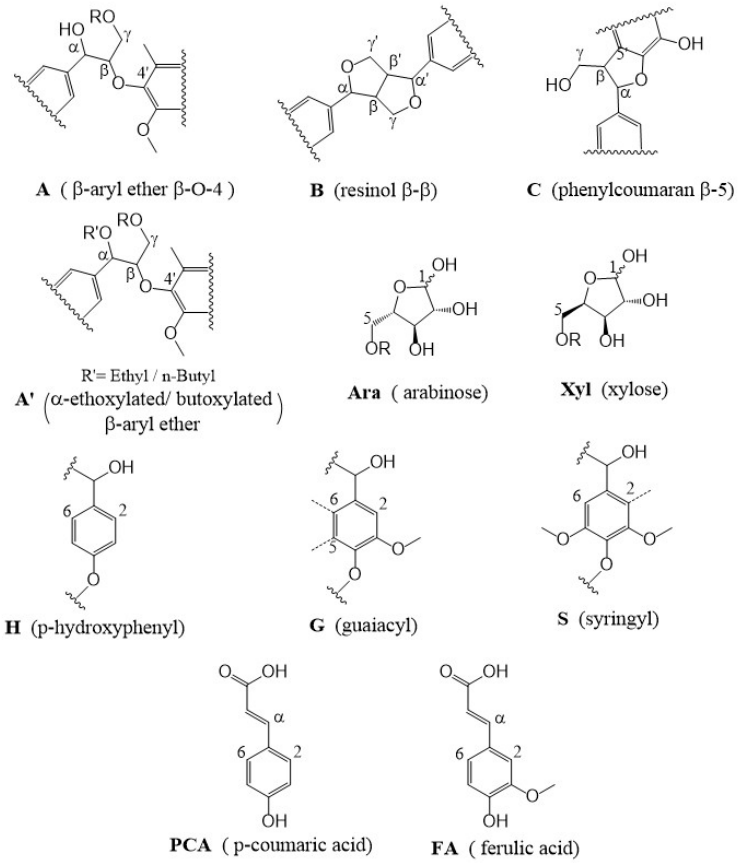


Figure 3. 8 key lignin substructures found in recovered lignin from Miscanthus pretreatment

The HSQC NMR spectra could be separated into three regions, the aliphatic, the side chain and the aromatic region. All the major lignin subunits and functionalities are located either in the side chain region (δ_C 50-90 ppm δ_H 2.5-5.8 ppm) or the aromatic region (δ_C 110-130 ppm δ_H 6.0-6.9 ppm). A semi-quantitative analysis for all the lignin subunits was conducted via volume peak integration to quantify the abundance of these subunits, presented in bar chart. The signal intensities for all subunits were presented as percentages relative to the sum of G_2 and G_{2cond} integrals. G_2 and G_{2cond} integrals were confirmed to maintain unchanged for all lignin isolated from pretreatments regardless their conditions.[2] The degree of condensation could be quantified by the signal intensity G_{2cond} relative to G_2 and G_{2cond} integrals.[60] The S/G ratio could also obtained though Equation 1, detailed below:

$$\frac{S}{G}\text{ratio} = \frac{0.5\cdot(S_{2,6} + S_{2,6\ \text{cond.}})}{(G_2 + G_{2\ \text{cond.}})} \qquad \text{(eq. 1)}$$

Where $S_{2,6}$, $S_{2,6\ cond.}$, G_2, $G_{2cond.}$ stand for the integrated volume peak intensities for uncondensed and condensed syringyl units, uncondensed and condensed guaiacyl units.

3.2.1.1 Lignin extracted by anhydrous ionic liquid with different organic co-solvents

For the 6 organic-IL lignins (ethanol 40 wt%, 60 wt%, butanol 40 wt%, 60wt %, acetone 40 wt%, 60 wt%), the coloured side chain region and aromatic region of the spectra were shown in Figure 3.9 and the semi-quantitative analysis for these 6 lignins was presented in Figure 3.10. Their subunits compositions were compared with an ionoSolv lignin.

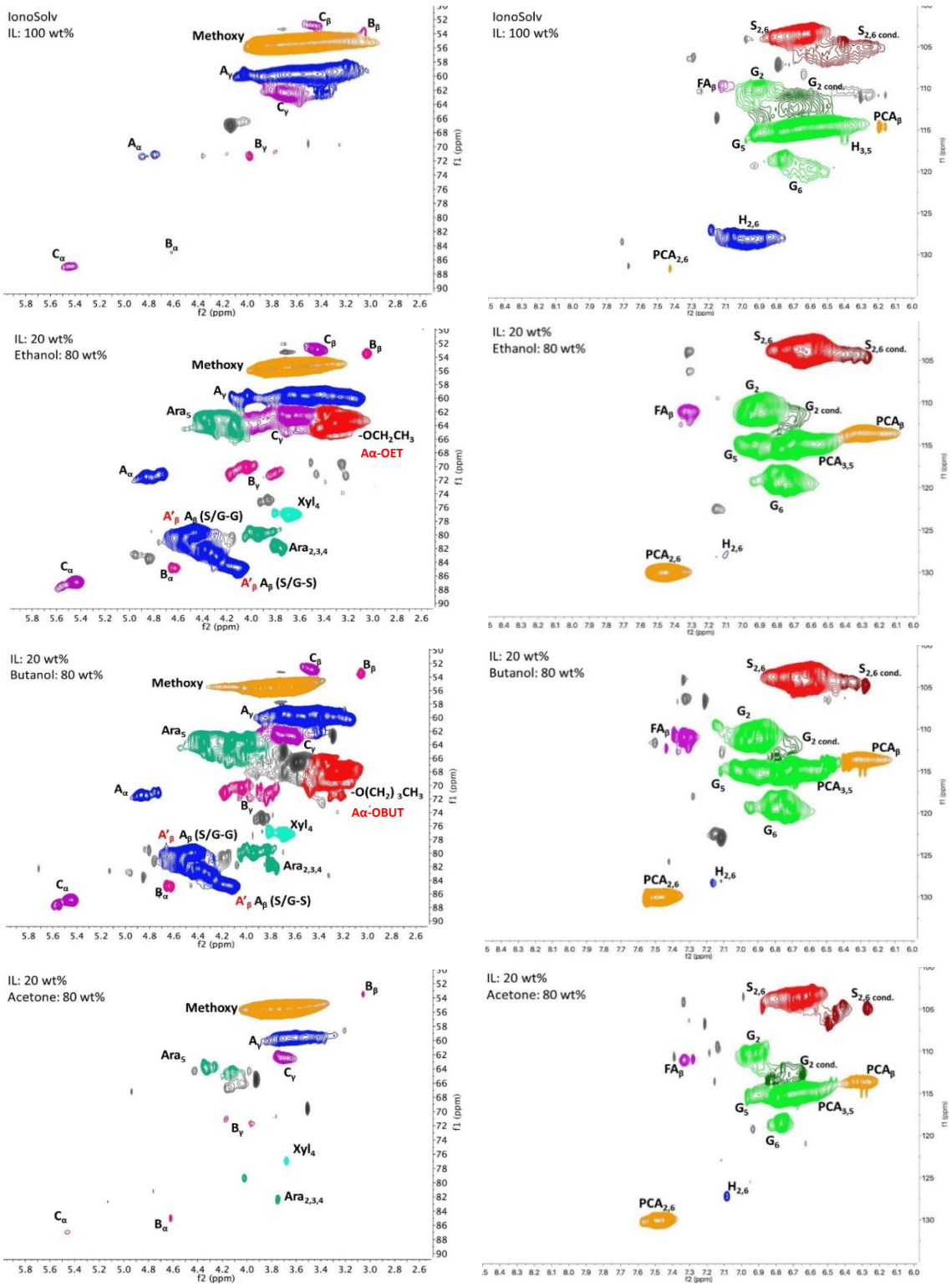


Figure 3. 9 HSQC NMR spectra of Miscanthus lignin recovered from ionoSolv, [TEA][HSO4] and organosolv-ionoSolv processes with different organic choices, ethanol, butanol, acetone, pretreatments were all performed at 120°C, for 8 hours and with a 1:10 g g^{-1} biomass loading , their organic concentration was 80 wt% (left) Side chain region of the HSQC NMR spectra (right)Aromatic region of the HSQC NMR spectra

Comparing the lignin subunit compositions between the ionoSolv process and 6 different organic-IL processes, the key information discovered in the side chain region of the spectra are: 1) the appearance of the α-butoxylated/ethoxylated β-*O*-4 ether signals; 2) the increased peak signals for those carbohydrates crosslinked with lignin units originated from the hemicellulose fraction, presented in Figure 3.9. Lancefield *et al* and Bauer *et al* have demonstrated that one type of lignin modification led by butanol/ethanol took place during organoSolv pretreatment. They have classified this type of chemical modification as α- butoxylation/ethoxylation.[204,205] α-alkoxylation not only could significantly prevent the lignin condensation taking place at α carbon position, but also could tune the lignin solubility towards a better lignin removal. For organic-IL processes, particularly butanol/ethanol-IL processes, α-butoxylated/ethoxylated β-*O*-4 ether linkages were detected, indicating butanol/ ethanol function in a same way in the organic-IL pretreatments as the organosolv pretreatment. For organic-IL processes with different organic contents, it was observed that increasing the organic content would result in a proportional increase in the signal intensities of these α-alkoxylated ether linkages. However, this α-alkoxylated ether linkages were not detected for acetone-IL lignins, as the literature reported that acetone dose not interact with any lignin subunits directly but dose interact with selective *cis*-vicinal hydroxyl group coming from the carbohydrate subunits, forming acetonide.[204]

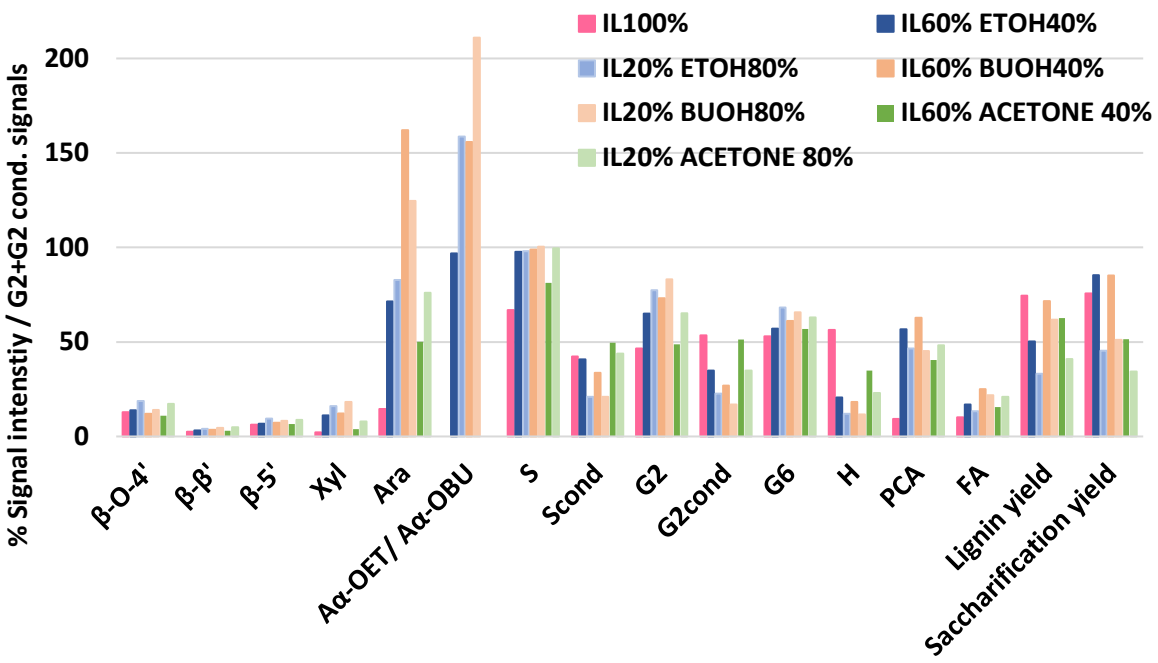


Figure 3. 10 Abundance of key lignin substructures in Miscanthus lignins recovered from organosolv-ionoSolv processes with different organic choices according to HSQC NMR spectroscopy. Signal intensities are presented in percentages relative to the sum of signal intensities for G2 and G2cond.

Different from ionoSolv lignin, a noticeable amount of carbohydrate residues was detected for organic-IL lignins, in a form of arabinose/xylose crosslinking with other lignin subunits. This was proofed by the increase signal intensities of arabinose and xylose. Especially for arabinose, its peak intensity increased by up to 11-fold, when increasing the organic content of the organosolv-ionoSolv pretreament, detailed in Figure 3.10: up to 162% for organic-IL lignins compared to 15% for ionoSolv lignin, relative to the sum of G_2 and G_{2cond} integrals. For organosolv lignin, a significant amount of sugar residues was also reported, and the major sugar subunit detected was arabinfuranose, connected to other lignin subunits via p-coumarate units.[204] The signals for unalkoxylated ether linkages were less than 20% for ionoSolv and organic-IL lignins, comparing 40% abundance for the native lignin. This suggests that β-*O*-4 ether linkages were the most readily cleaved during the lignin fractionation for all pretreatment conditions. The signal intensities of β-β and β-5 linkages rose slightly when increasing the organic content. This is in line with the literature: resinol and phenylcoumaran units were most

likely to be chemically modified rather than cleaved during pretreatment with mild conditions.[67] The slight increase in the peak intensities suggested that fewer resinol and phenylcoumaran units were modified by the organic-IL mixture comparing to aqueous IL.

Two interesting observations were made in the aromatic region of the spectra for organic-IL lignins: 1) compared to ionoSolv lignin, the degree of lignin condensation was reduced; 2) the degree of PCA to H unit conversion was also reduced, relative to the ionoSolv process. According to Figure 3.9, a much smaller peak for the G_{2cond} was observed for all organic-IL lignins, compared to ionoSolv lignin, suggesting guaiacyl units in organic-IL lignins are less condensed. This is further evidenced by lignin's degree of condensation see Table 3.8: for ionoSolv lignin, 53% of G_2 and G_{2cond} combined signal intensity was contributed by G_{2cond}, meaning little more than half of the G_2 units were taking parts in condensation during fractionation. However, this intensity dropped to 22% and even 16%, when lignins were extracted by an organic-IL mixture with an 80 wt% ethanol/butanol content.

Table 3. 8 Degree of condensation based on HSQC spectrum integrals for lignins extracted from miscanthus using different organic solvent-IL mixtures

Pretreatment solvent composition	IL100%	IL60% ETOH40%[a]	IL20% ETOH80%	IL60% BUOH40%[b]	IL20% BUOH80%	IL60% ACE40%[c]	IL20% ACE 80%
G_2	46	65	77	73	83	48	65
$G_{2con.}$ [d]	53	34	22	26	16	51	34
$G_{2con.}/G_2+G_{2con.}$ in %	**53**	**34**	**22**	**26**	**16**	**51**	**34**

[a] ETOH is short for ethanol

[b] BUOH is short for butanol

[c] ACE is short for acetone

[d] $G_{2con.}$ stands for condensed G_2 peak

The S/G ratio of the lignins characterised by the HSQC, presented in Figure 3. 11, suggested that the S/G ratio did not directly correlate with the extent of the lignin condensation. The ionoSolv lignin had a S/G ratio of 0.55. The values for the organosolv-ionoSolv lignins varied from 0.65 to 0.72, while those for Organosolv lignins extracted by aqueous ethanol or acetone ranged from 0.54 to 0.61.[204] In several studies for ionoSolv process, a correlation between S/G ratio and the degree of lignin condensation was repeatedly suggested: more condensed lignin tends to have a higher S/G ratio. [70,204] The increasing S/G ratio could be blamed for increase amount of condensed G_2. The G_2 units are more susceptible to lignin condensation, comparing to $S_{2,6}$ units, as the aromatic carbon 3 and 5 positions on the $S_{2,6}$ units are occupied by methoxy groups, and those two carbon positions on G_2 units are free and not steric hindered, ready for condensation. In our study, the organic-IL lignins were confirmed to be less condensed than ionoSolv lignin, but their S/G ratios are significantly higher than ionoSolv lignin. Hence, any changes in the S/G ratio could not be used as an indication for the rise of lignin condensation.

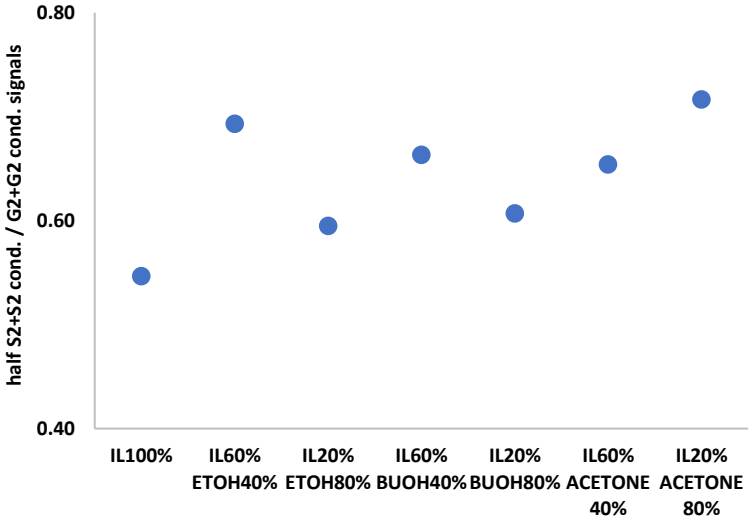


Figure 3. 11 The calculated S/G ratio of the extracted lignin based on HSQC NMR spetra for lignin extracted from miscanthus pretreated with different organic solvent-IL mixtures, organic solvent concentration ranged from 0 to 80 wt%, organic solvents used were ethanol, butanol, acetone

In several ionoSolv pretreatment studies, almost quantitative PCA unit conversion into hydroxyphenyl groups (H) were observed, suggesting this conversion is one of the typical chemical modifications occurring in the ionoSolv fractionation processes and is also feedstock-independent.[2,67,60] It is reflected in by a nearly quantitative drop in the signal intensity of the PCA units and a large rise in the H units' intensity. According to Figure 3.9 and 3.10, for organic-IL pretreatments, instead of polymerizing into H units, PCA units bridge the hemicellulose residue, mostly arabinose with other lignin subunits. Therefore, the PCA signals were more intense for organic-IL lignins, compared to ionoSolv lignin.

3.2.1.2 Lignin extracted by ethanol-ionic liquid mixtures with different ionic liquid acidities

Two series of lignins extracted from ethanol-IL mixtures with different IL acid/base ratios were characterised by HSQC NMR spectra, see Figure 3. 12 with the semi-quantitative analysis about the major integral intensities shown in Figure 3. 13. Changing the acid/base ratio of the IL did not change the types of lignin chemical modification during the fractionation. Similar to the pretreatment using ethanol-IL mixture (acid/base=1), α-alkoxylation was the major chemical modification happening to the lignin, resultantly hindering the condensation at the α carbon. However, using a more acidic IL resulted in a faster β-O-4 ether cleavage in the ionoSolv process compared to its corresponding ethanol-IL process. For the IL with an acid/base ratio of 1.02, 11.7% of β-*O*-4 linkages remained in the lignin recovered from the ionoSolv process, while 35.4 % of β-O-4 ether units remained in the recovered lignin for ethanol-IL process (40 wt% ethanol). When a less acidic IL was used (acid/base=0.98), the ionoSolv process was able to maintain 32.7% ether linkages while the ethanol-IL process (40 wt% ethanol) could reserve 35.9% β-*O*-4 units.

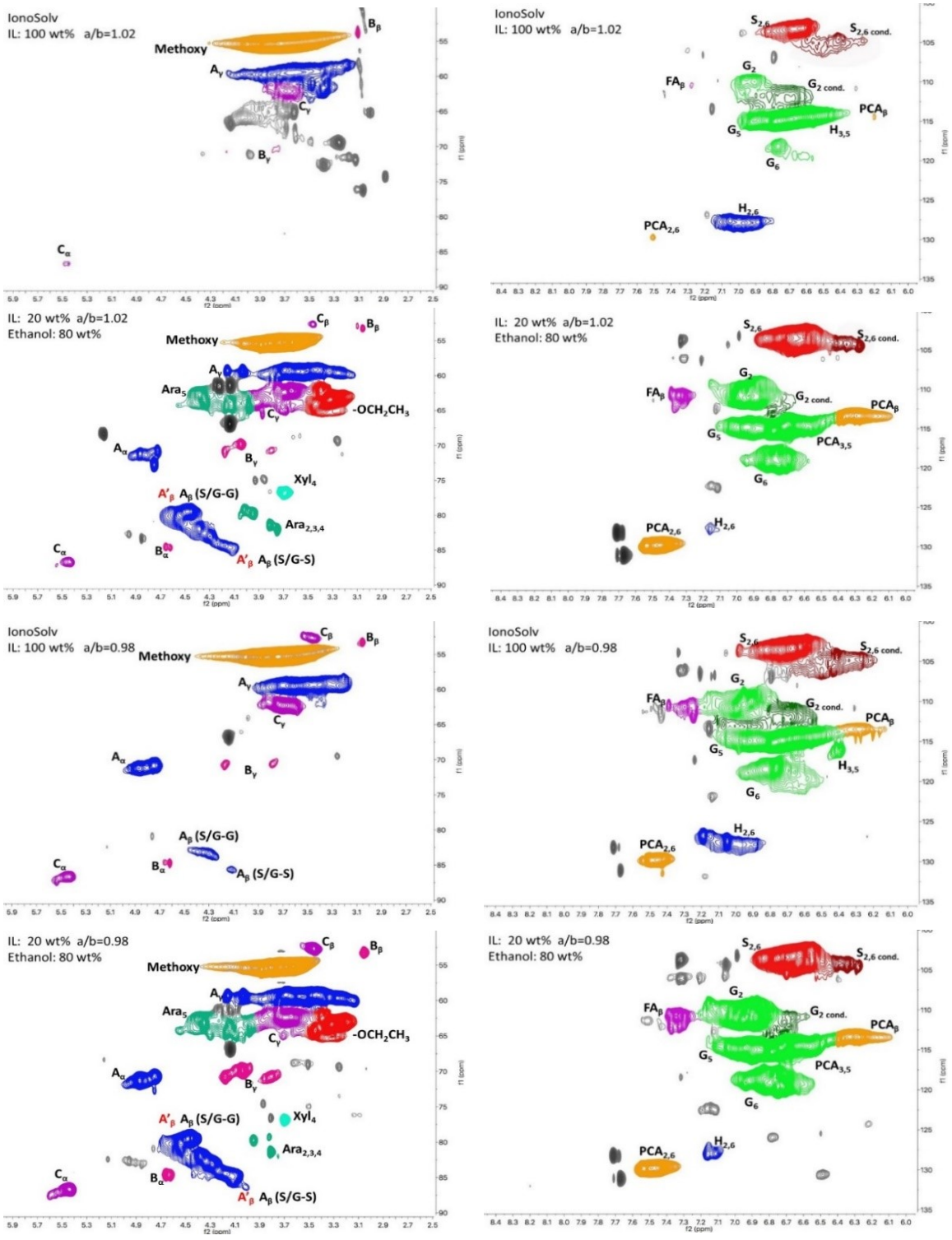


Figure 3. 12 HSQC NMR spectra of Miscanthus lignin recovered from ionoSolv, [TEA][HSO4] and organosolv-ionoSolv processes with two ionic liquid acidities, a/b=1.02, 0.98, pretreatments were all preformed at 120°C, for 8 hours and with a 1:10 g g-1 biomass loading , the organic concentration for organosolv-ionoSolv processes was 80 wt% (left) Side chain region of the HSQC NMR spectra (right)Aromatic region of the HSQC NMR spectra

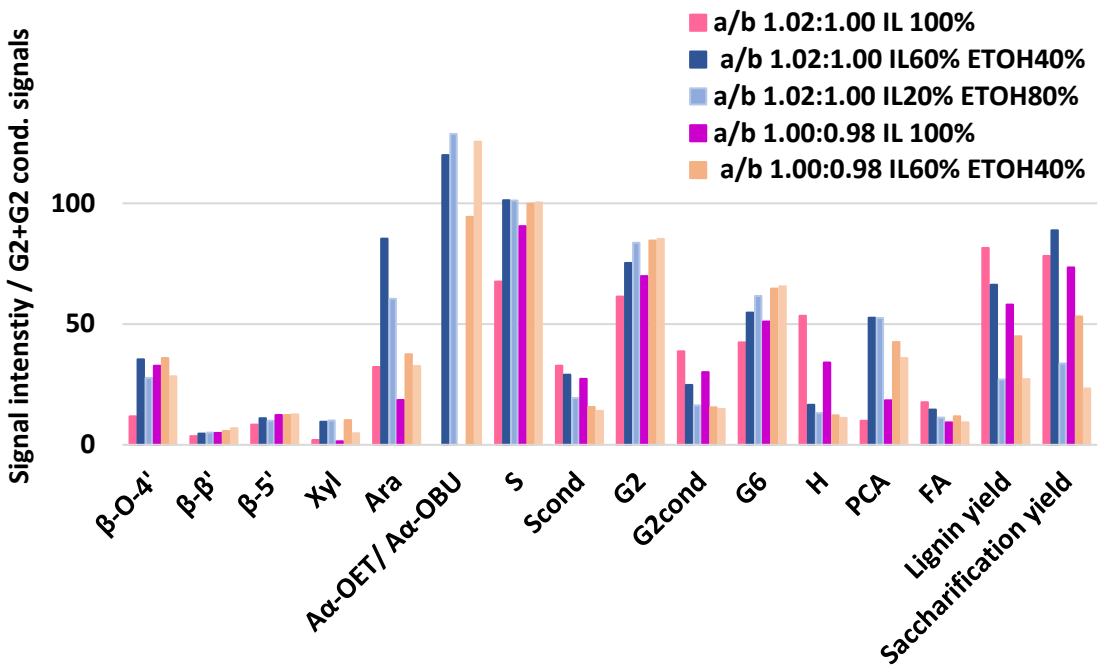


Figure 3. 13 Abundance of key lignin substructures in Miscanthus lignins recovered from organosolv-ionoSolv processes with different ionic liquid's acidities according to HSQC NMR spectroscopy. Signal intensities are presented in percentages relative to the sum of signal intensities for G_2 and $G_{2cond.}$

Table 3. 9 Degree of condensation based on HSQC spectrum integrals for lignins extracted from miscanthus using ethanol-IL mixtures with different IL acidities

Pretreatment solvent composition	IL 100% [a]	IL60% ETOH40%[a]	IL20% ETOH80%[a]	IL 100% [b]	IL60% ETOH40%[b]	IL20% ETOH80%[b]
G_2 peak integral intensity	61	75	83	69	84	85
$G_{2con.}$ [c] peak integral intensity	38	24	16	30	15	14
$G_{2con.}/G_2+G_{2con.}$ in %	**38**	**24**	**16**	**30**	**15**	**14**

[a] Pretreatments used IL with an acid to base ratio of 1.02

[b] Pretreatments used IL with an acid to base ratio of 0.98

[c] $G_{2con.}$ stands for condensed G_2 peak

IonoSolv lignin extracted by IL with excess acid (acidity 1.02) was most condensed, with a degree of condensation of 39%. The value for lignin condensation reduced to 30% when lignin was extracted with a less acidic IL (acidity 0.98). The condensation was reduced by half when 80 wt% ethanol was incorporated with 20 wt% IL during lignin fractionation, regardless the IL acidity. The degree of condensation for lignins extracted by IL with excess acid/base is listed in Table 3.9. The S/G ratio for all lignins (detailed in appendix Figure S3.1) did not provide any direct information about the lignin condensation happening during fractionation.

3.2.2 GPC analysis

The molecular structure of the lignin isolated was studied, any changes in lignin's molecular weights were also monitored by the GPC. GPC analysis provides information about lignin's average molecular weight and their weight distribution.

According to Figure 3.14, for ethanol-IL and butanol-IL processes, the number average molar mass, Mn, for extracted lignin kept constant for all organic contents, while the weight average molar mass, Mw, had an upward trend along with the increased organic content. Mw reached 7691 Da at 80 wt% ethanol and 6971 Da at 80 wt% butanol. Mw for the ionoSolv lignins (the pretreatments with three different pulp washing solvents) were much lower, ≤ 4000 Da. The upward trend for Mw was steeper after 60 wt% organic content. This could be explained by the growing fraction of α-alkoxylated lignin oligomers, of which their molecular weight is higher than the unmodified lignin units. We made an assumption that the average molecular weight for monolignol is 200 Da. This is based on the molecular weight of sinapyl alcohol, 208. The syringyl units of lignin were generated from sinapyl alcohol via an enzymatic polymerisation. Based on the assumption just made, the molecular weight of the α-alkoxylated lignin unit is potentially higher than that of the native lignin unit by 14% to 28%. When the organic content of the pretreatment is fairly high (> 60 wt%), α-ethoxylation/butoxylation is taking

place at more α carbon positions, and more lignin units have an increased molecular weight, these lignin units then aggregated to form larger oligomers and precipitated. The Polydispersity Index (PDI) was in line with the Mw for ethanol-IL and butanol-IL lignins. The PDI of ethanol-IL lignin reached 5 at 80 wt% ethanol and that of butanol-IL lignin was 5.6. For acetone, Mn and Mw maintained stable for all acetone concentrations and PDI kept around 2.5. According to Figure 3.15, No clear correlation was observed between IL acidities and lignin's molecular weight. Further investigation is needed to understand the correlation between lignin molecular weight and IL acidity. Increasing the biomass loading did not affect the recovered lignin's molecular weight and its weight distribution (detailed in Figure 3.16), suggesting the pretreatment generates the lignin fraction with the equal quality regardless the biomass loadings.

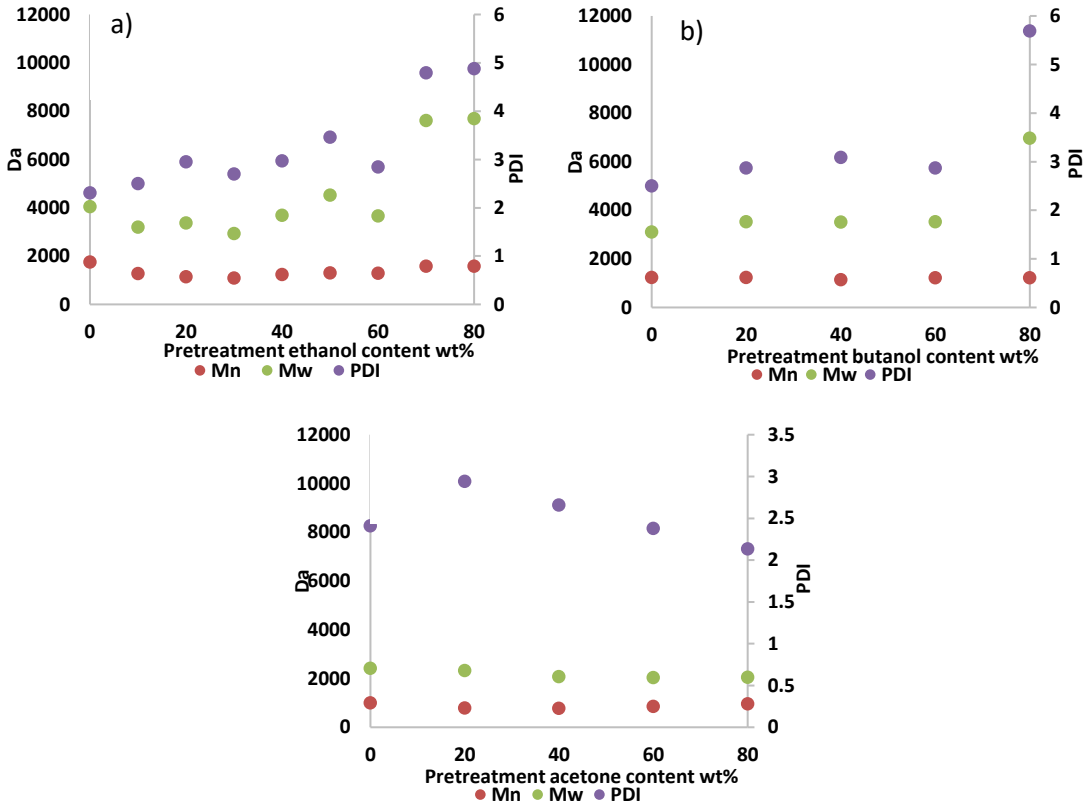


Figure 3. 14 Average molar mass and polydispersity of lignin extracted with different organic solvent-IL mixtures a) ethanol-IL mixtures b) butanol-IL mixtures c) acetone-IL mixtures

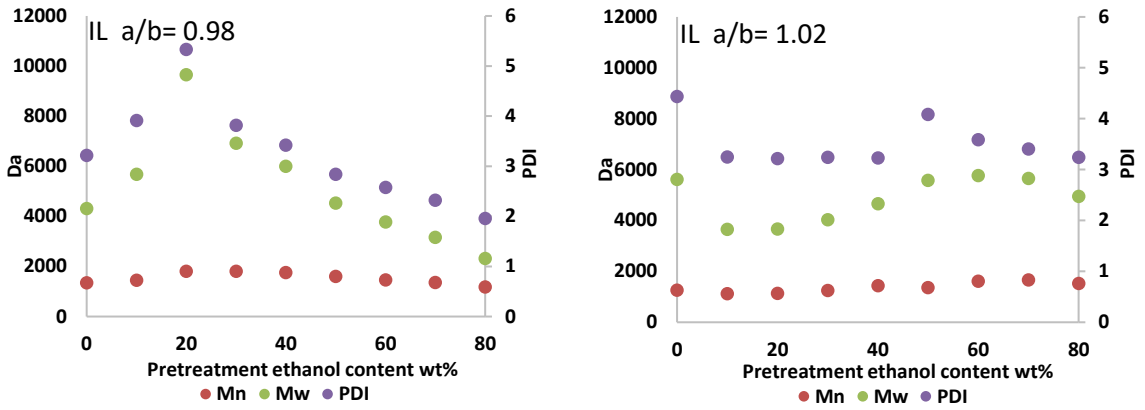


Figure 3. 15 Average molar mass and polydispersity of lignin extracted with different ethanol-IL mixtures a) IL acidity a/b=0.98 b) IL acidity a/b=1.02

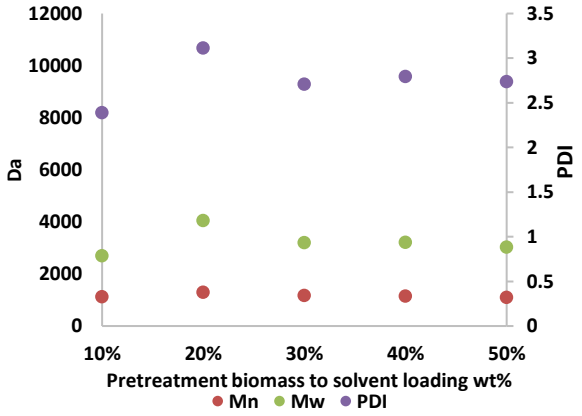


Figure 3. 16 Average molar mass and polydispersity of lignin generated after pretreatments with different biomass loadings

3.3 Technoeconomic analysis of the organosolv-ionoSolv pretreatment

An industrial scale ionoSolv process could be separated into four steps: 1) biomass mill, 2) pretreatment, 3) lignin precipitation and separation and 4) pretreatment solvent recovery (water-IL seperation) , where IL is regenerated from aqueous IL mixture.[248] Brandt et al. have highlighted that the IL regeneration process can be the most energy intensive step and consequently the most expensive step in the ionoSolv process.[3] As the organosolv-ionoSolv pretreatment could achieve a

better pretreatment effectiveness, replacing conventional ionSolv pretreatment with organosolv-ionoSolv pretreatment in industrial biorefineries becomes possible. Biomass will therefore be pretreated by organic-IL-water mixture instead of water-IL mixture. This means an additional organic solvent recovery process will be needed in step 4 (IL-water seperation). How this addition solvent recovery process affects the overall process cost will be a piece of important information for commercialising organosolv-ionoSolv pretreatment technology. Here, an economic analysis was carried out to get more insights of the solvent regeneration cost for organosolv-ionoSolv pretreatment and the energy produced by the lignin isolated from this pretreatment if 100% lignin is subjected to burning. Both capital cost CAPEX and operational cost OPEX are studied for the solvent regeneration (detailed process graph is presented in Appendix Figure S3.2), and CAPEX was presented in equation 2:

$$CAPEX = Heat\ exchanger + Vessel + Pump + (organic\ solvent\ regenration\ unit) \quad \text{eq (2)}$$

where Heat exchanger stands for the price of the heat exchanger used for heating up the organic-IL-water mixture, Vessel stands for the price of the vessel used to separation IL from aqueous organics, Pump stands for the price of the pump used for pumping organic-IL-water mixture into the vessel, organic solvent regeneration unit stands for the overall cost for regeneration pure organics from the aqueous organic mixture. It is important to note that the organic solvent regeneration unit could be costly in some cases, e.g. separating ethanol from aqueous ethanol, as azeotropic distillations might be involved.

The energy requirements for drying the IL have been modelled by a flash distillation model using software HYSYS V8.8, with the two assumptions: 1) the diluted aqueous solution contains 3 equivalents of water per equivalent of IL in mass basis, based on our laboratory pretreatment process protocol; 2) the IL was pre-dried and the IL moisture after drying was 20 wt% for ionoSolv processes and 2 wt% for organosolv-ionoSolv processes. Further details about the IL drying process model are

provided in the appendix. The energy requirements to regenerate the IL from aqueous post-pretreatment solvent mixture for the biorefinery process with a 100% glucose conversion to ethanol, are shown in Figure 3.17. According to the IL regeneration model, the organic-IL process with an organic concentration ≤40% has a higher energy input than that for ionoSolv process, due to the IL used in the hybrid process need to be extremely dry. For the organic-IL process with an organic concentration ≥40%, a clearly reduced energy requirement was needed for recovering the IL, comparing to ionoSolv process. It is worth mentioning that the comprehensive equilibrium data of the post-pretreatment solvent mixture, containing water, IL and organic solvent, have not been collected yet, and the pretreatment process conducted was not optimised in the aspect of optimal water usage for precipitating the lignin and regenerating the solvents. Therefore, we are more interested in the trend rather than the actual calculated values of the IL-regeneration energy input. Ethanol shows the highest energy consumption among the three organic solvents, especially at high solvent content. The savings in energy from using acetone will likely be offset by the increased operating cost (OPEX), due to the logistics of importing these substances into the biorefinery, given that the facility is already producing ethanol. Furthermore, if there is excess heat available, there will not be any benefits of using other solvents unless they are already produced or used in the industrial facility as there would not be a need to reduce the energy consumption.[249] Hence, the organoSolv-ionoSolv process with the optimal IL regeneration process may very likely be using ethanol or butanol as the IL co-solvent.

The economic analysis model not only looked into the energy consumption for IL recycling process, but also investigated the energy generation from the side product stream, lignin, of the biorefinery. If the lignin generated from the biorefinery is burnt to generate power for the facility, an assumption was made: its efficiency in the boiler is 100%, corresponding to 24.6 ± 0.9 MJ/kg, the average HHV value for ionoSolv Miscanthus lignins.[67] The energy regenerated by lignin

was normalized to the energy consumption of recycling the IL, and the calculated values are presented as triangles in Figure 3.17. For three organic solvent choices, with the increased organic content in the pretreatment process, the energy generation of the lignin isolated from the biorefinery were higher and peaked at 80 wt%, making the biorefinery more energy-sufficient at high organic content. It is important to know that the simulated process is not completely energy autonomous regardless the organic content, suggesting it is important to optimise the process when scaling up. Once again, we are more interested in the trend of the lignin energy production rather than the actual values. In general, ethanol and butanol displace similar trends between 20-60 wt% content. The acetone-IL lignin produces less energy at organic concentrations, compared to ethanol and butanol. At 80% organic content, the ethanol shows a highest energy production among the three solvents.

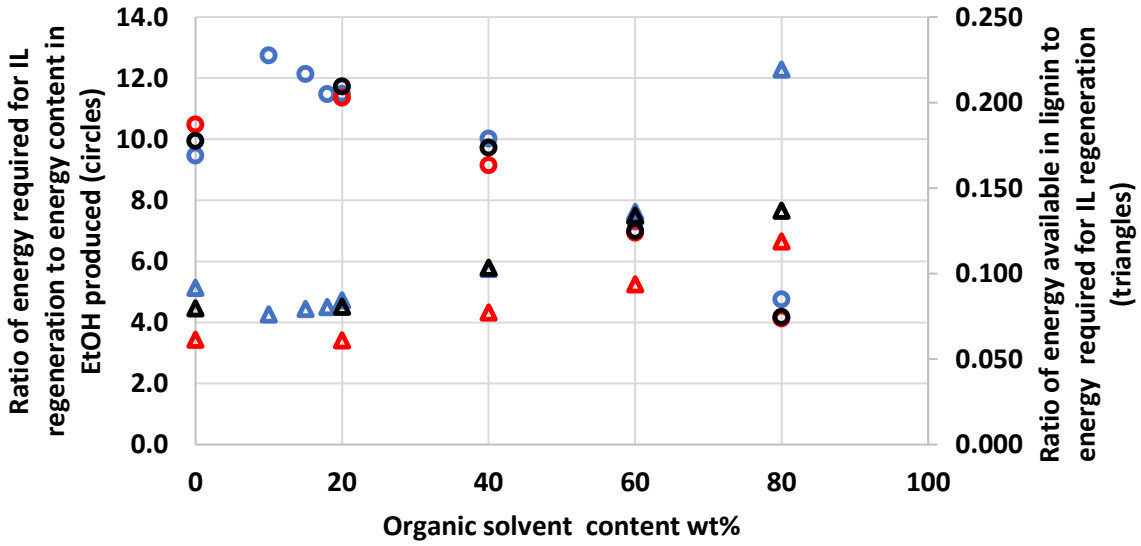


Figure 3. 17 Energy consumption ratios for recovering IL from the aqueous post-pretreatment mixture for energy content in produced ethanol (circles) and energy production ratios via lignin incineration (triangles) as a function of organic solvent content. Blue, black and red stand for ethanol, 1-butanol and acetone, respectively.

A detailed techno-economic analysis is essential for investigation of the impact on CAPEX of the biorefinery with different pretreatment step. For pretreatment step, the reactor and solid handling facilities are expected to remain the same as the solid loading, temperatures and pulp yields are all similar for all the cases, suggesting these features of the process has a little or no effect on the overall CAPEX. If IL is regenerated by a flash distillation, the heat exchanger cost will be in line with the trend of the energy consumption, hence, the IL recovering process should be cheaper at high organic content. For the case of organic-IL pretreatment, after separating IL from the post-pretreatment solvent mixture, the left-over mixture contains both water and organic solvent, requiring an azeotropic distillation to recycling the organic solvent. Compared to the ionoSolv process, complexity will be added to the pretreatment, therefore CAPEX and OPEX will be increased. It will be a different study if the biorefinery plant is producing ethanol or butanol as a main product stream, the ethanol/ butanol recovery unit for separating ethanol after the fermentation step could also be used in the pretreatment step to separate the ethanol-water mixture. It is also important to consider the organic solvent loss when doing the OPEX predictions, as the organic solvent are highly volatile. If the biorefinery is producing ethanol as one of its main products, a comprehensive techno-economic analysis is important for obtaining the minimum ethanol selling price (MESP), a key indicator of industrial-feasibility for the biorefinery process.

The boiling point temperature and energy of vaporization of the different organic solvents used in this work are listed in appendix. The difference in these properties could potentially lead to different conclusions if other process schemes, heat integration or order drying technologies are used to regenerate the IL. The techno-economic analysis could be extended if other characteristics of the process which could potentially impact the CAPEX are investigated, e.g. the water tolerance. *i.e.* the amount of water that can be left in the IL-organic solvent recycle for the organosolv-ionoSolv process.

Chapter conclusion

An organosolv-ionoSolv pretreatment process was developed based on the organosolv and ionoSolv processes. The new process using a mixture of organic solvent and [TEA][HSO_4] was conducted for miscanthus and its fractionation effectiveness and the composition of the pulp generated were compared with the ionoSolv process. The process using 40% ethanol or butanol with 60% IL had a glucose yield of 85%, 10% higher than that of ionoSolv process, due to the improved delignification of the biomass during fractionation. Incorporating acetone with IL to pretreat biomass did not change the overall fractionation effectiveness, compared to ionoSolv process. This new hybrid process was further tested with two different IL acidities (acid/base=1.02, 0.98) to investigate its operational IL acidity range. The process kept the same fractionation ability at acid/base ratio between 1 to 1.02. This pretreatment also was also conducted with different biomass loadings and the process maintained a compatible fractionation ability up to 50 wt% loading. Isolated lignin was subjected to HSQC NMR and GPC analysis. The results suggested that the organic-IL lignins were less condensed compared to ionoSolv lignin and α-alkoxylation was the most important lignin modification during this hybrid pretreatment. The less condensed lignin structure improved the economic value of the lignin fraction generated as the side product of the pretreatment process, bringing more possibility for high-value added application. According to the economical analysis, comparing to typical ionoSolv process, organosolv-ionoSolv process using ≥40 wt% organic solvent has a lower solvent regeneration cost and the extracted lignin fraction could generate more energy if subjected to burning. The solvent regeneration cost could be further reduced if organic solvents like ethanol, butanol are used during pretreatment, as these organics are the targeted end-products of the biorefinery.

This organosolv-ionoSolv pretreatment has provided evidence for a biomass fractionation process, which can generate a highly enzyme accessible sugar fraction and also produce side products including

high-quality lignin for value-added applications. This could be a milestone for applying the current ionoSolv pretreatment technology in the industrial biorefinery process.

4 Results: Pine organosolv-ionoSolv fractionation

Chapter summary

In the previous chapter, a new hybrid pretreatment method was developed, combining the main features of ionoSolv and organosolv pretreatments, and was tested on a grassy feedstock, miscanthus. In this chapter, we continued to test this newly developed hybrid process on a more recalcitrant softwood feedstock, pine. Pine is one of the most commonly studied softwood feedstocks, and is considered forest residues with a large abundance worldwide.[190] The IL selected was [DMBA][HSO_4] and the organic solvent used was ethanol. Pretreatments were conducted with 3 different ethanol contents: 20, 40, and 80 wt% . An ionoSolv pretreatment using [DMBA][HSO_4] with 20 wt% water was also carried out for comparison. Following that, the fractionation process using an ethanol (40 wt%) and IL (60 wt%) mixture was subjected to a series of biomass loading experiments to see whether the hybrid process could maintain a decent fractionation ability at loadings for recalcitrant feedstocks, such as pine. The overall pretreatment effectiveness for all processes were evaluated by the composition analysis and the saccharification assay of the pulp (treated biomass). HSQC NMR and GPC analysis were conducted for the extracted lignin fraction, to monitor any changes in lignin's internal structure and molecular weight. The full content of the chapter was also included in the written paper 'Design of an organosolv-ionoSolv biomass fractionation process for biofuel production and high value-added lignin valorisation' which is ready for submission, as detailed in **Publications**.

4.1 Effect of organic solvent concentrations on fractionation

The newly developed pretreatment, oganosolv-ionoSolv process, has previously been performed using miscanthus, presented in Chapter 3. The process achieved a high cellulose to sugar conversion, and a lignin side-product fraction with improved quality in terms of lignin structure. However, an ideal pretreatment process for industrial-scale biorefinery must be feedstock-independent is necessary, as the lignocellulosic biomass varies from region to region. For examining whether this organosolv-ionoSolv process could also function well with other feedstocks, especially more recalcitrant biomass, the pretreatment process was repeated with one type of softwood*, Pinus sylvestris* (pine). As [DMBA][HSO_4] was reported to be one of the best preforming protic ILs for pine in ionoSolv pretreatments, anhydrous [DMBA][HSO_4] was used in this organosolv-ionoSolv process and ethanol was the co-solvent. [190] Pine was fractionated by three different ethanol-IL mixtures with ethanol contents of 20, 40, 80 wt%. The ionoSolv process using [DMBA][HSO_4] with 20 wt% moisture was also conducted in parallel for comparison.

4.1.1 Biomass fractionation in reactors

The ionoSolv pretreament of pine has previously been carried out a much higher temperature than for miscanthus, 170°C.[190] In order to operate the organosolv-ionoSolv process at this temperature, the Hydrothermal Autoclave Reactors were used rather than pressure tubes (used for all ionoSolv pretreatments). This was, due a much higher operational pressures needed to fractionate biomass at the required temperature due to the organic solvent fraction. As the autoclave reactors had not previously been used for pretreatment, a time-course ionoSolv pretreatment was performed to determine the optimal pretreatment duration for pine in these reactors, and compare performance to pressure tubes. Saccharification assay were used to analyse and compare the pulps pretreated in pressure tubes and reactors. The saccharification

results in Table 4.1 shows that conducting pretreatment in a reactor for 80 minutes has a similar fractionation effectiveness as conducting in a pressure tube for 30 minutes. All pretreatments in Section 2.1.2 were carried out in triplicate at 170°C for 80 minutes with a solid to liquid loading of 1:10 g g^{-1} .

Table 4. 1 A list of saccharification data for pine [DMBA][HSO_4] pretreatment using different pretreatment apparatuses with different pretreatment durations

For hydrothermal autoclave reactor[a]		For pressure tube [a]	
Pretreatment time (min)	**Saccharification yield**	**Pretreatment time (min)**	**Saccharification yield**
40	41	**30**	63
60	48		
80	64		
100	46		
120	43		
a all pretreatments were conducted in triplicate and the average sugar yield was listed			

4.1.2 Impact of ethanol contents on pretreatment effectiveness

4.1.2.1 Saccharification yields

The trend of sugar yield as a function of ethanol content in the organosolv-ionoSolv pulps is displayed in Figure 4.1 and the actual values are presented in Table 4.2. Relative to the ionoSolv pretreatment, the ethanol-IL pretreatments have shown a better fractionation effectiveness, especially for an ethanol-content between 20% and 40 wt%. For ethanol-IL pretreatments, the peak sugar yield, 74%, which was reached at 20 wt% ethanol, was 12% higher than that for ionoSolv pretreatment. The sugar yield for 40 wt% ethanol was 70%, but this was down to 5% when ethanol content of the process increased to 80 wt%, showing that the organosolv-

ionoSolv process for pine requires at least 60% IL content, whereas miscanthus requires at least 40%, due to more recalcitrant nature of pine. For a standard Organosolv pretreatment (using water-ethanol mixture 50: 50 w/w, 1% sulfuric acid), a peak glucose yield, 75%, was reported for pitch pine.[199]

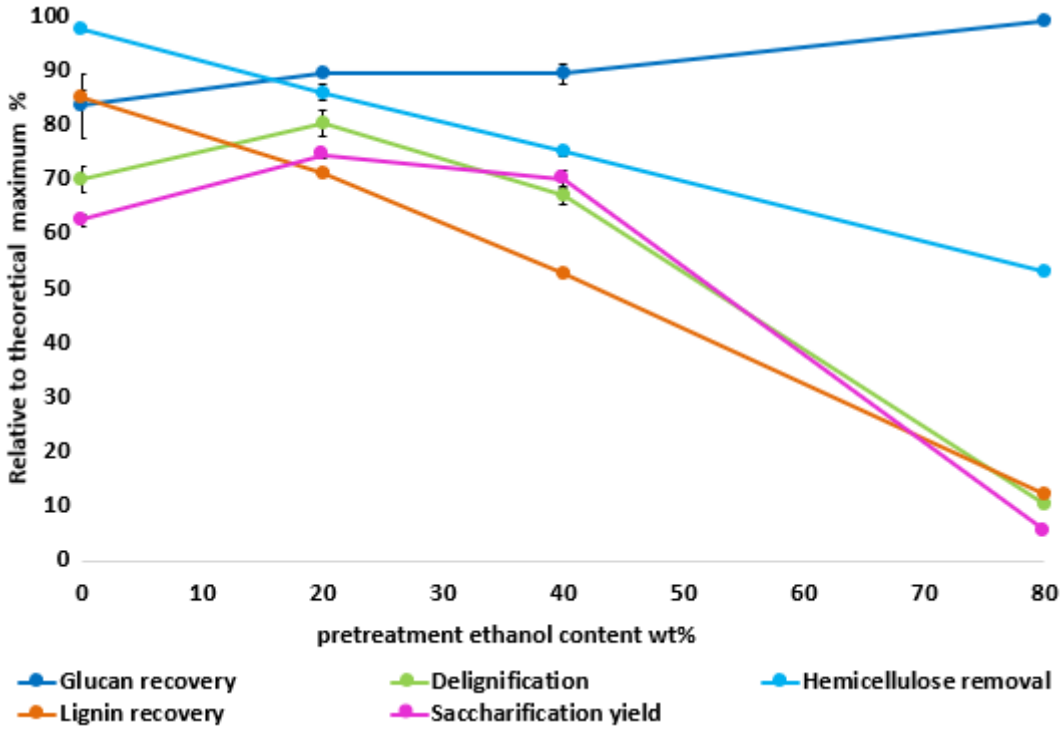


Figure 4. 1 Key indicators of fractionation effectiveness for ethanol-[DMBA][HSO_4] pretreatment at 170°C for varying organic solvent content in pretreatment solvent with 1:10 g g^{-1} biomass loading. All data points with 0% organic solvent represent corresponding ionoSolv processes, using [DMBA][HSO4] containing 20 wt% water. Error bar is included.

Table 4. 2 A list of compositional and saccharification key indicators for pine fractionation process using a mixture of ethanol and [DMBA][HSO_4]

wt% of ethanol in pretreatment solvent (wt% IL+water)	Glucan recovery[a]	hemicellulose removal[a]	lignin recovery yield[a]	Delignification[a]	Saccharification yield[a]
0[b] (100)	84	98	85	70	63
20 (80)	89	86	71	80	75
40 (60)	89	75	53	67	70
80 (20)	99	53	12	11	6

a the yield is presented in percentages of the theoretical maximum, relative to untreated biomass

b The pretreatment with 0% ethanol content represents the ionoSolv process where the pretreat biomass is subjected to the ethanol pulp washing process.

4.1.2.2 Pulp compositions

Apart from the saccharification assay, compositional analysis of the pulp also delivers useful information of the overall fractionation effectiveness of the pretreatment, including the degree of glucose degradation, hemicellulose and lignin removals. Figure 4.1 shows the trends of glucose recovery, hemicellulose removal, delignification and lignin recovery yield for pretreatments using 4 different ethanol-IL mixtures, with the actual values of the pulp compositions listed in Table 4.2.

The ionoSolv pulp only recovered 80% glucan, relative to the glucan content of the untreated pine, meaning 20% glucose was degraded after being fractionated by IL with 20 wt%. water. The degree of glucose degradation was less severe for miscanthus, ≤10% degradation was detected for ionoSolv pulp(detailed in Table 3.1). The degree of glucose degradation was inversely related to the organic concentration of the fractionation process, and a quantitative glucose recovery was achieved by 80 wt% ethanol. A quantitative hemicellulose removal was

achieved by the ionoSolv process, and for three ethanol-IL process, hemicellulose removal peaked at 85%, with 20 wt% ethanol. The peak hemicellulose removal of the ethanol-IL process for miscanthus was also found to be around 85%. It has been repeatedly reported that lignin is one of the biggest obstacles for effective biomass fractionation, and that the trend of the saccharification yield follows the trend of lignin removal.[190 71 67 70 60] Lignin removal reached a maximum at 20 wt% ethanol, with a peak value of 80%, and the corresponding lignin removal for ionoSolv process was 70%, again this is in line with the saccharification result. Another type of pine, Loblolly pine, was reported to achieve a 61% lignin removal in the organosolv pretreatment, and its glucose recovery reported was 79%.[250]

The lignin recovery yield (85%) exceeded the lignin removal (70%) when pine was fractionated by aqueous IL, suggesting the unwanted formation of condensed lignin and pseudo-lignin (lignin-like polymers, made of lignin and sugar degradation products) possibly took place during ionoSolv fractionation process. Delignification was higher than lignin recovery yield for all ethanol-IL process, indication there is no obvious sign of the lignin condensation. The lignin recovery reached a maximum of 74%, at 20 wt% ethanol. This kept dropping as the ethanol content increased.

In summary, the optimal solvent composition of the organosolv-ionoSolv pretreatment for pine requires a higher IL content, relative to miscanthus (optimal composition: 60% IL with 40% ethanol).For pine, 80% IL with 20% ethanol achieved the highest delignification, 80%, and highest saccharification yield, 74%. Its glucan recovery and hemicellulose were the highest as well. This indicates that the process with the optimal solvent composition was able to achieve a selective removal of the lignin and hemicellulose and leave a clean fraction of cellulose which

is highly enzyme-accessible. The fractionation effectiveness for 40 wt% ethanol was lower by little, compared to 20 wt%.

4.2 Effect of feedstock loading on fractionation

In the previous section, the ethanol-IL pretreatment has been proven to have a better fractionation performance than ionoSolv process. In this section, the hybrid process was further tested, investigating its performance as a function of biomass to liquid loadings. The ethanol-IL pretreatment for pine was performed at three biomass loadings, 10 wt% (1:10 g g^{-1}), 30 wt% (3:10 g g^{-1}), 50 wt% (5:10 g g^{-1}). The solvent composition used was 40 wt% ethanol and 60 wt% IL. The reasons for choosing this solvent composition over the optimal composition concluded in the previous section are:

1. The fractionation effectiveness differed by little between 20 wt% and 40 wt% ethanol, e.g. saccharification yield for 20 wt% ethanol was 74% whereas 70% for 40 wt% ethanol
2. IL regeneration (separate water from IL) is the most energy-intensive step of the pretreatment. Recycling low boiling-point organic solvent like ethanol (separating ethanol from IL)requires less energy, compared with recycling water from the aqueous IL mixture after the ionoSolv process ; hence, using organic solvent-IL mixture as the pretreatment solvent could potentially reduce the energy requirement for the industrial-scale fractionation, making the overall process more cost-effective.

All the pretreatments were characterised by saccharification assay and compositional analysis. The key information of the overall pretreatment effectivenessare presented in Figure 4.2 and in Table 4.3.

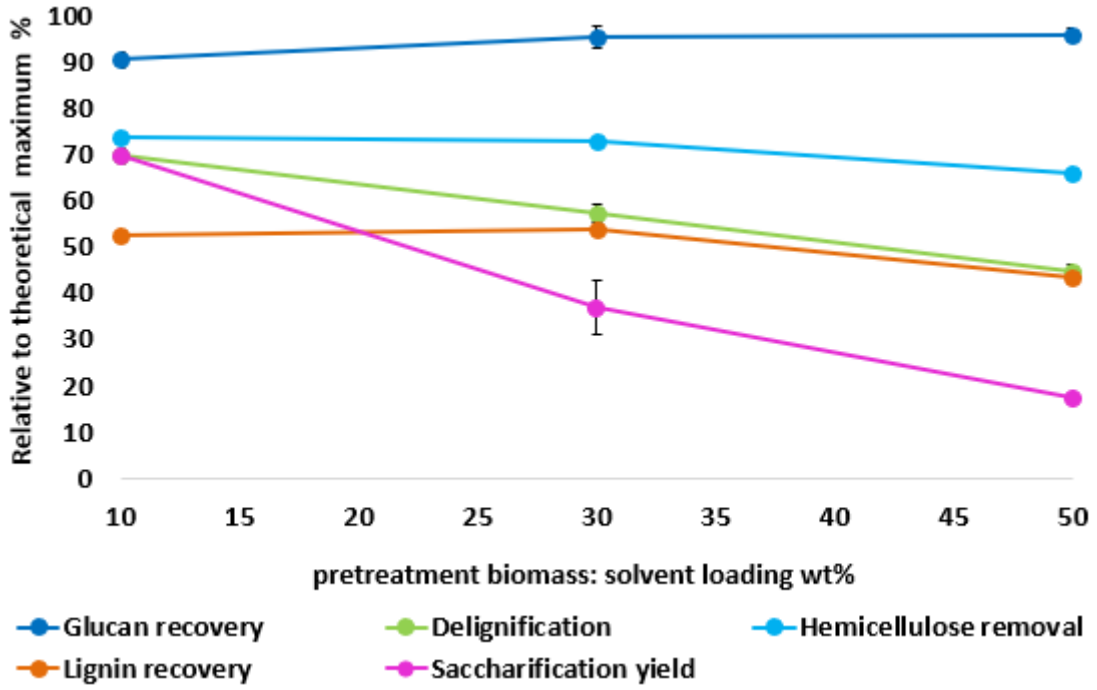


Figure 4. 2 key indicators of fractionation effectiveness for ethanol-[DMBA][HSO_4] pretreatment at 170°C as a function of biomass loading , pretreatment solvent composition was 60 wt% IL and 40 wt% ethanol. Error bar is included.

Table 4. 3 A list of compositional and saccharification key indicators for pine fractionation process using an ethanol-[DMBA][HSO_4] mixture with 40% wt. ethanol content with three different biomass loadings

wt% biomass to solvent loading	Glucan recovery[a]	hemicellulose removal[a]	lignin recovery yield[a]	Delignification[a]	Saccharification yield[a]
10	91	74	53	70	70
30	96	73	54	57	37
50	96	66	44	45	18

a the yield is presented in precentages of the theoretical maximum, relative to untreated biomass

Unlike for miscanthus, the hybrid process could maintain fractionation effectiveness up to 50 wt% biomass loading, the overall performance of the ethanol-IL mixture was less successful at high loadings for pine. More specifically, in terms of sugar releasing yield, a significant reduction of the sugar yield was observed: the saccharification yield for 10% wt. biomass loading was 70%, while the yield was only 17% when the biomass loading increased by 5-fold. At 10 wt% loading, 70% lignin removal was achieved, but the delignification was only 43% when the loading rose to 50 wt%. Gschwend also investigated the ionoSolv process efficacy as a function of biomass loading.[190] She reported that the saccharification yield for 10% loading was 55% and decreased by only 15% at 50 wt% loading, suggesting that the ionoSolv pretreatment could achieve a better fractionation at high loadings compared to organoSolv-ionoSolv pretreatment. The heavily condesned lignin structure and formation of pseudo-lignin could be blamed for the weaker preformance of the ionoSolv process at high loadings.[190]

In general, at low biomass loadings, using organosolv-ionoSolv process to pretreat pine could selectively fractionate lignin, hemicellulose and effectively prevent glucose and lignin degradation, resulting in an improved pretreatment performance. However, as pine is fairly recalcitrant in nature, the organosolv-ionoSolv process with selected conditions is not powerful enough to deliver an effective fractionation at high loadings, and a process with more hash conditions may be needed, e.g. increasing the IL content of the pretreatment.

4.3 Characterisation of isolated lignin

Lignin fractionation is strongly correlated with the overall pretreatment effectiveness.[67] An ideal biomass fractionation process requires effective lignin removal and little or no undesired lignin

modification taking place during the pretreatment. Each pretreatment process will lead to different chemical modifications at various position of the lignin inter-structure, and different degrees of lignin degradation. This has a significant effect on the structure of the lignin extracted during pretreatment, and consequently determines the suitable applications of the extracted lignin fraction. Therefore, it is important to understand the intermolecular of the isolated lignin. The isolated lignin was characterised by HSQC NMR and GPC analysis. HSQC NMR spectra reveals the inter-structure of the lignin, while GPC analysis provides information about lignin's molecular weight.

4.3.1 HSQC NMR analysis

Three lignins extracted with different ethanol-[DMBA][HSO_4] mixtures (ethanol content 0 wt%, 40 wt%, 80 wt%) were characterised by HSQC NMR analysis. A list of common lignin subunits appearing in softwood lignin, in this case pine, is presented in Figure 4. 3. In the side chain region (δ_C 50-90 ppm δ_H 2.5-5.8 ppm) of the spectra, β-O-4 ether (A), β- β resinol (B), β- 5 phenylcoumaran (C), α-alkoxy ether (A') and Lignin-carbohydrate linkages (lignin crosslinked arabinose (Ara)/xylose (Xyl)) have drawn the most attention. For the aromatic region (δ_C 110-130 ppm δ_H 6.0-6.9 ppm), uncondensed and condensed guaiacyl units (G_2, G_5, G_6, $G_{2cond.}$), are the most common lignin subunits. One type of subunits containing 8 membered ring, also named as dibenzodioxocin (DB) was also detected in pine.

229 81

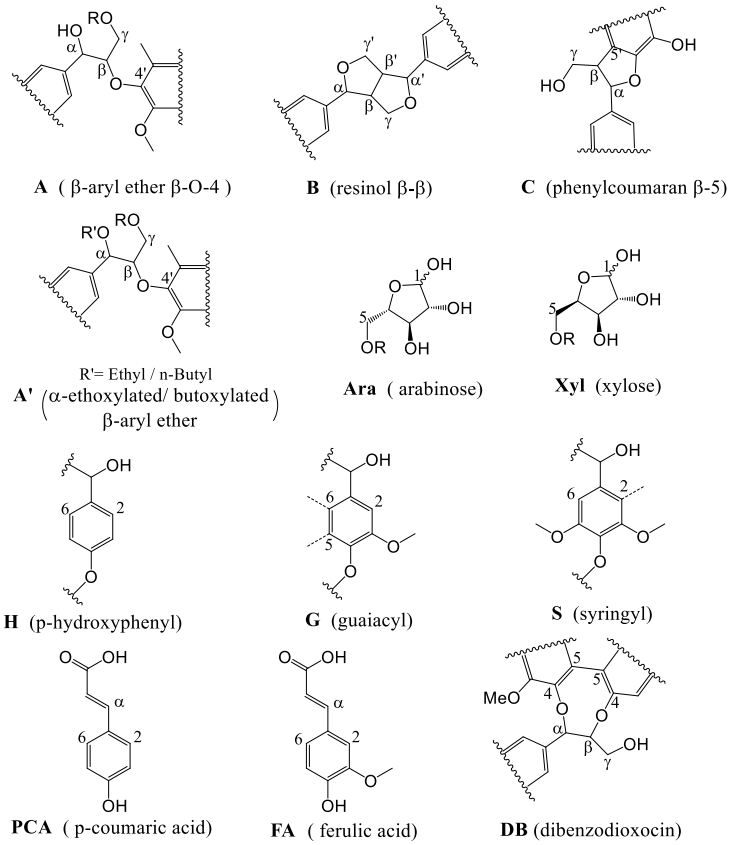


Figure 4. 3 Key lignin substructures found in recovered lignin from Pine pretreatment

The coloured HSQC NMR spectra for two lignins (ionoSolv and ethanol 80 wt%) are shown in Figure 4.4 a. A semi-quantitative analysis was conducted for the integrated volume peaks of all the major subunits, presented in Figure 4.4 b. The peak intensities were calculated as percentages of the combined G_2 and G_{2cond} volume peak. G_2 and G_{2cond} peak intensities of the lignin were reported to be the same for all pretreatments.[2] The degree of condensation could be quantified by the signal intensity G_{2cond} relative to G_2 and G_{2cond} integrals.[60]

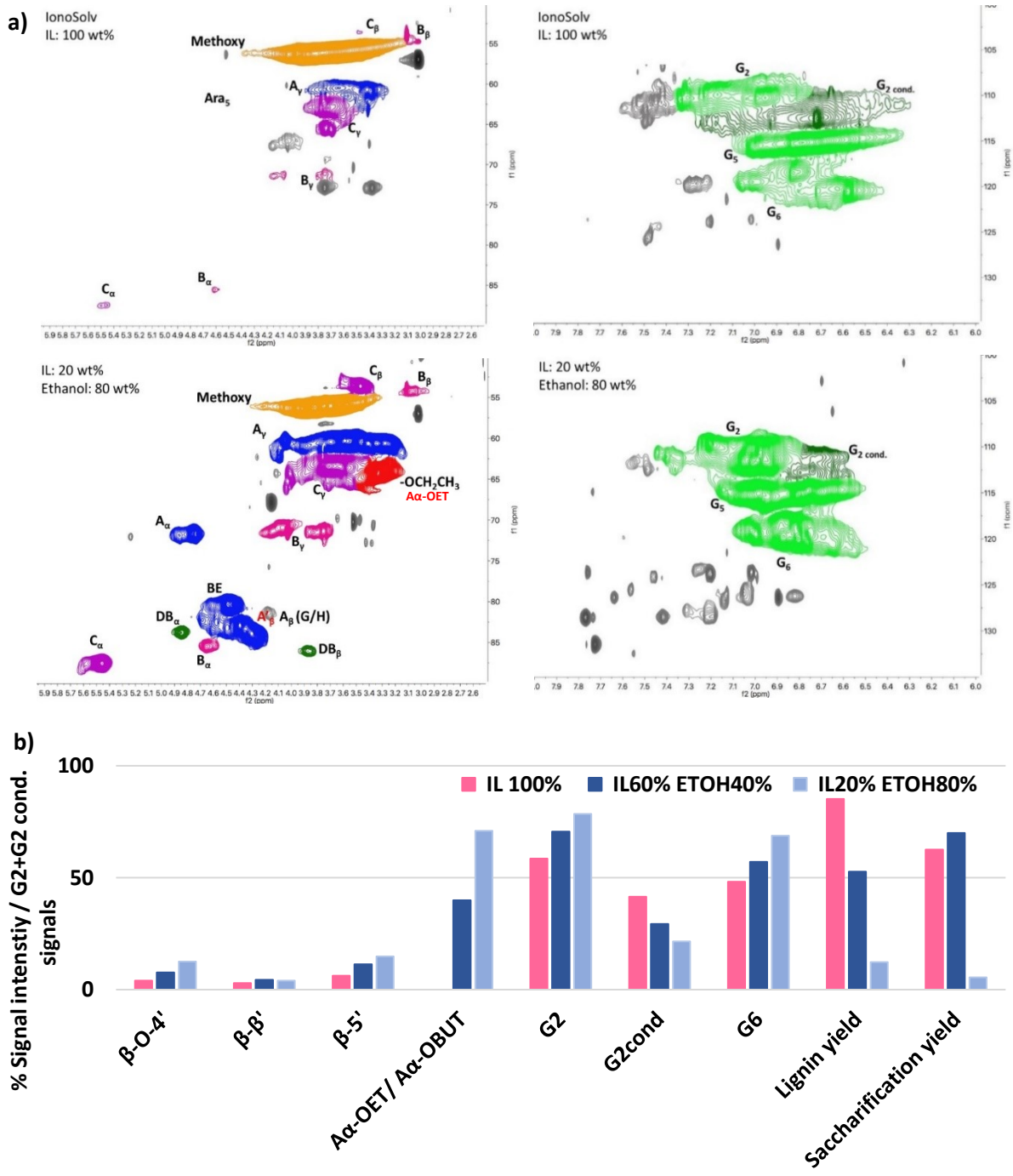


Figure 4. 4 a) HSQC NMR spectra of pine lignins recovered from ionoSolv, [DMBA][HSO_4], and ethanol-ionoSolv processes with the organic concentration of 80 wt% (left) Side chain region of the HSQC NMR spectra (right) Aromatic region of the HSQC NMR b) a semi-quantitative analysis for signal intensities of the key lignin subunits in pine lignins recovered from ionoSolv and ethanol-ionoSolv processes with different ethanol contents, 40 wt% 80 wt% , according to HSQC NMR spectroscopy. Signal intensities are presented in percentages relative to the sum of signal intensities for G_2 and $G_{2cond.}$

In the semi-quantitative HSQC analysis, there was less β-O-4 ether cleavage happening in the organosolv-ionoSolv process, compared to ionoSolv process. The peak intensity of uncleaved β-O-4 ether bond for the ionoSolv lignin was less than 5%, where this was 12% for ethanol-IL lignin, suggesting more aryl ether bond remained unchanged during the ethanol-IL process. Both values were much lower than the ether bond abundance for the native lignin, 35 to 60%.[229] Similar to miscanthus lignin, α-ethoxylation was the major chemical modification taking place at the lignin side chain region during the organosolv-ionoSolv fractionation process. This lignin modification led to a rise in the abundance of α- ethoxylated β-O-4 linkages, which was proportional to the ethanol content of the pretreatment. The modification also significantly hindered the lignin condensation, which should happen at the α carbon position of the lignin side chain.[204] The degree of condensation was halved when ethanol concentration increased to 80 wt%, compared to ionoSolv lignin, according to Table 4.4. For both processes, the process conditions were not harsh enough to break the stable carbon single bond linkages, resinol (β- β) and phenylcoumaran (β-5), which are fairly stable up to 200°C, therefore, these linkages were likely to be modified.[247] Similar amount of resinol (β- β) units was modified during ionoSolv and ogranosolv-ionoSolv processes. This was not the case for phenylcoumaran (β-5) linkages, for which fewer linkages were modified by the ethanol-IL mixture as the ethanol content of the pretreatment increased.

Table 4. 4 Degree of condensation based on HSQC spectrum integrals for lignins extracted from pine using different ethanol-IL mixtures

Pretreatment solvent composition	IL80% Water 20%	IL60% ETOH40% [a]	IL20% ETOH80%
G_2 peak integral intensity	58	70	78
$G_{2con.}$ [b] peak integral intensity	41	29	21
$G_{2con.}/G_2+G_{2con.}$ in %	**41**	**29**	**21**
a ETOH is short for ethanol			
b $G_{2con.}$ stands for condensed G_2 peak			

4.3.2 GPC analysis

Pine lignins extracted by different ethanol-IL mixtures and the lignins recovered from pretreatments with different biomass loadings were subjected to GPC analysis. Number average molecular weight, Mn, stayed below 2000 Da. Weight average molecular weight, Mw, for ionoSolv lignin was 4715 Da, but this rose to 6595 Da at 60 wt% ethanol. An increased fraction of α-alkoxylated lignin oligomers in the precipitated lignin could be to the blame for this molecular weight increase. More specifically, these α-alkoxylated lignin oligomers were made of α-alkoxylated monolignols, which have a higher molecular weight than the native monolignols by 14% at least, based on the assumption that each monolignol has a molecular weight of 200.[26] Mw dropped to 2487 Da when ethanol content increased to 80 wt%. The reason for this molecular increase is not fully understood. It is likely due to the reduced effectiveness of the lignin fractionation: there are fewer lignin units being modified, and also less lignin being recovered. The PDI has the same trend as Mw, and peaked at 40 wt% ethanol with a value of 3.88.

For the biomass loading experiments, Mn stayed below 1700 Da up to 50 wt% biomass loading, Mw increased from 4715 Da (10 wt% loading) to 6908 Da (30 wt% loading), the dropped to 6047 Da (50 wt% loading). The trend of PDI was in line with Mw. Further investigation is needed here for understanding the relationship between the weight molecular weight and the biomass loading.

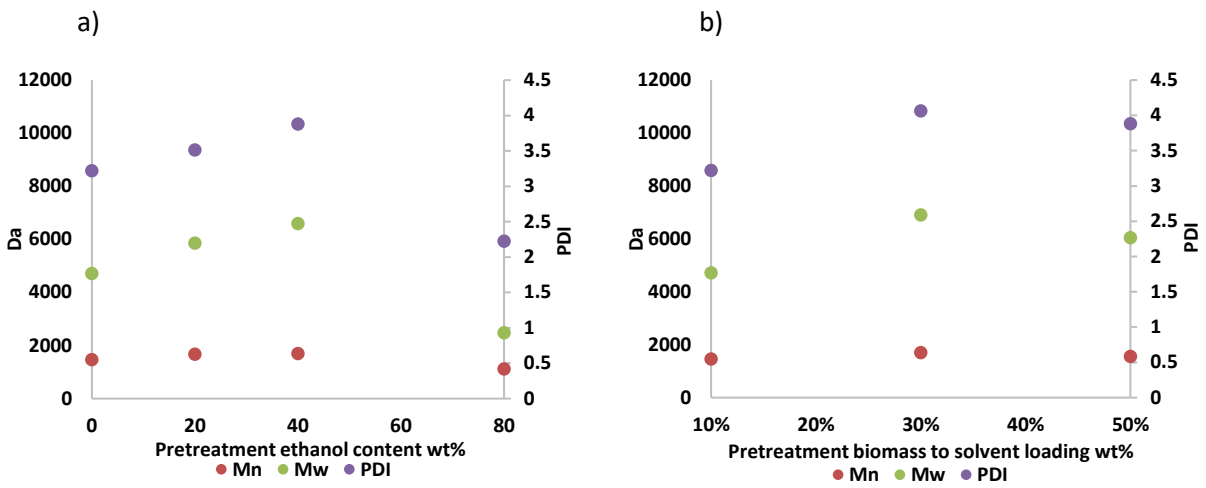


Figure 4. 5 Average molar mass and polydispersity of lignin extracted from pine a) lignin extracted with different ethanol-IL mixtures a) extracted lignin generated after pretreatments with different biomass loadings.

Chapter conclusion

In this chapter, the organosolv-ionoSolv (hybrid) pretreatment using ethanol and [DMBA][HSO_4] was tested on pine at 170 °C for 80 minutes in Hydrothermal Autoclave Reactors. The optimal solvent composition determined was 20 wt% ethanol and 80 wt% IL. Compared to the ionoSolv process, the hybrid process with optimal solvent composition was able to achieve a 12% increase in the saccharification yield (the most important fractionation performance indicator for the process), due to an increased delignification (10% increase). This suggested that the organosolv-ionoSolv pretreatment process is feedstock-independent. For recalcitrant feedstocks like pine, an improved fractionation performance could easily be achieved by adjusting the solvent composition, e.g. increasing the IL concentration of the pretreament.

According to the HSQC NMR analysis, the pine lignin recovered from the hybrid pretreatment was less condensed compared to ionoSolv lignin. The major chemical modification happening during the lignin fractionation was the α-alkoxylation, forming α-alkoxylated ether linkage on side chain of each monolignol, which could improve lignin's solubility in water to achieve a better lignin removal and also inhibit lignin condensation to a large extent. The lignin fraction produced by organosolv-ionoSolv pretreatment is expected to have a higher economical value than the ionoSolv lignin as the lignin produced by this pretreatment is less condensed than ionoSolv lignin.

5 Results: Agricultural residues fractionation

Chapter summary

Agricultural residues are the main side products for food production, often referred to as wastes, and commonly used in power generation. Direct burning of these agricultural wastes has induced some environmental issues, such as air pollution, due to the high ash content of the feedstocks.[60] The annual availability of these feedstocks is at gigaton-scale, an annual production of 975 million tons could achieved merely by rice straw, which have a great potential of being a biorefinery feedstock.[44] Using agricultural residues for bioethanol production will be beneficial for relieving the problem related to the direct burning. This chapter started with a time course experiment for rice husk to determine the optimal ionoSolv process condition. Using the optimal process conditions for rice husk, predictions were made for rice straw, wheat straw and bagasse, and pretreatments were conducted at predicted optimal conditions to investigate the pretreatment effectiveness towards these four feedstocks. organosolv-ionoSolv processes were also preformed and their performances were compared to the ionoSolv. Pretreatment effectiveness was determined by saccharification assay and compositional analysis of the pulps. The ionoSolv lignins were characterised by HSQC NMR spectroscopy, their major linkage compositions were compared, where bagasse EMAL was used as the reference. Organosolv-ionoSolv lignin were characterised by HSQC and GPC analysis and compared to ionoSolv lignin. The ionoSolv process with predicted optimal conditions for four feedstocks and the EMAL bagasse lignin were conducted by Dr. Clementine Chambon. The rice husk time course experiments with the ionoSolv process for three other feedstocks were in the published paper 'Efficient Fractionation of Lignin- and Ash-Rich Agricultural Residues Following Treatment With a Low-Cost Protic Ionic Liquid'. The section for the Organosolv-ionoSolv process was included in the written paper 'Integrated

fractionation of lignin- and ash-rich agricultural residues by a hybrid pretreatment method', as detailed in **Publications**.

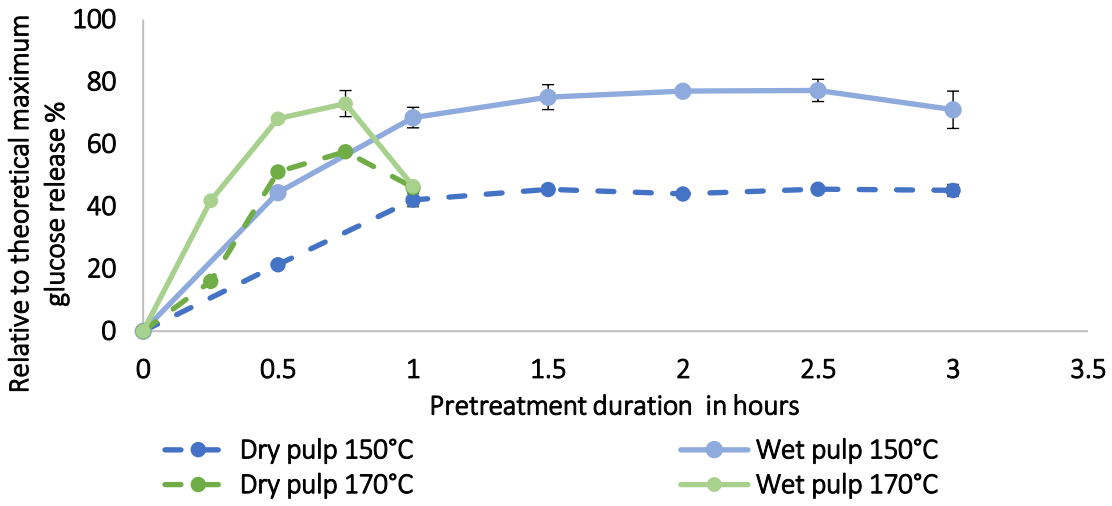
5.1 Rice husk ionoSolv process

Comparing the chemical composition of the agricultural residues studied in this chapter, rice husk has the highest lignin (27%) and second highest ash contents (11% mainly containing inorganic matters).[60] The high lignin and ash content makes rice husk the most recalcitrant feedstock among the four agricultural residues, rice straw, rice husk, wheat straw and sugarcane bagasse, studied in this chapter. Therefore, we decided to start by investigating the optimal pretreatment conditions for rice husk. The optimal conditions for rice husk would be the reference for estimating the suitable pretreatment duration and temperature for the other three feedstocks. A series of rice husk pretreatments as a function of pretreatment durations were carried out at 150°C and 170°C using a protic ionic liquid [TEA][HSO_4], which has already shown brilliant fractionation ability towards miscanthus.[71] All pretreatments was repeated two times, producing two type of pulps, air-dried(dry) and non-air-dried(wet). Enzymatic saccharification was conducted for both dry and wet pulp, to understand how the pulp saccharification yields would be effected by hornification, which was found to largely influence the glucose yield of softwood pulps.[190] The dry pulps were then subjected to compositional analysis. The compositional information of the dry pulp along with the saccharification yields of the wet pulp were used as the key fractionation parameters, when investigating the optimal pretreatment duration and temperature for rice husk.

5.1.1 Impact of pulp hornification on saccharification yields

For an industrial-scale biorefinery, the pulps are usually subjected to hydrolysis process right after the biomass pretreatment step without being air-dried, as the pulp air drying process is often energy-

intensive and hence capital-intensive as well. Hornification induced by air-drying the pretreated biomass was reported for several feedstocks in both literature and Chapter 3.[190] Drying the pulps caused the biomass cell wall to shrink and collapse irreversibly, resulting in a decreased sugar releasing ability of the dry pulps during enzymatic saccharification. However, how much the sugar releasing yield deceases due to hornification varies with feedstock, and no work has been done to investigate this phenomenon in rice husk pulp. Therefore, a series of pretreatments were conducted to generate both dry and wet pulps to study the impact of air-drying pulps on the saccharification yields for the rice husk ionoSolv process. Pretreatments were conducted at 150°C for 0.5h, 1h, 1.5h, 2h, 2.5h, 3h and at 170°C for 15min, 30min, 45min, with a biomass loading of 1:10 g g^{-1}. The trends of saccharification yields for dry and wet pulps at two operational temperatures are displayed in Figure 5.1. The actual values of the sugar yields are listed in Table 5.1 and 5.2, along with the increase factor of the dry pulps, i.e. by how much, in terms of a factor, the saccharification yield increases due to eliminate the pulp drying process.



TFigure 5. 1 The saccharification yields of dry and wet pulps produced from rice husk ionoSolv pretreatment at 150 and 170 for various pretreaetment durations. Yields are presented in percentages, relative to the glucose content of the untreated rice husk. Error bar is included.

Table 5. 1 A comparison of glucose releasing yields between air-dried pulps and non air-dried pulps for rice husk pretreated at 150°C

Time (h)	Dry pulp 150 [a] (%)	Wet pulp 150 [b] (%)	Increase Factor [c]
0.5	21	44	2.1
1	42	68	1.6
1.5	45	75	1.7
2	44	77	1.7
2.5	45	77	1.7
3	45	71	1.6

[a] **The sugar releasing yield for air-dried pulp pretreated at 150°C relative to the glucose content of the untreated biomass**

[b] **The sugar releasing yield for pulp pretreated at 150°C without going through a air-drying process relative to the glucose content of the untreated biomass**

[c] **By how much the sugar yield of wet pulp is increased relative to dry pulp**

Table 5. 2 A comparison of glucose releasing yields between air-dried pulps and non air-dried pulps for rice husk pretreated at 170°C

Time (h)	Dry pulp 170 [a] (%)	Wet pulp 170 [b] (%)	Increase Factor [c]
0.25	16	42	2.6
0.5	51	68	1.3
0.75	57	73	1.3
1	46	46	1.0

[a] **The sugar releasing yield for air-dried pulp pretreated at 170°C relative to the glucose content of the untreated biomass**

[b] **The sugar releasing yield for pulp pretreated at 170°C without going through a air-drying process relative to the glucose content of the untreated biomass**

[c] **By how much the sugar yield of wet pulp is increased relative to dry pulp**

The sugar yields for pulps pretreated for short durations were impacted the most by air-drying (hornification), and the impact gradually decreased as the pretreatment time increased. After the sugar yield reached the maximum, the effects of hornification was even less significant. The two highest increase factors discovered were 2.1 and 2.6 for 150°C, 30 minutes and 170°C, 15min, respectively. For pulp pretreated at 170°C, the increase factor remained around 1.3 after 30 minutes (detailed in Table 5.2), while this was around 2.1 for 150°C after 30 minutes (detailed in Table 5.1), indicating higher temperature lead to a less significant effects of hornification. This could be attributed to lignin condensation and the presence of pseudo-lignin: increasing pretreatment temperature is equal to increase the severity of the ionoSolv process; the degree of lignin condensation and pseudo-lignin formation is positively correlated to the process severity; the lignin residues with more condensed structure in the pulp would reduce the enzymatic digestibility of the pulp, especially for wet pulp. Similar experimental findings were reported by Gschwend *et al*.[190]

In summary, the process of air-drying pulp exerted an obvious effect on the pulp's enzymatic saccharification, and the saccharification yield differences between wet and dry pulps were up to 26%. For pretreatments with hasher conditions (higher temperature or longer pretreatment time), the discrepancies in the sugar yields were smaller, ≥ 0.3%, due to the wet pulp's saccharification was significantly hindered by a highly condensed lignin residue.

5.1.2 Optimisation of pretreatment duration and temperature

In order to determine the optimal pretreatment conditions for rice husk, compositional analysis was conducted onthe dry pulps. Key information such as glucan recovery, hemicellulose removal, delignification and lignin recovery yield were obtained and presented in Figure 5.2 and 5.3, as percentages, relative to the theoretical maximum, i.e. composition of the untreated rice husk. Along

with the other key process-performance indicators, such as, saccharification yields for the wet pulps are also presented.

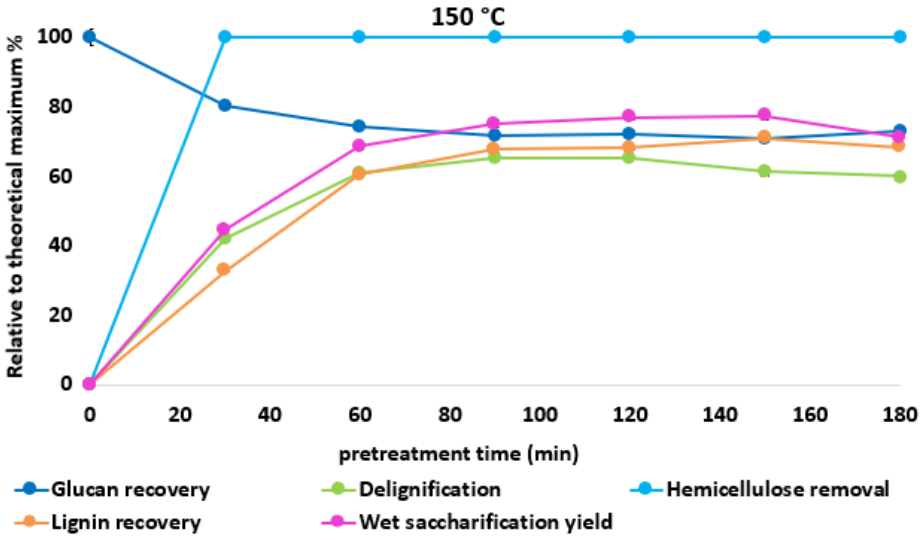


Figure 5. 2 Key indicators of fractionation effectiveness for rice husk [TEA][HSO_4] pretreatments at 150°C and 1:10 g g^{-1} biomass loading. Error bar is included.

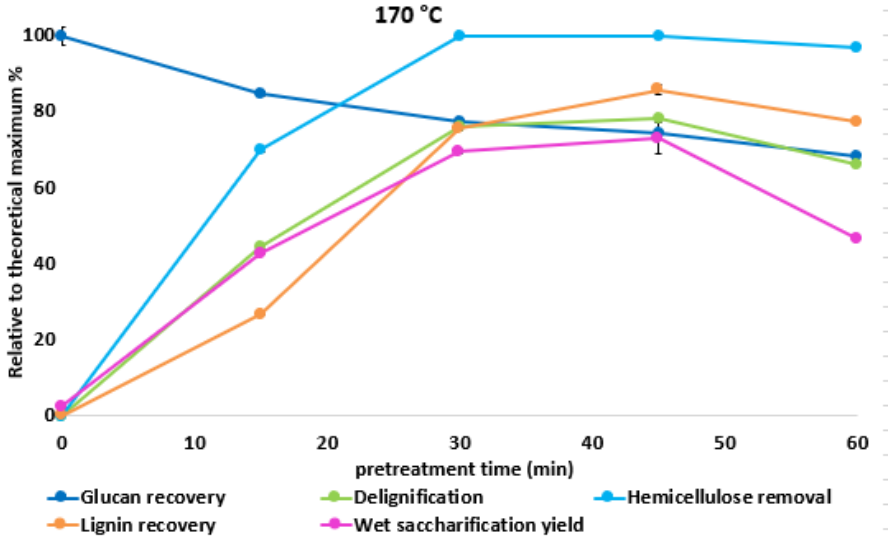


Figure 5. 3 Key indicators of fractionation effectiveness for rice husk [TEA][HSO_4] pretreatments at 170°C and 1:10 g g^{-1} biomass loading. Error bar is included.

Table 5. 3 A list of compositional and saccharification key indicators for rice husk pretreatment at 150°C and 1:10 g g^{-1} biomass loading.

Pretreatment duration (min)	Glucan recovery[a]	Hemicellulose removal[a]	Lignin recovery yield[a]	Delignification[a]	Saccharification yield[a]
0	100	0	0	0	0
30	80	100	33	42	45
60	74	100	60	61	69
90	72	100	68	65	75
120	72	100	68	65	77
150	71	100	71	65	77
180	73	100	68	60	71

[a] The yield is presented in precentages of the theoretical maximum, relative to untreated biomass

Table 5. 4 A list of compositional and saccharification key indicators for rice husk pretreatment at 170°C and 1:10 g g^{-1} biomass loading.

Pretreatment duration (min)	Glucan recovery[a]	Hemicellulose removal[a]	Lignin recovery yield[a]	Delignification[a]	Saccharification yield[a]
0	100	0	0	0	0
15	86	73	27	44	43
30	77	100	76	76	69
45	74	100	86	79	73
60	69	97	86	66	47

[a] The yield is presented in precentages of the theoretical maximum, relative to untreated biomass

The saccharification yield reached its maximum at 45min for 170°C and at 120 minutes (2 hours) for 150°C, the actual values were 77% and 73%, respectively, detailed in Table 5.3 and 5.4. The sugar

yields for both temperatures dropped after reaching their peak values, 120 minutes at 150°C or 45 minutes at 170°C. Several studies on the ionoSolv process have reported trends of saccharification and delignification are in line with each other and a strong correlation between lignin removal and saccharification yields has been observed repeatedly.[67,70,71,190] This was further confirmed by our study: The delignification for 170°C at 45min was 79%, and was also the peak value achieved for 170°C .The lignin removal for 150°C at 120 minutes (2 hours) was only 61%. This relatively lower lignin removal could be the reason for the pulp treated at 150°C, 120 minutes (2 hours) having a 4% lower sugar yield, compared to pulp treated at 170°C, 45min.

After the sugar yield and lignin removal reached the peak, the lignin recovery yield exceeded that of delignification. The increased lignin condensation and the undesired pseudo-lignin formation could be cause of these unreasonably high lignin recovery yields. Peak sugar yield and delignification, in some extent, indicated the fractionation process reach its optimal conditions. After reaching the optimal conditions, increasing severity of the pretreatment in term of pretreatment duration would cause the undesired lignin structural modifications. The lignin condensation refers to small water-soluble lignin fragments crosslinking with each other forming insoluble lignin oligomers with high molecular weight.

The amount of residual glucose decreased at both temperatures, but the decreasing trend was steeper at 150°C. Significant glucan degradation was observed after 15 min at 170°C and 60 minutes (1 hours) at 150°C. The more severe glucose degradation at 150°C may potentially limit the pulps' sugar release during saccharification as less amount of glucose remained in the pulps. Quantitative hemicellulose dissolution by the IL was achieved after 30min at 170°C and 30min at 150°C, indicating the hemicellulose polymer matrix was quickly disrupted during ionoSolv fractionation and hemicellulose was hydrolysed fairly easily.

Here, we could conclude that the optimal ionoSolv process conditions are at 170°C for 45min. It is worth pointing out that using a higher operational temperature with a short pretreatment duration is more economically favourable, as a smaller reactor volume are required and consequently a lower capital cost.

5.2 Optimised ionoSolv process for different agricultural residues

After successfully determining the optimal pretreatment conditions for rice husk, optimal pretreatment conditions were suggested, based on the optimal condition of rice husk, and confirmed for other agriculture feedstocks, sugarcane bagasse, rice straw and wheat straw, listed in Figure 5.4. All feedstocks were fractionated at 170°C for either 45min or 30min with a biomass loading of 1:10 g g^{-1} For bagasse and rice husk, a longer pretreatment duration, 45min, was used, as both these feedstocks have relative higher lignin contents (27% for rice husk, 24% for bagasse). A more severe fractionation was needed to achieve decent lignin removal, which would significantly influence the enzyme digestibility of the pretreated biomass. Rice straw and wheat straw have less dense and highly porous cell wall structure and their lignin contents are lower (21% for wheat straw, 18% for rice straw), therefore a shorter pretreatment duration was needed, 30 min. Again, for each feedstock, the pretreatment process was repeated twice, once with air-drying the pulp, a second time without drying the pulp. Both dry and wet pulps were subjected to enzymatic hydrolysis to determine the effect of hornification. Dry pulps were subjected to compositional analysis, the results alone with wet pulp saccharification yields were used to confirm the predication of optimal pretreatment conditions for the feedstocks studied. As one of the intrinsic characteristics of these four feedstocks is high ash content, any changes in the ash content of the biomass before and after pretreatment were carefully observed. The recovered lignin was characterised by HSQC NMR analysis, enzymatic mild acidolysis lignin (EMAL) extracted from bagasse was used as a reference when comparing the major lignin-subunits composition.

5.2.1 Saccharification yields

Enzymatic saccharification was performed for untreated feedstocks, wet and dry pulps. The sugar yield for four feedstocks were high. A sugar yield close to 90% was achieved for bagasse, wheat straw and rice straw, while rice husk's sugar yield was relatively lower, 73%, due to the more recalcitrant nature of its cell wall, more specifically, denser cell wall structure and higher lignin content. All the sugar yields for wet and dry pulp was presented in Figure 5.4 and the corresponding values are listed in Table 5.3. The sugar releasing yields listed in Table 5.3 are presented as percentages relative to the glucan content of untreated and treated biomass.

Compared to raw biomass, the saccharification yield for rice husk after pretreatment achieved a 30-fold increase, whereas the other three feedstocks only managed to obtain an increase of up to 8-fold. The massive increase in the sugar yield for rice husk along with extremely low sugar yield, 2%, for untreated rice husk further confirmed that the native rice husk cell wall is highly dense and ionoSolv process successfully disrupt cell wall structure by removing hemicellulose and lignin to leave the cellulose-rich pulp that is highly enzyme-accessible.

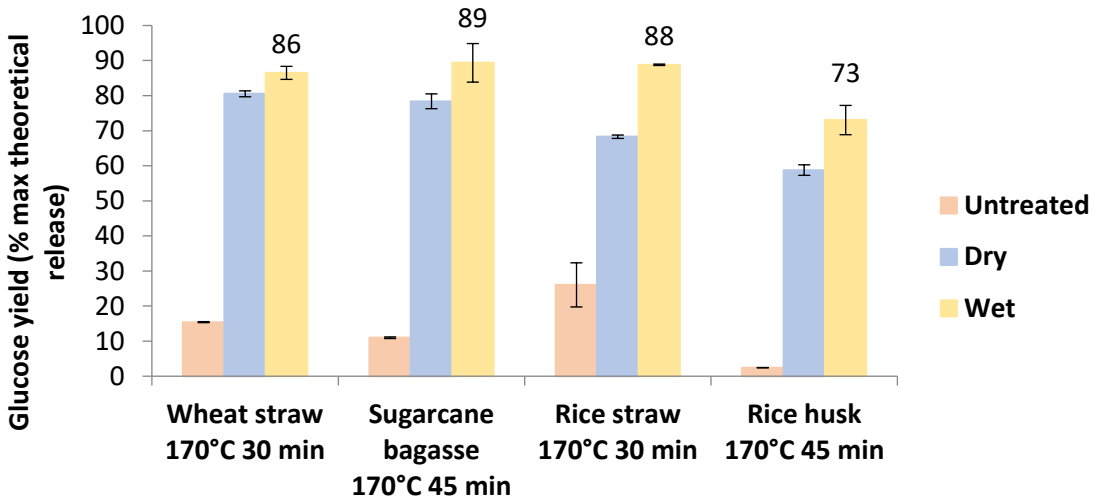


Figure 5. 4 A comparison of saccharification yields for untreated feedstocks and pulps for agricultural residues. Error bar is included.

Table 5.5 Percentage yields of sugar released during fractionation relative to untreated or treated biomass for four agricultural residues

	Glucose releasing yield for wet pulps based on enzymatic hydrolysis			
Feedstock type and Pretreatment condition	**% yield of untreated biomass**	**% yield of glucan in untreated biomass**	**% yield of pretreated pulp**	**% yield of glucan in pretreated pulp**
Wheat straw untreated	6±0.1	15±0.2	n/a	n/a
Wheat straw 170 °C 30 min	37±1.0	86±1.9	90.4±3.7	98±0.2
Rice straw untreated	11±3.4	26±6.3	n/a	n/a
Rice straw 170 °C 30 min	38±0.1	88±0.2	88.0±5.3	102±0.2
Rice husk untreated	1±0.02	2±0.0	n/a	n/a
Rice husk 170 °C 45 min	31±1.4	73±4.2	57.5±2.4	98±0.2
Bagasse untreated	4±0.1	11±2.8	n/a	n/a
Bagasse 170 °C 45 min	38±2.9	89±5.5	100.1±5.6	109±5.6

According to Table 5.3, bagasse obtained a quantitative glucose release, relative to the glucan content of the post-pretreatment solid, (from wet pulp) during saccharification. For two straw feedstocks, nearly 90% of glucose of the pulp was hydrolysed during saccharification. This suggested the biomass fractionated by IL have extremely high enzyme digestibility. Although rice husk pretreated IL had a better digestibility than the untreated one, only 57% of glucose of the fractionated rice husk was released during saccharification hydrolysis. Again, this relatively lower glucose release was attributed to the recalcitrant nature of rice husk.

The effects of hornification was observed for all agricultural residues. Rice husk and rice straw had the largest and second laregst discrepancy of the saccharification yields between air-died pulp and non-air-dried pulp, 20% and 15% difference, respectively. The dry and wet pulps for wheat straw only differed by 6%. This indicated that the rice-related residues are affected by the hornification more significantly.

It is also worth mentioning that the rice husk and rice straw fractionated by a bench mark IL [Emim][OAc] were reported to obtain sugar releasing yields of 75% and 40%.[43] [Emim][OAc] fractionates the biomass in a different fashion, compared to protic [TEA][HSO_4], where the cellulose was dissolved in [Emim][OAc] along with other biomass components but was recovered in the later stage of the pretreatment. [3,176] The estimated price for [TEA][HSO_4] was only around 2.5% of [Emim][OAc].[177]

5.2.2 Pulp compositions

All the key indicators of the pretreatment effectiveness for agricultural residues' ionoSolv processes are presented in Figure 5.5, including the glucose recovery, hemicellulose and lignin removal and lignin recovery.

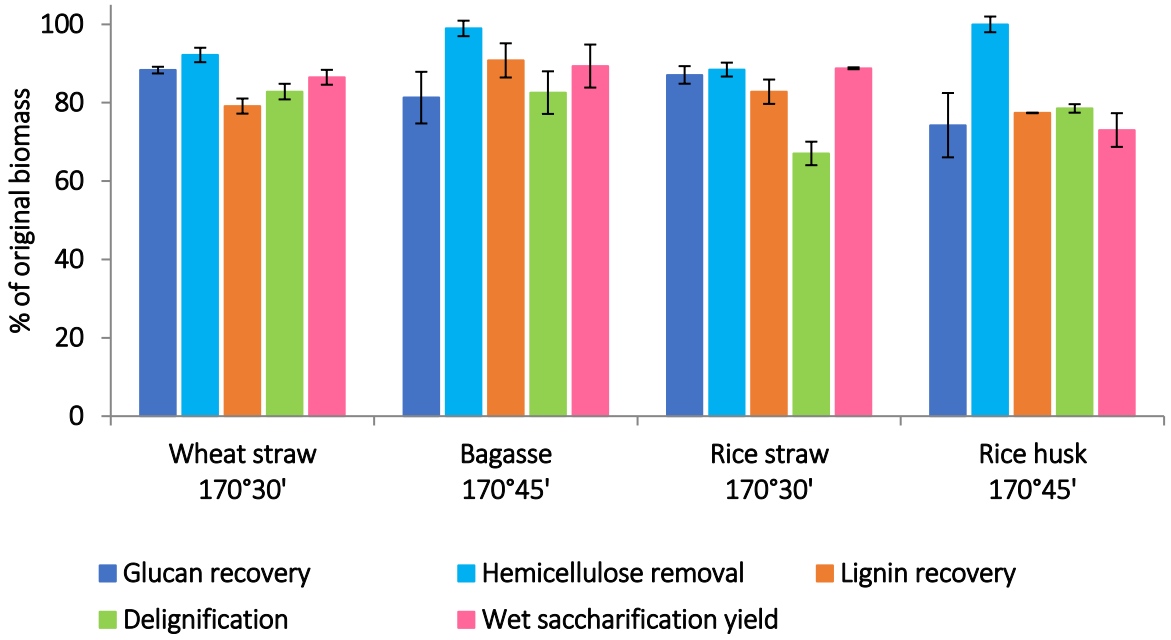


Figure 5. 5 Key indicators of fractionation effectiveness derived from compositional analysis for all agricultural residue using ionoSolv processes at 170°C with a 1:10 g g^{-1} biomass loading. Error bar is included.

Different degrees of glucan degradation were observed for these four feedstocks, where the degradation was the most severe for rice husk. Rice husk pulp preserved 74% glucose, relative to the glucose content of the untreated biomass, while the straws managed to keep around 90% glucose and bagasse recovered 81%. The lower glucan recovery for rice husk was thought to be responsible for the lower saccharification yield of the rice husk. Approximate 90% hemicellulose removal was reported for wheat straw, rice straw and bagasse, and rice husk achieved quantitative hemicellulose dissolution.

Regardless of the feedstock, the delignification and enzymatic saccharification of the pulp are believed to be positively related to each other. Very decent lignin removal was recorded for all feedstock, ranging from 67% to 82%. It is worth noticing that rice straw achieved a nearly 90% saccharification yield while its lignin removal was only 67%, suggesting the lignin removal was not the major liming effect of pulp's enzymatic hydrolysis in the case, as the feedstock is low in lignin and has less packed

cell wall. However, it was not the case for rice husk, its delignification was 78%, this is accompanied by a severe glucose degradation, the saccharification of rice husk at 170°C, 45min was significantly hindered. Lignin recovery yields for bagasse and rice straw were higher that their corresponding lignin removal, suggesting lignin degradation including pseudo-lignin formation may possibly happen during fractionation of these two feedstocks. For the other two feedstocks, rice husk and wheat straw, the possibility of lignin condensation and pseudo-lignin formation should not be ruled out, further analysis about the structure of lignin extracted from the pretreatments should be done to confirm this.

Process optimization is needed here, especially for rice husk. Although all feedstocks obtained fairly high glucose releasing yields, thanks to decent lignin removals, proving the ionoSolv fractionation process was effective for agricultural residues with high ash and lignin contents. All feedstocks suffered from different degrees of glucose and lignin degradations, especially rice husk. In order to achieve an even better sugar release (close to 100%) in the enzymatic hydrolysis, further pretreatments with a series of pretreatment durations (between 20min to 45min) should be done for all feedstocks, with a duration interval smaller than 15min, as in this study, the duration interval was set to be 15min. At high operation temperature like 170°C, heat transfer during the pretreatment is fast and 15-minutes interval might be too wide.

5.2.3 Ash contents of pulps

The high ash-content of these agriculture residues is one of the reasons behind the development of effective fractionation process for these feedstocks studied here, as the ash component in the biomass is high in inorganic matters, such as silica, which could be potentially isolated and used in value-added applications, such as cement production. [44,46,213] Therefore, where the ash fraction of the agriculture feedstock would end up during pretreatment was investigated.

According to compositional analysis, nearly quantitive ash recovery was achieved for all feedstocks, including rice husk (11%) and rice straw (13%) which are extremely high in ash content, compared with wheat straw and bagasse.[60] 92% and 84% of ash remained in the rice husk and rice straw pulps, respectively, detailed in Appendix, Figure S5-3. It is important to know that the acid insoluble fraction of the recovered ash was only detected, and the acid soluble fraction was either dissolved in the acidic IL solution during pretreatment or dissolved in the aqueous acid solution during composition analysis. The remaining ash residues in the pulps did not limit pulps' ability to release sugar during enzymatic hydrolysis, nearly 90% glucose of the pulps was released for rice straw, wheat straw and bagasse, according to Table 5.3. For rice husk, only half of the glucose in the pulp was released during saccharification, relatively denser cell wall and higher lignin residue of the pulp would be responsible for this rather than the high amount of ash preserved in the pulp. In terms of recovering the ash for further utilisations, most of the carbohydrates in the pulps are able to be hydrolysed, the post-hydrolysis solids should be high in ash (slica) and low in sugars, therefore simple and cheap separation will be needed to recover the ash from the post-hydrolysis residues.

5.2.4 Summary

Based on the saccharification assay and compositional analysis, the ionoSolv process could effectively fractionate rice straw, wheat straw, bagasse and rice husk. As expected, severe process conditions (longer duration) were needed for feedstocks with high lignin content. The sugar releasing yields up to 89% was achieved by these agriculture residues, due to decent lignin removals (up to 83%). The ash fractions for all feedstocks were quantitatively preserved in the post-pretreatment residues, and can be easily separated after hydrolysing the remaining carbohydrates in the pulp. Glucan degradation and lignin condensation was observed, suggesting a further process optimization, more specifically, adjusting the pretreatment duration, is necessary.

5.2.5 Characterisation of isolated lignin

In earlier sections, some indications for the presence of lignin degradation was observed when conducting compositional analysis on the pulps. This was further confirmed by analysing the major subunit composition of the lignins isolated from the pretreatments. HSQC NMR spectroscopy provides information about all the carbon-hydrogen linkages appearing in the lignin inner structure, providing direct evidence for chemical modification happening at the lignin during fractionation. This will provide guidance for the lignin usage, as the lignins' structure determines its suitability for application and the additional modifications needed for high value-added utilisations. IonoSolv lignins extracted from rice husk, rice straw, wheat straw, and bagasse were subjected to HSQC NMR analysis. Enzymatic mild acidolysis lignins (EMAL) extracted from these four feedstocks were attempted, but only bagasse EMAL was successfully obtained. Therefore, four ionoSolv lignins' structural information was compared, using bagasse EMAL as a reference, as the chemical composition of the feedstocks studied are similar.[60]

The HSQC NMR spectra were divided in regions, and our main focus was the side chain region and the aromatic region. The side chain regions reveal the composition of the aryl ether, resinol and phenylcoumaran, while the aromatic region provides information about guaiacyl and syringyl units. All the major lignin subunits are listed in Figure 5.6. In the spectra, any Lignin-carbohydrate linkages were labelled as Ara or Xyl, representing the lignin fragment crosslinked with arabinose or xylose. The condensed guaiacyl and syringyl units were marked as $G_{2cond.}$ and $S_{2,6\ cond.}$.

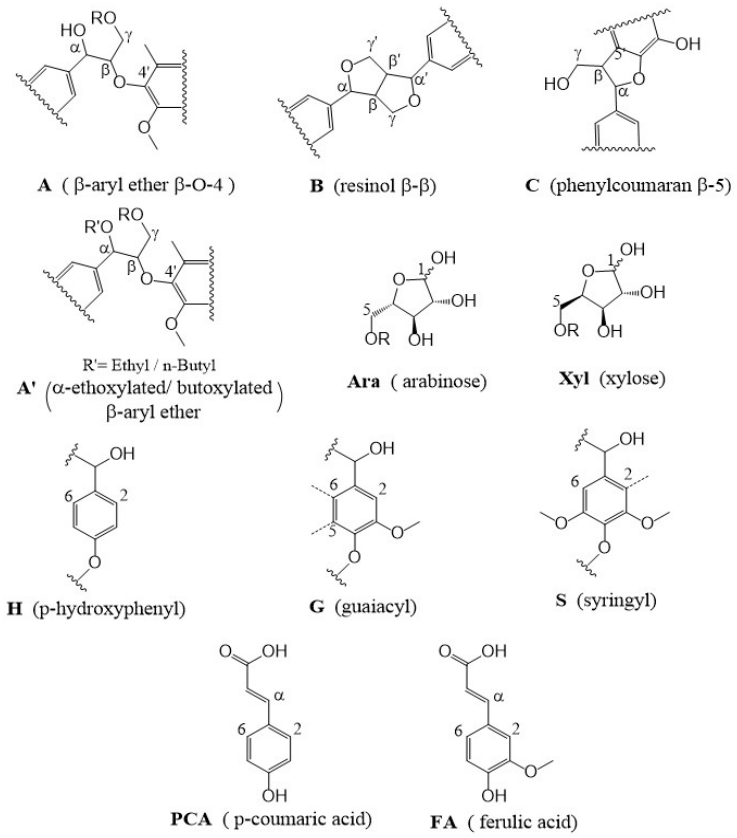


Figure 5. 6 Key lignin substructures found in recovered lignins from the ionoSolv processes for agricultural residues

The major subunits were semi-quantitatively analysed by integrating volume peaks to quantify their abundances in percentages, relative to the sum of G_2 and G_{2cond} integrals, displayed in Figure 5.7. G_2 and G_{2cond} integrals were repeatedly reported to be constant for the lignin isolated from all ionoSolv process and the newly developed organosolv-ionoSolv processes (detailed in earlier chapters).[60 2 190] The degree of condensation and S/G ratio could also be derived based on this semi-quantitative study, listed in Table 5.4. The degree of condensation could be expressed as the percentage of the signal intensity for G_{2cond} divided by the combined signal intensity for G_2 and G_{2cond}.[60] The S/G ratio could be expressed as equation 1:

$$\frac{S}{G}\text{ratio} = \frac{0.5\cdot(S_{2,6} + S_{2,6\ \text{cond.}})}{(G_2 + G_{2\ \text{cond.}})} \qquad \text{(eq. 1)}$$

Where $S_{2,6}$, $S_{2,6\ cond.}$, G_2, $G_{2cond.}$ repesent the signal intensities for uncondensed and condensed fractions of syringyl and guaiacyl units.

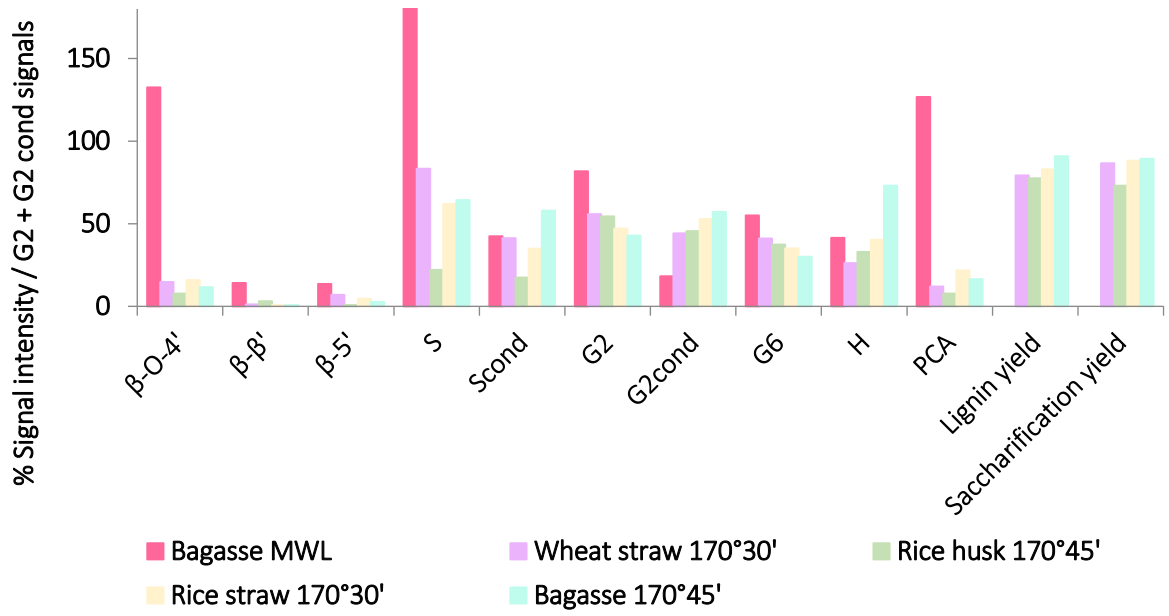


Figure 5. 7 Abundance of key lignin substructures in lignin recovered from ionoSolv processes for agricultural residues according to HSQC NMR spectroscopy. Signal intensities are presented in percentages relative to the sum of signal intensities for G_2 and $G_{2cond.}$

Table 5. 6 Degree of condensation and S/G ratio based on HSQC spectrum integrals for agricultural residue lignin

Pretreatment solvent composition	Bagasse EMAL	Wheat straw [a]	Rice straw [b]	Rice husk [c]	Bagasse [d]
G_2 peak integral intensity	81	55	47	54	42
$G_{2con.}$ [e] peak integral intensity	18	44	52	45	57
Degree of condensation[f]	18	44	52	45	57
S/G ratio	1	0.62	0.48	0.20	0.61
[a] Lignin extracted from wheat straw pretreated at 170 for 30 minutes					
[b] Lignin extracted from rice straw pretreated at 170 for 30 minutes					
[c] Lignin extracted from rice husk pretreated at 170 for 45 minutes					
[d] Lignin extracted from bagasse pretreated at 170 for 45 minutes					
[e] $G_{2con.}$ stands for condensed G_2 peak					
[f] Calculated based on $G_{2con.}/G_2+G_{2con.}$ in %					

In the side chain region, the signals representing carbohydrates completely disappeared, indicating any lignin-carbohydrate linkages were not maintained after fractionation and the extracted lignins were carbohydrate-free. In the literature, the lignin removal for any ionoSolv processes was believed to be achieved by fast cleavage of aryl ether (β- O-4) linkages and chemical modifications of resinol (β-β) and phenylcoumaran (β- 5) units.[177] This was further confirmed by experimental observations: the signal intensities for β-O-4 ether, β- β and β-5 integrals dropped significantly, comparing to bagasse EMAL. For bagasse, the integral intensity of β-O-4 ether for ionoSolv lignin was only 1/12th of the intensity for EMAL.

In the aromatic region, the presence of lignin condensation was confirmed, which is in line with the predictions based on the compositional analysis, in Section 5.2.2. The signal intensities of $S_{2,6}$, G_2, and G_6 significantly reduced, while that for $S_{2,6\ cond.}$ and $G_{2cond.}$ increased, relative to the combined signal

intensity of G_2 and G_{2cond}. This is due to the condensation at the unsubstituted carbon 2 and 6 positions of the aromatic ring, and this condensation happened at both syringyl and guaiacyl units. The degree of condensation calculated from G_2 and $G_{2cond.}$ integrals shown the extent of lignin degradation was much severe for ionoSolv lignin comparing to EMAL. The degree of condensation for EMAL was 18%, but that for ionoSolv lignins extract from agricultural residues ranges from 44% to 57%, suggesting condensation happened at half or over half of the guaiacyl units. The calculated S/G ratio for EMAL was higher than that for ionoSolv lignins, no clear correlation was found between the change in S/G ratio and the extent of lignin condensation. Additionally, a large drop in abundance of the PCA units accompanied with a huge increase in the abundance of H units was observed. This could be explain as during ionoSolv process, the PCA units underwent the conversion into H units in acidic IL medium.

In summary, the large amount of aryl ether cleavage indicates the effective lignin removal during the ionoSolv process. The disappearance of the carbohydrates in the lignin suggests that ionoSolv process was able to fractionate lignin and sugars selectively. Heavy lignin condensation was observed for all ionoSolv lignin, proof the predication made by compositional analysis was true. Process optimization is needed in order to generate the lignin fraction which can be used for high value-added applications, such as carbon fiber production(less condensed lignin).[251]

5.3 Primary alcohol–[TEA][HSO_4] pretreatment for agricultural residues

In earlier section, four agricultural residues were fractionated via the ionoSolv process using [TEA][HSO_4]. Lignin condensation and glucose degradation was discovered for all feedstocks (rice husk, rice straw, wheat straw, bagasse), which hindered the enzymatic saccharification for these feedstocks. To overcome these issues, another fractionation process, newly developed organosolv-ionoSolv (hybrid) pretreatment, was applied to these four feedstocks. As reported in Chapter 4 and 5, organosolv-ionoSolv (hybrid) process could selectively fractionate lignin and keep isolated lignin less condensed than ionoSolv process. The hybrid process was also reported to be superior in preventing glucose degradation.

It is important to note that cost-effective is crucial for an ideal lignocellulosic biomass pretreatment operating at industrial-scale. The whole fractionation process is not cost effective if bioethanol/biobutanol is generated merely from the cellulose fraction of the feedstock. Apart from the main product stream, the cellulose fraction, side product stream including hemicellulose and lignin are also required to have decent quality, so that they could be subjected to value- added applications.[37,51] Recovered hemicellulose fractions can be potentially used to generate bioethanol or other organic alcohol via fermentation, or produce other value-added chemicals such as xylitol, furfural.[252,253] Depending on the purity of the sugar fraction recovered, purification process, detoxification, is very likely needed for majority of the current pretreatment process. Especially for ionoSolv process, high hemicellulose removal can be easily achieved, and the polymeric hemicellulose matrix is broken forming oligomers and potentially some degradation products.[37] The hemicellulose oligomers along with degraded sugar products was left with IL at the end of the ionoSolv process; an additional separation step is needed for recovering the hemicellulose fraction. The recovery of

hemicellulose has not been well studied up untill now. The Isolated lignin fraction is most commonly subjected to powering, but is the potential starting material for value added applications such as phenolic chemicals for pharmaceutical and food industry, cost effective carbon fiber composites.[254]

In this Section, organosolv-ionoSolv pretreatment was performed for rice husk, rice straw, wheat straw and bagasse. The organic solvent used were ethanol and butanol and the IL used was [TEA][HSO_4]. The pretreatment composition was 40%wt. organic alcohol with 60% wt. IL, identified as the optimal solvent composition in Chapter 3. Pretreatments were conducted at 170 °C with a solid to liquid loading of 1:10 g g^{-1} in Hydrothermal Autoclave Reactors due to high operational pressure requirement. For rice husk and bagasse which are richer in lignin, a longer pretreatment duration was used 100 min, while rice straw and wheat straw were pretreated for 80 min. In Section 4.1.1, the pretreatment preformed in reactors for 80 min was reported to have the same overall effectiveness as the process in pressure tubes for 30 min. All pulps were subjected to the air-drying process following the pretreatment protocol set up in our laboratory, for the ease of handling Enzymatic saccharification and compositional analysis were conducted for pulp to understand the pretreatment effectiveness, and isolated lignin was subjected to HSQC NMR and GPC analysis.

5.3.1 Glucose and hemicellulose releasing yields for enzymatic saccharification

Figure 5.8 presented the amount of monomeric sugar, including both glucose and hemicellulose, released during enzymatic saccharification. The enzyme choice in the saccharification was the same as the other studies conducted in this and other chapters, Cellic® CTec 2, a cellulase enzyme blend. This enzyme was not designed for hemicellulose hydrolysis. The glucose yields, relative to the pulp glucan content, and untreated biomass glucan content are listed in Table 5.5. The hemicellulose yields for two straws are also presented in Table 5.5, as percentages, relative to the hemicellulose content

of the pulp and untreated biomass. The total sugar yield refers to the total monomeric pentoses and hexoses released during enzymatic hydrolysis and is presented in Table 5.5 as percentage yields relative to the sum of glucose and hemicellulose content for the untreated biomass.

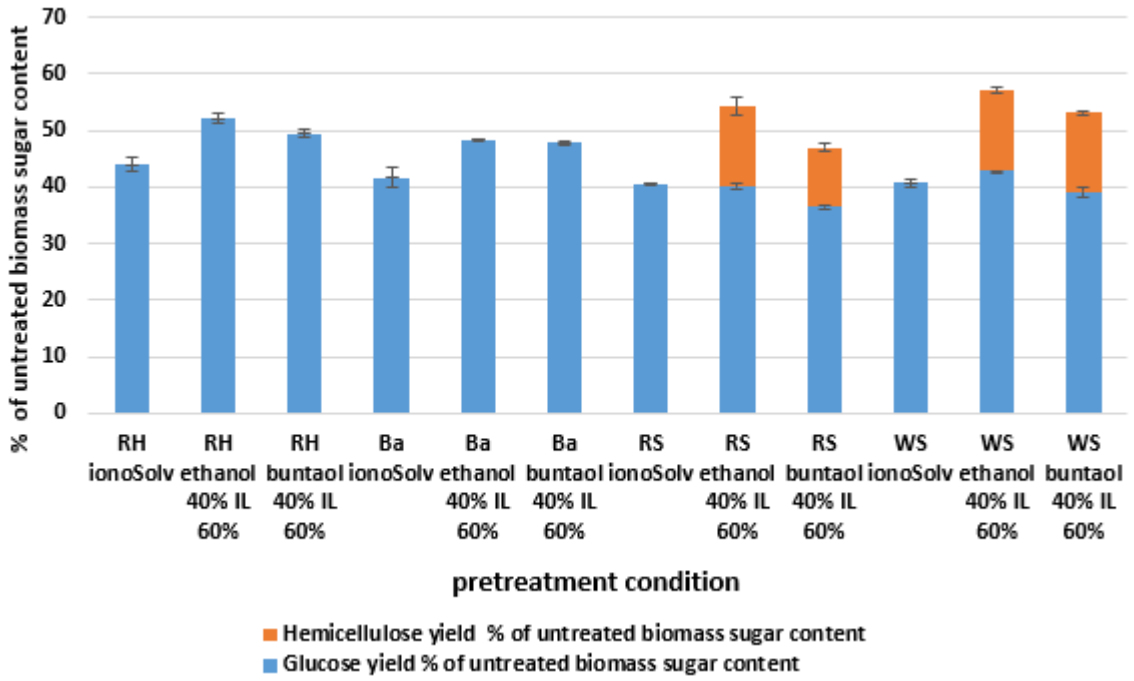


Figure 5. 8 Combined monomeric cellulose and hemicellulose releasing yields for agricultural residues fractionated by organic-IL mixtures, yields are presented as percentages relative to the sum of glucan and hemicellulose contents for the untreated biomass. RH, BA, RS, WS stand for rice husk, bagasse, rice straw and wheat straw. Error bar is included.

For the organic-IL processing of more recalcitrant feedstocks (rice husk and bagasse), higher glucose releasing yields was obtained, compared to the ionoSolv process. For the organic-IL processes of straws, glucose yield remained stable, but an additional hemicellulose release was observed, compared to ionoSolv process, detailed in Table 5.7. Comparing to ionoSolv process (zero hemicellulose yield), the hemicellulose yields for the ethanol/butanol-IL processes increased up to 14%, relative to the sugar content of the untreated biomass (the sum of glucose and hemicellulose content for untreated biomass). This led to an increase in the total sugar yield (the sum of glucose and hemicellulose content for pretreated biomass) up to 14% for the rice straw, 17% for wheat straw, relative to the total sugar content of the untreated biomass (the sum of glucose and hemicellulose content for untreated biomass).

Table 5. 7 A list of Glucose, hemicellulose and total sugar yield derived from saccharification assay for agricultural residues.

	Glucose yield			Hemicellulose yield			Total sugar yield [b]
	% of pulp glucose content	% of untreated biomass glucose content	% of untreated biomass sugar content [a]	% of pulp hemicellulose content	% of untreated biomass hemicellulose content	% of untreated biomass sugar content [a]	% of untreated biomass sugar content [a]
RH ionoSolv [c]	72	58	44	0	0	0	44
RH ethanol 40% IL 60%	767	71	52	0	0	0	52
RH butanol 40% IL 60%	70	66	50	0	0	0	49
BA ionoSolv [d]	89	71	41	0	0	0	42
BA ethanol 40% IL 60%	92	82	48	0	0	0	48
BA butanol 40% IL 60%	88	80	48	0	0	0	48
RS ionoSolv [e]	72	64	41	0	0	0	41
RS ethanol 40% IL 60%	65	64	40	68	39	14	54(14% higher than ionoSolv process)
RS butanol 40% IL 60%	60	60	36	53	29	11	47
WS ionoSolv [f]	78	67	41	0	0	0	40
WS ethanol 40% IL 60%	78	72	43	82	35	14	57(17% higher than ionoSolv process)
WS butanol 40% IL 60%	71	66	39	72	35	14	53

[a] Sugar content si the sum of glucose and hemicellulose contents

[b] Precentage sugar released including glucose and hemicellulose relative to the sugar content of the untreated biomass

[c] RH is short for rice husck

[d] BA is short for bagasse

[e] RS is short for rice straw

[F] WS is short for wheat straw

When an aqueous IL solvent medium was replaced with ethanol/butanol-IL solvent mixture (40% wt. organic content) in rice husk and bagasse fractionation, an improved overall process effectiveness was observed, in the aspect of sugar releasing yield. For rice husk, the glucose yields for the ionoSolv process, ethanol-IL process and butanol process were 58%, 71% and 66%, respectively, relative to the glucose content of the untreated biomass, listed in Table 5.7. The total sugar yield including cellulose and hemicellulose for the ionoSolv process was 44%, while this for ethanol-IL process was 52%. Similar

experimental observations were made for bagasse. The glucose yield increased from 71% to 82% when aqueous IL medium was switched to an ethanol-IL mixture for fractionation the feedstock, where the corresponding total sugar yield increase was 7%, listed in Table 5.7. This was in line with the experimental observation for miscanthus, where the ethanol-IL process has a glucose releasing yield 10% higher than the ionoSolv process, as detailed in Chapter 3.

A organosolv study reported a approximated enzyme digestibility of 50% for rice husk.[107] In this study, rice husk was pretreated with an aqueous ethanol mixture (60% ethanol) at 180°C for 12 hours, with 0.25 wt% concentrated sulphuric acid. It also reported that the digestibility dropped below 20%, if the process was not catalysed by acid. Compared to the ethanol-IL process, the aqueous ethanol process was conducted at a higher temperature for a longer duration, but achieved a less profound effectiveness in terms of glucose yield. For bagasse, several studies about its organosolv fractionation were conducted. Zhang *et al* conducted a series of bagasse pretreatment with or without the catalyst, $FeCl_3$.[255] They set the preatment conditions to 160°C for 90min, the solvent composition was 60 to 40 ethanol to water and the concentration of catalyst if present was 0.05M. The glucose yield reported was around 25% for the process without $FeCl_3$, and 90% with $FeCl_3$. Another study on hot aqueous ethanol pretreatment for bagasse reported a 29 g glucose released from every 100g of the feedstock after being preated at 195°C for 1 hour.[45] For the ethanol-IL process developed in our study, the saccharification assay suggested 35.1g of glucose was released from every 100g of the bagasse.

Different from rice husk and bagasse, the two straws did not obtain a glucose yield increase when switching the pretreatment solvent medium from aqueous IL to ethanol/butanol-IL mixture, but they both obtained an additional hemicellulose release during enzymatic saccharification. The glucose yields (relative to the untreated biomass's glucose content) of ionoSolv and organic-IL process for rice

straw ranged from 60% to 64%. The yields for wheat straw fluctuated around 67%. The actual numerical data is presented in Table 5.7.

Nearly 40% of hemicellulose was released (relative to the untreated biomass hemicellulose content) for rice straw pretreated by ethanol-IL mixture during saccharification, and a 30% hemicellulose release was reported for butanol-IL process. Similar results were found for wheat straw. Both ethanol-IL and butanol-IL achieved a 35% hemicellulose yield. The actual numerical data is presented in Table 5.7. This hemicellulose release was not discovered for the ionoSolv process of the straws. Quantitative hemicellulose removal of the ionSolv process may be the reason for this disappearance of the hemicellulose release. Because of the additional hemicellulose release during the enzymatic hydrolysis, the total sugar yields (including glucose and hemicellulose) increased, ≥ 14% for rice straw, ≥ 17% for wheat straw. According to the total sugar yield, using ethanol as the IL co-solvent could obtain a better fractionation effectiveness than butanol. Significantly increased in hemicellulose contents of the straws was noticed by conducting the saccharification assay, but these large amounts of residual hemicellulose did not inhibit the enzyme approaching and digesting glucose, they could also be hydrolysed by the enzyme which is one type of cellulase and is not specifically designed for hemicellulose hydrolysis.

The hemicellulose preserved in the post-pretreatment solid was in a form which could directly covert into monomeric sugars along with glucose hydrolysis, without the need for the detoxification. This could make the organosolv-ionoSolv process more cost-effective than ionoSolv process where the hemicellulose isdissolved into the IL.

A aqueous glycerol (70%) pretreatment study was conducted for wheat straw, at 220°C for 3 hours with a biomass loading of 0.5:10 g g^{-1} , by Sun *et al*.[207] Sun *at al* reported that the wheat straw achieved a 75% glucose yield for dry pulp and 63% glucose release for wet pulp, suggesting the organosolv process was also influenced by pulp hornification due to cellulose matrix dehydration during the drying process[106,207] A glucose yield of 52% was reported by Khaleghian *et al* for rice straw.[19] In this study, rice straw was fractionated by 75% aqueous ethanol at 180°C for 1 hour, where the process was catalysed by 1% sulfuric acid.

It is also worth pointing out that the pulps produced from the organosolv-ionoSolv processes are more enzyme-digestible, compared to ionoSolv pulps. 77% glucose in the Rice husk pulp generated from ethanol-IL pretreatment was released, while 72% glucose (relative to the pulp glucan content) was released from ionoSolv pulp, listed in Table 5.7. A nearly quantitative glucose release was achieved by bagasse pulp fractionated by ethanol-IL mixture. For straws which their pulp digestibilities in terms of glucose were not significantly improved, a high hemicellulose digestibility was achieved when the pretreatment solvent medium switched from aqueous IL to organic-IL mixture, up to 68% for rice straw, and up to 82% for wheat straw, relative to the hemicellulose content of the pretreated biomass.

In summary, the organosolv-ionoSolv process has a significantly improved fractionation performance than ionoSolv process towards all agricultural residues studied here. The integrated performance was achieved by either improving the glucose releasing yield of the pulp, making the treated biomass more enzymatic-digestible or hydrolysing the residual hemicellulose along with the cellulose.

5.3.2 Pulp compositions

Although saccharification yield is the most important pretreatment effectiveness indicator, compositional analysis also delivers useful information about the pretreatment, it could describe the process in other perspectives, such as lignin and hemicellulose removal, lignin recovery, which are also relative to the pulp saccharification to some extent. The pulp compositions for four agricultural feedstocks investigated are presented in Figure 5.9.

5.3.2.1 Delignification and lignin recovery

Decent lignin fractionation was observed for all feedstocks, especially for bagasse. For rice husk and bagasse, improved lignin removal was achieved by organic-IL process, relative to the ionoSolv process. The delignification reached 85% for rice husk fractionated in ethanol-IL solvent medium, compared to 77% lignin removal achieved by ionoSolv process, presented in Figure 5.9. The trend of lignin removal was in line with the trend of glucose releasing yield, confirming that lignin was one of the biggest limiting effects for effective enzymatic hydrolysis.[175] Similar observations were made for bagasse, a 10% increase in delignification was reported when fractionation solvent changed from aqueous IL to ethanol-IL mixture. A quantitative lignin removal (94%) was achieved for bagasse. For all feedstock, the improvement in the lignin fractionation performance was less profound when butanol was used as the co-solvent for the IL during pretreatment, compared to ethanol.

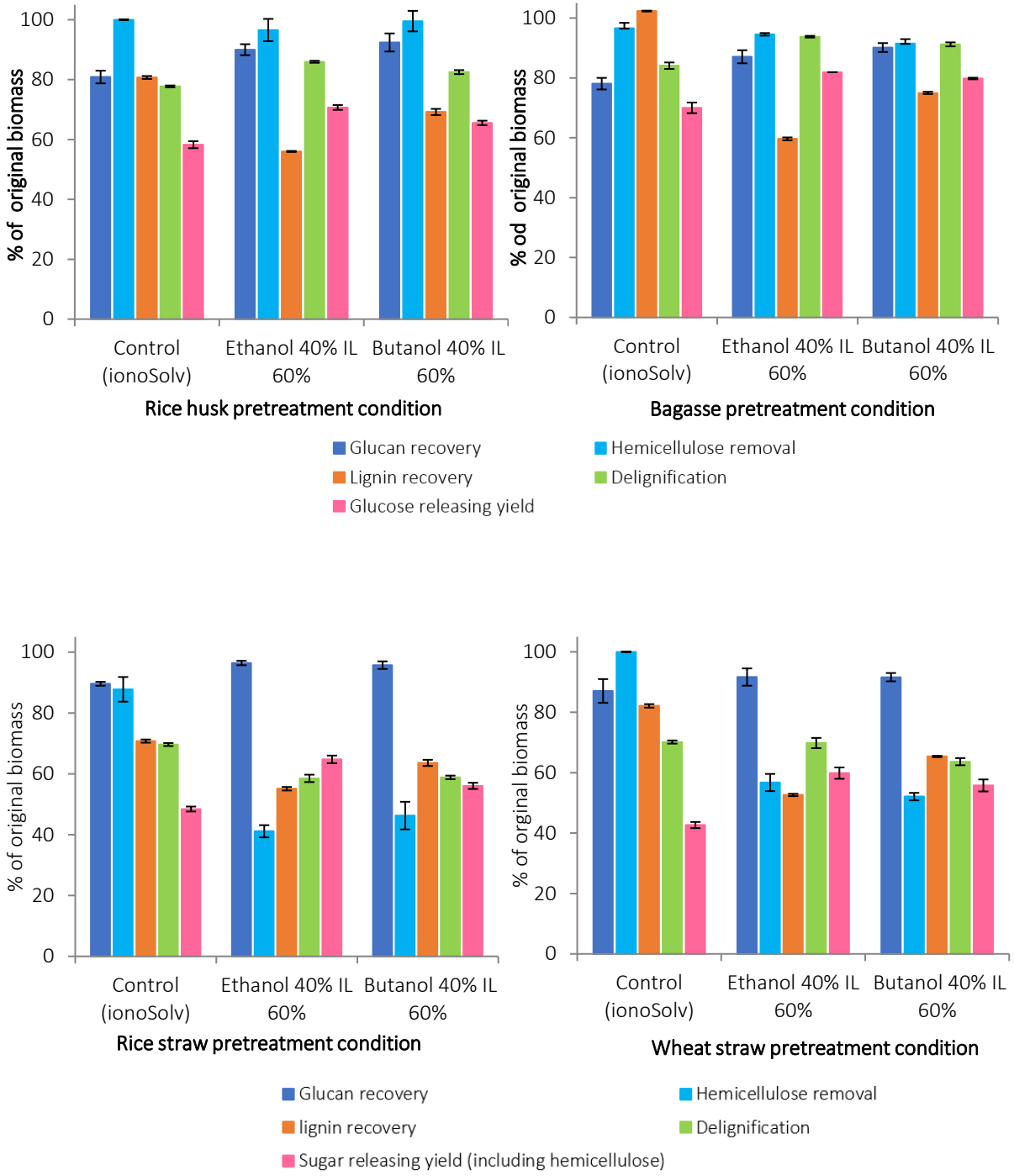


Figure 5. 9 Key indicators of fractionation effectiveness derived from compositional analysis for agricultural residue fractionated by aqueous IL, ethanol-IL and butanol-IL mixtures at 170°C with a 1:10 g g^{-1} biomass loading. Yields are presented as percentages, relative to the untreated biomass composition. Error bar is included.

The delignification stayed around 70% for wheat straw regardless the pretreatment type. For rice straw, a decreased deligninfication was observed when the pretreatment solvent changed from aqueous IL to an organic-IL mixture. Ethanol/butanol-IL processes achieved 59% delignification, while the ionoSolv process achieved 69%, presented in Figure 5.9. This is consistent with the slightly lower glucose releasing yield achieved by butanol-IL (60%), compared to the ionoSolv process (64%), detailed in Table 5.5. This trades off with the additional hemicellulose release, and the overall fractionation effectiveness was still improved by using a butanol-IL mixture (47% sugar release, relative to the sum of glucose and hemicellulose contents for untreated rice straw) instead of aqueous IL (41% sugar release, relative to the sum of glucose and hemicellulose contents for untreated rice straw). As ethanol was more powerful than butanol in term of improving the enzyme accessibility of the pulp, the glucose yield of the ethanol-IL process (64%) was not largely affected by the lower lignin removal, which was the same as the ionoSolv process.

For all ionoSolv processes, lignin recovery yields exceeded their corresponding deligninfication, regardless of the feedstock type. This suggested that lignin condensation accompanied with pseudo-lignin formation appeared for all ionoSolv pretreatments. However, this unexpected high lignin recovery was not observed for any organosolv-ionoSolv pretreatments. This could be attributed to the different lignin modification taking place during the lignin fractionation. Incorporating ethanol/butanol with anhydrous IL during biomass fractionation induced the α-ethoxylation/butoxylation at side chain region of the lignin. This lignin modification inhibited the condensation reaction yielding insoluble lignin fragments with larger molecular weight. The reduced lignin recovery for organic-IL process might be explain as α-butoxylated/ethoxylated β-O-4 ether linkages improved lignin solubility in water, less lignin fragments was precipitated.

For organosolv pulping, 86% xylan removal and 77% lignin removal was reported for rice husk, where the feedstock was pretreated at higher temperature, 195°C, compared with the hybrid process.[107] 97% xylan removal and up to 62% delignification was reported for bagasse.[255]

5.3.2.2 Glucose recovery and hemicellulose removal

Different levels of glucan degradation were observed for the ionoSolv process and was more severe for rice husk and bagasse. 80% and 78% glucose were persevered in the pulp for rice husk and bagasse, respectively, relative to the glucan content of untreated biomass, presented in Figure 5.9. The issue of glucose degradation was largely relieved in organic-IL process. For both feedstocks, Less than 10% glucan lost was observed for all organosolv-ionoSolv pretreatments.

Quantitative hemicellulose removal was achieved for rice husk and bagasse regardless the pretreatment type. This was not the case for straws. Approximately 100% hemicellulose (relative to hemicellulose of untreated biomass) was removed during ionoSolv processing of straws, but only around 40% and 50% hemicellulose were removed in the organic solvent-IL process for rice straw and wheat straw, i.e. the remaining hemicellulose in the pulps was 60% and 50%, presented in Figure 5.9. The high hemicellulose residues positively influenced the enzymatic hydrolysis, where the hemicellulose was easily hydrolysed by cellulase into monomeric sugars alone with the cellulose fraction in the pulp.

For organosolv pretreatment reported in the literature, wheat straw was able to achieve a 70% hemicellulose removal and a 65% lignin dissolution into the aqueous glycerol solvent medium, whereas the hot ethanol solution was able to reduce the lignin content of rice straw from 18% to 11%, before and after fractionation.[19,208]

According to compositional analysis, the glucan degradation was reduced in organic-IL pretreatments, compared to ionoSolv ones. Quantitative hemicellulose removal was achieved by ionoSolv pretreatments, regardless the feedstock type, and organic-IL pretreatments for rice husk and bagasse. Significantly lower removal was achieved by organic-IL process for rice straw (≤ 40%) and wheat straw (≤ 50%), but residual hemicellulose could be easily hydrolysed. Lignin condensation was believed to take place in ionoSolv processes but not in the organic solvent-IL ones.

5.3.3 Characterisation of isolated lignin

As lignin is one of the major side products generated by the pretreatment, the economic value of the lignin produced has a large impact on the overall cost of the process. Both quantity and quality of the lignin define its value, therefore lignin's characters, such as chemical structure and molecular weight, are useful to know. The lignins extracted by aqueous IL and ethanol/butanol-IL mixtures were subjected to HSQC NMR analysis and GPC analysis. HSQC NMR analysis is able to provide structural information about the isolated lignins, while GPC analysis defines the molecular weight and polydispersity of the lignins.

5.3.3.1 HSQC NMR analysis

The side chain region and the aromatic region of all HSQC NMR spectra were coloured and presented in Figure 5.10, 5.11, 5.12, 5.13 for bagasse, rice husk, rice straw and wheat straw, respectively. The side chain regions reveal the composition of the aryl ether, resinol, phenylcoumaran and Lignin-carbohydrate linkages. Lignin-carbohydrate linkages in spectra were labelled as Ara or Xyl, representing the lignin fragments crosslinked with arabinose or xylose. The aromatic region provides information about guaiacyl (condensed and uncondensed), syringyl (condensed and uncondensed), *p*-hydroxyphenyl and *p*-coumaric units.

The semi-quantitative analysis about the signal intensities of the major lignin linkages was conducted for lignin isolated from all four agricultural feedstocks, presented in Figure 5.10, 5.11, 5.12 and 5.13. All subunits were semi-quantitatively analysed by integrating volume peaks to quantify their abundances in percentages, relative to the sum of G_2 and G_{2cond} integrals. G_2 and G_{2cond} integrals were comfirmed to be unchanged for the lignin isolated from all ionoSolv process and the newly developed organosolv-ionoSolv processes (detailed in Chapter 3 and 4).[2,60,190] For all lignins, the degree of condensation and S/G ratio could be derived from the semi-quantitative analysis, presented in Table 5.6. The degree of condensation could be derived from equation 1, presented in Section 5.2.5.

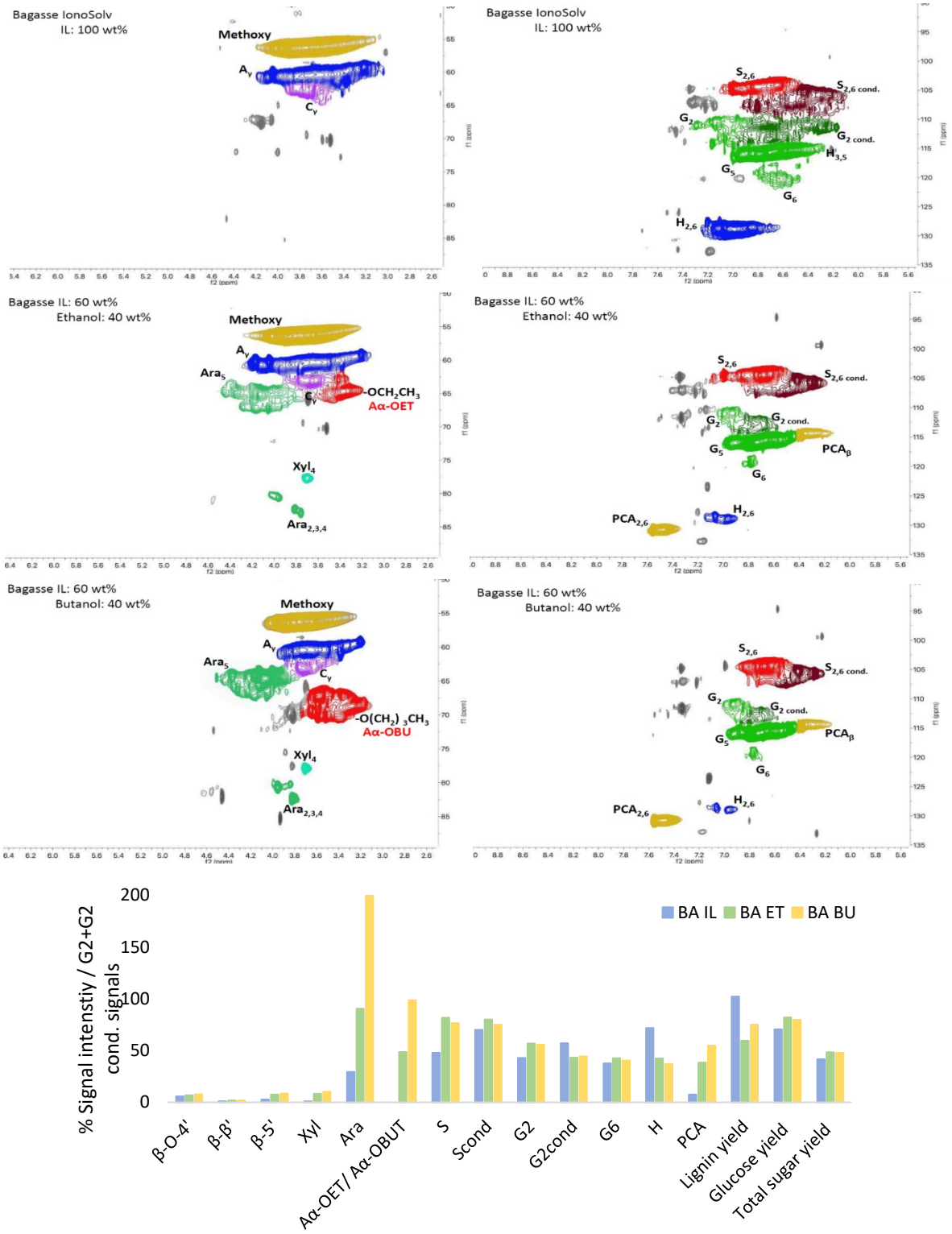


Figure 5. 10 HSQC NMR spectra of lignins recovered from Bagasse fractionated by aqueous [DMBA][HSO_4] and ethanol/butanol-[DMBA][HSO_4] mixture. (top left) Side chain region of the HSQC NMR spectra (top right) Aromatic region of the HSQC NMR (bottom)a semi-quantitative analysis for signal intensities of the key lignin subunits in bagasse lignins according to HSQC NMR spectroscopy. Signal intensities are presented in percentages relative to the sum of signal intensities for G_2 and $G_{2cond.}$

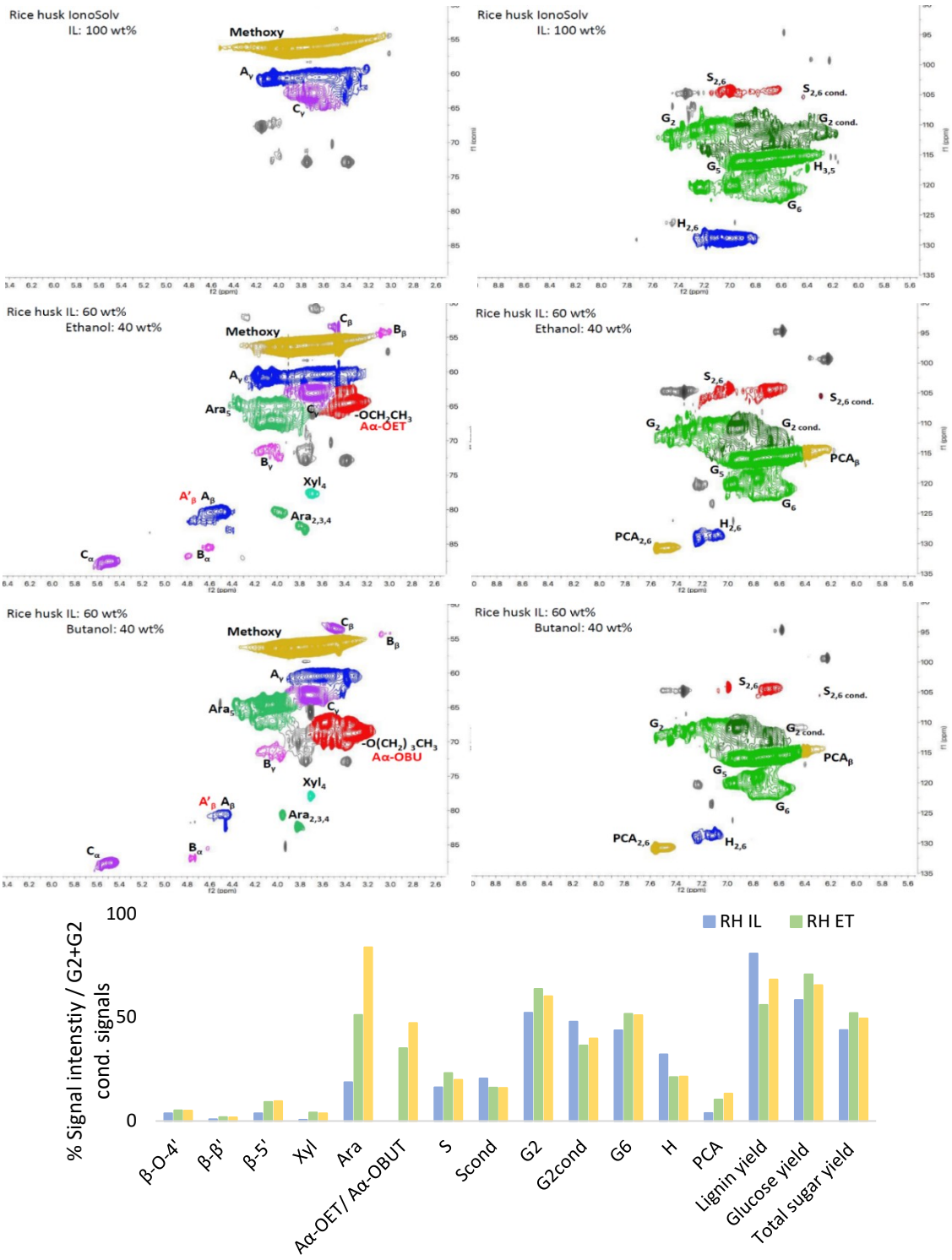


Figure 5. 11 HSQC NMR spectra of lignins recovered from rice husk fractionated by aqueous [DMBA][HSO_4] and ethanol/butanol- [DMBA][HSO_4] mixture. (top left) Side chain region of the HSQC NMR spectra (top right) Aromatic region of the HSQC NMR (bottom)a semi-quantitative analysis for signal intensities of the key lignin subunits in rice husk lignins according to HSQC NMR spectroscopy. Signal intensities are presented in percentages relative to the sum of signal intensities for G_2 and $G_{2cond.}$

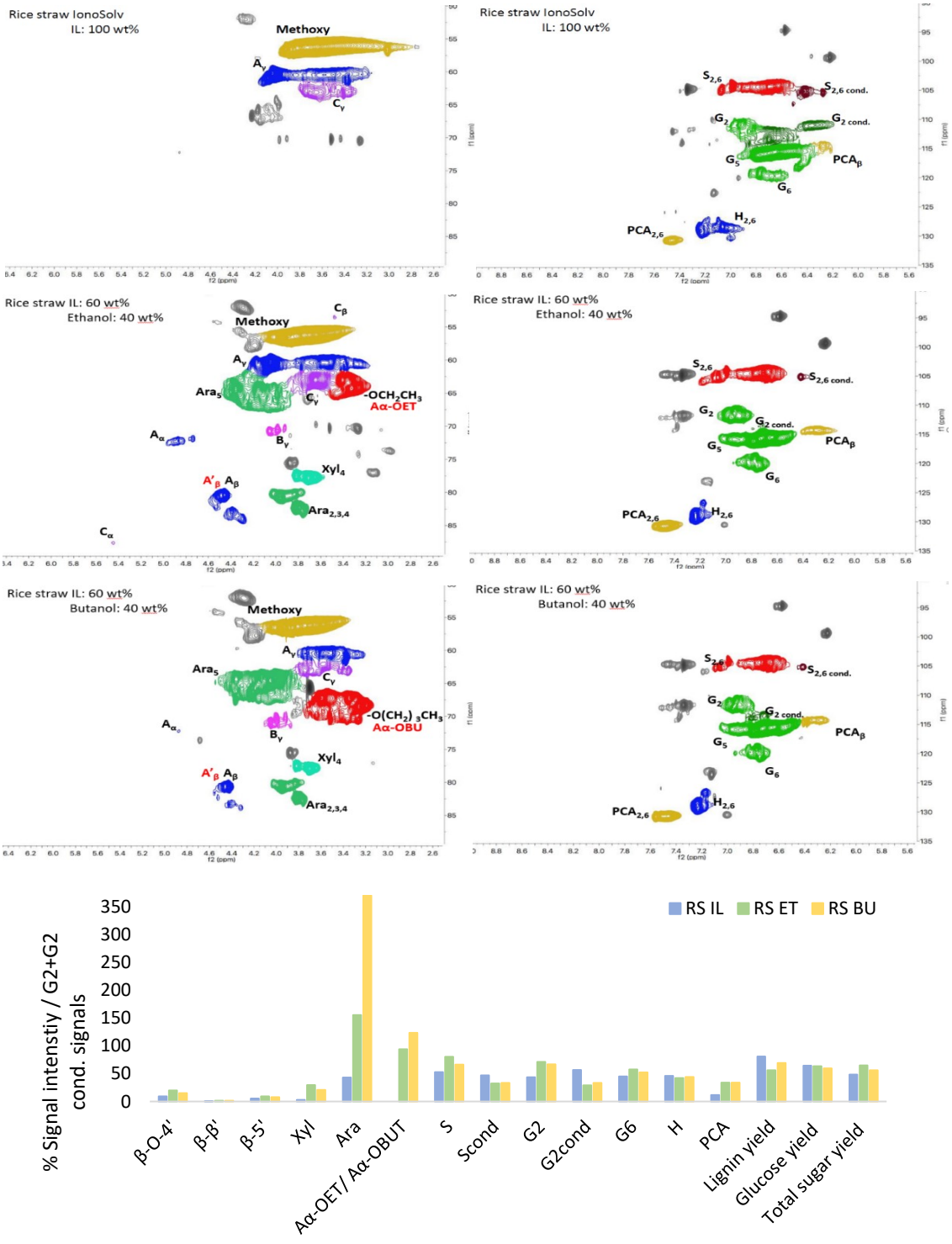


Figure 5. 12 HSQC NMR spectra of lignins recovered from rice straw fractionated by aqueous [DMBA][HSO_4] and ethanol/butanol- [DMBA][HSO_4] mixture. (top left) Side chain region of the HSQC NMR spectra (top right) Aromatic region of the HSQC NMR (bottom)a semi-quantitative analysis for signal intensities of the key lignin subunits in rice straw lignins according to HSQC NMR spectroscopy. Signal intensities are presented in percentages relative to the sum of signal intensities for G_2 and $G_{2cond.}$

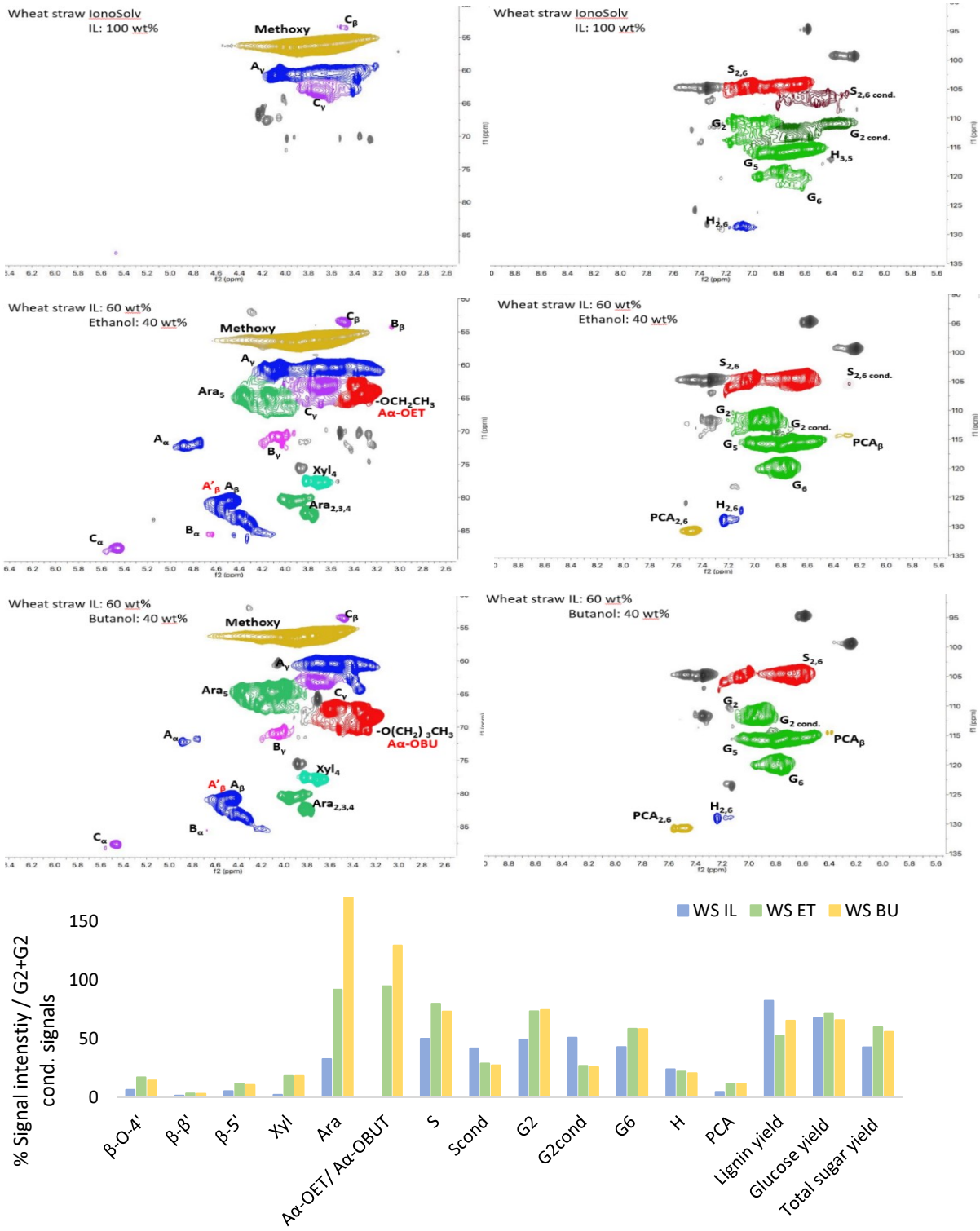


Figure 5. 13 HSQC NMR spectra of lignins recovered from wheat straw fractionated by aqueous [DMBA][HSO_4] and ethanol/butanol- [DMBA][HSO_4] mixture. (top left) Side chain region of the HSQC NMR spectra (top right) Aromatic region of the HSQC NMR (bottom)a semi-quantitative analysis for signal intensities of the key lignin subunits in wheat straw lignins according to HSQC NMR spectroscopy. Signal intensities are presented in percentages relative to the sum of signal intensities for G_2 and G_{2cond}.

Table 5. 8 Degree of condensation and S/G ratio based on HSQC spectrum integrals for lignins extracted from pine using different ethanol-IL mixtures

Pretreatment condition	BA [a] IL [b]	BA ET [c]	BA BU [d]	RH [e] IL	RH ET	RH BU	RS [f] IL	RS ET	RS BU	WS [g] IL	WS ET	WS BU
G_2 peak integral intensity	43	56	55	52	63	60	43	71	66	49	73	74
$G_{2con.}$ [c] peak integral intensity	57	43	44	47	36	39	56	29	33	50	26	25
Degree of condensation [h]	57	43	44	47	36	39	56	29	33	50	26	25
S/G ratio	0.59	0.81	0.76	0.18	0.20	0.18	0.50	0.56	0.50	0.46	0.54	0.50

[a] BA is short for bagasse

[b] IL represents the lignin isolated from ionoSolv pretreatment

[c] ET represents the lignin isolated fron ethanol-IL pretreatment

[d] BU represents the lignin isolated fron butanol-IL pretreatment

[e] RH is short for rice husk

[f] RS is short for rice straw

[g] WS is short for wheat straw

[h] Calculated based on $G_{2con.}$ /G_2+$G_{2con.}$ in %

According to Figure 5.10, 5.11, 5.12 and 5.13, useful lignin structural information obtained from the side chain region of the spectra is 1) the appearance of the integrated volume peak standing for the α-ethoxylated/butoxylated aryl ether linkages, the peak intensity was higher for butoxylated ether linkages, relative to the sum of G_2 and $G_{2cond.}$ peak intensities; 2)+ the presence of peaks for carbohydrates originated from hemicellulose, suggesting the lignin generated from organosolv-ionoSolv pretreatment was not carbohydrate-free, unlike the ionoSolv lignins. All information obtained is highly similar to the structural information obtained from the miscanthus study, detailed in Section 3 as well as the organosolv studies for miscanthus and beech. [63,205] α-ethoxylation/ butoxylation was suggested to be one of the signature chemical reaction happening during fraction. It took place at the α carbon position on the side chain of the lignin and modified β-O-4 ether into α-alkoxy ether (A'). [205] At the α-carbon position, lignin condensation might also take place, therefore α-alkoxylation was competing with condensation during fractionation. For all ethanol/butanol-IL processes regardless the feedstock, α-alkoxylation induced by ethanol/ butanol significantly inhibit

lignin crosslinking at the α carbon position and the resultant α-ethoxylated/butoxylated aryl ether changed the lignin solubility in order to achieve a better lignin fractionation.[205] The tuned lignin solubility also changed the amount of lignin recovered via precipitation as a larger amount of the lignin fraction turned to be water soluble, comparing to ionoSolv lignin.

Significant amounts of residual arabinose and xylose were observed in the spectra for all agricultural residue lignins, suggesting the carbohydrates are with other lignin fragments but was not true for ionoSolv lignin. No sugar residues were detected for ionoSolv lignin. The highest signal intensity for residual carbohydrates was for rice straw, 369%, relative to the sum of G_2 and $G_{2cond.}$ peak intensities, whereas the signal intensities for rice husk, wheat straw and bagasse were 83%, 177%, 200%. This could be explained as for organosolv or organosolv-related lignins, sugar subunits, arabinfuranose, were crosslinked with lignin subunits via *p*-coumarate acid.[63]

For all feedstocks, the abundances of β-O-4 ether, β- β resinol and β- 5 phenylcoumaran were low, no more than 16%, where these subunits were the most common linkages appeared in native lignins. This suggested that organosolv-ionoSolv process fractionate lignin via breaking aryl ether bonds and modifying resinol and phenylcoumaran linkages. Compared to ionoSolv lignin, the peak intensity for phenylcoumaran linkages was higher for organic-IL lignin, indicating less amount of β- 5 units were chemically modified in the organic-IL solvent medium.

The key information delivered by the aromatic region is 1) lignin condensation was less severe when the pretreatment solvent medium switched from acidic aqueous IL to an organic solvent-IL mixture; 2) larger amounts of PCA subunits were preserved by the organic-IL mixture, instead of converting into H subunits. According to the coloured HSQC spectra, the signal intensities for G_2, G_6, $S_{2,6}$, increased

and that for $G_{2cond.}$ $S_{2,6\ cond.}$ decreased, when solvent composition changed from aqueous IL to an organic solvent-IL mixture. This indicated a lower extent of lignin condensation took place at the S and G units. The increased G_6 integral suggested that the condensation could also happen at the carbon 6 position of the aromatic ring for G units. These observations were in lignin with the calculated degree of condensation. For bagasse, 57% of G units were condensed for ionoSolv lignin; this decreased to 43% for ethanol-IL lignin. A 12% drop in the degree of condensation was observed for bagasse ethanol-IL lignin, compared to the corresponding ionoSolv lignin. For straw lignins, the extent of condensation was twice as severe as for the ionoSolv process. As PCA units acted as a bridge to link the carbohydrates to other lignin subunits, it is reasonable to observe a higher amount of PCA units remained in the organic-IL lignins, relative to ionoSolv lignin where PCA units to H units conversion was one of the signature lignin modification occurring in the fractionation.

The S/G ratio kept constant for rice husk (around 0.2), rice straw (around 0.5), and wheat straw (around 0.5), regardless of the fractionation process type. However, the S/G ratio increased to 0.81 for rice husk when ethanol was used as a co-solvent for IL during fractionation; for ionoSolv lignin was 0.59. According to the literature, the degree of condensation has a positive correlation with the S/G ratio but this was not the case here. This suggested that comparison of the S/G ratio for lignin extracted by different methods do not provide a reliable indication for any changes in the degree of lignin condensation.

5.3.3.2 GPC analysis

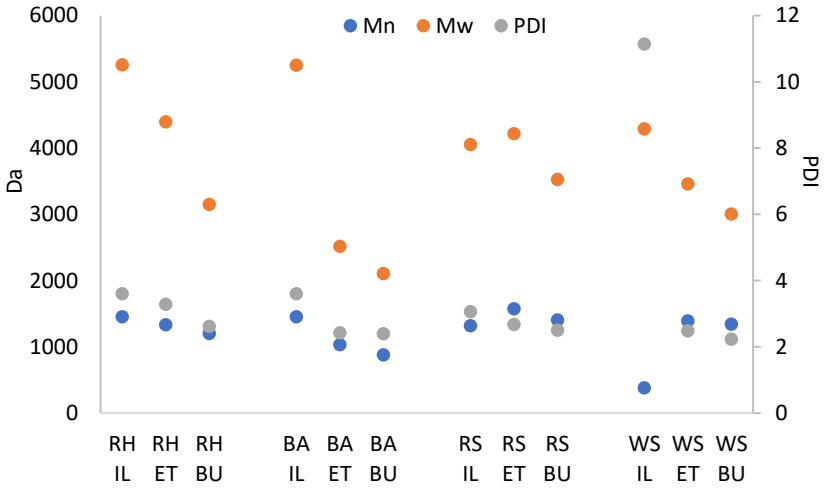


Figure 5. 14 Average molar mass and polydispersity of lignins extracted from agricultural residues via the ionoSolv or the organosolv-ionoSolv fractionation process

Similar trends of molecular weight and lignin polydispersity were observed for all feedstocks, ionSolv > ethanol-IL > butanol-IL, presented in Figure 5.14. The data points for ionoSolv lignin isolated from wheat straw did not follow the trend. Excluding that data point, all average number molecular weight ranged between 1000 to 1500 Da. The highest Mn was 1577 for ethanol-IL lignin isolated from rice straw, whereas the lowest was 879 Da for butanol-IL lignin isolated from bagasse. Further, the PDI was fairly narrow, ranging between 2 to 3, which is suitable for carbon fiber production.[63,233]

Chapter conclusion

The time course experiments were conducted at 170°C and 150°C for rice husk. The optimal glucose yield achieved was 77% at 45min, 170°C, and 73% at 2 hours, 150°C. The former was superior as the process has a shorter pretreatment time at higher temperature and is believed to be more cost-effective. The appearance of the lignin condensation was suggested by the results of the

compositional analysis. Process integration is needed to yield a lignin fraction with a better quality. The ionoSolv process for rice husk was limited by hornification. The dry and wet pulp glucose yield difference was up to 26%, this difference got smaller for harsher conditions, as the dry pulp's glucose yield was limited by lignin condensation and the lignin redeposited on to the cellulose surface.

Based on the study of rice husk, predictions of the optimal ionoSolv process was made for two straws (30 min, 170°C) and bagasse (45min, 170°C). Fractionation processes with the predicted conditions were conducted including rice husk. The experimental results confirmed the prediction. The ionoSolv process successfully fractionated these feedstocks, achieving glucose yield up to 90% (for wet pulp) and lignin removal up to 83%. The ash was quantitatively preserved in the treated biomass, based on the compositional analysis. Glucose degradation was detected, and lignin condensation were observed in compositional analysis and further confirmed by HSQC, suggesting these two issues could potentially limit the glucose release of the biomass during enzymatic hydrolysis. Further optimization of the process might be needed.

The organosolv-ionoSolv process was carried out to fractionate these agricultural feedstocks. An optimized fractionation was obtained for all feedstocks in terms of saccharification yields. The enzymatic hydrolysis was conducted for air-dried pulp, for the ease of handling. For recalcitrant rice husk and bagasse, approximately 12% glucose yield increase was obtained, compared to ionoSolv process. For rice straw and wheat straw with less dense cell wall, the glucose yield increase was not observed, but an additional hemicellulose release (up to 14%) was achieved, resultantly increasing the feedstock's total sugar yield up to 17% (compared to ionoSolv pretreatment), relative to the hemicellulose and glucose content of the untreated biomass. Compositional analysis suggested that the degree of glucan degradation and lignin condensation was reduced in organic-IL process, relative to ionoSolv process. The decreased lignin condensation was confirmed by HSQC. HSQC analysis

suggested that ethanol/butanol-IL lignins contain traces of hemicellulose, and the signature chemical reaction happened during lignin fractionation was α-alkoxylation induced by ethanol/butanol. The α-ethoxylation/ butoxylation prevented the lignin condensation and changed the lignin solubility for all agricultural lignins, which also happens with miscanthus (detailed in data chapter 1 miscanthus organosolv-ionoSolv fractionation).

6 Results: Monolignol synthesis and radical polymerisation of lignin-like polymers

Chapter summary

In this chapter, the main focus was to develop a synthetic route for three major lignin monomers, in which the route should be easy to handle and use cheap reagents. The major monolignols synthesised were sinapyl alcohol, coniferyl alcohol and *p*-coumaryl alcohol, corresponding the syringyl, guaiacyl and *p*-coumaryl/hydroxyphenyl units in the polymeric lignin structure. All the phenylpropane units were synthesised from their corresponding carboxylic acids via an esterification followed by a reduction. The monolignols were then subjected to a homogeneous radical polymerisation catalysed by horseradish peroxidase to product lignin-like polymers, where the polymers were subsequently fractionated by the degree of polymerisation. The polymers were also subject to GPC analysis, and the results were compared with the polymeric lignins (oligomers) isolated from nature feedstocks via pretreatments.

6.1 Monolignol synthesis

The dominant lignin building blocks are a group of phenylpropane units with different degrees of methoxylation, derived from sinapyl alcohol, coniferyl alcohol and *p*-coumaryl alcohol. Within the cell wall, these alcohol monomers are derived from its corresponding carboxylic acids via a series of chemical reactions including hydroxylation and methylation, and then polymerised by peroxidase, but this chemical pathway of monolignols and its subsequent polymerisation has not been fully understood due to the complexity of the lignin structure.[81] Understanding how the lignin structure is

formed would influence the current development of the lignin depolymerisation for value added valorisations.[257] Therefore, the investigation for the synthesis pathway of these alcohol monomers has lasted for decades.

Chemically synthesising lignin model compounds like sinapyl alcohol from its corresponding carboxylic acid often requires two or more steps: esterification and reduction, involving three types of intermediates, methyl/ethyl esters and a ketone. The esters have been reported with the highest frequency. Gangar *et al.* investigated the anti-selective glycolate aldol reactions for various substituted allyl aldehydes.[258] In their work, the ethyl ferulate was successfully synthesised from ferulic acid via an acid-induced esterification, with a conversion of 94%. The ethyl ferulate and ethyl sinapate were subjected to a lithium aluminium hydride ($LiAlH_4$) reduction in the mixed solvent medium of tetrahydrofuran (THF) and benzyl chloride to yield coniferyl and sinapyl alcohols, where both esters achieved a yield of 85%. A similar $LiAlH_4$ reduction of carboxylic esters to allylic alcohols was demonstrated by Wang *et al* in 2008.[259] The ethyl ferulate, ethyl sinapate and ethyl *p*-coumarate were reduced to their corresponding alcohols with a conversion rate of 83% to 85%. Wallace *et al.* conducted the reduction reaction for ethyl ferulate and ethyl p-coumarate in a different solvent medium, diethyl ether.[260] Lancefield *et al* demonstrated the synthetic pathway of lignin model compounds like coniferyl and *p*-coumaryl alcohols which were generated from their corresponding aldehydes via a ester intermediate.[205] The aldehydes underwent a menthol esterification facilitated by acetyl chloride. Lancefield also studied the dehydrogenation polymerisation of those lignin model compounds. By varying the lignin monomers, lignin-like polymers were successfully synthesised, where their polymeric structure could be a good structural model for hardwood (rich in β-*O*-4 and β-β) and softwood lignins (rich in β-*O*-4 and β-5). He also compared the synthetic lignin-like polymers to lignin isolated from birch and Douglas fir by HSQC. In another work on one-pot microwave-assisted

synthesis of phenylpropanpoid units, the methyl ferulate, sinapate and *p*-coumarate was reduced to monolignols by DIBAL-H in dry dichloromethane.

Monolignols were also reported as generated from the ketone by Duran *et al.* back in 1984.[261] Kim *et al.* represented a simple synthetic method to prepare coniferyl and sinapyl alcohols, where the alcohols were generated from aldehydes via a ketone intermediate using borohydride exchange resin.[262]

6.1.1 Monolignol synthesis

Two synthetic routes for the monolignols were used according to the work of Chand *et al.* and Quideau *et al*. [263] [94] The synthesis pathway is shown in Figure 6.1. Both routes are a two-step process consisting of an esterification (step 1, step a and b in Figure 6.1) and reduction (step 2, step c in Figure 6.1), but differ from each other by the ester intermediate formed. For route 1 (step a in Figure 6.1), an esterification is induced by concentrated sulfuric acid, forming a methyl ester; while in the first step of the route 2 (step b in Figure 6.1), acetyl chloride undergoes a nucleophilic addition with the ethanol presenting in the reaction mixture, forming hydrochloric acid which then catalyses the subsequent esterification between ethanol and cinnamic acid. The mechanisms of these two esterification are detailed in Figure 6.2. For both synthetic routes, the second step, reduction, is conducted using DIBAL-H as a reducing agent rather than $LiAlH_4$ for the ease of handling.

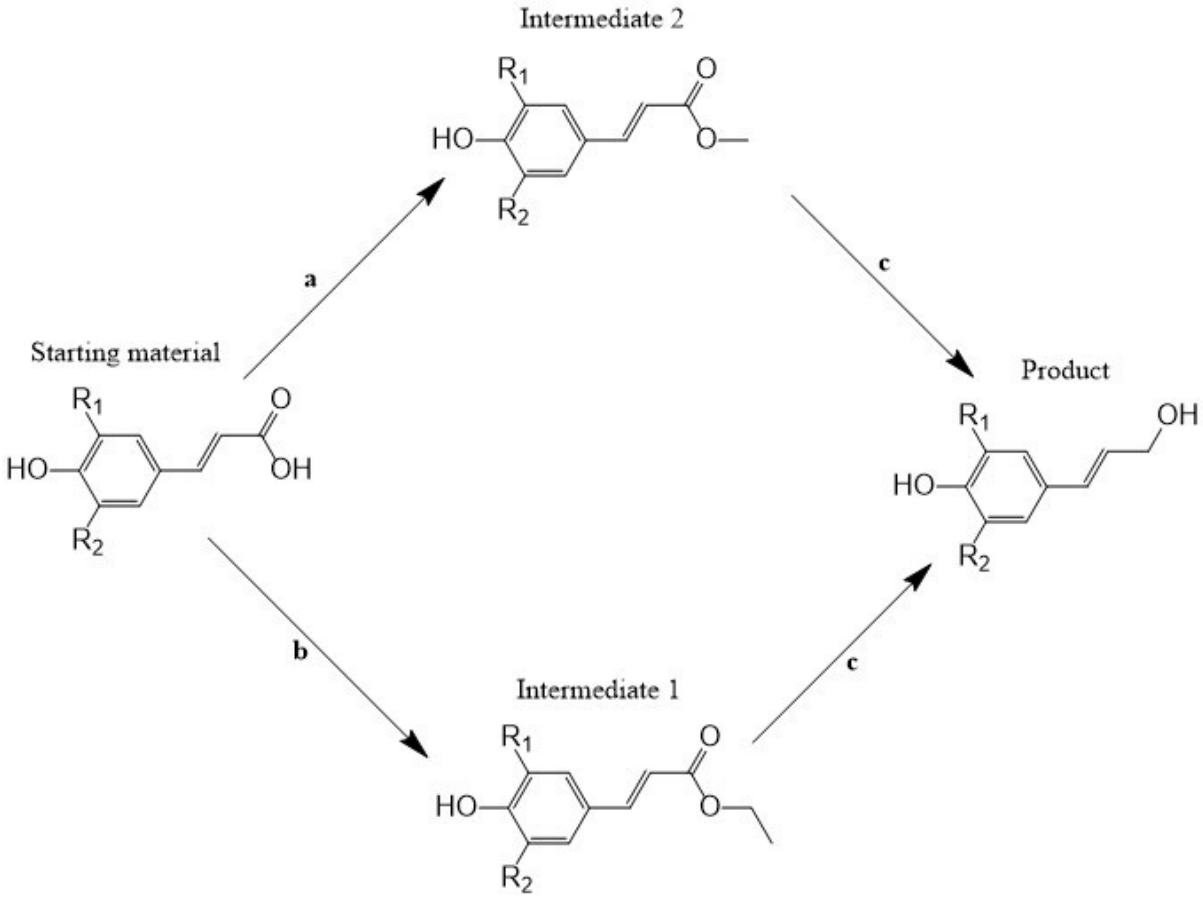


Figure 6. 1 Reaction scheme of monolignols

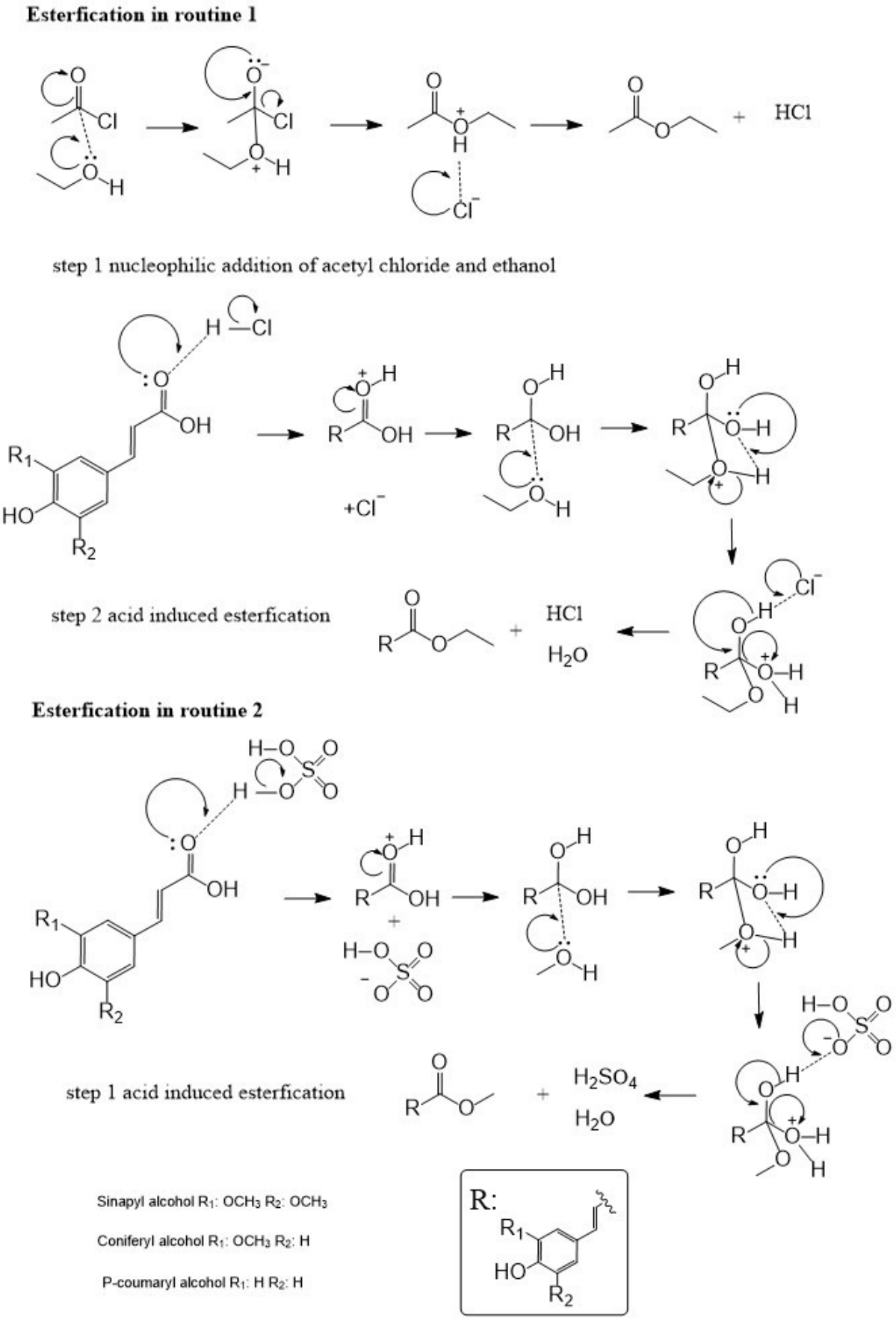


Figure 6. 2 The mechanisms of the esterification reactions for both synthesis routes

Using synthetic route 1, sinapyl alcohol and coniferyl alcohol were successfully produced with high yields for both process steps, and *p*-coumaryl alcohol was synthesised according to route 2. According to route 1, ethyl sinapate was converted from sinapic acid with a yield of 58%, which was further reduced to sinapyl alcohol with a conversion of 86%. The conversion of acid to the ethyl ester was not ideal, therefore the sinapyl alcohol synthesis was repeated using route 2. The conversion of acid to methyl ester was much higher, 87%, and the alcohol conversion was maintained around 80%. Here, we could see that route 2 is a better synthesis pathway for sinapyl alcohol. However, both synthetic routes suffered from the same issue. The final product, sinapyl alcohol, was obtained in the form of an oil, which was in line with the experimental observation from Chand *et al* 's work;[263] even after purification using a mixture of polar/non-polar solvents , a clean crystalline solid could not be produced, which was consistent with most of the literature. [57,81,92,] The low purity of sinapyl alcohol is potentially responsible for the failure of the polymerisation for this monolignol. Repeated experiment for route 2 was attempted for 5 times, a crystalline solid (final alcohol product) was achieved once with a conversion (from the intermediate) less than 10%, which was not enough to undergo the enzymatic polymerisation. In summary, the synthesis process for sinapyl alcohol needs improvement.

Different from sinapyl alcohol, a crystalline form of coniferyl alcohol was obtained using synthetic route 1, where the conversion rates of the 1st and 2nd steps were 81% and 113%. A solid form of *p*-coumaryl alcohol was also synthesised, where its conversion rate was lower, 70% for the esterification step and 75% for the reduction step. Both monolignols were subjected to dehydrogenation (polymerisation) induced by horseradish peroxidase, detailed below.

6.2 Radical polymerisation in organic aqueous media

Within the cell wall, the biosynthesis of the polymeric lignin was rather complex and difficult to control, therefore, investigations have been conducted to chemically polymerise the monolignols gaining a polymeric structure close to the native lignin, where the synthetic polymerisation allows more control on the structure and properties of the product (polymer). Dehydrogenative polymerisation methods have been widely used to polymerise monolignols. This type of polymerisation has two synthetic modes, Zulaufverfahren (ZL) and Zutrophverfahren (ZT), where the former is a bulk reaction and the latter requires a dropwise addition of the monomers into the reaction mixture. The peroxidase, such as horseradish peroxidase (HRP), is one of the commonly used enzyme to induce the reaction, phosphate and dioxane is the commonly selected solvent medium for the polymerisation.[257,264] Moon *et al* demonstrate the HRP polymerisation in a mixed solvent medium, phosphate buffer and dioxane for a mixture of sinapyl and coniferyl alcohols, where the process was operated in the ZT mode and hydrogen peroxide H_2O_2 was the oxygen resource.[265] The polymer produced had a number average molecular weight Mn ranging from 2300 Da to 3400 Da, a weight average molecular weight Mw ranging from 4000 Da to 5000 Da, and its polydispersity index PDI (Mw/ Mn) was around 1.5. Sasaki *et al* was able to conduct the same polymerisation process in in the ZL mode for coniferyl alcohol only, generating a homopolymer which is rich in aryl ether linkages.[263]

The polymerisation conducted in this study was based on Guan *et al* 's work and was in a ZT mode, monolignols (in phosphate buffer), oxygen resource H_2O_2 (in phosphate buffer and dioxane) and the HRP enzyme (in phosphate buffer) were separately dropwise introduced to the reaction over a period of few hours depends on the amount of reagents.[34] The monolignols used were coniferyl alcohol and *p*-coumaryl alcohol. Two poly (coniferyl alcohol) and one poly (*p*-coumaryl alcohol) were synthesised successfully. As the polymerisation produced a mixture of polymer, oligomers and traces of unreacted monomers, the crude product of the process, obtained from the reaction mixture after being

concentrated, needed to be fractionated via a methanol and a DMF dissolution. The crude product was dissolved firstly in the methanol, where monomers and oligomers would be completely dissolved, and the polymer would remain insoluble towards methanol. The methanol insoluble fraction of the product was then dissolved in the DMF again, where the polymer with highest degree of polymerisation would remain insoluble.

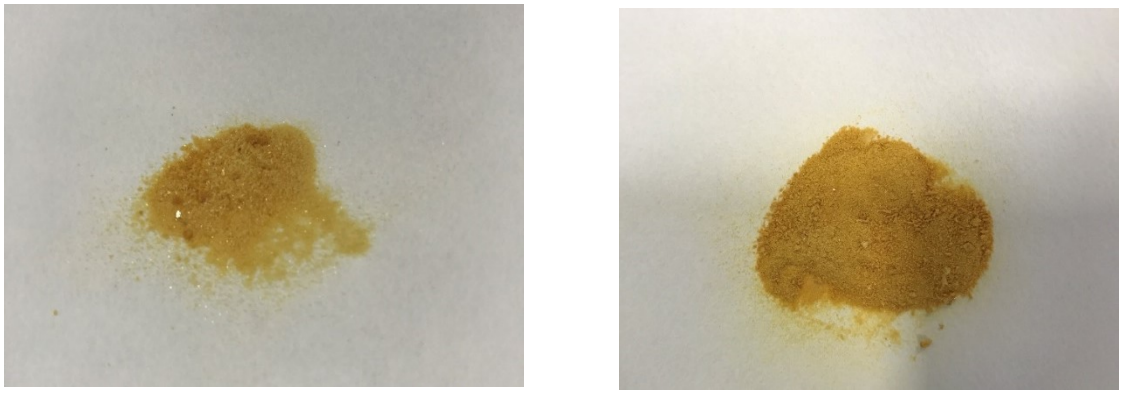


Figure 6. 3 Poly (coniferyl alcohol) left: methanol soluble fraction, right: methanol insoluble but DMF soluble fraction

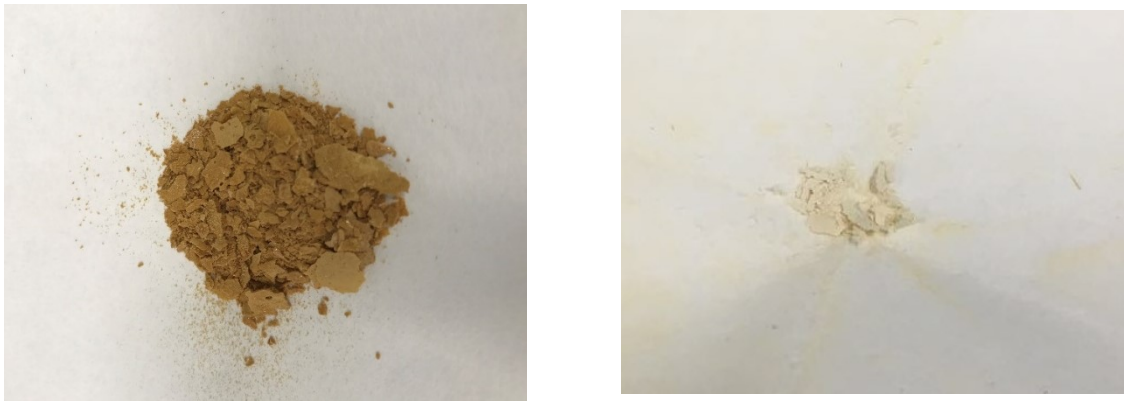


Figure 6. 4 Poly (*p*-coumaryl alcohol) left: methanol soluble fraction, right: methanol and DMF soluble fraction

poly (coniferyl alcohol), also named as G polymer, was generated twice, and the crude products of both batches were able to generate a methanol soluble fraction and a methanol insoluble/ DMF soluble fraction. However, poly(*p*-coumaryl alcohol), also called H polymer, was synthesised once, the crude product was fractionated into a methanol soluble, a methanol insoluble/DMF soluble, and a methanol insoluble/ DMF insoluble fraction. All the polymeric products synthesised were in a solid form, detailed in Figure 6.3 and 6.4.

6.2.1 GPC analysis

For the two G polymer crude products and the H polymer crude products, the product yields were presented in Table 6.1. Yields for fractions of the polymer with different ethanol and DMF solubilities are presented as percentage, relative to the amount of monomer incorporated the polymerisation. These fractions of the polymer products were subjected to GPC analysis, detailed in Table 6.2.

Table 6. 1 the yields of fractionated product for the dehydrogenative polymerisation of coniferyl alcohol and p-coumaryl alcohol

The yield of lignin-like polymer solubilised by different solvents	**Poly (coniferyl alcohol) G polymer**		**Poly(*p*-coumaryl alcohol) H polymer**
	1st experiment	2nd experiment	1st experiment
MeOH soluble[a]	24	9	54
MeOH insoluble /DMF soluble[a]	74	80	45
MeOH insoluble /DMF insoluble[a]	0	0	0.4
Monomer conversion rate	99	89	99

a: The lignin production yields are presented in percentages, relative to the amount of monolignols participating the polymerisation.

The two G polymers were produced with two batches of monomers. Although the identical polymerisation process was applied for both polymers, but the end products generated were different in terms of monomer conversion rate and the yield of methanol soluble polymer fraction, suggesting the precision of the experiement could be improved. More repeated polymerisation should be carried out in order to provide accurate experimental outcomes. The monomer conversion rates (the sum of MeOH soluble polymer yield, DMF soluble polymer yield and DMF insoluble polymer yield) were 99% for the 1st experiment and 90% for the 2nd one, where there were significant amounts of monomer remained unreacted in 2nd batch, listed in Table 6.1. The methanol-soluble fraction (polymer) has the shortest polymer chain, compared with two other polymer fractions, and is mainly made up by unreacted monomer and oligomers.[81] For the 1st batch, the amount of methanol-soluble fraction occupied 25% of the total amount of polymer synthesised, while this only occupied around 10% in the 2nd batch. As the monomers were completely used up during polymerisation, the methanol soluble fraction in 1st batch was made up by the oligomers. However, this fraction was made up by both the coniferyl alcohols and its corresponding oligomers. The amounts of methanol insoluble and DMF soluble fraction obtained were similar for two experiments, 75% for 1st batch and 80% for 2nd batch, which was the dominant polymer fraction.

For H polymer, the overall monomer conversion reached 100%. Over half of the monomers converted into polymers (oligomers) which were soluble in methanol, while just below half of the monomers converted to polymers with a relative longer chain length. It is important to note that an extremely small fraction of the polymer synthesis was unable to dissolve in DMF, indicating this polymer fraction could have a much higher degree of polymerisation then the methanol-soluble and methanol-insoluble DMF-soluble fractions. As this organic-insoluble fraction is too little to be characterized, the polymerisation for H polymer should be repeated in the future. A solubility test in mixtures of DMSO/DMF and lithium bromide should be attempted, if this organic-insoluble fraction is produced

in a larger quantity. If it dose dissolve in DMSO/DMF-lithium bromide solvent medium, that means this organic-insoluble polymer could be subjected to GPC analysis, which provides information of molecular weight, molecular weight distribution and potentially degree of polymerisation.

A previous study conducted in our research group reported that the poly (coniferyl alcohol) and poly (*p*-coumaryl alcohol) were synthesised in a ZL mode dehydrogenative polymerisation, using HRP as the enzyme, H_2O_2 as the oxygen resource.[83] The poly (coniferyl alcohol) produced was completely methanol soluble and only 14% of poly (*p*-coumaryl alcohol) synthesised was insoluble in methanol.

According to the GPC analysis, the G polymer produced from two batches of experiments displayed different molecular weights and polydispersities. The Mn for both batches were ≤ 500 Da, suggesting the oligomers built up by less than 3 monomers were generated during the polymerisation. The Mw was 36292 Da for the methanol-insoluble DMF-soluble fraction of the 1st batch, while this was only 7599 Da for the 2nd batch. The reasons for this difference could be: 1) different amounts of monomer used let to a reagent addition time, therefore the actual reaction time for two polymerisations were different, which might lead to a change in the polymeric product; 2) the two polymerisation used different batches of monomers, where the quality of the monomers differed from batch to batch, in other words, two batches of monomers may contain different amounts of impurities. The first batch of poly(coniferyl alcohol) synthesised has extremely high PDI, due to the large discrepancy between the Mn and Mw. Repeats are needed here to confirm the accuracy of this GPC analysis. Process optimization is also required in order to develop a repeatable polymerisation process for coniferyl alcohol.

Table 6. 2 Information about molecular weight and polysidpersity index of the polymer synthesised

	G polymer				H polymer
	1st experiment		2nd experiment		1st experiment
	MeOH soluble	DMF soluble	MeOH soluble	DMF soluble	MeOH soluble
Mw (Da)	8856	36292	3481	7599	6295
Mn (Da)	251	336	516	485	467
PDI	35	107	6	15	15

For H polymer, the Mn recorded for methanol soluble fraction was 467 Da and Mw was 6295 Da, PDI was around 15. The methanol-insoluble DMF-soluble fraction was contaminated during drying, therefore no reliable GPC data was obtained. The DMF insoluble fraction where was expected to have a high degree of polymerisation only achieved a yield less than 0.5%, corresponding to 0.0054g, GPC analysis was attempted but failed as the polymer was insoluble towards GPC solvent DMSO. Repeats are needed here to confirm the experimental results are reliable and possible process integration is needed for increasing the yield of DMF insoluble fraction. According to the previous study, poly(*p*-coumaryl alcohol) achieved a smaller Mw 3011. But larger Mn 2091, and smaller PDI 1.28. This suggests that the poly(*p*-coumaryl alcohol) synthesised here was a mixture of long polymers and small oligomers, while the polymer synthesised previous was more uniform in term of molecular weight.

Compared to the polymeric lignin isolated from miscanthus and pine, the polymer synthesised using monolignols had a much bigger Mw, a lower Mn, larger PDI. Miscanthus and pine lignin usually has a Mn below 1000, a Mw below 5000 and a PDI around 3. A fractionation would be needed for the synthesised lignin-like polymer before being subjected to any industrial valorisation.

6.2.2 HSQC analysis

HSQC analysis reveals structural information about the polymer synthesised, e.g. the linkages between the adjacent monolignols within the polymer structure. Compared to the native lignin, the polymers synthesis here were similar in terms of major linkages. All the major linkages are in the side chain region (δ_C 50-90 ppm δ_H 2.5-5.8 ppm) or the aromatic region (δ_C 110-130 ppm δ_H 6.0-6.9 ppm). The structure of the linkages or important subunits of the polymer is presented in Figure 6.5, β-*O*-4 ether, β- β resinol, β-5 phenylcoumaran for side chain region and guaiacyl units (G_2, G_5, G_6), p-hydroxyphenyl units ($H_{2,6}$, $H_{3,5}$) and p-hydroxycinnamyl alcohol end groups (I_α, I_β) for the aromatic region.[81] The HSQC spectra of G and H polymers produced are presented in Appendix, Section 'HSQC spectra for G and H polymers synthesised'.

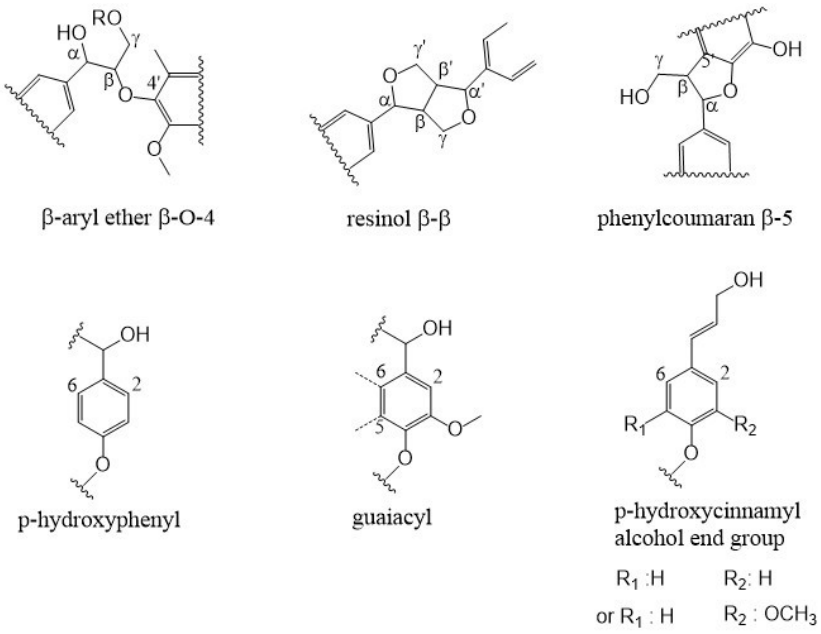


Figure 6. 5 Major linkages and subunits observed in Poly (coniferyl alcohol) and Poly (*p*-coumaryl alcohol)

In the side chain region, for both G and H polymers, the dominate linkages recorded by the HSQC were aryl ether, resinol and phenylcoumaran linkages, which is in line with the experimental observation

for lignin extracted from nature feedstocks (see chapters 3 and 4). During the dehydrogenative polymerisation, the enzyme HRP and the hydrogen peroxide reacted, forming a complex which subsequently reacted with monomers to produce phenoxyl radicals. For each radical, the single electron density was originally located at the phenol group (where a proton was removed from the phenol group). Due to the delocalised electron density around the aromatic ring, the single election density was able to relocate to carbon 5 and carbon 1 of the ring and carbon β on the side chain of the monomer. The phenoxyl radical and its resonance forms are presented in Figure 6.6. Monomers with their single electrons located differently could interact with each other forming different linkages between the two, the possible linkages were β-*O*-4, β-β, and β-5, as detailed in Figure 6.7. The HSQC has confirmed the formation of these three linkages.

In aromatic region, three signals for uncondensed guaiacyl units (G_2, G_5, G_6) were detected for G polymer and two signals for *p*-hydroxyphenyl units ($H_{2,6}$, $H_{3,5}$) were recorded for H polymer. Two peaks representing the p-hydroxycinnamyl alcohol end groups (I_α, I_β) were observed in both polymers.

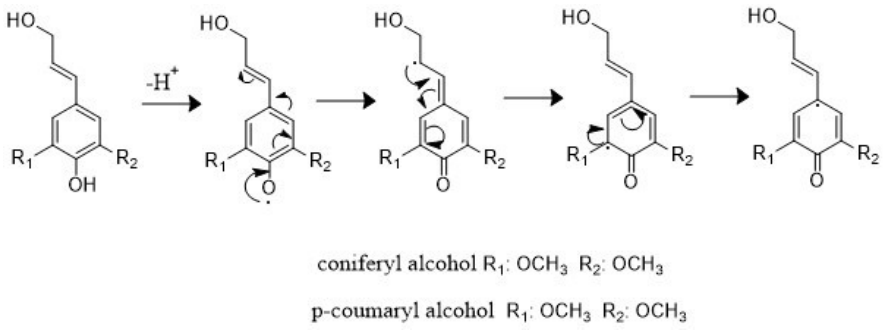


Figure 6. 6 Possible resonance forms of the phenoxyl radicals generated during dehydrogenative polymerisation

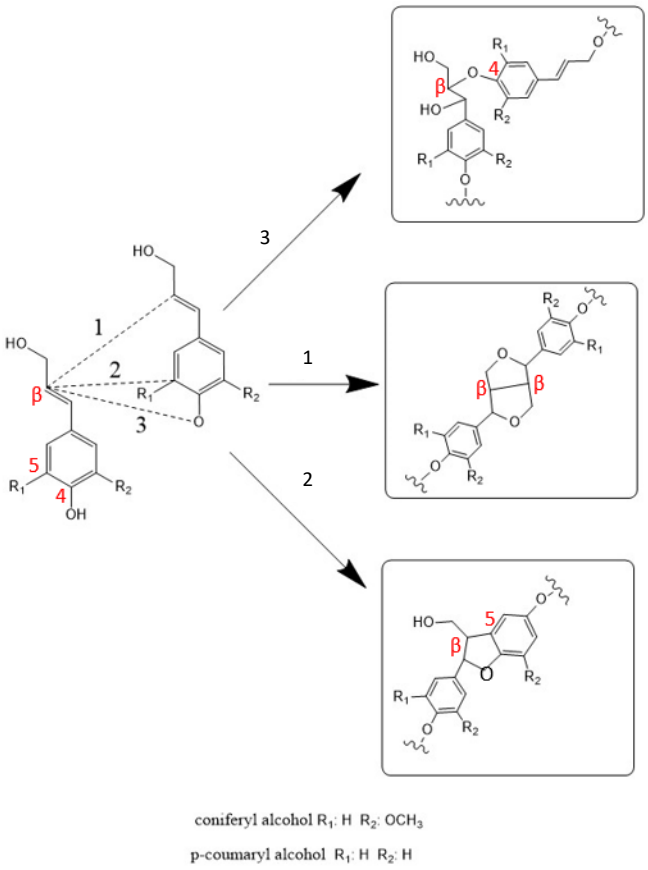


Figure 6. 7 Possible linkage formation between the monolignols at carbon β position

Chapter conclusion

A two-step synthesis pathway was developed here to produce three monolignols, sinapyl alcohol, coniferyl alcohol and *p*-coumaryl alcohol. The latter two were successfully synthesised while the first one was produced in form of oil which could not be used in the later polymerisation process. Optimization of the current synthesis method or design of a new method is needed for sinapyl alcohol. Dehydrogenative polymerisation using horseradish peroxidase (HRP) and hydrogen peroxide in a mixture of phosphate buffer and dioxane was carried out for coniferyl alcohol and *p*-coumaryl alcohol, and a polymeric form of these two alcohols was generated. According to GPC, poly(coniferyl alcohol) and poly(*p*-coumaryl alcohol) were generated as a mixture of short oligomers and polymers with

longer chains, corresponding to the methanol soluble and DMF soluble fractions of the polymer. Poly(*p*-coumaryl alcohol) was able to generate a small fraction of DMF insoluble polymer fraction, which could not be characterised by GPC. Repeat experiments are needed to confirm the experimental observation made in this study. Elaborating the polymerisation process are needed to developing a reliable (repeatable) process with high quality product (high monomer conversion, high yield for polymers with high degree of polymerisation, low yield for oligmers). Other polymer characterisation analysis should be used to reveal more physical and chemical properties of the poly(monolignol), e.g elemental analysis, ^{31}P NMR(describing the functionalities within the polymer structure, relate to the chemical activity and physical properties of the polymer, influencing its melting performance in carbon fiber production), TGA (describe the thermal decomposition of the polymer), fiber spinning process (determining the thermostablisation and carbonization behaviour for the polymer, relate to the performance of the polymer for carbon fiber production). [251,257,265,266]

Conclusion and future work

Organosolv-ionoSolv pretreatment: a summary

Comparing to conventional ionoSolv pretreatment, the new hybrid pretreatment developed, also named as organoSolv-ionoSolv pretreatment, is superior in terms of: 1) larger amount of sugar, including glucose and hemicellulose, is released during saccharification assay; 2) the lower degree of lignin condensation; 3) aryl ether linkages are partially preserved via taking a part of alcohol-induced α-alkoxylation, instead of chemically transforming into carbon-carbon single bonds.

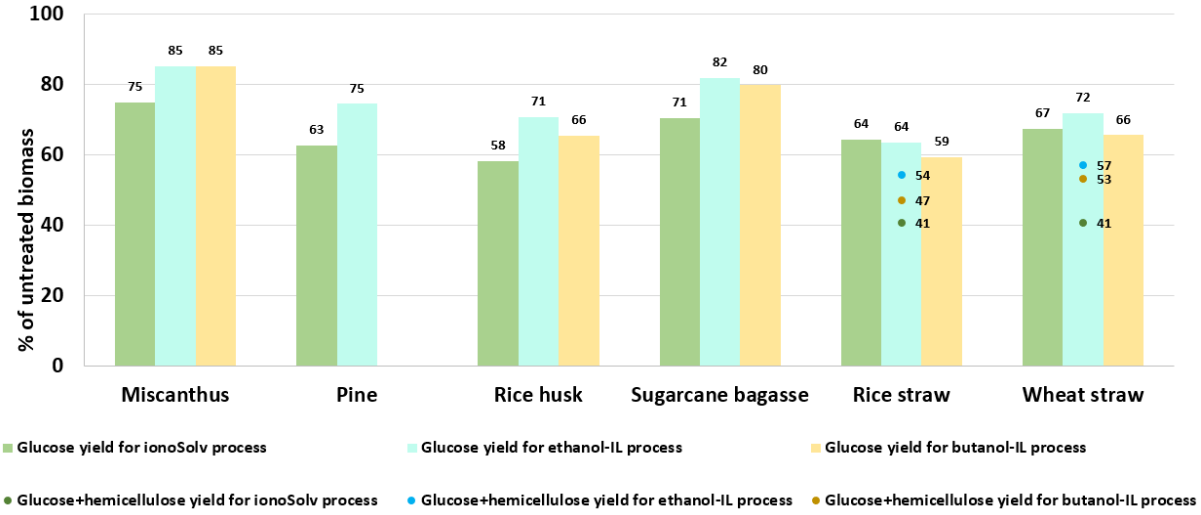


Figure 7. 1 A summary of glucose and hemicellulose releasing yields for both ionoSolv and organosolv-ionoSolv pulps. For straws, glucose+hemicellulose yield is referred to the total sugar releasing yield of the pulp.

According to Figure 7.1, it clearly shows that the organosolv-ionoSolv process is more powerful towards fractionating biomass, comparing to ionoSolv process. For grassy feedstock (miscanthus), softwood (pine), rice husk and bagasse, replacing aqueous IL with ethanol-IL mixture resulted in a ≤13% glucose yield increase. The fractionation ability of the butanol-IL mixture was less profound, as the glucose yields of butanol-IL processes were only ≤10% higher than those of ionoSolv processes. For straws, switching pretreatment solvent from aqueous IL to organic-IL mixture did not result in an increase of glucose yield but an increase of hemicellulose yield. If counting in both glucose and

hemicellulose releasing yields, the total sugar yields of IL-pretreated straws were around 41%, but the yields of organic-IL-pretreated straws were ≤57%, around 16% higher.

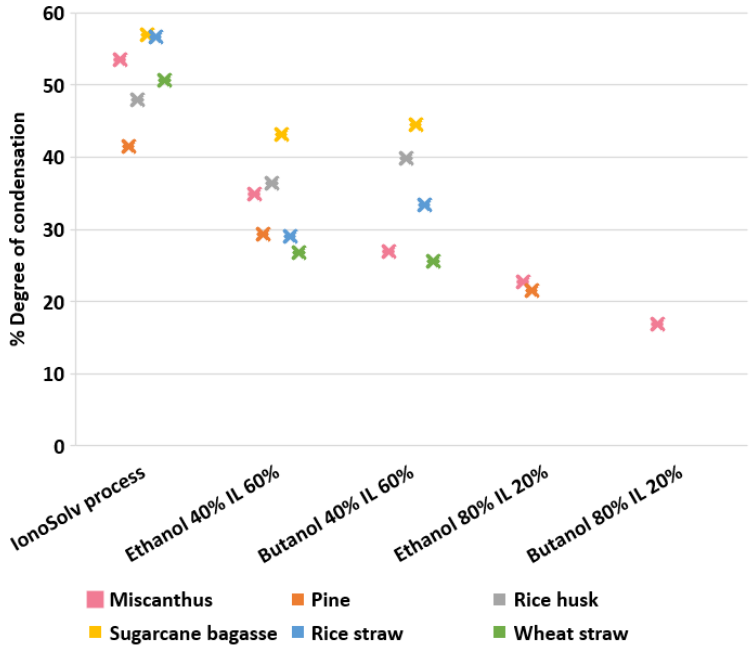


Figure 7. 2 The degree of lignin condensations for ionoSolv and organosolv-ionoSolv pretreatments

As a pretreatment solvent, organic-IL mixtures are more powerful than aqueous IL. This is because the former could not only produce more enzyme-accessible pulps, but also generate lignins with a less condensed structure. Figure 7.2 provides the information about the degree of lignin condensation for All processes. IonoSolv lignins had the highest degree of lignin condensations, regardless the feedstocks. For miscanthus and pine, the ethanol content of pretreatment was negatively related to the degree of lignin condensation. For miscanthus, butanol-IL lignins was less condensed than ethanol-IL lignins, but this was not the case for agricultural residues. The reason for ethanol/butanol-IL lignins are less condensed is: during organosolv-ionoSolv pretreatment, aryl ether linkages reacted with ethanol/butanol and formed new ether linkages via α-alkoxylation; while these linkages were leaved and forming new carbon-carbon single bonds via condensation. According to Figure 7.3, the amount of aryl ether bonds involved in α-alkoxylation reactions was positively related to the ethanol/butanol content of pretreatment.

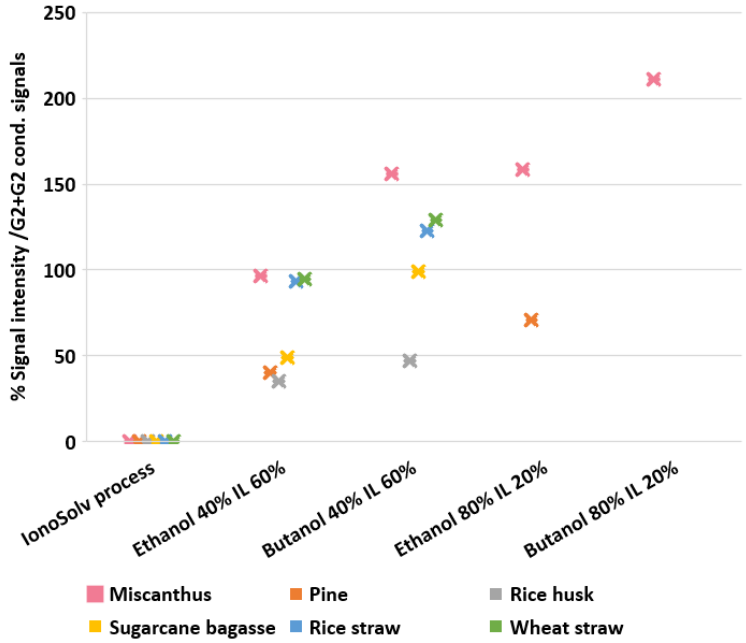


Figure 7. 3 The percentage signal intensities of alkoxylated aryl ether linkages for all processes based on the semi-quantitative analysis of the HSQC spectra

Miscanthus

An organosolv-ionoSolv fractionation process was built based on the organosolv and ionoSolv processes. The hybrid process used a mixture of organic solvents and [TEA][HSO_4] to pretreat miscanthus, and its fractionation performance were compared to the conventional ionoSolv process. The process using 40% ethanol/ butanol achieved the highest glucose yield, 85%, 10% higher than the ionoSolv process. Incorporating acetone with IL to pretreat biomass did not improve the overall process effectiveness. This new process was further tested with different IL acidities (acid/base=1.02, 0.98), suggested that the process outcomes were identical at acid/base ratio between 1 to 1.02. This hybrid pretreatment maintained a compatible preformance up to 50% wt. biomass loading. Isolated lignin was characterised by HSQC NMR and GPC. The organic-IL lignins were less condensed relative to ionoSolv lignin due to the change in the dominant lignin modification during fractionation, and α-alkoxylation induced by primary alcohols was found out to the signature modification happening in this hybrid process. The less condensed lignin structure could effectively rise the economic value of

the lignin fraction, where the lignins isolated would be more suitable for high value-added applications. This organosolv-ionoSolv pretreatment has provided proofs for a biomass fractionation process, which could generate a highly accessible pulp and high-quality side products. This is vital for the development of current ionoSolv technology in the large scale biorefinery.

Although acetone is excellent organosolv solvent due to its high lignin dissolution ability, it is a bit surprise for acetone-IL pretreatment not to achieve a more profound results than the aqueous acetone/ IL processes, further investigation should be done understanding this unexpected observation. As ionoSolv process is highly sensitive to the acid base ratio of the IL, it has a narrow acidity range (less than 2% acid) for optimum fractionation performance. Based on the experimental observations, the hybrid process seems to be less sensitive, therefore it is possible to conduct this hybrid process using more aciditc ILs, e.g. ILs with 5% , 10% or even 20% acid excess. The upside of the using ILs with excess acid is the lower solvent generation cost, relative to IL with a acid to base ratio of 1.

Pine

The hybrid process using ethanol and [DMBA][HSO_4] was repeated with pine, one type of softwood. The optimal solvent composition was anhydrous IL with 20% wt. ethanol. This process increased the saccharification yield by 12% due to an improved lignin removal, suggested that this newly built fractionation is feedstock-independent. For recalcitrant biomass, an improved fractionation performance could be obtained by increasing the IL content. Increasing the IL acidity increases the harshness of the process, and softwoods are generally harder to pretreat, comparing to hardwood and grasses. ILs with excess acid could be test for this recalcitrant feedstock.

Agricultural residues

The time course experiments for rice husk were conducted at 170°C and 150°C. The optimal fractionation effectiveness was achieved by 45min, 170°C. Based on this time-course study, the optimal ionoSolv process conditions were suggested for rice straw, wheat straw (30 min, 170°C) and bagasse (45min, 170°C). The experimental results confirmed that those ionoSolv processes successfully fractionated all four feedstocks, by obtaining glucose yields up to 90%. Most of the ash was preserved in pulps for these high ash-content feedstocks. lignin degradation was suspected to happen according to compositional analysis and was confirmed by HSQC. Further optimization of the process for better lignin fractionation might be needed. A time course with a smaller time interval < 15 min should be carried out, as the sign of the overtreatment was detected for less dense feedstocks, such as straws. By further optimizing the pretreatment duration, the lignin condensation could be effectively prevented.

The organosolv-ionoSolv process was also conducted for these agricultural residues. Overall, an improved fractionation was detected for all feedstocks. For rice husk and bagasse, approximately 12% glucose yield increase was observed relative to the corresponding ionoSolv process. For straws, an additional hemicellulose release was detected by saccharification assay and their cellulose releases maintained at the same level, resulting in an increased total sugar yield. The decreased lignin condensation was suggested by HSQC. Ethanol/butanol-IL lignins isolated from all four feedstocks possessed a similar lignin subunits composition, which were highly α-ethoxylated and not hemicellulose free. For those feedstocks having hemicellulose releases during the hydrolysis, the fermentation process could be carried out to check whether the hemicellulose could be easily converted into biofuel alone with the cellulose, and no specific microbial enzyme would be needed. This has been a issue for hemicellulose fermentation, as many current biorefinery process require the

recovered hemicellulose fraction to be fermented separate to the cellulose, as different enzymes are needed for these fractions.

Synthesis of monolignols and lignin-like polymer

A synthesis protocol was built for sinapyl alcohol, coniferyl alcohol and *p*-coumaryl alcohol. Apart from sinapyl alcohol, the other two alcohols were successfully synthesised. Sinapyl alcohol was obtained as an orange oil, and its correspond polymerisation was a failure as no polymer was successfully generated. Dehydrogenative polymerisation using horseradish peroxidase (HRP) was preformed for coniferyl and *p*-coumaryl alcohols, subsequently two homopolymers were generated. GPC suggested that poly(coniferyl alcohol) and poly(*p*-coumaryl alcohol) synthesised were mixtures of monomers, short oligomers and polymers, where the polymer and oligomers had larger molecular weights comparing to the isolated lignin from nature feedstocks. Poly(*p*-coumaryl alcohol) was able to generate a small fraction of DMF insoluble polymer fraction (polymer with super long chain), which could not be characterised by GPC.

Repeats are needed to improve the accuracy and reliability of the experiments. Adjustments of the current synthesis route for sinapyl alcohol are required. The polymerisation protocol should be carefully altered in order to make the dehydrogenation process repeatable. Other characterisation techniques should be applied to describe the physical and chemical properties of these synthetic lignin-like polymers, e.g ^{31}P NMR, TGA, in order to find suitable utilization for these lignin -like polymers.

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Technology Enhancing enzymatic hydrolysis of sugarcane bagasse by ferric chloride catalyzed organosolv pretreatment and Tween, *Bioresour. Technol.*, 2018, **258**, 295–301.

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Appendix

- **s/g ratio for lignin extracted with ethanol-IL mixtures with different IL acidities**

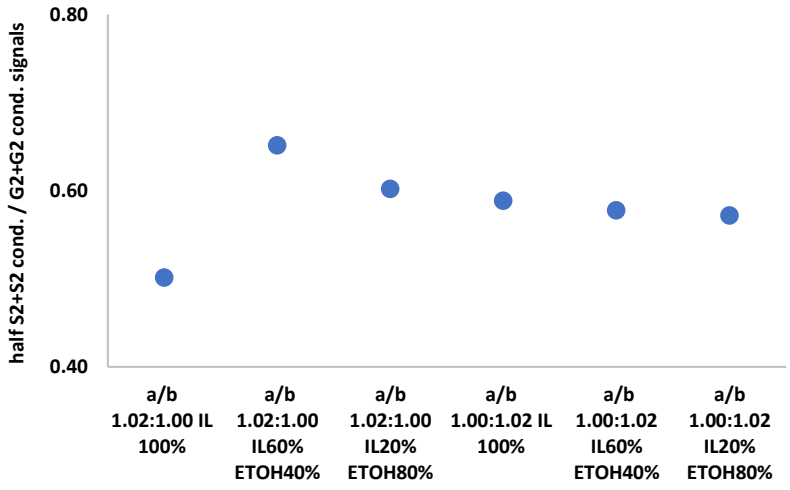


Figure S3.1 The calculated S/G ratio of the extracted lignin based on HSQC NMR spetra for lignins extracted from miscanthus pretreated with ethanol-IL mixtures where two IL acidities were used, a/b=1.02, 0.98.

- **Technoeconomic analysis of the Organosolv-ionoSolv pretreatment (also included in the Electronic Supplementary Information for the paper 'Design of hybrid Organosolv-ionoSolv pretreatment processes for biofuel production and high value-added lignin valorisation ')**

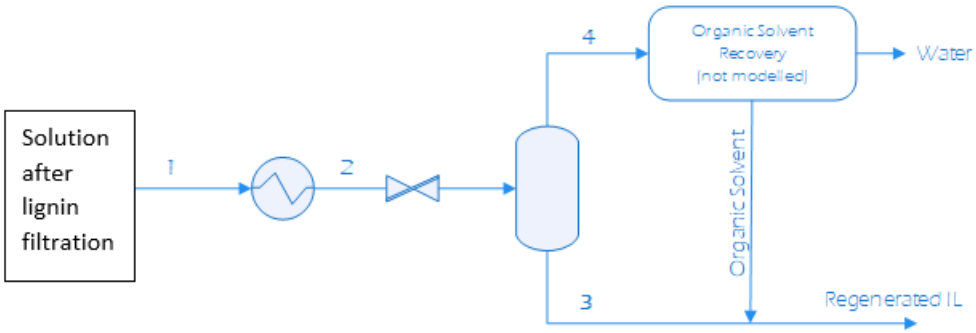


Figure S3.2. Simplified process flow diagram

The energy requirements for drying the IL were modelled as a simple but energy-intensive flash distillation with minimum capital investment cost (CAPEX). The system was modelled in HYSYS V8.8 (thermodynamic package glycol package) with the following assumptions: 1) the diluted solution contains 3 equivalents of water per equivalent of IL in mass basis; 2) IL were dried to 20% wt. water for the IonoSolv processes (0% organic solvent) and to 2% wt for the hybrid processes. The ionic liquid was modelled as triethylene glycol (TEG). This compound has been selected due to its high boiling point, detailed in Table S3.2. Despite the high boiling point and that operating temperatures remain below the normal boiling point, detailed in Table S3.1, traces of TEG was found on stream 4. In the actual system, it had been expected that no trace of IL would present in stream 4 as its vapour pressure is so low, but this should be further confirmed by experimental observations. The heat capacity for TEG is 3.052 kJ/kg°C, which is lower than the experimental value for [TEA][HSO_4], 3.792kJ/kg°C. As we are more interested in the trend rather than the absolute energy consumption, the actual Cp value and the presence of TEG in stream 4, should not impact the conclusion of this analysis. The Cp value for [TEA][HSO_4] is higher than the reported Cp for an imidazole hydrogen sufate IL (1-ethyl-3-methylimidazolium hydrogen sulfate) 1.419 kJ/kg°C.

Table S3.1. Process conditions

Stream	Temperature [°C]	Pressure [barg]	Remarks
1	25	4	Diluted IL for lignin precipitation
2	200-221 (*)	3.5	
3	174-209 (*)	0	Dried Il
4	174-209 (*)	0	Organic Solvent-water mixture

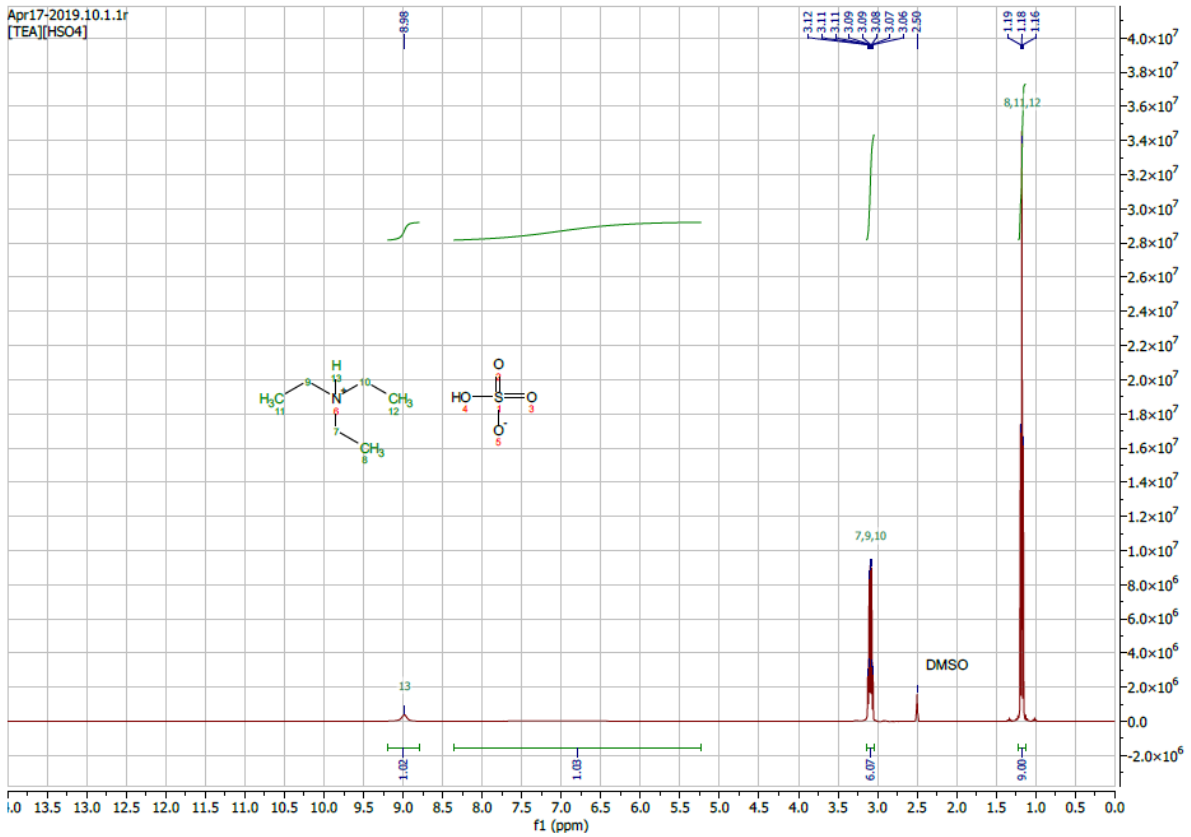
(*)Temperature is a function of the composition

Table S3.2. Compound properties predicted by HYSYS V8.8 with the thermodynamic package glycol package.

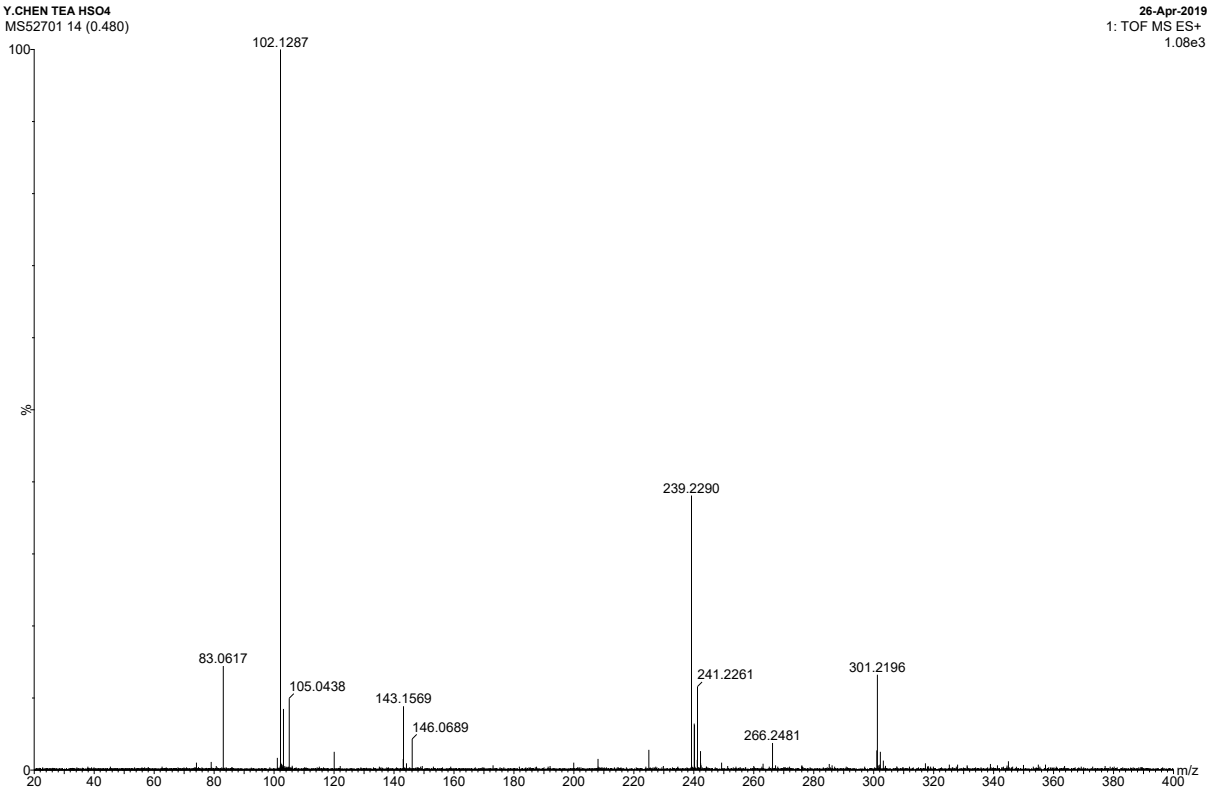
Compound	Molecular Weigh [g/mol]t	Mass Heat Capacity [kJ/kg°C]	Mass Heat of Vap. [kJ/kg]	Boiling Point [°C]
Acetone	58.1	2.022	512.1	56.1
1-Butanol	74.1	2.934	574.4	119.3
Ethanol	46.1	2.726	846.3	78.2
Water	18.0	4.217	2269.8	100.0
TEG	150.2	3.052	396.1	289.5

- **Proton NMR and Mass spectrometry spectra for [TEA][HSO$_4$]**

- ^{1}H NMR for [TEA][HSO4]

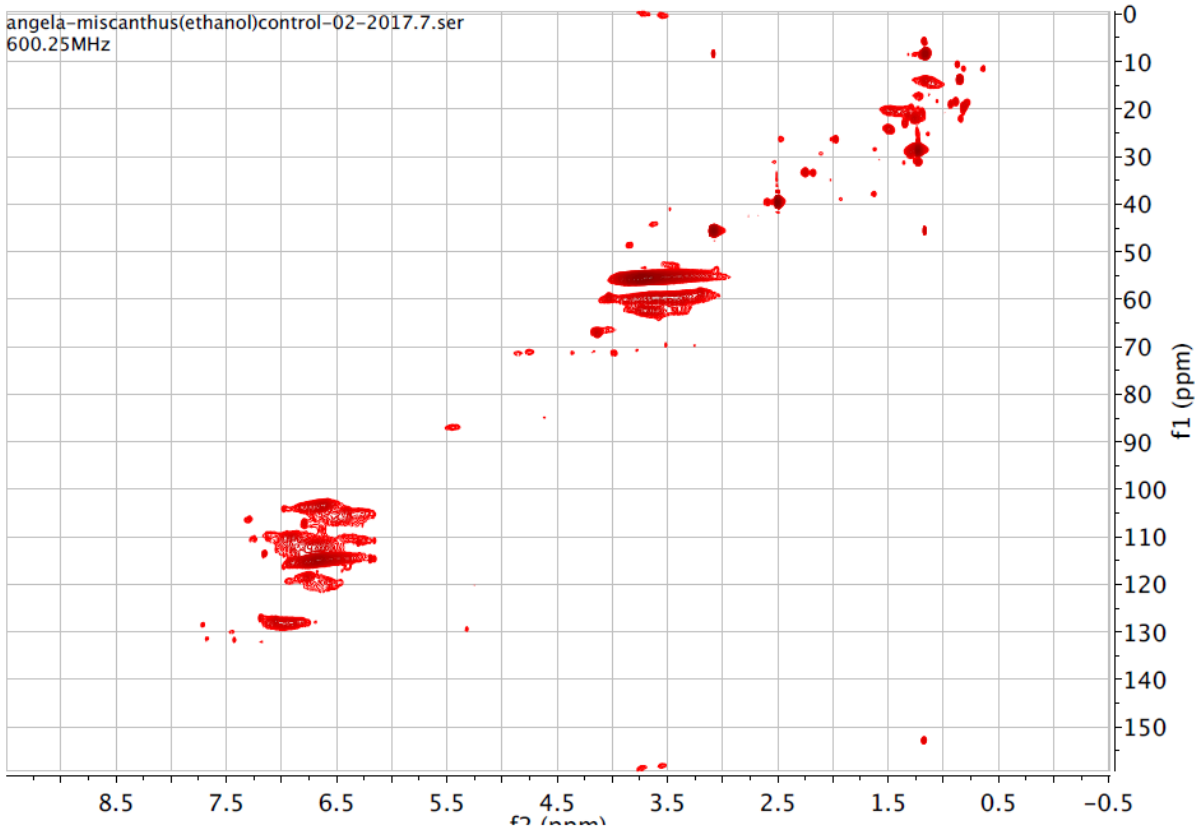


- Mass spectrum for [TEA][HSO4]

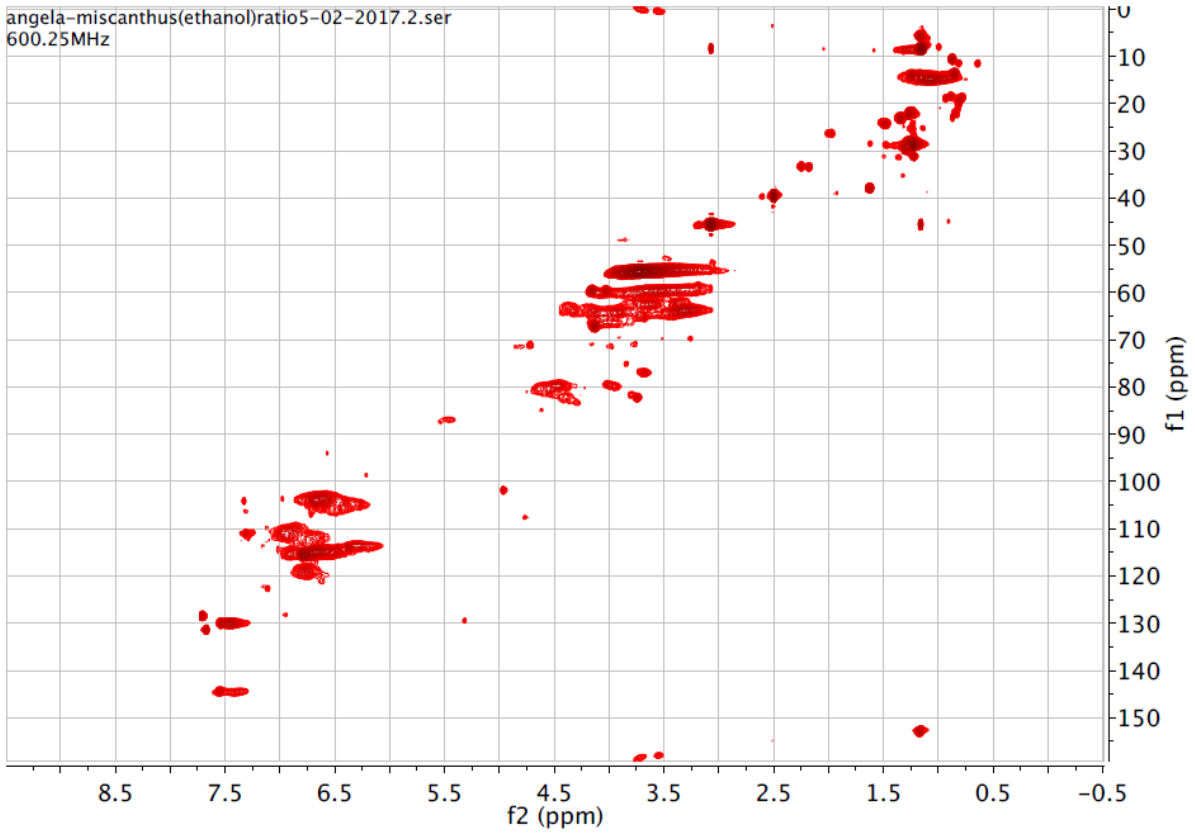


- **HSQC NMR spectra for isolated miscanthus lignins**

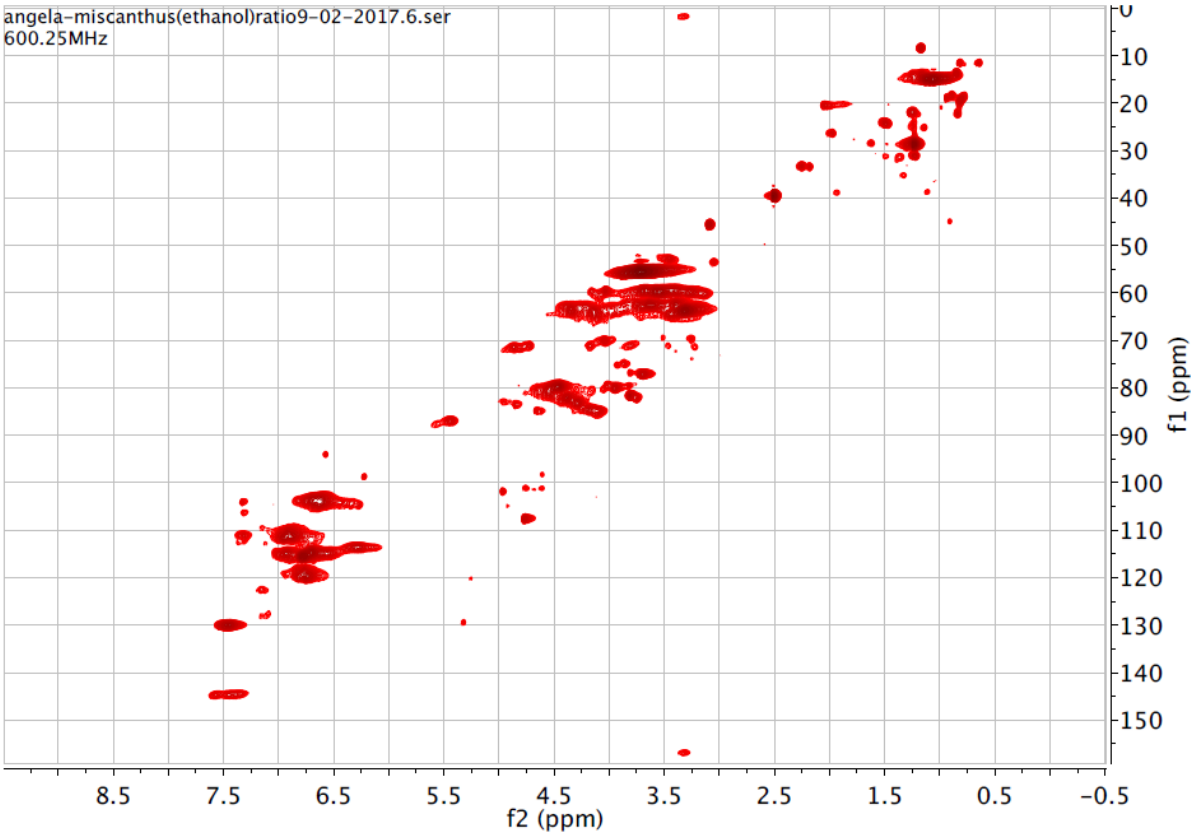
- IL 100% a/b=1



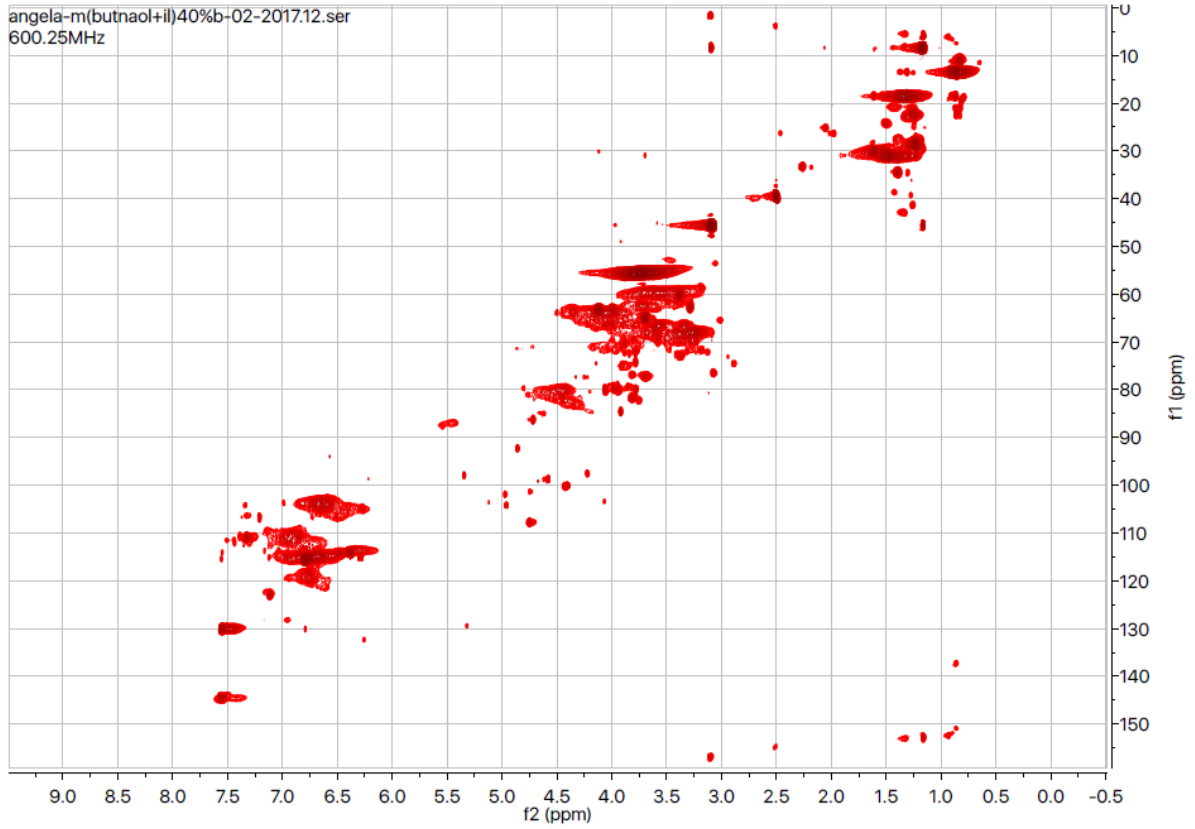
- Ethanol 40% IL 60% a/b=1



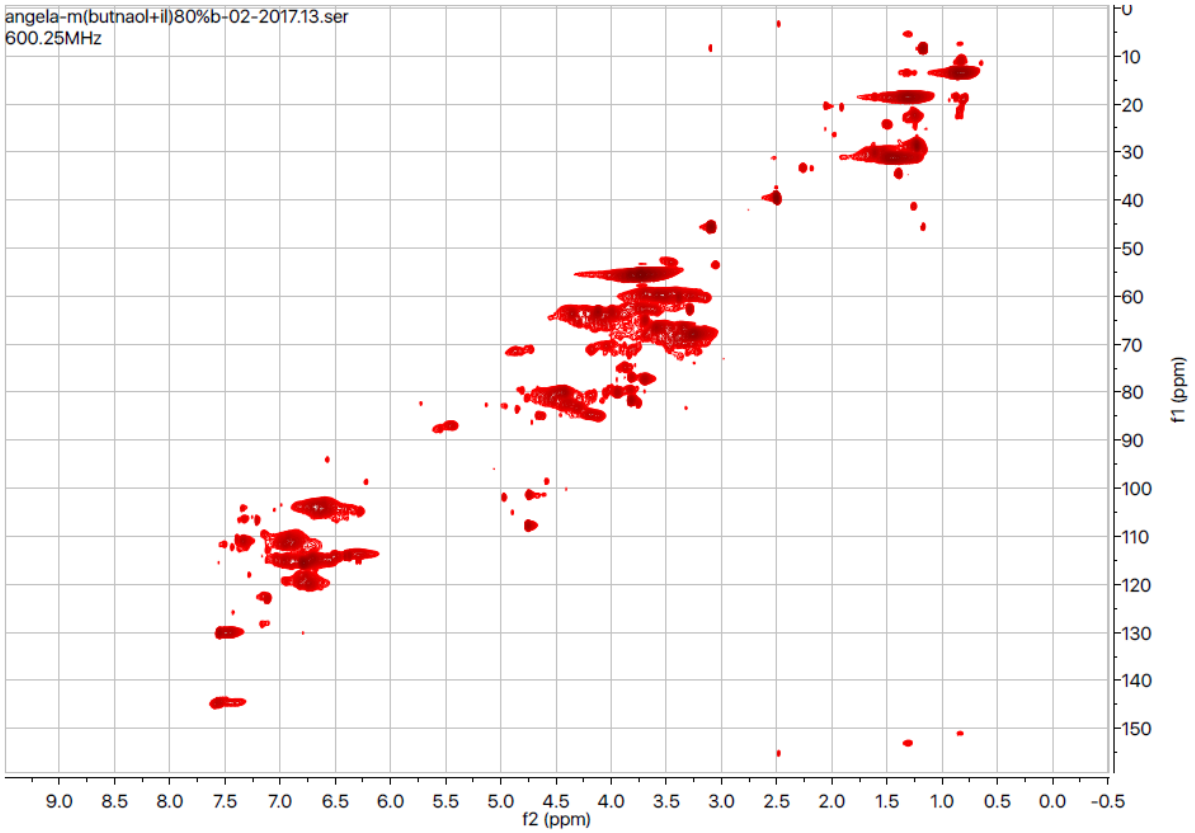
- Ethanol 80% IL 20% a/b=1



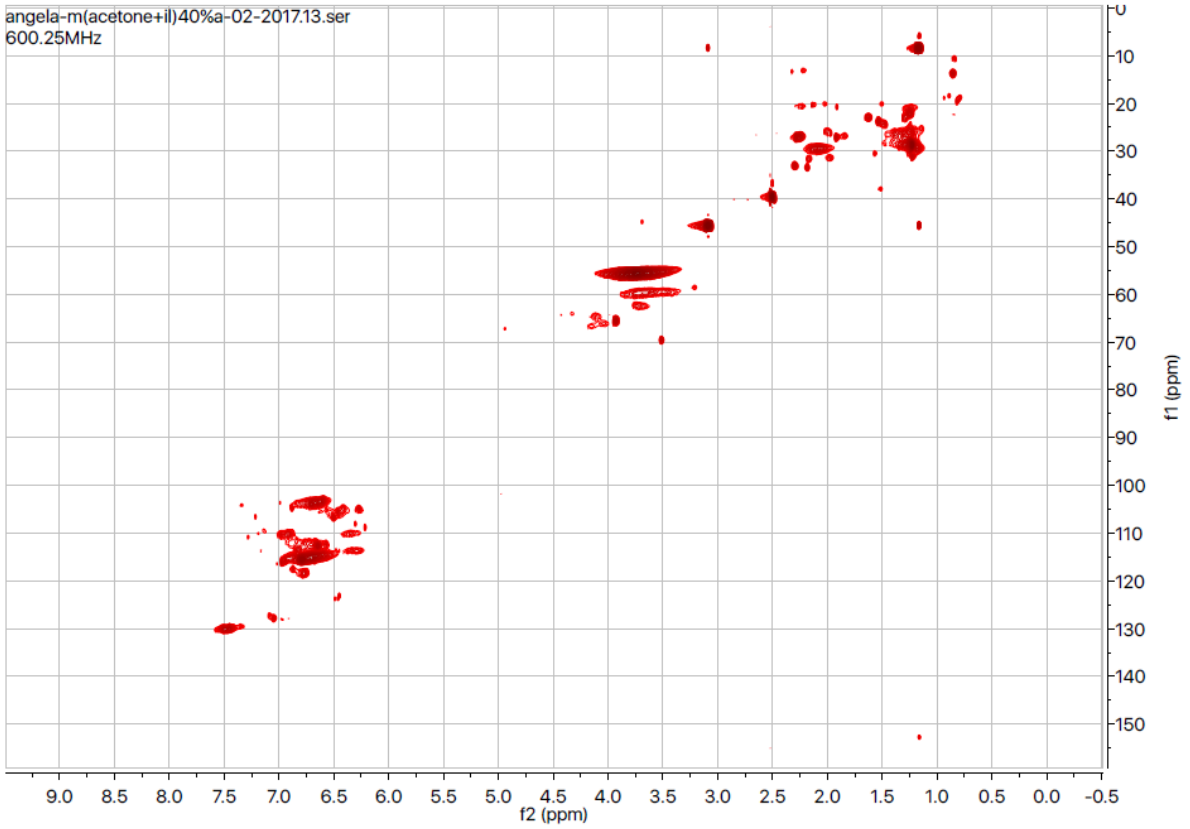
- Butanol 40% IL 60% a/b=1



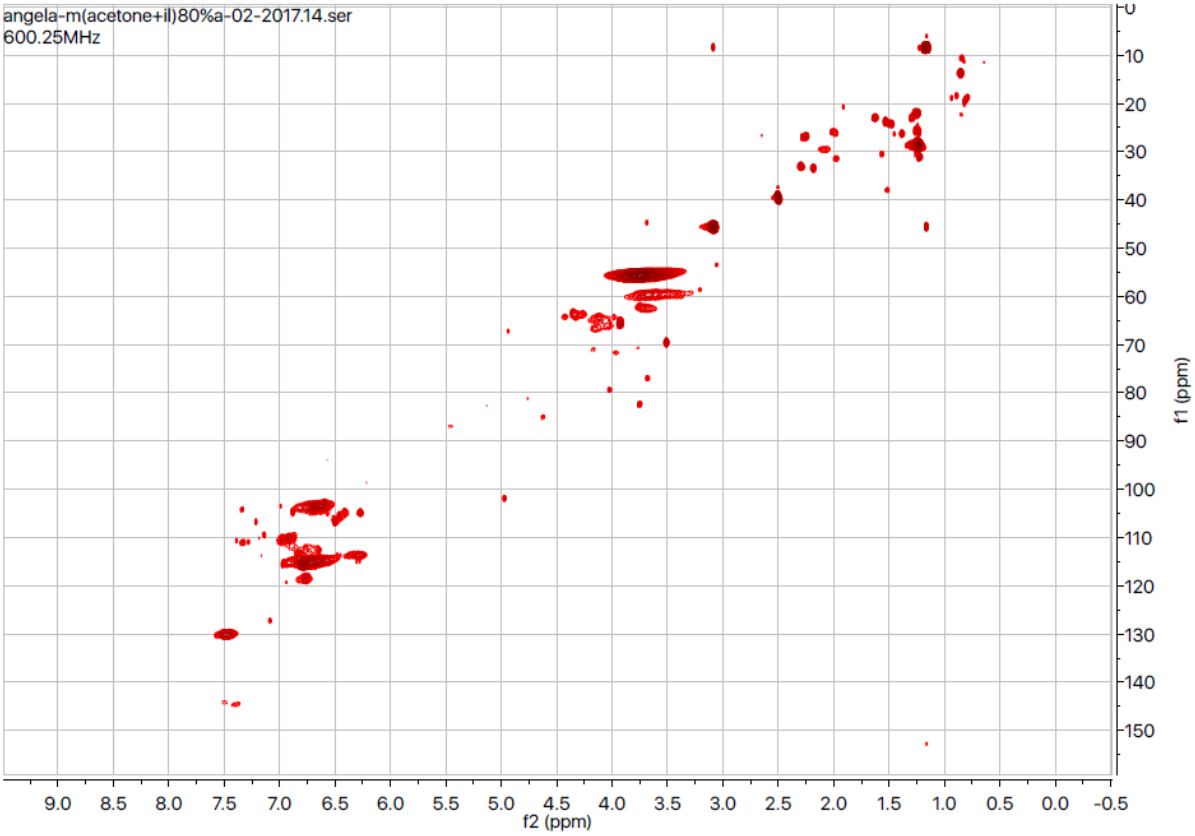
- Butanol 80% IL 20% a/b=1



- Acetone 40% IL 60% a/b=1

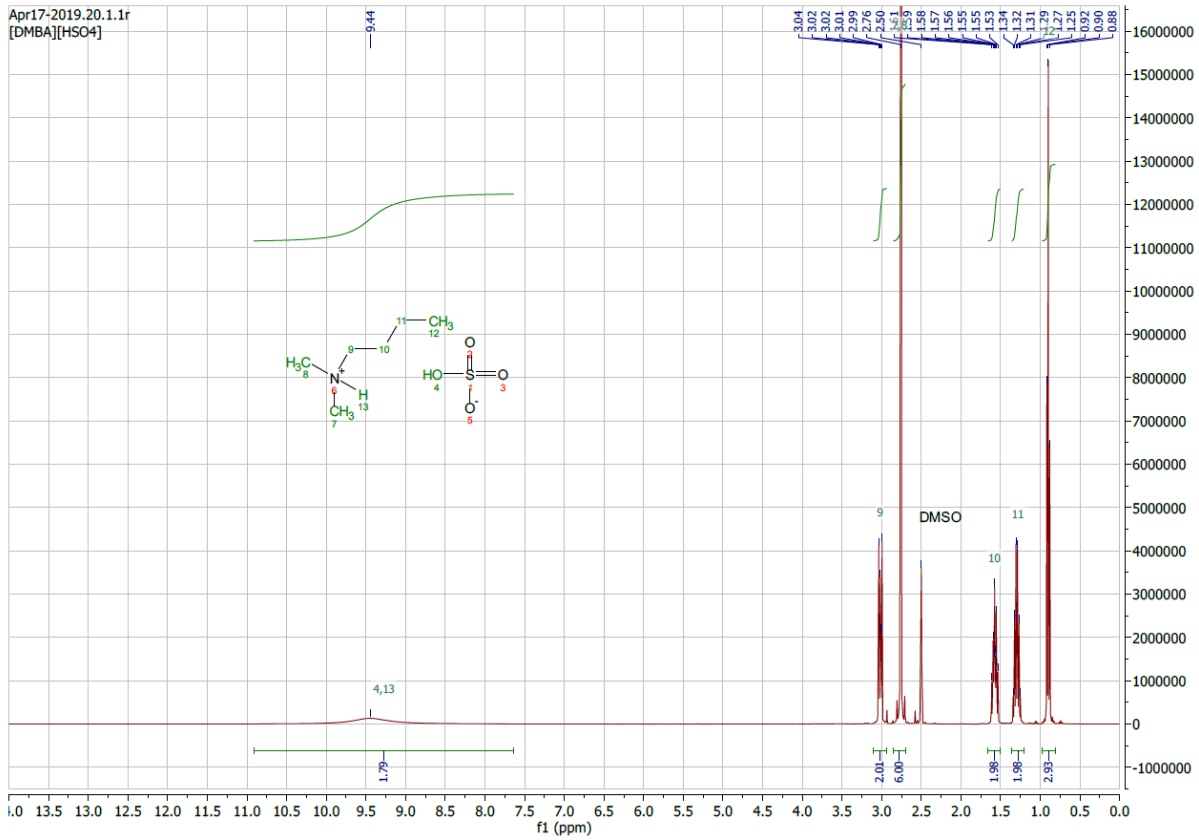


- Acetone 80% IL 20% a/b=1

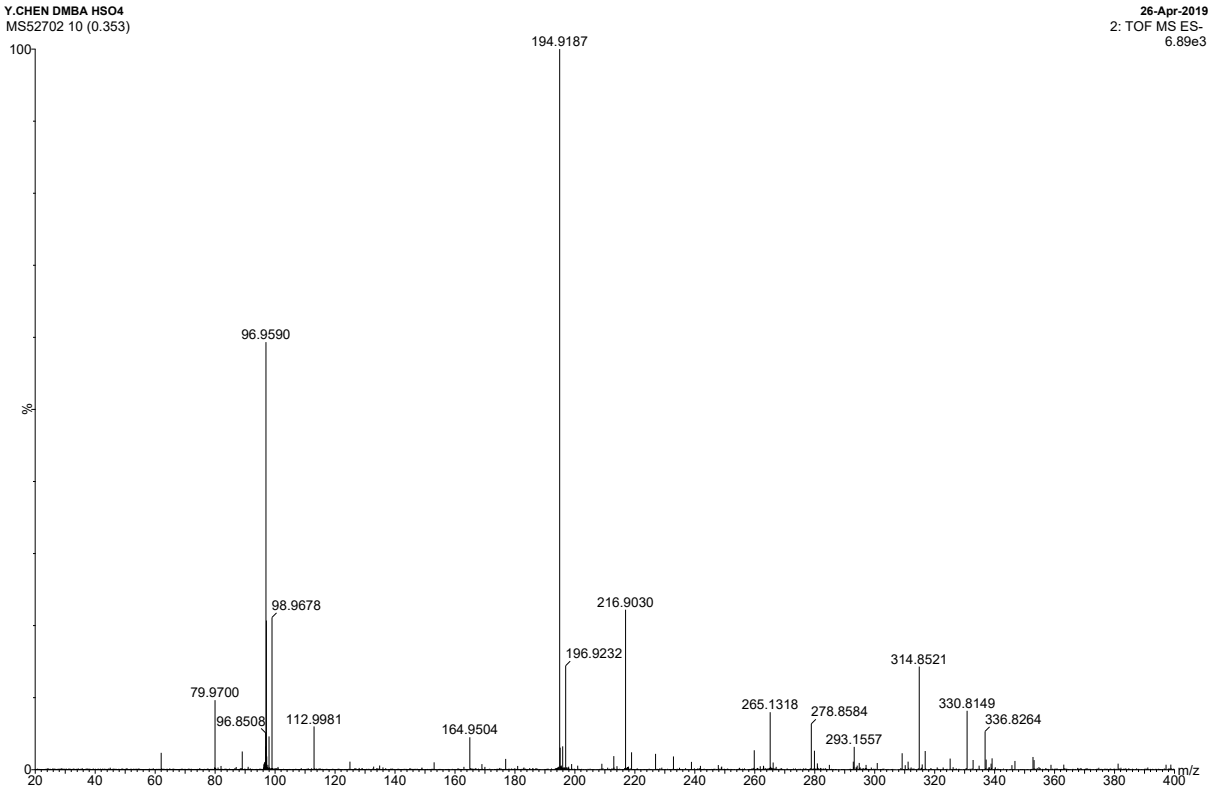


- **Proton NMR and Mass spectrometry spectra for [DMBA][HSO4]**

- ^{1}H NMR for [DMBA][HSO4]

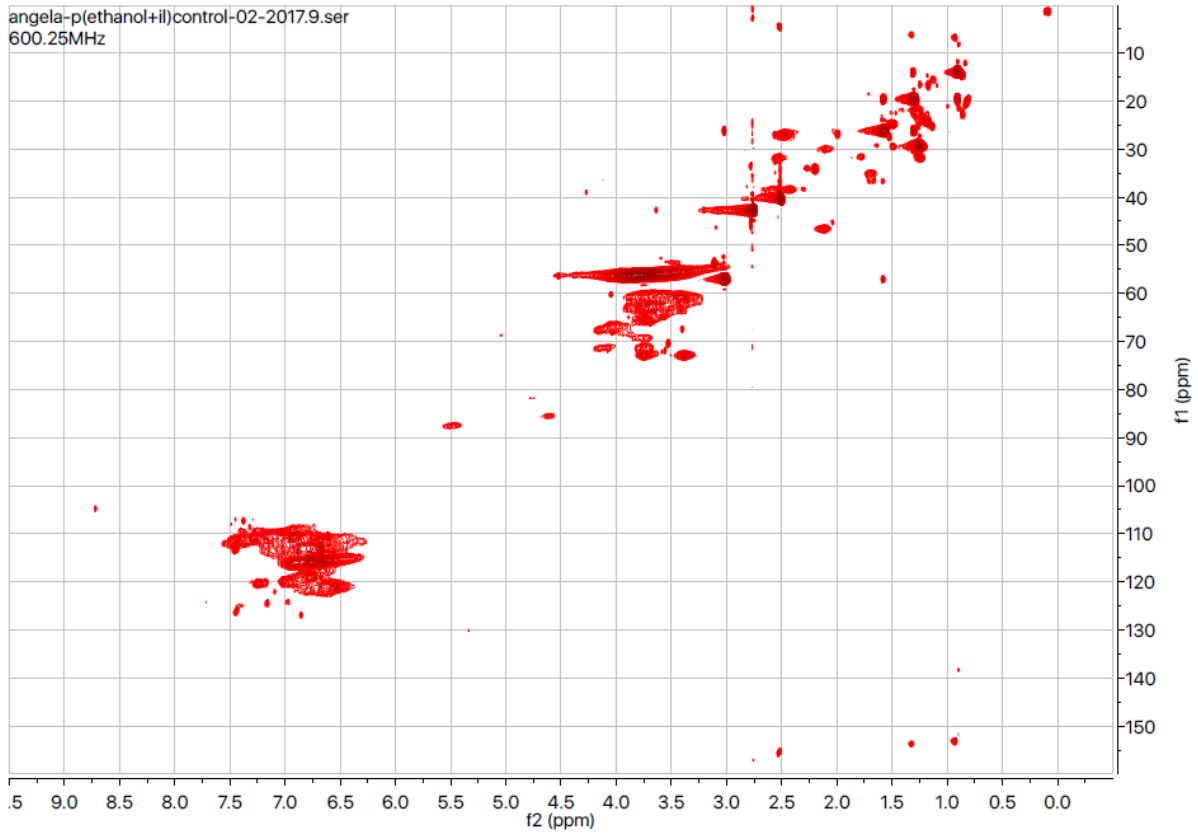


- Mass spectrum for [DMBA][HSO4]

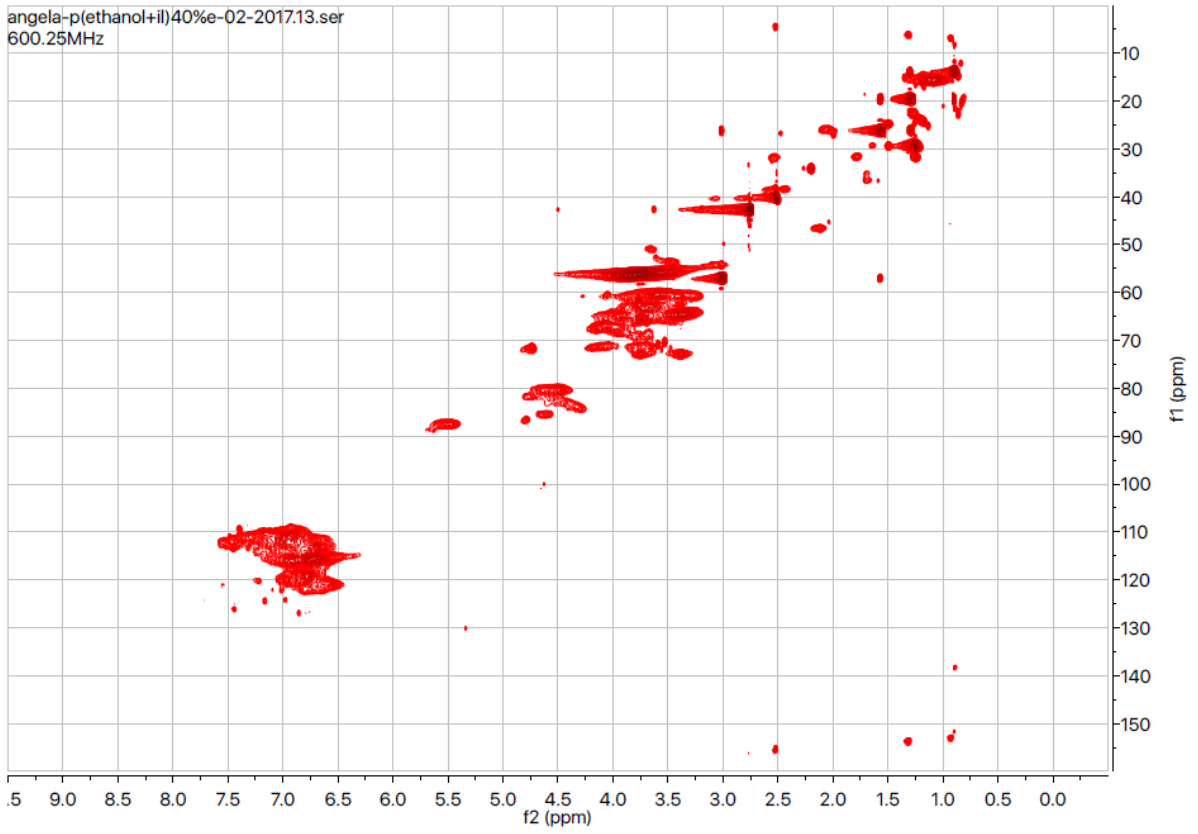


- **HSQC NMR spectra for isolated pine lignins**

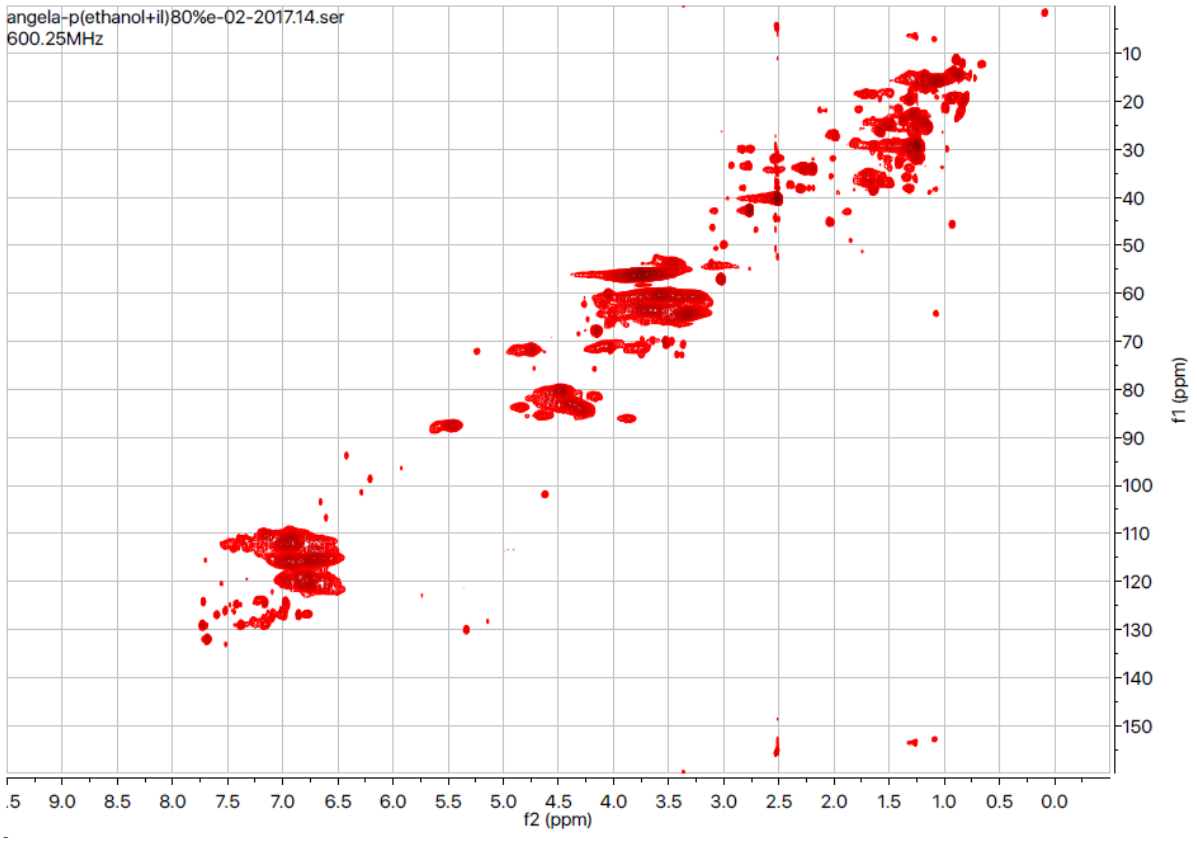
- IL 100% a/b=1



- Ethanol 40% IL 60% a/b=1



- Ethanol 80% IL 20% a/b=1



- **Pulp composition for rice husk pretreated at 150°C and 170°C**

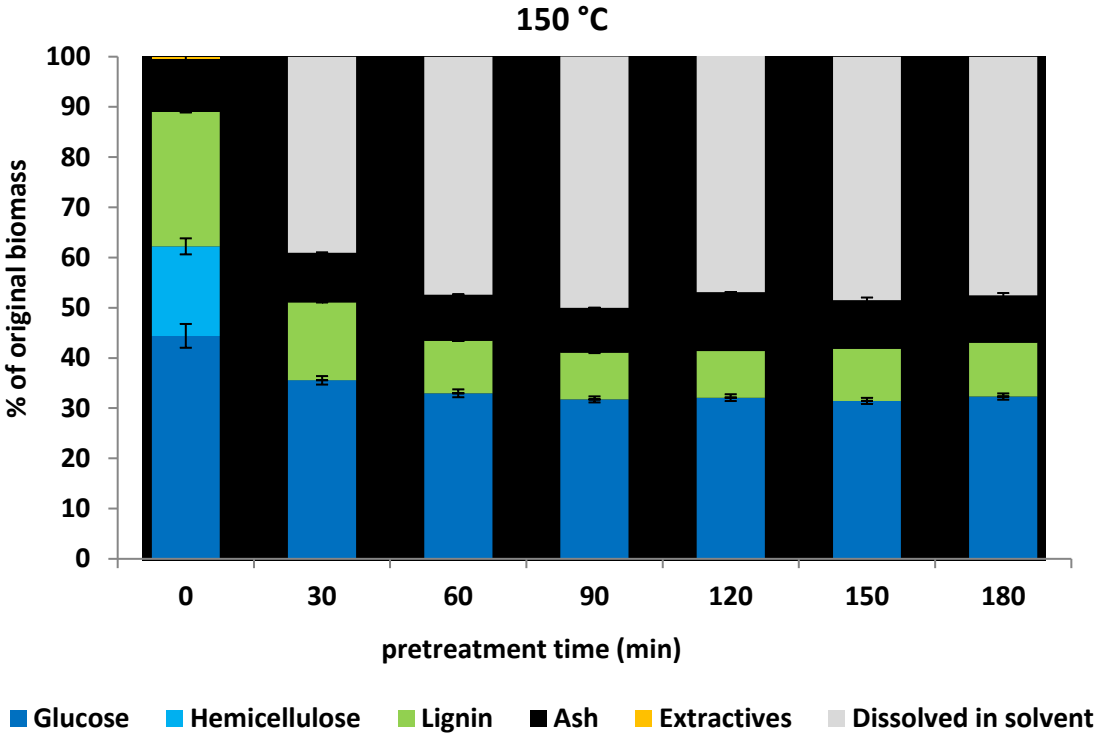


Figure S5-1 Pulp composition of ionoSolv pretreatments for rice husk at 150°C

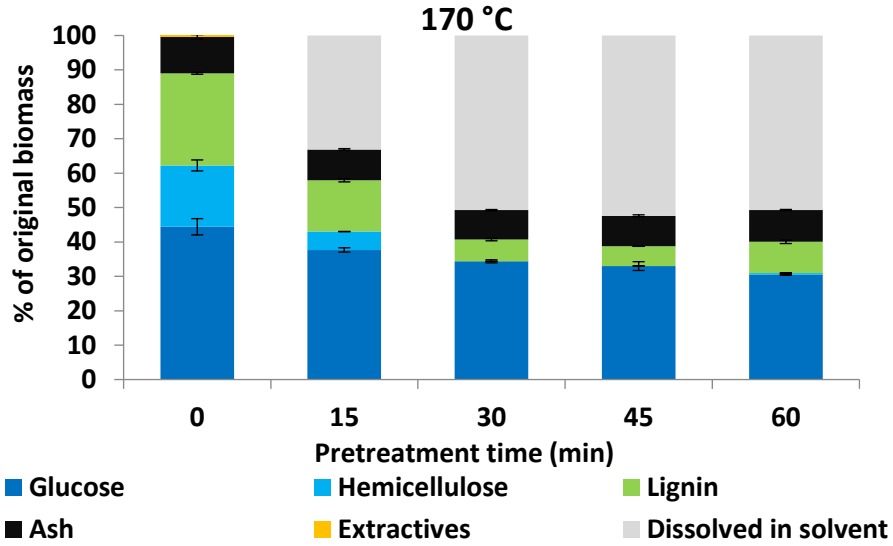


Figure S5-2 Pulp composition of ionoSolv pretreatments for rice husk at 170°C

- **Pulp composition for four agricultural residues pretreated at estimated optimal conditions**

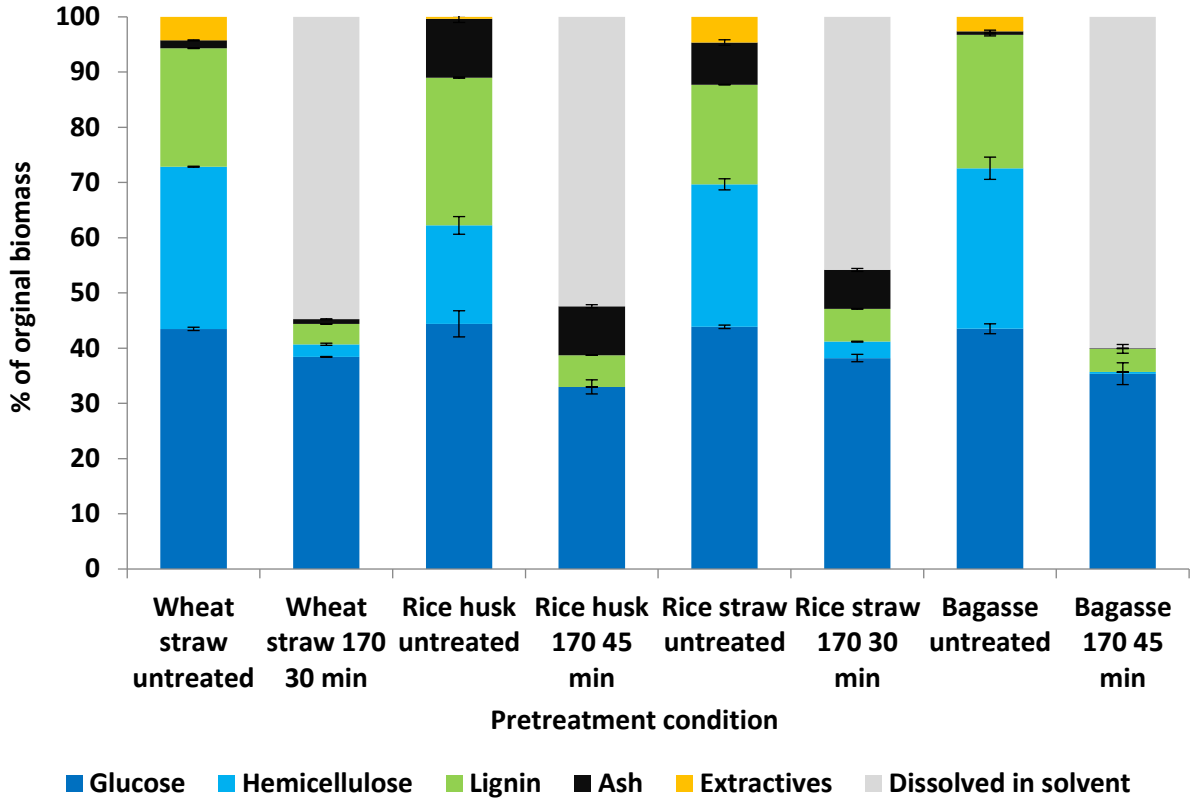


Figure S5-3 Pulp composition of agricultural residues pretreated at 170°C with optimal durations

- **S/G ratio for lignins extracted from agricultural residue**

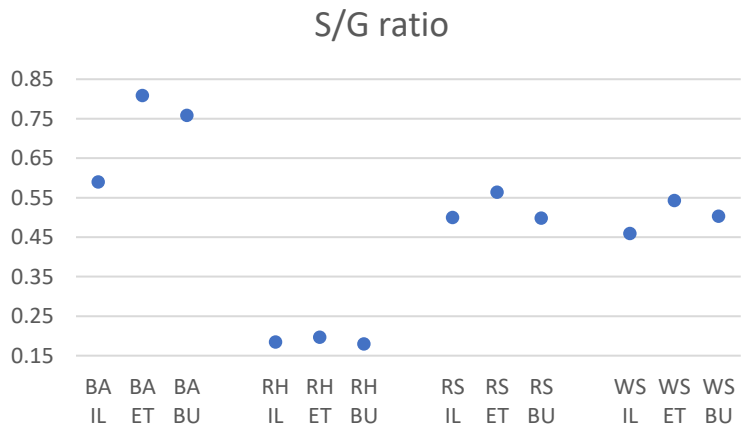
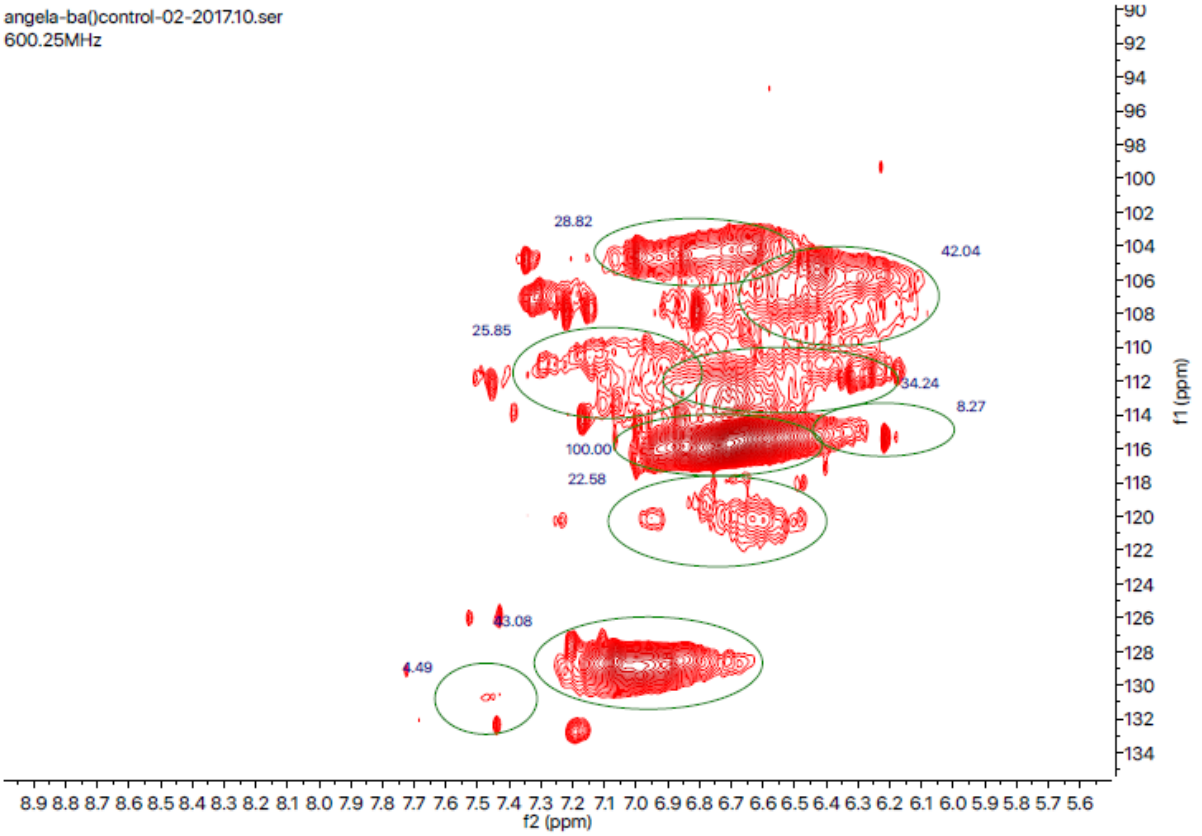
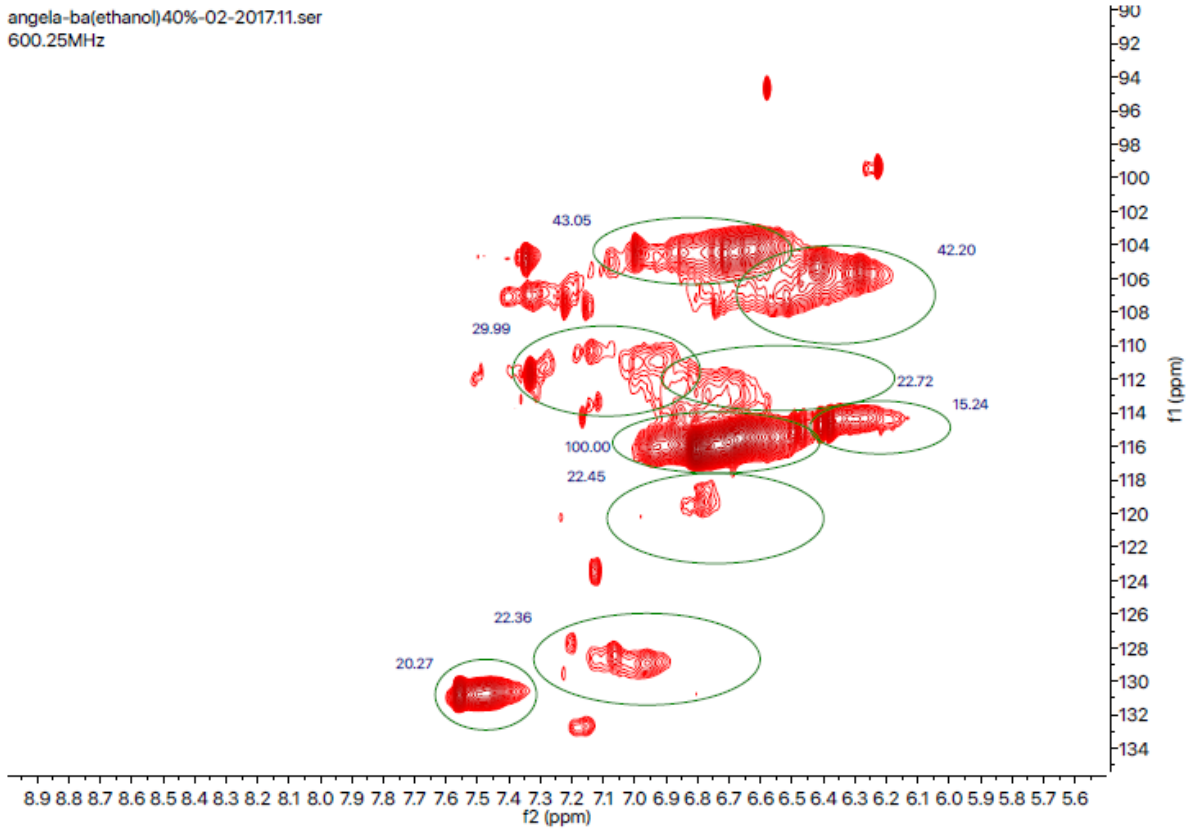
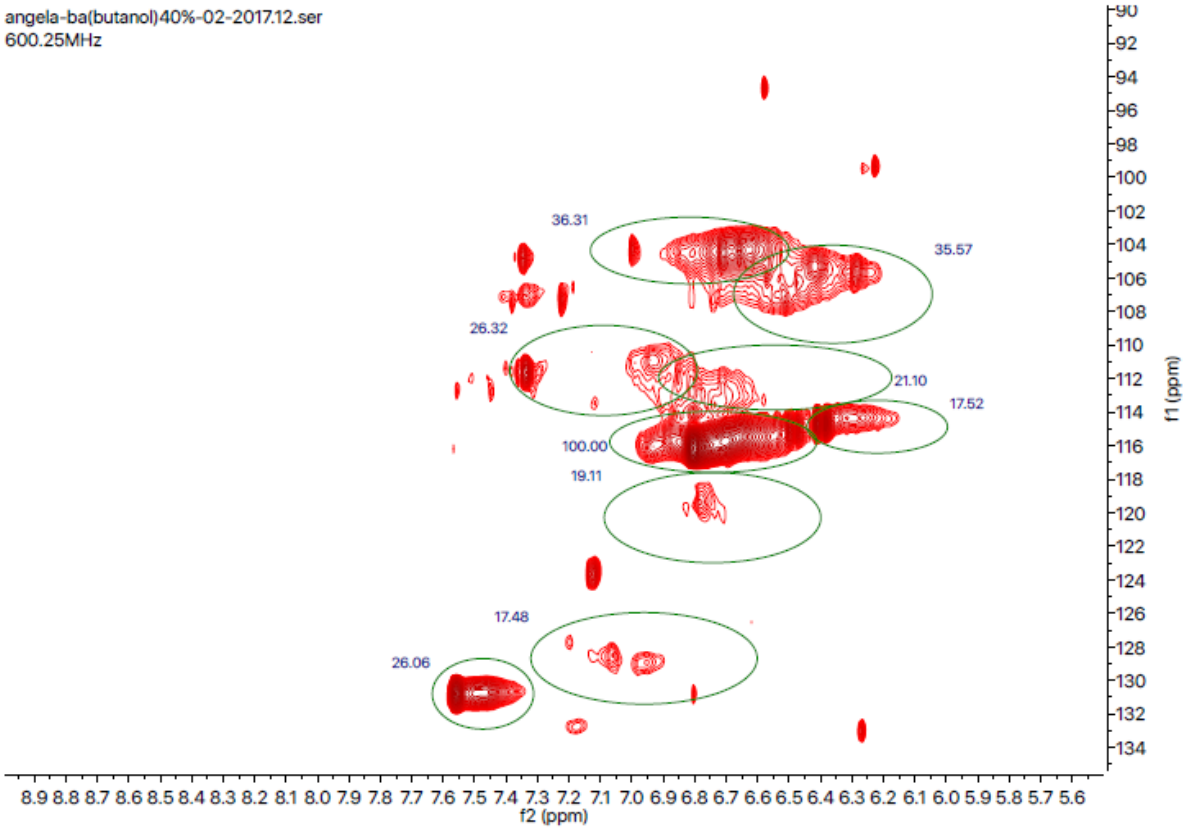


Figure S5-4 S/G ratio for lignins extracted from agricultural residue

- **HSQC NMR spectra for isolated lignins**



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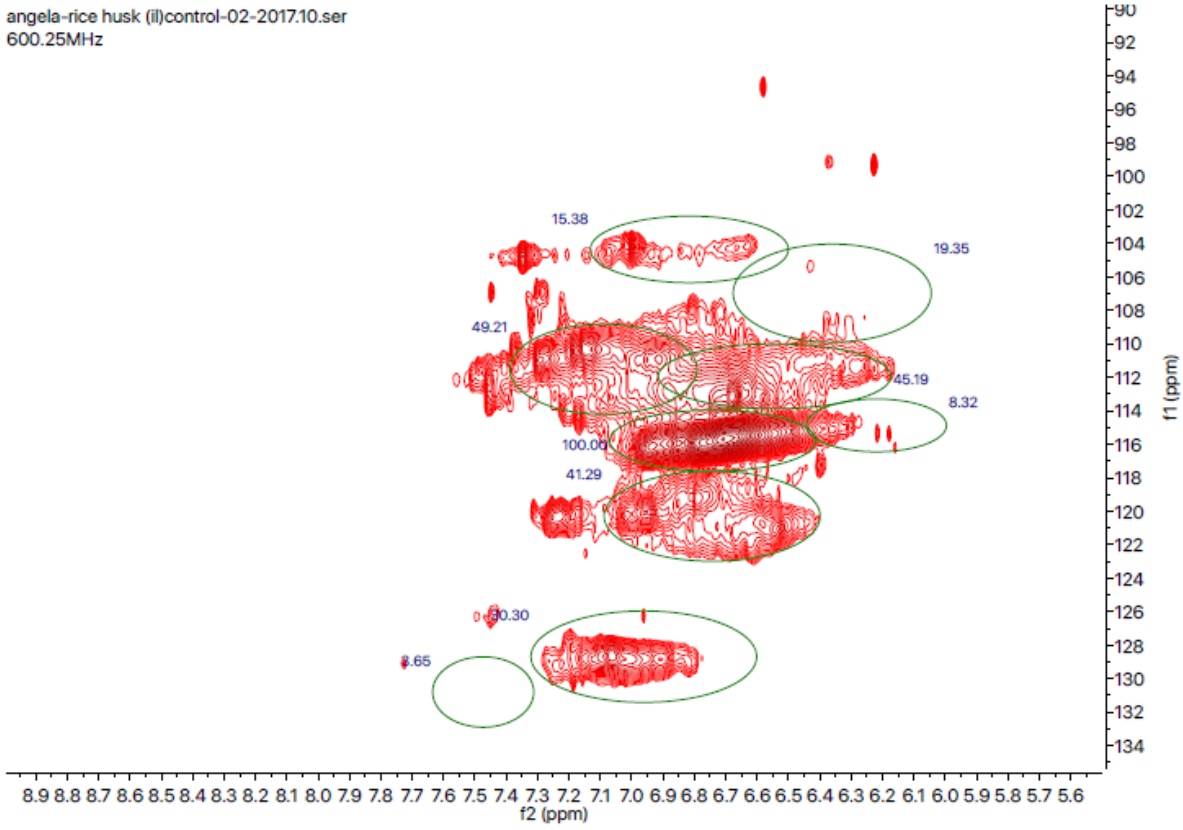
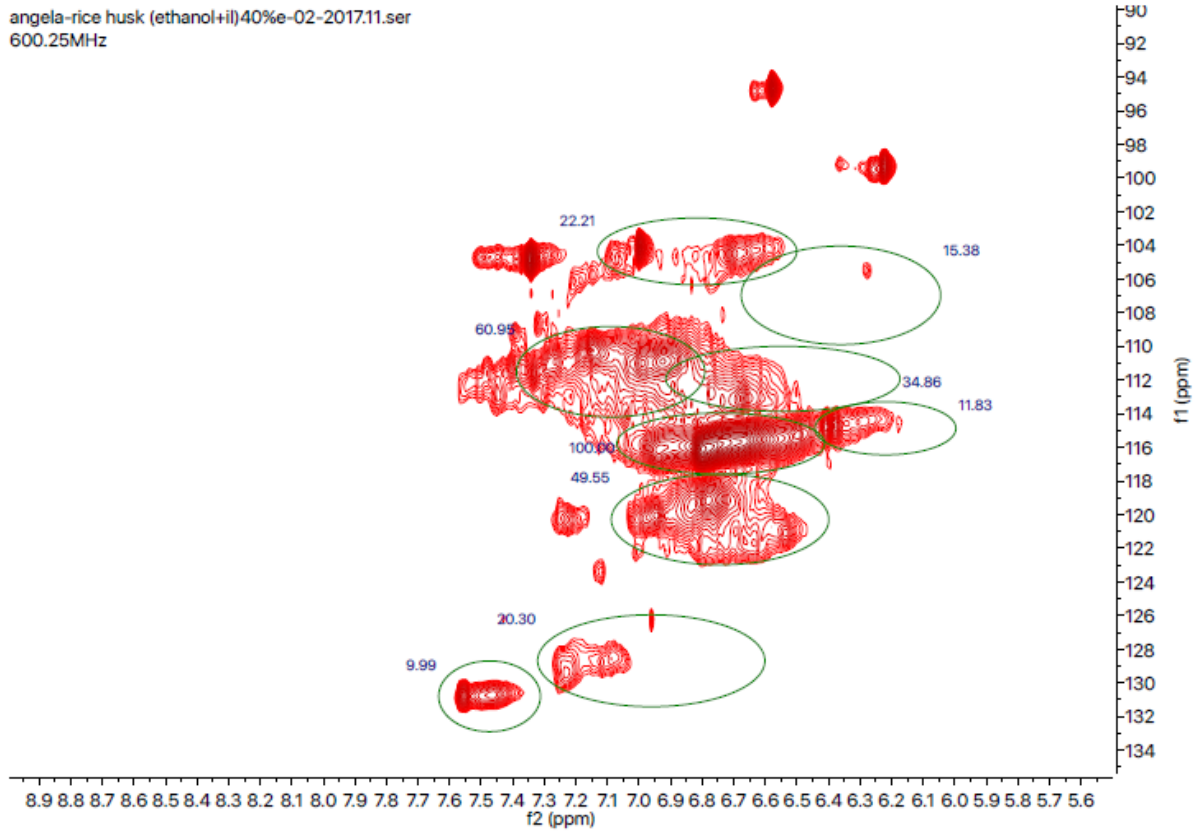
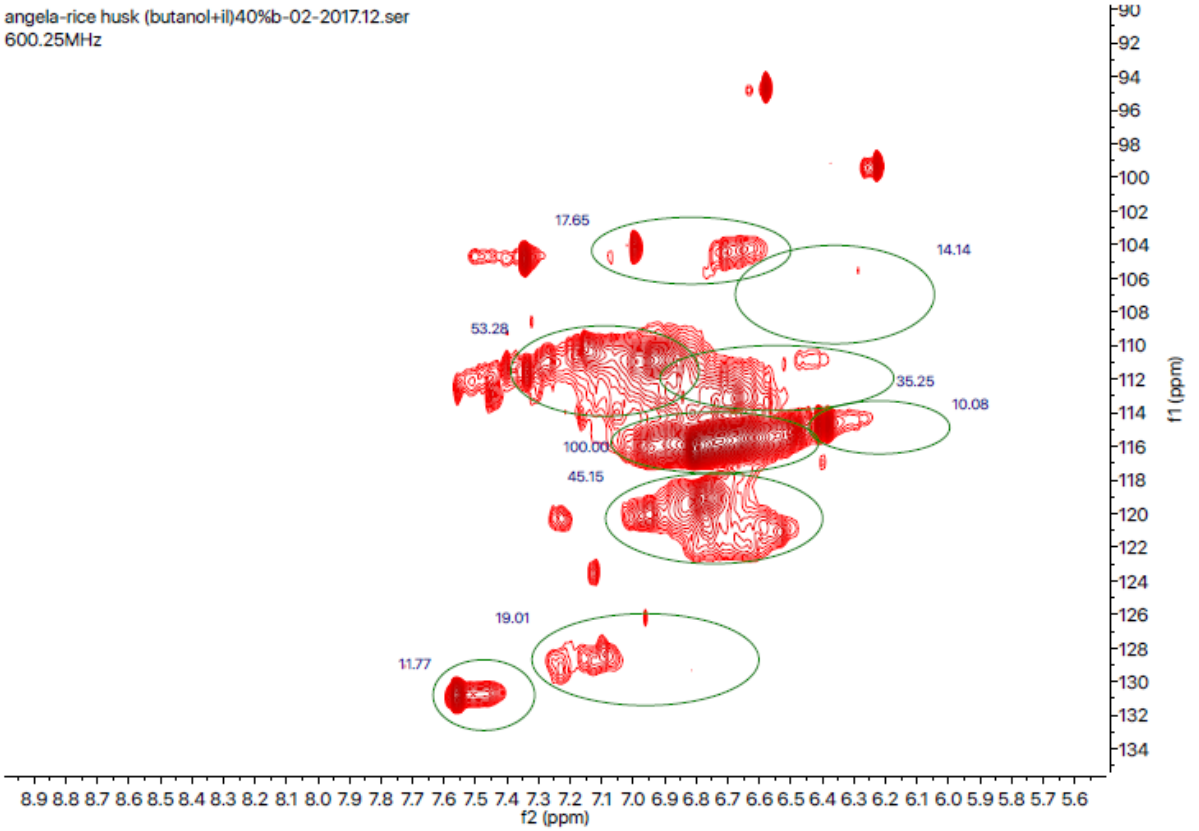
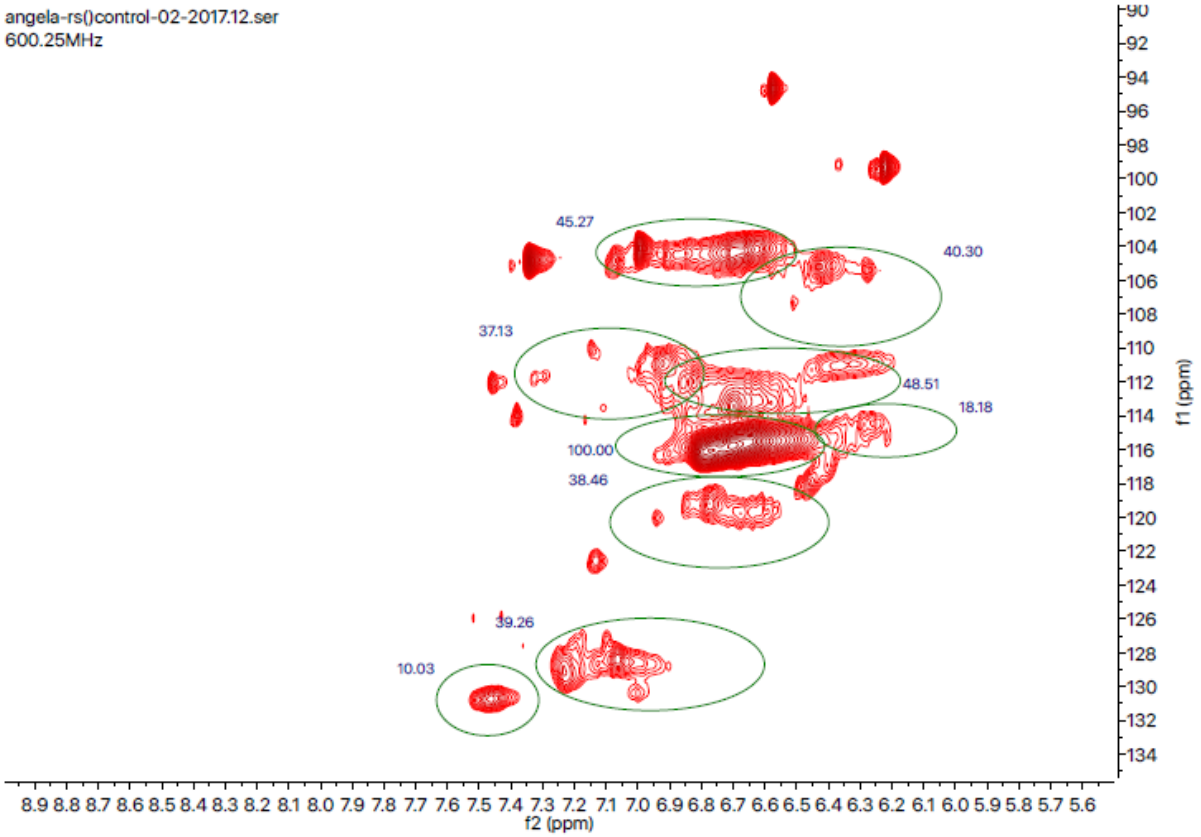
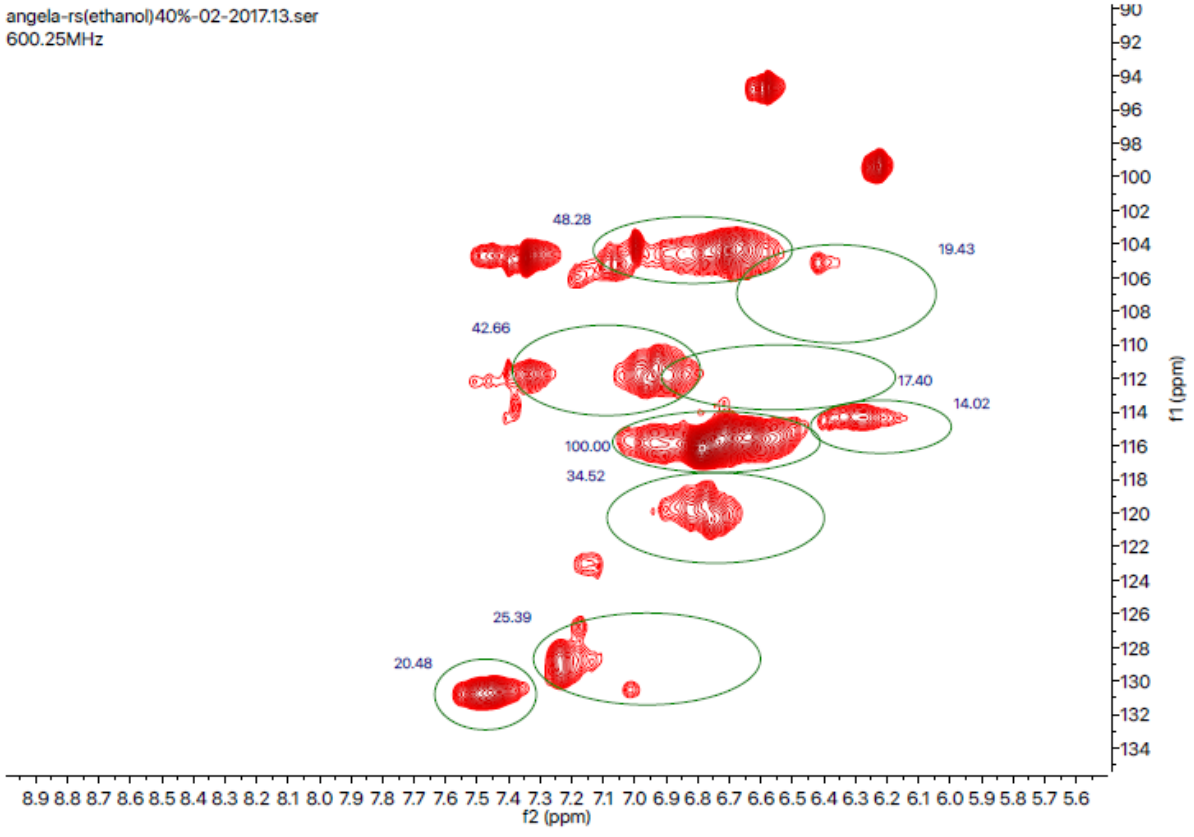
21.10
17.52
100.00
19.11
17.48
26.06
f2 (ppm)
f1 (ppm)

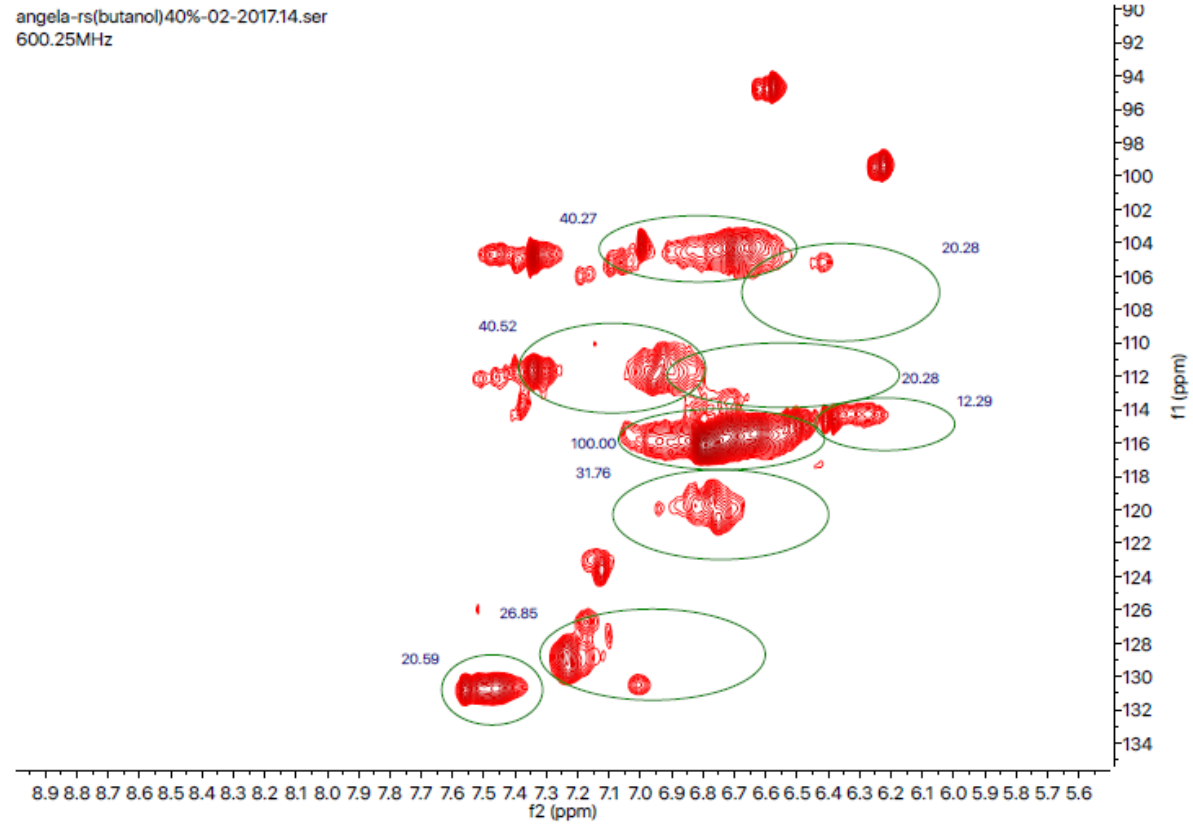

angela-rice husk (il)control-02-2017.10.ser
600.25MHz
15.38
19.35
49.21
45.19
8.32
100.00
41.29
f2 (ppm)
f1 (ppm)

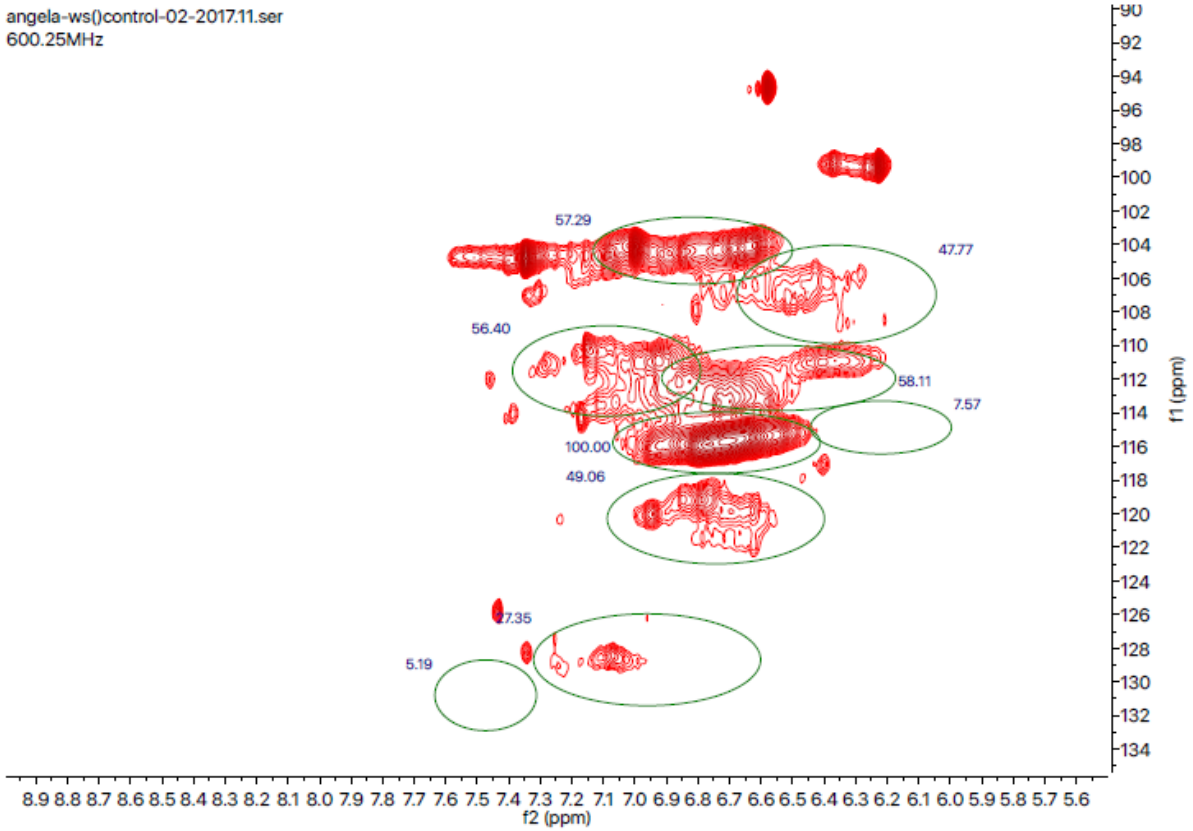

angela-rice husk (ethanol+il)40%e-02-2017.11.ser
600.25MHz
22.21
15.38
60.99
34.86
11.83
100.00
49.55
20.30
9.99
f1 (ppm)
f2 (ppm)

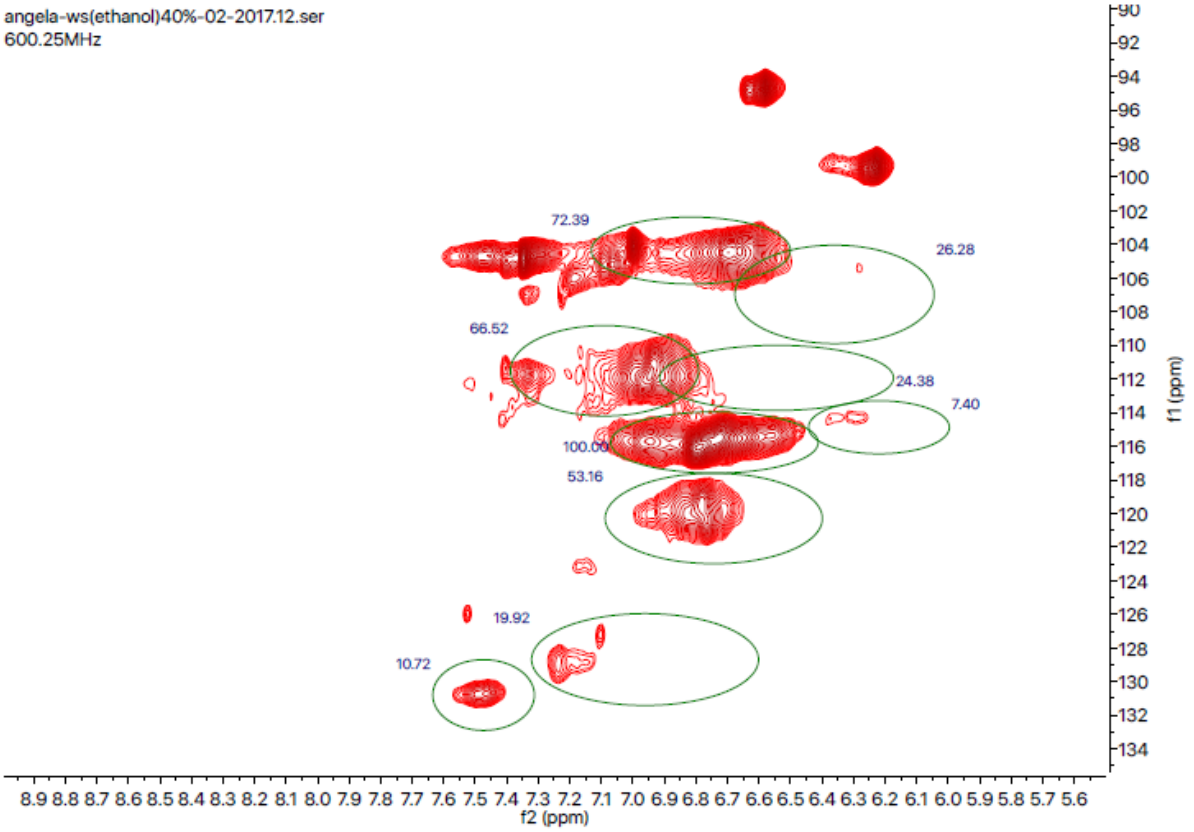

angela-rice husk (butanol+il)40%b-02-2017.12.ser
600.25MHz
17.65
14.14
53.28
35.25
10.08
100.00
45.15
19.01
11.77
f1 (ppm)
f2 (ppm)

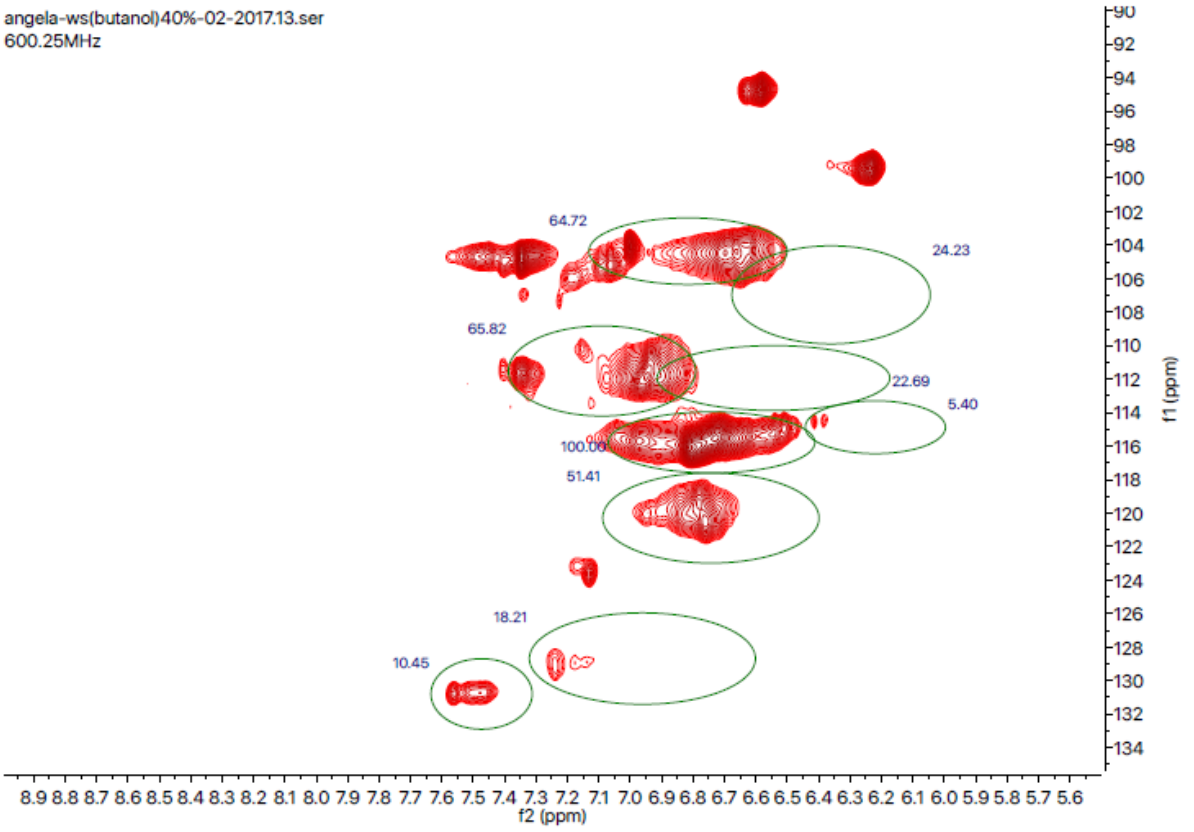

angela-rs()control-02-2017.12.ser
600.25MHz
45.27
40.30
37.13
48.51
18.18
100.00
38.46
39.26
10.03
f1 (ppm)
f2 (ppm)


angela-rs(ethanol)40%-02-2017.13.ser
600.25MHz
48.28
19.43
42.66
17.40
14.02
100.00
34.52
25.39
20.48
f1 (ppm)
f2 (ppm)

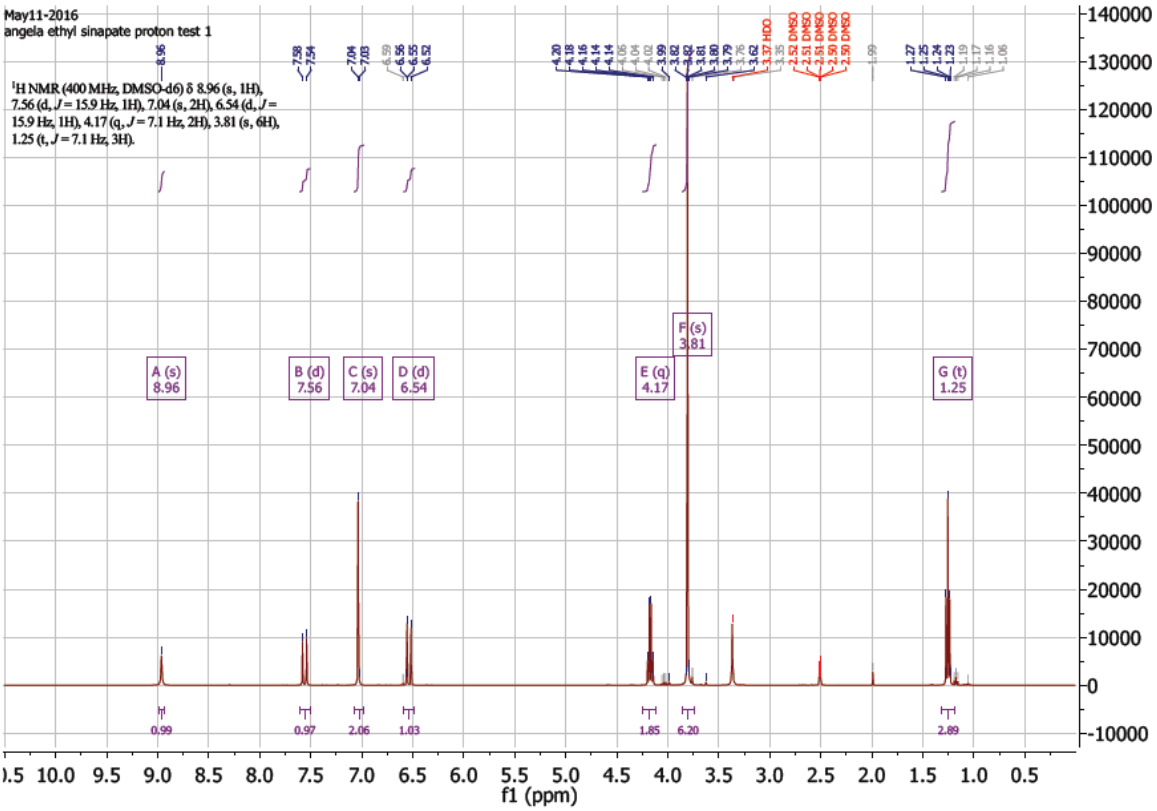

angela-rs(butanol)40%-02-2017.14.ser
600.25MHz
40.27
20.28
40.52
20.28
12.29
100.00
31.76
26.85
20.59
f1 (ppm)
f2 (ppm)

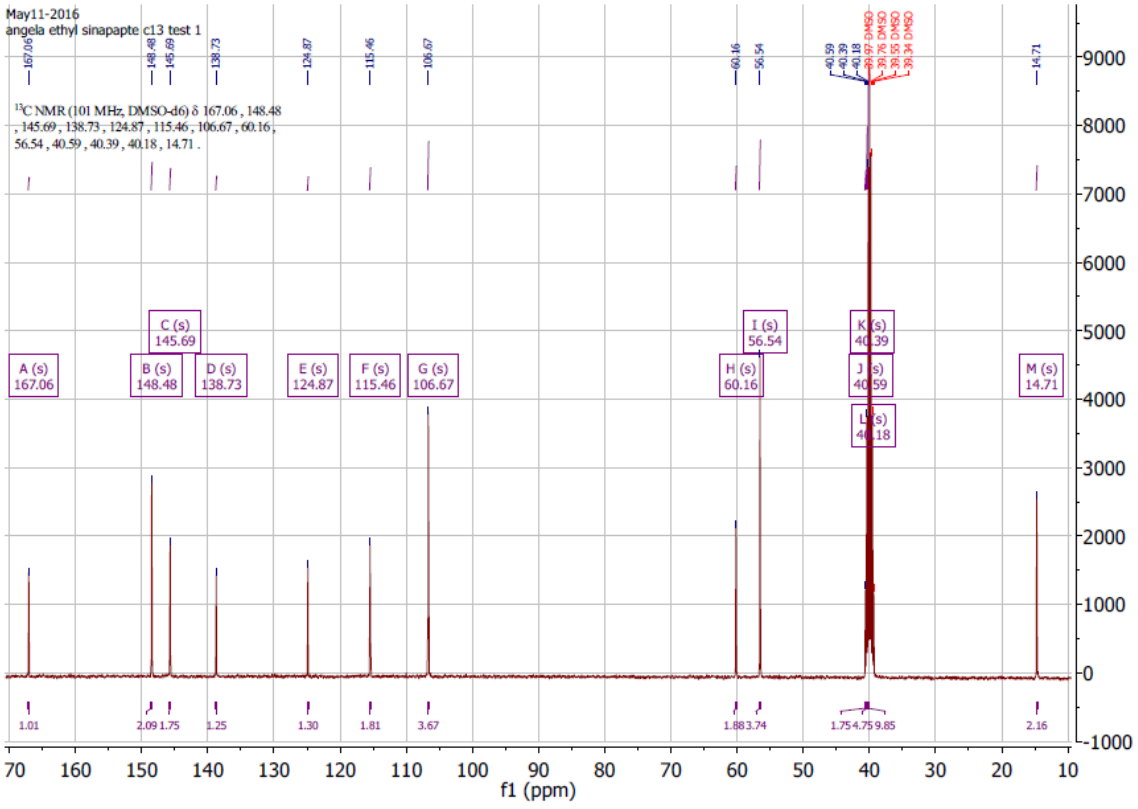

angela-ws()control-02-2017.11.ser
600.25MHz
57.29
47.77
56.40
58.11
7.57
100.00
49.06
27.35
5.19
f1 (ppm)
f2 (ppm)

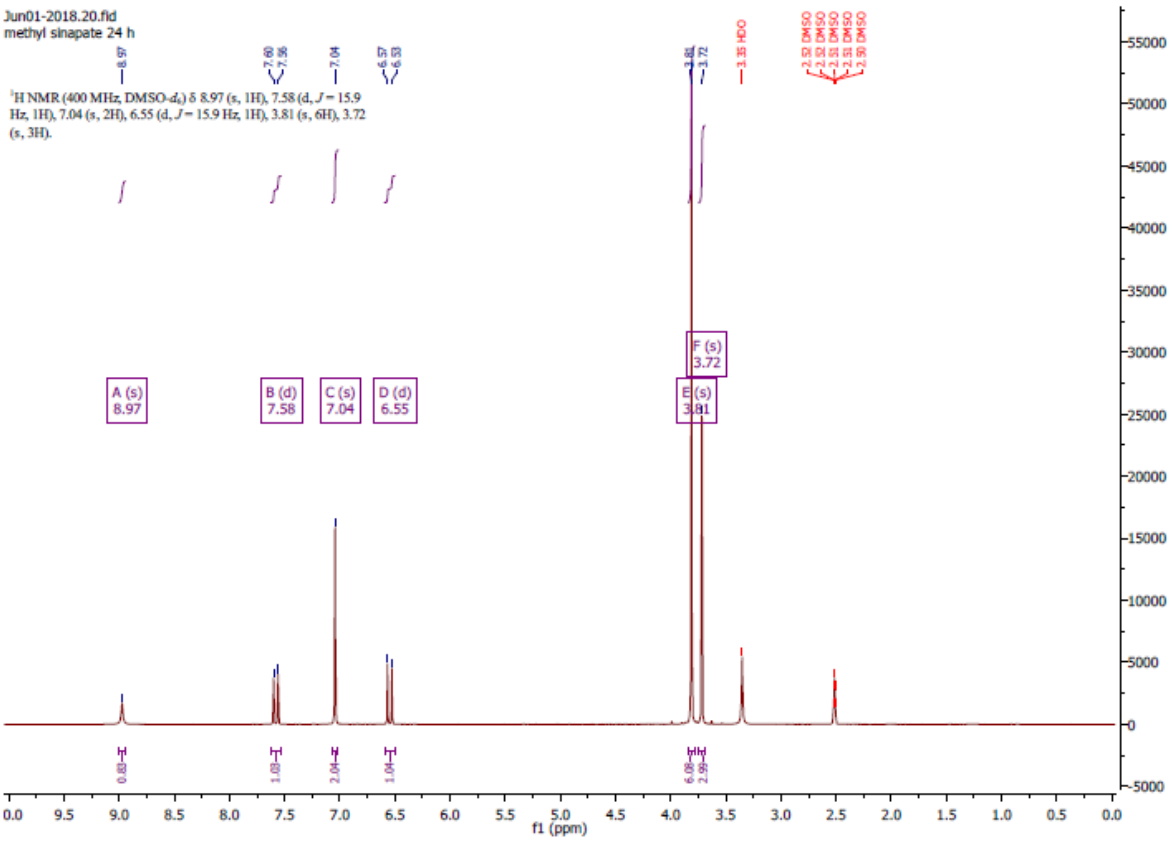

angela-ws(ethanol)40%-02-2017.12.ser
600.25MHz
72.39
26.28
66.52
24.38
7.40
100.00
53.16
19.92
10.72
f1 (ppm)
f2 (ppm)


angela-ws(butanol)40%-02-2017.13.ser
600.25MHz
64.72
24.23
65.82
22.69
5.40
100.00
51.41
18.21
10.45
f1 (ppm)
f2 (ppm)

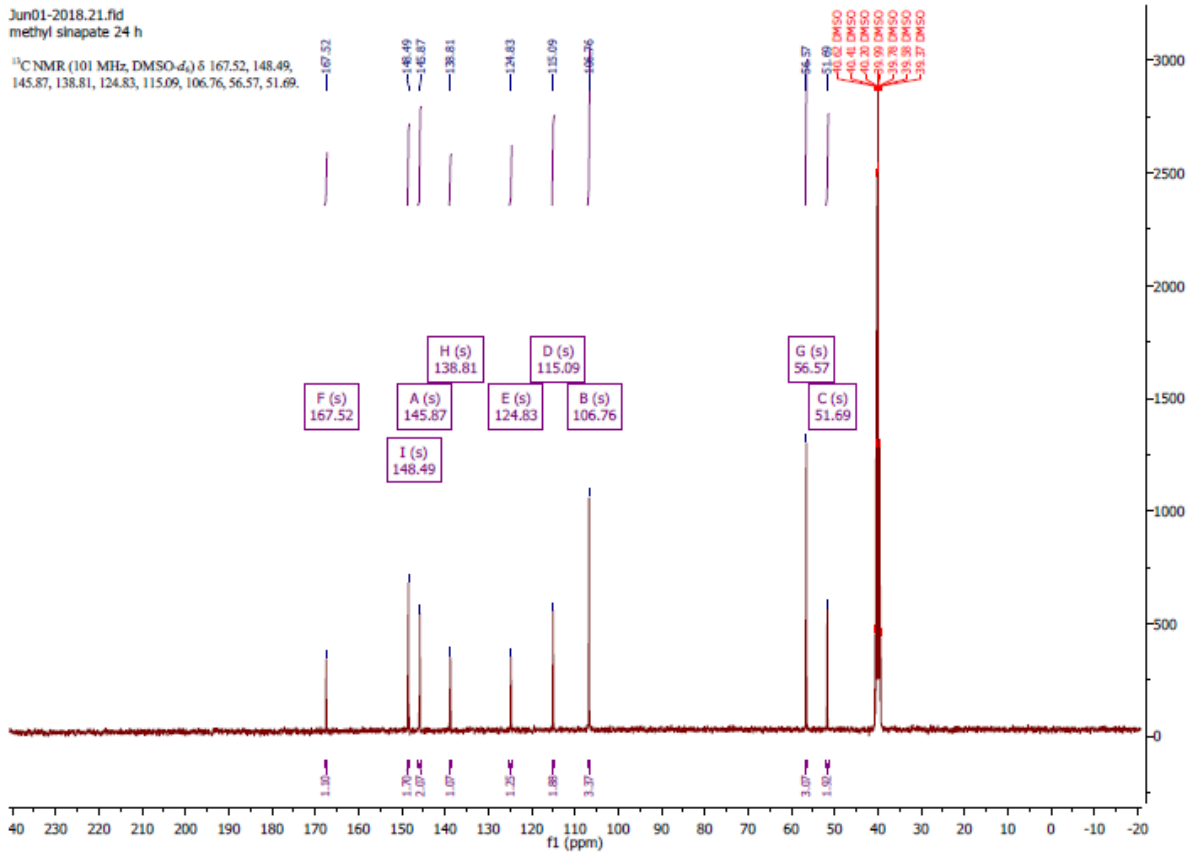
- **Proton and carbon NMR spectra for monolignols**



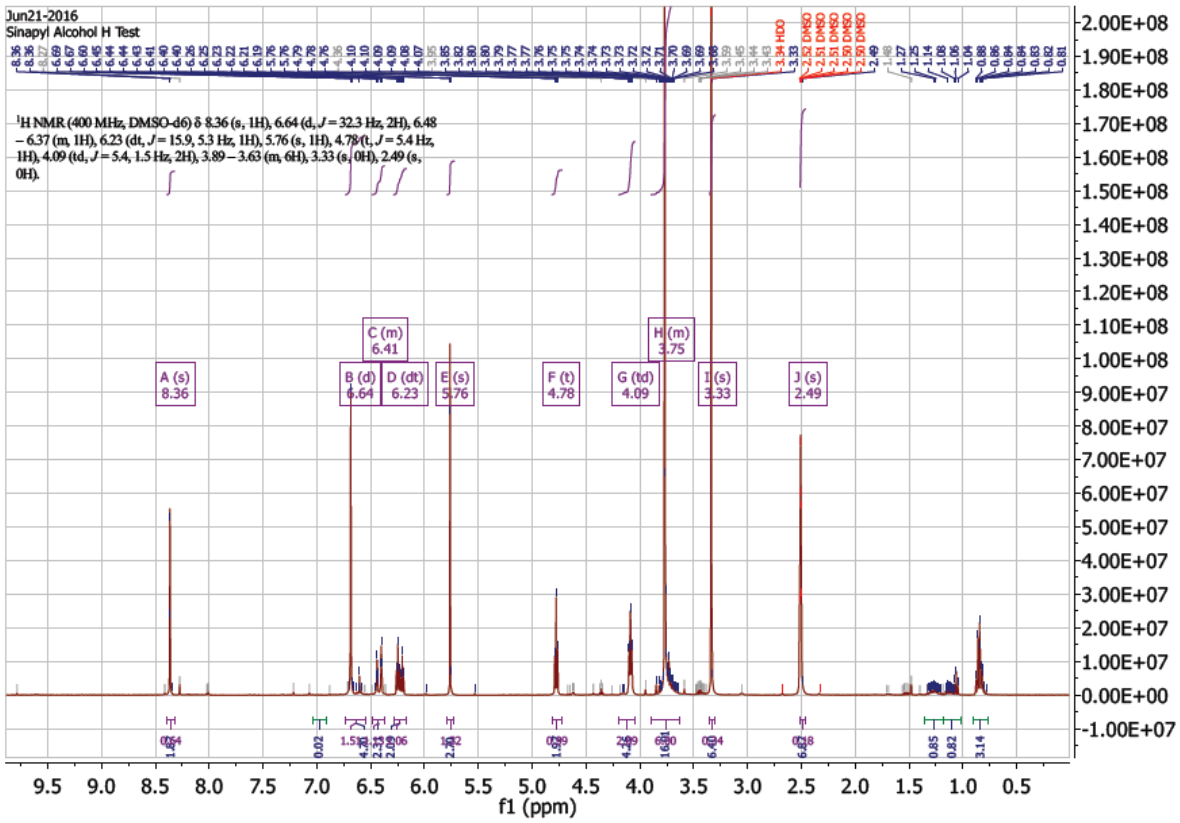


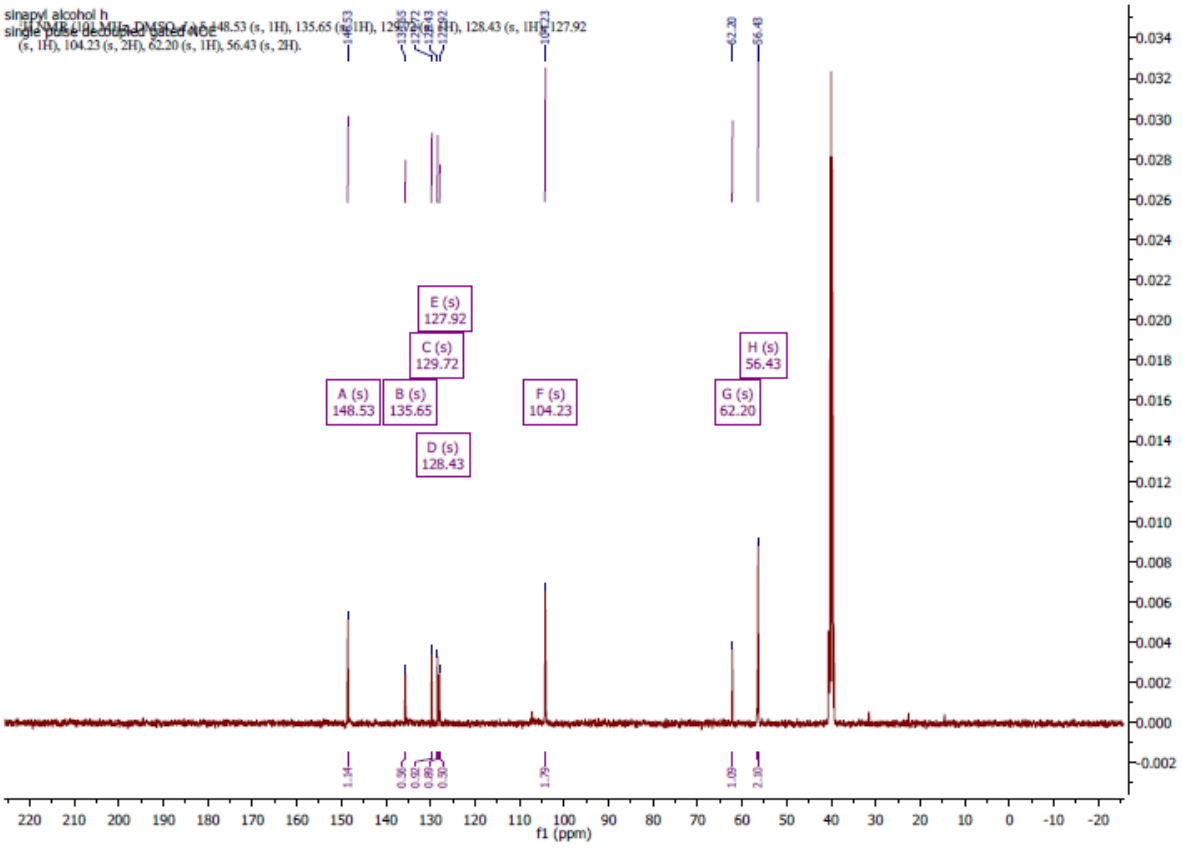


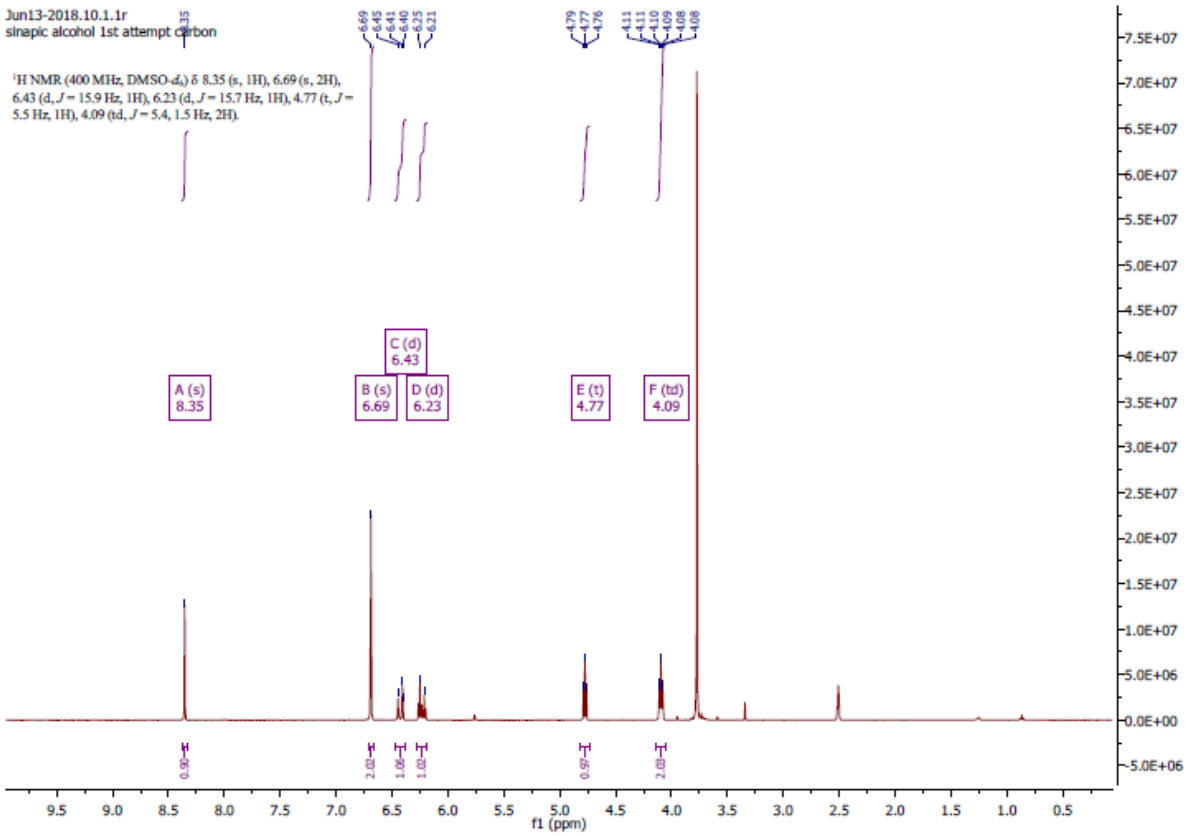
^{1}H NMR (400 MHz, DMSO-d_6) δ 8.97 (s, 1H), 7.58 (d, J = 15.9 Hz, 1H), 7.04 (s, 2H), 6.55 (d, J = 15.9 Hz, 1H), 3.81 (s, 6H), 3.72 (s, 3H).

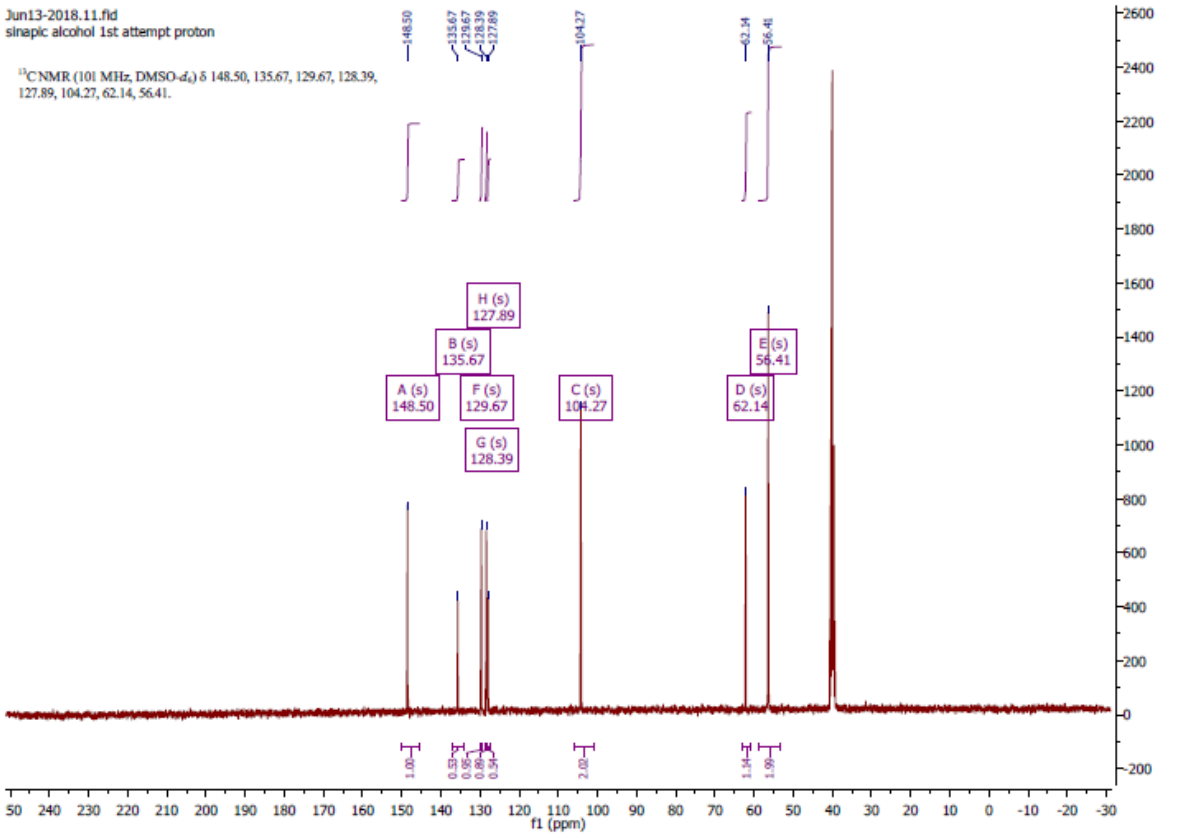


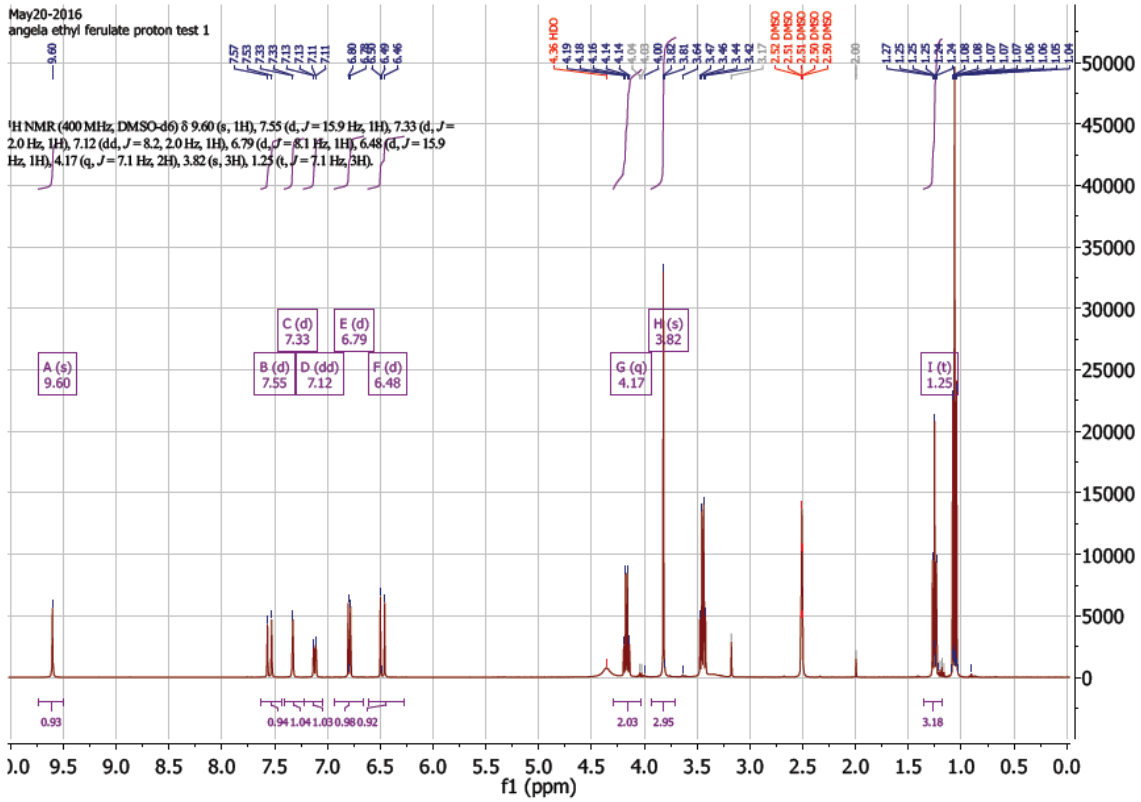
^{13}C NMR (101 MHz, DMSO-d_6) δ 167.52, 148.49, 145.87, 138.81, 124.83, 115.09, 106.76, 56.57, 51.69.

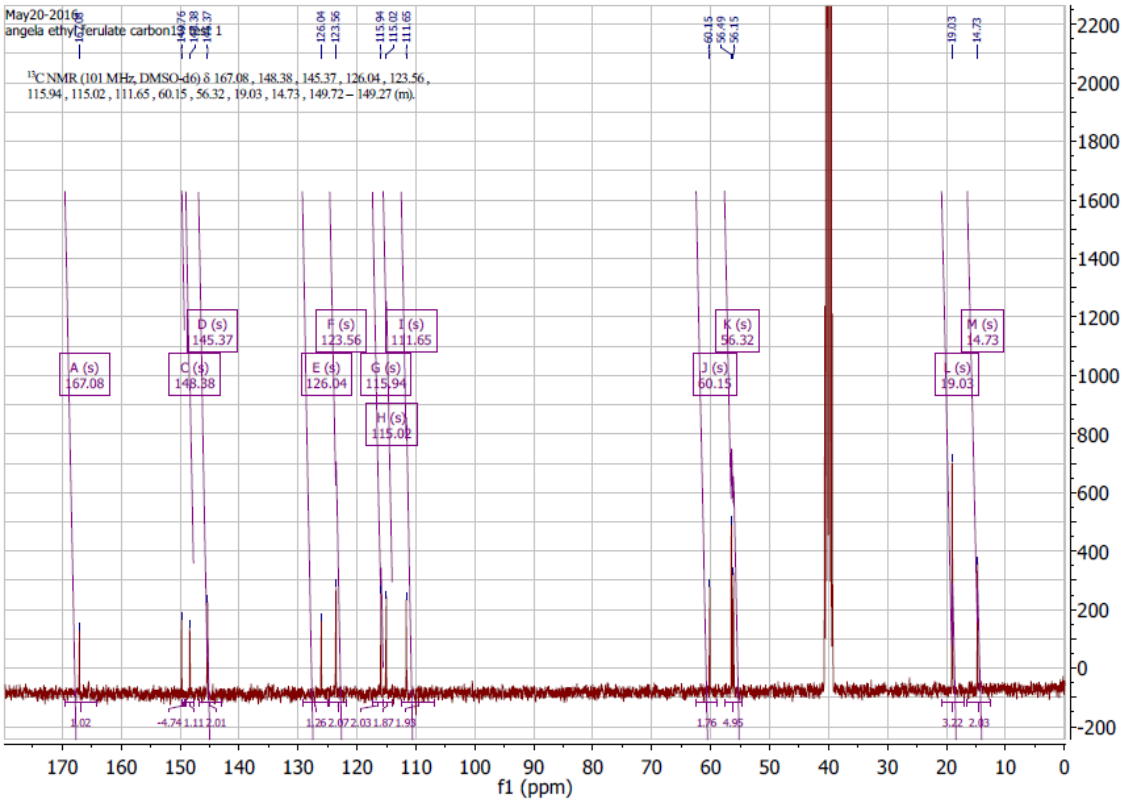

Jun21-2016
Sinapyl Alcohol H Test
¹H NMR (400 MHz, DMSO-d6) δ 8.36 (s, 1H), 6.64 (d, J = 32.3 Hz, 2H), 6.48 – 6.37 (m, 1H), 6.23 (dt, J = 15.9, 5.3 Hz, 1H), 5.76 (s, 1H), 4.78 (t, J = 5.4 Hz, 1H), 4.09 (td, J = 5.4, 1.5 Hz, 2H), 3.89 – 3.63 (m, 6H), 3.33 (s, 0H), 2.49 (s, 0H).
A (s) 8.36
B (d) 6.64
D (dt) 6.23
C (m) 6.41
E (s) 5.76
F (t) 4.78
G (td) 4.09
H (m) 3.75
I (s) 3.33
J (s) 2.49
f1 (ppm)

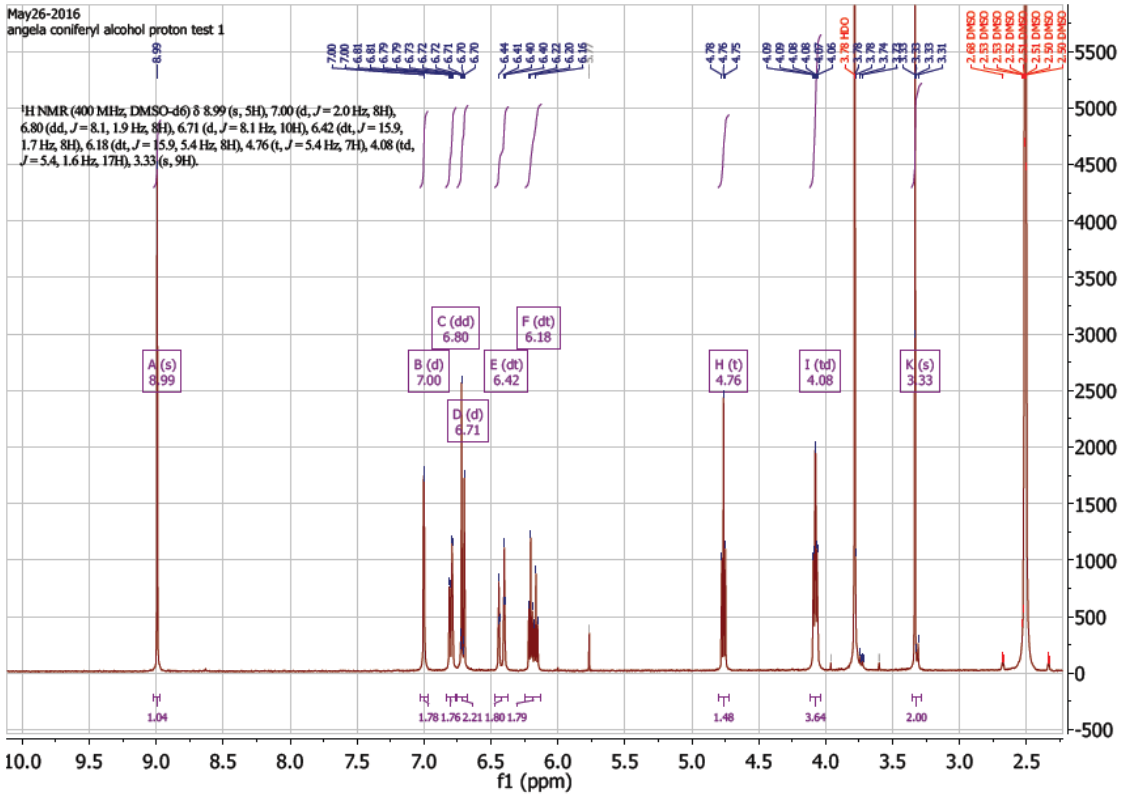

sinapyl alcohol h
single pulse decoupled gated noe
(s, 1H), 104.23 (s, 2H), 62.20 (s, 1H), 56.43 (s, 2H).
A (s) 148.53
B (s) 135.65
C (s) 129.72
D (s) 128.43
E (s) 127.92
F (s) 104.23
G (s) 62.20
H (s) 56.43
f1 (ppm)

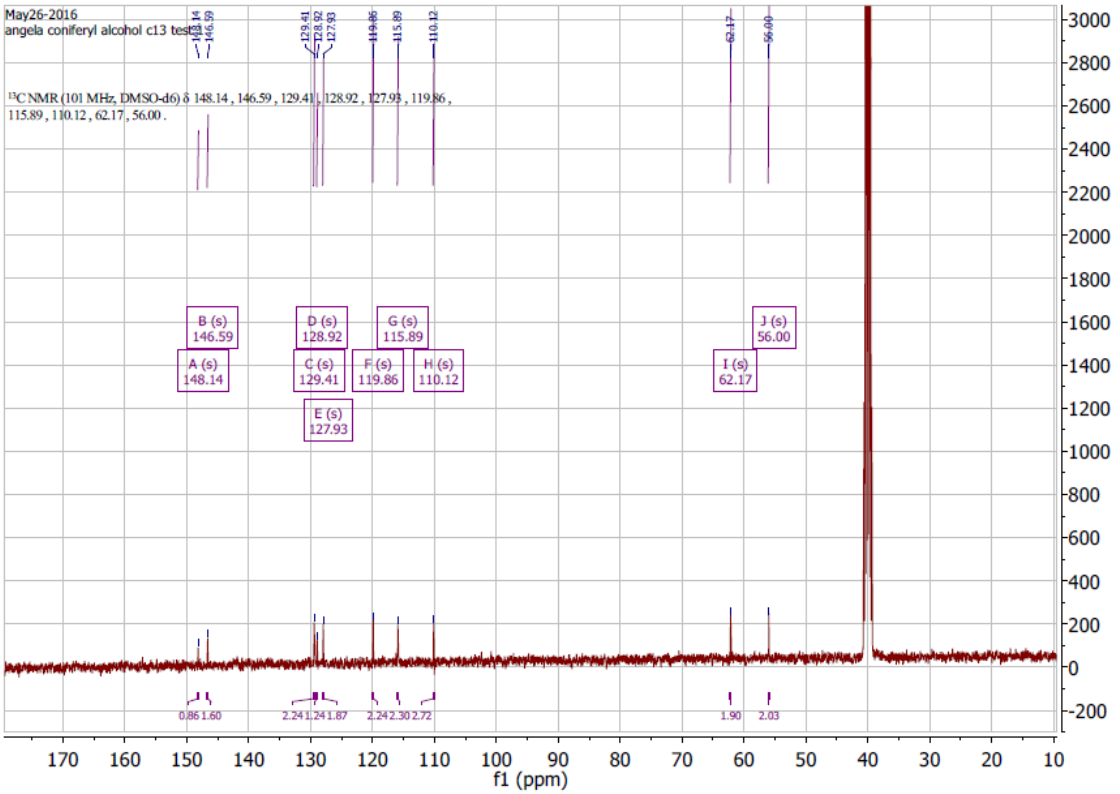

Jun13-2018.10.1.1r
sinapic alcohol 1st attempt carbon
^{1}H NMR (400 MHz, DMSO-d_6) δ 8.35 (s, 1H), 6.69 (s, 2H), 6.43 (d, J = 15.9 Hz, 1H), 6.23 (d, J = 15.7 Hz, 1H), 4.77 (t, J = 5.5 Hz, 1H), 4.09 (td, J = 5.4, 1.5 Hz, 2H).
A (s) 8.35
B (s) 6.69
C (d) 6.43
D (d) 6.23
E (t) 4.77
F (td) 4.09
f1 (ppm)

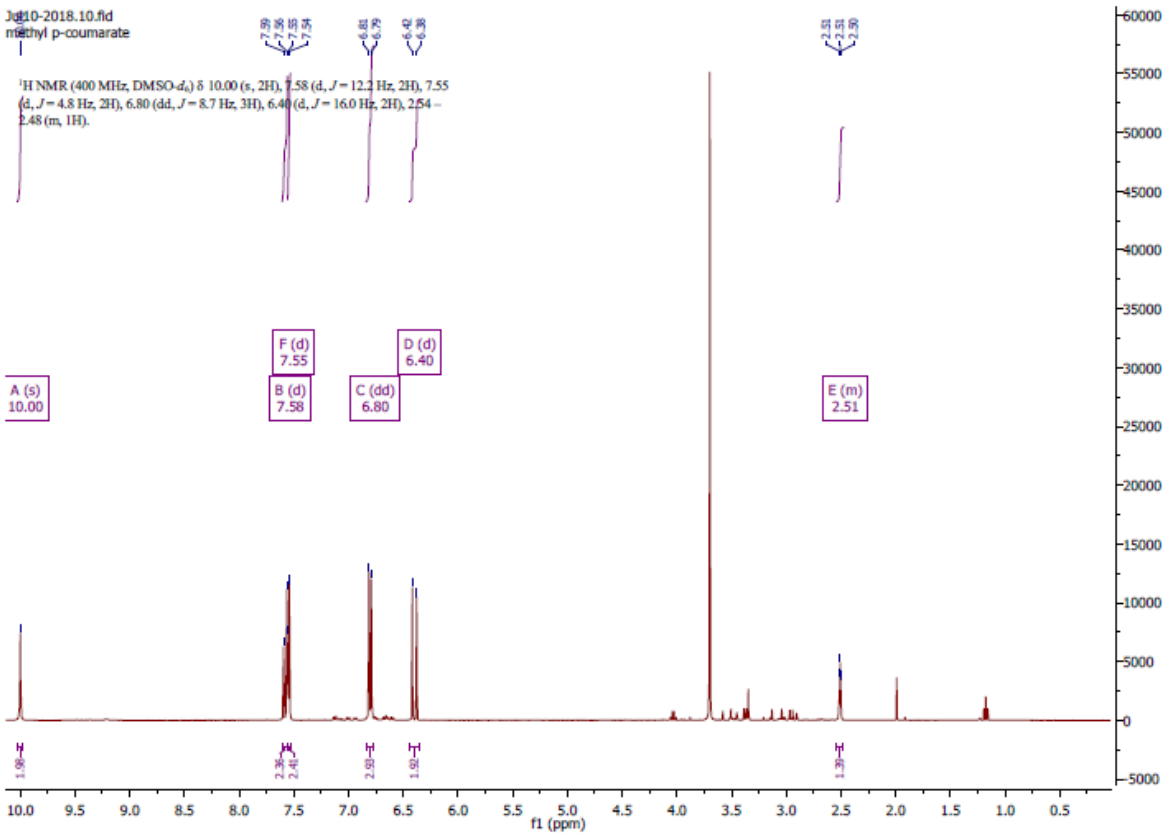

Jun13-2018.11.fid
sinapic alcohol 1st attempt proton
^{13}C NMR (101 MHz, DMSO-d_6) δ 148.50, 135.67, 129.67, 128.39, 127.89, 104.27, 62.14, 56.41.
A (s) 148.50
B (s) 135.67
C (s) 104.27
D (s) 62.14
E (s) 56.41
F (s) 129.67
G (s) 128.39
H (s) 127.89
f1 (ppm)

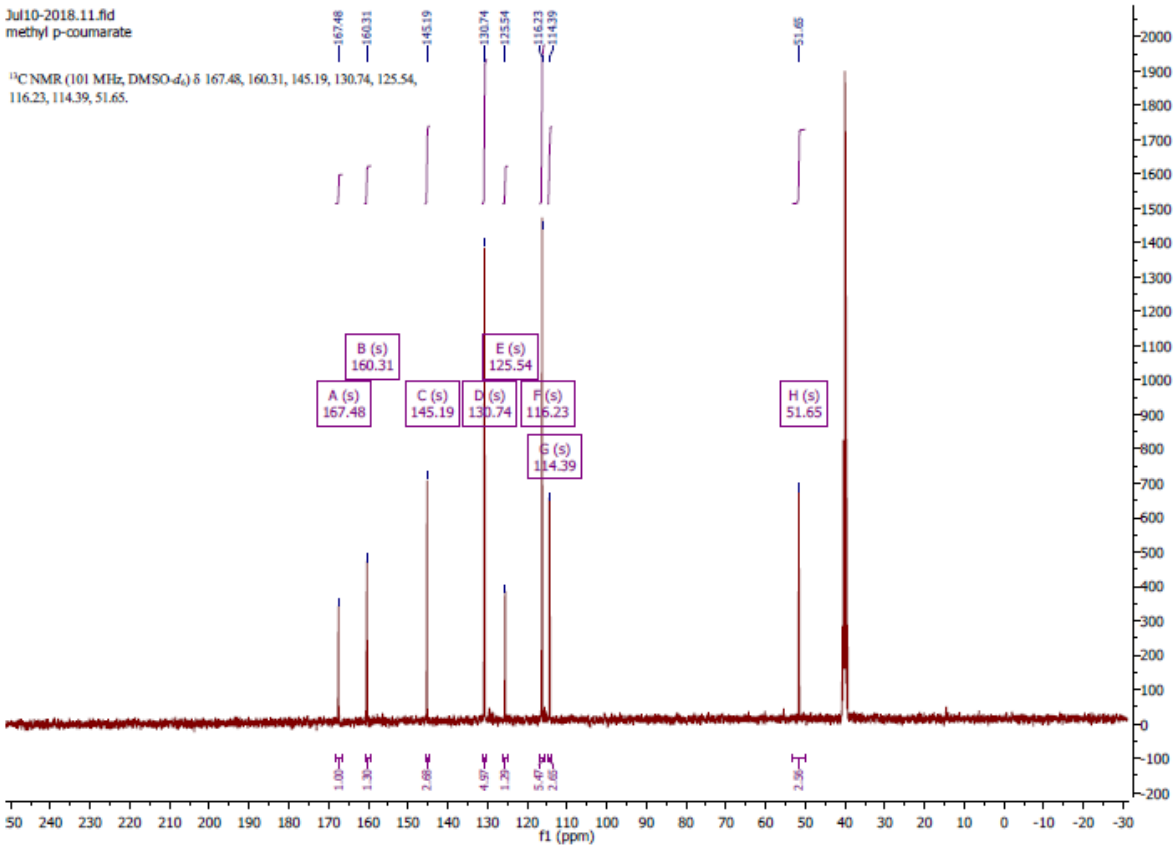

May20-2016
angela ethyl ferulate proton test 1
¹H NMR (400 MHz, DMSO-d6) δ 9.60 (s, 1H), 7.55 (d, J = 15.9 Hz, 1H), 7.33 (d, J = 2.0 Hz, 1H), 7.12 (dd, J = 8.2, 2.0 Hz, 1H), 6.79 (d, J = 8.1 Hz, 1H), 6.48 (d, J = 15.9 Hz, 1H), 4.17 (q, J = 7.1 Hz, 2H), 3.82 (s, 3H), 1.25 (t, J = 7.1 Hz, 3H).
A (s) 9.60
B (d) 7.55
C (d) 7.33
D (dd) 7.12
E (d) 6.79
F (d) 6.48
G (q) 4.17
H (s) 3.82
I (t) 1.25
f1 (ppm)

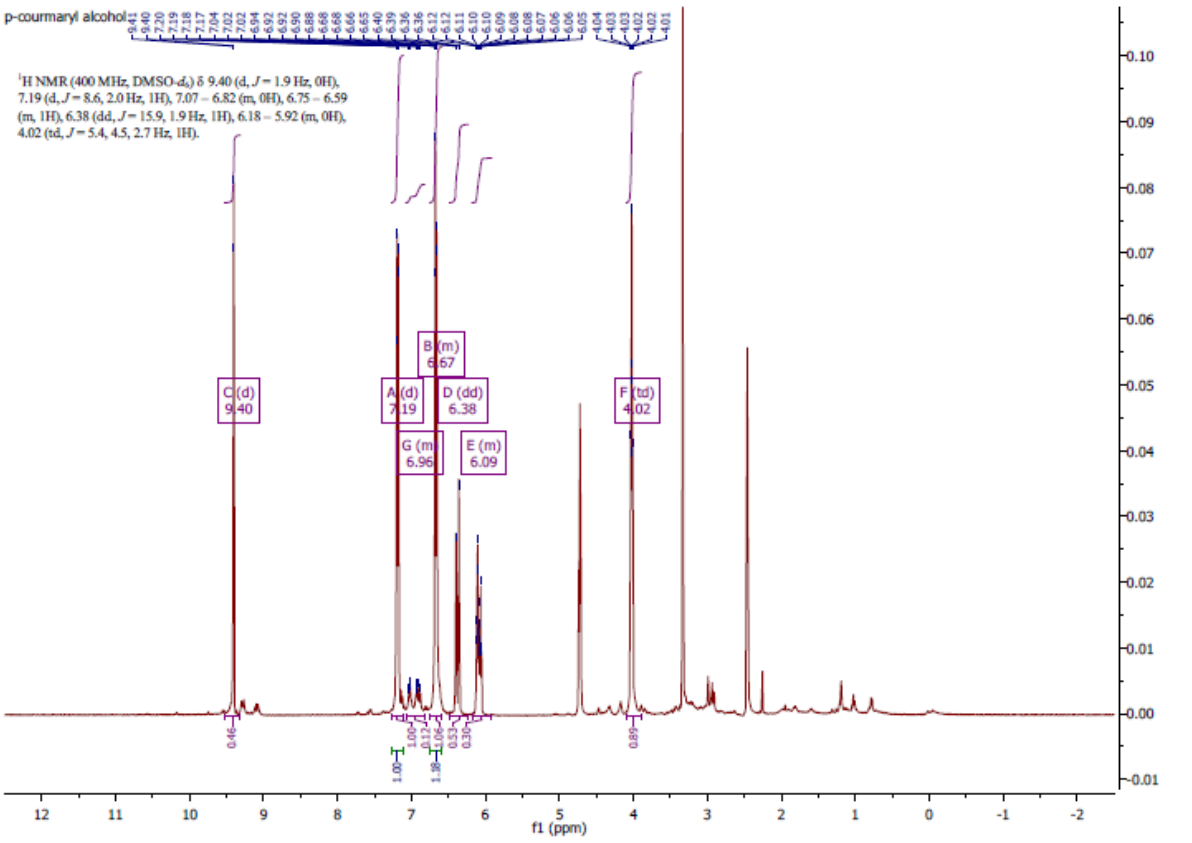

May20-2016
angela ethyl ferulate carbon1
¹³C NMR (101 MHz, DMSO-d6) δ 167.08, 148.38, 145.37, 126.04, 123.56, 115.94, 115.02, 111.65, 60.15, 56.32, 19.03, 14.73, 149.72 – 149.27 (m).
A (s) 167.08
C (s) 148.38
D (s) 145.37
E (s) 126.04
F (s) 123.56
G (s) 115.94
H (s) 115.02
I (s) 111.65
J (s) 60.15
K (s) 56.32
L (s) 19.03
M (s) 14.73
f1 (ppm)

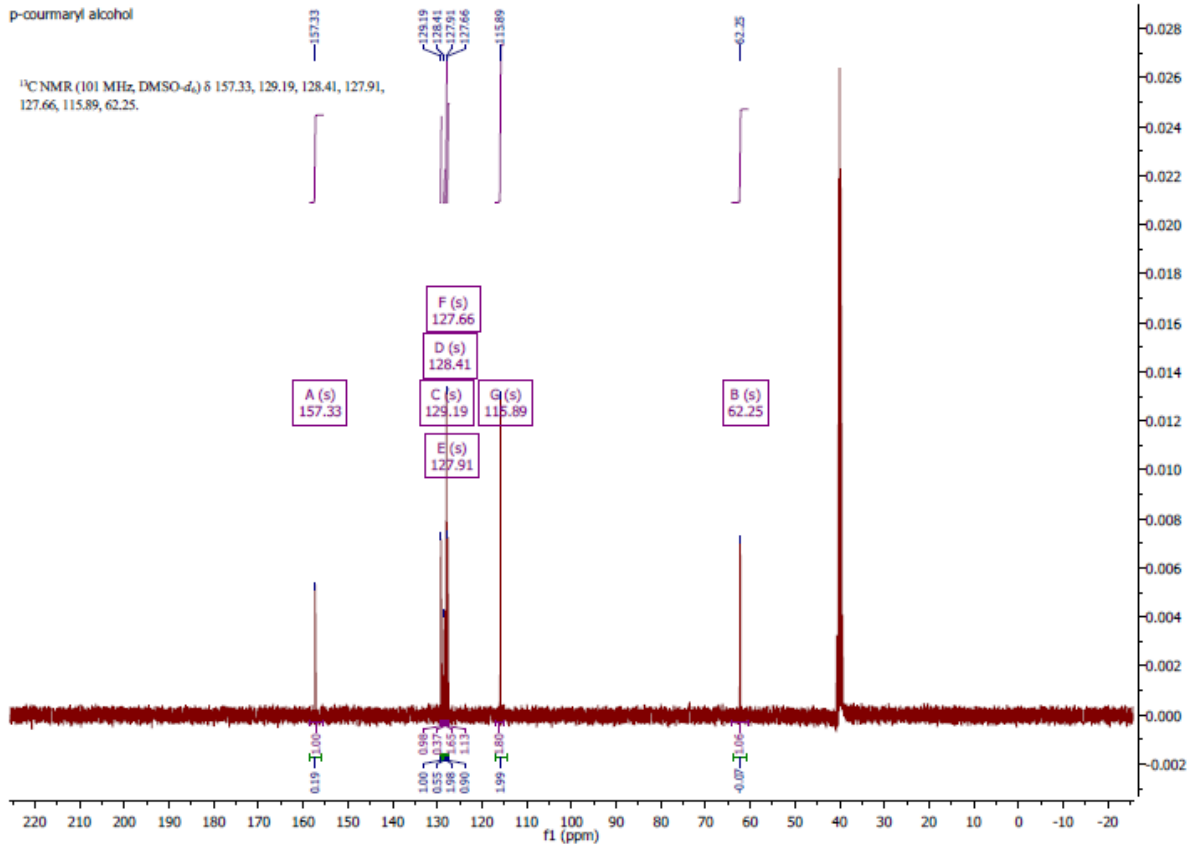

May26-2016
angela coniferyl alcohol proton test 1
¹H NMR (400 MHz, DMSO-d6) δ 8.99 (s, 5H), 7.00 (d, J = 2.0 Hz, 8H), 6.80 (dd, J = 8.1, 1.9 Hz, 8H), 6.71 (d, J = 8.1 Hz, 10H), 6.42 (dt, J = 15.9, 1.7 Hz, 8H), 6.18 (dt, J = 15.9, 5.4 Hz, 8H), 4.76 (t, J = 5.4 Hz, 7H), 4.08 (td, J = 5.4, 1.6 Hz, 17H), 3.33 (s, 9H).
A (s) 8.99
B (d) 7.00
C (dd) 6.80
D (d) 6.71
E (dt) 6.42
F (dt) 6.18
H (t) 4.76
I (td) 4.08
K (s) 3.33
1.04
1.78 1.76 2.21 1.80 1.79
1.48
3.64
2.00
f1 (ppm)


May26-2016
angela coniferyl alcohol c13 test
¹³C NMR (101 MHz, DMSO-d6) δ 148.14 , 146.59 , 129.41 , 128.92 , 127.93 , 119.86 , 115.89 , 110.12 , 62.17 , 56.00 .
B (s) 146.59
A (s) 148.14
D (s) 128.92
C (s) 129.41
E (s) 127.93
F (s) 119.86
G (s) 115.89
H (s) 110.12
J (s) 56.00
I (s) 62.17
0.86 1.60
2.24 1.24 1.87
2.24 2.30 2.72
1.90
2.03
f1 (ppm)

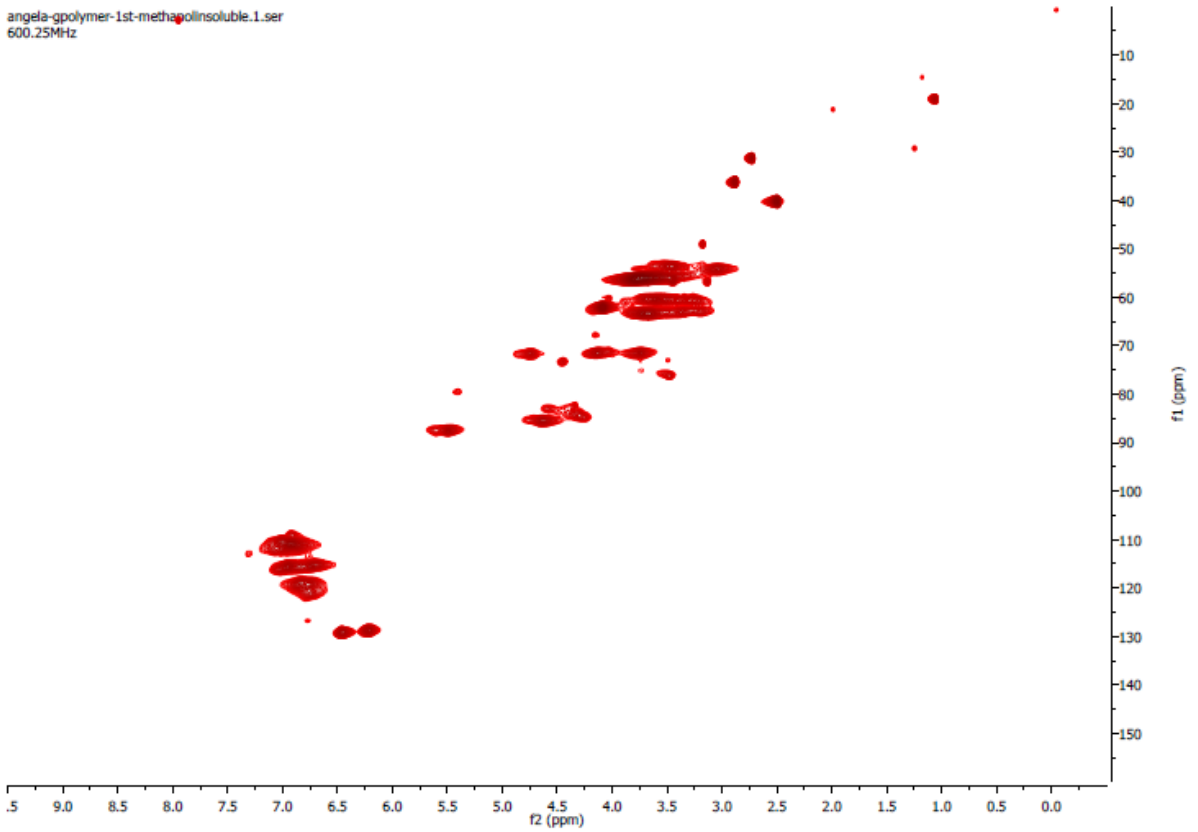

Jul10-2018.10.fid
methyl p-coumarate
¹H NMR (400 MHz, DMSO-d_6) δ 10.00 (s, 2H), 7.58 (d, J = 12.2 Hz, 2H), 7.55 (d, J = 4.8 Hz, 2H), 6.80 (dd, J = 8.7 Hz, 3H), 6.40 (d, J = 16.0 Hz, 2H), 2.54 – 2.48 (m, 1H).
A (s) 10.00
B (d) 7.58
F (d) 7.55
C (dd) 6.80
D (d) 6.40
E (m) 2.51
f1 (ppm)

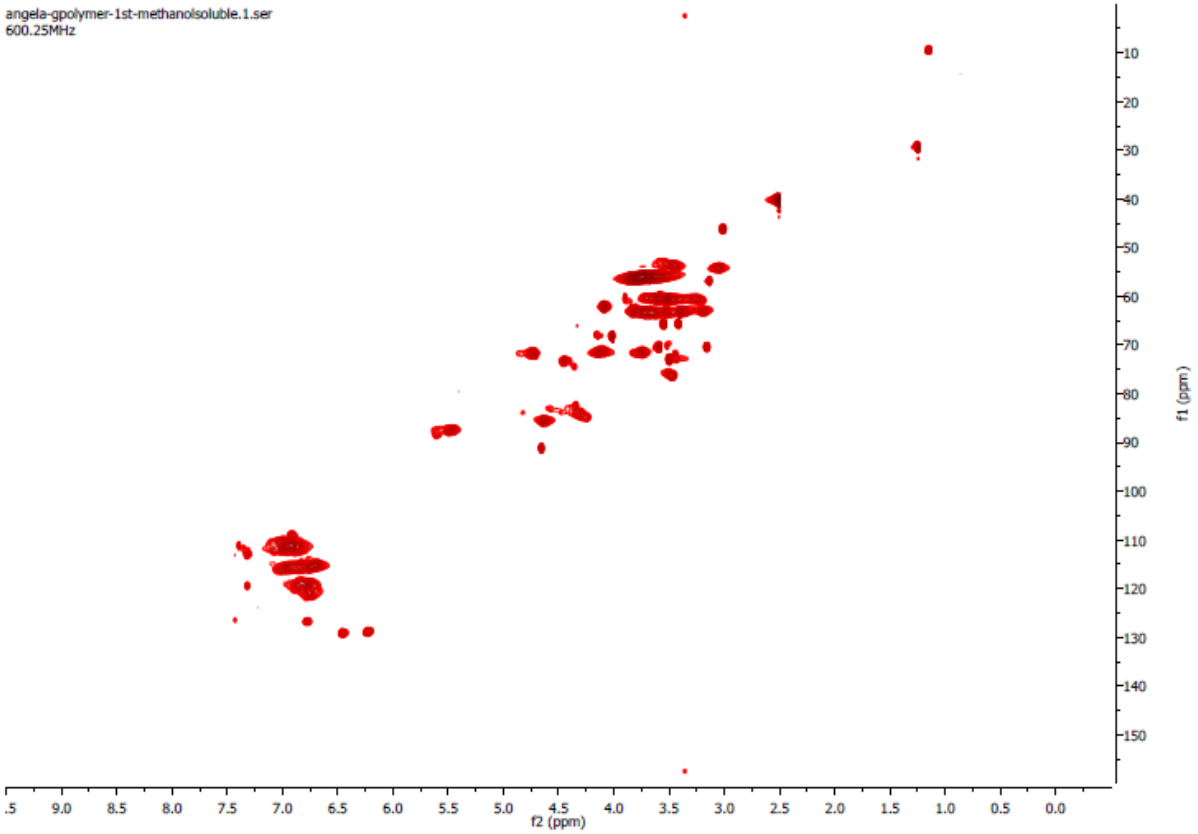

Jul10-2018.11.fid
methyl p-coumarate
¹³C NMR (101 MHz, DMSO-d_6) δ 167.48, 160.31, 145.19, 130.74, 125.54, 116.23, 114.39, 51.65.
A (s) 167.48
B (s) 160.31
C (s) 145.19
D (s) 130.74
E (s) 125.54
F (s) 116.23
G (s) 114.39
H (s) 51.65
f1 (ppm)

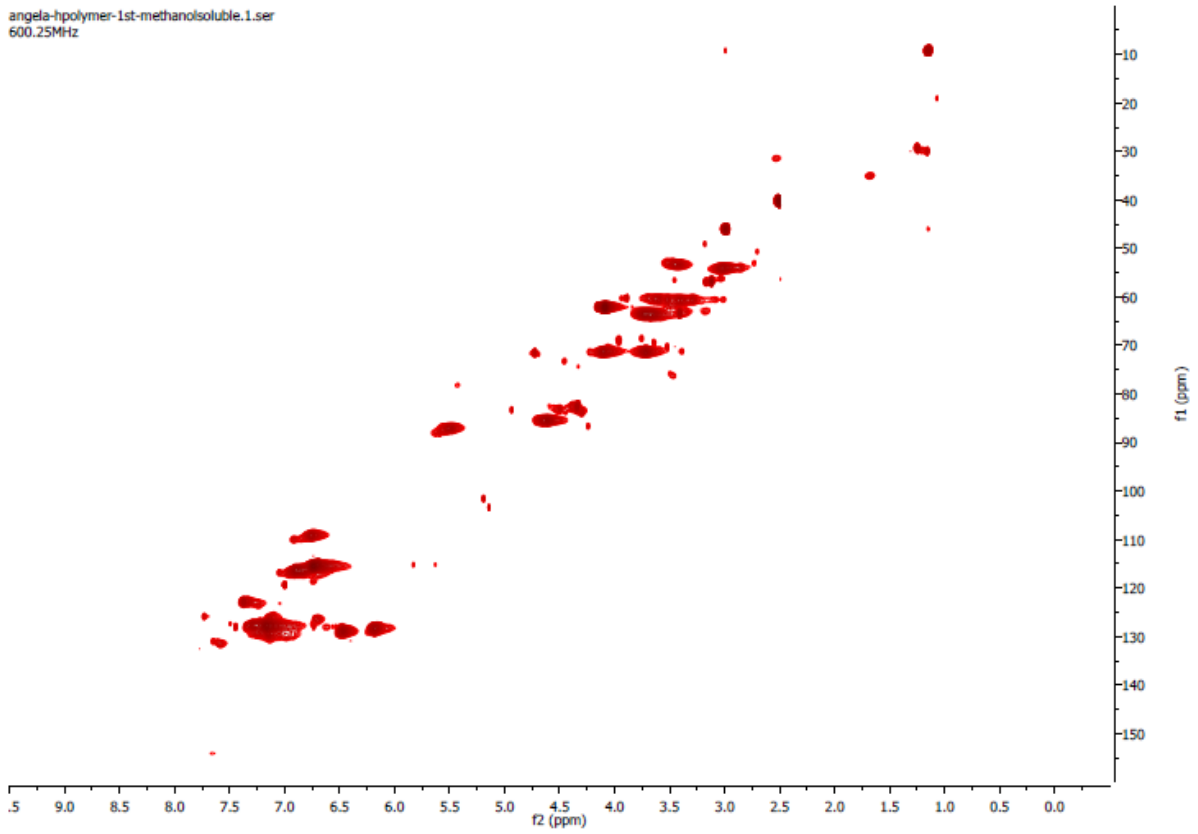

p-courmaryl alcohol
^{1}H NMR (400 MHz, DMSO-d_6) δ 9.40 (d, J = 1.9 Hz, 0H), 7.19 (d, J = 8.6, 2.0 Hz, 1H), 7.07 – 6.82 (m, 0H), 6.75 – 6.59 (m, 1H), 6.38 (dd, J = 15.9, 1.9 Hz, 1H), 6.18 – 5.92 (m, 0H), 4.02 (td, J = 5.4, 4.5, 2.7 Hz, 1H).
B (m) 6.67
C (d) 9.40
A (d) 7.19
D (dd) 6.38
F (td) 4.02
G (m) 6.96
E (m) 6.09
f1 (ppm)


p-courmaryl alcohol
^{13}C NMR (101 MHz, DMSO-d_6) δ 157.33, 129.19, 128.41, 127.91, 127.66, 115.89, 62.25.
F (s) 127.66
D (s) 128.41
A (s) 157.33
C (s) 129.19
G (s) 115.89
B (s) 62.25
E (s) 127.91
f1 (ppm)

- **HSQC spectra for G and H polymers synthesised**






angela-hpolymer-1st-methanolsoluble.1.ser
600.25MHz
f2 (ppm)
f1 (ppm)