Microwave-Assisted Treatments of Biomass: Lignin Isolation from Lignocellulose and Natural Products Recovery from Bilberry Presscake

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Abstract

Microwave thermal treatment has generated an increasing interest in biomass valorisation. In this research, softwood, hardwood and straw are processed by microwave-assisted acidolysis, producing high quality residual lignin without significant modification, especially softwood (purity 93%, yield 82%). Under equivalent conditions, microwave treatment produces lignin with higher yield and purity than conventional treatment. The aqueous hydrolysate is fermented by two oleaginous yeasts, *Cryptococcus curvatus* and *Metschnikowia pulcherrima*. Both yeasts could grow on the hydrolysate and produce an oil with similar properties to palm oil. This preliminary work demonstrates new protocols of microwave-assisted acidolysis and therefore offers an effective approach to produce high purity lignin and fermentable chemicals, which is a key step towards developing a zero-waste lignocellulosic biorefinery.

In addition, microwave conversions (lab and pilot scale) of bilberry presscake, aiming to fulfill multiple chemicals recovery, were carried out using only water as the solvent, ensuring all products are suitable for food grade status applications. Microwave hydrolysis gives much higher yield of mono-/disaccharides than conventional extraction, with the yield of rhamnose particularly high (10.8%). Pilot scale microwave conversions are also carried out with high conversion. It is believed microwave hydrolysis offers an efficient and green approach to convert bilberry presscake into value-added products for food industry and biorefinery.

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你们得救在乎归回安息，你们得力在乎平静安稳

Declaration

Some of the results of this thesis were obtained by, or in collaboration with other workers, who are fully acknowledged in the text. I declare that this thesis is a presentation of original work and I am the sole author. This work has not previously been presented for an award at this, or any other, University. All sources are acknowledged as References.

Long Zhou

March 2018

Collaborator	**Work conducted**
Dr. Hannah Briers	HPLC data
Dr. Raymond Sloan	Py-GC-MS data
Dr. Christopher J. Chuck Dr. Fabio Santomauro	Experimental, data and analysis of fermentation part in Chapter 3
Mr. Yann Lie	Soxhlet extraction data in Chapter 4
Dr. Hannah Briers and Biorenewables Development Centre	Experimental and report writing of pilot trials of conversion of bilberry presscake in Chapter 4

Contents of three published papers are included in this thesis:

1.Zhou, L., Budarin, V., Fan, J., Sloan, R. and Macquarrie, D., 2017. Efficient Method of Lignin Isolation Using Microwave-Assisted Acidolysis and Characterization of the Residual Lignin. ACS Sustainable Chemistry & Engineering, 5(5), pp.3768-3774.(Chapter 2)

2.Zhou, L., Santomauro, F., Fan, J., Macquarrie, D., Clark, J., Chuck, C.J. and Budarin, V., 2017. Fast microwave-assisted acidolysis: a new biorefinery approach for the zero-waste utilisation of lignocellulosic biomass to produce high quality lignin and fermentable saccharides. Faraday discussions, 202, pp.351-370.(Chapter 3)

3.Zhou, L., Lie, Y., Briers, H., Fan, J., Remón, J., Nyström, J., Budarin, V., Macquarrie, D. and McElroy, C.R., 2018. Natural product recovery from bilberry (Vaccinium myrtillus L.) presscake via microwave hydrolysis. ACS Sustainable Chemistry & Engineering, 6(3), pp.3676-3685.(Chapter 4)

Introduction

Chapter 1

1.1 Biomass valorisation

World energy resources can be divided by type into fossil fuel, nuclear fuel and renewable resources. [1–3] Among all renewable energy resources (biomass, hydropower, solar, geothermal, wind and marine energies), biomass is very attractive as the only sustainable carbon carrier,[4] which can be also converted into value added chemicals. Fuels and chemicals have been produced from biomass for more than one century.[5] Nowadays, increasing concerns are drawn to the use of biomass, because currently fossil fuels (oil, coal and gas) are still the main source of energy and chemical production, in which oil supplies are assumed to be depleted in 50-150 years.[6] In addition, the use of fossil fuel has resulted in severe environmental problems, including, most notably, global warming.[7] Facing such crisis, it is vitally important that biomass becomes a sustainable resource to replace fossil fuel. Compared with fossil energy, biomass is a promising renewable resource for several reasons, including the vast availability, conversion to secondary energy carrier without high capital investment, low emission of greenhouse gas (Figure 1-1) and degradibility.[8,9] The consumption of biomass recourse even contributes to the social aspect of sustainability due to employment creation in rural area.[10]

Cellulose, hemicellulose and lignin are the three major components of lignocellulosic biomass.[11] Substantial efforts have been invested in valorising the polysaccharide (cellulose and hemicellulose) *via* hydrolysis / fermentation to fuels and chemicals, such as monosaccharide (glucose, xylose etc)[12], bio-ethanol[13] and platform molecules (HMF, furfural, levulinic acid etc.)[14]. Lignin, due to its recalcitrance to thermal degradation, is

produced in these processes as a low value by-product or waste. In terms of structure, lignin could be a potential source for the production of aromatic compounds. However, the poor quality of 'by-product' lignin significantly hinders downstream applications. Hence, an effective method for lignin isolation is a key and first step for lignin valorisation.

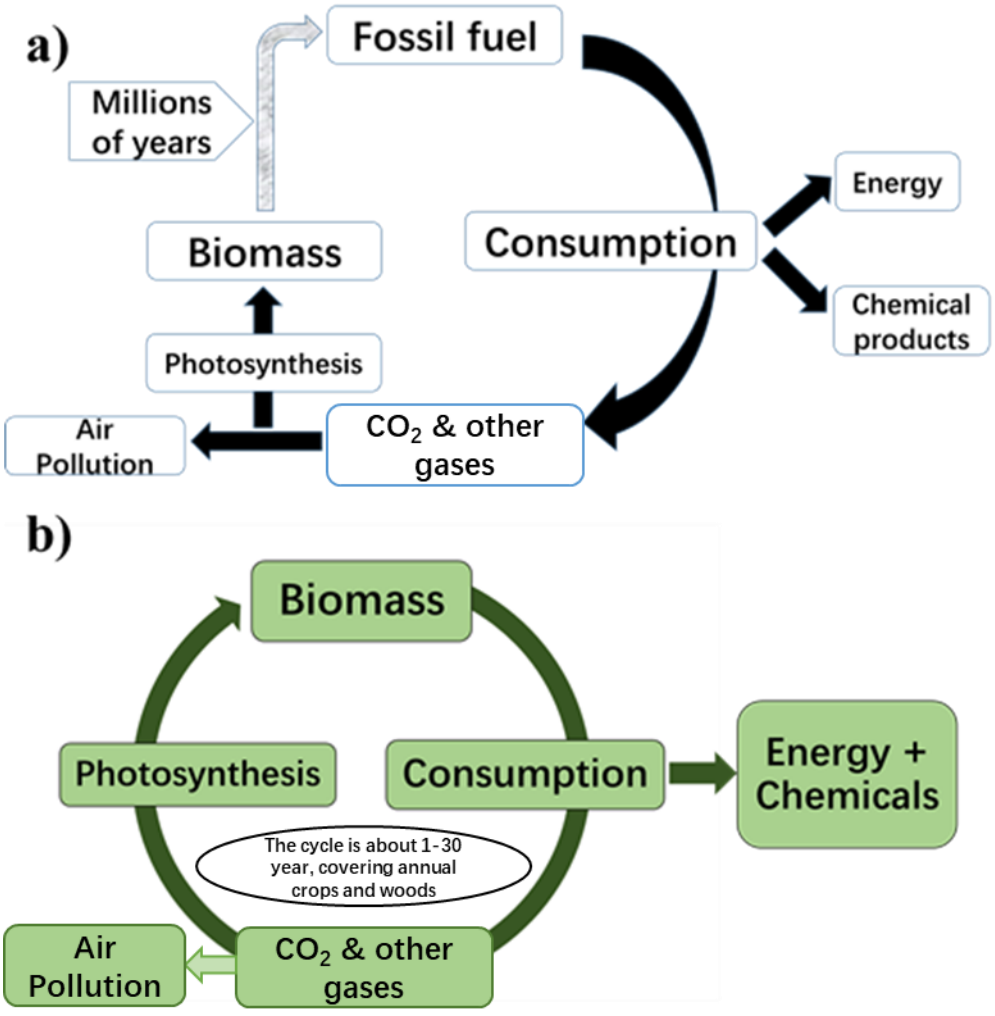


Figure 1-1 Consumption of fossil fuel *vs* biomass. a) circulation of fossil fuels; b) circulation of biomass

1.2 Lignin and its isolation from biomass

In 1838 Anselme Payen first found ‘encrusting material’ that was later named ‘lignin’ embedded between cellulose and hemicellulose (acting as glue to adhere the plant cellular layers together[15]), numerous studies have been carried out to investigate the structure and characteristics of lignin. Lignin ranks second in quantity in the terrestrial regions of the earth's surface in terms of biomass components, playing an important role in plants allowing water conduction and protecting them against pathogen attacks.[16] It is a main component of lignocellulose (cellulose accounting for ca 40%, hemicellulose ca 20%-30% and lignin ca 20%-30%[17]). From the viewpoint of chemical structure, lignin can be a potential source of valuable phenolic compounds by degradation.[18,19] Compared with other sustainable carbon-based resources, these vast resources constitute a potential advantage for lignin utilisation.

1.2.1 Lignin structure

Lignin is a three-dimensional amorphous phenolic polymer composed of three major phenylpropanoid unit (C9-unit): hydroxyphenyl (H-unit), guaiacyl (G-unit), and sinapyl(S-unit). As shown in Figure 1-2, the difference among the three units is the number of methoxy groups that they contain, where S-unit contain two methoxy group, G-unit one and H-unit none.

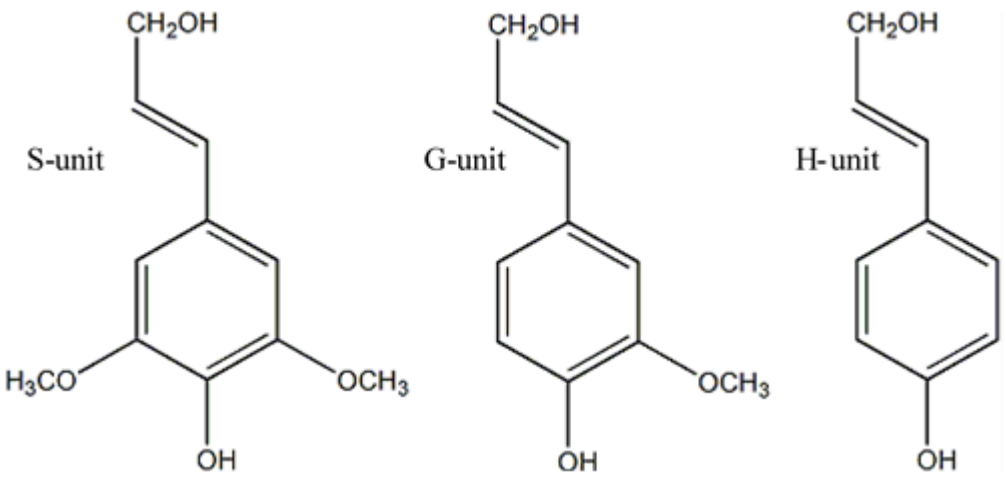


Figure 1-2 monomers of lignin (sinapyl, guaicyl and hydroxyphenyl from left to right)

The random coupling process between the three monomers will form various interunit linkages, such as aryglycerol-β-ether dimer (β-O-4), resinols (β-β′), phenylcoumarin (β-5) and spirodienone (β-1) (Figure 1-3), thereby leading to irregular three dimensional reticulate structures.[20] Among these linkages, β-O-4 ether bond is of great importance in the condensation procedure of forming lignin.[21] Veratrylglycerol-β-guaiacyl ether (Figure 1-4) is widely used as a model compound to investigate behaviour of β-O-4 ether bond.

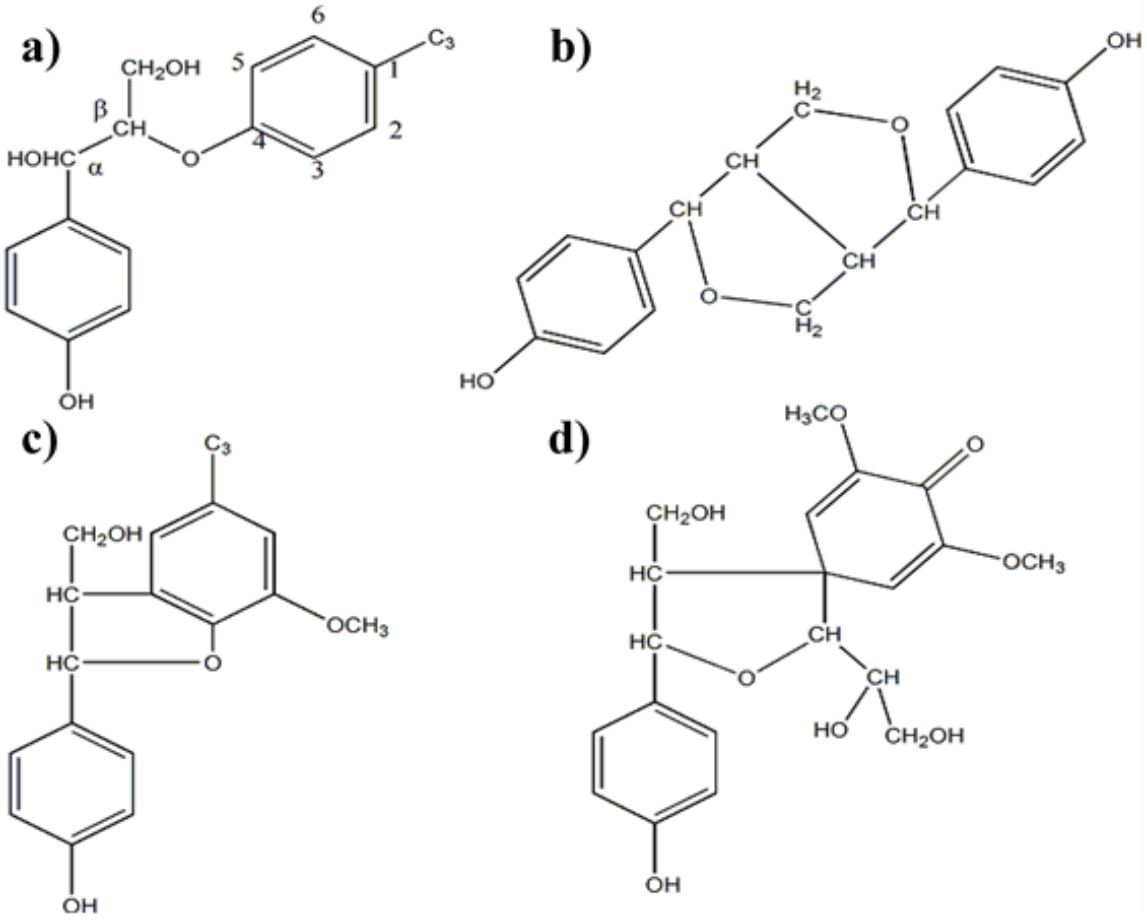


Figure 1-3 Typical ether bonds in lignin. a) β-O-4; b) β-β′; c) β-5; d) β-1

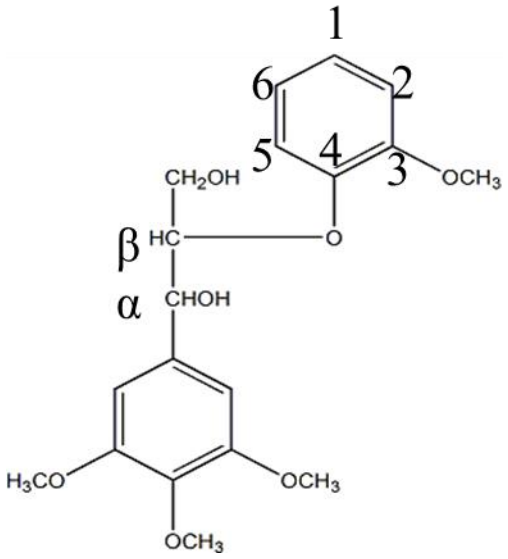


Figure 1-4 Veratrylglycerol-β-guaiacyl ether

The structural integrity of lignin polymers varies by species, subcellular location, and plant tissue.[22] Lignin is commonly defined as woody type lignin and herbaceous crop lignin (grass lignin), respectively. Within woody type plants, gymnosperm (softwood) lignin is composed by G units mostly,[23] whereas angiosperm (hardwood) lignin is composed of G and S units in different proportions.[24] The herbaceous crops lignin contains all three types of units.[20] Even within one plant, the distributions of monomers and lignin structure varies among lignin in different parts of the plant.[25]

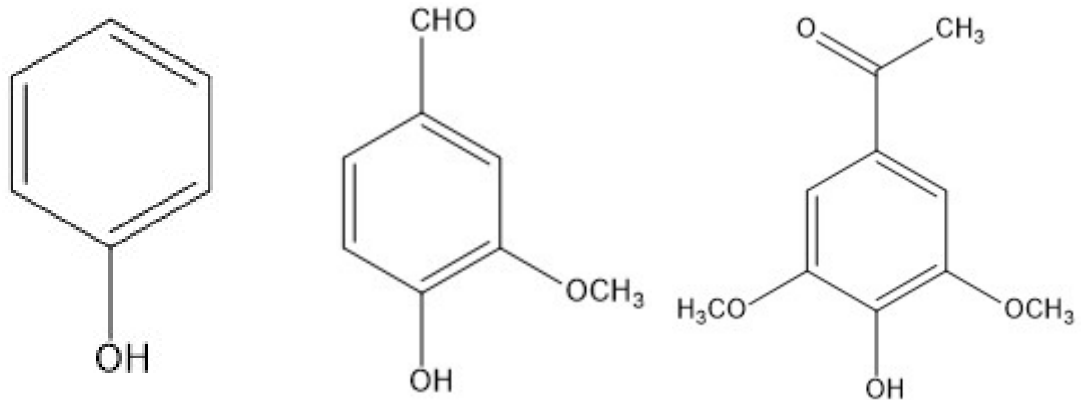
1.2.2 Lignin application

Nowadays, lignin, as a byproduct of pulping process, is still mainly (nearly 99% of commercial lignin) burnt as an internal fuel for pulping process. However, the pulping efficiency has continued to improve, less energy is needed, in turn allowing more lignin to be converted into high value-added products. Lignin, due to its functions in biomass and chemical characteristics, can be used in various high value-added applications rather than simply as an internal heat source for the pulping process.[26] Lignin has a high concentration of reactive oxygen-containing functional groups, especially the lignin obtained in acidolysis. Therefore, it can be used as low-toxicity binding agent for production of wood panel materials with good strength characteristics.[27] It was reported sulphur-free lignin can be used as a substitute for phenol formaldehyde resin.[28] Compared with the binder of 100% phenol formaldehyde resin, the lignin containing binder (10-20 % lignin content) offered great improvement, including stability of the friction coefficient to temperature variations and improved wear behaviour.

Lignin antioxidant is another hot spot in lignin applications. Because there are large number of active functional groups, including hydroxyl groups, methoxyl groups and carbonyl groups, lignin exhibit a powerful antioxidant activity.[29] The antioxidant activity of lignin are greatly depended by the original material and the extraction method, which significantly influence the amount and proportions of active functional groups. Conventional industrial methods for lignin isolation, like Kraft protocol, involve processes using harsh conditions and sulphur-containing reagents, resulting in the decrease of active functional groups and antioxidant properties. Thus, a mild method that is able to extract lignin in its original form without structure damage and condensation is vitally important for the downstream application of lignin to act as an antioxidant agent. Till now it is reported lignin from different type of biomass, including bamboo,[30] corn stover[31] and grass[32] have exhibited strong antioxidant activities. Furthermore, because lignin could protect plant against pathogen attacks,[33] it is found lignin, while working as a strong antioxidant, also has some antimicrobial properties,[31] making lignin very attractive in the application of preservatives.

Lignin, as a polyphenol compounds, is a vast potential source for aromatics *via* depolymerisation. As lignin is composed by three type of monomers (G, H, S units), multiple building-blocks, like vanillin, phenol and acetosyringone (Figure 1-5) can be achieved after lignin degradation. A protocol of vanillin recovery from lignin *via* membrane and ion exchange technologies was illustrated in Figure 1-6.[34] A mixture of multiple phenolic compounds can be achieved via oxidation in alkaline medium. Vanillate compounds can be separated from the mixture using membrane process. Ion

exchange process could convert vanillate into vanillin. After evaporation and crystallisation, solid vanillin can be obtained. In addition to vanillin, the unconvertable lignin can still be used as biofuels and other applications in this protocol. These building-blocks can be further used in the food, beverage, perfume, pharmaceuticals et al.[35]



Phenol Vanillin Acetosyringone

Figure 1-5 Three typical aromatic products from lignin

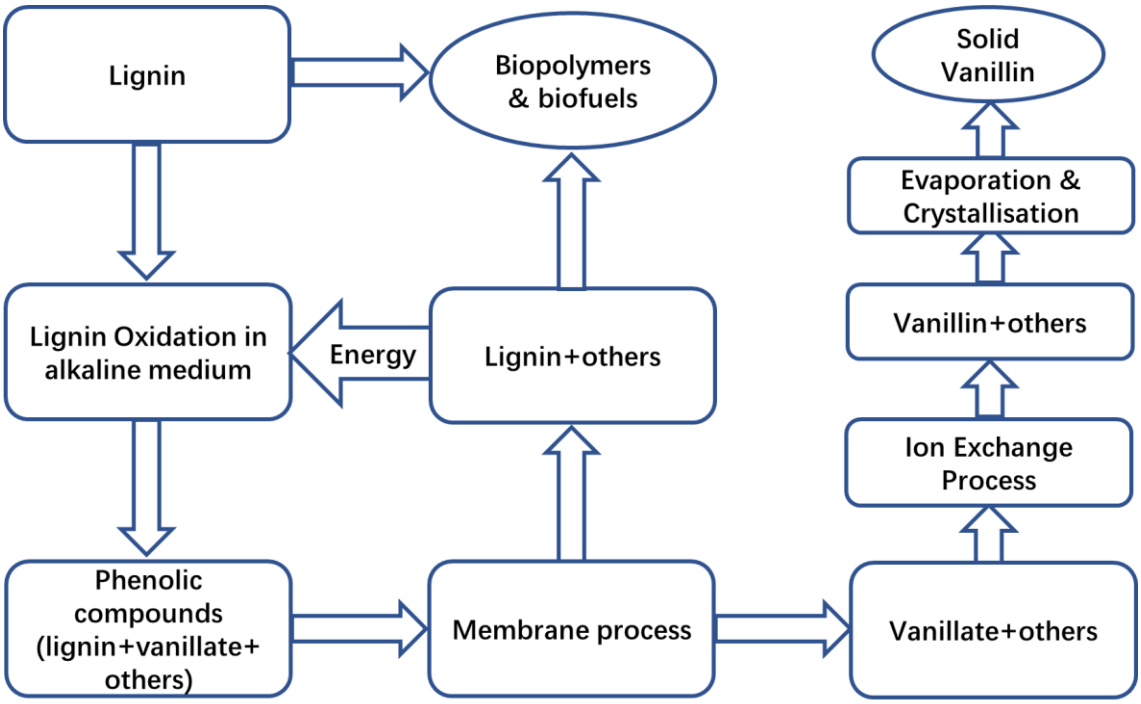


Figure 1-6 A protocol of vanillin production from lignin[34]

Based on the discussion above, the application of lignin can be broken down into three groups (Table 1-1): fuels, macromolecules and aromatic small molecules.[36] Unfortunately, till now the majority of lignin is still low-efficiently used as fuel. Only a small portion of lignin is applied for aromatic small molecules production. The lignin isolation method is a main reason for this situation. Industrial pulping involves harsh conditions that significantly damages the structure and changes the characteristics of lignin. Take the production of vanillin for example: Before 1980, 80% of all vanillin produced came from lignin; but in 2010, only 20% of vanillin is produced from lignin, the remaining 80 % from crude oil.[36] Compared with natural and lignin-based vanillin, crude-oil-based vanillin is a non-renewable end product. This is because, beginning in the 1980s, the era of modified Kraft pulping (originally called extended delignification) was founded on chemical principles intended to make Kraft pulping more selective for delignification over polysaccharide degradation.[37] This change in the pulping process made vanillin production alongside pulp environmentally unfriendly. In 2010, a report showed the annual vanillin demand was growing at a stable annual growth rate of 2% in Europe and the USA; however, the annual growth rate reaches 10% in China. This rising demand, combined with uncertainties on supply (50% of vanilla is exported from Madagascar) is pushing up the prices of both natural and synthetic vanillin. [36] These facts suggest the huge market of vanillin. However, typical pulping process, like Kraft pulping, hinders vanillin production from lignin, because the harsh conditions and sulphur-containing reagents cause severe condensation and contamination of lignin.

Table 1-1 Categories of lignin applications[36]

GROUP	VOLUME	VALUE	APPLICATION EXAMPLE
Power/Fuel	High	Low	●Carbon source for energy production
Macromolecules	Medium	Medium	●High molecular mass applications like wood adhesives (binders) ●Carbon fibres ●Polymers, e.g., polyurethane foams ●Antioxidant
Aromatics	Low	High	●Polymer building blocks ●Aromatic monomers, e.g., benzene, toluene and xylene (BTX) ●Phenol ●Vanillin

From the example of vanillin production, the importance of lignin extraction methods upon lignin downstream application can be manifested. An efficient and sustainable protocol to extract low sulfur lignin from biomass is a key and the first step for high-ending applications. In the next section, several typical lignin extraction methods will be introduced, including both lab-based and industrial scales methods.

1.2.2 Classical methods of isolation of lignin

The methods of isolation (or extraction) are of great importance in lignin study, as the characteristics of lignin (including purity, yield and structure modification) are significantly affected by the isolation approaches. The ideal method of lignin isolation, which should be applied for mass production, would provide a high yield of lignin, no change in lignin structure and non-lignin contaminants would be absent.[38] However, till now none of the existing isolation methods can achieve the goal of both quality and quantity. Commercial and industrial lignin is achieved in papermaking and pulping as a

by-product. Various methods are applied to extract lignin from the lignin-rich liquid waste in pulping progress—black liquor. Among these industrial scale protocols, the Kraft lignin protocol (detailed protocols in Section 1.2.2.4) seems to be the most well-known method.[39] In a Kraft pulping, NaOH and Na_2S are used for delignification to solublise and degrade lignin in a high temperature cooking. After cooking, lignin and hemicellulose enriched solution, the black liquor is achieved. Raw Kraft lignin can be recovered by adding acids into black liquor for precipitation. However, industrial lignin is featured with severe saccharide contamination and structure modification due to the intensive conditions, causing significant degradation of lignin and incomplete cleavage of lignin-carbohydrate linkages.

Compared with industrial lignin isolation methods, which are in favor of mass production, the lab-based lignin protocols care more about quality, which focus on reducing carbohydrate contaminant and keeping structure intact. The early studies tend to use strong acid to cleave the linkage between lignin and polysaccharide to isolate lignin, such as Klason lignin (KL).[40] Because concentrated sulfuric acid is applied in Klason protocol, structure of Klason lignin is severely modified from native lignin in biomass. Therefore, several milder protocols have been subsequently proposed to isolate lignin from wood, including milled wood lignin(MWL) protocol, enzymatic mild acidolysis lignin (EMAL) protocol, cellulolytic enzyme lignin (CEL) protocol and Brauns lignin protocol. Theses lignin are all characterised a high level of consistency with native lignin, especially MWL and CEL, which are believed to be the best matrices for lignin structure analysis.[41] However, all these methods share a same disadvantage of cumbersome protocols. Take

MWL protocol for example, the ball milling step alone takes more than 48 hours, not mentioning the pretreatment and post-processing after milling. With modem milling machine, the milling time could be shortened, but still it lasts for many hours and the intensive milling is rather energy consuming. A brief introduction of these isolation methods is provided in following text.

1.2.2.1 Klason Lignin (KL)

In the early 1900's, Peter Klason established a lignin isolation method, by which acid insoluble lignin could be obtained using a two-step hydrolysis.[42] The first step is to soak biomass in concentrated sulfuric acid (primary hydrolysis, PH). Klason claimed that 61-68 wt% H_2SO_4 at room temperature was the optimum condition in this stage. The concentrated acid help with the cleavage of the bond between lignin and carbohydrate. The second step is to dilute acid solution and then reflux the mixture for a few hours, which is the secondary hydrolysis (SH). Researchers had made several modifications based on this original protocol and finally formed the standard Klason method protocol (Figure 1-7)[43]: in PH, soak 1.0 g feedstock in 15ml 72wt% H_2SO_4 in 20 °C for 2 h; then in SH, dilute the acid to 3wt% with de-ionised water, reflux this mixture for 4 h. This method and its modified version have been widely used as standard methods in lignin content determination, such as the standard TAPPI T222 (acid-insoluble lignin in wood and pulp),[44] because compared with other isolation protocol KL has the least carbohydrate contamination (especially for softwood feedstock). Till now many studies are focused on these protocols and changes have been made to fit various feedstock. Matsumoto et al.

investigated the sugar contents of KL.[45] They found KL from softwood contained 0.75% neutral sugars, while for KL from hardwood, this value is 0.95%. Bose et al.[46] found that in PH step if mixing was inadequate, cleavage between lignin-carbohydrate (L-C) would be incomplete. Nicholson[42] pointed out in SH, the cleavage of L-C was incomplete in typical conditions, such as using 3% H_2SO_4 refluxing for 4 h at 100 °C[43] or 4% H_2SO_4 for 1 h at 121 °C (in pressured autoclave)[47]. To achieve a better lignin isolation, he designed a two step-SH (Figure 1-8): in SH-1, 72 wt% H_2SO_4 was diluted to 40 wt% and hydrolysis took place at 80℃ for 1 hour; in SH-2, mixture was further diluted to 3% H_2SO_4 and refluxed for 1 hout at 100 °C.

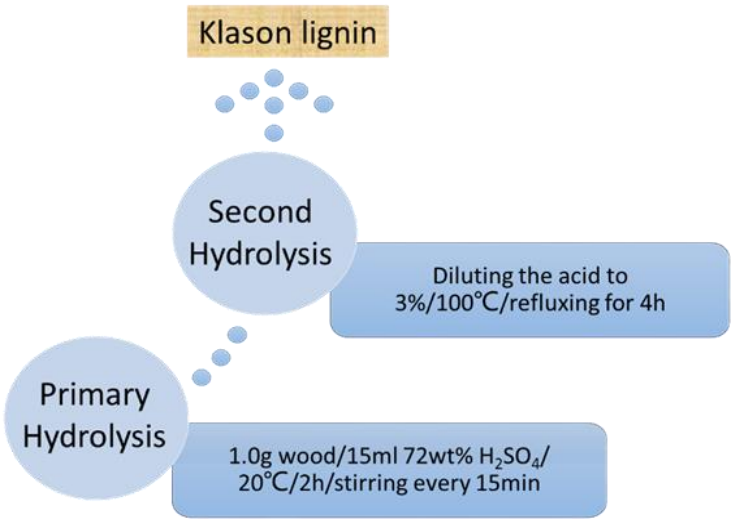


Figure 1-7 Klason Lignin Protocol

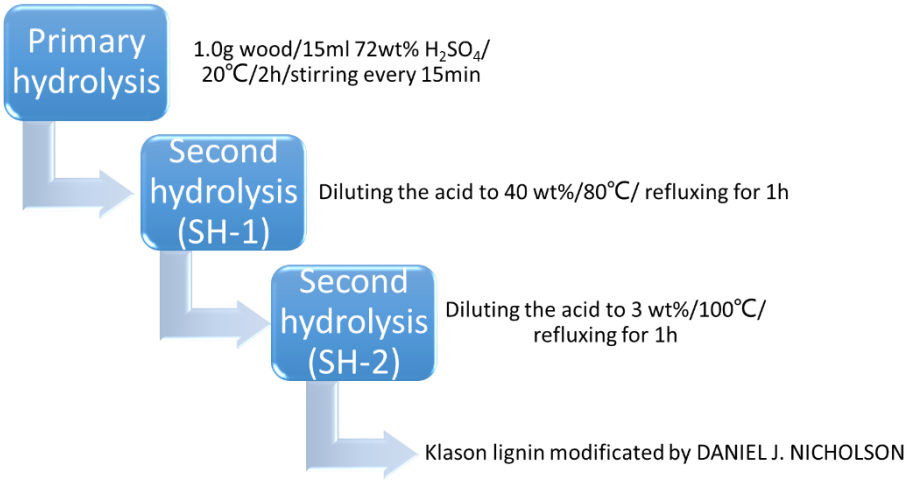


Figure 1-8 Nicholson Modified Lignin Isolation Protocol

1.2.2.2 Milled wood lignin (MWL) and cellulolytic enzyme lignin (CEL)

Among all types of lignin, milled wood lignin (MWL or Bjorkman lignin) is believed to be the best matrix to represent native lignin in original biomass. It marks a profound milestone as lignin is extracted in neutral solvent without elevated temperature.[48] MWL protocol has long been used as a standard method for lignin structure studies. According to Björkman's protocol, the key step to cleave the lignin-carbohydrate complex (LCC) is achieved when extractive-free meal of a woody species is ground for 48 hours or more in a vibratory ball mill under nitrogen atmosphere. The detailed MWL protocol is as follow (Figure 1-9):

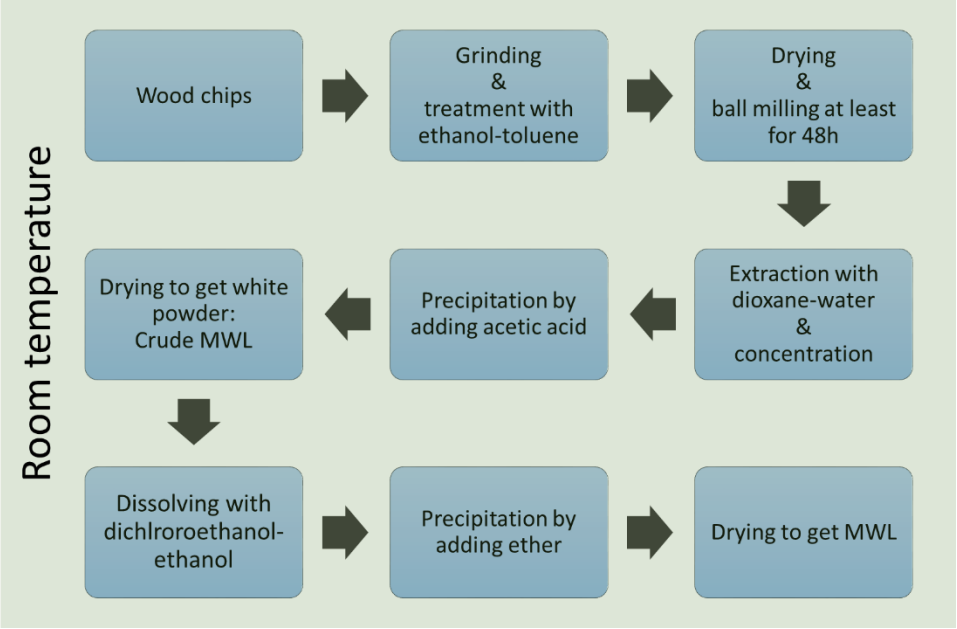


Figure 1-9 MWL Lignin Protocol

To start, woody biomass is ground into powder of 40 mesh and then refluxed (about 4-6 h) with ethanol-toluene (1:2) to prepare extractive-free meal. To conserve the structure of lignin to the maximum, vacuum drying rather than drying oven is used for sample drying throughout the whole procedure. The dried meal is processed in a vibrational ball mill in the presence of an organic solvent with a non-swelling agent, such as ethanol-toluene (ethanol as solvent and toluene as non-swelling agent). This milling lasts for 48 hours at room temperature. Milling time could affect the characteristics of MWL significantly.[49] For different woods the milling time should be modified and optimised accordingly. During ball milling the cell structure of the wood is destroyed. Dioxane-water (9:1) is used to extract lignin after the milling. The extraction liquid is concentrated and then added to acetic acid to precipitate of the acid-insoluble lignin. After drying, the obtained white powder is crude MWL. To purify this crude lignin, dichlroroethanol-ethanol is used

to dissolve it, diethyl ether is then added into solvent to bring about precipitation. After drying in vacuum dryer again, final MWL is recovered.

As shown in Figure 1-9, because the whole procedure is using mild conditions, such as room temperature and non-acid solution, MWL minimises damage to the lignin structure and therefore is believed the best matrix to represent native lignin. However, one drawback of MWL is low yields compared to other protocol. It was found using MWL method only 34 % of lignin can be recovered after 28 days milling.[50] Some other disadvantages include low purity, contamination of polysugar and time/energy consuming.[41] The lignin isolated is the lower molecular weight more soluble components and therefore less representative of the whole structure

To overcome the weakness of MWL protocol, in 1975 cellulolytic enzyme lignin (CEL) method was proposed.[51] The main improvement is the utilisation of a mixture of cellulase/hemicellulase in order to degrade polysaccharides. Therefore, the obtained lignin is less contaminated by cellulose and hemicellulose. Compared to MWL procedure, CEL procedure is featured with a higher yield and time-saving (using same extraction time, CEL protocol produce lignin with higher yield and purity than MWL protocol)[52], and CEL is also highly representative of native lignin as MWL. However, because LCC can hardly be cleaved completely under mild conditions of both CEL and MWL methods, it still faces contamination of carbohydrate somehow. CEL, same to MWL, is recovered from dissolved lignin in extraction solution rather than from biomass directly, resulting

in a similar problem of low molecular weight. Moreover, it was found the protein from enzyme became an additional source of contaminant.[41]

1.2.2.3 Enzymatic mild acidolysis lignin (EMAL)

Acidolysis and neutral hydrolysis are two main branches for lignin isolation. However, each of them has some drawbacks respectively. As concentrated acids used in Klason protocol could change structure of lignin severely, it is only suitable in lignin content determination. Therefore, to isolate lignin, mild acidolysis is more widely used. Wu etc.[52] proposed one famous mild acidolysis protocols is to reflux the pulp under a nitrogen atmosphere with acidic dioxane–water solution (0.1 N HCl) and recover the lignin from this solution. The hydrolysis residue (HR) is lignin of high purity; but the yield is less than 50 %. Moreover, it still faces the same problem of KL: some internal linkages of lignin are changed somehow, such as β-aryl ether bond.[53]

As discussed above, CEL protocol has the advantages of higher efficiency and yield compared with MWL protocol due to the application of enzymes, hence being widely used for lignin isolation. In this method, carbohydrates are digested by cellulases and hemicellulases, leaving lignin intact in the HR. This method gives high yield of lignin. But the isolated fraction contains huge amount of carbohydrates and some protein from the enzyme.[53]

Combining mild acidolysis and CEL protocols, Wu etc. designed a two-step enzymatic mild acidolysis lignin (EMAL) isolation method.[52] The first step is to remove carbohydrates by an enzymatic treatment, and in second step a mild acidolysis is applied.

The enzymatic treatment could expose the fiber structure, thus reduce the diffusion in the acidolysis of second step. Because EMAL method is optimised to have the advantages of both acidolysis and CEL methods, a high lignin yield of 70% is achievable without obvious structure alteration, and contamination is relatively low. However, EMAL method is the most tedious among all lignin isolation protocols. The key step of two-step EMAL protocol is as follow (Figure 1-10):

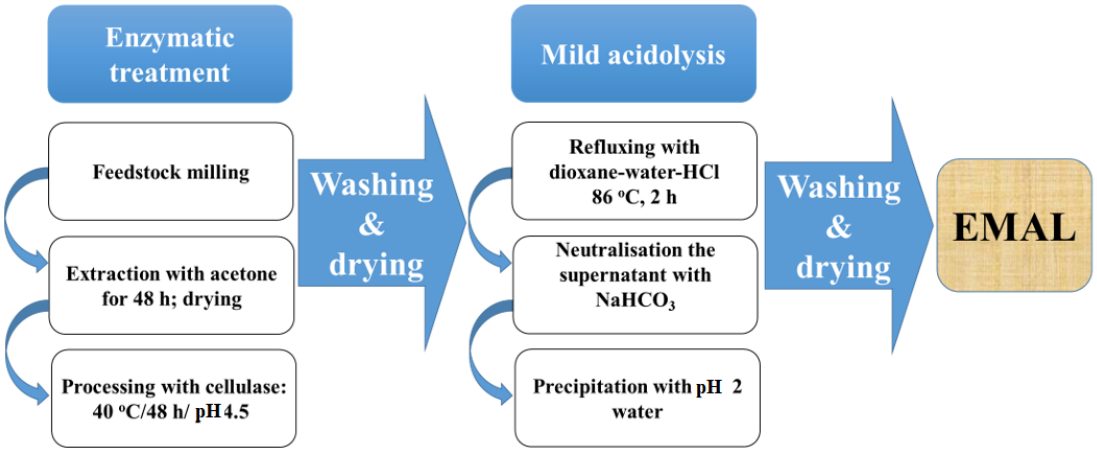


Figure 1-10 the key steps of EMAL protocol

Step 1: enzymatic treatment

Feedstock (poplar and black spruce) is milled in Wiley mill. The milled feedstock is extracted in acetone for 48h and then dried to get the wood meal. Wood meal is treated with cellulase at 40 ℃ for 48 h at pH=4.5, followed by washing and drying to be prepared for acidolysis.

Step 2: mild acid hydrolysis treatment.

The residue from Step 1 is suspended in acidic dioxane-water (85: 15 v/v and 0.01M HCl) mixture. The residue/liquid ratio is 5 g and 100 ml, respectively. This mixture is refluxed for 2h at 86℃ under nitrogen atmosphere. After centrifuge (or filtration), the supernatant is withdrawn, neutralised with sodium bicarbonate and finally added dropwise to the final volume of 1 L with diluted HCl (pH=2). Then the precipitation is dried to powder, washed by hexane and vacuum dried at room temperature.

1.2.2.4 Industrial lignin-Kraft lignin (lignin from black liquor)

The lignin isolation methods mentioned above are lab-based because the tedious protocols are not suitable for large scale production. The main applications of these lignin are structure investigation and property study but not for commercial use. Till now, pulping is still the main source for commercial lignin. In paper making and pulping industry, lignin is an unwanted material and always discarded as waste (sometimes burned for energy).[54] Black liquor is a main by-product in paper making and pulping, containing about 50% lignin. On industrial scale, black liquor is the predominant source for lignin isolation. It is formed when digesting wood into paper pulp *via* removing lignin, hemicelluloses and other extractives from the wood to free the cellulose fibers.[55] In different pulping processes the component of black liquor changes, resulting in various methods for lignin extraction, such as Kraft lignin from Kraft pulping, lignosulphonate lignin from sulphite pulping etc. However, nowadays 99% of this lignin is used as a low value internal fuel in pulping process.[26] As pulping efficiency has continued to improve, less energy is needed, in turn allowing more exploitation of industrial lignin for high

value-added products. As Kraft pulping is a dominant chemical pulping progress,[39] here only Kraft lignin protocol is discussed among several industrial lignin protocols. The progress can be divided into two steps (Figure 1-11):

Step 1 black liquor generation[56]

In a typical Kraft cook, an aqueous solution of sodium hydroxide and sodium sulfide, also known as white liquor, is reacted with the wood chips in a large pressure vessel called a digester. The mixture of white liquor and wood is heated to 170 ℃ and held for 2 hours.[57] During this treatment, lignin is degraded into smaller fragment and dissolved into the white liquid. The whole cook can be divided into three phases[56]: the initial phase (room temp-150 ℃), the bulk phase (150-170℃ and cooking treatment) and the final phase. At the start of final phase 90 % of lignin is removed and further cooking will significantly degrade carbohydrate.

Step 2 lignin extraction from black liquor

The pH of black liquor is lowered by 1M sulfuric acid with stirring at 50 ℃. When the pH of black liquor reaches 9.0, sodium hydroxide and sodium sulfide are greatly removed (converted into Na_2SO_4 and H_2S gas). Lignin starts to precipitate at pH 6.0-6.5 and end at pH 3.0. When lower than pH 4.0, more sulfuric acid will only achieve a little more precipitation meanwhile increase cost. After filtration, precipitation is washed by acidified water. After drying, Kraft lignin is isolated. The sodium can be recycled with further treatment.

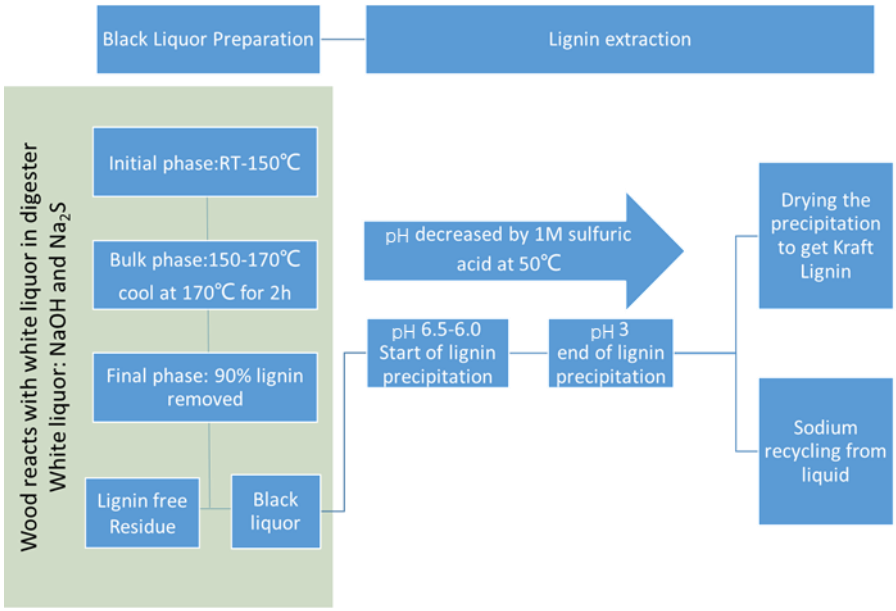


Figure 1-11 Kraft Lignin Protocol

Similar to Kraft lignin, there are several other types of lignin isolated from black liquor, including alkali lignin and soda lignin. All these lignins from black liquor are typical commercial lignin on industry scale. Compared to lab-based lignin, industrial lignin protocols are easy to operate and suitable for mass production for commercial use. However, on the other hand, these lignin structure is far from the native lignin due to the intensive conditions.[41] During the process of pulping, lignin is significantly degraded and contaminated by saccharides, reducing the quality of commercial lignin significantly and hindering for value-added applications.

1.2.2.5 A comparison of lignin isolation methods.

Based on the discussion above, a comparison was made between these typical lignin extraction methods. The characteristics of lignin, including the purity and structure, are greatly dependent on the isolation method. The ideal lignin isolation protocol should be

able to remove all non-lignin compounds (polysaccharides, ash, pectin…) from biomass while keep the original structure of lignin. Unfortunately, none of the existing methods could achieve this goal.

Table 1-2 A comparison of different lignin isolation methods[41]

Name	Principle of the method	Advantages	Disadvantages
Klason lignin (KL, quantitive measurement of lignin)	Residue after treatment of sample with 72% sulphuric acid	Suitable for a wide range of samples, the least sugar contamination	Structure different from that of lignin in biomass Only suitable for content determination
Milled wood lignin (MWL)	Ultra-milling of wood and extraction with neutral solvents followed by precipitation in water and ethyl ether	Best representation of native lignin, macromolecular	Low yield, preparation timeconsuming and cumbersome, some contaminating sugars
Cellulase enzyme lignin (CEL)	Cellulase enzyme treatment	Good representation of native lignin, macromolecular	Low yield, contains protein impurities
Enzymatic mild acidolysis lignin (EMAL)	Enzyme treatment followed by mild acidolysis	Purity and yield: MRL<CEL<EMAL Structure preservation: EMAL<CEL<MRL	
Kraft lignin	Cooking in the presence of NaOH and Na_2S	Easy availability, suitable for industrial-scale production	Structure far from native lignin, not entirely macromolecular, contaminating sugars

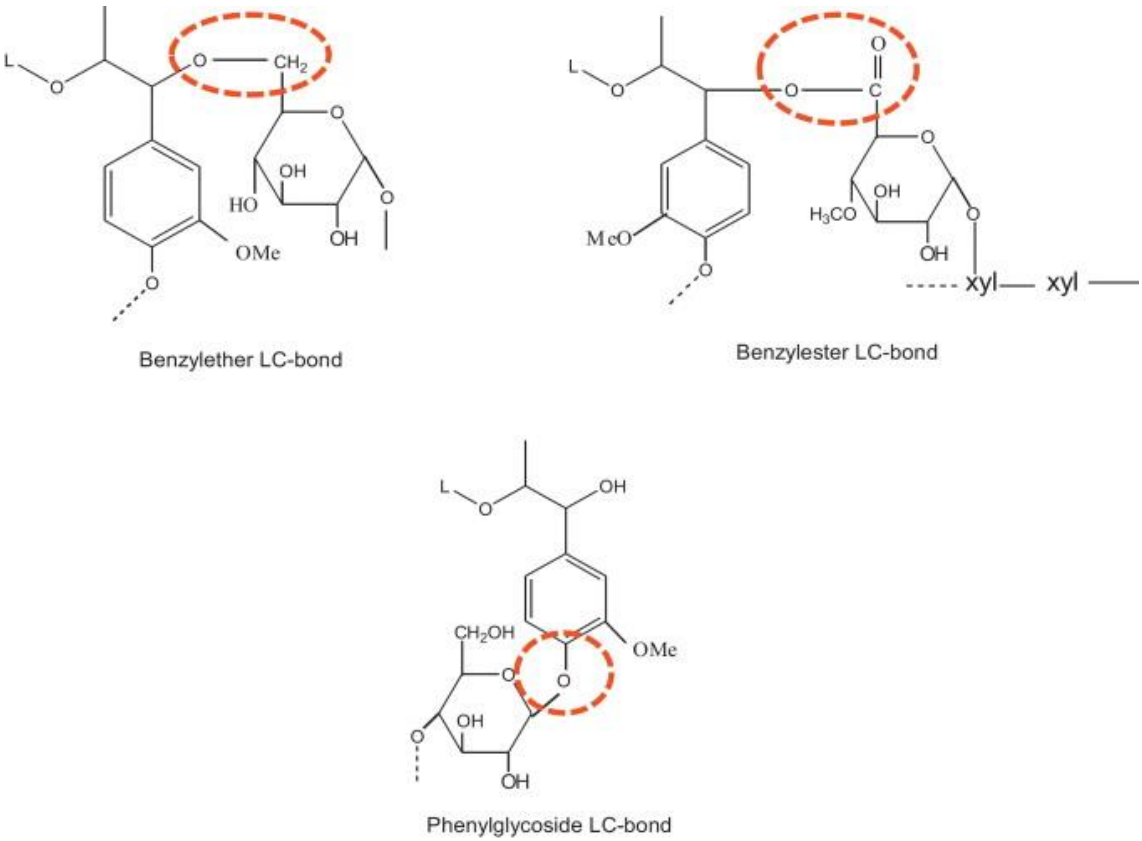


Figure 1-12 Three typical LCC bonds in biomass[58]

The cleavage of lignin-carbohydrate complex (LCC, Figure 1-12), which is the linkage between lignin and carbohydrates, is a key process in lignin isolation. This can be achieved by both chemical and physical methods. In the Klason protocol, chemical treatment using 72 % sulfuric acid is applied to cleave LCC thoroughly; however, this strong concentrated acid destroys the lignin structure. Hence, the Klason method and its modified versions are only regarded as standard methods of lignin content determination, but are seldom used in lignin isolation. Physical milling is less efficient in the cleavage of LCC compared with chemical methods, hence severe sugar contamination occurs in MWL. After milling process, extracted lignin is still largely chemically linked to hemicellulose by LCC bonds,[59] which significantly reduces the lignin purity. As a result,

MWL protocol can only produce lignin with purity around 80%. [52,60] Moreover, a considerable portion of lignin is lost due to the incomplete cleavage of LCC. According to relative studies, the lignin yield of MRL protocol is only 20-30%,[52] which indicate the majority of lignin in biomass is lost along with the removal of polysaccharides. Compared with MWL, CEL and EMAL protocols could produce lignin with remarkably higher yields as a result of the application of enzyme treatment and mild acidolysis, especially for wood biomass as shown in Table 1-2. Using enzymes with cellulolytic and hemicellulolytic activities, such as Onozuka SS1500 Cellulase,[51,61] a substantial amount of the carbohydrate fraction is removed before lignin extraction. Similar to enzyme treatment, mild acidolysis is another effective method to cleave to covalent linkage between lignin and carbohydrate, in turns increase the lignin yield dramatically.[50] As shown in Table 1-3, the EMAL procedure yields are approximately 2 to 5 times greater than the MWL or the CEL procedure yields. In addition to yields, enzyme and mild acid processes could also increase the molecular weight of isolated lignin (Table 1-4), because after enzyme and mild acid treatment lignin is more extractable than that in MWL procedure, where only physical milling is applied for the cleavage of LCC bond. While CEL and EMAL procedures exhibit a remarkable advantage in lignin yield, on the other hand, the structure modification is more severe in these two protocols. As shown in Table 1-4, the dispersity indices (Đ) of CEL and EMAL are higher compared to those of MWL, suggesting CEL and EMAL are more heterogeneous due to the structure damage occurring during lignin isolation. It is widely believed many biological polymers, including native lignin in biomass, have low dispersites. A uniform structure of lignin is

in favor for research and downstream applications. Therefore, despite the low yield, MWL is generally viewed as the best representative of native lignin's chemical structure and reactivity.

Table 1-3 Typical lignin yields from various biomass sources isolated by MWL, CEL, and EMAL[50,62]

Biomass	Isolation Method Yields (%)		
	MWL	CEL	EMAL
Norway Spruce (milled)	11.4	23.4	44.5
Douglas Fir	1.4	7.1	24.8
Redwood	15.7	13.2	56.7
White Fir	11.3	11.5	42.9
E. globulus	34.0	32.5	63.7
Southerm Pine	11.9	12.4	56.3

Yield determined by Klason method; MWL, milled wood lignin; CEL, cellulolytic enzyme lignin; EMAL, enzymatic mild acidolysis lignin

Table 1-4 Average molecular weights and dispersity indices of MWL, CEL and EMAL of various biomasses[50,62]

Biomass	MWL		CEL		EMAL	
	M_w	Đ	M_w	Đ	M_w	Đ
Norway Spruce (milled)	23500	3.7	53850	5.7	78400	8.8
Douglas Fir	7400	3.0	21800	4.0	38000	5.0
Redwood	5900	2.5	23000	4.2	30100	6.4
White Fir	8300	3.0	21700	4.6	52000	8.2
E. globulus	6700	2.6	17200	3.1	32000	3.7
Southerm Pine	14900	3.2	29600	3.9	57600	5.9

M_w, average molecular weights, g/mol; Đ, dispersity indices

The characteristics of industrial lignin from pulping, like the typical Kraft pulping, is significantly different from lignin above due to the harsh conditions in pulping process. The Kraft pulping process generally fragments lignin, thereby decreasing its molecular weight (Table 1-5). During Kraft pulping, wood chips are added to an aqueous solution of sodium hydroxide and sodium sulfide (white liquor), which facilitates the cleavage of select bonds in lignin and results in depolymerisation of lignin.[56,63] In addition to

depolymerisation, condensation of lignin during Kraft pulping also changes the lignin structure and reduces the solubility of lignin.[56] Figure 1-13 illustrates a typical condensation reaction of phenolic units during the alkaline treatment of Kraft pulping. The lignin structure modification caused by depolymerisation and re-condensation results in a non-uniform lignin product, hindering the downstream applications significantly.

Table 1-5 Average molecular weights and dispersity indices of Kraft lignin

Biomass[a]	M_w	Đ
Spruce+Pine[64]	4500	4.5
Birch[65]	19650	2.7
Eucalyptus globulus[64]	2300	4.3

a: Lignin is extracted from the black liquor of Kraft pulping of biomass, not from biomass directly
M_w, average molecular weights, g/mol; Đ, dispersity indices

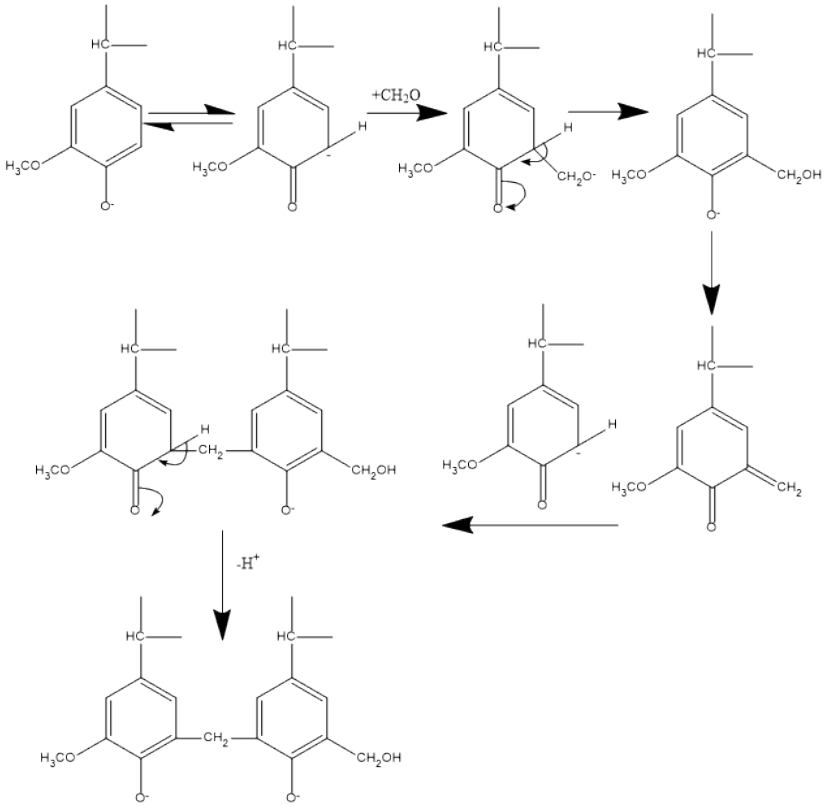


Figure 1-13 Alkali-promoted condensation reactions in phenolic units during Kraft pulping[56]

Till now, Kraft lignin is mainly used as fuels for pulping process. However, the combustion of Kraft lignin can be a source for SO_x pollution. In addition to carbohydrate contamination that occurs in all types of lignin (except Klason lignin), sulphur content is also considerable in Kraft lignin as a result of the action of NaOH and Na_2S used for separating cellulose from the other wood constituents in pulping. It is reported that the sulphur content can reach 1.5-4.5 % in Kraft lignin.[66,67] The high content of sulphur in Kraft lignin is an obstacle for its downstream applications. In addition to a potential source of pollution, the high sulphur content is also an inhibitor in the production of aromatics from Kraft lignin, as it is found that sulphur can negatively affects the reactivity of lignin in degradation.[67,68] In general, it is widely believed industrial lignin has more structure modification and impurity. Different from MWL, CEL and EMAL that are obtained by 'lignin-first' processes, industrial lignin is the by-product from pulping that is designed to achieve cellulose fibre. This fact determines that industrial lignin can hardly have the same quality as good as lab-based lignin, probably rendering it less attractive in high-value added applications.

1.3 Applications of microwave thermal treatment in valorisation of biomass

Microwave thermal treatment has risen increasing interests in the recycling and conversion of solid waste and biomass. Due to effective and selective heating of microwave, coupled with the nature of lignin (low polarity), microwave treatment could hopefully become a powerful tool in lignin isolation.

1.3.1 Microwave: dielectric heating

Microwave is a type of electromagnetic wave which contains electric and magnetic field components. The electric field applies a force on charged particles as a result of which the charged particles start to migrate or rotate. Due to the movement of charged particles further polarisation of polar particles takes place. The concerted forces applied by the electric and magnetic components of microwaves are rapidly changing in direction causing warming because the assembly of molecules, e.g., a liquid or a semi-solid, cannot respond rapid enough to the changing direction of the field and this creates friction which manifests itself as heat.(Figure 1-14)[69] In addition to the dipolar polarisation, the ionic polarisation of ions in microwave could also contribute to the energy conversion from microwave into heat as a result of the fast movement of ions due to their inherent charge (Figure 1-14). The polarisation of polar molecules and ions creates 'internal heat source' or 'hot spot' in a microwave process. Thus, different to conventional heating that energy is transferred from outside to inside, in a microwave heating, polar molecules and charged particles react with microwave directly and instantly, resulting a volumetric and easy-controllable heating. A comparison of conventional and microwave heating is shown in Table 1-6. Claimed effects of microwave dielectric heating can be divided into two kinds: thermal effects and non-thermal effects.[69] Thermal effects are those which are caused by the different temperature regime which can be created due to microwave dielectric heating. Non-thermal effects are effects which are caused by effects specifically inherent to the microwaves and are not caused by different temperature regimes.

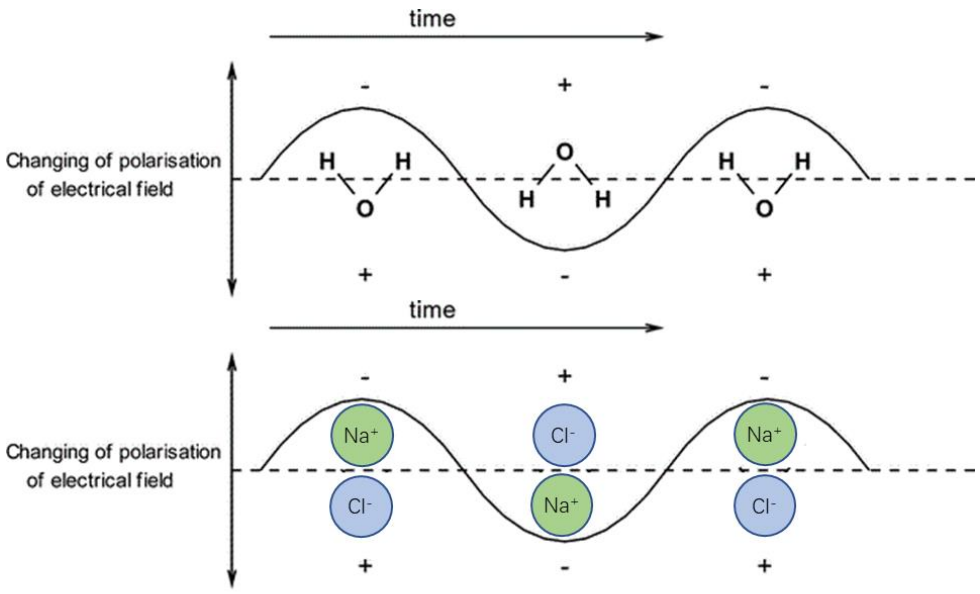


Figure 1-14 Dipolar polarisation (top)[70] and ionic polarisation (bottom) in microwave

Table 1-6 A comparison between microwave and conventional heating[71]

Microwave dielectric heating	**Conventional thermal heating**
Conversion of energy	Transfer of energy
In-core volumetric and uniform heating: the whole material heated simultaneously, energetic coupling at molecular level	Superficial heating: via convection/ conduction
Rapid and efficient, e.g., methanol can be rapidly super-heated to above 100 °C; ionic liquids can gain temperature jumps of 200 °C within seconds	Slow, inefficient, limited by material thermal conductivity
Selective: rapid intense heating for polar substances while ineffective for apolar substances	Non-selective
Hot spots: an effect due to inhomogeneities of microwave field or dielectric properties within a material, resulting in local temperatures in the material being much higher than the temperature measured in the bulk	No hot spot
Dependent on material's properties	Less dependent
Precise and controlled heating: the energy input starts and stops immediately when the power is turned on or off, respectively	Less controllable

Microwave heating is significantly dependant on the dielectric properties of the feedstock, including dielectric constant, dielectric loss and loss factor tanδ (or dissipation factor).[72] Dielectric constant describes the ability of molecules to be polarised by the electric field.

Dielectric loss is indicative of the efficiency with which electromagnetic radiation is converted into heat. Loss factor tanδ describes the ability of a specific substance to convert electromagnetic energy into heat at a given frequency and temperature. Loss factor tanδ is expressed as the following equation:

$$\tan\delta = \frac{\varepsilon''}{\varepsilon'}$$

tanδ, loss factor; ε'', dielectric loss; ε', dielectric constant

Due to the dielectric mechanism of microwave heating, molecules with high polarity (or dielectric constant) have the priorities to react with the external electromagnetic wave in a microwave reactor. However, this priority of reaction is not equal to an efficient heating. A reaction medium with high tanδ value and dielectric loss are required for efficient heating. The loss factors of some common organic solvents are summarised in Table 1-7. In general, solvents can be classified as high (tanδ > 0.5), medium (tanδ 0.1–0.5), and low microwave absorbing (tanδ < 0.1).

Table 1-7 Loss factors (tanδ) of different solvents (2.45 GHz, 20 ºC)[72]

Solvent	Tanδ	Solvent	Tanδ
Ethylene glycol	1.350	Water	0.123
Ethanol	0.941	Chlorobenzene	0.101
2-Propanol	0.799	Chloroform	0.091
Formic acid	0.722	Acetonitrile	0.062
Methanol	0.659	Ethyl acetate	0.059
Nitrobenzene	0.589	Acetone	0.054
1-Butanol	0.571	Tetrahydrofuran	0.047
2-Butanol	0.447	Dichloromethane	0.042
1,2-Dichlorobenzene	0.280	Toluene	0.040
Acetic acid	0.174	Hexane	0.020
1,2-Dichloroethane	0.127		

Water is a good microwave solvent in terms of loss factor, which allows water a fast temperature ramping in microwave heating. Thus, it is widely used as a solvent in microwave for biomass valorisation. In addition to loss factor, water as a solvent has several other advantages in microwave heating: 1) Water has advantages of being environmentally-friendly and cheap; 2) The boiling point of water is higher than most organic solvents, facilitating the microwave treatment at relatively high temperatures; 3) The majority of metal ions in biomass are soluble in water. When an electrical field is applied to solutions containing ions, the ions move at an accelerated pace due to their inherent charge. The resulting collisions between the ions cause the conversion of microwave energy of the moving ions into thermal energy, which increases the heating efficiency remarkably; 4) Water becomes relatively 'microwave-transparent' at high temperatures, ensuring the selective heating of feedstock based on its dielectric properties. As shown in Figure 1-15 (2.45 GHz is the frequency used in most microwave reactors), at low temperature, the dielectric constant and dielectric loss of water are high enough to ensure a rapid temperature ramping in microwave heating; on the other hand, the dielectric constant and dielectric loss of water drop drastically at relatively high temperature, allowing more microwave energy to work directly on biomass and less energy on the water solvent. The dielectric constant drops from 80 to 20 when temperature increases from 20 ºC to 300 ºC and it can be even lower at supercritical condition.[73] This dielectric property of water ensures a rapid temperature ramping at low temperature range and reduces the energy loss at high temperature range during a microwave heating process.

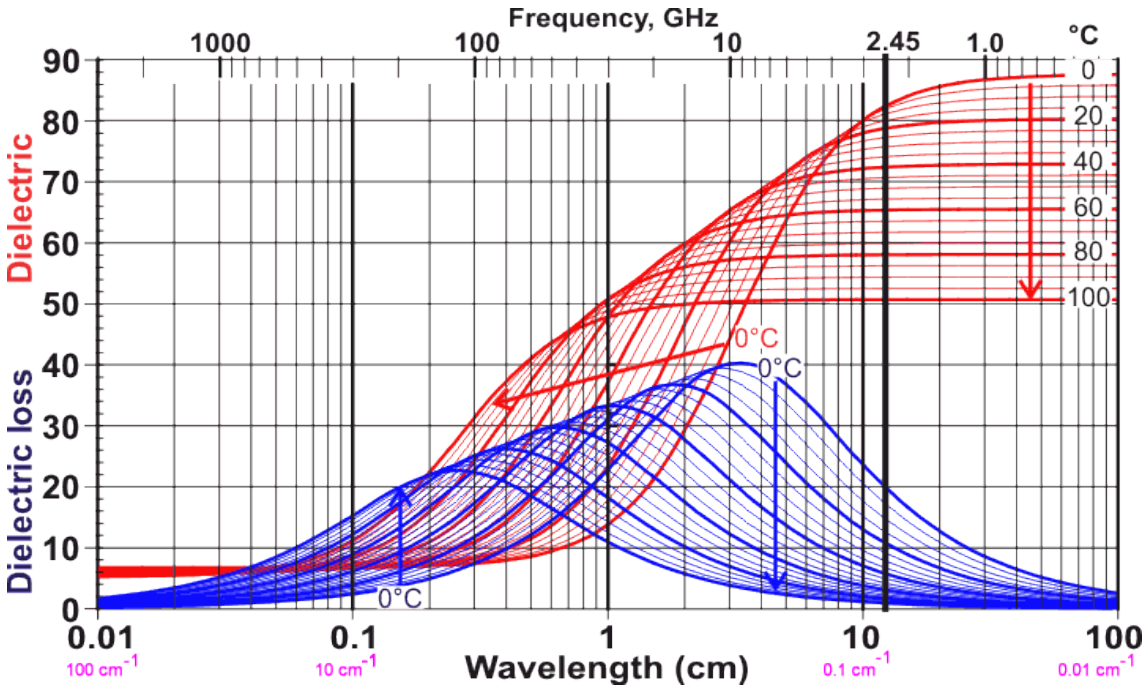


Figure 1-15 The dielectric constant and dielectric loss of water under different temperatures and frequencies (2.45 GHz is the frequency used in domestic microwave ovens.)[74]

The frequencies allotted for microwave dielectric heating are 918 MHz and 2.45 GHz, with the latter frequency being mostly used. The latter is also applied in domestic microwave ovens. Microwave oven is commonly designed for individual purposes, ranging from simple household oven to large scale batch or continuous oven. Among these, large scale continuous microwave ovens are used frequently in industry, especially food chemistry.

Different from the design of lab-scale reactor, where pressure and temperature tolerance are the main concern, in the design of pilot and industrial reactor, microwave penetration depth should also be taken into consideration. The dielectric properties and microwave

frequencies determine the penetration depths of certain materials in microwave. As mentioned earlier, loss factor is an index of the ability to convert electromagnetic energy into heat. While material with high loss factor could be heated effectively in a microwave process, the microwave penetration depth of this material is narrow due to the efficient energy absorption of microwave energy. As a consequence, for high loss factor material, the sample size should be reduced to achieve a uniform heating. On the other hand, non-polar or less-polar materials, like oil, have larger penetration depths, though these materials cannot be heated fast. A comparison between water and corn oil is shown in Table 1-8. Because of the high dielectric constant and dielectric loss, the penetration depth of water is relatively shallow. Thus, tubular microwave reactors are often designed for industrial applications when water or other materials with high loss factor are used as reagent. Notably, the loss factors of water and most organic solvents decrease with increasing temperature, i.e. the absorption of microwave radiation in water decreases at higher temperatures. In turn, the penetration depth of microwaves for water increases.[70]

Table 1-8 Dielectric properties of water and corn oil (2.45 GHz, 20 °C)[75]

	Water	Corn Oil
Specific heat, $J \cdot kg^{-1} \cdot K^{-1}$	4190	2000
Thermal conductivity $W \cdot m^{-1} \cdot K^{-1}$	0.609	0.168
Density, $kg \cdot m^{-3}$	1000	900
Penetration depth, cm	3.6	36.8
Dielectric constant	79.5	2
Dielectric loss	9.6	0.15

To increase the energy efficiency, 'microwave-transparent' materials are commonly used as reactor walls, ensuring the least energy loss on the reactor itself. As shown in Figure 1-16, glass, Teflon and quartz all have low loss factors and high penetration depths, hence making them suitable for microwave reactors. In addition to the high penetration depths,

Teflon and quartz also have high temperature and pressure tolerance. Since glass and quartz react with base at high temperature, when alkaline solvent or other corrosive solution are involved in reactions, Teflon reactor is preferred as a non-reactive material. Figure 1-17 shows the Teflon and quartz tubes used in two microwave reactors (CEM Mars and CEM Discover microwave reactor, respectively).

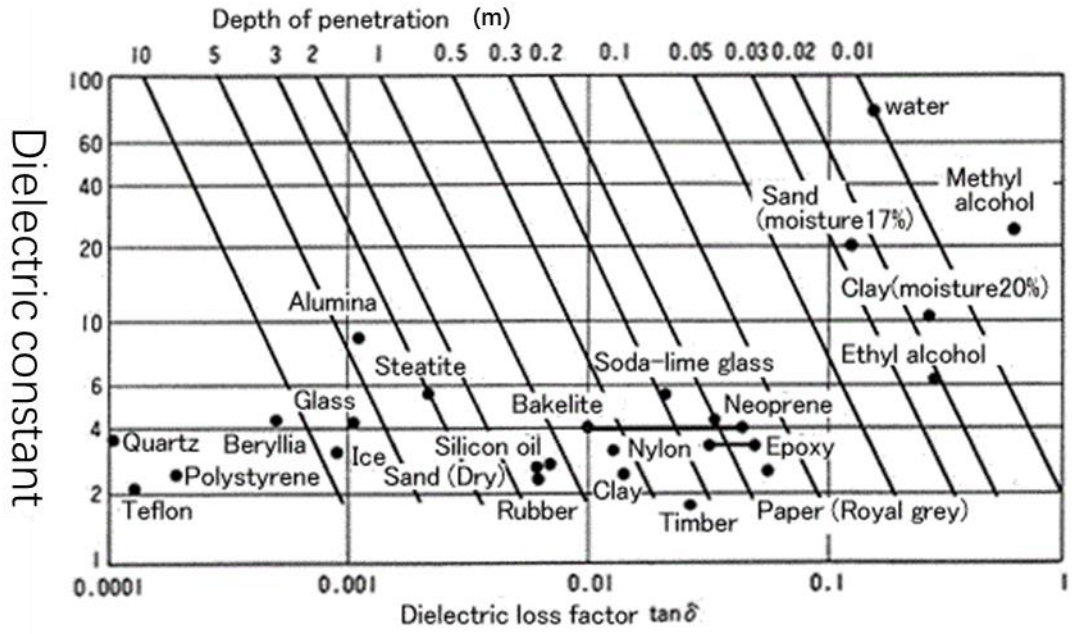


Figure 1-16 The dielectric properties and penetration depths of different materials (2.45 GHz, 20 °C)[76]

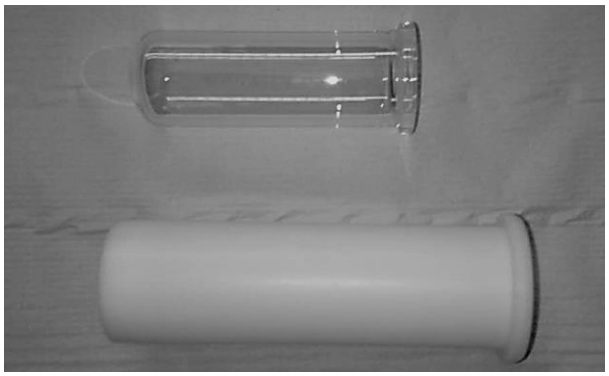


Figure 1-17 Microwave reactor tubes. top, quartz tube (30 ml) for CEM Discover microwave reactor; bottom Teflon tube (60 ml) for CEM Mars microwave reactor

The first application of microwave in chemistry was reported in the early 1970s, when gas-phase discharge was applied to realise decomposition of simple organic compound.[77] Since 1990s, microwave chemistry has been extensively reviewed.[78–80] These articles reported how microwave heating can be effectively used in analytical and food chemistry, organic and inorganic reactions. Due to the unique heating mechanism, microwave heating provides an efficient, flexible, controllable and powerful tool for chemistry investigation of various topics.

1.3.2 Valorisation of lignocellulosic biomass using microwave

The world is facing an energy crisis due to constantly increasing demand for fossil fuel, making it vitally important to maximise the potential of biomass to power the future.[81] Any future bio-refinery process has to become more effective and flexible than the existing processes, while not interfering with food supplies. Nowadays, microwave, due to its ability to shorten treatment time and hence reduce the energy consumption,[82] has

been widely applied for the valorisation of lignocellulosic biomass, including pyrolysis,[83] hydrolysis,[84] gasification[85] and pre-treatment like simply drying.[86]

The microwave thermal treatment of biomass can produce a wide range of products according to the type of feedstock and the microwave treatment conditions. (Figure 1-18) Generally, intensive conditions, like high temperature and power input, are in favour for the conversion of biomass polymer into products with small molar mass. For example, Fan[87] checked the degradable products of microwave hydrothermal degradation of cellulose from 150-230 ºC and it was found that the highest glucose yield of 14 % can be achieved at 220 ºC. The main reason accounting for this fact is the increase of mobility properties of cellulose alongside the temperature ramping as shown in Figure 1-19: 1) at temperatures below 180 °C the CH_2OH groups are hindered from interacting with microwaves while they are strongly involved in hydrogen bonding within both the amorphous and crystalline regions[88]; 2) Above the softening temperature (180 °C) these CH_2OH groups of amorphous content could be involved in a localised rotation in the presence of microwaves.[89] As such they could act similarly to "molecular radiators" allowing for the transfer of microwave energy to their surrounding environment[90–92]; 3) at temperature higher than 220 ºC, the crystalline content of cellulose also becomes active,[93,94] allowing more reaction of cellulose in microwave. This example indicates the significant influence of temperature to microwave treatment of lignocellulosic biomass. Lignocellulose, which is mainly composed by carbohydrate polymer (cellulose and hemicellulose) and aromatic polymer (lignin), can hardly affected by microwave irradiation at low temperature range due to the limited molecule (or functional group)

mobility; however, when temperature increases to the softening temperature, for example 180 °C for cellulose as mentioned above, localised rotation of functional groups occurs to create collisions and in turn produce heat. In this situation, simple low temperature microwave treatment could hardly achieve a thorough degradation of biomass. Moreover, dry biomass as a sole feedstock in microwave cannot be rapidly heated to the softening temperature due to its low loss factor.

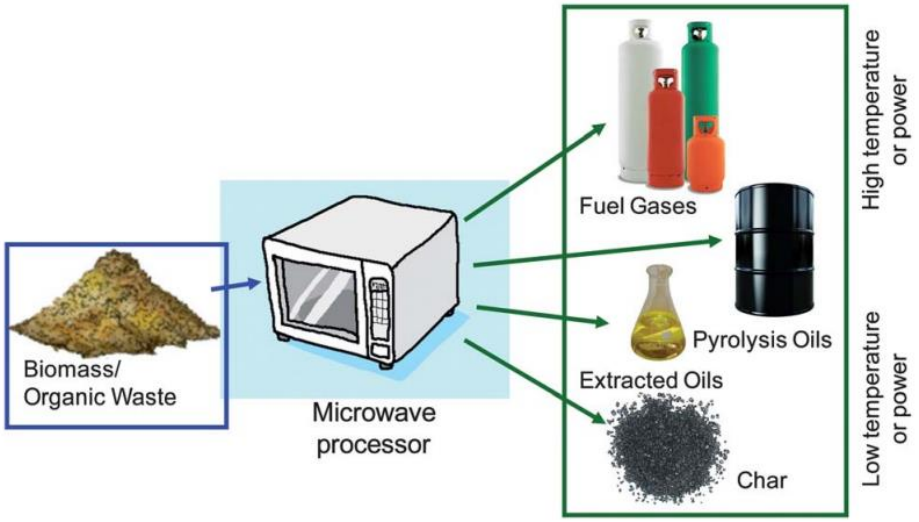


Figure 1-18 The microwave-assisted thermal treatment concept[95]

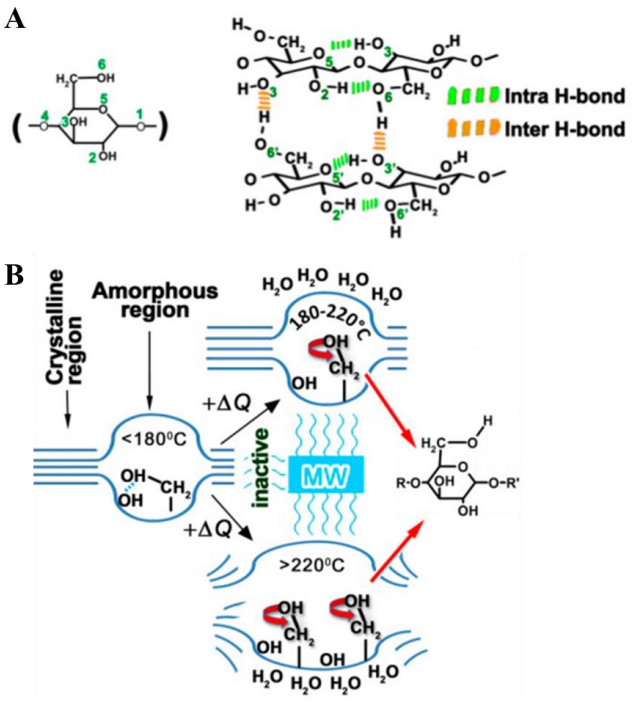


Figure 1-19 Cellulose in microwave. a) the hydrogen bond in cellulose; b) the interaction between cellulose and microwave in at different temperature[87]

To overcome the slow heating rate, microwave hydrolysis is widely used in lignocellulose treatment. In biochemistry, hydrolysis is a chemical process used for degradation of polymers that results in smaller polymer or monomers with the existence of water. Water, as mentioned earlier, is an excellent solvent in microwave treatment. The high dielectric constant and loss factor of water at low temperature could help to achieve a rapid temperature ramping; a considerable amount of inorganic salts in biomass are soluble to become charged ions in water and hence ionic polarisation can take place to promote the heating. At the high temperature range where the softening point of biomass is reached, the dielectric constant and loss factor of water drop dramatically, allowing more electromagnetic energy to work on biomass. Compared with hydrolysis using

conventional heating, microwave-assisted hydrolysis is simpler, more rapid and more controllable.[81] Microwave-assisted hydrolysis of lignocellulosic biomass, due to the different natures of its three main components, can be roughly divided into two types: microwave-alkali hydrolysis that aims to mainly solubilise lignin, and microwave-acid hydrolysis (or microwave acidolysis) that aims to solubilise hemicellulose. Sodium hydroxide and sulfuric acid are most commonly used species in the two types of hydrolysis respectively.[96–98] Considering the different solubility natures of polysaccharide and lignin in acidic environment, it is expected that microwave acidolysis could potentially be applied in lignin isolation from lignocellulosic biomass *via* the degradation of polysaccharides. It is widely known that acidolysis is able to convert hemicellulose to soluble sugars and facilitates the subsequent enzymatic treatment of cellulose.[99–101] However, this process is complex and very dependent on the acidolysis conditions. The concentration of sulfuric acid solution is vitally important. Researchers found that higher acid concentration in microwave acidolysis could result in not only more solubilisation of hemicellulose, but also slight degradation of cellulose at relatively high concentration (0.02M).[102] For herbaceous biomass like sugarcane bagasse and wheat straw, lignin could also be partly degraded according to researches.[98,102,103] These facts indicate there is a possibility to isolate lignin *via* microwave acidolysis, however, systematic experimental work and analysis are needed to investigate and optimise this process for the production of high yield and high purity lignin.

1.4 Aim of this study

Although there are various methods of lignin isolation, till now none of these methods could achieve quantity and quality goals at the same time. Industrial methods are suitable for mass production. However, as a by-product, industrial lignin is modified and contaminated severely, thus often only suitable in low value applications as fuel[104] or as a binding agent[105]. Lab-based methods result in higher quality lignin, but their time-consuming protocols make it uneconomical and inefficient in industrial production. Lacking proper isolation methods is one of the main reasons for the undervaluation of lignin.

Currently, microwave-assisted treatment of waste and biomass has drawn an increasing interest.[106–108] Compared with conventional treatment, microwave treatment offers a rapid, efficient and controllable heating which could also minimise the inner temperature gradient.[109–111] The mechanism of microwave heating is significantly different to conventional heating, which is based on the high-frequency rotation of polar molecules. Thus, polar compounds, such as water, have the priorities to be treated by microwave, resulting in a selective heating of microwave treatment.

This study proposes a novel method of fast microwave hydrolysis for the valorisation of biomass (three types of lignocellulose and bilberry). Different from conventional processes, which only care about a certain fraction (lignin or polysaccharides) of biomass, this study aims to achieve a zero waste utilisation of biomass, which means isolating purity lignin with little structural modification and meanwhile producing fermentable sugar for the bio-refinery. The microwave-assisted hydrolysis is not only suitable for lab-

scale investigation and research, but also hopefully meets the needs for industrial-scale production due to the simple and fast protocols. Based on systematic analysis using various feedstocks, this study is intended to offer a new possibility and approach for lignin isolation and biomass valorisation *via* microwave-assisted hydrolysis.

Lignin isolation from softwood and the characterisation of the residual lignin

Chapter 2

2.1 Introduction

The vast amount of lignin is a potential source of valuable phenolic compounds. However, lignin, among the three main components (cellulose, hemicellulose and lignin) of lignocellulose, is currently the most undervalued. One key reason is that extraction of polysaccharide-free lignin with high efficiency using conventional methods is still a challenge, because in biomass lignin acts as 'glue' adhering the plant polysaccharides layers together with strong covalent bonding to cellulose and hemicellulose.[112] Extraction of lignin is accompanied by structural damage and polysaccharide contamination.[41] The lack of high quality lignin on the market coupled with difficulties in degrading it selectively and efficiently into useful low molecular weight products make it undervalued and underdeveloped compared with cellulose and hemicellulose.[20] Therefore, lignin is still widely used as an internal energy source in chemical pulp and paper mills and in some industrial bio-refinery processes.[113,114] One of the advancements in pure lignin isolation was proposed by Klason. The two-step Klason acidolysis protocol and its modified versions have been mostly used as standards of lignin content and purity determination, as in the TAPPI T222 method.[44] The drawback with the Klason protocol is that as concentrated sulfuric acid is applied, the structure of Klason lignin (KL) is modified from that in the original biomass. In a KL procedure, lignin condenses to become insoluble in water. As a result, the re-polymerisation is serious. Another commonly used lignin in laboratory studies is milled wood lignin (MWL). This milder protocol that uses neutral solvents for isolation affords a product that is widely regarded to offer the best material for the structural analysis of the 'native lignin' originally present

in the plant tissue.[41,115] The linkage in lignin-carbohydrate complexes (LCC) are broken by milling and then lignin is extracted by dioxane-water solvent. The disadvantage is that the intensive and lengthy milling (taking between 1 hour to 3 weeks depending on milling machine) is energy-consuming, in turn increasing the cost of the isolation. Low lignin yield[41], polysaccharide contamination[52] and the tendency for dioxane-water to dissolve only the lower molecular weight fractions of the lignin are also drawbacks of MWL protocol. MWL is generally representative of total lignin in wood except that phenolic content is higher than native lignin, because MWL is only partially extracted by dioxane-water solvent. Based on MWL protocol, cellulolytic enzyme lignin (CEL) protocol was proposed to increase lignin yield, but yields are still low at 27-29 wt%.[52] Furthermore, CEL protocol requires high dosage of enzymes and the process is time and energy consuming. Therefore, both MWL and CEL methods are used mainly in lab-scale research, but are not suitable for industrial production.[41,52] Although there are many improvements based on these methods, efficient lignin isolation with high yield and low contamination remains a difficult task and calls for new protocols.

Due to the efficient and selective heating, microwave heating provides a promising approach in thermal treatment of bio-waste, especially lignocellulose.[87,116,117] Until now there have been only a few studies focusing on microwave-assisted lignin isolation. Zhou[118] investigated microwave-assisted lignin extraction from birch in formic acid and compared it with conventional isolation methods. A higher delignification was achieved by microwave heating than oil bath heating. Li[30] also performed microwave lignin extraction from bamboo at 90 ºC and 109 ºC separately. It was found that increasing

temperature would benefit lignin extraction. Zoia[119] performed microwave-assisted lignin isolation in inorganic acid solution (fixed power mode, 240 minuts) and a high yield of 68 wt% (total amount of acid soluble and insoluble lignin) was achieved. All these studies prove the advantages of microwave-assisted lignin isolation, especially lignin purity and processing time. However, these studies only focus on relatively low temperature isolation. Higher temperature isolation still needs investigation. With elevated temperature, better performance is expected to be achieved, because acidolysis lignin is more stable to thermal degradation than cellulose and hemicellulose.[120,121] This thermal stability can be expected to be further enhanced during microwave treatment because of the selectivity of microwave. Microwave heating is a type of dielectric heating that is based on the high frequency rotation of polar molecules.[122] Therefore, rotatable compounds with high polarity are more rapidly heated during microwave irradiation. As mentioned in Chapter 1.3.2, polysaccharides (like cellulose) are softened at higher temperature and localised rotation happens in microwave, becoming easily degradable.[87] Lignin, with higher aromaticity and lower polarity than polysaccharide,[123,124] is therefore likely to degrade less severely in a microwave isolation than conventional acidolysis under equivalent conditions of total energy input.

Based on the discussion above, in this chapter a new method for fast microwave-assisted lignin isolation is proposed. Dilute sulfuric acid is used for acidolysis, as previous studies have shown that lignin-carbohydrate complexes (LCC) are reduced to negligible levels when acidolysis is conducted in this medium.[125] High temperature isolation (160-210 °C) is carried out to ensure LCC can be cleaved in short duration of only 10 min. Systematic

analysis is performed to investigate lignin quality. A new analysis approach based on Py-GC/MS is applied to check the structure of lignin after isolation. It is reported that softwood, among three main types of lignocellulose (hardwood, softwood and herbaceous biomass), contains the least acid-soluble lignin. Therefore, in this chapter, milled mixed softwood pellet was used for lignin isolation (MSP, milled into 40 mesh powder). The experiment details of microwave hydrolysis are given in Chapter 6.1.

2.2 Mass ratios and C/H contents of hydrolysis residues

Microwave hydrolysis were carried out in CEM Discover reactor using dynamic mode to produce microwave hydrolysis residual lignin. The holding times and temperature are 5-20 min and 160-210 ºC, respectively. Conventional hydrolysis was carried out using Anton PAAR Monowave 50 at 190 ºC and 10 min holding time to produce conventional acidolysis residual lignin (AL). Using TAPPI T222 protocol, Klason lignin (KL) was isolated from MSP for comparison.

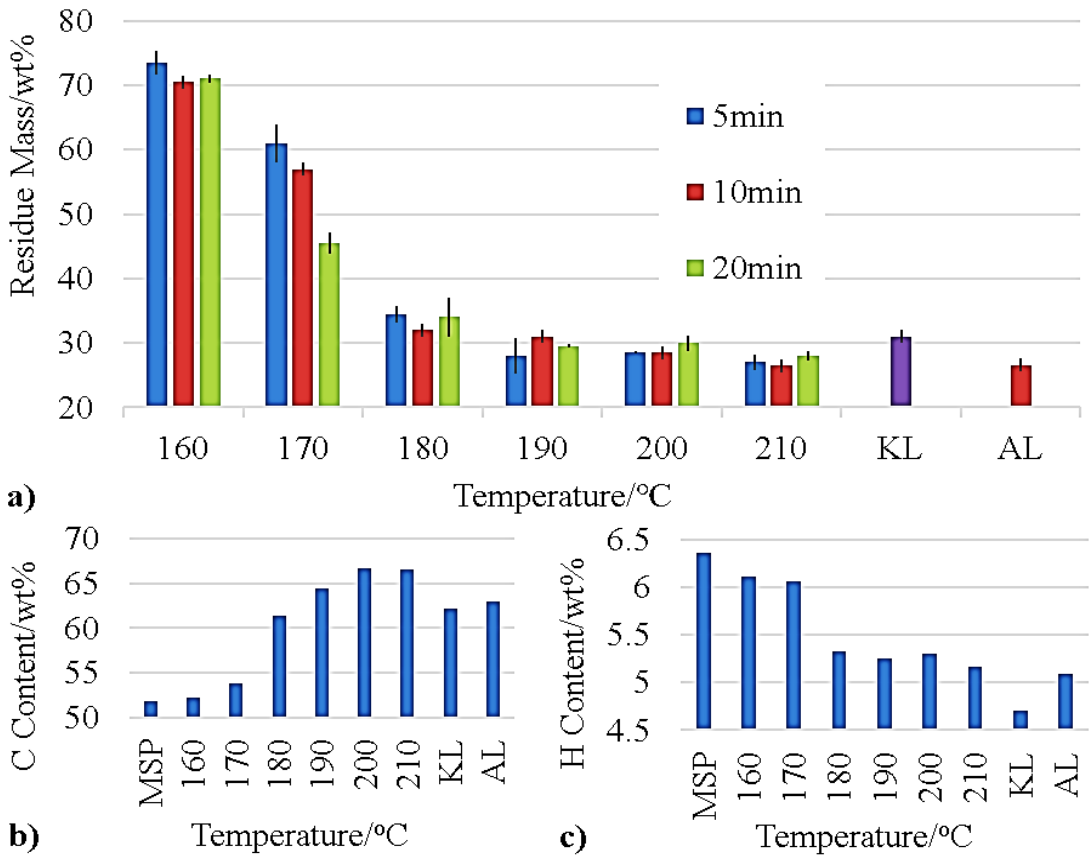


Figure 2-1 Comparisons of KL, 190° C AL and microwave lignin isolated with different conditions. a) mass balance; b)&C) C/H content of hydrolysis residues. The experiments were carried out using CEM DISCOVER microwave reactor. Temperature and holding time are shown in figure. Mixed softwood pellets were feedstock. In each run, 0.2 g and 15 ml H_2SO_4 (0.2 M) were loaded in reactor tube. After each run, the solid residue was filtered and washed to separated from hydrolysate for further analysis.

note: 1) KL, Klason lignin from MSP;2) AL, acidolysis lignin from MSP achieved *via* conventional heating at 190 °C, 10 min holding time; 3) no C/H results of 5 or 20 min residues in b&c, and in the following analysis except mass balance, only the result of 10 min residues were analysed, because in mass balance section it was found 10 min was the optimal holding time; 4) in the following text MRL only refers to 10 min residues 5) triple repeated experiments were done in residue mass calculation. The values in a) are average values and the error bars were calculated from the repeated experiments.

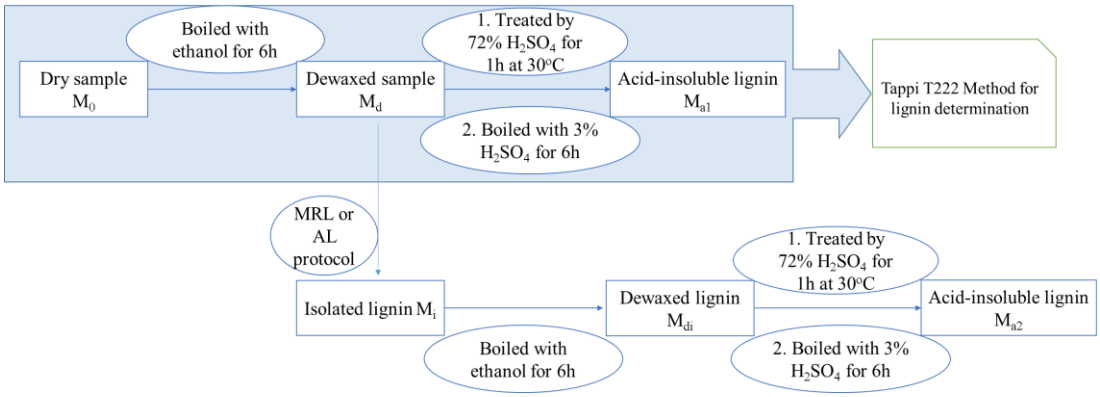
Figure 2-1 shows the influence of temperature and holding time on the yield of residue and C/H content. Due to the structure difference, the element distributions of

polysaccharides and lignin are different. With fewer hydroxy functional groups, lignin is featured with lower oxygen content and higher carbon content than those of polysaccharides. As lignin and polysaccharides are the main components of lignocelluloses, simple carbon content can effectively reflect the lignin content in hydrolysis residue qualitatively. It was found major changes of residue mass and C/H content took place from 160 ºC to 190 ºC. At 170 ºC, the residue mass could be still affected by holding time; at temperature above 190 ºC the masses of the residues obtained did not vary significantly with holding time beyond 5 minutes, showing the high efficiency of microwave heating. The residue mass of KL was 31 wt%, which was close to that of 190/200 ºC residues. Adler[48] reported that the equivalent formula of the purest softwood lignin he could produce from spruce was $C_9H_{7.92}O_{2.40}(OCH_3)_{0.92}$, which suggested the C/H content of pure softwood lignin should be close to 65.1/5.5 wt%. Lin[126] also measured the C/H content of an industrial lignin which was 65.0/6.4 wt%. Compared with these values, the C/H content of microwave-assisted hydrolysis residue lignin (MRL) at 190 ºC was similar to those isolated lignins. As a comparison, the C content of polysaccharides is lower than that value of lignin, for example it is 44.4 wt% for cellulose with a formula of $(C_6H_{10}O_5)_n$. (The H content of cellulose is 6.2 %, making it less indicative of the nature of the material than C%). The higher C/H ratio of MRL suggested the that removal of polysaccharide is extensive. The H content of KL was low because concentrated sulfuric acid was likely to dehydrate and/or sulfonate the lignin. The C/H of acidolysis lignin using conventional heating (AL) at 190 ºC was similar to that of 190 ºC MRL, however the residual mass was lower, showing conventional acidolysis at high

temperature caused more mass loss than microwave treatment. Given the similarity in C/H content, this suggests a similar loss of polysaccharides in both situations but a greater loss of lignin in the AL case. This suggests that lignin is indeed more thermally stable under microwave heating than when subjected to conventional heating as expected.

2.3 Purities (acid-insoluble lignin content) and yields

The purity and yield was calculated by TAPPI T222 method.[44] The method is shown schematically in Figure 2-2. About 0.1 g of dewaxed sample was treated with 10 g of sulfuric acid (72 wt%) at 20 °C for 2 hours. The solution was then diluted with deionized water to 3 wt% sulfuric acid and refluxed for 4 hours. The insoluble residue (lignin) was isolated by filtration. After washing with hot water, the residue was dried at 105 °C for 24 h. This dried residue is Klason lignin (KL). The purity and yield were calculated according to the equation in Table 2-1. The purity result was adjusted by subtracting the ash content measured by TG analysis.



$$lignin\ content_{dry-basis}=\frac{M_{a1}}{M_0}$$

$$lignin\ content_{extractive-free-basis}=\frac{M_{a1}}{M_d}$$

$$purity\ of\ lignin\ isolated = \frac{M_{a2}}{M_{di}}$$

$$lignin\ yield = \frac{M_{a2}}{M_{a1}}$$

Figure 2-2 Tappi T222 method for purity and yield calculation

After 10 min microwave treatment at 190 °C, the isolation produced lignin with high purity (93 wt%) and yield (82 wt%), both of which were higher than lignin obtained using MWL methods. Wu and Argyropoulos[52] produced MWL with a 14-day milling process on the softwood, black spruce (*Picea mariana*). The extractive-free basis purity and yields were 88.3 wt% / 28.5 wt% respectively. Compared to MWL methods, the much shorter duration required by microwave heating is probably the main reason for the higher lignin yields obtained during this study. Within such a short processing time, lignin loss is reduced to a great extent. Sulfuric acid offers an environment where carbohydrate can be hydrolysed and solubilised, while most lignin is insoluble.

Table 2-1 Purity and yield of MSP and 190 ºC MRL/AL

	Purity / wt%		Yield
	Dry basis	Extractive-free	wt%
MSP	30.4[a]	39.1[b]	-
190 ºC MRL	80.6[c]	92.9[d]	82.3[e]
190 ºC AL	75.9[c]	87.5[d]	65.6[e]

Note: a=M_{a1}/M_0 b= M_{a1}/M_d c= M_{a2}/M_i

M_0, M_d, M_{a1}, M_i, M_{di}, M_{a2} and the definitions of purity/ yield see Figure 2-2

Another reason accounting for high purity and yield may relate the selectivity of microwave treatment.[87,116,117] Different from conventional heating, microwave heating is achieved by the high frequency rotation of polar molecules. Compared to non-polar compounds, polar molecules and functional groups are treated more intensively and faster in microwave radiation. Lignin has higher aromaticity and lower polarity compared with carbohydrate.[123,124] Therefore, carbohydrate and lignin can be expected to behave in significantly different ways under microwave radiation, particularly in the presence of dilute aqueous sulfuric acid as lignin can hardly be solubilised in acidic solution. Such a hypothesis explains why in Table 2-1 lignin yield of 190 ºC AL was much lower than that of 190 ºC MRL under same conditions except the heating methods.

It is worth noting that the solvent water (dielectric constant 81, loss factor 0.123 at room temperature) is far more polar, better microwave-absorbing and heat-producing than both lignin and polysaccharides in lignocellulosic biomass (dielectric constant less than 10) in microwave irradiation, indicating water could receive more energy. However, this does

not change the fact that within biomass carbohydrate is more polar than lignin, thus, carbohydrate is more readily to degrade in microwave heating than in conventional heating of parallel conditions. Moreover, the existence of water as excellent microwave absorber makes it possible to achieve a rapid heating, in turn reduce the energy consumption. On the other hand, at dielectric constant and loss factor of water drop drastically alongside the increase of temperature. For example, the dielectric constant of water drops to 34.5 at 200 °C. This allows more electromagnetic energy work on the biomass. Most importantly, it is reported the the softening point of polysaccharides in microwave is about 180 °C.[87] At temperature higher than 180 °C, the localised rotation of CH_2OH of cellulose and hemicellulose takes place, increasing the polarity of polysaccharides. and accelerating the degradation of polysaccharides. Last but not the least, a considerable amount of inorganic salts in biomass are soluble in water to become charged ions, causing ionic polarisation which could promote heat production and accelerate the degradation of polysaccharides. These facts also possibly account for the better performance of microwave acidolysis than the conventional alternative.

2.4 Analysis of the hydrolysate

GC/MS and liquid ^{13}C NMR test of the hydrolysate after microwave acidolysis were carried out to check the hydrolysis products in aqueous phase. Since the goal is to isolate residual lignin with high yields, it is believed that the lower the amount of aromatic products in hydrolysate, the better the isolation of lignin during microwave acidolysis. According to the acid solubility, lignin can be divided into acid-soluble lignin and acid-

insoluble lignin. Compared with hardwood and herbaceous lignin, softwood lignin contains the least proportion of acid-soluble lignin (only ~0.5 %)[127]. However, during the degradation and solubilization of polysaccharides, a small portion of acid-insoluble lignin unavoidably degrades to become soluble, in turn to increase the aromatics in hydrolysate. GC/MS and liquid ^{13}C NMR spectroscopy can be used to check the dissolved lignin according to relevant studies.[128,129]

Table 2-2 The list of main compounds of GC/MS analysis in liquid phase (hydrolysate of MSP after microwave lignin isolation at 190°C)

Name	Source	RT/ min	Peak area /%
4-Hydroxy-2-butanone	Sugar	4.50	2.9
Dihydro-5-methyl-2(3H)-furanone	Sugar	7.64	12.8
1,2,3-Butanetriol	Sugar	8.34	1.1
1,2-Cyclopentanedione, 3-methyl-	Sugar	9.15	1.6
4-Hydroxy-2-pentenoic acid,	Sugar	10.09	10.4
1,2-Epoxy-3-propyl acetate	Sugar	10.25	13.6
4-Oxo-pentanoic acid	Sugar	10.36	13.7
5-(Hydroxymethyl)-2-furancarboxaldehyde	Sugar	12.57	3.1
Vanillin	Lignin	14.49	1.9
1-Propyl-2,5-pyrrolidinedione	Protein	15.04	3.7
Homovanillyl alcohol	Lignin	15.71	1.6
1-(4-Hydroxy-3-methoxyphenyl)-2-propanone	Lignin	16.70	18.0
3-Hydroxy-1-(4-hydroxy-3-methoxyphenyl)-1-propanone	Lignin	17.48	6.8
4-(Ethoxymethyl)-2-methoxy-phenol,	Lignin	18.57	2.3

Notable, GC/MS analysis is not suitable for quantitative analysis. Thus, here peak area % is used for a semi-quantitative analysis, which could roughly indicate the products distribution in hydrolysate. After isolation at 190 °C (10 min), the hydrolysate after microwave treatment was analysed by GC/MS and liquid ^{13}C NMR. The GC/MS

compounds list (main products) of aqueous phase are showed in Table 2-2. The GC/MS results showed that the majority of compounds in solution were chemicals derived from sugars characterised by the presence of ketone, aldehyde and furan groups, while aromatic compounds occurred in lower proportions. The total peak area % of aromatic compounds in Table 2-2 is ca. 30 %, while Table 2-1 suggests that only 18 % of lignin of MSP is solubilised judging from the yield of 190 MRL, indicating phenolics might ionise more readily than other compounds in GC/MS. Figure 2-3 shows liquid ^{13}C NMR spectrum**.** The peaks in 20-40 ppm were ascribed as saturated carbon which were mainly from polysaccharide-derived products. The peaks of ketone were located in 205-220 ppm, suggesting dehydrated sugars were probably the main products in liquid phase. Of greatest significance is the absence of intense peaks between 100-150 ppm, where carbons in benzenoid rings typically resonate, indicating that aromatic compounds remained predominantly in the insoluble solid residue. The absence of these peaks provides further evidence for lack of thermal depolymerisation of lignin, when wood is heated by microwaves for 10 min at 190 ^{o}C.

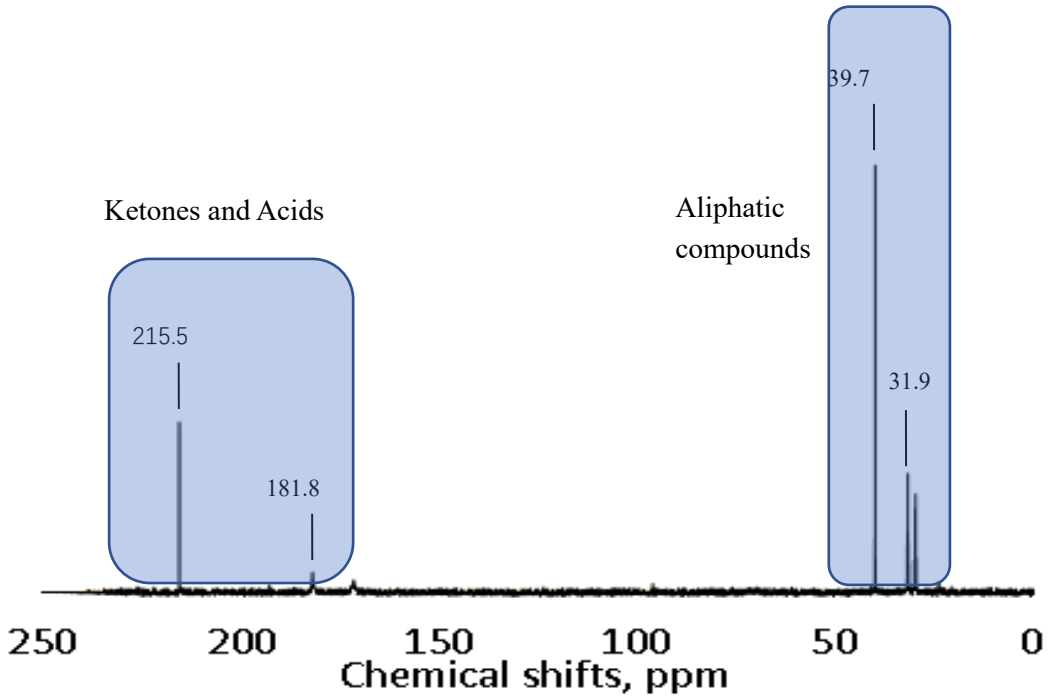


Figure 2-3 Liquid ^{13}C NMR spectrum of liquid sample (hydrolysate of MSP after microwave lignin isolation at 190 °C, deuterated water as solvent)

2.5 Thermal gravimetric analysis

Thermal gravimetric analysis is widely used in biomass research, because the various components in biomass degrade at different temperature ranges. Thus, the thermo-gravimetric (TG) and derivative thermo-gravimetric (DTG) curves can help to qualitatively identity the components in biomass.[130,131] Figure 2-4 shows the TG curves of MSP, KL, 170/190 °C MRL and 190 °C AL. For MSP, the DTG curve had a very strong peak at around 350 °C that corresponds to the decomposition of cellulose.[130,131] This peak was accompanied with a well-pronounced shoulder at around 300 °C, attributable to hemicellulose decomposition.[130,131] For 170 °C MRL, the final mass loss was lower than that of MSP. These results illustrated that a microwave isolation at 170 °C was already

able to remove the carbohydrate to some extent. However, there were two strong DTG peaks (294 ºC & 330 ºC) in the range of 290-350 ºC, showing 170 ºC MRL was still severely contaminated by cellulose and hemicellulose.

The mass and DTG curves of KL and 190 ºC MRL had similar trend in general. Compared with linear structure of cellulose and hemicellulose (with some branches), the complex 3D structure of lignin and predominance of aryl-alkyl ether linkages make it recalcitrant to thermal degradation. These factors resulted in the 190 ºC MRL and KL samples having high residual mass at 600 ºC, a higher peak zone for degradation. The final residual masses were high at 52 wt% and 55 wt% respectively, showing fewer degradable compounds existed in these two samples than those in 170 ºC MRL. Their DTG peaks were located between 370-410 ºC, where pure lignin displays its DTG peak according to previous studies.[130,131] Unlike the DTG curves of MSP and 170 ºC MRL, the DTG curves showed no peaks between 290 ºC to 350 ºC, confirming that polysaccharides were mostly removed in the 190 ºC MRL and KL samples. A subtle difference between KL and 190 ºC MRL was that the degrading peak of 190 ºC MRL was slightly lower, which was either caused by structural changes brought about by the 190 ºC treatment, or by dehydrations/oxidations/sulfonation promoted by the 72 wt% sulfuric acid used in the Klason protocol.

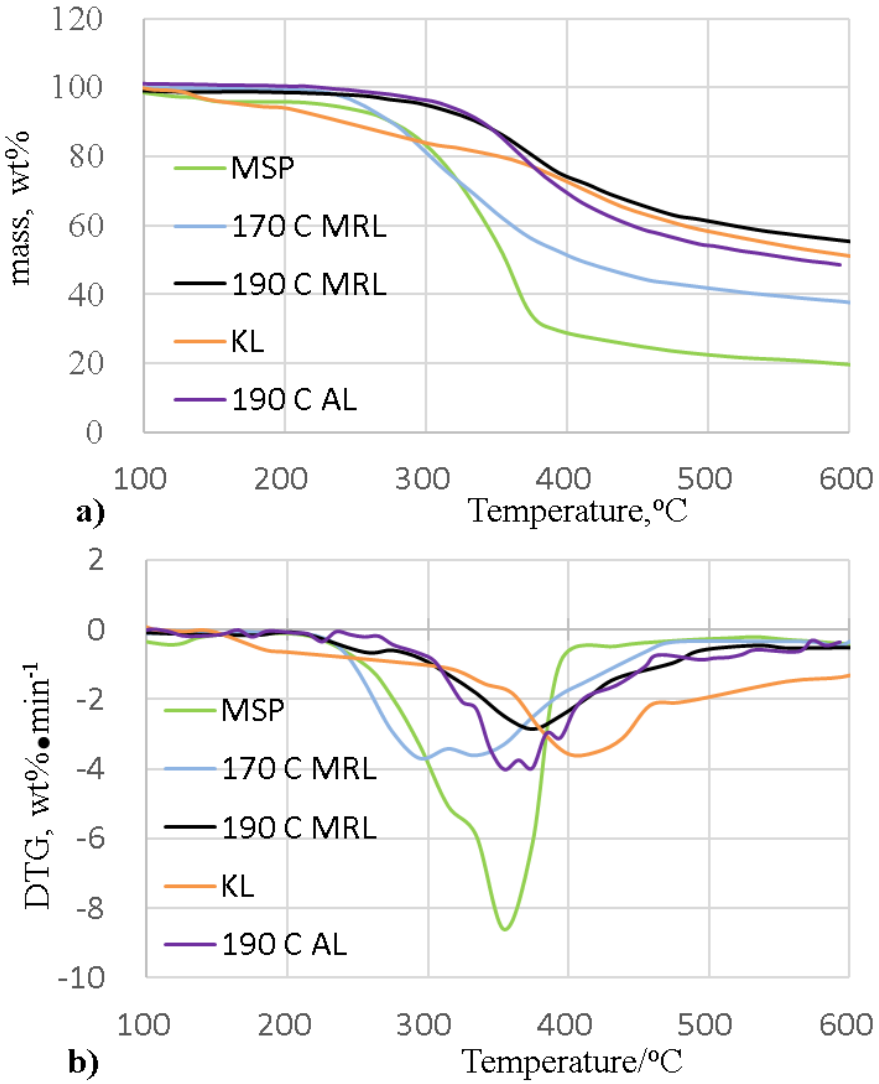


Figure 2-4 Pyrolysis Curves of samples (MSP, KL, 190° C AL and 170/190 °C MRL):

a) TG curves b) DTG curves (N_2 as carrier gas)

Comparing 190 °C MRL and 190 °C AL, it was found there was more polysaccharide in 190 °C AL sample. The two DTG curves both had peaks at around 375 °C, showing lignin was a main component in both isolated residues. However, for the DTG curve of 190 °C AL, there was also a pronounced peak at 354 °C, that was attributable to the degradation of polysaccharide.[130,131] Furthermore, the DTG peaks of 190 °C AL were stronger than those of 190 °C MRL, showing 190 °C AL was less thermally stable. The thermogravimetry data indicate that 190 °C MRL is less contaminated by polysaccharides

than lignin produced by conventional acidolysis at 190 ºC.

2.6 Fourier-transform infrared spectroscopy analysis

Fourier-transform infrared spectroscopy (FTIR) is a simple but powerful tool in biomass investigation to offer informative results about the nature of functional groups of the sample.[132,133] Compared with polysaccharides (cellulose and hemicellulose), a distinguished difference of lignin is the high proportion of aromatic rings, which can be clearly reflected in FTIR spectra. Figure 2-5 shows the FTIR spectra of MSP and residual lignin. As the treatment temperatures were increased, the bands assigned as aromatic skeleton (1601/ 1508/ 1451/ 1424 cm^{-1})[59,134–136] were strengthened significantly. These strong peaks suggest high aromaticity of the residues after treatment. The peak at 1030-1060 cm^{-1} was assigned as C-O stretching of primary alcohol.[134–136] It weakened as temperature rose, indicating a better removal of polysaccharide at high temperature. The overall trend of the FTIR spectra demonstrated that temperature acts as an important factor in lignin isolation. At treatment temperatures higher than 190 ºC, the spectra of MRL were very similar to that of KL. From 190 ºC to 210 ºC, the peak at 1114 cm^{-1} (secondary alcohol)[137] and 1030 cm^{-1} were further weakened slightly. This may suggest that 210 ºC MRL was purer than 190 ºC MRL. However, as shown in Table 2-1, the 190 ºC treatment rendered 18 wt% of the lignin acid soluble. Therefore, an isolation at 210 ºC would be expected to solubilise more lignin, resulting in lower lignin yield and perhaps further structural changes away from native lignin. Furthermore, the tube pressure of 210 ºC experiment was 100 psi higher than that of 190 ºC. (Figure 2-6) Therefore, due to

lignin yield and safety reasons, 190 ºC seemed a suitable temperature for this current protocol.

The FTIR spectra of 190 ºC MRL and 190 ºC AL showed similar general trends. However, the peak at 1260 cm^{-1} was stronger in 190 ºC AL than that in 190 ºC MRL. This peak could be ascribed to ether bonds, especially alkyl aryl ethers.[134]

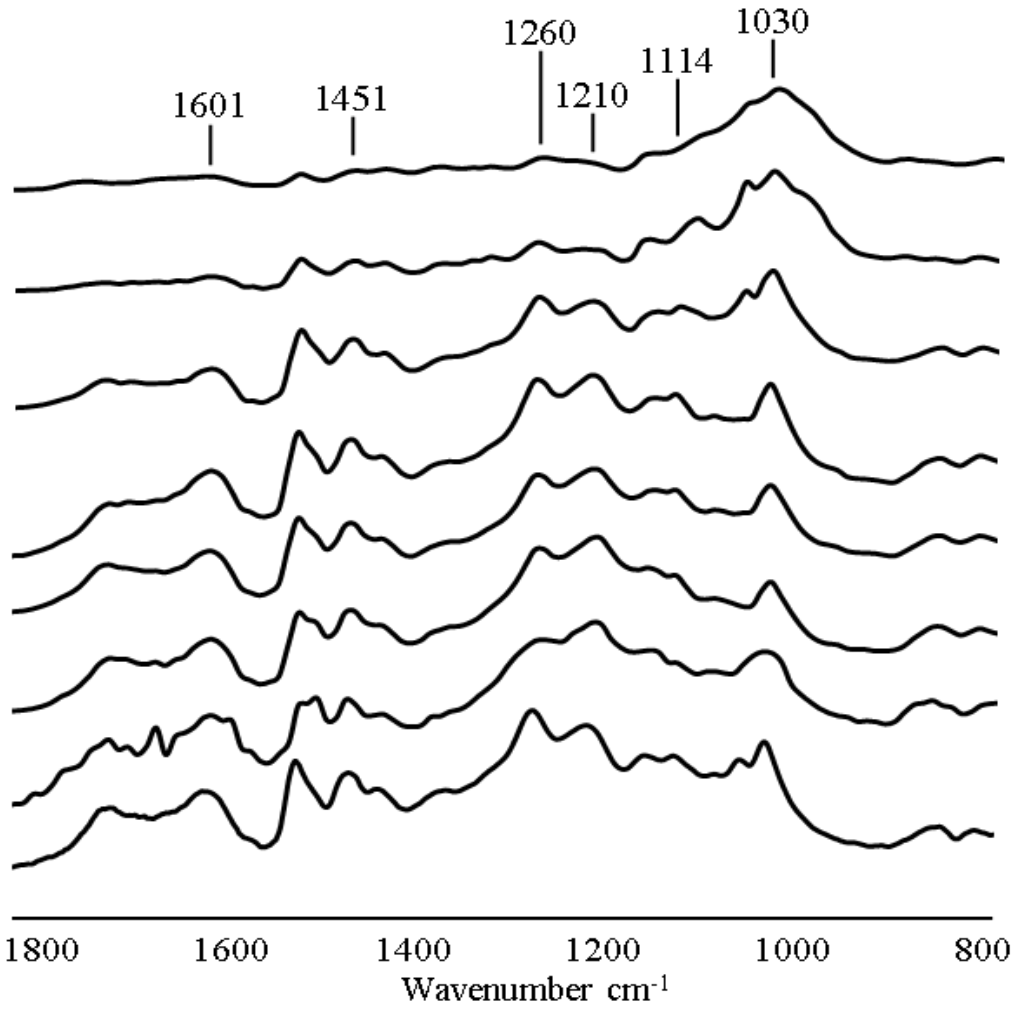


Figure 2-5 FTIR spectra of MSP and isolated lignin. From top to bottom: MSP, 170/180/190/200/210 ºC MRL, KL,190 ºC AL

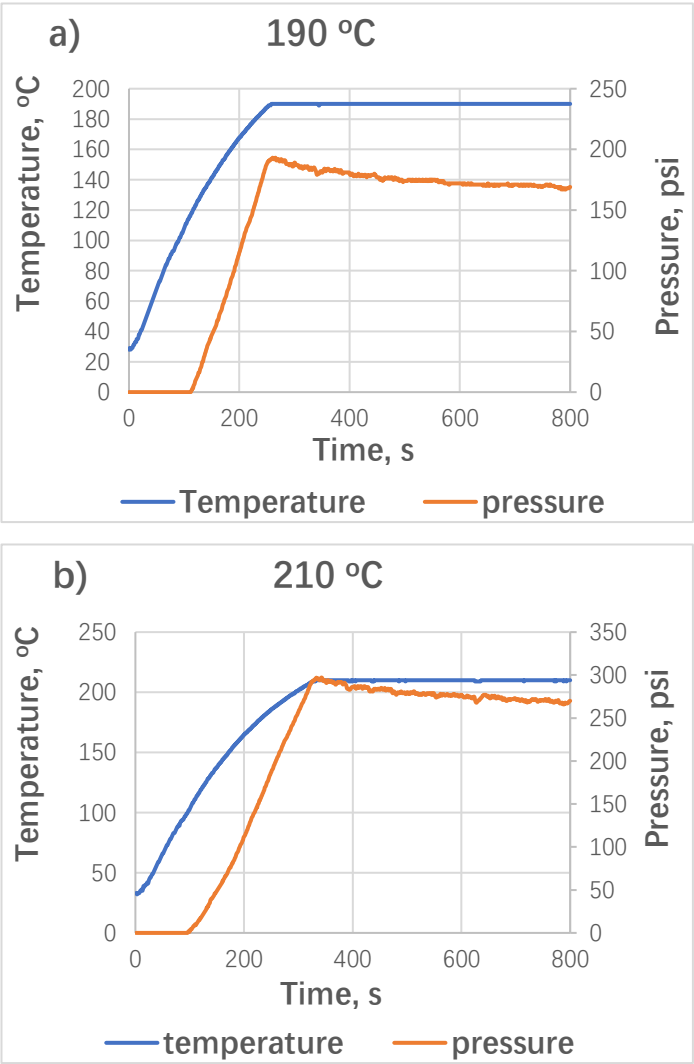


Figure 2-6 Comparisons of temperature and pressure during microwave experiments at 190 / 210 °C

2.7 Solid state ^{13}C NMR spectroscopy analysis of residual lignin

Solid state ^{13}C NMR (SSNMR) is widely used in the investigation of various types of biomass.[138,139] In this research, lignin is produced in hydrolysis residue, thus solid state NMR spectroscopy offers an easier and more effective tool to check the residue properties than liquid NMR. Lignin is an aromatic polymer, hence can be distinguished from carbohydrate easily on SSNMR spectra, because aromatic chemicals have pronounced

peaks at chemical shift range higher than sugars. Figure 2-7 shows the spectra of solid state ^{13}C NMR (SSNMR) of MSP and the isolated lignin samples. The peak at 55 ppm was attributable to methoxyl carbons.[137,140] This peak intensified in isolated lignin samples, because the monomer of softwood lignin, guaiacyl unit (G-unit), contains one methoxyl sidechain. Comparing the spectra of MSP and 170/190 ºC MRL, it was obvious that the peaks between 109-162 ppm were much stronger after microwave treatment. According to Jindong Mao,[137] the peaks in the range between 108-60 ppm can be attributed to aliphatic carbons that are mainly derived from carbohydrates and sidechains of lignin, such as the peaks at 72 ppm and 105 ppm which were the characteristic of C-2, C-3, C-5 and C-1 carbon of cellulose[141]; while peaks between 162-109 ppm were attributed to carbon atoms in benzenoid rings. The major peaks in this zone were located at 147 ppm (aromatic C-O),[140,142] 130 ppm (aromatic carbon bearing alkyl group),[142] 125 ppm (hydrogen-bearing aromatic carbon not adjacent to oxygen functionalities),[142] and 114 ppm (aromatic carbon ortho to phenolic C-OH moieties).[142]

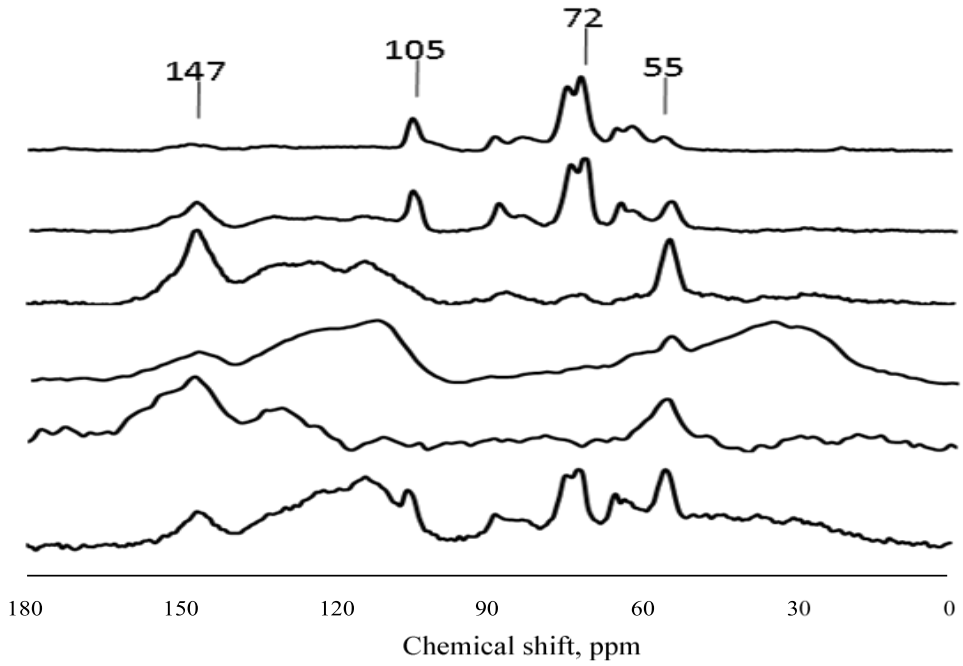


Figure 2-7 SSNMR spectra of MSP and isolated lignin. From top to bottom: MSP, 170 ºC MRL, 190 ºC MRL, KL, KL (CPNQS) and 190 ºC AL

The spectra of 190 ºC MRL and KL showed significant differences. There were two wide bands at 30-50 ppm and 120-135 ppm for KL spectrum. Researchers[137,143,144] showed these two bands can be attributed to CH_2 carbons and CH carbons respectively. When processing the KL spectrum using the CPNQS methodology that suppresses the CH_2/CH band, the spectrum became similar to that of 190 ºC MRL. This data suggested microwave isolation can keep the aromatic part of lignin intact, however it appears to remove the aliphatic part to some extent. The monomers of lignin are phenylpropanoid in structure. They are based on a C_6-C_3 structure that contains both aliphatic and aromatic carbons. Compared with the aromatic C_6 moieties, the C_3 aliphatic sidechains of lignins are characterised by higher polarities, having higher O/C ratios than the aromatic parts of the structure. Microwaves are more efficient in heating polar compounds.[87] So the sidechain

is more likely to be modified or cleaved during lignin isolation. As a result, lignin isolated using microwave heating has a higher proportion of intact aromatic rings and lower intact side chains than the lignin isolated using conventional heating at the same temperature. This fact will benefit the application of isolated lignin as a potential source for production of low molecular weight aromatic compounds.

The SSNMR spectrum of 190 ºC AL showed a strong peak at 72 ppm, suggesting severe sugar contamination. Similar with the spectrum of KL (normal CP spectrum), there was a band at 120-135 ppm, suggesting a high content of CH_2 group in lignin isolated by conventional acidolysis. These data add further evidence to the hypothesis that MRL has a proportionately higher aromatic carbon content and that microwave heating at 190 ºC results in cleavage of the side chains of this type of lignin.

2.8 Pyrolysis gas chromatography mass spectroscopy (Py-GC/MS) analysis

Pyrolysis-GC/MS (Py-GC/MS) allows direct analysis of the pyrolytic products of biomass samples.[145,146] By comparing the contents of pyrolytic products, especially aromatic products of the original feedstock and the isolated lignin samples, the structure modification of lignin during isolation can be checked. MSP and the isolated lignin samples (190 ºC MRL, KL, 190 ºC AL) were analysed by Py-GC/MS (GC spectra in Figure 6-4). From the changes of peak area % of typical pyrolytic products (compounds lists in Table 6-3), especially phenolic compounds, the structure change and degradation

extent during lignin isolation can be investigated. Because most polysaccharide had been removed, the phenolic compounds were dominant in pyrolytic products of the three lignin sample, while there were more pyrolytic products from cellulose and hemicellulose in MSP, such as 1-hydroxypropanone / furfural / cyclopentane-1,2-dione.

Table 2-3 Comparisons of phenolic compounds peak area (%) of MSP and isolated lignin

NO	Compounds	MSP	190 ºC	KL
1	4-Methyl-1,2-benzenediol	0.4	2.5	2.6
2	4-Ethyl-2-methoxy-phenol	1.2	3.7	3.7
3	2-Methoxy-4-methylphenol	6.2	18.7	13.9
4	2-methoxy-phenol	3.2	8.5	9.7
5	4-Hydroxy-3-methoxybenzaldehyde	1.4	2.7	1.3
6	2-methoxy-5-(1-propenyl)-, (E)-phenol	1.0	1.9	1.4
7	2-methoxy-4-(1-propenyl)-, (Z)-phenol	0.4	0.7	0.3
8	2-Methoxy-4-vinylphenol	4.0	4.9	3.6
9	2-methoxy-4-(1-propenyl)-, (E)-phenol	3.8	3.2	1.4

In Table 2-3, nine typical phenolic compounds from the Py-GC/MS (Table 6-3) are listed together with their measured ion current peak areas. In Table 2-1 it was shown that the lignin content of 190 ºC MRL (80.6 wt%, dry basis) was 2.67 times that of MSP (30.4 wt%, dry basis). Thus, it is to be expected that, if the lignin is isolated without significant modification, that a plot of the peak areas for py-GCMS of the isolated lignin vs. MSP should have a slope of 2.67. When the ratios between the ion current peak areas for the 190 ºC MRL and those for MSP are compared as shown in Figure 2-8a, it is apparent that the trend line has a slope of 2.99 which is in acceptable agreement with the expected ratio of 2.67, suggesting lignin was well preserved without significant degradation. Notably, two of the nine compounds were significant outliers from the trend line, 2-methoxy-4-

vinylphenol and (E)-isoeugenol. There are two possible reasons that can explain why these two compounds do not conform to the expected trend: 1) the precursors for these compounds are concentrated around the periphery of the 3D lignin structure and are bonded covalently to carbohydrates as part of the LCC, resulting in chemical modification of the alkene groups during acid hydrolysis, or 2) the compounds are more or less evenly distributed through the 3D structure of the lignin and do not survive the acidic conditions at 190 ºC for reasons that cannot be explained at present. The fact that compound numbers 6 and 7, that also contain double bonds in the side chain do fit closer to the trend line may be seen as evidence favouring the former explanation. By way of the trend line between KL and MSP (Figure 2-8b) was somewhat lower than that in Figure 2-8a, showing there were proportionately fewer aromatic compounds in pyrolytic products of KL than that of 190 ºC MRL. This was probably because there were more aliphatic compounds in KL due to less sidechain modification than 190 ºC MRL, which is consistent with the results of SSNMR analysis presented above. The fit of the trend line in Figure 2-8b was worse than that in Figure 2-8a, suggesting there might be more severe degradation of lignin using Klason method, where concentrated sulfuric acid was used.

When the volatile products produced by Py-GC/MC of 190 ºC AL were compared with those obtained from 190 ºC MRL, it was evident that pyrolysis products derived from carbohydrates, such as 5-(hydroxymethyl) furfural (0.6% in 190 ºC AL, 0.2% in 190 ºC MRL) and D-allose (2.1% in 190 ºC AL, undetectable in 190 ºC MRL), were evident with higher peak areas in the case of 190 ºC AL. An interesting fact was that one of main pyrolytic products, creosol, showed higher peak area % in 190 ºC AL (25.0%) than 190

ºC MRL (18.7%), though the latter was purer lignin and less contaminated with carbohydrates. Fleck[147] observed that some model lignin dimers, such as conidendrin and di-isoeugenol (Figure 2-9) in which the two monomers are linked by a saturated ring, did not produce creosol during pyrolysis. Fleck found that certain inter-linkages, such as an benzocyclopentane ring, could effectively prevent the formation of creosol under pyrolytic conditions. Furthermore, Fleck[147] pointed out that creosol was one of the main pyrolytic products of coniferin which is a glucoside of coniferyl alcohol. So sugar contamination actually could increase the yield of creosol to some extent. It is arguable that these two factors explain why 190 ºC AL with the higher carbohydrate content produced more creosol. It is also possible that some of the structural changes in the side chains of the lignin promoted by microwave heating lead to formation of new cyclic aliphatic inter-linkages between monomeric units that are in close proximity within the 3D structure and that these changes also serve to reduce creosol yields from Py-GC/MS of 190 ºC MRL.

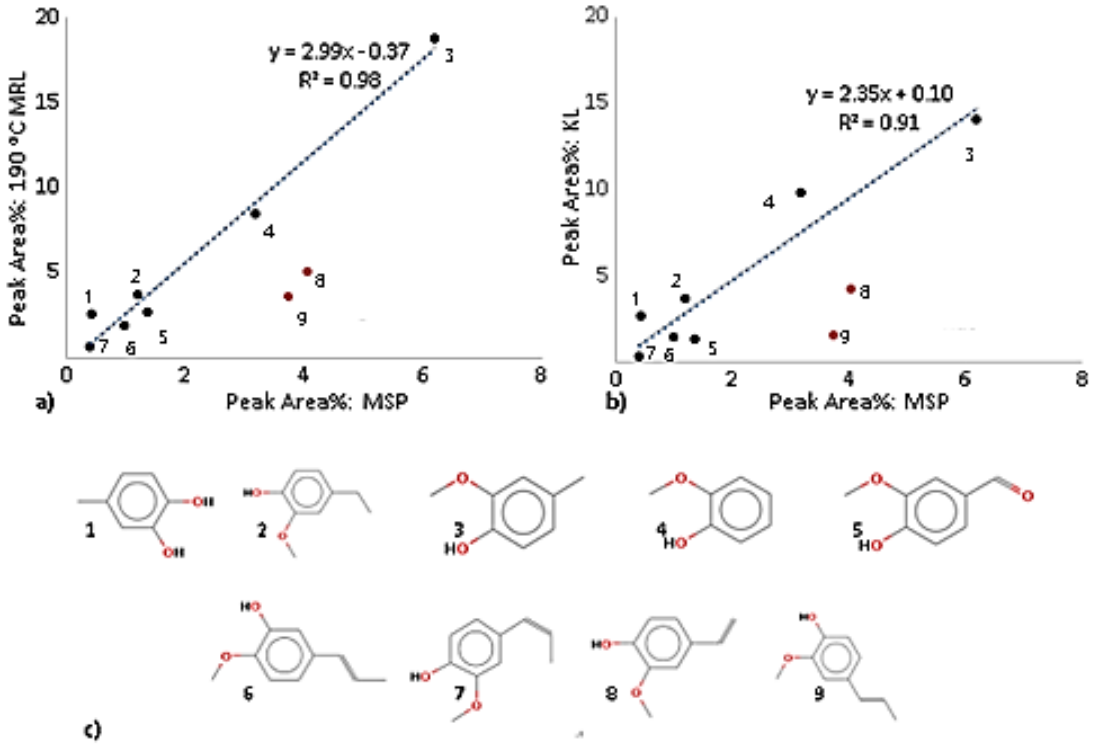


Figure 2-8 Peak area % of phenolic compounds according py-GC/MS analysis.

a) MSP vs 190 ºC MRL; b) MSP vs KL; c) compounds structures (compounds sequence according to Table 2-3)

Products name: 1. 1,2-Benzenediol, 4-methyl-; 2. Phenol, 4-ethyl-2-methoxy-; 3. Creosol; 4. Phenol, 2-methoxy-; 5. Vanillin; 6. Phenol, 2-methoxy-5-(1-propenyl)-, (E)-; 7. Phenol, 2-methoxy-4-(1-propenyl)-, (Z)-; 8. 2-Methoxy-4-vinylphenol; 9. trans-Isoeugenol

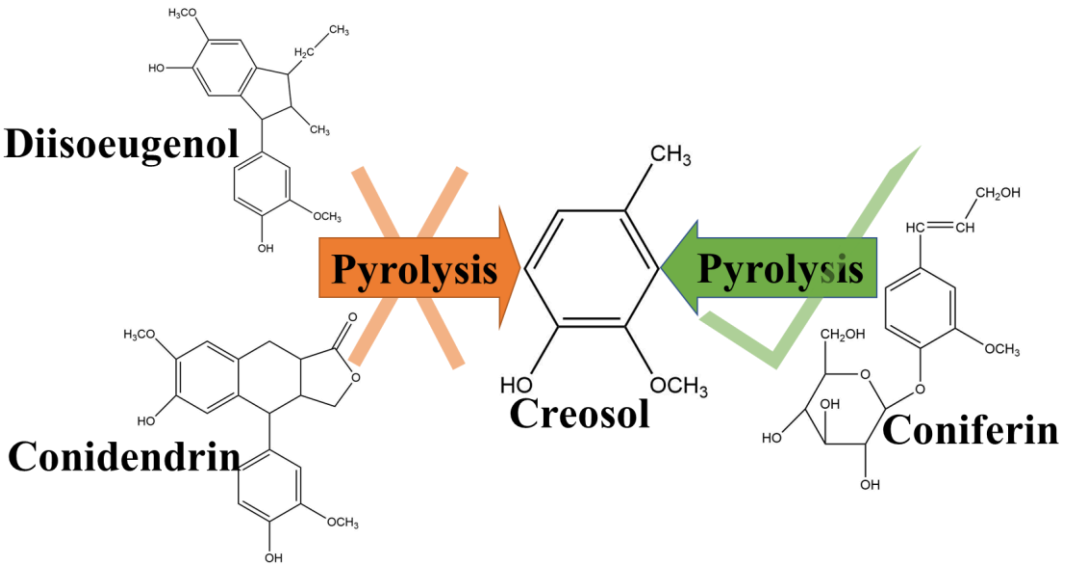


Figure 2-9 Structure of diisoeugenol, conidendrin, coniferin and creosol (pyrolysis temperature at 400 ºC)

2.9 Conclusions

It has been demonstrated that a pure form of lignin, relatively uncontaminated by residual carbohydrates can be produced rapidly and efficiently by brief (10 min) microwave heating of mixed softwood pellets (MSP) at 190 ºC in dilute aqueous sulfuric acid. The type of lignin produced by this new method, designated 190 ºC MRL (microwave residual lignin), has both higher yield and purity than equivalent material produced by conventional heating to 190 ºC in aqueous sulfuric acid of the same concentration in an autoclave for the same time. The latter material has been designated 190 ºC AL (acidolysis lignin). It has been shown that 190 ºC MRL is of high aromaticity due to the modification of lignin side chains. The Py-GC/MS results from the two types of lignin indicate that some formation of cyclic aliphatic linkages occurs between the side chains of monomeric units that are in close proximity when microwave heating at 190 ºC is applied. However, the structure modification is very limited, as the Py-GC/MS result suggests the peak area % of most pyrolytic phenolic products from 190 ºC MRL is consistent with what would be expected if the lignin was not modified during the removal of the polysaccharides from MSP, showing lignin is indeed isolated without significant degradation. The analytical techniques applied using comparative Py-GC/MS on lignin samples obtained by differing reaction conditions have general application in identifying structural changes occurring during lignin isolation.

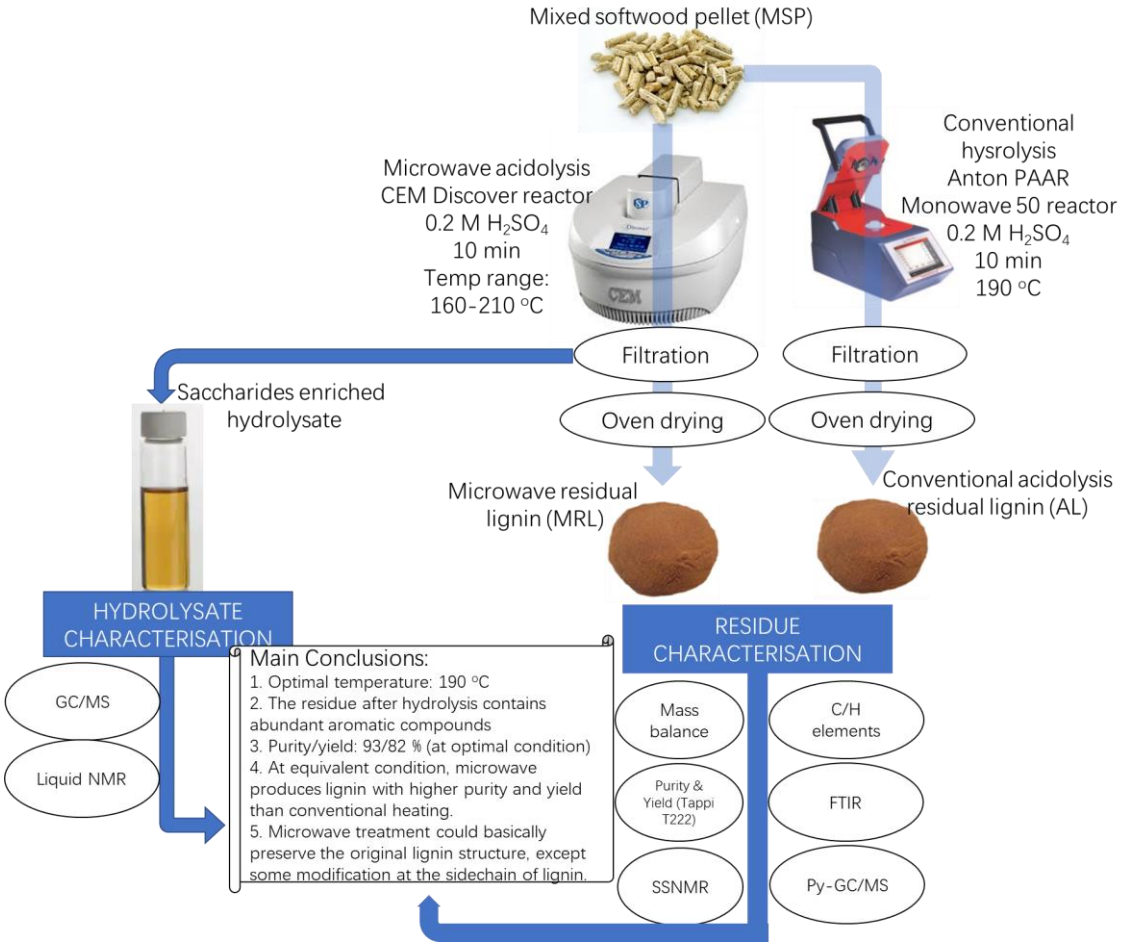


Figure 2-10 The content summary of Chapter Two: microwave acidolysis of softwood for lignin isolation

In general, the research results show that high temperature microwave treatment is a powerful tool for lignin isolation. High efficiency, simple protocol and high lignin yield are its most significant advantages. It is potentially a very promising method for high quality lignin preparation.

Towards a zero waste biorefinery using microwave acidolysis to achieve high purity lignin isolation and saccharide for fermentation

Chapter 3

In Chapter 2, a new method of microwave-assisted acidolysis is proposed to isolate lignin from biomass. Softwood is chosen as feedstock because it contains the less acid-soluble lignin compared with hardwood and herbaceous crops. In this chapter, microwave acidolysis of hardwood and herbaceous crops are investigated to check the feasibility of this lignin isolation protocol on other lignocellulosic substrates. In addition to residual lignin, the hydrolysate containing mono-/di-saccharides is used as broth for fermentation, in order to achieve a multiple-goal and zero waste utilisation of biomass.

3.1 Introduction

Due to fluctuations in crude oil prices, the finite nature of the resource and need for national energy reserves, there has been an increasing interest in developing lignocellulosic biorefineries for fuels and chemicals in the last few decades.[148–150] Lignocellulose is mainly constituted of cellulose, hemicellulose and lignin, and can be converted both chemically or biologically into a range of value added chemicals and fuels.[151,152,153] For example, in conventional biorefinery processes, cellulose and hemicellulose can be depolymerised to produce mono- and di-saccharides such as cellobiose, glucose, xylose and furanose, (Figure 3-1) which can be fermented to alcohols or other microbial products or chemically converted into furans.[154,155] However, the current utilisation of lignocellulosic biomass is still relatively inefficient.[156] In industrial processes, such as paper pulping, commonly only one product is targeted at the expense of large waste streams.[157,158] Side products such as lignin are commonly discarded or used to provide low value energy for the process, and not valorised into suitable phenolic

components,[159] as these processes are designed only to achieve high quality cellulose. The cellulose purification protocols damage the lignin structure and produce lignin with significant polysaccharide contamination. However, pure lignin, with little degradation is a valuable source of aromatic functionality and could be converted into high value fuel additives or chemicals.[112] For example, it has drawn an increasing interest to produce vanillin from lignin (lignin-based vanillin) to become a substitute for natural vanillin.[34] In addition to vanillin production, the degradation of lignin is also a vast source for other phenolic compounds (like phenol and syringaldehyde) and bio-fuels.[160] Therefore, a more selective pre-treatment to cleave the bonds connecting polysaccharide and lignin efficiently is of interest. Classical lignin isolation protocols, such as the Klason method or milled wood lignin (MWL), take hours or even days according to the intensity, and involve a range of additional acids and chemicals. As such, conventional separation methods cannot effectively isolate lignin with both high yield and low structural modification at the same time.

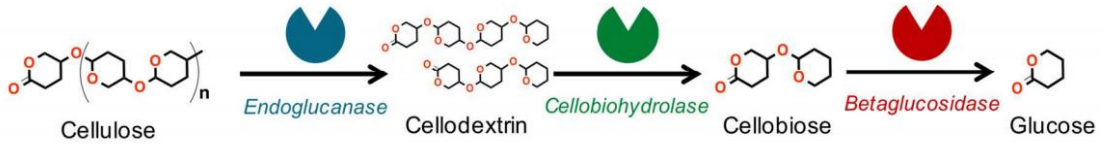


Figure 3-1 A schematic depicting cellulose degradation via three enzymatic steps [161]

Compared with conventional thermal treatment, microwave-assisted treatment has the characteristics of high efficiency and selectivity, making it an efficient tool in biomass processing and solid waste recycling. Microwave heating has been widely used in pyrolysis, gasification as well as delignification of biomass.[83,162,85] Microwave heating has a dramatic effect on the reaction kinetics[163] and reduces overall reaction times

substantially.[164] Cellulose and hemicellulose are more polar than lignin,[123,124] therefore they receive more energy input on microwave irradiation. Based on this selective heating, a highly efficient separation could potentially be achieved through microwave treatment, with far shorter reaction times. A drawback of microwave treatment of biomass is the low dielectric constant and loss factor of biomass, making the temperature ramping slow when biomass is the sole feedstock in microwave. To solve this problem, microwave hydrolysis, which is thermal treatment with the existence of water, is often used, as water is an excellent electromagnetic energy absorber and heat producer with high dielectric constant and loss factor (81 and 0.123 at room temperature). Additionally, the considerable amount of inorganic salts in biomass are soluble in water to become charged ions, rendering ironic polerisation that could further accelerate the temperature ramping. At relatively high temperature range, water interacts less with microwave as its dielectric constant drops significantly, allowing more electromagnetic energy to work on biomass, of which the polysaccharides already start to soften and localised rotation in microwave at certain temperature (~180 ^{o}C) becomes possible.[87] In this situation, polysaccharides are readily degraded and solublised, in turn being separated from lignin. Thus, an effective and thorough isolation of lignin can be expected with microwave hydrolysis.

However, to further develop the zero waste biorefinery concept, various products must be produced alongside the lignin[165,166,167,168] To this end, a new protocol of rapid microwave-assisted acidolysis was examined to separate lignin effectively in three types of lignocellulosic biomass. The saccharide-rich aqueous phase was then investigated as a fermentation source for two oleaginous, inhibitor-tolerant yeast.

3.2 Analysis of the acidolysis residue

3.2.1 Conversion ratio and C/H analysis

CEM Discover microwave reactor was used for hydrolysis of three types of biomass. After treatments under different conditions, the solid residues and liquid were collected for further analysis. The experimental details are in Chapter 6.2. Figure 3-2 shows the influence of temperature on microwave lignin isolation (Figure 3-2a, b, c). Clearly, for MSP and willow edging (WE, hardwood biomass) the residual mass decreased sharply from 170 °C to 180 °C. For WE, residual mass kept decreasing until 190 °C and remained at 20 % at higher holding temperatures. In contrast to woody biomass, wheat straw (WS, herbaceous crops) had a relatively low residual mass even at 170 °C. The C/H results show similar trends. For MSP and WE, C/H content was relatively stable when ≥190 °C holding temperature was applied; while for WS, carbon content stabilised at 180 °C but hydrogen content kept decreasing with increasing temperature. For all three feedstocks, the residue colour kept getting darker when holding temperature increased. It is important to note that after 200 °C treatment, samples became totally black, suggesting that these temperatures may be causing the destruction of the lignin, and may therefore be too high to be of value.

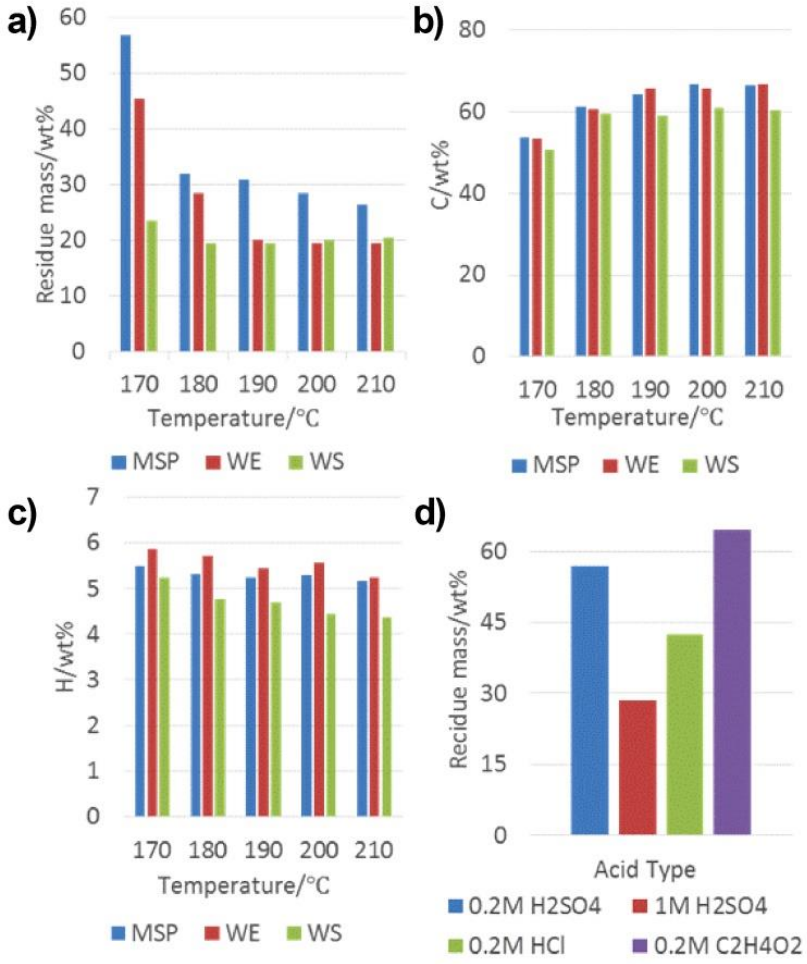


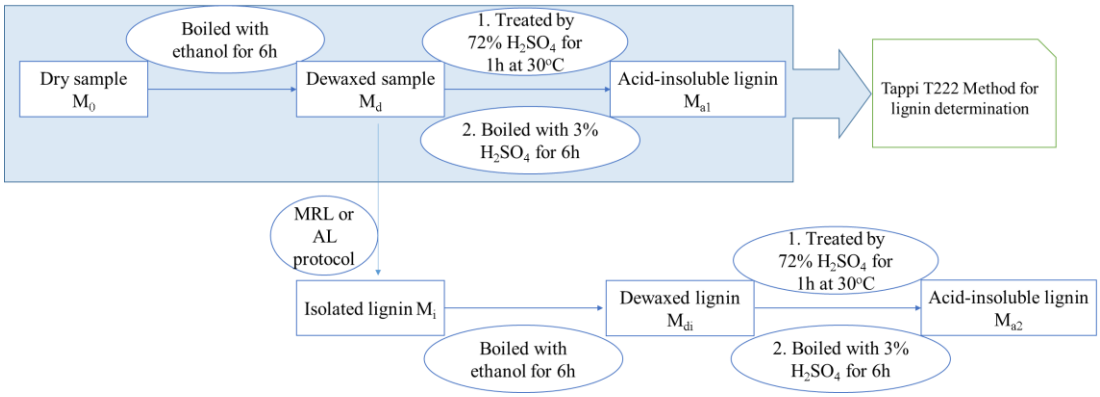
Figure 3-2 Influence of temperature preparation on a) residue yield, b) carbon and c) hydrogen content. Experiment details for a)b)&c): CEM DISCOVER reactor (dynamic mode), 0.2 g different types of biomass: 15 ml sulphuric acid solution (0.2 M) holding time 10 min, after hydrolysis, the residues were filtered, washed and weighed for mass balance calculation and further characterisation. The aqueous phase was used for fermentation. d) Nature of acid influence on residue mass yield of MSP, (sulphuric acid pKa=-9; hydrochloric acid pKa=-7; acetic acid pKa=4.8) Experiment details for d): CEM DISCOVER reactor (dynamic mode), 0.2 g biomass: 15 ml acid solution (different types and concentration) holding time 10 min, after hydrolysis the residues were filtered, washed and weighed for mass balance calculation and further analysis. MSP: mixed softwood pellet; WE willow edging; WS, wheat straw

Microwave-assisted acidolyses were also performed with different types of acid and solution concentrations (Figure 3-2d). It was found that the concentration of acid had a significant influence on the acidolysis process. When more concentrated H_2SO_4 (1 M)

was applied, the residual mass was much less than that of diluted H_2SO_4 (0.2 M), showing that a higher concentration of solution could accelerate the reaction even at relatively low temperature. The mass of residue formed by acetic acid (0.2M) was only slightly higher than that by sulphuric acid, even though it is a much weaker acid. This suggested there was potential to perform acidolysis by acetic acid. Acetic acid can be produced from biomass, which is a more benign and potentially more attractive option than sulphuric acid. Among the three residues treated by diluted acid solution (0.2M), the one from hydrochloric acid has the lowest residual mass, indicating hydrochloric acid can cause a higher extent of solubilisation of biomass in acidolysis. This result is out of expectation, because, as shown in the footnote of Figure 3-2, the pKa of the three acids rank as: pKa(Acetic acid)>pKa(HCl)>pKa(H_2SO_4). This suggests that the solubilisation and conversion of biomass in microwave acidolysis are not only determined by the strength of acid. Transition metal chlorides, especially $FeCl_3$, $CuCl_2$, $AlCl_3$ and $CrCl_3$, have been reported as effective catalysts in conversion of polysaccharides according to Peng.[169] He found that the conversion of cellulose to glucose, HMF and levulinic acid were significantly promoted when it was catalysed by these metal chlorides at 180 ºC (2 wt% cellulose, 0.01 M metal chlorides). As shown in Table 3-3, MSP contains certain amount of transition metal elements (Al 6.1×10^{-3} wt%, Fe 5.2×10^{-3} wt%, Cu 0.9×10^{-3} wt% and Zn 3.3×10^{-3} wt%), thus the higher extent of solubilisation in HCl trial may be aided by more degradation of polysaccharide due to the existence of metal chlorides. This might explain why HCl has a middle pKa among three acids, but gives the lowest residual mass. However, more work is needed to verify this hypothesis in the future.

3.2.2 Purities (acid-insoluble lignin content) and yields

By the method showen in Figure 2-2, the purities and yields were calculated in Table 3-1. As shown in Figure 2-2, the purity indicates the lignin content in the sample; the yield indicates how much lignin (compared with the original lignin in biomass) can be preserved in the hydrolysis residue after microwave treatment. In terms of purity and yield, MSP was a very suitable feedstock for the microwave-assisted acidolysis at 190 °C (10 min holding time, 0.2 M sulphuric acid), but for WE and WS the protocol still needs improvement. Compared with hardwood and herbaceous biomass, it was reported that softwood has the lowest content of acid-soluble lignin, only 0.2-0.5% of total lignin.[44] For MSP, 82 wt% of acid-insoluble lignin could be retained in the residue and, after the removal of soluble matter, the purity of the lignin residue can reach as high as 93 wt%. Compared to MSP, the yield of WE was much lower at 30%, but the purity was still relatively high at 87%. Wu and Argyropoulos[52] produced MWL with a 14-day milling process on black spruce (softwood) and poplar (hardwood). The yields were 28.5wt%/28.7wt% and purities were 88.3wt%/84.7wt%. Compared with these data, the microwave lignin isolation could compete with MWL protocol in terms of both the yield and purity, especially when the feedstock was softwood where both high yield and purity was achieved. More importantly, the microwave lignin isolation only took 10min, which was far more efficient than the MWL method.



$$lignin\ content_{dry-basis}=\frac{M_{a1}}{M_0}$$

$$lignin\ content_{extractive-free-basis}=\frac{M_{a1}}{M_d}$$

$$purity\ of\ lignin\ isolated = \frac{M_{a2}}{M_{di}}$$

$$lignin\ yield = \frac{M_{a2}}{M_{a1}}$$

Figure 2-2 Tappi T222 method for purity and yield calculation

Table 3-1 Purity and yield of residue lignin (wt%) obtained from different biomasses.

Conditions: 0.2 M H_2SO_4 solution, 10 min, 190°C

	Lignin content in feedstock % (M_{a1}/M_d)	Purity % (M_{a2}/M_d)	Yield % (M_{a2}/M_{a1})
MSP	39.0	92.9	82.4
WE	40.6	87.3	30.3
WS	33.8	63.1	31.2

Note: All the results are based on extractive-basis.

M_0, M_d, M_{a1}, M_i, M_{di}, M_{a2}, see Figure 2-2

The purity of WS derived lignin was 63%, lower than that of MSP and WE derived lignin. However, the low purity was caused not only by sugar contamination, but also ash content. The ash content (extractive-free basis) of 190 °C WS residue was as high as 25wt% while

the ash contents of MSP and WE residues are both < 3 wt%. Table 3-2 shows the WS had an ash content of 14 wt%. Researchers have reported that straw contains considerable amount of ash (such as SiO_2, which is one of the very few inorganics that are insoluble in acid conditions)[170,171], and the ash content in wheat straw is even higher than that in straw of other types (such as corn straw).[172] This indicates the ash content of the feedstock could greatly affect the quality of residual lignin. Most herbaceous energy crops contain high amount of ash,[173,174] suggesting an ash-wash is necessary for effective lignin isolation from herbaceous biomass.

Another possible reason accounting for the low yield and purity in the lignin isolation from WS is the high content of metal elements in WS. As shown in Table 3-3, most metal elements contents in WS are higher than the other two biomasses, especially potassium. These metals become the source of charged ions when the biomass is mixed with water, in turn rendering intensive ionic polarisation caused by the interaction of the ions with the electromagnetic field in microwave irradiation. It is assumed that the microwave isolation of lignin might be affected by this intensive ionic polarisation. Consequently, lignin is expected to experience more degradation in microwave hydrolysis, reducing the yield and purity of the residual lignin significantly. As discussed in Chapter One, the metals in biomass could accelerate the temperature ramping in microwave heating due to ionic polarisation; however, from the example of WS residual lignin, it is presumed that too high a content of metal in biomass might become a disadvantage in lignin isolation which could promote the depolymerisation of lignin, reducing the lignin purity and yield severely. As discussed before, for biomass with high ash content like wheat straw, a pre-

treatment of ash wash may be necessary to increase the purity and yield in lignin isolation.

Table 3-2 Proximate and ultimate analysis of feedstocks (wt%)

	Moisture	Dry basis					
		Ash	C	H	N	S	O[a]
MSP	6.6	1.1	51.8	5.6	0.0	1.3	40.2
WE	4.7	3.7	48.1	5.9	0.3	0.2	41.9
WS	6.1	14.4	44.7	5.4	0.3	0.2	35.0

a. Oxygen=100-Ash-C-H-N-S

Table 3-3 ICP analysis of feedstock ($\times 10^{-2}$ wt%)

	MSP	WE	WS
Na	17.0	<0.0	0.3
Mg	1.4	5.6	8.0
Al	0.6	0.9	1.2
Si	10.5	2.2	10.6
P	1.2	8.3	5.2
K	7.8	30.4	115.4
Ca	18.2	53.2	54.8
Fe	0.5	1.3	2.7
Co	<0.0	<0.0	<0.0
Ni	<0.0	0.1	<0.0
Cu	0.1	0.1	<0.0
Zn	0.3	0.7	0.4

3.2.3 Thermal gravimetric analysis

Thermal gravimetric analysis is widely used in biomass research, because the various components in biomass degrade at different temperature ranges. Thus, the thermo-gravimetric (TG) and derivative thermo-gravimetric (DTG) curves can help to qualitatively identity the components in biomass.[130,131] Figure 3-3 showed the mass and DTG curves of three feedstocks and their isolated residues at 170 / 190 °C. The TG curves of the three feedstocks and treated residues shared some similar trends: 1) for all the feedstocks, the maximum DTG peaks occurred in the temperature range of 330-360 °C. According to the literature[130,131], the strong peaks are attributed to the pyrolysis of cellulose. There were also well-pronounced shoulders at 280-330°C, related to the pyrolysis of hemicellulose;[130,131] After microwave treatment, the residues were more recalcitrant to pyrolysis. After acidolysis, polysaccharides were removed and lignin was retained in the residues. The 190 °C residues had higher final mass than those produced at 170 °C, showing higher temperature benefited the acidolysis. 190 °C WS residue had the highest final residual mass and the lowest maximum mass loss rate, which was perhaps caused by the much higher ash content in wheat straw (Table 3-2). For 170 °C residues, the peaks at 330-360 °C were weakened significantly, but the peaks between 280-330 °C were still sharp, indicating a microwave-acidolysis at 170 °C could remove cellulose to a certain level, but was not able to solubilise hemicellulose effectively. This was most obvious for 170 °C WE residue, showing it had abundant polysaccharide among

the three 170 °C residues; for the three 190 °C residues, the TG and DTG curves were very similar. The DTG curves only showed weak peaks around 370-410 °C, which were caused by the pyrolysis of lignin. This demonstrated that the microwave acidolysis at 190 °C could solubilise the saccharide component and lignin was predominant in the residues. This provided evidence that a relatively thorough separation between lignin and polysaccharides could be achieved by acidolysis at 190 °C using microwave assisted heating.

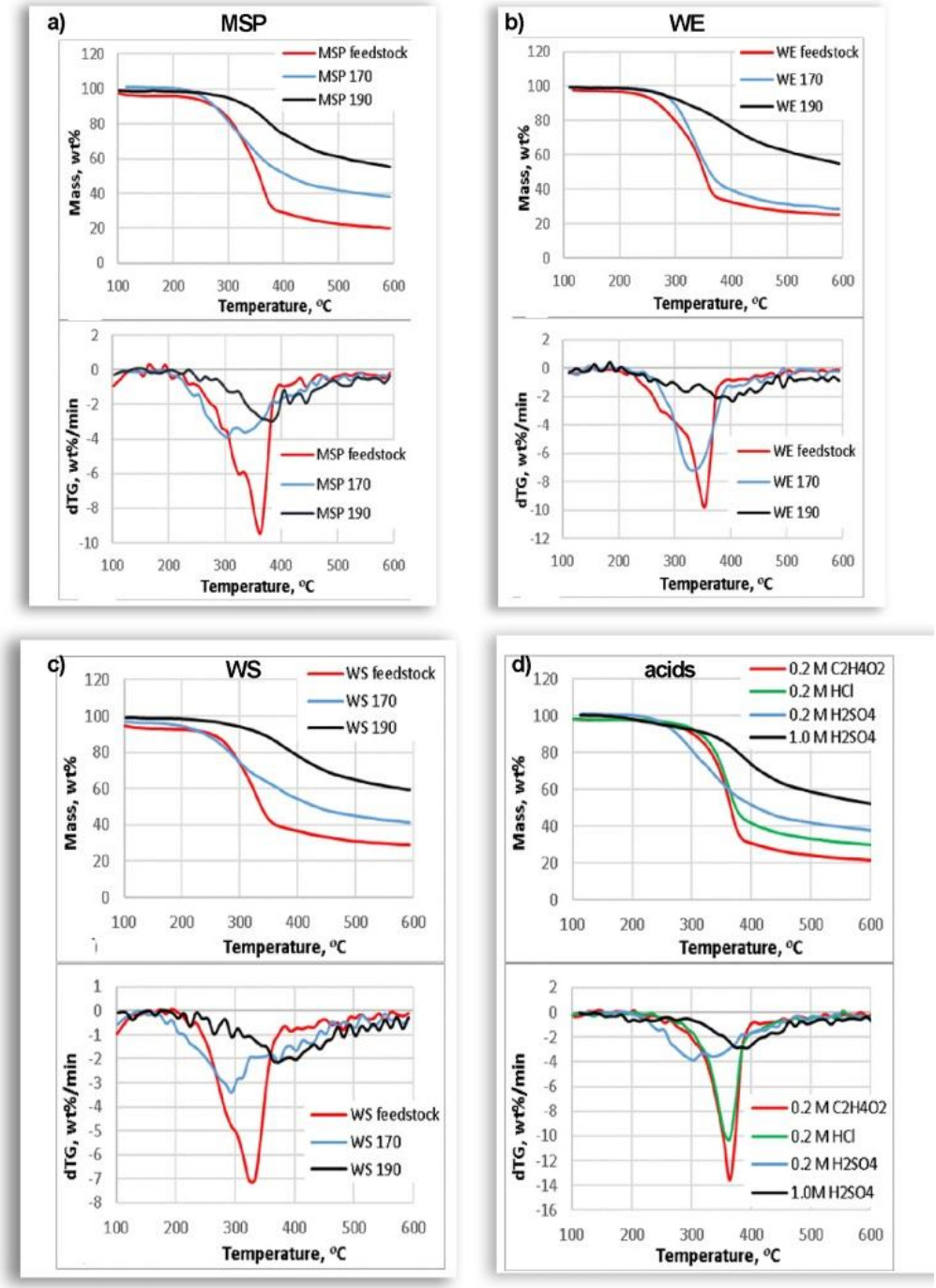


Figure 3-3 Comparison of TG and dTG curves for (a) MSP, (b) WE and (c) WS; (d) influence of the acid nature on the properties of MSP. Conditions for (a)–(c): holding time of 10 min, dynamic mode, 300 W, treatment temperatures of 170/190 °C. Conditions for (d): treatment temperature of 170 °C (50 ml/min N_2 carrier gas)

Figure 3-3d shows TG curves of four 170 °C residues processed by different acid solution. The peak of DTG curve of 1M H_2SO_4 was at 393.9 °C, thus showing that it contained highly pure lignin. Therefore, its final residual mass of 49.9% was the highest. It illustrated the lignin could be efficiently isolated at relatively low temperature with concentrated sulphuric acid. In Figure 3-3d, the DTG curves of HCl and acetic acid residues showed sharp peaks at 350 °C, while the curve in H_2SO_4 residue only had two broad peaks at 294.2 °C and 334.0 °C. Furthermore, the final residual mass of the H_2SO_4 residue was shown to be lower than that of the HCl and acetic acid residues. The higher thermal stability of the H_2SO_4 residue suggested that it contained more lignin. This indicated sulphuric acid was more effective in lignin isolation. Although dilute HCl and acetic acid could dissolve similar amounts of biomass as diluted H_2SO_4 (as shown in Figure 3-2), the fact that their DTG curves are somewhat similar to that of original feedstock suggests that a selective dissolution has not been achieved, possibly due to the different solubility of lignin in various type of acids. It is well known that lignin (especially softwood lignin) has little solubility in sulfuric acid, resulting in sulfuric acid being widely used for lignin determination in many methods, including the standard TAPPI T222 method.[44] As a comparison, it has been reported that hydrochloric acid and acetic acid have a certain ability to solubilise lignin and are able to remove the 80 wt% lignin from feedstock (newsprint) in optimum conditions *via* acidolysis.[175] Thus, it is deduced lignin is partly solubilised during microwave hydrolysis with the existence of hydrochloric acid and acetic acid; while using sulfuric acid in hydrolysis, lignin is mostly kept in residue. This also demonstrates that further optimisation and mechanistic

investigation are needed for the use of HCl or acetic acid in microwave acidolysis.

3.2.4 Fourier-transform Infrared Spectroscopy Analysis

Figure 3-4 shows the FT-IR spectra of MSP and its residual lignin isolated from microwave treatment using different temperatures. For the three biomasses, as the holding temperature rose, the bands at 1601 cm^{-1}, 1508 cm^{-1} , 1451 cm^{-1} and 1424 cm^{-1} (assigned as aromatic skeleton bands[59,135,136]) intensified. These strong peaks suggested high aromatic content in the residues after treatment. Similarly, the peak at 1208–1215 cm^{-1} which was ascribed to the OH deformation of phenol was strengthened as the processing temperature rose, thus indicating that more phenolic units were present in the higher temperature residues. The peak at 1030 cm^{-1}, assigned to the primary alcohol of polysugars,[136] weakened as the holding temperature increased, thus suggesting the better removal of polysaccharide at higher temperatures. At temperatures higher than 190 ºC, the weakening of this peak became less obvious, thus showing that the residues were relatively stable after processing by microwave acidolysis at temperatures of 190 ºC or higher. Besides the general trends discussed above, there were also some minor differences among the three lignin residues (Figure 3-4b), such as at 1250–1270 cm^{-1} (C–O stretching of the guaiacyl unit[135,136]) and at 1100–1125 cm^{-1} (C–H in-plane deformation of the syringyl unit[135,136]). This is caused by the variety of lignin, because softwood lignin contains only guaiacol units, while hardwood and herbaceous lignin contain both guaiacyl and syringyl units in different proportions. Based on this, lignin from softwood, hardwood and herbaceous biomass could be used for the production of different chemicals after

further activation. For example, softwood lignin could produce more vanillin, while if the target product is acetosyringone or phenol, lignin from hardwood and straw would be a better raw material. (Figure 3-5) These phenolic compounds can be used in various value-added downstream applications. (link back to Section 1.2.2 about lignin applications) For example, vanillin is frequently used as an additive in food products like ketchup[176] and ice-cream[177]; the conversion of phenol produces an important precursor for plastic[178,179]; phenol is also a versatile precursor to a large collection of drugs, including the most notably aspirin[180]; acetosyringone can promote the plant transformation effectively[181,182]. Of note, in the spectrum of residual lignin of WS, there is a strong band between 1000-1150 cm^{-1}, which is likely to be ascribed as Si-O bond[134]. This is because, as discussed earlier, WS residual lignin contains more acid-insoluble ash (especially SiO_2) than other two types. Figure 3-4c shows the spectra of residues when different acid types and concentrations were applied at 170 ºC. The spectrum of the residue treated by concentrated sulphuric acid showed notable differences compared with the spectra of the residues treated by dilute acid. The sugar peak (1030 cm^{-1}) was much weaker compared to that of the other three spectra. On the other hand, the aromatic peaks (1601 cm^{-1}, 1508 cm^{-1} , 1451 cm^{-1} , 1424 cm^{-1} and 1213 cm^{-1}) were very sharp. These signals showed that the processed residue was highly pure lignin, which proved that concentrated acid could accelerate the separation between the lignin and sugar at a relatively low temperature. This result is consistent with the TG analysis, which showed that lignin was dominant in the residue processed by 1 M sulphuric acid.

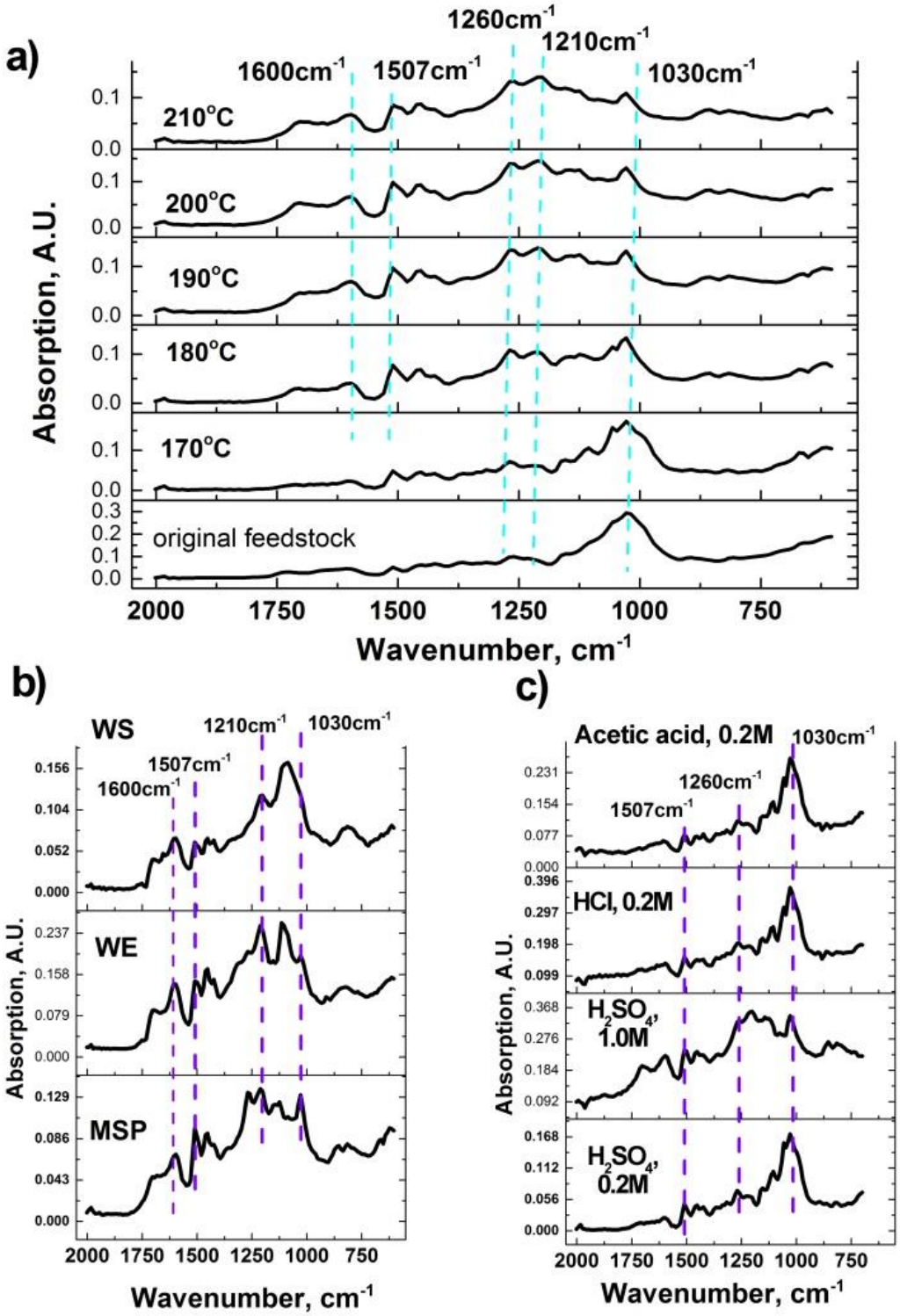


Figure 3-4 FTIR analysis of feedstocks and the processed residues. (a) Temperature influence on the spectra of the MW treated MSP residue. The conditions are: holding time of 10 min, dynamic mode, 300 W, MSP, 0.2 M H_2SO_4 solution; (b) influence of feedstock nature on the spectra of the MW residues obtained at 190 ºC. The conditions are: holding time of 10 min, dynamic mode, 300 W, MSP, 0.2 M H_2SO_4 solution; (c) influence of acid nature on the spectra of the MW residues obtained at 170 ºC. The conditions are: holding time of 10 min, dynamic mode, 300 W, MSP.

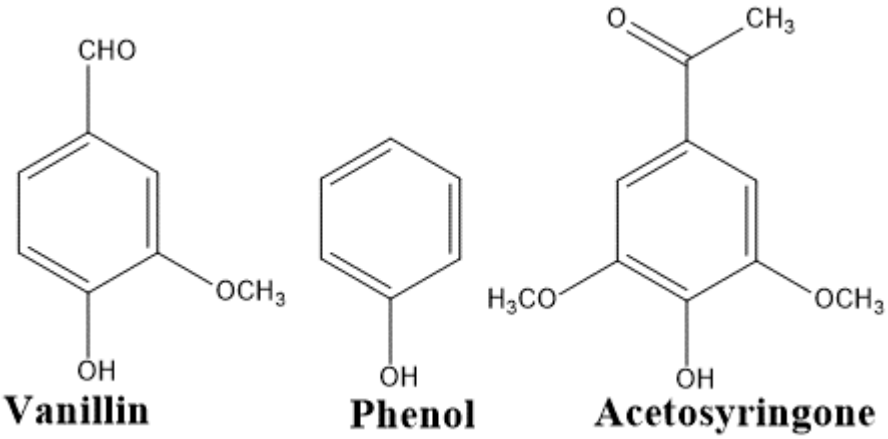


Figure 3-5 The structure of lignin degradable products: vanillin, phenol and acetosyringone

3.2.5 Solid State ^{13}C NMR Spectroscopy Analysis of Residual Lignin

Solid State ^{13}C NMR (SSNMR) spectra are shown in Figure 3-6. Obviously, after microwave acidolysis, the peaks between 109–162 ppm were much stronger. According to Jingdong Mao et al.,[137] the peaks in the range between 60–108 ppm can be ascribed to aliphatic carbon mainly from carbohydrate (a portion of the side-chain of lignin also contributes to these peaks) while the peaks between 109– 162 ppm are aromatic. Thus the spectra clearly show the existence of lignin as a major component of the material.[137,141] This demonstrated that lignin was well isolated from the biomass, especially at a holding temperature of 190 ºC. Comparing the SSNMR spectra of the 190 ºC residues with those of the 170 ºC residues, the aromatic peaks were seen to be stronger, while the carbohydrate peaks were weaker at 190 ºC, thus proving that the isolation of lignin by

microwave-assisted acidolysis is affected greatly by the holding temperature. At high temperature (190 ºC), microwave acidolysis could dissolve most cellulose and hemicellulose, while at a relatively low temperature (170 ºC) the treated residues still contained severe sugar contamination. The peak at 55 ppm was attributed to the methoxy groups of lignin.[137,140] The change of this peak at 55 ppm proved that lignin was well isolated at a holding temperature of 190 ºC. The SSNMR spectra of the three types of biomass also showed some differences besides the general trend observed above. One of the most obvious differences was the peaks located in the range from 50–15 ppm that were attributed to aliphatic carbon.[137,143] Compared with those of the MSP residues, these peaks in the spectra of the WE and WS residues were much stronger, indicating the residual lignin from WE and WS contained more aliphatic compounds, for example the sidechains of lignin. A difference was also found for the peak at ~105 ppm for the three 190 ºC residue spectra. The 190 ºC residues of hardwood and straw had well-pronounced shoulders at 105 ppm which was not the case for softwood. This peak was assigned as the C2 and C6 carbon in the syringyl unit.[143] Because softwood lignin only has G units, this peak was weak in the spectrum of the 190 ºC MSP residue.

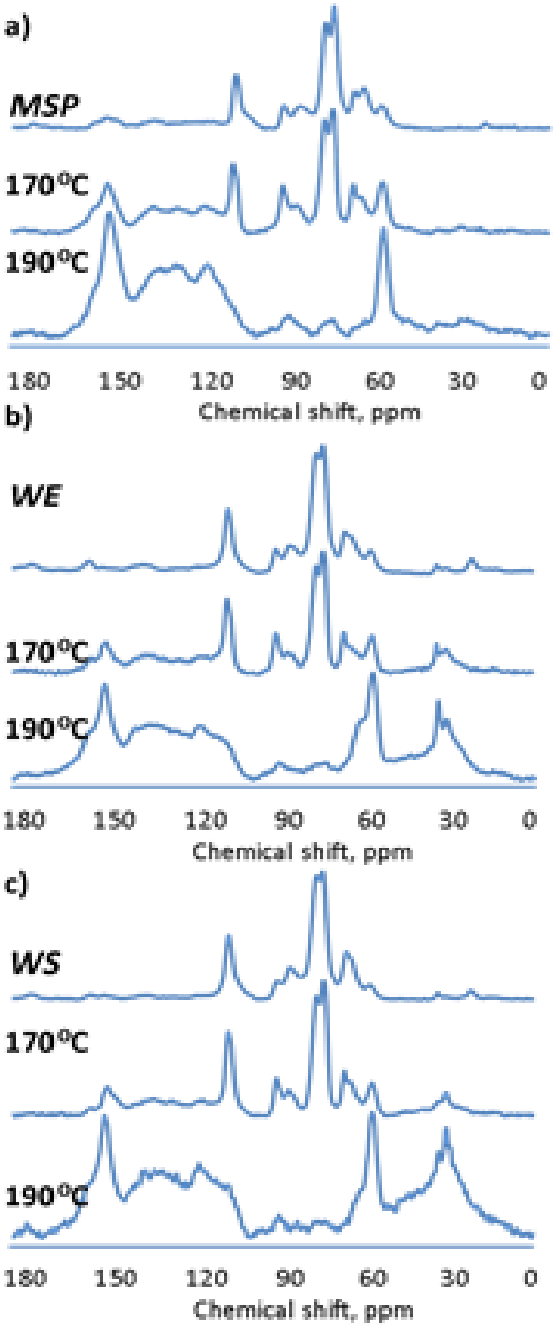


Figure 3-6 Solid State NMR spectra of three feedstocks and their processed residues. The conditions are: holding time of 10 min, dynamic mode, 300 W, MSP, 0.2 M H_2SO_4 solution. (a) Spectra of MSP and its processed residue; (b) spectra of WE and its processed residue; (c) spectra of WS and its processed residue.

3.2.6 Pyrolysis gas chromatography mass spectroscopy (Py-GC/MS) analysis

Figure 3-7 shows the contents of the pyrolytic compounds (phenolic) of MSP, WE and WS and their 190 ºC residual lignin obtained by py-GC/MS analysis. After microwave acidolysis, the content of the phenolic compounds increased significantly as indicated in Figure 3-7. For the feedstocks, compounds from polysaccharides, such as furfural and mannose, were dominant. On the other hand, for the residue lignins, phenolic compounds were the main pyrolytic products. As expected, the three types of residual lignin produced pyrolytic products with different distributions, due to softwood, hardwood and herbaceous lignin having different monomers. Softwood was mainly composed of G-units, hardwood of G-units and S-units, and herbaceous lignin of all three units (G/S/H-units). Thus, the 190 ºC MSP residual lignin had more vanillin (G-unit pyrolytic product), while the residual lignin of WE and WS had more phenol (H-unit pyrolytic product) and acetosyringone (S-unit pyrolytic product). In each of Figure 3-7a–c, a trend line was drawn to show the linear relationship between the content of phenolic compounds of the feedstock and the residual lignin. The gradient of the line is approximately the mass ratio of the biomass to the residue. Thus, if the phenolic compounds are stable during microwave acidolysis, they should be concentrated in the residues to the extent that the mass is reduced, and thus they should sit on the line; if not, it suggests that structure modification occurred during the treatment. Therefore, the coefficient of determination (R^2) of the trend line can be used as an index to show the extent of lignin structure modification during isolation. Obviously, most phenolic compounds obeyed the trend

except for only one or two compounds in each figure, thus showing that the lignin structure was generally well maintained during microwave acidolysis. Comparing the three trend lines, the R^2 value of Figure 3-7b was the lowest (0.76), thus indicating that WE lignin experienced relatively more modification during microwave treatment, while for MSP and WS, the R^2 values were higher at 0.97 and 0.95, respectively. This suggests that the microwave acidolysis protocol can preserve lignin structure very well for softwood and herbaceous biomass, but might damage the lignin structure of hardwood to some extent. It is worth noting that in the purity calculation section, the WS microwave isolation protocol produced lignin with a much lower purity and yield than for WE. However, from the results of py-GC/MS, it seems that the WS lignin structure was better maintained than that of WE lignin, although WS lignin experienced more dissolution during microwave treatment. This also implies that the soluble component reflects the residual component very closely in terms of overall composition.

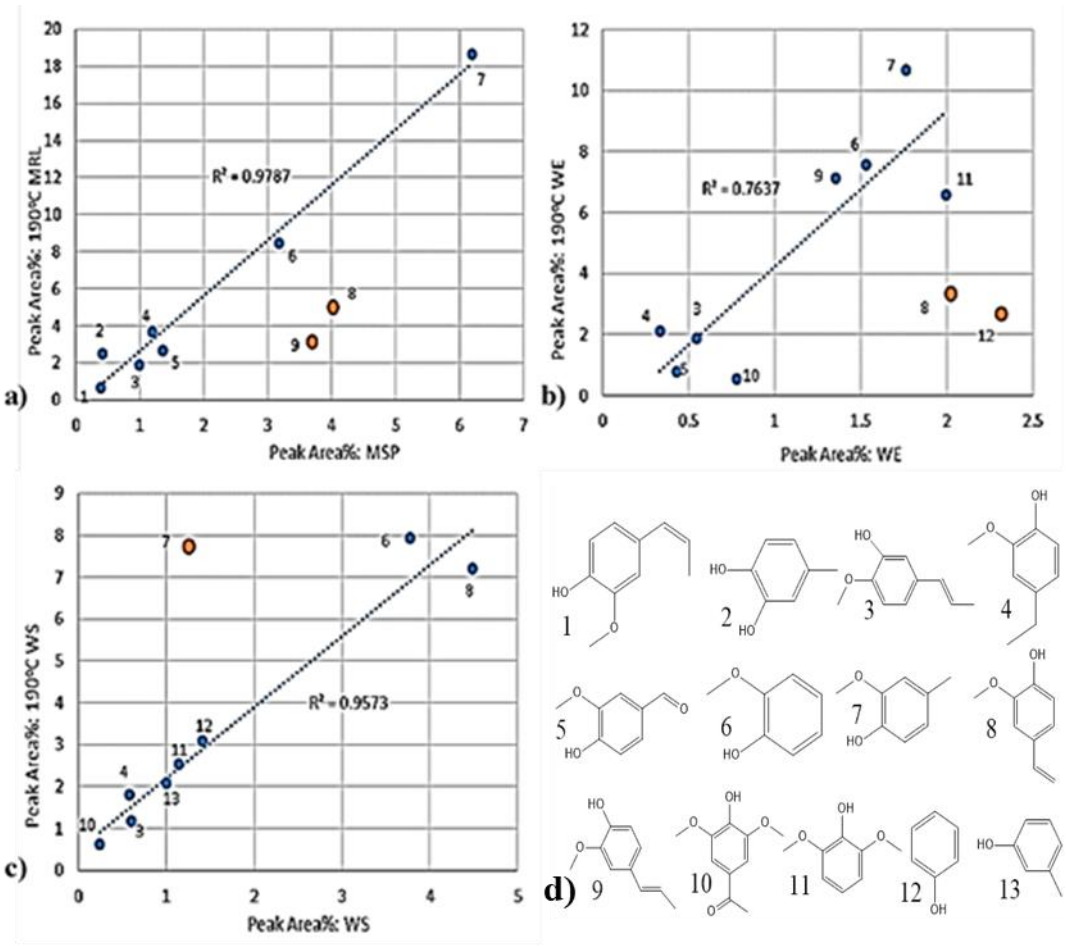


Figure 3-7 Py-GC/MS analysis. Comparisons of the GC peak areas of the phenolic compounds of the feedstock and the 190 ºC residue. Conditions: holding time of 10 min, dynamic mode, 300 W, MSP, 0.2 M H_2SO_4 solution, 190 ºC. (a) MSP samples; (b) WE samples; (c) WS samples; (d) structures of the standard compounds plotted in the figure. (1) cis-Isoeugenol; (2) 4-methylcatechol; (3) isochavibetol; (4) 4-ethylguaiacol; (5) vanillin; (6) guaiacol; (7) 2-methoxy-4-methylphenol; (8) 4-vinylguaiacol; (9) trans-isoeugenol; (10) acetosyringone; (11) syringol; (12) phenol; (13) m-cresol (the compounds represented by red dots are outside of the trend line).

By combining the residue analysis above, it was found that MSP was the most suitable material for microwave-acidolysis among the three feedstocks, and produced lignin in the

highest yield, and with the least ash and sugar contamination. The lignin and polysaccharide of softwood biomass could be separated thoroughly by a 10 min microwave treatment while the lignin structure was well preserved in the residue, thus making softwood the most potentially useful feedstock for a zero-waste microwave biorefinery. Therefore, the aqueous phase after acidolysis of MSP was used for fermentation.

3.3 Fermentation of the aqueous phase

Chapter 3.3 is a collaborative work with researchers from University of Bath (details shown in Declaration). A proportion of the saccharide feedstock partitions into the aqueous phase; this is a dilute solution that contains some monosaccharide and disaccharide sugars (DP1 and DP2), organic acids, furans, and a small amount of lower molecular weight oligosaccharides (DP3–DP5), larger oligosaccharides (DP6+) and some phenolic compounds. To quantitatively investigate the soluble fraction of biomass during hydrolysis, (high-performance liquid chromatography) HPLC analysis was carried out to calculate the yields of these compounds. HPLC analysis is a rapid and accurate tool for the qualitative determination of sugars and organic acids concentrations, in turns their yields can be calculated.[183,184] Conventional yeast based processes, such as the production of bioethanol using *S. cerevisiae*, require inhibitor concentrations of below 10 mM and high hexose sugar concentrations.[185] Larsson[186] reported that acidolysis of softwood could produce several by-products, which can be inhibitors to fermentation. These inhibitors include: furfural, 5-HMF[187], acetate[188], formic acid, levulinic acid[189] and

low molecular weight phenolics degraded from lignin[190]. Compared with WE and WS, MSP in microwave hydrolysis produced lignin with highest yield, in turn produced the least inhibitors of soluble phenolics. Furthermore, the ethanol and acetic acid produced in fermentation contribute to the inhibition.[191] However, several oleaginous yeasts are known to be able to survive in highly inhibitory conditions and can even metabolise some of these types of carbon sources.

For example, *Cryptococcus curvatus* has been demonstrated to have a high threshold for furfural inhibitors,[192] while being able to metabolise acetate and some oligosaccharides.[193] Similarly the yeast *Metschnikowia pulcherrima* was recently reported as being able to grow in extremely acidic conditions[194] and has been shown to produce a range of cellulases.[195]

Yeast derived lipids have generated an increasing amount of interest in recent years, and have been demonstrated to be suitable for a range of applications including as a feedstock for biodiesel,[196] advanced biofuels[197] and alternative chemical transformations.[198] However, one of the most sought after applications could be in the replacement of terrestrial crops, such as palm oil, which lead to widespread deforestation. Palm oil is a lipid extracted from the oil palm tree (*Elaeis guineensis*).[199] Palm oil has diverse applications and can used as a cooking oil, a food additive, and in cosmetics, industrial lubricants and biofuels.[200] From 1980 to 2000, the global production of palm oil increased 4.6-fold from 4.5 million to 20.9 million tonnes per year.[200] The increasing demand for palm oil results in a severe deforestation. For example, in Malaysia, the world's largest producer and exporter of palm oil, only about 38,000 ha of land have been used in 1950,

whereas 4,050,000 ha have been used in year 2005 for oil palm plantation, proving its rapid expansion.[201] In this situation, yeast derived lipid potentially offers an alternative product to palm oil and in turn mitigates the deforestation in tropical areas. For this application the product must be highly saturated, with the majority of the rest of the lipid being composed of oleic acid.[202] To determine the suitability of the solubilised fraction for fermentation, *C. curvatus* (Cc) and *M. pulcherrima* (Mp) were cultured on the solubilised fraction produced from the microwave processing (Figure 3-8). Both yeasts grew reasonably well on the material depolymerised at 170 ºC with 2 $g \cdot L^{-1}$ of yeast biomass produced for both species. Under these conditions, 1.5 $g \cdot L^{-1}$ of mono- and disaccharides were available for fermentation but less than 0.5 $g \cdot L^{-1}$ of acids and furfurals were produced. The co-efficient in Figure 3-8b indicates the proportion of hydrolysate that can be used in yeast cultivation. It is calculated as:

$$\text{co} - \text{efficient} = \frac{yeast\ biomass\ concentration\ after\ fermentation}{total\ hydrolysate\ products\ concentration\ before\ fermentation}$$

*hydrolysate products include mono-/di-saccharides, organic acids, sugar degradable products

The high yeast co-efficients strongly suggest that both yeasts are using the larger oligosaccharides in the broth as well. Upon increasing the processing temperature to 190 ºC, the acid and furfural concentrations were increased at the expense of the monosaccharide concentration. *C. curvatus* was able to metabolise some of this feedstock, although the impact on *M. pulcherrima* was more pronounced. Upon increasing the loading of initial wood biomass to 33 $g \cdot L^{-1}$, the mono- and di-saccharide concentrations were only increased partially, although over 2.5 $g \cdot L^{-1}$ of organic acids were now present

in the hydrolysate. Presumably as a result, the growth of *M. pulcherrima* was reduced substantially, though *C. curvatus* continued to thrive on this feedstock, producing 2.8 $g \cdot L^{-1}$ yeast biomass. A co-efficient of 0.54 on the available acids, furans and saccharides (DP1–DP5) in the media was applied. While higher loadings of biomass promoted higher sugar concentrations, the furfural and acids produced also increased substantially. With initial wood biomass loadings of 67 $g \cdot L^{-1}$ and 100 $g \cdot L^{-1}$, little yeast biomass was produced from either yeast. Both yeasts metabolised the majority of the mono- and di-saccharides over the 7 days, though *M. pulcherrima* was more capable of metabolising the small amount of DP3–DP5 oligosaccharides in the broth than *C. curvatus* (Figure 3-9a and b). Both yeasts demonstrated some ability to break down some of the larger oligosaccharide feedstock too (Figure 3-9c). *M. pulcherrima* appeared to reduce some of the larger oligosaccharides, thus presenting a different oligosaccharide profile of the broth after the fermentation was complete. More strikingly, *C. curvatus* seemed able to convert a proportion of the larger oligosaccharides, presumably producing saccharides in the DP6 and smaller ranges in the process. This ability supports the higher levels of yeast biomass observed with *C. curvatus*, and demonstrates why the yeast could grow so well on the hydrolysed feedstock.

Interestingly, both yeasts seem to have similar mechanisms for dealing with the inhibitory compounds. At a 33 $g \cdot L^{-1}$ loading, neither *M. pulcherrima* nor *C. curvatus* converted the acids in the hydrolysate, and the majority present at the start of the fermentation remained in the broth. However, at higher concentrations, where both yeasts grew poorly, the acids were seemingly metabolised. The furfural concentrations in the mixtures were found to

be below the detectable limits for both yeasts, under all conditions, while the 5-HMF concentration was also severely reduced. This suggests that the yeasts were converting the furan compounds and producing a less toxic substrate, which is a mode of action commonly seen with these types of yeasts.[203]

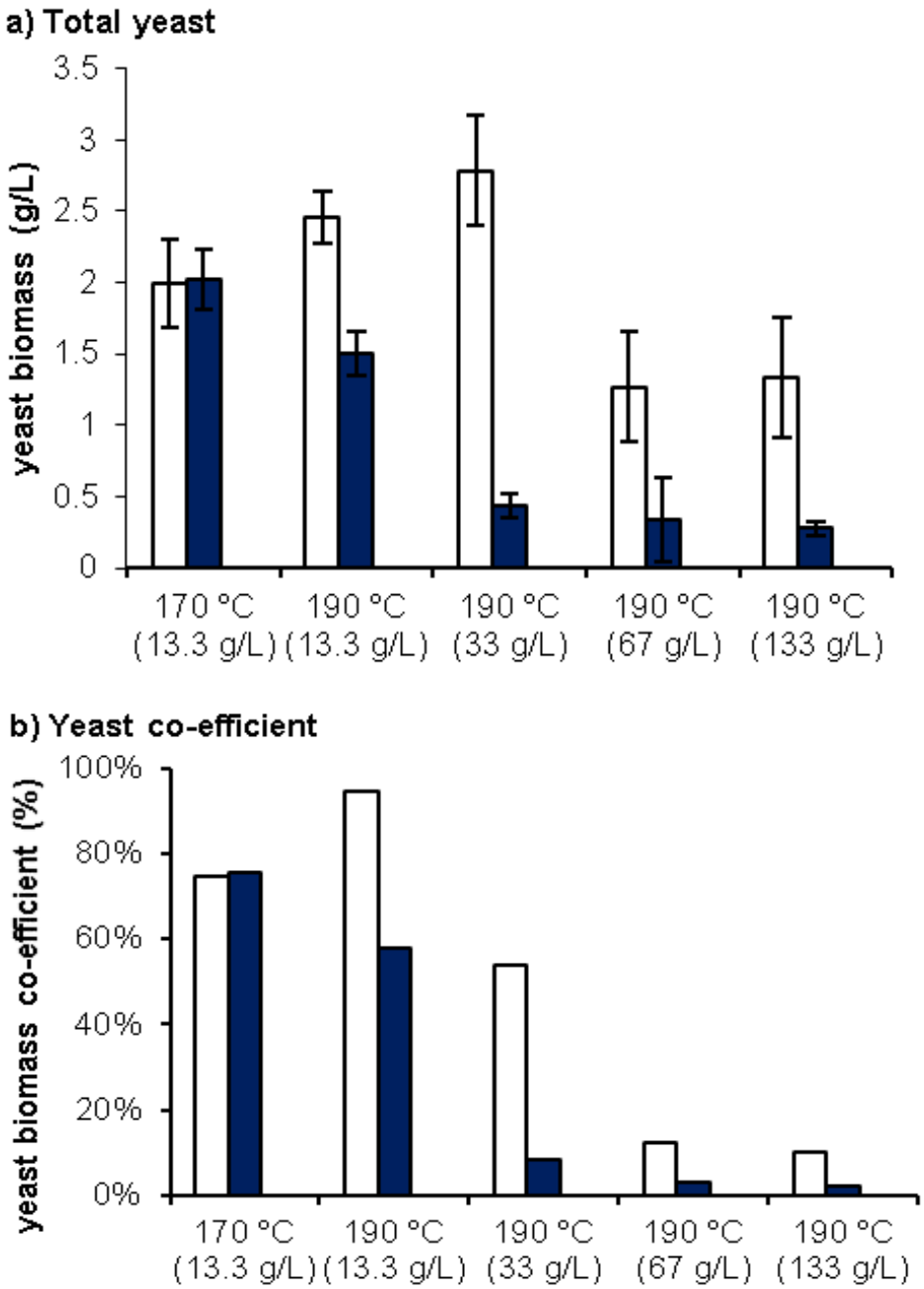


Figure 3-8 Yeast biomass produced from the various hydrolysate samples, given as the initial loadings of wood biomass at the temperature it was depolymerised, for *C. curvatus* (white bars, pH 6.5, 180 rpm, 168 h) and *M. pulcherrima* (blue bars, pH 4, 180 rpm, 168 h), where (a) is the total yeast biomass and (b) is the yeast biomass coefficient, based on the proportion of the yeast grown compared to the initial level of saccharides (DP1–DP5), organic acids and sugar dehydration products inhibitors.(results partially from University of Bath)

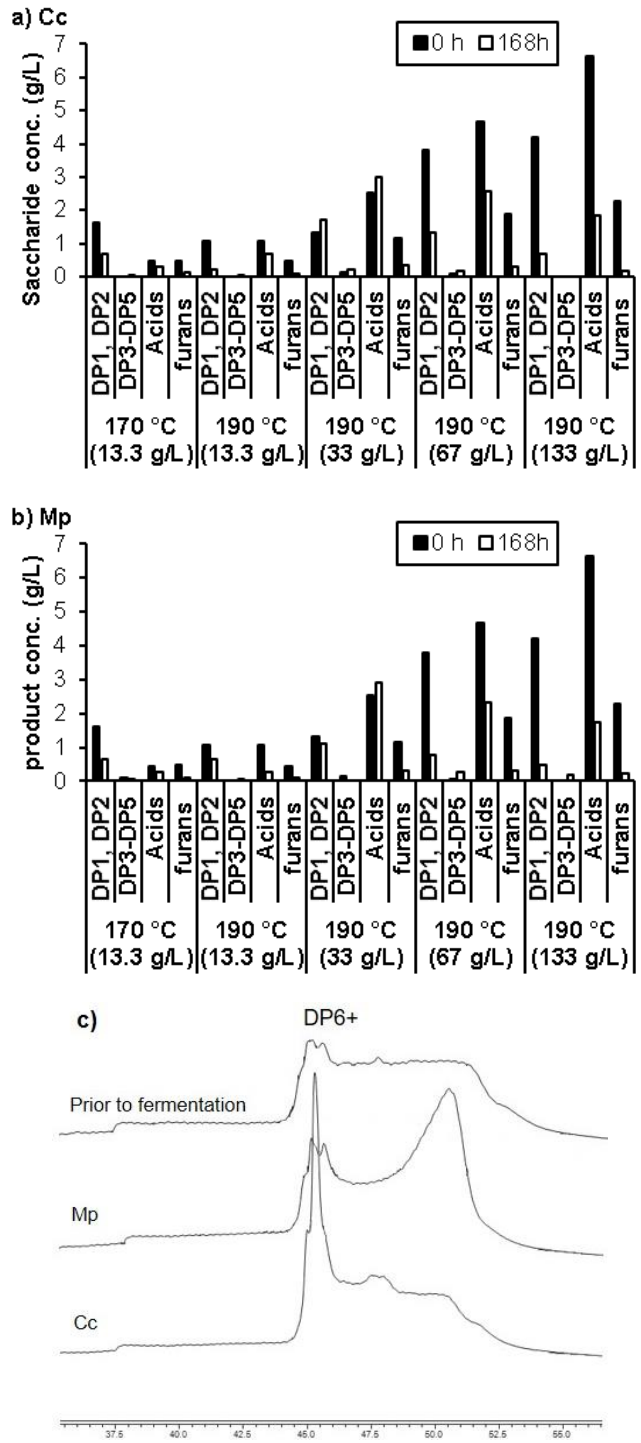


Figure 3-9 The quantification of saccharides (DP1–DP5), acids (formic, lactic and acetic) and sugar dehydration products (5-HMF and furfural) before and after fermentation over the range of hydrolysates for (a) *C. curvatus* (pH 6.5, 180 rpm, 168 h) and (b) *M. pulcherrima* (pH 4, 180 rpm, 168); (c) qualitative oligosaccharide chromatograph demonstrating DP6 and larger oligosaccharides for the original hydrolysate (depolymerised at 170 ºC, 13.3 $g \cdot L^{-1}$ wood biomass) and those left after fermentation with *M. pulcherrima* (pH 4, 180 rpm, 168 h) and *C. curvatus* (pH 6.5, 180 rpm, 168 h). (results partially from University of Bath)

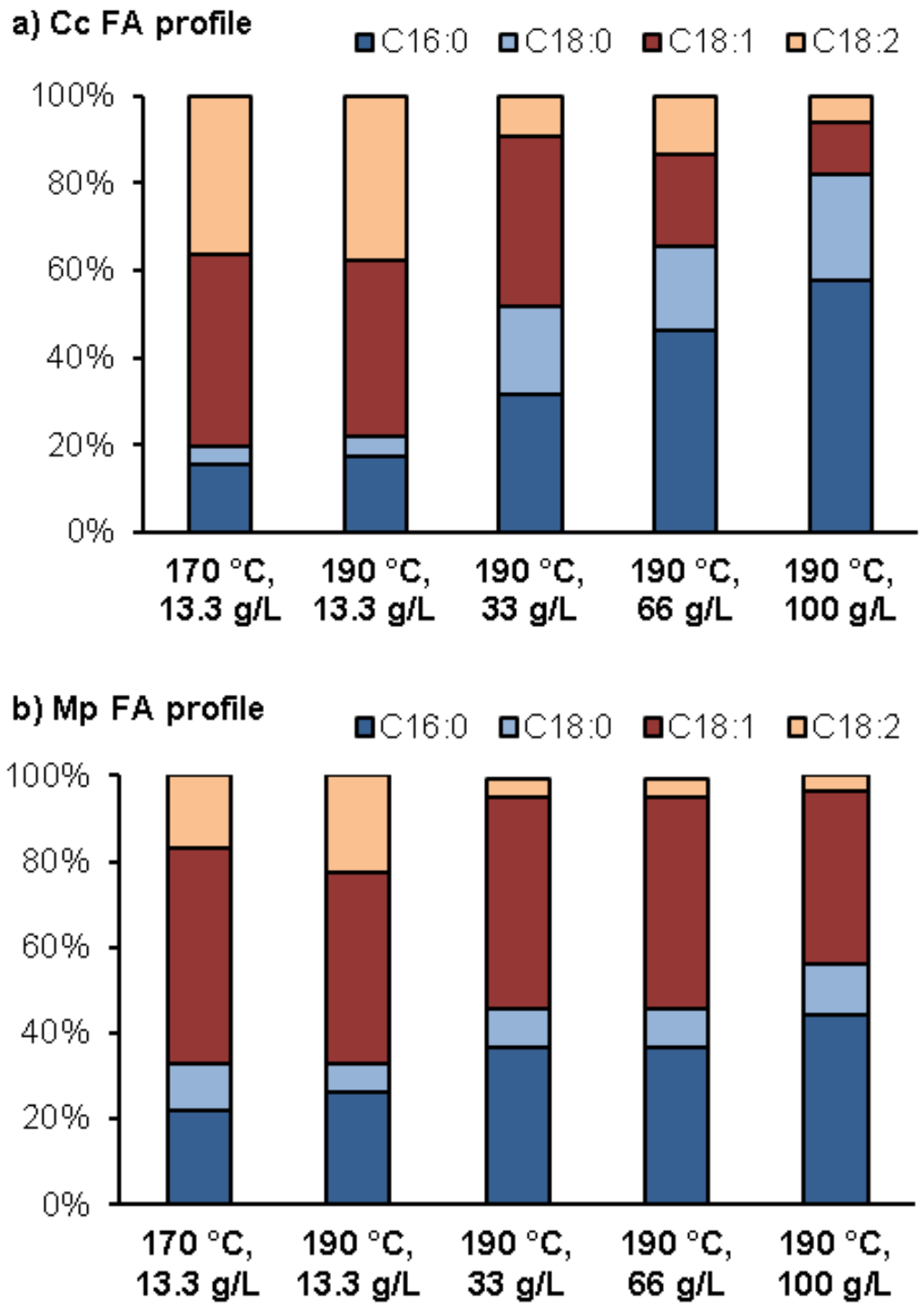


Figure 3-10 Fatty acid profile of the lipids extracted from the yeasts (a) *C. curvatus* (pH 6.5,180 rpm, 168 h) and (b) *M. pulcherrima* (pH 6.5, 180 rpm, 168 h). (results partially from University of Bath)

Both yeasts are oleaginous, and as such the lipid produced was investigated as a possible palm oil substitute (Figure 3-10). Despite both yeasts having been reported to be effective producers of saturated lipid, less than 20% of the cell weight was recovered under any of the conditions analysed. In addition, the lipid profile was found to be highly dependent on the feedstock processing temperature. Under these conditions *C. curvatus* produced a lipid that was more similar to rapeseed oil at low loadings of initial biomass, but the saturation increased substantially upon increasing the initial biomass loading. Similarly, *M. pulcherrima* produced a lipid that was analogous to palm oil at increased loadings of biomass. This suggests that highly stressful fermentation conditions can contribute to increasing saturation. This opens up the possibility of being able to stress these yeasts into producing desirable lipid profiles for industrial production, although more work would be necessary to increase the lipid content up to suitable levels.

3.4 Conclusions

A new method of rapid microwave-assisted acidolysis for a potential biomass biorefinery is proposed to achieve the zero-waste utilisation of lignocellulosic biomass. Three types of lignocellulosic biomass were investigated for the separation of lignin and polysaccharides. Compared with WE and WS, MSP was the most suitable feedstock for microwave acidolysis, and produced high quality lignin with a high yield (82%) and purity (93%). According to the py-GC/MS results, it was found that the lignin structure could be well preserved during acidolysis, thus making this unmodified lignin suitable for phenolic compound production. Sulphuric acid treatment could isolate lignin with the

highest quality. If concentrated sulphuric acid was used in acidolysis, lignin could be isolated at a relatively low temperature in comparison with that required for dilute acid, although it is likely that the use of this more concentrated acid caused extensive degradation of the saccharide feedstock. To convert the inhibitor rich aqueous phase, two yeasts known for their inhibitor tolerance were selected (*Cryptococcus curvatus* and *Metschnikowia pulcherrima*). Both yeasts could grow on the saccharide feedstock and metabolise a range of acids, furans, sugars and oligosaccharides. However, at high inhibitor loadings the yeast biomass was severely reduced. Both yeasts are known to be oleaginous and produced an oil that was akin to palm oil from this feedstock. Based on these results, microwave-assisted acidolysis offers a potentially powerful tool for lignocellulosic biomass utilisation, especially for softwoods. Due to the efficiency and selectivity of microwave heating, 10 minutes is enough time to produce high quality lignin and a fermentable aqueous phase.

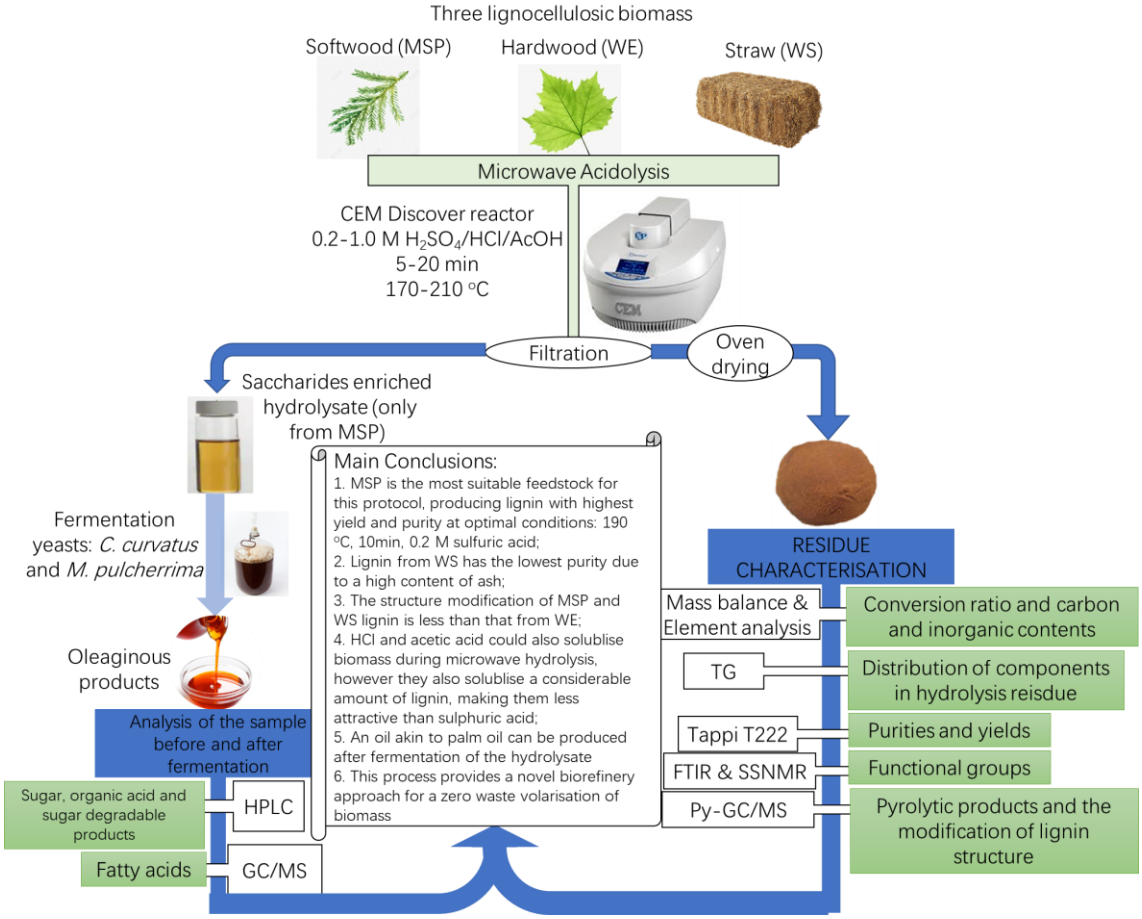


Figure 3-11 The content summary of Chapter Three: a novel approach for zero waste utilisation of biomass *via* microwave treatment

Natural product recovery from bilberry (*Vaccinium myrtillus L.*) presscake via microwave hydrolysis

Chapter 4

In Chapter 2 & 3, it is found that microwave-assisted hydrolysis offers an efficient method for lignin isolation through the dissolution of the polysaccharides fraction. The results suggest polysaccharides can be effectively degraded to produce mono-/ di-sugars *via* microwave hydrolysis. Based on this fact, in this chapter, another application of natural products recovery from bilberry press-cake is investigated using microwave hydrolysis.

4.1 Introduction

Bilberries (*Vaccinium myrtillus L.*) are a significant wild fruit harvested for numerous applications including cold pressing to produce bilberry juice.[204] It is one of the most economically important wild berry species in several European countries[205] where bilberry is collected for both household consumption and sale.[206] In late 1970s, the total annual bilberry production in Sweden, which is a major producer of bilberry product, reached 307 million kilogram fresh weight.[207] There is intense interest in the health benefits of fresh berry fruit and related products due to their high antioxidant capacity, impact on vision, and potential cancer suppression, all of which highly correlates to the content of anthocyanin and other phenolic compounds.[208,209] Bilberries are one of the richest dietary sources of anthocyanins;[210] thus bilberry juice is often used as a major constituent of functional food and beverages.[211] Of note, as bilberries are very soft and juicy, making them difficult to transport, bilberry juice is a more common product than fresh bilberry fruits, especially outside Scandinavia where bilberries are mostly harvested. However, a by-product of juice production is bilberry presscake, a currently under-utilised resource rich in dietary fiber.[212] Bilberry presscake, with further exploitation, is a

potential source for various applications as it contains abundant healthy components to human. For example, the presence of cellulose, hemicellulose (mostly xyloglucan), and pectin in the presscake[213–215] makes it a promising feedstock for use in the food industry. These dietary fibres have multiple healthy benefits to human, including reducing the risk of cardiovascular diseases and cancer, diabetes control, providing a regularity/laxative benefit, appetite control, body weight control, altering the composition of the intestinal flora and improving immune function.[216] Additionally, as the presscake retains the fruit skins and remains highly colored, it contains high levels of anthocyanins, which can be extracted and further processed into healthcare products.[217] Anthocyanins is a soluble phenolic compound with antioxidant function and has the healthy benefits for improving eyesight, reducing the risk of cancer and reduction of blood pressure.[218–220] The high contents of these healthy components indicate the potential of bilberry presscake for high value-added applications, including pharmaceuticals and food industries.

Due to the facts stated above, recently researchers started to concern the natural products recovery from bilberry presscake. The existing studies looking to exploit bilberry presscake mainly focused on the recovery of anthocyanins (Figure 4-1). Ethanol, acetone, and their water mixtures are widely used for the extraction of anthocyanins.[221–225] In addition, supercritical carbon dioxide with ethanol as a polar modifier has been investigated as a more environmentally friendly alternative with food grade status, to extract anthocyanins from bilberry presscake.[217,225,226] Fidaleo et al.[223] demonstrated that common food products such as yogurt and condensed milk can be easily fortified with phenolic extracts from bilberry presscake *via* ethanol extraction. Aaby et al.[221] reported

that bilberry presscake contained high concentrations of anthocyanins (458 mg 100 g^{-1}) that could be effectively extracted by water.

Compared with the extraction of anthocyanins, the recovery of saccharides offered another path for utilisation of bilberry presscake. As efforts to optimise cold press conditions to give greater anthocyanin content in the juice are constantly underway, the anthocyanin content in bilberry presscake will decrease greatly in the future; however, polysaccharide loading will still remain high. These polysaccharides can degrade into mono- and disaccharide with thermal treatment. According to Aura[212] and Hilz[214], bilberry presscake produced abundant glucose, xylose, and mannose (Figure 4-1) *via* hydrolysis by using concentrated sulfuric acid (72 wt%). Thus, the extraction of saccharide appears to be a feasible, economical, and promising approach for the valorisation of bilberry presscake. However, to the best of our knowledge, there is still no mature technology and systematic research to convert bilberry presscake into mono-/disaccharide which is suitable for food grade status applications.

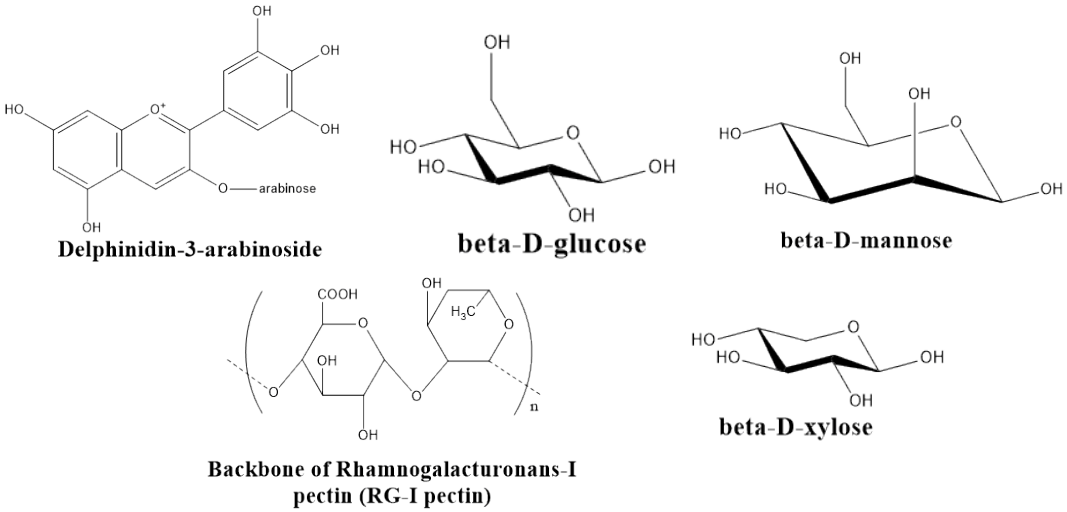


Figure 4-1Several value-added chemicals in bilberry presscake

(delphinidin-3-arabinoside is a typical anthocyanins in bilberry)

Compared with conventional thermal treatment, microwave assisted treatment has the characteristics of high efficiency and selectivity, making it an efficient tool in biomass processing and solid waste recycling.[87,103,227] It has dramatic effect on the reaction kinetics[163] and reduces overall reaction time substantially[164]. Several researches have demonstrated the excellent performance of microwave thermal treatment in recovery of chemicals (mainly anthocyanins) from fresh berry fruit and berry pomace.[228–230] According to our previous studies, polysaccharide could be effectively degraded via hydrolysis using diluted acid[103] or even just water[87]. Thus, microwave hydrolysis is expected to offer an effective approach to obtain low-molecular-weight chemicals from bilberry presscake.

In this chapter, microwave thermal hydrolysis is, for the first time, used in saccharide recovery from bilberry presscake. Rather than focusing only on anthocyanins, the main aim of this study is to design a novel, efficient approach to obtain various value-added products (saccharides, anthocyanins, protein, and even inorganic salts) from bilberry presscake *via* microwave treatment. Instead of conventional approaches using organic solvents or mineral acids, water is used for the depolymerisation and chemicals recovery of bilberry presscake, making the extracts more suitable for food applications. Statistical analysis using a Box−Behnken design is carried out to optimise the conditions to achieve maximum conversion. Furthermore, pilot scale microwave extraction is also carried out to compare with lab scale results and discuss the feasibility of industrial application.

4.2 Optimisation of Microwave Conversion

A CEM Mars 6 microwave reactor (USA) was used for the experiments. Dried bilberry presscake (DBP) was combined with deionised water (60 mL) at different ratios in microwave vessels prior to microwave hydrolysis. Samples were heated to their target temperature, with a set ramping time of 5 min (variable ramping rate). Following each trial, the samples were filtered to obtain the solid residue and liquid phase for further analysis.

Table 4-1 Box-Behnken design and the conversion ratios (response value)

	Parameters			Conversion	
Trial	**Temp, °C (A)**	**Time, min (B)**	**Solid, % (C)**	**Tested value, wt%[a]**	**Calculated value, wt%**
13	80	0	5.5	22.2	20.9
14	80	30	5.5	27.3	27.5
15[b]	80	15	1.0	34.9	34.9
16	80	15	10.0	22.2	23.9
17	110	0	1.0	33.3	34.3
18	110	30	1.0	46.8	47.1
19	110	0	10.0	25.7	25.0
20	110	30	10.0	26.5	25.2
21	110	15	5.5	33.0	32.1
22	110	15	5.5	32.7	32.1
23	110	15	5.5	31.0	32.1
24	140	0	5.5	31.3	32.3
25	140	30	5.5	38.2	38.8
26	140	15	1.0	52.3	50.9
27[b]	140	15	10.0	30.3	30.6

a.Equations supporting this data can be found further in Experimental chapter

b.Pressure and temperature traces can be found in Experimental chapter

The conversions are listed in Table 4-1 (conditions in Table 4-1 are based on Box-Behnken design in Table 4-2). Notably, high temperature, long holding time, and low solid content all benefit conversion. According to Table 4-1, the variance analysis was made in Table 4-3.

Table 4-2 The factors and the levels of Box-Behnken design

Factor	**Level**		
	-1	**0**	**1**
A: temp (°C)	80	110	140
B: Time (min)	0	15	30
C: solid content (%)	1	5.5	10

Table 4-3 The variance analysis using Box-Behnken design

Source	Sum of Squares	D_f	Mean Square	F Value	p-value Prob > F
Model	953.70	9	105.97	42.85	0.0003
A	258.90	1	258.90	104.70	0.0002
B	85.54	1	85.54	34.59	0.0020
C	491.57	1	491.57	198.79	< 0.0001
AB	0.85	1	0.85	0.34	0.5839
AC	21.86	1	21.86	8.84	0.0311
BC	39.82	1	39.82	16.10	0.0102
A^2	0.34	1	0.34	0.14	0.7243
B^2	17.43	1	17.43	7.05	0.0451
C^2	33.22	1	33.22	13.44	0.0145
Residual	12.36	5	2.47		
Lack of Fit	10.04	3	3.35	2.88	0.2685
Pure Error	2.33	2	1.16		
Cor Total	966.06	14			

The P value of lack of fit was 0.2685 > 0.05, showing the lacking sources were not significant. The P value of the model was 0.0003 < 0.05, indicating the model was of high significance. The P values of source A (temperature), B (holding time), and C (solid content) were very low; this indicated that the microwave hydrolysis temperature, holding time, and solids content all had significant influence. According to the P values, the influences ranked as solid content > temperature > holding time. Most sources had P value lower than 0.05, except AB and A^2, so the variance analysis was optimised by excluding AB and A^2 in Table 6-8. The P value of the model (<0.0001) was further lowered, showing

the model was more significant after optimisation. A quadratic regression equation was made based on Table 6-8:

Quadratic regression equation:

$$Conversion\ \% = 5.5323 + 0.2849A + 0.7617B - 0.7784C$$
$$0.0173AC - 0.0467BC - 0.0096B^2 + 0.1493C^2 \quad (1)$$

where A is temperature; B, holding time; and C, solid content.

The high R^2 of this equation at 0.986 indicates the degree of error to be minimal. Using the above equation, the calculated conversions are presented in Table 4-1, showing the predicted and observed conversions to be very similar. Based on statistical software, within the range of the three factors, the following conditions theoretically should produce the highest conversion (55.21%): temperature 140 °C, holding time 30 min, solid ratio 1%.

4.3 Soxhlet Extraction.

The Soxhlet extraction (extraction using conventional heating, not microwave hydrothermal treatment as above) results are presented in Table 4-4. Notably, the polarity of solvent could affect the extraction significantly. More highly polar solvents benefited the extraction of dried bilberry presscake (DBP), suggesting that DBP contains less fatty acids, wax esters, or oil (usually extractable with low-polarity solvent) but more sugars, pigments, and phenols (extracted with high polarity solvent). Within 4 hours, ethanol extraction could achieve the highest extraction among all organic solvents, whereas heptane, with the lowest polarity, achieved the least. A 24 hours Soxhlet extraction using

deionised water (Trial SW) was carried out to compare with conversion via microwave hydrolysis. A longer extraction time with water was required to give the same number of fresh solvent cycles as compared to ethanol, acetone, and heptane.

Table 4-4 Soxhlet Extraction of using different solvents. Number of fluxes as 4, this needs about 4 hours for organic solvent extraction and 24 hours for water trial (the distribution of extractive products will be discussed in Chapter 4.9)

Solvent	Relative Polarity[231] (compared to water)	Extraction Ratio, wt%
Acetone	0.355	2.8
Ethanol	0.654	6.2
Heptane	0.012	1.8
Water (Trial SW)	1.000	30.5

conversion ratio calculated by method in Table 4-1

4.4 Microwave Conversion

The conversions of microwave trials are listed in Table 4-5. As suggested in condition optimisation, solid content could affect the conversion significantly. For example, with the same treatment temperature and time, the conversion was still low at 30.0 wt % in Trial 8, whereas it was 44.2 wt % in Trial 4. This indicated that, under lab scale conditions without pre-treatments, DBP cannot adequately mix with water, hindering the hydrolysis of the DBP. In addition to solid content, increasing temperature and hydrolysis duration can also increase conversion, which is consistent with condition optimisation. Comparing Tables 4-4 and 4-5, it is easy to observe that microwave hydrolysis provides a more

efficient approach for DBP hydrolysis than conventional methods. In Trial 4, a 30 min microwave hydrolysis converted 44.2% of feedstock into water-soluble compounds, whereas in Trial SW, a 24 hours Soxhlet extraction only converted 30.5%, proving microwave hydrolysis is an effective method in waste recycling of DBP. In turn, this fast processing by microwave should result in reduced energy consumption, as has been previously demonstrated in various applications.[122,232–234]

Table 4-5 Experimental parameters for microwave hydrolysis of bilberry presscake and the conversion ratio (wt%), Trials 9r-12r are repeated experiment. The experiments were carried out using CEM MARS microwave reactor. After each run, (conditions as shown in table), the solid residue and hydrolysate were separated by filtration for further analysis

Trial	Temp (°C)	Time (min)	Solids (%)	Conversion (wt%)
1	80	0	1	14.8
2	140	0	1	32.7
3	80	30	1	35.6
4	140	30	1	44.2
5	80	0	10	13.1
6	140	0	10	22.9
7	80	30	10	21.5
8	140	30	10	30.0
9r	110	15	5.5	26.9
10r	110	15	5.5	33.0
11r	110	15	5.5	32.7
12r	110	15	5.5	31.0

conversion ratio calculated by method in Table 4-1

4.5 Elemental Analysis.

Table 4-6 shows the elemental content of DPB and the processed residues. DBP contains ~40 wt % of other elements in addition to C, H, and N, which were expected to be oxygen and a considerable amount of trace elements, especially K, Ca, P, Mg, and Mn.[235,236] Trace elements exist in biomass mainly in the form of inorganic salts, of which the concentrations are the highest in the peel for fruits.[237–239] These inorganic salts would preferentially be solubilised in the aqueous phase during the microwave hydrolysis. This observation is most obvious when insufficient mixing of solvent and feedstock occurs, resulting in a lower content of other elements in Trial 5-12r than in Trial 1-4. The higher solid content in Trial 5-12r resulted in a greater amount of compounds from the surface of the DBP being hydrolysed as compared to those from within the fruit. This hypothesis is in agreement with condition optimisation that showed solid content had significant influence on DBP microwave hydrolysis. Therefore, to carry out hydrolysis with high solid contents, pre-processing to homogenise before hydrolysis or more vigorous agitation is necessary to ensure adequate mixing of DBP with the solvent.

Table 4-6 Element contents of feedstock and residue (Trial 1 to Trials 12r in the table mean the hydrolysis residues in different trials)

Samples	C, wt%	H, wt%	N, wt%	Other, wt%
DBP	52.2	6.7	2.0	39.1
Trial 1	51.4	6.3	2.0	40.4
Trial 2	52.1	6.2	2.3	39.4
Trial 3	52.8	6.6	2.2	38.4

Trial 4	52.0	6.3	1.4	40.3
Trial 5	54.0	6.6	2.0	37.3
Trial 6	53.3	6.7	2.2	37.9
Trial 7	54.1	6.5	1.6	37.8
Trial 8	54.6	6.5	2.1	36.8
Trial 9r	53.7	6.5	2.2	37.6
Trial 10r	55.2	6.7	2.3	35.8
Trial 11r	56.5	7.2	2.4	33.9
Trial 12r	54.0	6.7	1.9	37.4

4.6 Thermal gravimetric Analysis.

The DTG curves of DBP and processed residues are shown in Figure 4-2. The major peaks of all curves appeared between 350 and 370 °C, which possibly corresponds to the decomposition of cellulose,[103,130,131] illustrating that, after hydrolysis, the processed residues were still polysaccharide-rich materials that can be potentially converted into mono-/disaccharides using more intensive conditions. The peaks above 400 °C correspond to the degradation of lignin.[131] As lignin is very recalcitrant to thermal treatment,[103] the low temperatures applied in this research (80−140 °C) would result in little or no lignin depolymerisation.

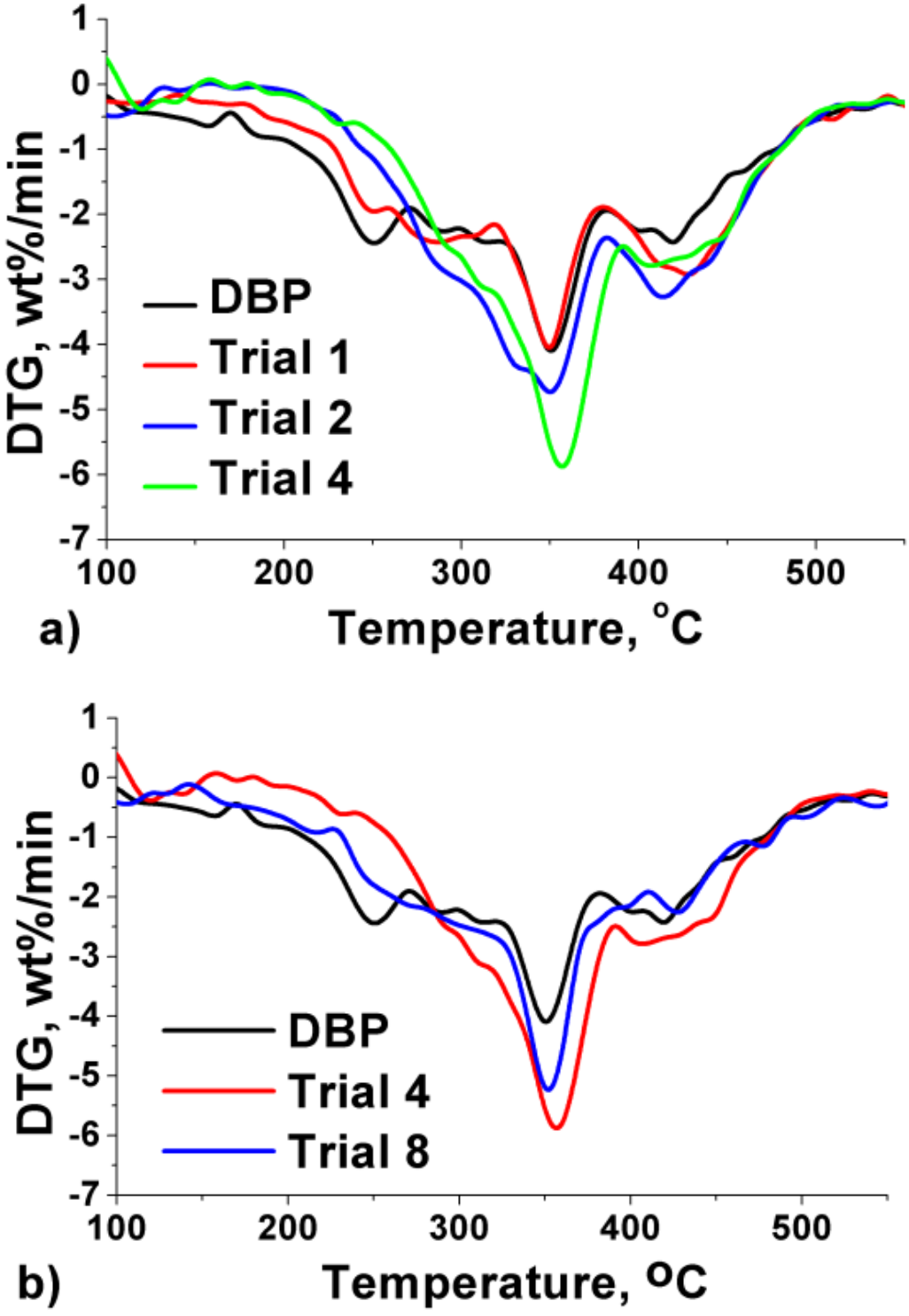


Figure 4-2 DTG curves of DBP and processed residues in different conditions: (a) different temperature and time, and (b) different solid content.

As is shown in Figure 4-2a, hydrolysis temperature and holding time could slightly influence the processed residues. The curve of Trial 1 is similar to that of DBP, suggesting

minimal hydrolysis takes place at low temperature (80 °C) and short holding time (0 min). This is in agreement with the very low conversion of 14.8% obtained in Trial 1 (80 °C). When the hydrolysis temperature was increased to 140 °C (Trial 2), the DTG trace showed two distinct changes (Figure 4-2a). First, between 100 and 270 °C, the mass loss rate was lower than DBP, suggesting that the more easily degradable compounds were significantly less abundant in the solid residue postprocessing. These thermally labile compounds are likely to be anthocyanins,[240] pectin,[241] oligosaccharide,[242] and some fatty acids. Second, from 270 to 390 °C, the DTG curve had a sharper peak compared with that of Trial 1, indicating that polysaccharides are the main component in residue. As stated above, the peak between 350 and 370 °C corresponds to the degradation of polysaccharide. These changes were even more apparent in the DTG curve of the residue from Trial 4 (Figure 4-2a), indicating a longer hydrolysis time (30 min) could further enhance the conversion of DBP to water-soluble compounds. Figure 4-2b shows the influence of solid content on processed residues. Where a lower solid content was used (1 wt %, Trial 4), the residue contained more polysaccharide and less easily degradable compounds than when a higher solids content of 10 wt % was applied (Trial 8). As such, DTG analysis suggests that hydrolysis employing a longer duration, higher temperature, and lower solid content results in a greater degree of water soluble compounds obtained from DBP. However, even using the best condition (Trial 4) the large quantities of polysaccharide remained within the solid residue, depolymerisation of which should be possible with treatment employing more intense conditions.

4.7 FTIR analysis

The FTIR spectra of feedstock and processed residues (Figure 4-3) show no significant difference before and after hydrolysis.

The peaks at 1025, 2923, and 3329 cm^{-1} are attributed to C−O, C−H, and O−H vibration, respectively,[227,243,244] which are all typical peaks of polysaccharide.[244] These strong peaks suggested the processed residues after hydrolysis still contained abundant polysaccharides, consistent with the differential thermal gravimetric analysis. The peaks at 1616, 1516, and 1461 cm^{-1} were attributed to aromatic ring,[59,135,136] possibly from lignin and anthocyanins.

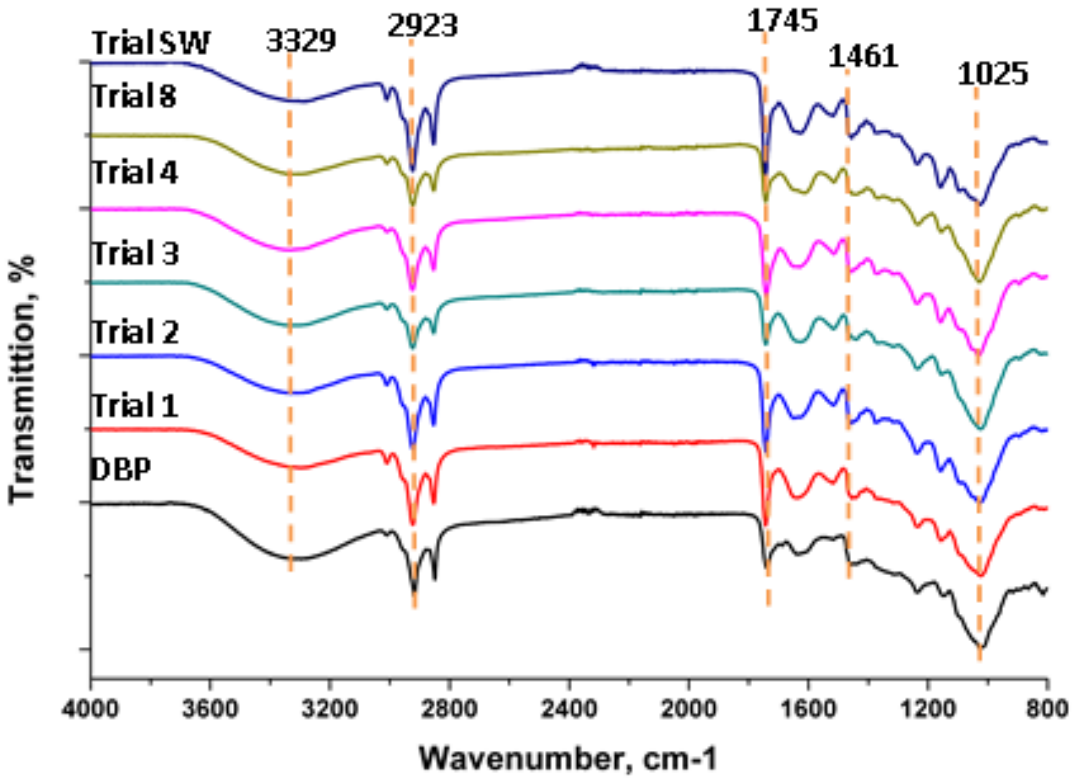


Figure 4-3 FTIR spectra of DBP and the processed residues.

The peak at 1745 cm^{-1} is attributed to carbonyl groups,[134] suggesting the presence of organic acids. Of note is the difference of the carbonyl peak between the control Trial SW

where it is very sharp and the microwave Trials 1−4, and 8, where it is less prominent, suggesting microwave hydrolysis extracts more organic acids, thus, lowering the content in the resulting residue. This hypothesis was confirmed by HPLC analysis that showed hydrolysate of microwave trials contained more organic acids than conventional extractions. The peak is stronger in all residues as compared to DBP which could be due to production of organic acids during hydrolysis.

4.8 UV−Vis spectra analysis

The aqueous phase ("hydrolysate") postprocessing was investigated using ultraviolet–visible spectroscopy (UV−vis) spectroscopy, HPLC, and ^{13}C NMR spectroscopy. UV-vis spectroscopy has been been widely used in anthocyanins pigment content determination, especially for the anthocyanins in berry fruits and their juices products. [245,246] Anthocyanins molecules contain abundant π-electrons that undergo orbital transitions in ultraviolet-visible spectral region, thus the absorbance of samples at certain wavelength in UV-vis spectroscopy can reflect the anthocyanins content quantitatively. Figure 4-4 shows the absorbance of hydrolysate at 530 nm of UV-Vis spectra, which is directly in proportion to the concentration of anthocyanins. The absorbances of hydrolysates of Trials 2 and 3 were much higher than that of Trial 1, indicating that increasing hydrolysis temperature or time results in greater recovery of anthocyanins. However, there was a significant decrease of absorbance in Trial 4. This is very likely caused by degradation of anthocyanins due to the overly intensive conditions. The literature suggests that anthocyanins readily convert to colorless derivatives and subsequently to insoluble brown

pigments.[240] This was observed in this research: the hydrolysate of Trials 1−3 was a clear purple (with intensity of color increasing from Trials 1 to 3), whereas the solution of Trial 4 was cloudy caused by the degradation of anthocyanins.

It is worth noting anthocyanins are water-soluble. A certain volume of anthocyanins is already transferred to juice during cold press. As already stated, improving cold press techniques in the future aims to increase the anthocyanins content in juice, thus lowering the content in the presscake. For fresh bilberry, the anthocyanins content is roughly 3−4 mg g^{-1},[247,248] and would require additional methodologies to isolate, which is outside the scope of this work. Furthermore, in this research it was found the best conditions for high conversion are not in favor of recovery of anthocyanins because of severe decomposition. A two-step hydrolysis could possibly be designed to extract both anthocyanins and saccharide, with mild conditions to extract the former and a second intensive step to give the latter. It is reported that the anthocyanins content could reach 60-80 g/kg in dry bilberry presscake,[226] making the recovery of anthocyanins from bilberry presscake very attractive. However, systematic optimisation and investigation are needed for the anthocyanins extraction in the future work.

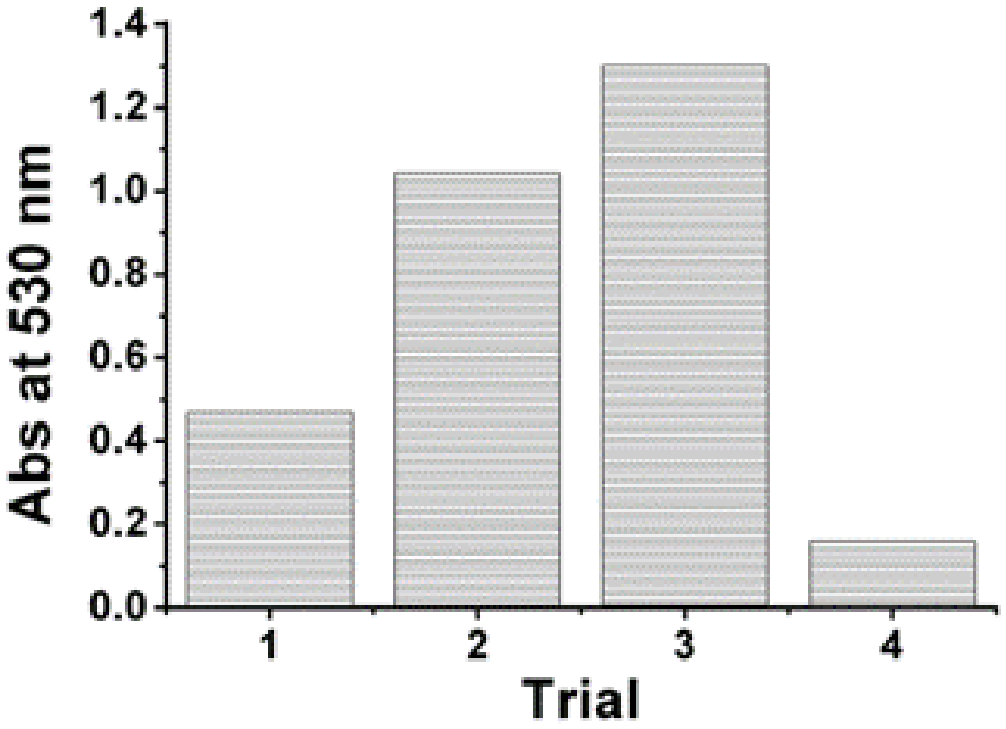
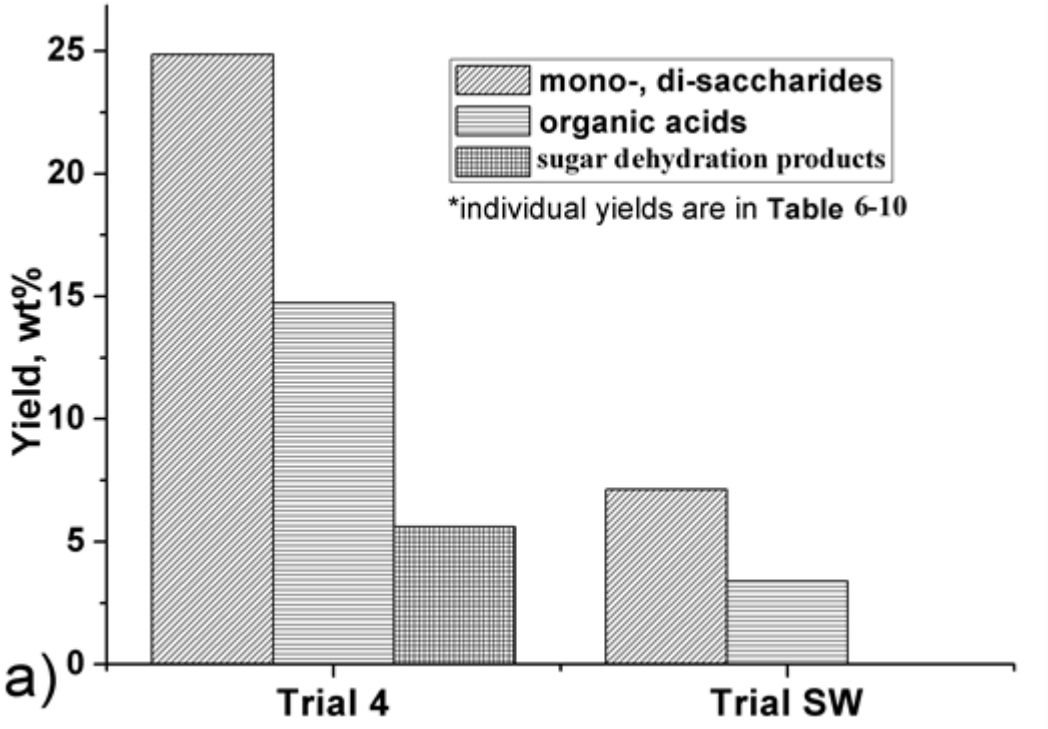


Figure 4-4 Absorbance of hydrolysate at 530 nm of UV−vis spectroscopy of different trials.

4.9 Chemical content determination *via* HPLC analysis

Table 6-10 and Figure 4-5 show the concentrations and yields of mono- and disaccharide, organic acids, and sugar dehydration products compounds in the hydrolysate. Of note, the repeated runs Trials 9r−12r were very consistent, showing good reproducibility. Table 6-10 indicates that the yields were much higher in 1% solid content trials than in 10% trials, in keeping with previous observations. However, higher water content results in lower concentrations, perhaps making it less attractive in further applications. The yield and concentration increased with higher temperatures and extended holding times during hydrolysis. Overall, Trial 4 resulted in the highest yields (also the highest conversion as shown in Table 4-5) for the individual compounds analysed by HPLC- with rhamnose

(10.8 wt %), acetic acid (7.5 wt %), formic acid (6.4 wt %), and levoglucosenone (5.4 wt %) yields being particularly high. As a contrast, the yields were much lower in Trial SW, with glucose (2.3 wt %), xylose (2.1 wt %), formic acid (1.3 wt %), and acetic acid (1.2 wt %) as high-yield compounds. Notably, a 30 min microwave hydrolysis could extract 24.9% of saccharides from DBP, more than 3 times that in Soxhlet extraction for 24 h (7.1%). Fan has pointed out that polysaccharides can be depolymerised *via* microwave-assisted hydrolysis using just water,[87] which can explain the high mono-/disaccharide yields obtained in this research.



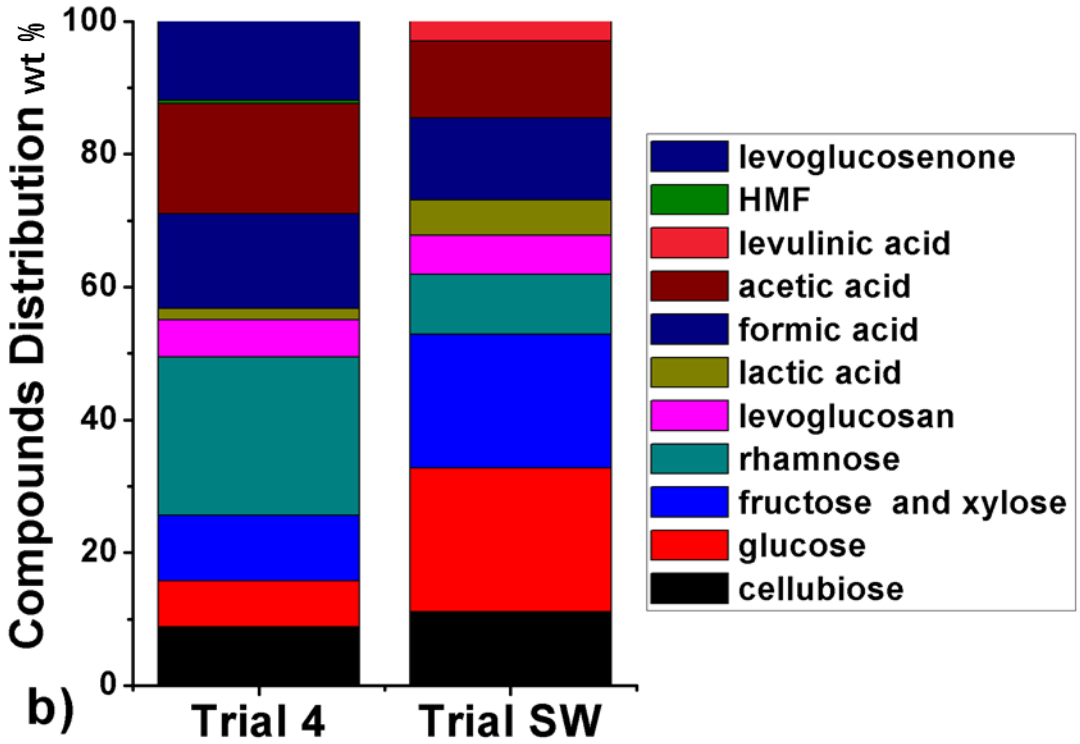


Figure 4-5 Compounds yield and distribution of hydrolysate. Trial 4 condition: microwave conversion, 140 ºC, 30 minutes, 1 % solid ratio. Trial SW condition: Soxhlet extraction, water as solvent, 24 hours, 2.4 % solid ratio. The 'Sugar degradation products' refers to the combination of levoglucosenone, HMF and levoglucosan. Although the degradation of sugar could also produce acids, here all the acids products are categorised as organic acid in Figure 4-5a

Of note, the yield of rhamnose was much higher in all microwave trials (4.7−10.8%) than Trial SW (1.0 wt %). This was perhaps due to the degradation of pectin, as rhamnose is in the backbone of some types of pectin, including rhamnogalacturonan I pectin (RG-I) and rhamnogalacturonan II pectin (RG-II). Compared with conventional heating, microwave hydrolysis appeared to cause more degradation of pectin, resulting in an extremely high yield of rhamnose. Rhamnose is a monosugar with a sweet taste but cannot (or only can partly) be metabolised by humans;[249] thus, it can be hopefully used as a low-

calorie sweetener. Rhamnose is also used in a widely accepted clinical test for the determination of intestinal permeability.[250] Therefore, rhamnose, considering the high yield, appears to be the most useful and profitable extractive in microwave processing of bilberry presscake in this research.

In addition to saccharides, the yields of organic acids and sugar dehydration products (including HMF, levoglucosenone and so on) in Trial 4 were both higher than those of Trial SW. In Trial 4, the high yields of formic acid and acetic acid are 6.42% and 7.52%, respectively. This is consistent with FTIR analysis that indicated microwave hydrolysis could extract more organic acids than conventional thermal treatment. In addition to the organic acids that already exist in feedstock, the degradation of monosaccharides can also result in the production of organic acid.[87] The high yield of organic acids in turn increases sugar dehydration products yields. This is because sugar dehydration products, such as HMF, are formed in acidic environments *via* dehydration of saccharides. Compared with microwave trials, the concentration of sugar dehydration products in the hydrolysate of Trial SW was much lower. It is worth noting that the yield of leveglucosenone is much higher than HMF in Trial 8, suggesting the conditions in Trial 8 are able to promote the dehydration of glucose to form leveglucosenone, but not able to achieve the conversion of leveglucosenone and glucose to HMF effectively. (Figure 4-6)

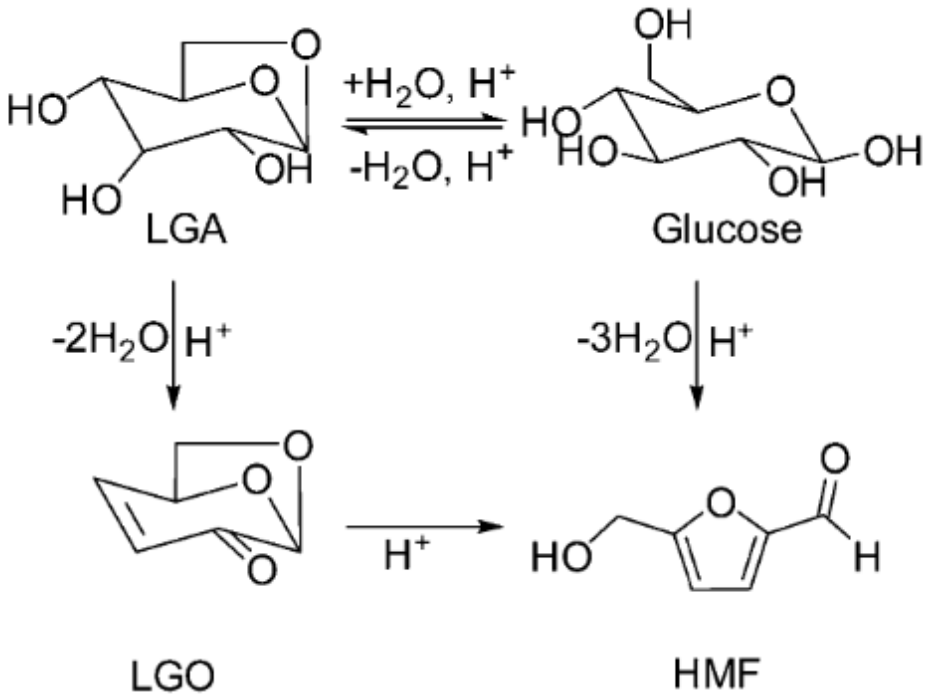


Figure 4-6 The conversion of glucose to leveglucosan (LGA), levoglucosenone (LGO) and hydroxymethylfurfural (HMF)[251]

4.10 Liquid Phase ^{13}C NMR Spectroscopy of Hydrolysate

Figure 4-7 shows the liquid ^{13}C NMR spectrum of the hydrolysate. The major resonances between 60 and 105 ppm are attributed to C−O bonds, indicating saccharides were the major compounds in the hydrolysate, for example the C2, C3, C4, C5, C6 (60−80 ppm), and C1 (90− 105 ppm) carbons of glucose.[252–254] The peaks between 35 a1nd 45 ppm are likely to be attributed to carbon bound to nitrogen, which is consistent with elemental analysis, Table 4-6, which indicated DBP contained nitrogen (2.03%). This in turn suggests DBP contains nearly 13% protein using a 6.25 nitrogen to protein conversion factor.[255,256] The ^{13}C NMR spectrum result suggested these proteins could potentially be extracted directly or extracted after degradation from the feedstock *via* microwave hydrolysis

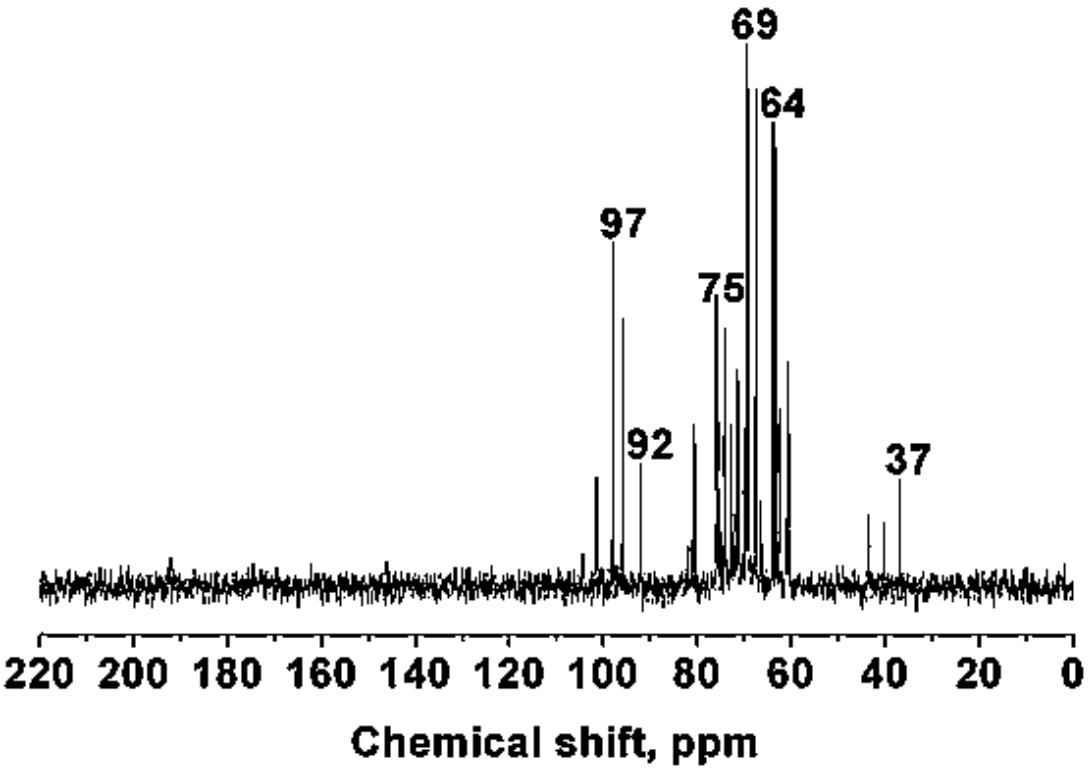


Figure 4-7 ^{13}C NMR spectrum of hydrolysate (Trial 8, deuterated water as solvent).

According to HPLC results, formic acid and acetic acid were the two main products in the liquid phase; however the peaks of carboxyl groups (appears between 160 and 180 ppm) were missing in the spectrum. This is perhaps because these acids (bp 100−101 °C formic acid, 117−118 °C acetic acid) were removed via volatilisation in rotary evaporator when preparing the NMR spectroscopy samples.

4.11 The four-stage conversion of bilberry presscake

Based on the systematic analyses above, a possible progress during microwave hydrolysis is presented in Figure 4-8. Depending on the hydrolysis conditions, microwave processing of bilberry presscake can be roughly divided into four stages as follows.

In stage I, the outer portions of the fruit, which contain abundant mineral elements and anthocyanins, are most easily solubilised, and inorganic salts and pigments are extracted

first. Due to the existence of anthocyanins and the weak acidic environment of the hydrolysate, the solution at this stage is clear and purple. Most organic compounds are still contained within the residue.

In stage II, the solid residue from stage I, releases mono-/ disaccharides, pectin, and protein into the solution. Monosaccharides (especially glucose and xylose as suggested in the literature[212,214]) are the main extractives as the results of degradation of polysaccharides. The mass of residue is relatively stable (40−60 wt % of original mass). Decomposition of anthocyanins already in the hydrolysate results in the aqueous phase darkening and becoming cloudy.

In stage III, rhamnose is released as a product from pectin degradation (Figure 4-9). Rhamnose yield increases significantly in this stage, probably making it the most attractive products in microwave hydrolysis. Rhamnose can be potentially applied as a sweetener,[257] determining intestinal permeability,[258] and in home and personal care.[259] It can be hardly metabolised by human, thus making it a low-calorie sweetener for food industries. Conventionally, rhamnose is recovered by the hydrolysis of rhamnose-containing glycosides. This process has several disadvantages including the labor intensity, the huge amount of toxic aromatic by-products and the toxic solvents needed for the rhamnose separation.[260] This research provides a novel and green approach for rhamnose production from microwave hydrolysis of bilberry presscake. After hydrolysis, rhamnose can be potentially separated from the mixture by membrane filtration or extraction. As shown in Figure 4-8, by controlling the hydrolysis conditions (temperature, solid ratio and holding time) in Stage III, a high rhamnose yield can be expected from

hydrolysis, which is in favor of the downstream separation and applications, However, more experimental work is needed to optimise the recovery of rhamnose from hydrolysate mixture.

In stage III, saccharides degrade to form organic acids, also giving rise to the formation of sugar dehydration products. Nitrogen containing compounds, presumably as a result of proteolysis, are detected in the hydrolysate.

In stage IV, the insoluble residue (~40% of original mass, mainly cellulose and lignin) theoretically decomposes under more intensive conditions (not investigated in this research), and the conversion of saccharides into organic acids and sugars continues.

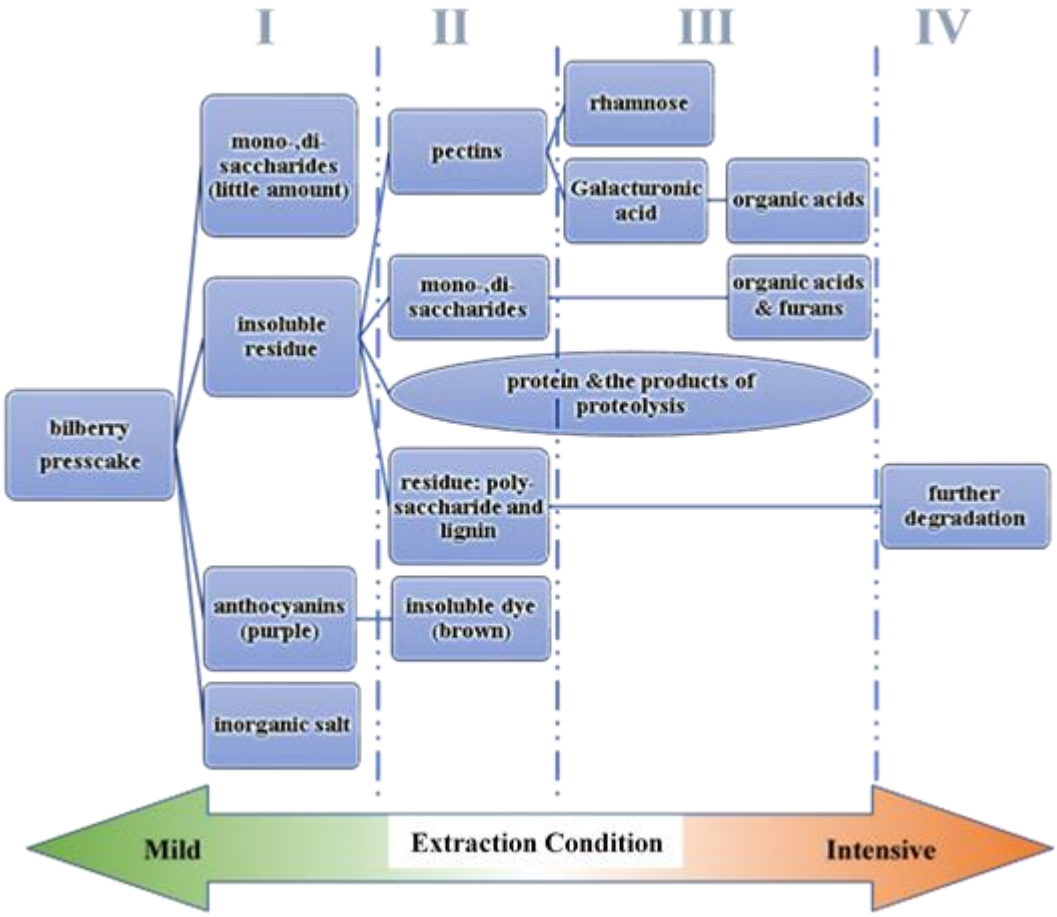


Figure 4-8 Bilberry Presscake Conversion Progress (The extraction condition is a combination of temperature, holding time and solid ratio, among those the solid ratio has a most significant influence to hydrolysis as shown in condition optimisation section. Intensive conditions refer to low solid ratio, high temperature and long holding time)

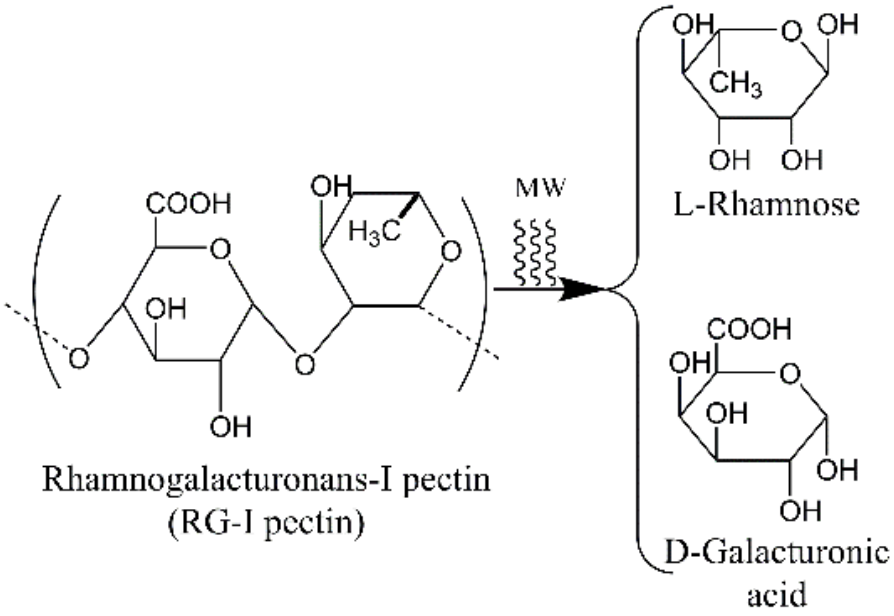


Figure 4-9 Degradation of Pectin

4.12 Higher Volume Processing of Bilberry Presscake Using Microwave

It is worth noting the "hydrolysis conditions" in Figure 4-8 are a combination of solid content, temperature, and time, among which solid content has the most significant influence according to condition optimisation. This is because, in lab scale trials, DPB and water were simply loaded into microwave without any pre-treatment and with a large particle size (10 mm), resulting in insufficient mixing of solvent and feedstock. However, in the higher volume (pilot scale) processing trials, pre-treatments were carried out to give a pumpable slurry with a maximum particle size of 4 mm, also ensuring a more homogeneous mixture. In each trial, 5 kg of bilberry presscake (the equivalent of 1.5 kg of dried bilberry presscake per run) was defrosted, macerated in a Robot Coupe Blixer 4vv instrument, and mixed with a total of 12 L of deionised water to form a slurry with a

maximum particle size of 4 mm diameter (chosen due to the solids handling capability of the pump). The slurry was charged in to the microwave and then recirculated for 10 minuts, at 230 L min^{-1} to ensure a homogeneous mixture. The trials were carried out in triplicate. Microwave power was applied, initially at 1 kW for microwave leakage tests to be carried out and then, following safety testing, at 6 kW until the target temperature of 95 °C was reached. The bilberry presscake/water slurry was recirculated at 95 °C for 60 min. Then, 400 mL samples were taken at 30 and 60 min time points from the three trials (Trials P1-30, P1-60, P2-30, P2-60, P3-30, and P4-60, respectively). The flow diagram of this higher volume processing is illustrated in Figure 4-10. Notably, the higher volume processing of bilberry presscake is not scaling up the lab-scale experiments using parallel conditions. Due to the equipment restrictions, the temperature used in pilot trials cannot exceed 100 ºC. Thus, to ensure extraction and conversion efficiency under this situation, sufficient mixing was carried out as stated above. With sufficient mixing, high conversion is hopefully achievable at relatively low temperatures (<100 °C) which will reduce capital expenditure (CapEx) in terms of equipment due to lower heat and pressure tolerances. The conversions are presented in Table 6-9. The average conversion was 57.6 % after 30 min of microwave processing at 95 °C; this then increased slightly (but not significantly) to 59.5 % after 60 min which indicates that longer hold times may not be advantageous. Because of the use of pre-treatment, milder conditions gave higher conversions for higher volume processing trials as compared to those of lab scale trials (Tables 4-1 and Table 4-5).

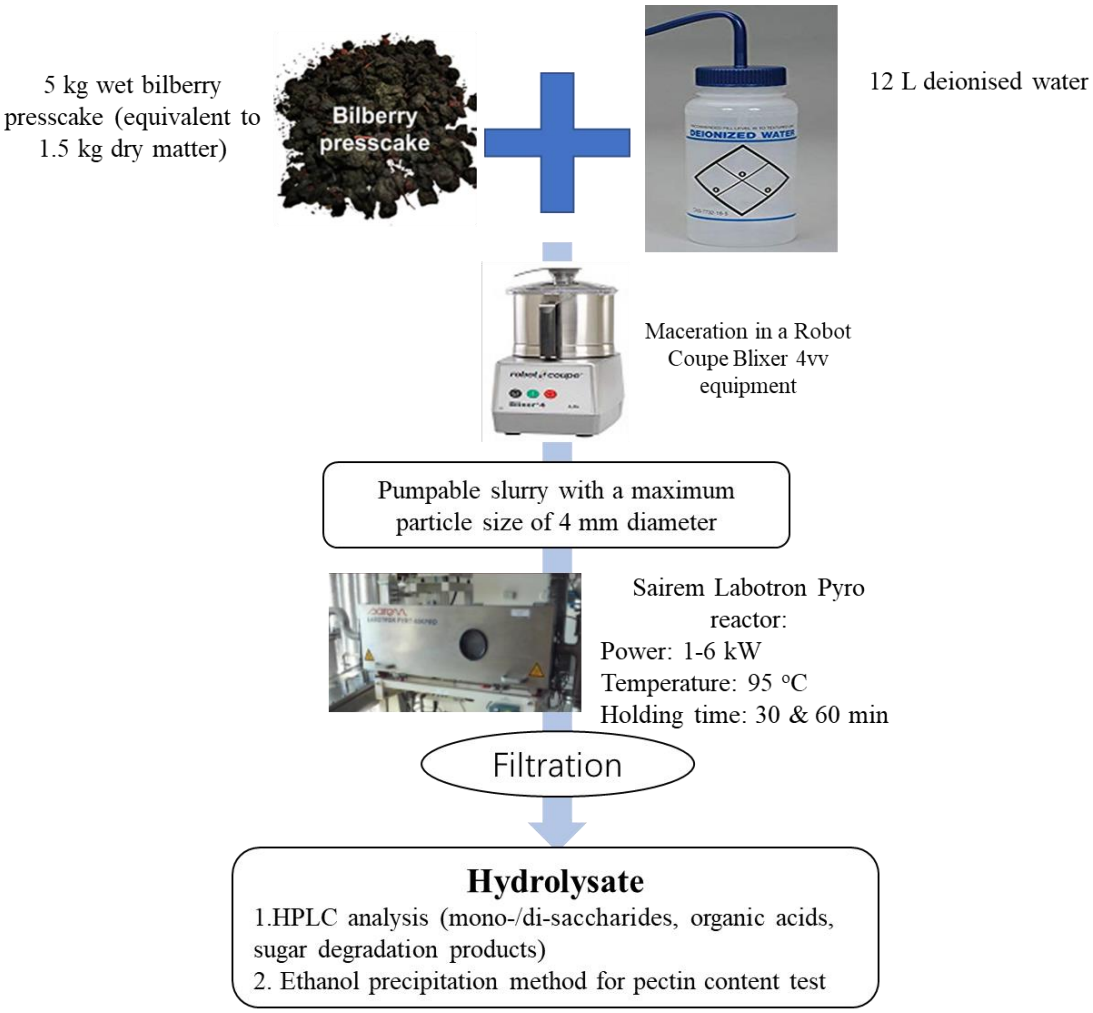


Figure 4-10 The flow diagram of higher volume (pilot trial) microwave processing of bilberry presscake

The HPLC results are presented in Table 6-11, and a comparison with lab-scale trial (Trial 4) is shown in Figure 4-11, where it is easy to see that, for higher volume processing, the yields of rhamnose, organic acids, and sugar dehydration products are lower. According to Figure 4-8, this indicates Trial 4 is at stage III, whereas the pilot scale trial is at stage II, i.e., with little degradation of pectin or monosaccharides. This is probably due to the lower temperature applied. This is confirmed by the pectin yield in Trial P2-60 being 6.3%. This high yield suggests pectin is perhaps one of main extractives in addition to monosaccharides, especially when low temperature is used.

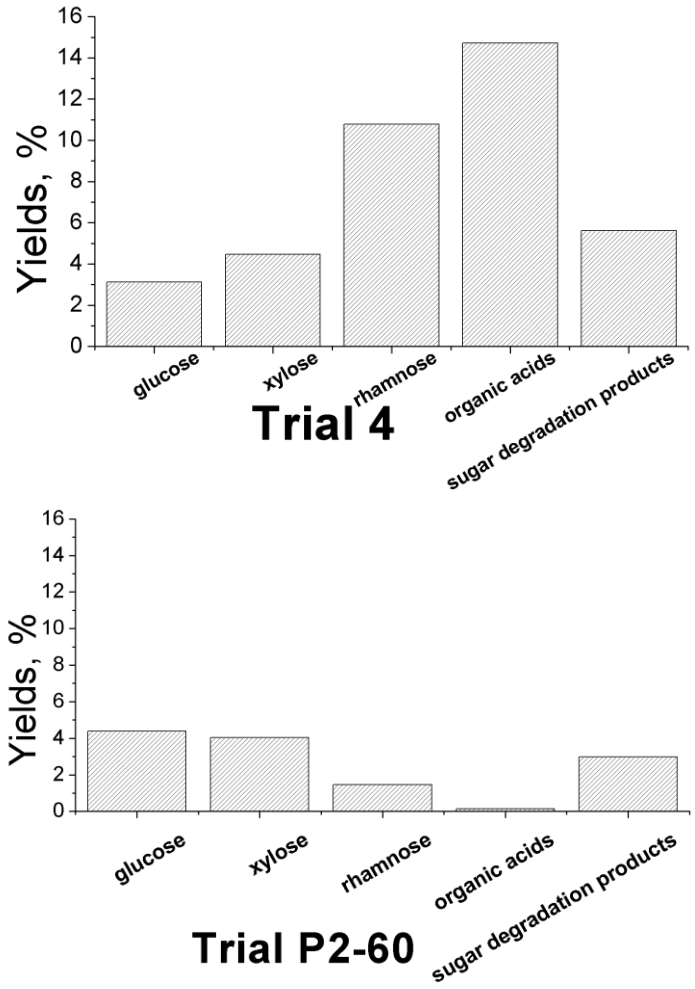


Figure 4-11 Chemical yields, lab scale (top) vs pilot scale (bottom) trials.

4.13 Conclusion

Microwave hydrolysis offers a powerful tool for chemicals recovery from bilberry presscake. Unlike conventional methods that used organic solvent, in this research water is used in microwave hydrolysis to ensure all extracts are suitable for food grade status applications. Using a Box−Behnken design, a quadratic regression equation with high accuracy was made to predict the conversions. Among the three factors (temperature, time, solid content), solids content has the most significant influence on conversion. Within the condition ranges of this research, the highest theoretical conversion obtainable is 55.2 %.

Microwave hydrolysis shows significant advantages compared with conventional extraction (Soxhlet extraction). A 30 minutes microwave hydrolysis could achieve high conversion of 44.2%, where for a 24 hours Soxhlet extraction only 30.5% was obtained. This indicates a clear advantage in efficiency for microwave thermal hydrolysis. Microwave hydrolysis is effective in extracting mono-/disaccharides (the highest yield of 24.9%), with rhamnose yield being particularly high (10.7%), which is perhaps caused by the degradation of pectin on microwave irradiation. Thus, rhamnose, considering its high yield, appears to be the most attractive and profitable extractive in microwave processing of bilberry presscake, with potential applications as a sweetener,[257] determining intestinal permeability,[258] and in home and personal care.[259]

Based on systematic analysis of lab scale experiments, for the first time a scheme is proposed to divide the conversion progress into four stages depending on conditions from mild to intensive. For stages I−III, the main extractives are stage I, anthocyanins, inorganic salts; stage II, mono-/disaccharides, pectin; and stage III, rhamnose, organic acids, and sugar dehydration products. The residue (40−50 wt % of original mass) of stages II and III contains abundant polysaccharides/cellulose and lignin that hopefully can be degraded with further treatment (stage IV). Compounds containing nitrogen are detected in the extractives, which are very likely to be protein and its degradable products. The scheme suggests that, with further optimisation, including multistep hydrolysis, it is possible to isolate fractions rich in various value-added products from bilberry presscake (Figure 4-12): monosaccharides can be used as food additives; pectin is a type of soluble fibre that has multiple healthy function to human body, like improving intestinal

microflora balance; anthocyanins has the function of antioxidant, vision improvement and anti-cancer; mineral salts are also the essential compounds for human. As water is the sole solvent in this research, the products hopefully meet the requirements of food-grade applications.

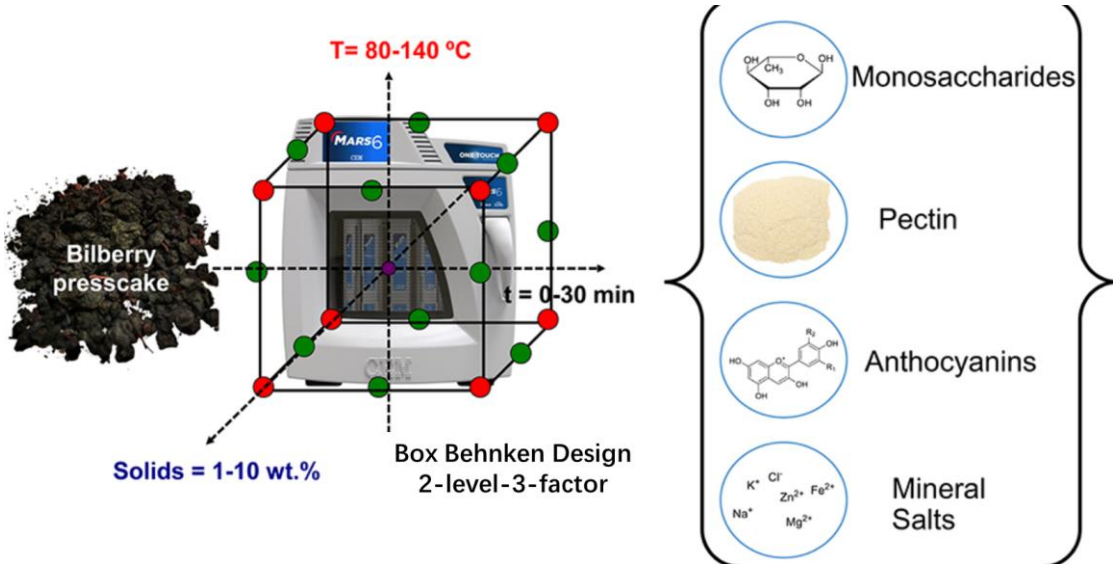


Figure 4-12 The content summary of Chapter Four: natural products recovery from bilberry presscake *via* microwave treatment

Pilot scale microwave conversions were carried out at low temperature (95 °C) due to a modified pyrolysis microwave being employed which has to operate at atmospheric pressure. Due to the pre-treatments, higher conversions are achieved than those of lab scale trials, with major products (average yield over 3 runs) of glucose (3.8%), xylose (3.9%), and pectin (6.3%). The yield of rhamnose is lower than lab scale conversion due to the low temperature unable to decompose pectin.

Greater correlation between lab and pilot scale work is required in future research; this includes a detailed investigation into pretreatment with regard to the effect of drying and

a 10 mm particle size as compared to wet maceration and a 4 mm particle size on conversion and selectivity. Additionally, large scale microwave hydrolysis at higher temperature and pressure is needed to provide like for like comparisons.

Conclusion and future work

Chapter 5

5.1 Conclusion

Valorisation of biomass has become increasingly important as a promising and sustainable solution to the issue of depletion and overexploitation of natural resources.[261,262] Conversion of abundant lignocellulosic biomass into biofuels and high value-added chemicals presents a viable option to improving energy security and reducing green gas emission.[263] Lignocellulosic biomass, such as wood, agricultural or forest residue, is mainly composed of cellulose, hemicellulose and lignin.[264] For a long time, researches have been carried out to enhance the effective conversion of cellulose and hemicellulose to ethanol,[265,266] methane[267,268] or even hydrogen[269,270], which offers a sustainable solution to the energy crisis nowadays. Lignin is also one of the most abundant polymers in nature. However, due to its inactive nature, complex 3D structure and recalcitrance to thermal treatment, lignin is greatly undervalued to the extent that most commercial lignin (almost 99%) is consumed as internal energy source in pulping and biorefinery process itself, thereby recovering energy.[26,271,272] Another important reason accounting for its undervaluation is the lack of proper method to separate lignin from biomass. Firstly, commercial lignin protocol is accompanied with severe saccharide contamination and structure alteration that hinder its further application. Lab-based methods could result in better quality lignin, but the tedious protocol and strict conditions make these methods hard to scale up for mass production. Furthermore, none of the existing methods could achieve high yield/purity and little structure modification at same time. Of note, almost all conventional methods for biomass utilisation are single-target processes that only focus on a certain fraction of biomass, which is a main reason

accounting for the poor quality of commercial lignin, because of cellulose being the only target product in pulping process and leading to less concern on lignin quality. All these problems call for new approaches for lignin isolation. Microwave thermal treatment is characterised by high efficiency, selective and homogeneous heating, drawing an increasing attention in solid waste and biomass valorisation. Thus, in this research, a novel and efficient method of microwave-assisted hydrolysis has been developed to produce highly pure lignin with little structure modification, meanwhile to use the saccharide fraction in fermentation, thus achieving a zero waste valorisation of biomass. (Figure 5-1)

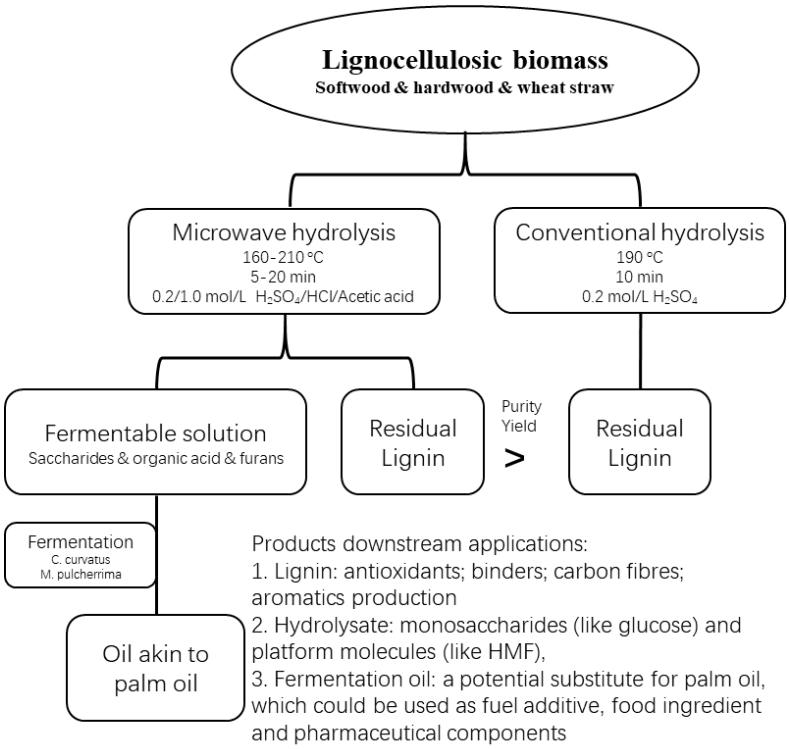


Figure 5-1 The valorisation of lignocellulosic biomass *via* microwave hydrolysis

Fast microwave-assisted acidolysis is used to process three types of biomass, mixed softwood pellet, willow edging and wheat straw, among which softwood produces lignin with the highest yield (82 wt%) and purity (93 wt%) at optimum condition for only 10

min, showing significant advantage compared with classic lignin isolation protocols. Acidolysis using conventional heating is performed at equivalent conditions but results in lignin with lower yield and purity. A new method for investigation of lignin structure modification is proposed using a coefficient of determination (R^2) of the trend line between feedstock and residual lignin based on pyrolysis gas-chromatography. The result indicates that the residual lignin from microwave acidolysis can basically keep the original structure to native lignin in biomass except some modification at side-chains. Compared with residual lignin achieved *via* conventional heating, microwave isolated lignin contains more aromatic fraction.

Systematic analyses suggest the processed residue contains mainly lignin and most saccharides have been removed into hydrolysate. Thus, fermentation using this hydrolysate has been carried out with two types of yeast, *Cryptococcus curvatus* and *Metschnikowia pulcherrima*, both of which have high tolerance for furfural inhibitors and acidic environment. Both yeasts could grow on the saccharide feedstock and metabolise a range of acids, furans, sugars and oligosaccharides, although at high loading of inhibitors the yeasts reduce somehow. The fermentation could produce an oil akin to palm oil.

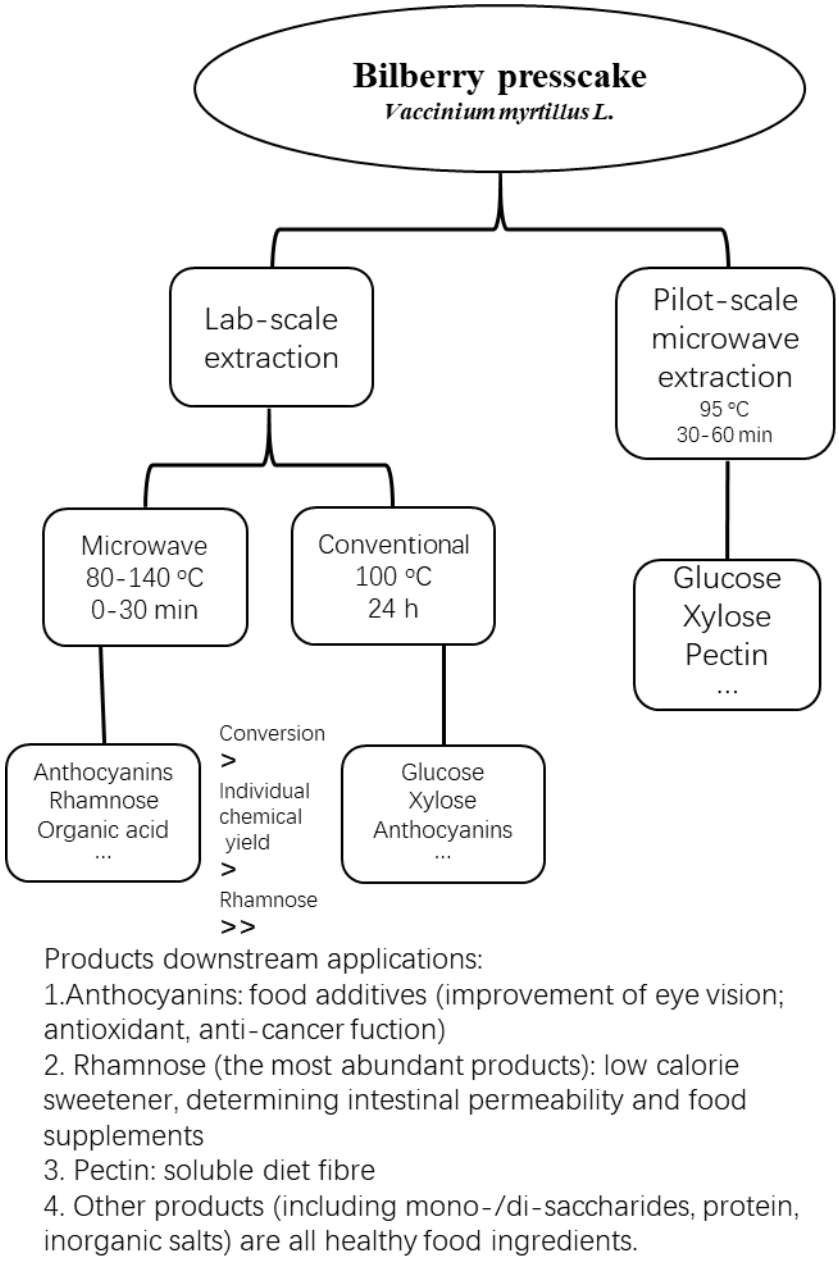


Figure 5-2 Natural products recovery from bilberry presscake *via* microwave hydrolysis

As microwave hydrolysis has shown a great ability of degradation and solubilisation of polysaccharide, another microwave application is investigated about the natural products recovery from bilberry presscake *via* microwave hydrolysis (Figure 5-2). Unlike conventional methods that used organic solvent, in this research water is used in microwave hydrolysis to ensure all extracts are suitable for food grade status applications. A set of experiments are carried out based on a Box-Benhken design and the results

suggest among all three parameters, holding time, temperature and solid content ratio, the last one has the most significant influence on product yields. By comparison, microwave conversion shows remarkable advantages compared with conventional extraction in terms of holding time, total convention ratio and individual saccharides yields. The abundant yield of rhamnose (10.7 wt% at the optimum condition) suggests rhamnose might be the most prospective product from bilberry presscake hydrolysis, which can be widely used as low calorie sweetener, determining intestinal permeability and food supplements. A hypothesis is proposed to divide the hydrolysis into four stages depending on the condition from mild to intensive. The extractable products of each stage vary from each other, which could help to design a multiple-step protocol to extract various chemicals from bilberry presscake in the future. Pilot-scale microwave hydrolysis is also carried out in addition to lab-scale conversion. Because of the pre-treatment, higher conversion ratio is achieved at lower temperature than lab-scale conversion. Pectin (6.3 wt%), xylose (3.9 wt%) and glucose (3.8 wt%) are the main products of pilot-scale conversion.

5.2 Future works

The future works will be focused on two topics: 1) Degradation of microwave isolated residual lignin (MRL). This thesis presents a new effective method for lignin isolation. However, the characteristics of lignin is greatly affected by isolation protocol, indicating more downstream work is needed to investigate the conversion of MRL into low molecular weight chemicals. Preliminary experiments (not included in this thesis) have been done, which suggest 95 wt% of MRL could be degraded and solubilised in alkaline

environment in the presence of an oxidant *via* microwave hydrolysis to produce several types of monophenols. But more condition optimisation and systematic investigation is required; 2) Multiple-step protocol design for various products recovery from bilberry presscake. In Chapter 4, the saccharide products are mainly focused, as plenty research has done about anthocyanins recovery while the recovery of saccharides for food status application is still blank. The results of Chapter 4 suggest anthocyanins and saccharides cannot be recovered with single step due to the degradation of anthocyanins at high temperature. Thus, a multiple-step protocol will solve this issue. Furthermore, in addition to anthocyanins and saccharides, more works need to be done focusing the content of mineral salts and protein in the extracts.

5.3 Outcomes of this work

5.3.1 Conferences and presentations

- Review: Isolation of lignin from biomass & lignin hydrolysis, Green Chemistry Centre of Excellence (GCCE), Department of Chemistry, University of York, 2/2015
- A fast lignin isolation method using microwave radiation, Green Chemistry Centre of Excellence (GCCE), Department of Chemistry, University of York, 8/2015 & 5/2016
- Faraday Discussions: Bio-resources: Feeding a sustainable chemical industry. 6/2017, London. Paper submitted and oral presentation made by Dr Vitaliy Budarin:

Zhou, L., Santomauro, F., Fan, J., Macquarrie, D., Clark, J., Chuck, C.J. and Budarin, V., 2017. Fast microwave-assisted acidolysis: a new biorefinery approach for the zero-waste utilisation of lignocellulosic biomass to produce high quality lignin and fermentable saccharides. Faraday discussions, 202, pp.351-370.

- A novel microwave-assisted method of lignin isolation. A poster presentation at the KMS Award Seminar, Department of Chemistry, University of York, 10/2015.

5.3.2 Publications

- Zhou, L., Budarin, V., Fan, J., Sloan, R. and Macquarrie, D., 2017. Efficient Method of Lignin Isolation Using Microwave-Assisted Acidolysis and Characterization of the Residual Lignin. ACS Sustainable Chemistry & Engineering, 5(5), pp.3768-3774.
- Zhou, L., Santomauro, F., Fan, J., Macquarrie, D., Clark, J., Chuck, C.J. and Budarin, V., 2017. Fast microwave-assisted acidolysis: a new biorefinery approach for the zero-waste utilisation of lignocellulosic biomass to produce high quality lignin and fermentable saccharides. Faraday discussions, 202, pp.351-370.
- Zhou, L., Lie, Y., Briers, H., Fan, J., Remón, J., Nyström, J., Budarin, V.L., Macquarrie, D.J. and McElroy, C.R., 2018. Natural product recovery from bilberry (Vaccinium myrtillus L.) presscake via microwave hydrolysis. ACS

Sustainable Chemistry & Engineering.

Paper in preparation

- Saccharides recovery from softwood *via* microwave hydrolysis using sulfuric acid and formic acid

Paper published during PhD period but work not included in this thesis:

- Javier Remon Nunez, Long Zhou, Jiajun Fan, Duncan Macquarrie, Vitaliy Budarin and James Clark, Production of high purity lignin from rapeseeds meal using a microwave-assisted hydrothermal process, Papers of the 25th European Biomass Conference: Setting the course for a biobased economy (2017)

Experimental and Data

Chapter 6

6.1 Chapter 2

6.1.1 Materials

Mixed softwood pellets (MSP, UK Biochar Research Centre, School of Geosciences, University of Edinburgh) were used as feedstock for lignin isolation. The elemental and ICP analyses are shown in Table 6-1 and 6-2. Compared with hardwood and herbaceous biomass, softwood has the least acid-soluble lignin, only about 0.2−0.5 wt%,[44] and thus is the most suitable for acidolysis lignin isolation. Sulfuric acid was purchased from Fischer Chemicals (>95 wt%). Creosol (99 wt%), vanillin (99 wt%), and 2-methoxy-phenol (98 wt%) were purchased from Sigma-Aldrich. trans-Isoeugenol (99 wt%) was purchased from Acros Organic.

Table 6-1 Proximate and ultimate analysis of MSP (wt%)

	Moisture	Dry-basis content					
		Ash	C	H	N	S	O[a]
MSP	6.5	1.1	51.8	6.4	<0.1	1.3	39.4

a. Oxygen=100-Ash-C-H-N-S

Analysis of Carbon, Hydrogen & Nitrogen (CHN) is carried out on an Exeter Analytical Inc. CE-440 analyser. The results is achieved from Microanalysis service from Chemistry Department, University of York

Table 6-2 ICP analysis of MSP (wt%)

Elements	Content/10^{-2}wt%
Na	17.0
Mg	1.4
Al	0.6
Si	10.5
P	1.2
K	7.8
Ca	18.2
Fe	0.5
Co	<0.0
Ni	<0.0
Cu	0.1
Zn	0.3

The results were achieved from ICP-MS service (GCCE) with the help from Dr. Andrea Muñoz García for running the sample test.

6.1.2 Experimental methods and Data

6.1.2.1 Experiment design

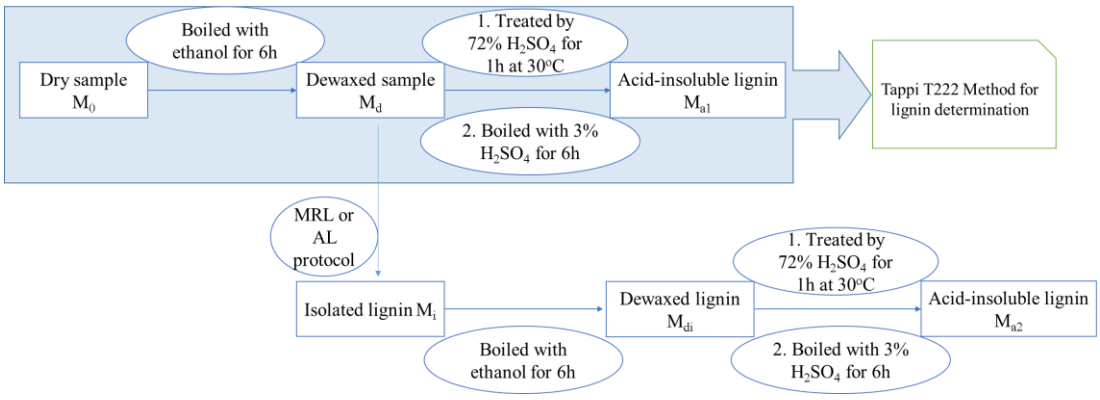
Experimental Methods. All biomass was milled to 60 mesh powders using a cutting mill (Retsch SM300, Germany) in Biorenewables Development Center (BDC), University of York. The microwave treatment was performed in a Discovery SP microwave reactor (CEM Corporation, USA) in capped vessels. Maximum power (300 W) of the microwave reactor was applied in all the experiments to make sure that the holding temperature could be achieved as quickly as possible. Diluted sulfuric acid (0.2 M) was applied for isolation. The processing temperature of 160−210 °C at intervals of 10 °C was used for isolation. The holding time was 5/10/20 min (in this paper, the abbreviation microwave residual lignin (MRL) only refers to the 10 min sample). During microwave treatment, 0.2 g of MSP and 15 mL of acid solvent were heated in a capped vessel with stirring. After microwave treatment, the residue was recovered by filtration. Then, the residue was washed several times with deionized water until the rinsed water was neutral. In order to prepare the microwave residual lignin obtained in this way for further analysis, the residue was dried (105 °C, 24 h) and then weighed. All the experiments were repeated 3 times.

Lignin isolation by conventional heating (acidolysis lignin, AL) was performed using a benchtop autoclave (Anton Paar Monowave 50). MSP (0.08 g) and aqueous sulfuric acid (0.2 M, 6 mL) were heated with stirring in a sealed vessel. The temperature was ramped up to 190 °C (within 5 min, similar to microwave experiments) and was held for 10 min. The residue (190 °C AL) after isolation was washed and dried as in the microwave residual lignin preparation. By comparing AL and MRL, the characteristics and

advantages of microwave treatment can be investigated. The conversion ratios of conventional hydrolysis and microwave hydrolysis are given in Figure 2-1.

6.1.2.2 Purity and yield calculation

The purity and yield was calculated by TAPPI T222 method.[44] The method is shown schematically in Figure 6-1. About 0.1 g of dewaxed sample was treated with 10 g of sulfuric acid (72 wt %) at 20 °C for 2 h. The solution was then diluted with deionized water to 3 wt % sulfuric acid and refluxed for 4h. The insoluble residue (lignin) was isolated by filtration. After washed with hot water, the residue was dried at 105 °C for 24 h. This dried residue is Klason lignin (KL). The purity and yield were calculated according to the equation in Table 2-1. The purity result was adjusted by subtracting the ash content measured by TG analysis.



$$lignin\ content_{dry-basis}=\frac{M_{a1}}{M_0}$$

$$lignin\ content_{extractive-free-basis}=\frac{M_{a1}}{M_d}$$

$$purity\ of\ lignin\ isolated = \frac{M_{a2}}{M_{di}}$$

$$lignin\ yield = \frac{M_{a2}}{M_{a1}}$$

Figure 6-1 Tappi T222 method for purity and yield calculation,

6.1.2.3 Elemental analysis

Elemental analysis and ICP analysis data were obtained from the analytical service offered by Department of Chemistry, University of York. The results are given in Table 6-1 and Table 6-2.

6.1.2.4 Thermal gravimetric analysis

Thermogravimetric (TG) analysis was performed using a Netzsch STA 409 analyzer (Germany). The following parameters were applied: temperature ramp rate 20 ºC/min, final temperature 600 °C, and carrier gas 50 mL/min pure nitrogen gas. To measure ash content, the following parameters were applied: temperature ramp rate 20 ºC /min, final temperature 625 °C holding for 1 h (or less providing a stable final mass is achieved), and carrier gas 50 mL/min N_2 and 100 mL/min O_2. The final mass % was used as the ash content. The data are given in Figure 2-4 and Figure 6-2 (unpresented data in Chapter 2).

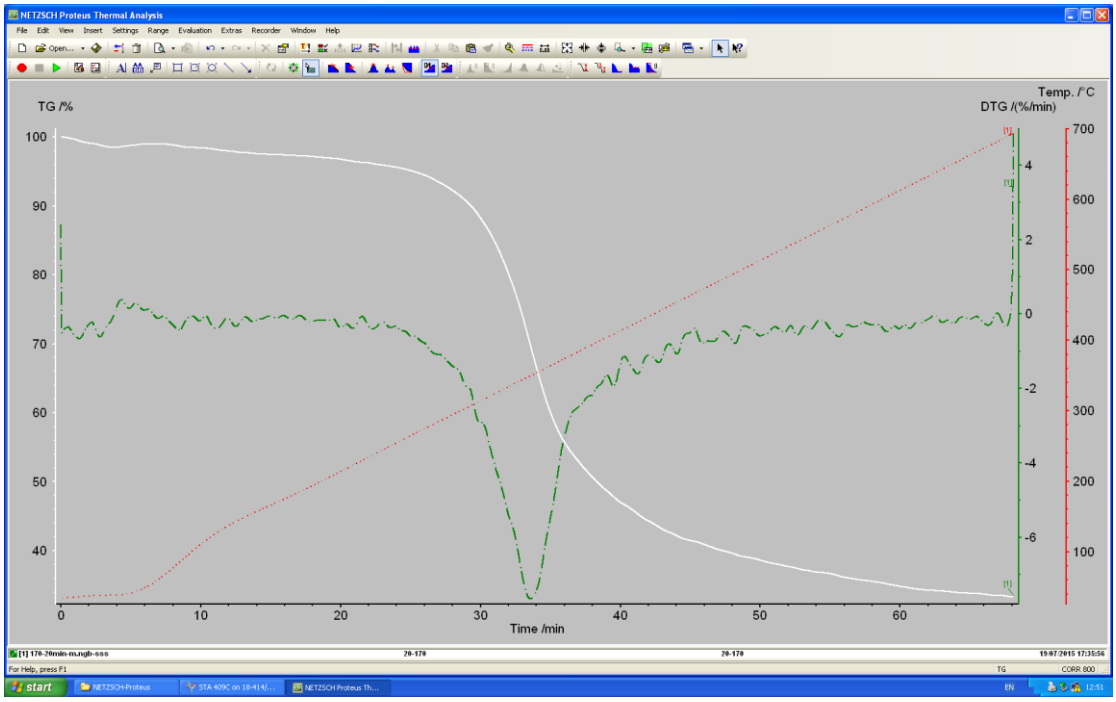


Figure 6-2a) TG curves of residual lignin, isolation condition: 170 ºC, 20min, 0.2 M sulfuric acid, 0.2 g feedstock in 15 ml solution. (white curve is TG curve, green curve is

DTG curve, red curve is temperature)

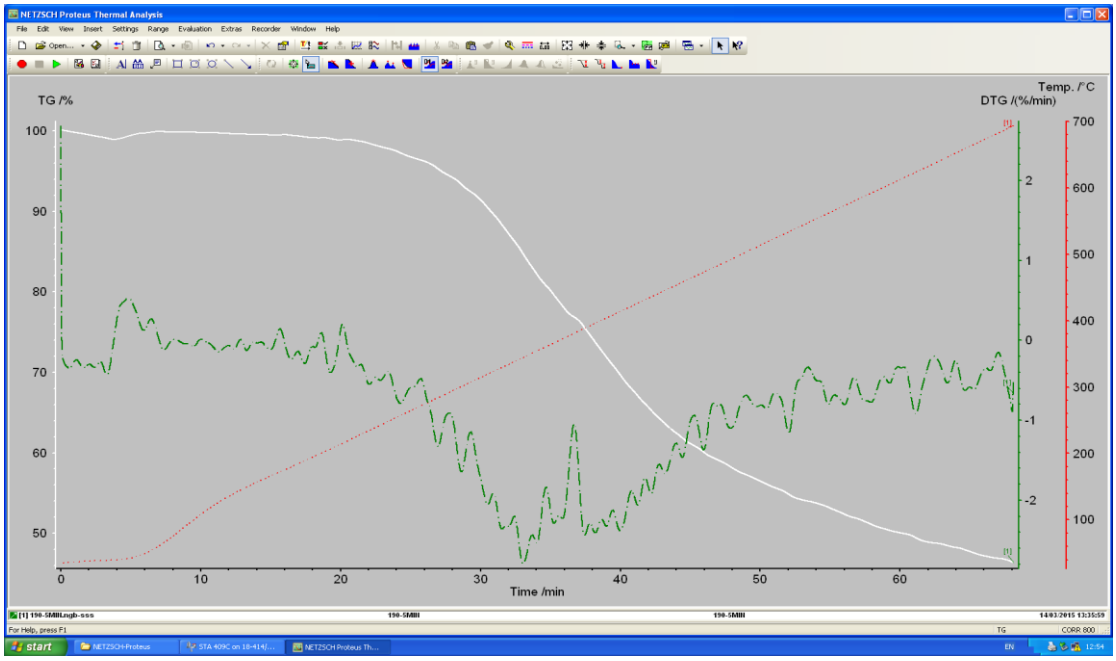


Figure 6-2b) TG curves of residual lignin, isolation condition: 190 ºC, 5min, 0.2 M sulfuric acid, 0.2 g feedstock in 15 ml solution. (white curve is TG curve, green curve is DTG curve, red curve is temperature)

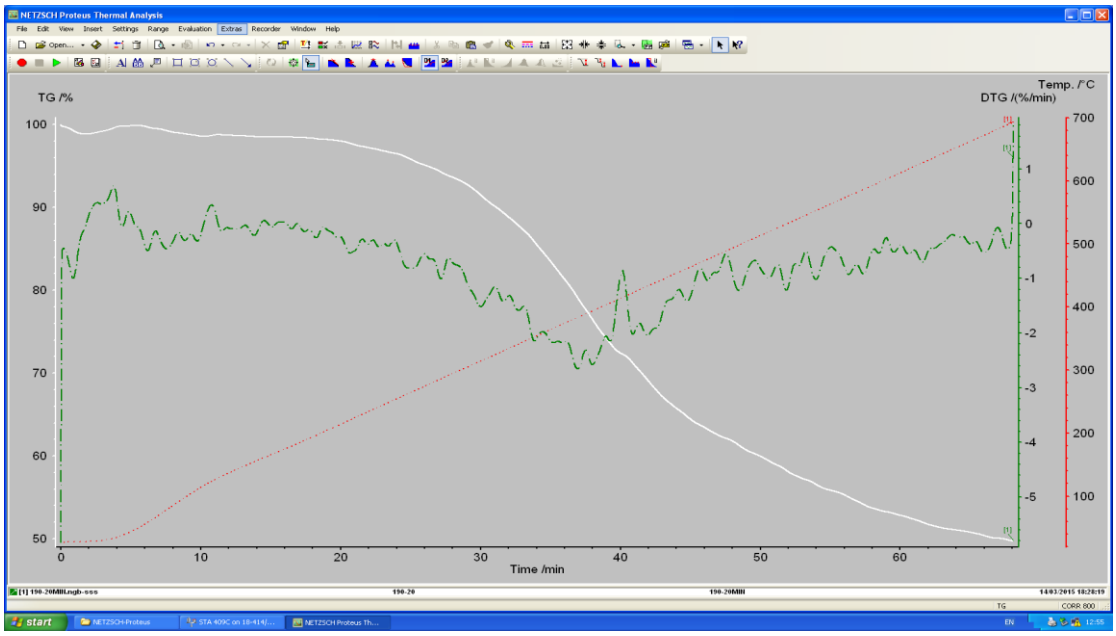


Figure 6-2c) TG curves of residual lignin, isolation condition: 190 ºC, 20min, 0.2 M sulfuric acid, 0.2 g feedstock in 15 ml solution. (white curve is TG curve, green curve is

DTG curve, red curve is temperature)

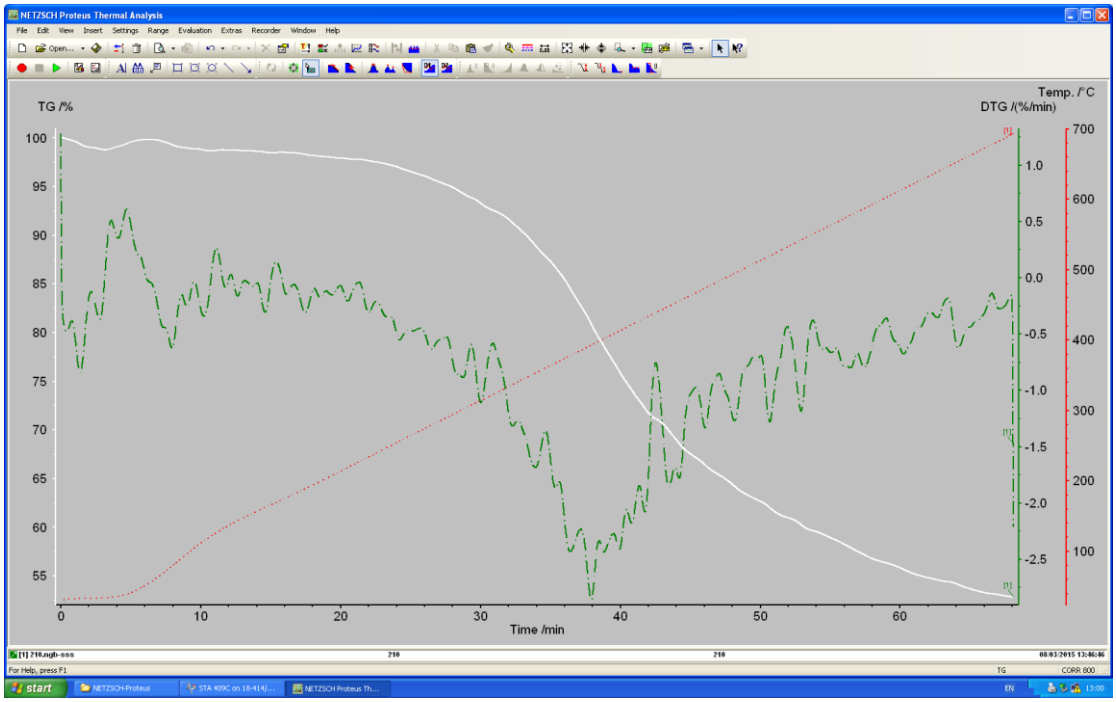


Figure 6-2d) TG curves of residual lignin, isolation condition: 210 ºC, 10min, 0.2 M sulfuric acid, 0.2 g feedstock in 15 ml solution. (white curve is TG curve, green curve is DTG curve, red curve is temperature)

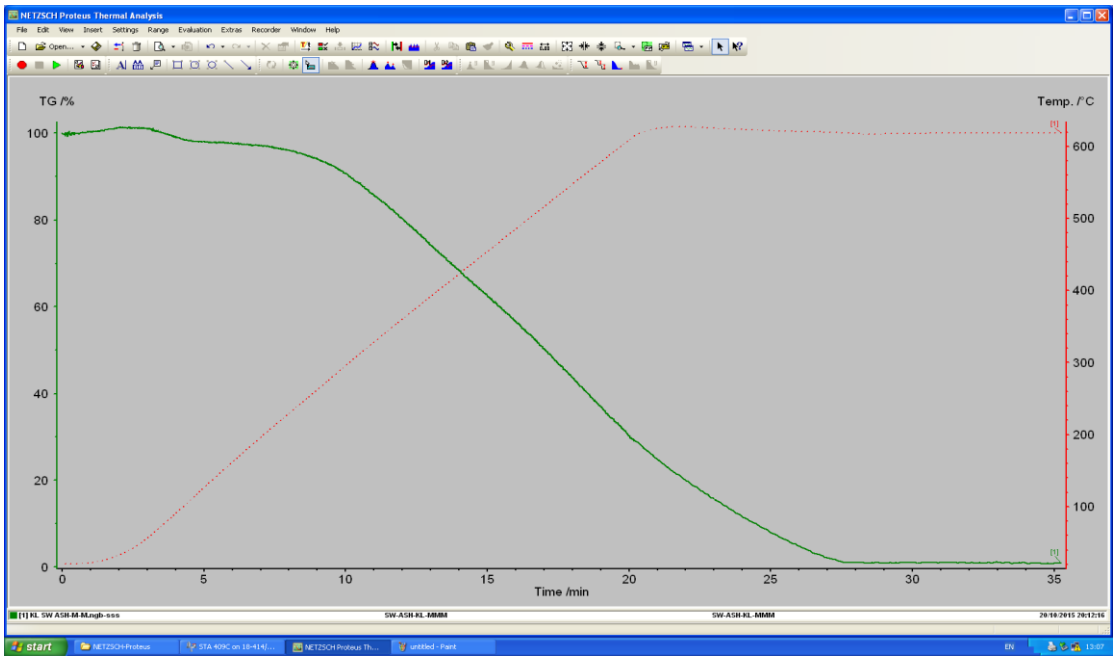


Figure 6-2e) Ash content determination (using TG analysis) of Klason lignin from MSP. (green curve is TG curve, red curve is temperature curve)

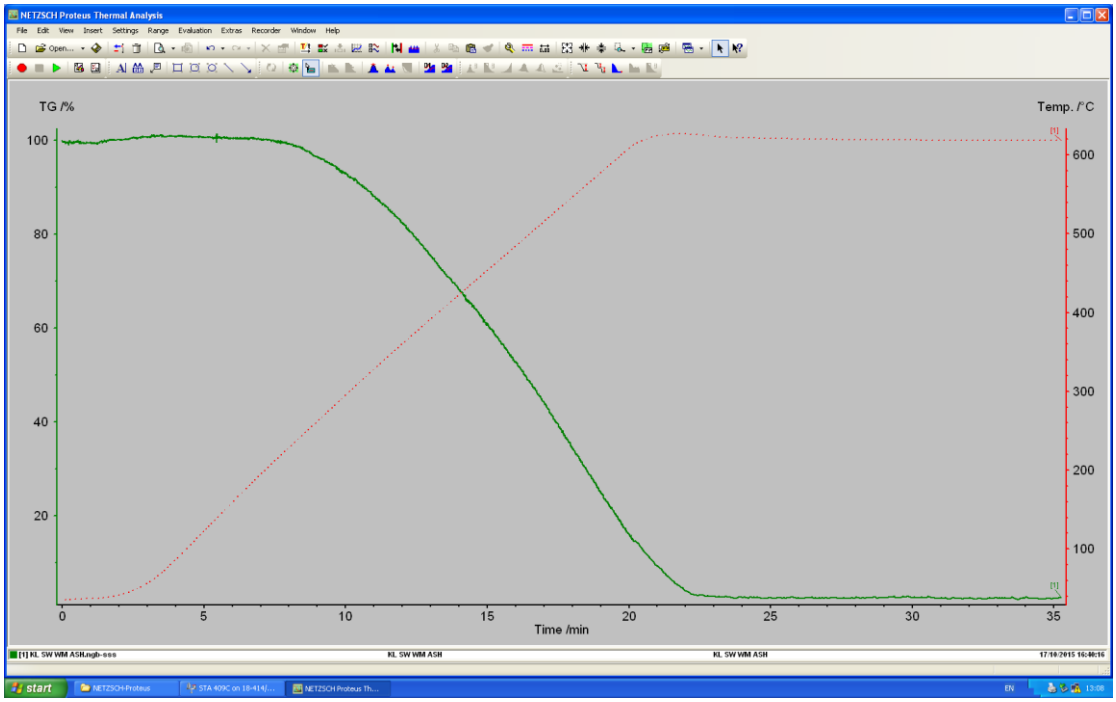


Figure 6-2f) Ash content determination (using TG analysis) of Klason lignin of microwave isolated residue from MSP, isolation condition: 190 ºC, 10min, 0.2 M sulfuric acid, 0.2 g feedstock in 15 ml solution. (green curve is TG curve, red curve is temperature curve)

6.1.2.5 FTIR analysis

FTIR data was obtained using a PerkinElmer FTIR/FTNIR Spectrum 400 analyzer (USA). The spectra were acquired between 700 and 4000 cm^{-1} with resolution of 2 cm^{-1} and scan time of 64 s. The spectra are shown in Figure 2-5 and Figure 6-3(unpresented results in Chapter 2).

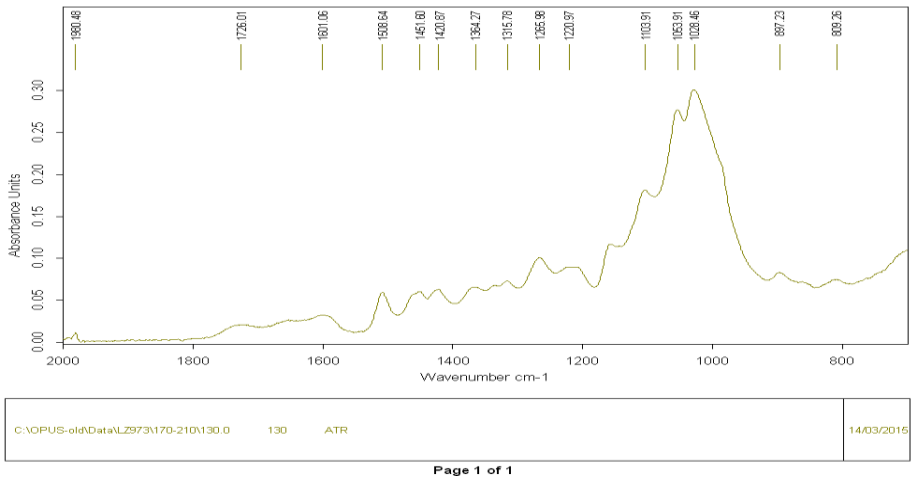


Figure 6-3a) FTIR spectra of residual lignin, isolation condition: 130 ºC, 10 min, 0.2 M sulfuric acid, 0.2 g feedstock in 15 ml solution.

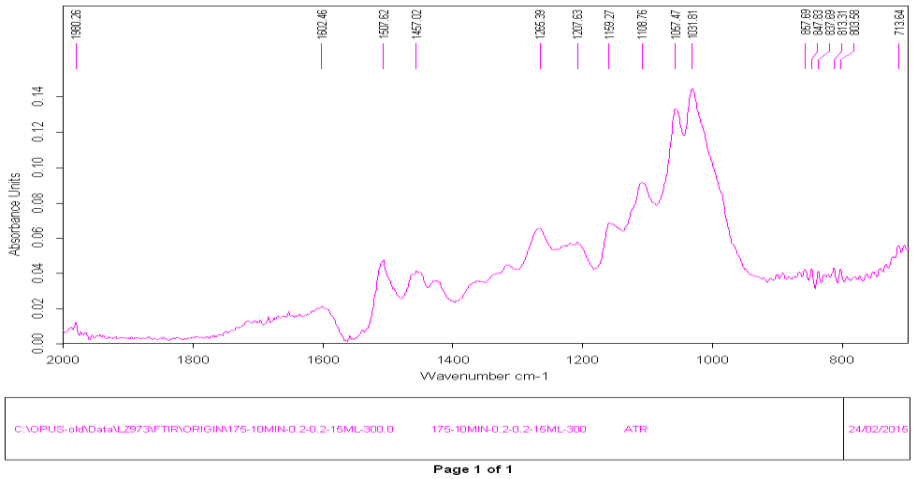


Figure 6-3b) FTIR spectra of residual lignin, isolation condition: 175 ºC, 10 min, 0.2 M sulfuric acid, 0.2 g feedstock in 15 ml solution.

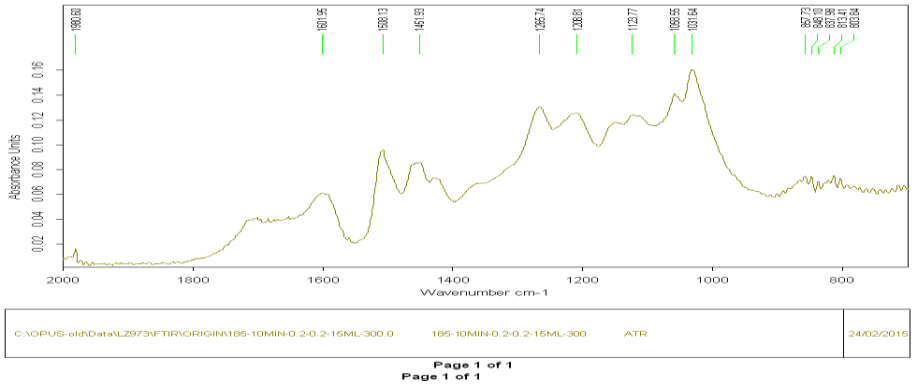


Figure 6-3c) FTIR spectra of residual lignin, isolation condition: 185 ºC, 10 min, 0.2 M

sulfuric acid, 0.2 g feedstock in 15 ml solution.

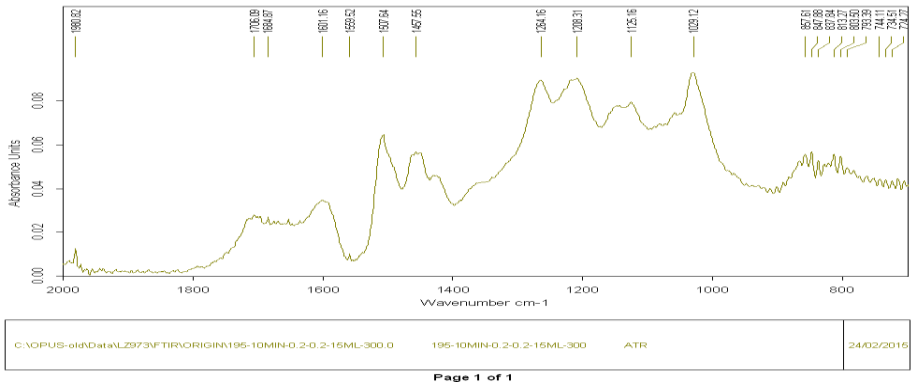


Figure 6-3d) FTIR spectra of residual lignin, isolation condition: 195 ºC, 10 min, 0.2 M sulfuric acid, 0.2 g feedstock in 15 ml solution.

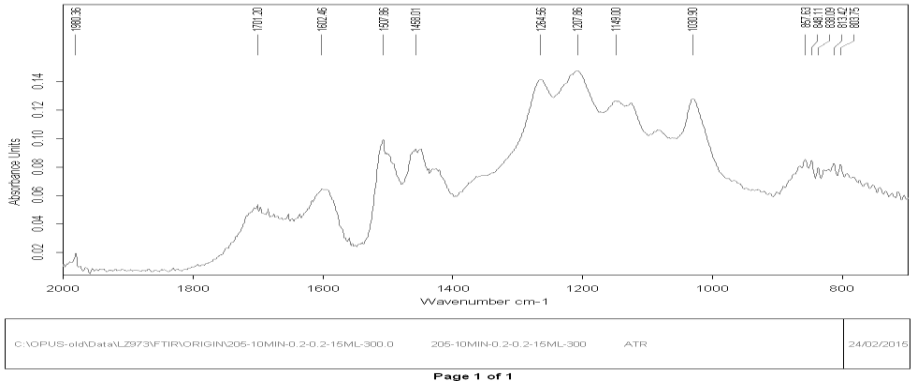


Figure 6-3e) FTIR spectra of residual lignin, isolation condition: 205 ºC, 10 min, 0.2 M sulfuric acid, 0.2 g feedstock in 15 ml solution.

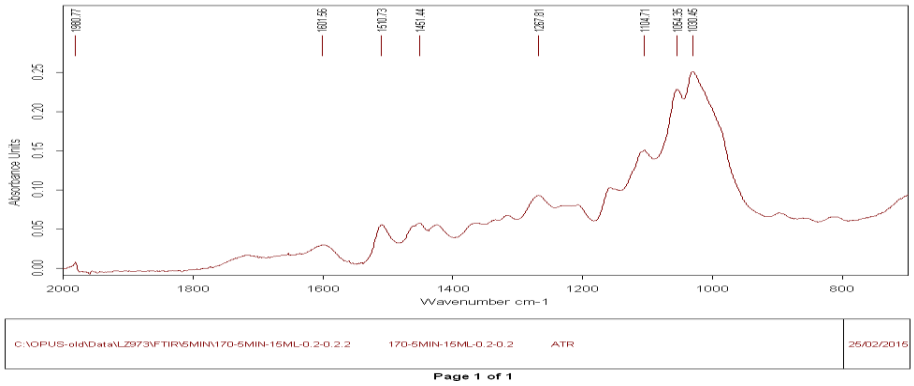


Figure 6-3f) FTIR spectra of residual lignin, isolation condition: 170 ºC, 5 min, 0.2 M

sulfuric acid, 0.2 g feedstock in 15 ml solution.

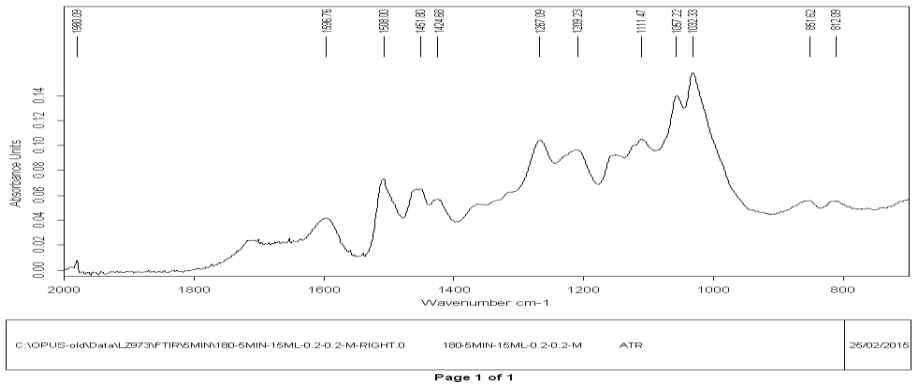


Figure 6-3g) FTIR spectra of residual lignin, isolation condition: 180 ºC, 5 min, 0.2 M sulfuric acid, 0.2 g feedstock in 15 ml solution.

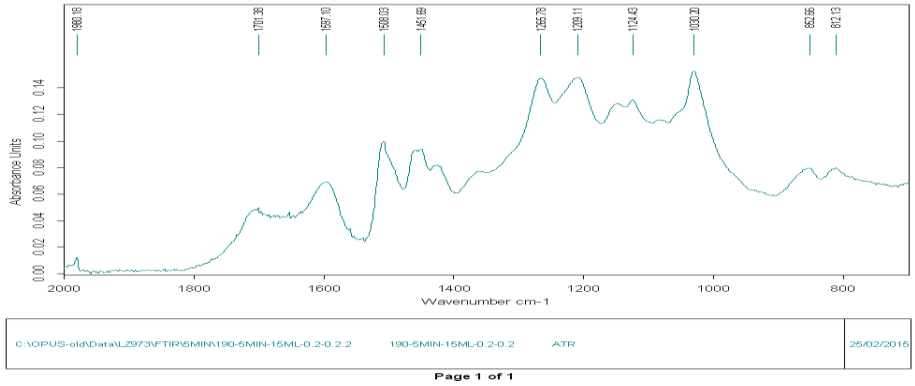


Figure 6-3h) FTIR spectra of residual lignin, isolation condition: 190 ºC, 5 min, 0.2 M sulfuric acid, 0.2 g feedstock in 15 ml solution.

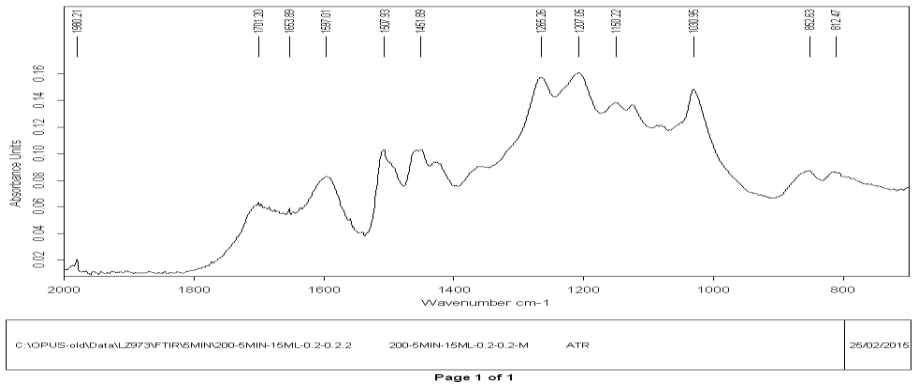


Figure 6-3i) FTIR spectra of residual lignin, isolation condition: 200 ºC, 5 min, 0.2 M sulfuric acid, 0.2 g feedstock in 15 ml solution.

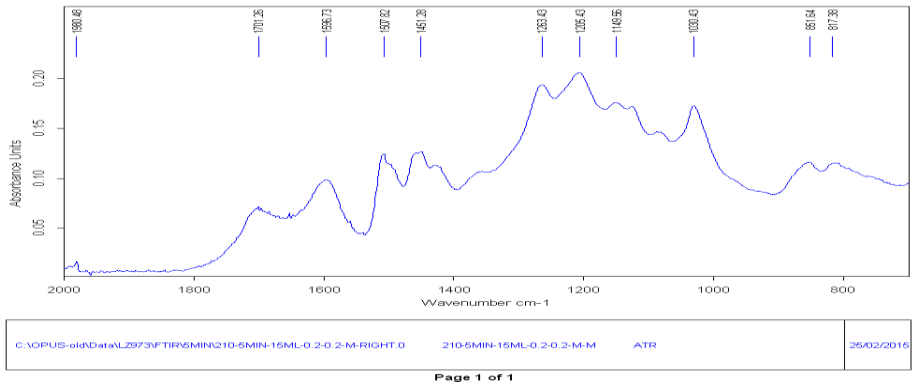


Figure 6-3j) FTIR spectra of residual lignin, isolation condition: 210 ºC, 5 min, 0.2 M sulfuric acid, 0.2 g feedstock in 15 ml solution.

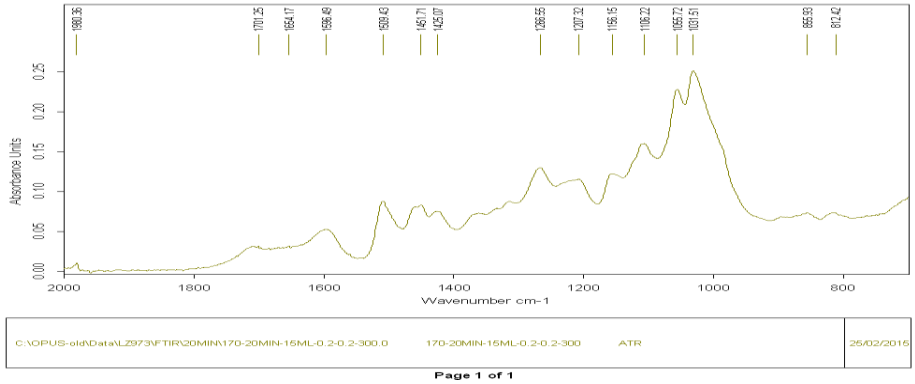


Figure 6-3k) FTIR spectra of residual lignin, isolation condition: 170 ºC, 20 min, 0.2 M sulfuric acid, 0.2 g feedstock in 15 ml solution.

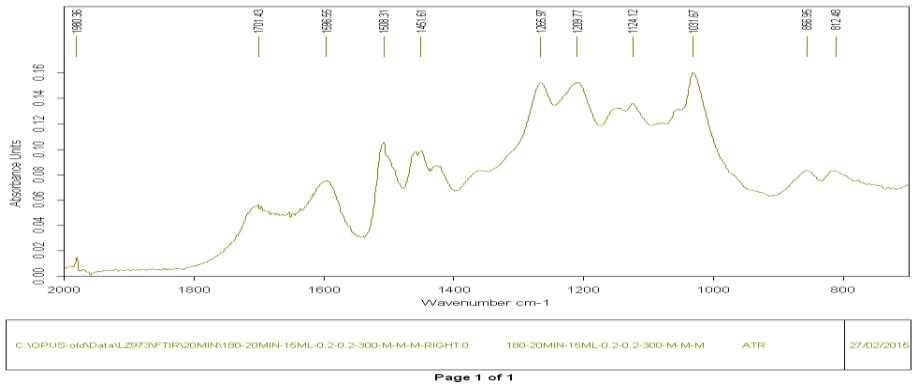


Figure 6-3l) FTIR spectra of residual lignin, isolation condition: 180 ºC, 20 min, 0.2 M sulfuric acid, 0.2 g feedstock in 15 ml solution.

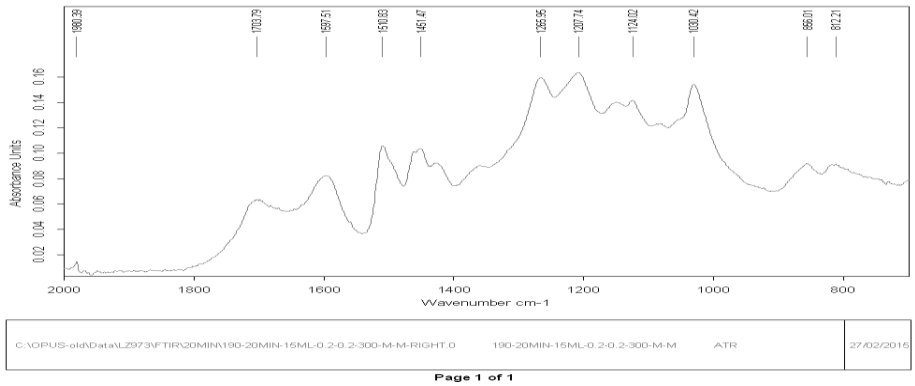


Figure 6-3m) FTIR spectra of residual lignin, isolation condition: 190 ºC, 20 min, 0.2 M sulfuric acid, 0.2 g feedstock in 15 ml solution.

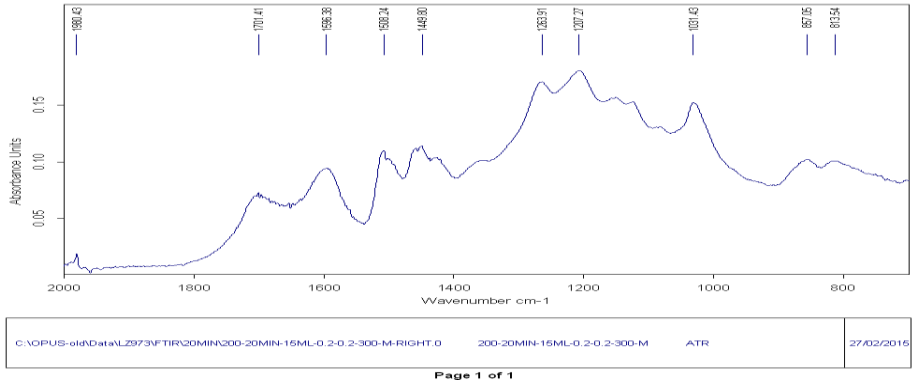


Figure 6-3n) FTIR spectra of residual lignin, isolation condition: 200 ºC, 20 min, 0.2 M sulfuric acid, 0.2 g feedstock in 15 ml solution.

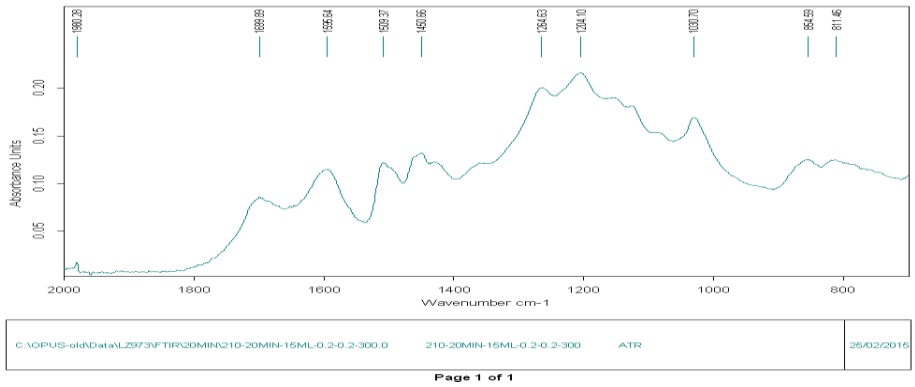


Figure 6-3o) FTIR spectra of residual lignin, isolation condition: 210 ºC, 20 min, 0.2 M sulfuric acid, 0.2 g feedstock in 15 ml solution.

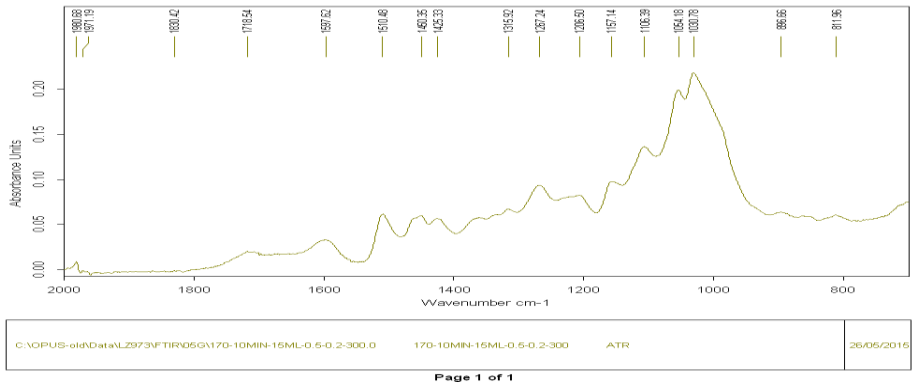


Figure 6-3p) FTIR spectra of residual lignin, isolation condition: 170 ºC, 10 min, 0.2 M sulfuric acid, 0.5 g feedstock in 15 ml solution.

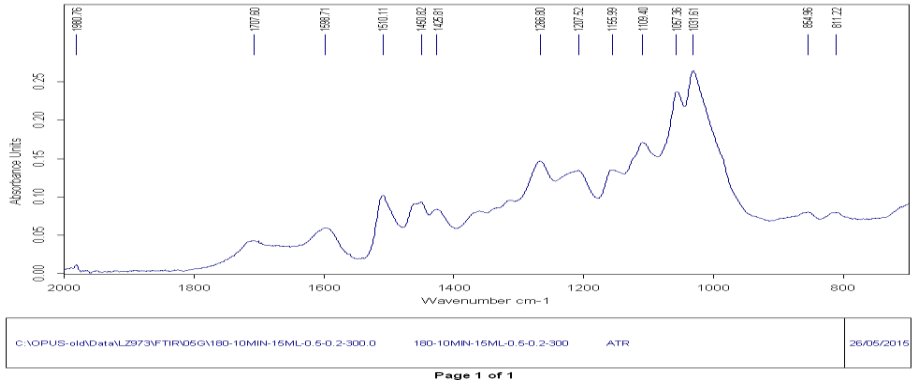


Figure 6-3q) FTIR spectra of residual lignin, isolation condition: 180 ºC, 10 min, 0.2 M sulfuric acid, 0.5 g feedstock in 15 ml solution.

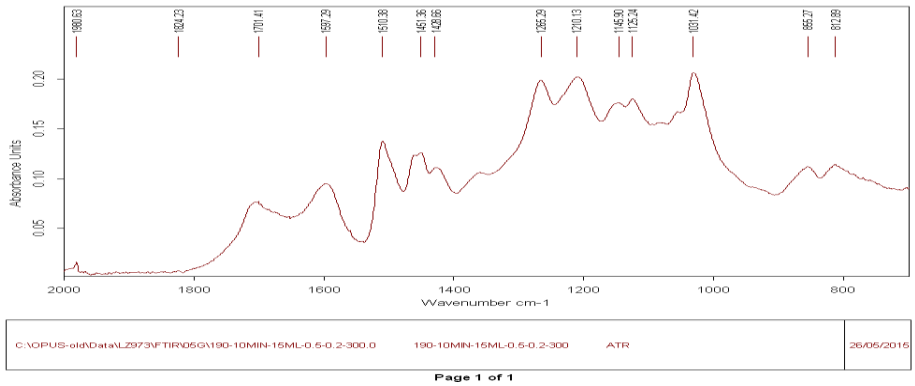


Figure 6-3r) FTIR spectra of residual lignin, isolation condition: 190 ºC, 10 min, 0.2 M sulfuric acid, 0.5 g feedstock in 15 ml solution.

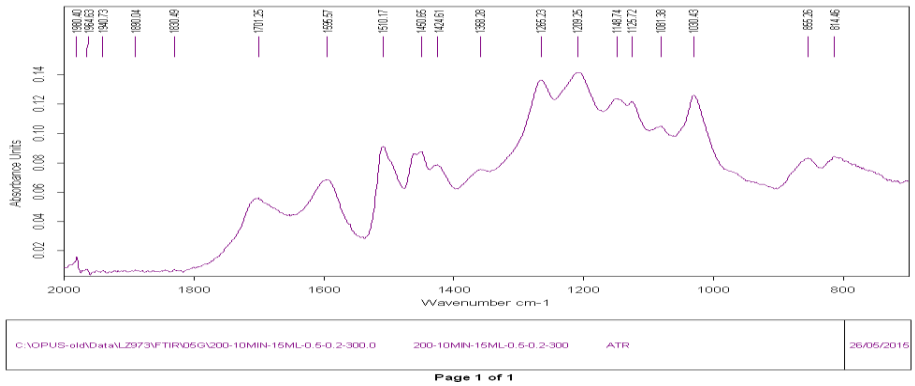


Figure 6-3s) FTIR spectra of residual lignin, isolation condition: 200 ºC, 10 min, 0.2 M sulfuric acid, 0.5 g feedstock in 15 ml solution.

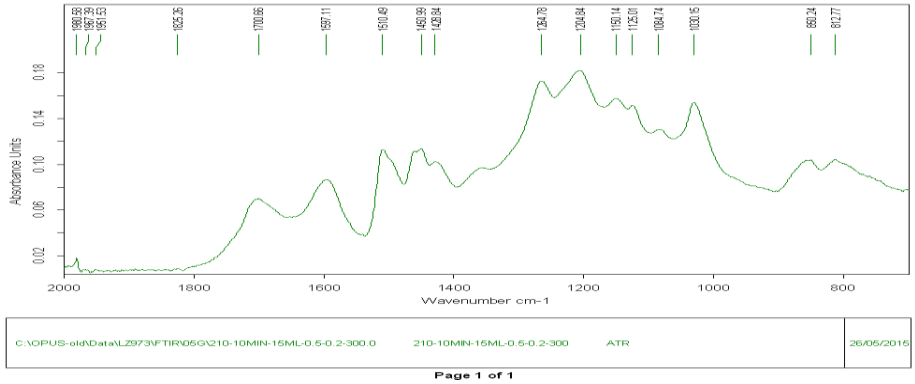


Figure 6-3t) FTIR spectra of residual lignin, isolation condition: 210 ºC, 10 min, 0.2 M sulfuric acid, 0.5 g feedstock in 15 ml solution.

6.1.2.6 Solid-state ^{13}C NMR spectroscopy analysis.

Solid-state ^{13}C NMR spectroscopy (SSNMR) results were obtained at the EPSRC UK National Solid-State NMR Service at University of Durham. The spectra were obtained at 100.562 MHz. The chemical shift range from 0 to 240 ppm was recorded. The spectra are given in Figure 2-7.

6.1.2.7 Pyrolysis-GC/MS analysis

Py-GC/MS results were obtained from BDC, University of York. The units used were

CDS Analytical 5250-T Trapping Pyrolysis Autosampler (UK) as the pyrolysis unit, Agilent Technologies 7890B GC System (USA) as gas chromatography unit, and Agilent Technologies 5977A MSD (USA) as mass spectrum unit. The sample was loaded into the pyrolysis unit and pyrolysed at 600 °C for 10 s. The volatile materials released were carried into the GC/MS unit by nitrogen for analysis. The following GC/MS parameters were applied: GC inlet temperature at 350 °C, initial temperature at 40 °C for 2 min, ramp rate at 10 ºC/min until 300 °C, holding at 300 °C for 30 min, and split ratio with 50:1. Volatile compounds were identified by comparing the mass spectra with NIST Lab database. The comparisons for compounds in Table 2-3 are given in Figure 6-5. A standard sample mixture of four compounds, creosol/vanillin/2-methoxyphenol (guaiacol)/isoeugenol, was also subjected to pyrolysis and GC/MS in order to verify the mass spectral identities. The GC spectra are given in Figure 6-4. The compounds lists are given in Table 2-3 and Table 6-3 (unpresented data in Chapter 2).

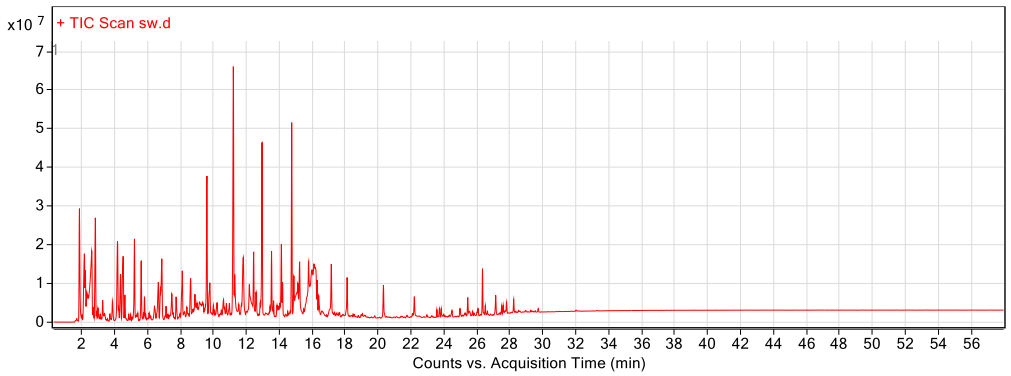


Figure 6-4a) The GC spectrum of MSP in Py-GC/MS. MSP, mixed softwood pellet. The sample was loaded into the pyrolysis unit and pyrolysed at 600 °C for 10 s. The volatile materials released were carried into the GC/MS unit by nitrogen for analysis. The following GC/MS parameters were applied: GC inlet temperature at 350 °C, initial temperature at 40 °C for 2 min, ramp rate at 10 ºC/min until 300 °C, holding at 300 °C for 30 min, and split ratio with 50:1 (all the py-GC/MS results are achieved using these setting)

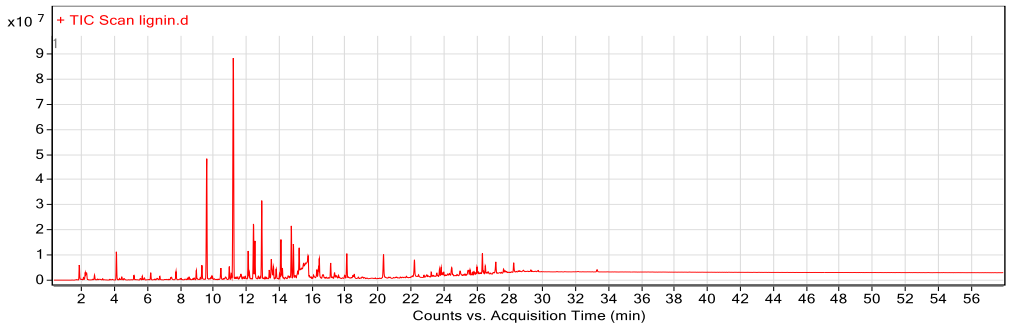


Figure 6-4b) The GC spectrum of microwave isolated lignin in Py-GC/MS, isolation conditions: 190 ºC, 10 min, 0.2 g feedstock in 15 ml 0.2 M H_2SO_4, in CEM discover microwave reactor

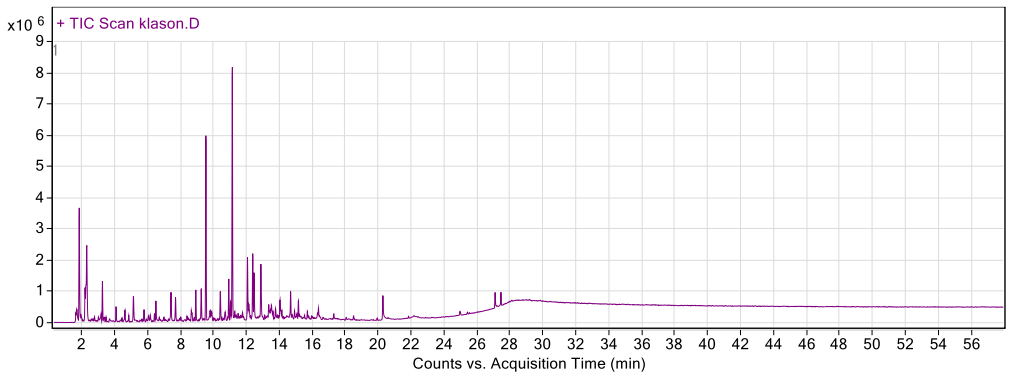


Figure 6-4c) The GC spectrum of Klason lignin from MSP in Py-GC/MS

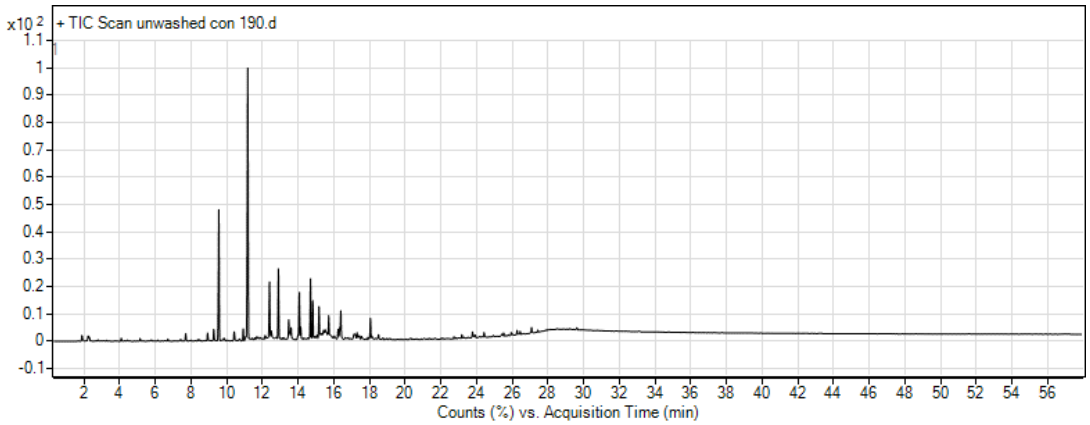


Figure 6-4d) The GC spectrum of conventional heating isolated lignin in Py-GC/MS, isolation conditions: 190 ºC, 10 min, 0.08 g feedstock in 6 ml 0.2 M H_2SO_4, in Anton Paar Monowave 50 reactor

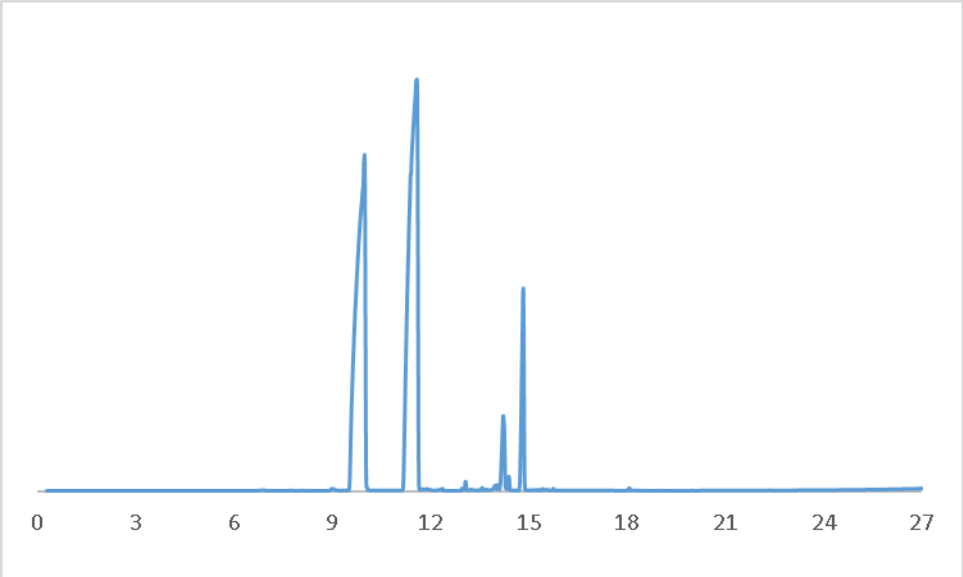


Figure 6-4d) The GC spectrum of mixed standard chemicals in Py-GC/MS, chemicals and retention time: 2-methoxyphenol (guaiacol, 10.002 min), creosol (11.602 min), vanillin (14.231 min), isoeugenol (14.848 min)

Table 6-3a Compounds list of Py-GC/MS analysis of MSP

Name	Peak area%	RT/min
Acetone	2.9	1.88
2,3-Butanedione	1.1	2.23
Acetic acid	1.6	2.32
1-Hydroxy-2-propanone	2.9	2.84
3,4-Dihydro-2H-pyran	0.7	3.88
Acetic acid	2.8	4.19
Succindialdehyde	1.4	4.37
Methyl 2-oxopropanoate	2.4	4.53
Furfural	2.0	5.21
2-Furanmethanol	1.4	5.63
5-Hexen-2-one	0.7	5.82
2(5H)-Furanone	1.5	6.67
1,2-Cyclopentanedione	3.0	6.87
9-Oxabicyclo[6.1.0]nonan-4-one	0.6	7.13
5-Methyl-2-furaldehyde	1.3	7.48
2-Hydroxy-3-methyl-2-cyclopenten-1-one	1.4	8.62
2-hydroxy-decanoic acid	0.6	8.77
1,2,6-Hexanetriol	1.7	9.16
9-Oxabicyclo[3.3.1]nonan-2-ol, acetate	1.1	9.34
2-methoxy-phenol	3.2	9.61
2-Nonen-1-ol	1.1	9.79
Creosol	6.2	11.21
5-Hydroxymethylfurfural	2.3	11.82
4-ethyl-2-methoxy-phenol	1.2	12.45
4-methyl-1,2-Benzenediol	0.4	12.60
2-Methoxy-4-vinylphenol	4.0	12.96
trans-m-Propenyl guaiacol	1.0	13.53
Vanillin	1.4	14.13
cis-Isoeugenol	0.4	14.20
trans-Isoeugenol	3.8	14.76
2-methoxy-4-propyl-Phenol	0.7	14.88
1,4-Dimethoxy-2,3-dimethylbenzene	1.1	15.24
Levoglucosan	2.4	15.99
(E)-4-(3-Hydroxyprop-1-en-1-yl)-2-methoxyphenol	0.4	16.31
Dihydroconiferyl alcohol	1.5	17.16
Coniferyl aldehyde	1.0	18.12
n-Hexadecanoic acid	0.8	20.32
Octadecanoic acid	0.6	22.20

Table 6-3b Compounds list of Py-GC/MS analysis of 190ºC MRL

Name	Peak Area%	RT/min
Acetone	1.0	1.86
Acetic acid	0.8	2.31
Toluene	1.7	4.12
2-methyl-Phenol	0.5	8.97
3-methyl-Phenol	0.9	9.31
2-methoxy-Phenol	8.5	9.60
3,5-dimethyl-Phenol	0.7	10.46
2-methoxy-3-methyl-Phenol	0.7	10.97
Creosol	18.7	11.21
3-ethyl-5-methyl-Phenol	0.6	11.68
3-methyl-1,2-Benzenediol	1.7	12.12
4-ethyl-2-methoxy-Phenol	3.7	12.44
4-methyl-1,2-Benzenediol	2.5	12.53
2-Methoxy-4-vinylphenol	4.9	12.94
2,6-dimethyl-1,4-Benzenediol	0.5	13.40
trans-m-Propenyl guaiacol	1.9	13.52
1,3-Benzenediol, 4-ethyl-	0.7	13.82
Vanillin	2.7	14.11
cis-Isoeugenol	0.7	14.20
trans-Isoeugenol	3.2	14.73
Phenol, 2-methoxy-4-propyl-	2.2	14.86
1,4-Dimethoxy-2,3-dimethylbenzene	2.3	15.21
(E)-4-(3-Hydroxyprop-1-en-1-yl)-2-methoxyphenol	0.7	16.28
1,2-Dimethoxy-4-n-propylbenzene	2.0	16.44
Dihydroconiferyl alcohol	1.0	17.12
1-[3,6-dihydroxy-2-(2-propenyl)phenyl]- 1-Ethanone	0.7	17.36
Coniferyl aldehyde	1.7	18.11
n-Hexadecanoic acid	2.0	20.34
Octadecanoic acid	1.5	22.21

Table 6-3c Compounds list of Py-GC/MS analysis of KL

Name	Peak Area%	RT/min
Sulfur dioxide	1.3	1.72
Acetone	6.5	1.86
2,3-Butanedione	1.8	2.22
Acetic acid	7.2	2.32
4,4-dimethyl-1,2-Pentadiene	0.6	3.19
Furan, 2,5-dimethyl-	1.9	3.27
Toluene	0.7	4.09
2,5-dimethyl-2,4-Hexadiene	0.7	4.64
Furfural	1.7	5.16
5-Hexen-2-one	0.7	5.80
1-(2-furanyl)-Ethanone	1.2	6.52
5-methyl-2-Furancarboxaldehyde	1.8	7.43
Phenol	1.3	7.71
2-methyl-Phenol	1.4	8.94
3-methyl-Phenol	1.8	9.27
2-methoxy-Phenol	9.7	9.55
2,3-dimethyl-Phenol	1.5	10.43
2-methoxy-3-methyl-Phenol	2.1	10.94
4-ethyl-1,3-Benzenediol	0.9	11.04
Creosol	14.0	11.16
3-methyl-1,2-Benzenediol	3.3	12.07
4-ethyl-2-methoxy-Phenol	3.7	12.39
4-methyl-1,2-Benzenediol	2.6	12.47
2-Methoxy-4-vinylphenol	3.6	12.88
2,6-dimethyl-1,4-Benzenediol	1.1	13.36
trans-m-Propenyl guaiacol	1.4	13.52
Vanillin	1.3	14.05
cis-Isoeugenol	0.3	14.16
trans-Isoeugenol	1.4	14.69
1,4-Dimethoxy-2,3-dimethylbenzene	0.7	15.17
1,2-Dimethoxy-4-n-propylbenzene	1.5	16.38
n-Hexadecanoic acid	2.0	20.29
Octadecanoic acid	1.1	22.18

Table 6-3d Compounds list of Py-GC/MS analysis of 190 ºC AL

Name	Peak Area%	RT/min
Acetone	0.6	1.88
2,3-Butanedione	1.0	2.22
Phenol	0.6	7.69
2-methyl-Phenol	0.6	8.91
p-Cresol	0.9	9.26
2-methoxy-Phenol	10.6	9.55
2,3-dimethyl-Phenol	0.8	10.41
3-ethyl-Phenol	0.2	10.71
2-methoxy-3-methyl-Phenol	0.8	10.91
Creosol	25.0	11.17
5-Hydroxymethylfurfural	0.6	11.87
1,2-Benzenediol, 3-methyl-	0.7	12.14
4-ethyl-2-methoxy-Phenol	5.3	12.39
4-methyl-1,2-Benzenediol	1.3	12.49
2-Methoxy-4-vinylphenol	5.9	12.88
trans-m-Propenyl guaiacol	2.2	13.47
4-ethyl-1,3-Benzenediol	1.2	13.59
Vanillin	4.1	14.06
cis-Isoeugenol	1.0	14.14
trans-Isoeugenol	4.6	14.69
2-methoxy-4-propyl-Phenol	3.1	14.81
1,4-Dimethoxy-2,3-dimethylbenzene	3.1	15.16
1,5,5-trimethyl-6-acetylmethyl-Cyclohexene	0.8	15.31
Guanosine	1.4	15.42
D-Allose	2.1	15.52
(E)-4-(3-Hydroxyprop-1-en-1-yl)-2-methoxyphenol	0.8	16.23
1,2-Dimethoxy-4-n-propylbenzene	3.2	16.38
Dihydroconiferyl alcohol	1.1	17.13
1-[3,6-dihydroxy-2-(2-propenyl)phenyl]-1-Ethanone	0.6	17.29
Coniferyl aldehyde	1.7	18.05

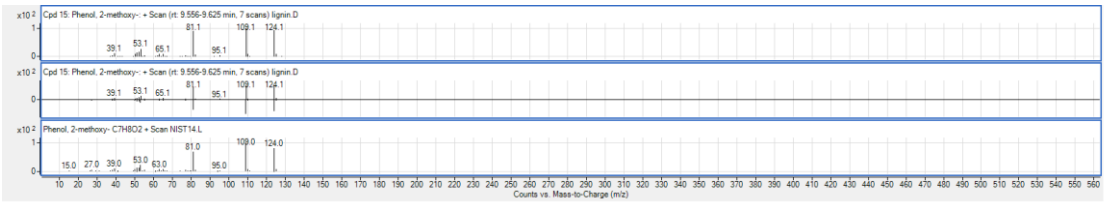


Figure 6-5a Identified sample phenolics MS (**Table 2-3**) and their best MS match from NIST Spectral library: 2-methoxy-Phenol (around 9.60 min)

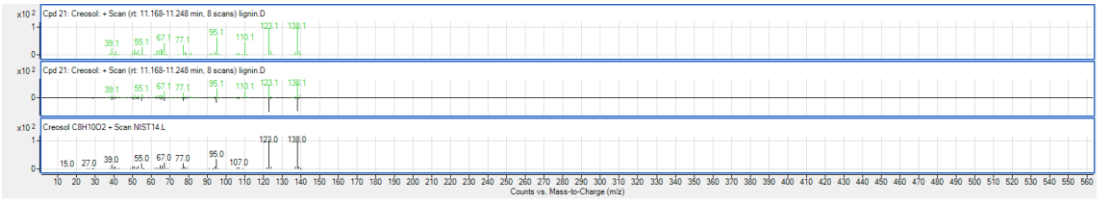


Figure 6-5b Identified sample phenolics MS (**Table 2-3**) and their best MS match from NIST Spectral library: Creosol (around 11.20 min)

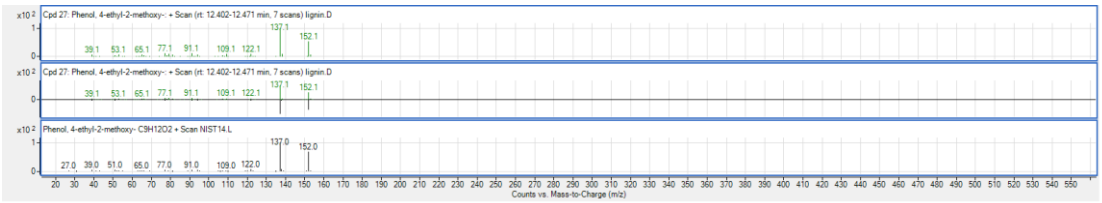


Figure 6-5c Identified sample phenolics MS (**Table 2-3**) and their best MS match from NIST Spectral library: 4-ethyl-2-methoxy- Phenol (around 12.42 min)

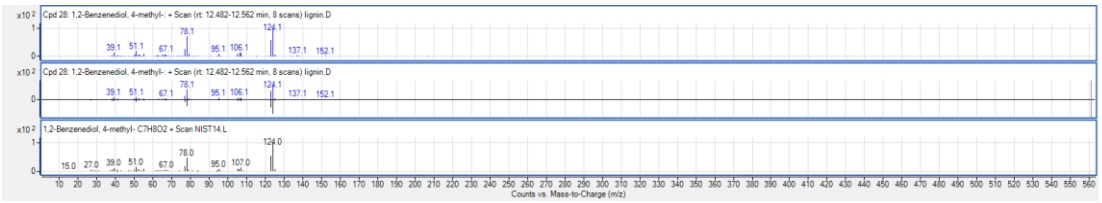


Figure 6-5d Identified sample phenolics MS (**Table 2-3**) and their best MS match from NIST Spectral library: 4-methyl-1,2-Benzenediol (around 12.51 min)

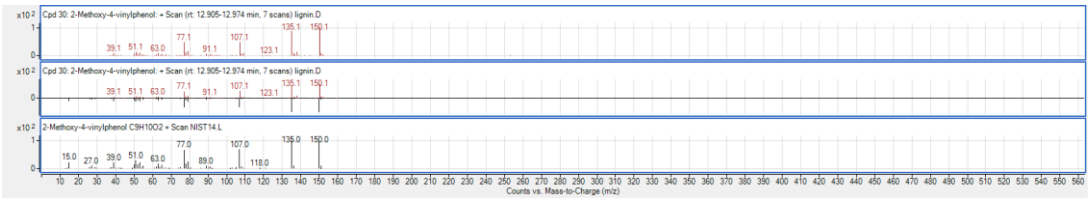


Figure 6-5e Identified sample phenolics MS (**Table 2-3**) and their best MS match from NIST Spectral library: 2-Methoxy-4-vinylphenol (around 12.92 min)

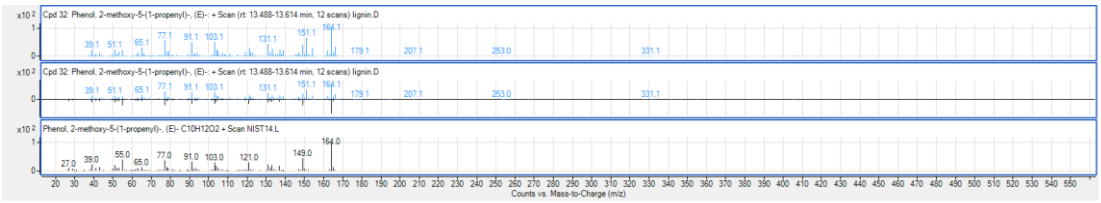


Figure 6-5f Identified sample phenolics MS (**Table 2-3**) and their best MS match from NIST Spectral library: trans-m-Propenyl guaiacol (around 13.52 min)

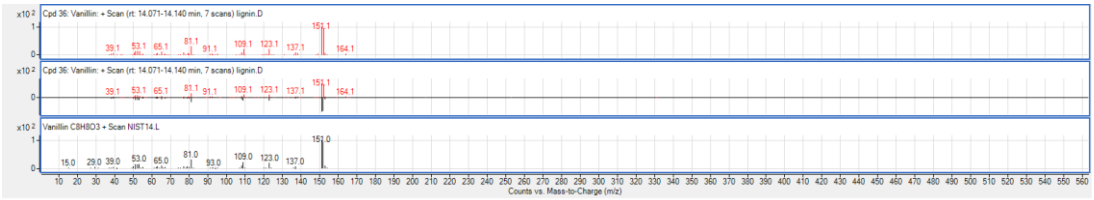


Figure 6-5g Identified sample phenolics MS (**Table 2-3**) and their best MS match from NIST Spectral library: Vanillin (around 14.10 min)

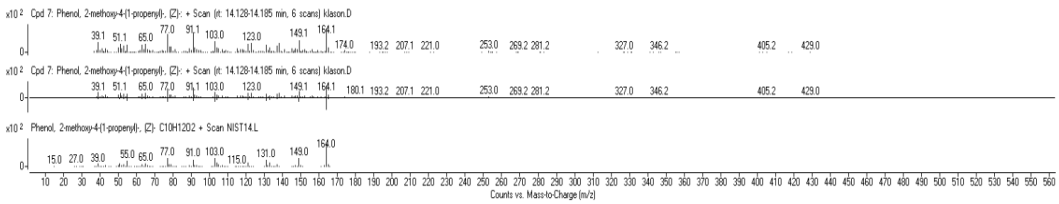


Figure 6-5h Identified sample phenolics MS (**Table 2-3**) and their best MS match from NIST Spectral library: cis-Isoeugenol (around 14.17 min)

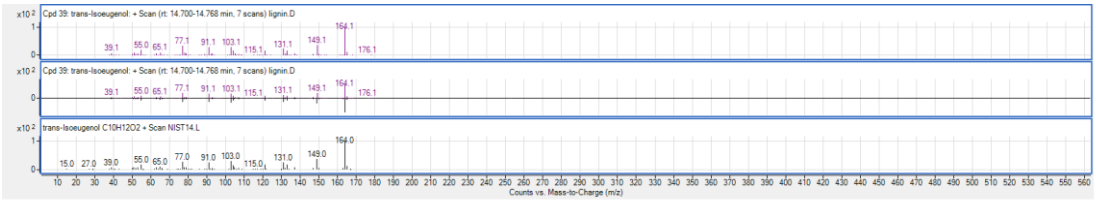


Figure 6-5i Identified sample phenolics MS (**Table 2-3**) and their best MS match from NIST Spectral library: trans-Isoeugenol (around 14.71 min)

6.1.2.8 Liquid state ^{13}C NMR spectroscopy

After microwave isolation (conditions: 190 ºC, 10 min, 0.2 g feedstock in 15 ml 0.2 M H_2SO_4, in CEM discover microwave reactor), the aqueous phase was neutralised and dried using freeze-dryer for 24 h, preparing for liquid state ^{13}C NMR and GC/MS analysis. Liquid-state ^{13}C NMR spectroscopy results were obtained by JEOL ECS 400 NMR Spectrometer (Japan). D_2O was used as the solvent for analysis. The number of scans was 8192. The spectrum is shown in Figure 2-3.

6.1.2.9 GC/MS analysis

GC/MS results were obtained using a PerkinElmer Clarus 500 GC/ MS (USA). Ethanol was chosen as solvent for analysis. The GC program used was as follows: initial temperature at 50 °C holding for 4 min, ramp rate with 10 K/min until 290 °C and holding for 10 min, split ratio with 5:1, and injector temperature at 290 °C. The GC spectrum is shown in Figure 6-6. The compounds list is given in Table 2-2. The identities of the compounds were determined by comparing the mass spectra with NIST lab database, which are shown in Figure 6-7.

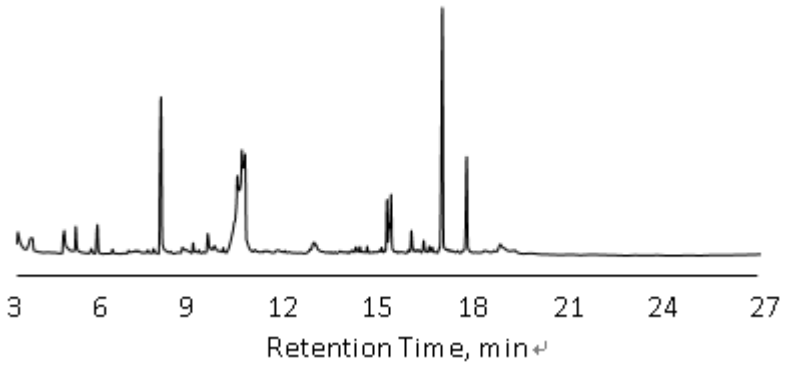


Figure 6-6 Gas chromatogram of liquid sample (solubilized products of MSP after microwave lignin isolation at 190ºC)

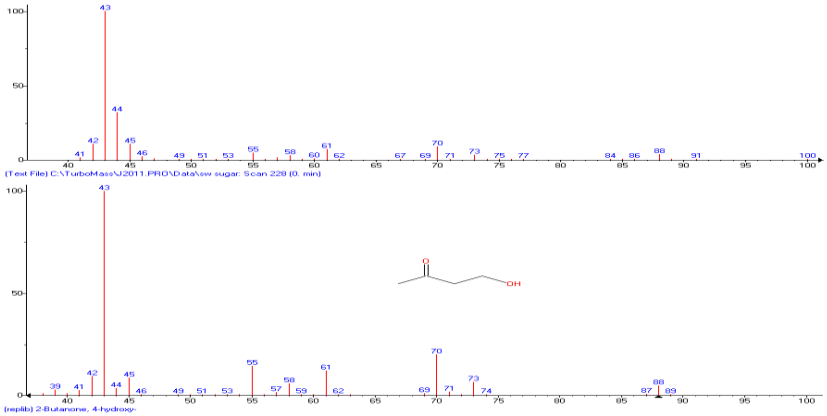


Figure 6-7a Identified sample MS (**Table 2-2**) and their best MS match from NIST Spectral library: 4-Hydroxy-2-butanone (4.5 min)

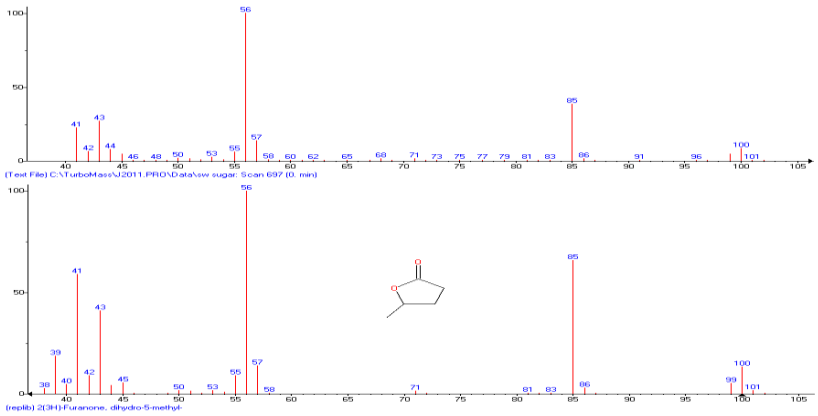


Figure 6-7b Identified sample MS (**Table 2-2**) and their best MS match from NIST Spectral library: Pentanoic acid (7.64 min)

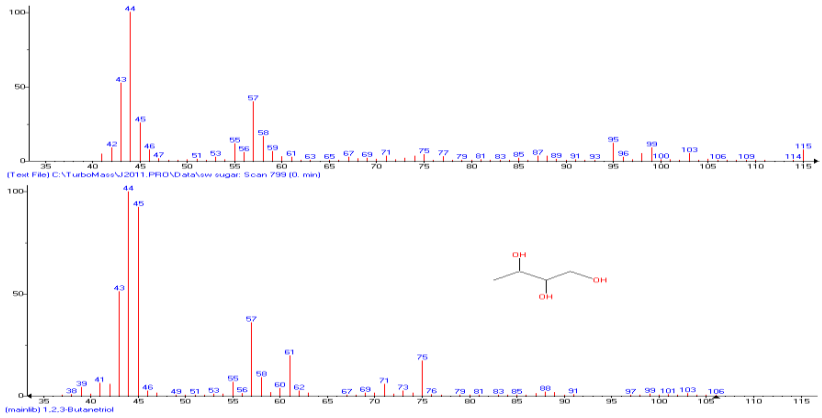


Figure 6-7c Identified sample MS (**Table 2-2**) and their best MS match from NIST Spectral library: 1,2,3-Butanetriol (8.34 min)

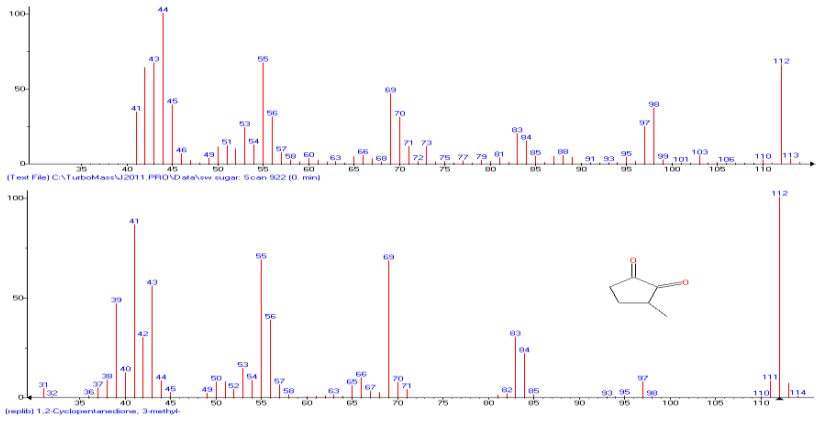


Figure 6-7d Identified sample MS (**Table 2-2**) and their best MS match from NIST Spectral library: 3-Methyl-1,2-cyclopentanedione (9.15 min)

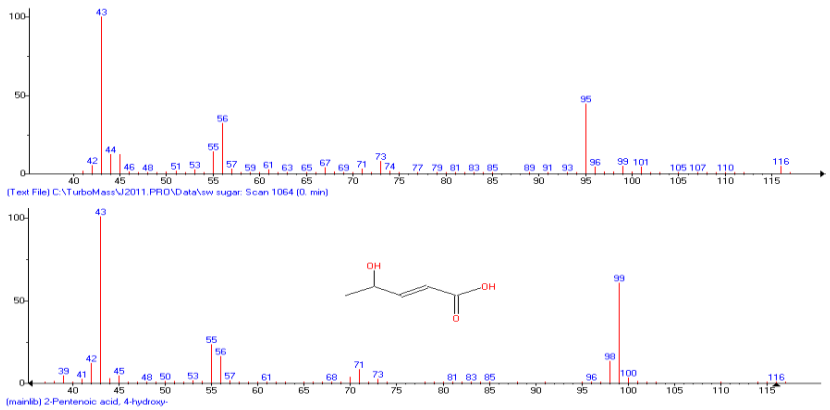


Figure 6-7e Identified sample MS (**Table 2-2**) and their best MS match from NIST Spectral library: 4-hydroxy-2-Pentenoic acid (10.09 min)

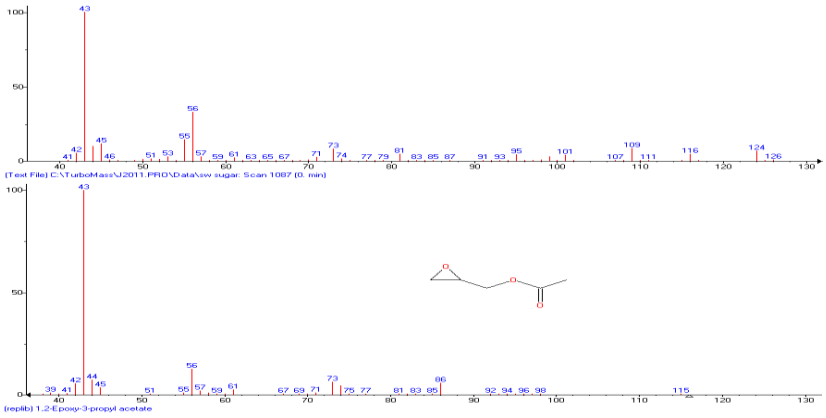


Figure 6-7f Identified sample MS (**Table 2-2**) and their best MS match from NIST Spectral library: 1,2-Epoxy-3-propyl acetate (10.25 min)

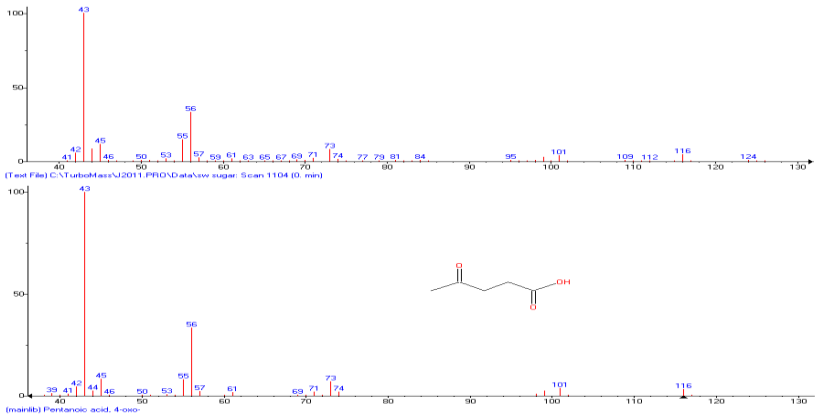


Figure 6-7g Identified sample MS (**Table 2-2**) and their best MS match from NIST Spectral library: Levulinic acid (10.36 min)

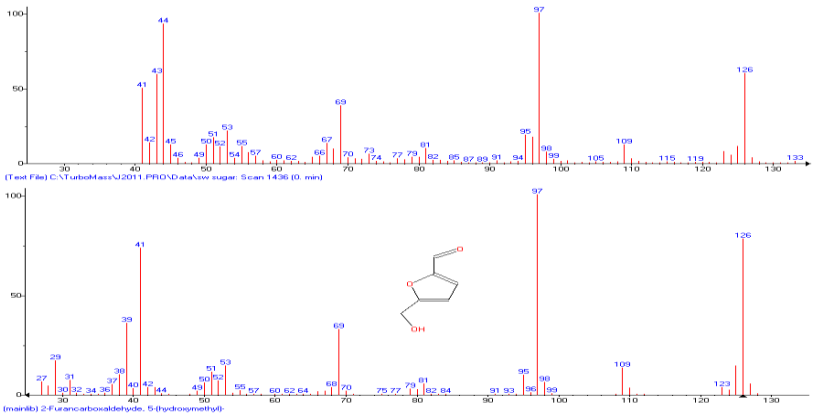


Figure 6-7h Identified sample MS (**Table 2-2**) and their best MS match from NIST Spectral library: 5-Hydroxymethylfurfural (12.57 min)

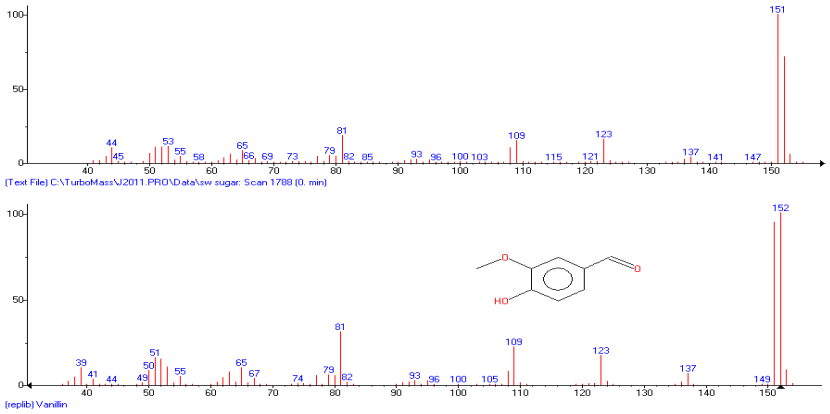


Figure 6-7i Identified sample MS (**Table 2-2**) and their best MS match from NIST Spectral library: Vanillin (14.92 min)

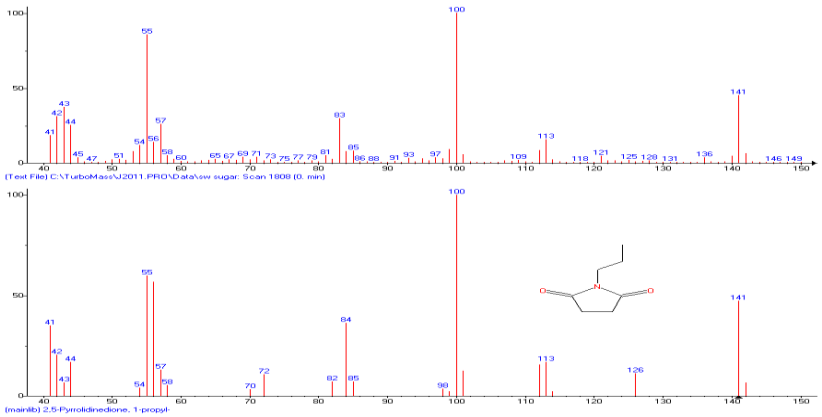


Figure 6-7j Identified sample MS (**Table 2-2**) and their best MS match from NIST Spectral library: 1-Propyl-2,5-pyrrolidinedione (15.04 min)

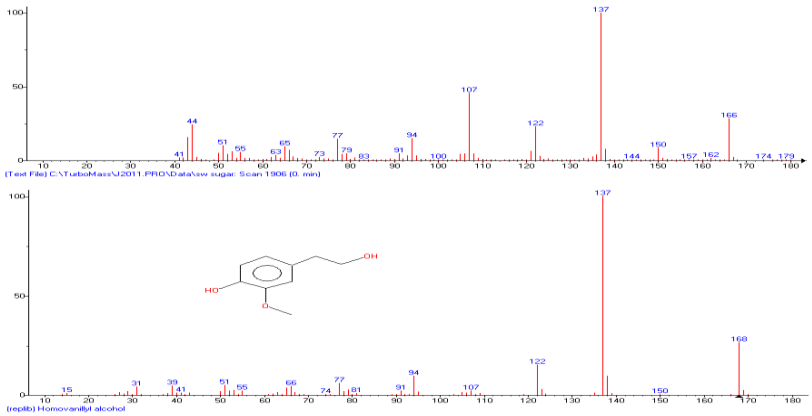


Figure 6-7k Identified sample MS (**Table 2-2**) and their best MS match from NIST Spectral library: Homovanillyl alcohol (15.71 min)

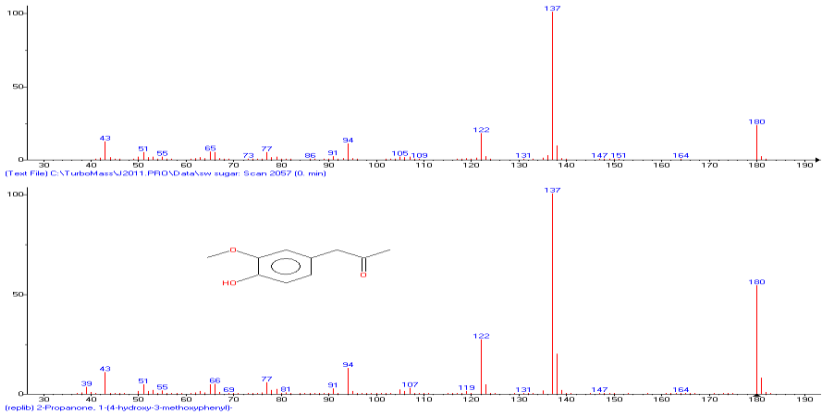


Figure 6-7l Identified sample MS (**Table 2-2**) and their best MS match from NIST Spectral library: Guaiacylacetone (16.70 min)

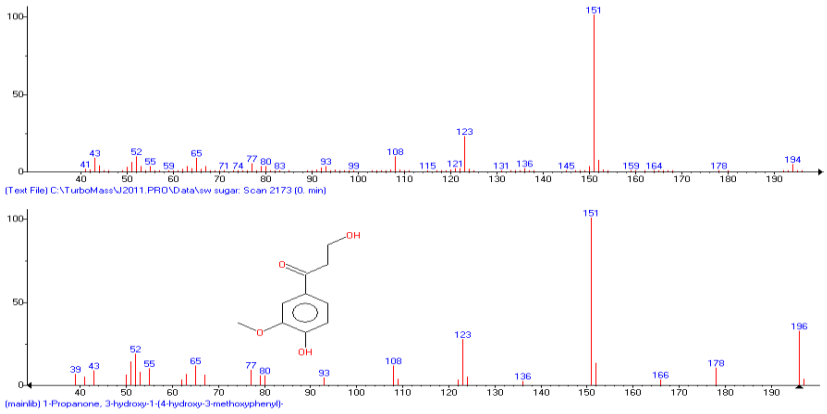


Figure 6-7m Identified sample MS (**Table 2-2**) and their best MS match from NIST Spectral library: Propiophenone (17.48 min)

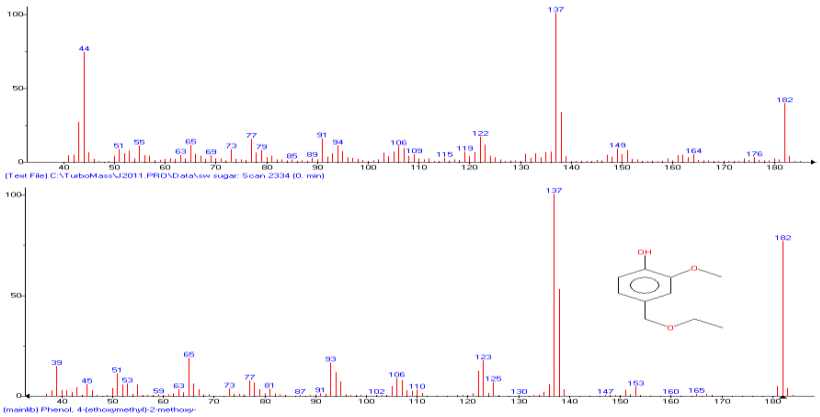


Figure 6-7n Identified sample MS (**Table 2-2**) and their best MS match from NIST Spectral library: Vanillyl ethyl ether (18.57 min)

6.2 Chapter 3

6.2.1 Materials

Three lignocellulosic biomass samples were investigated in this research: mixed softwood pellets (MSP, UK Biochar Research Centre, School of Geosciences, University of Edinburgh), willow edging (WE, Gardman Ltd) and wheat straw (WS, Biorenewables Development Centre, University of York), which represent softwood, hardwood and herbaceous biomass respectively. The elemental and ICP analysis results are given in Table 6-4 and Table 6-5.

Sulfuric acid and sodium hydroxide were purchased from Fisher Chemicals. Creosol, vanillin, 2-methoxy-phenol, glucose, fructose, rhamnose, formic acid and furfural were purchased from Sigma-Aldrich. *E*-Isoeugenol was purchased from Acros Organic. Levoglucosan and 5-hydroxymethylfurfural (HMF) were purchased from Carbosynth. Xylose was purchased from VWR. Cellobiose was purchased from Fluorochem. Lactic acid was purchased from Wardle. Acetic acid was purchased from Alfa Aesar. Levoglucosenone was purchased from Dextra.

Metschnikowia pulcherrima was isolated from the local area (named strain I1) and *Cryptococcus curvatus* (strain DSM-70022) was purchased from the German Collection of Microorganisms and Cell Cultures.

Table 6-4 Proximate and ultimate analysis of feedstocks (wt%)

	Moisture	Dry basis					
		Ash	C	H	N	S	O[a]
MSP	6.6	1.1	51.8	5.6	0.0	1.3	40.2
WE	4.7	3.7	48.1	5.9	0.3	0.2	41.92
WS	6.1	14.4	44.7	5.4	0.3	0.2	35.0

a. Oxygen=100-Ash-C-H-N-S

Analysis of Carbon, Hydrogen & Nitrogen (CHN) is carried out on an Exeter Analytical Inc. CE-440 analyser. The results is achieved from Microanalysis service from Chemistry Department, University of York

Table 6-5 ICP analysis of feedstock ($\times 10^{-2}$ wt%)

	MSP	WE	ST
Na	17.0	0.0	0.3
Mg	1.4	5.6	8.0
Al	0.6	0.9	1.2
Si	10.5	2.2	10.6
P	1.2	8.3	5.2
K	7.8	30.4	115.4
Ca	18.2	53.2	54.8
Fe	0.5	1.3	2.7

Co	0.0	0.0	0.0
Ni	0.0	0.1	0.0
Cu	0.1	0.1	0.0
Zn	0.3	0.7	0.4

The results were achieved from ICP-MS service (GCCE) with the help from Dr. Andrea Muñoz García for running the sample test.

6.2.2 Experimental methods and Data

6.2.2.1 Lignin isolation experiment design

All biomass was milled into 60-mesh powders using a cutting mill (Retsch SM300, Germany) in the Biorenewables Development Center (BDC), University of York. Microwave treatment was carried out using a Discovery SP microwave reactor (CEM Corporation, USA) in capped vessels. Maximum power (300 W) was applied in all of the experiments in order to achieve the holding temperature as quickly as possible. Acid solutions of different types and concentrations were applied for isolation. Processing temperatures from 170 °C to 210 °C in intervals of 10 °C were used for isolation. The holding time was 10 min. During microwave treatment, the biomass feedstock and acid solvent were heated in a capped vessel with stirring in the ratios of 13, 33, 67 and 100 g L^{-1}. After microwave treatment, the residue was recovered by filtration. The residue was then washed several times with deionized water until the rinsed water was neutral. The residue was dried (105 °C, 24 h) and then weighed. All of the experiments were repeated 3 times. The results are given in Figure 3-2.

6.2.2.2 Purity and yield calculation

The method is same to 6.1.2.2. Results are given in Table 3-1.

6.2.2.3 Element analysis

The element and ICP analysis method and apparatus are same to those in 6.1.2.3. The results are given in Table 6-4 and Table 6-5.

6.2.2.4 Thermal gravimetric analysis

The thermal gravimetric analysis method and apparatus are same to those in 6.1.2.4. The results are given in Figure 3-3, Figure 6-4 (unpresented data of MSP) and Figure 6-8 (unpresented data of WE and WS)

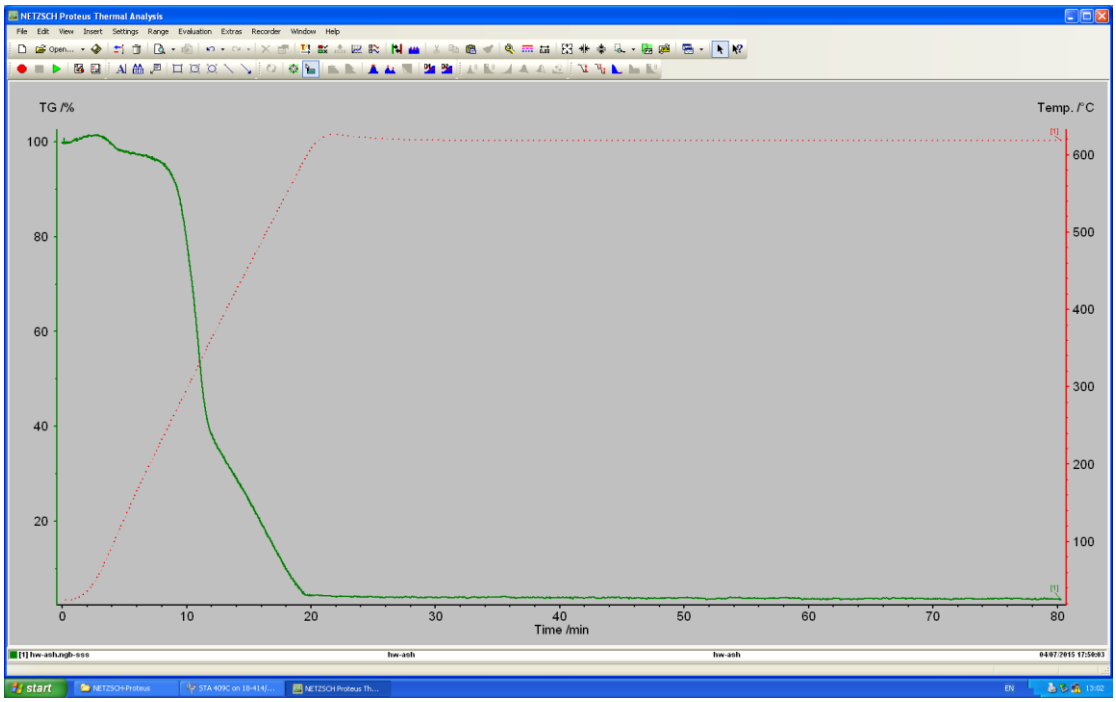


Figure 6-8a Ash content determination (using TG analysis) of WE. (The red curve is temperature curve, the green curve is TG curve)

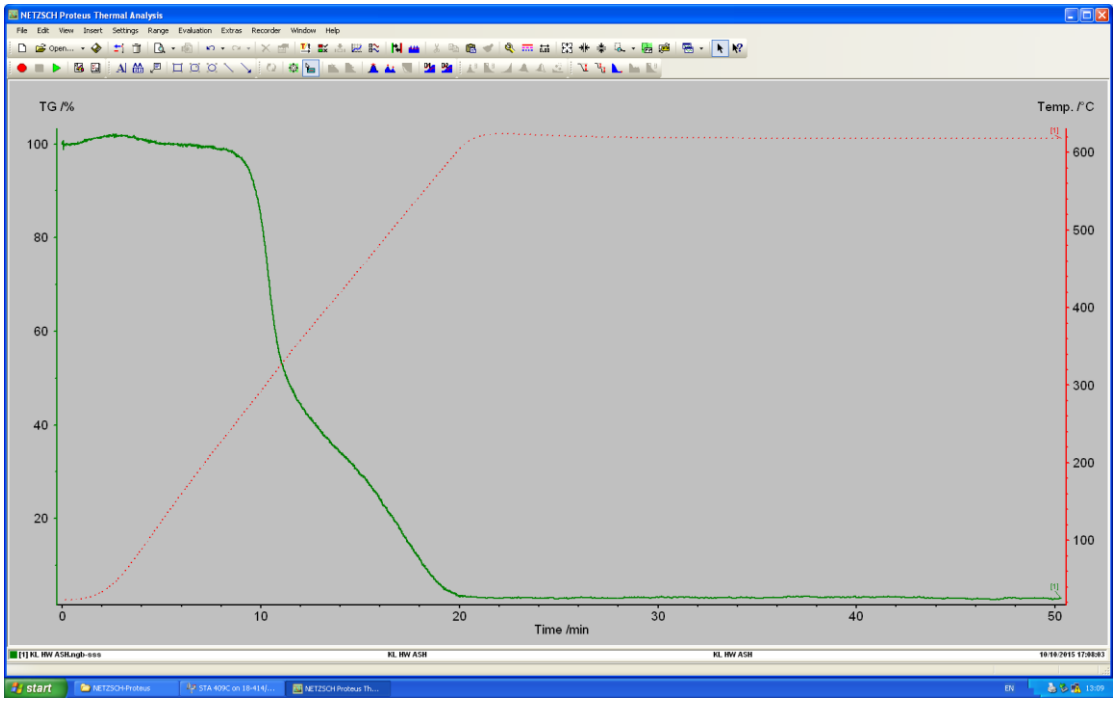


Figure 6-8b Ash content determination (using TG analysis) of Klason lignin from WE.

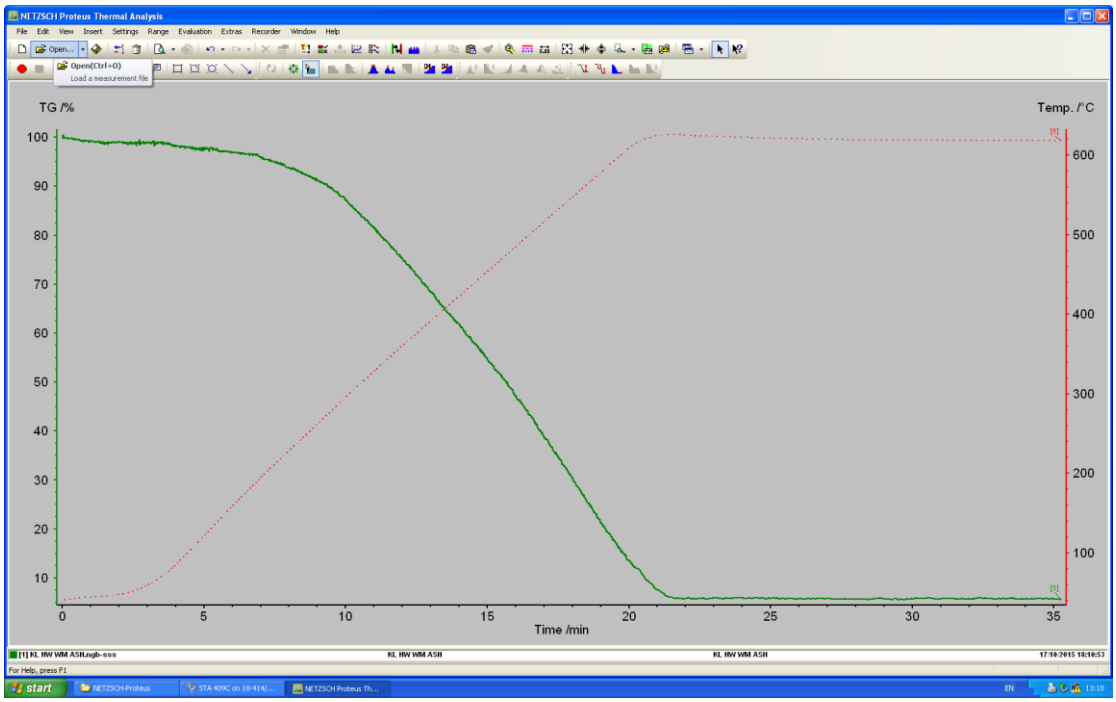


Figure 6-8c Ash content determination (using TG analysis) of Klason lignin of microwave isolated residue from WE, isolation condition: 190 ºC, 10min, 0.2 M sulfuric acid, 0.2 g feedstock in 15 ml solution.

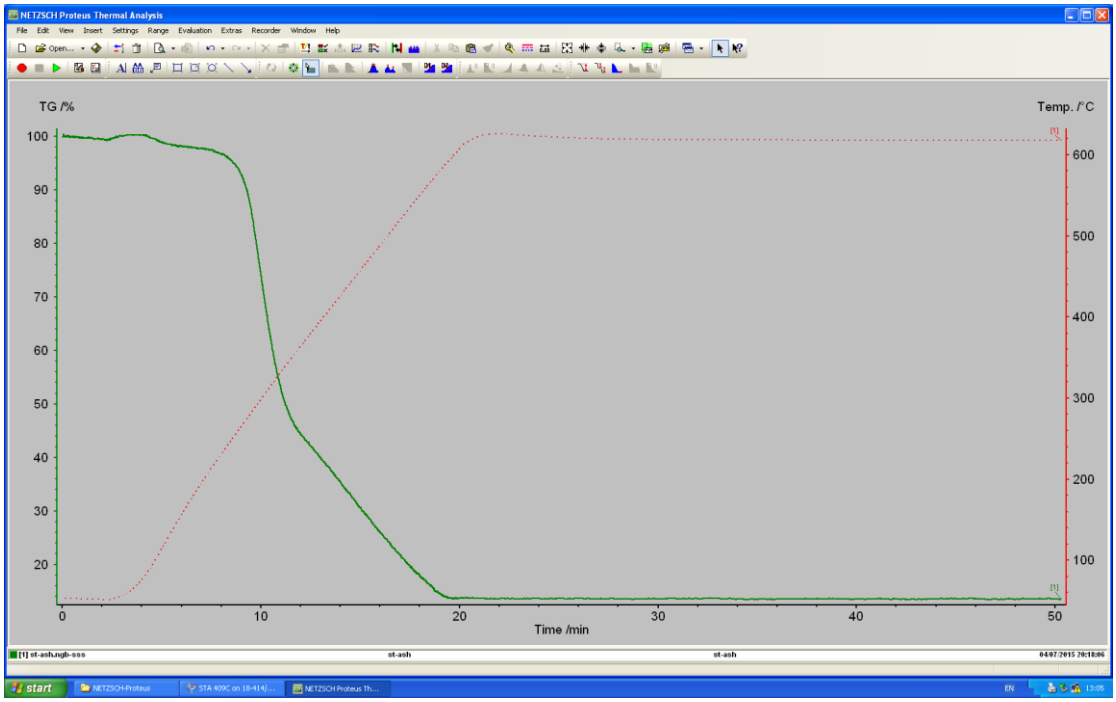


Figure 6-8d Ash content determination (using TG analysis) of WS.

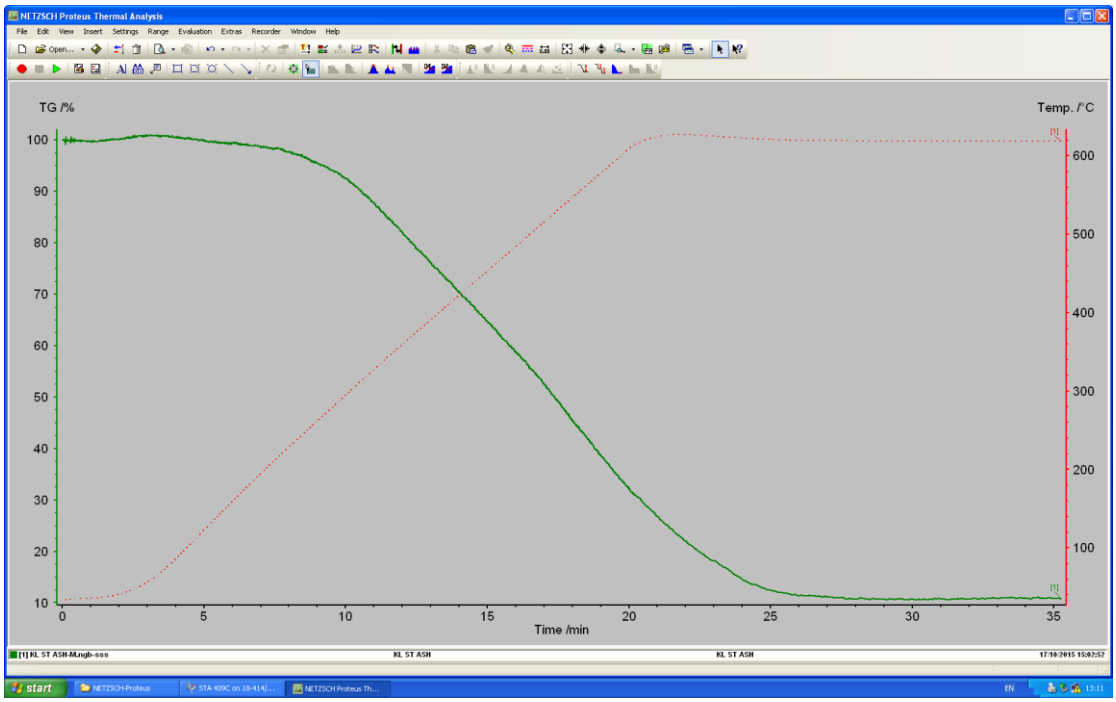


Figure 6-8e Ash content determination (using TG analysis) of Klason lignin from WS.

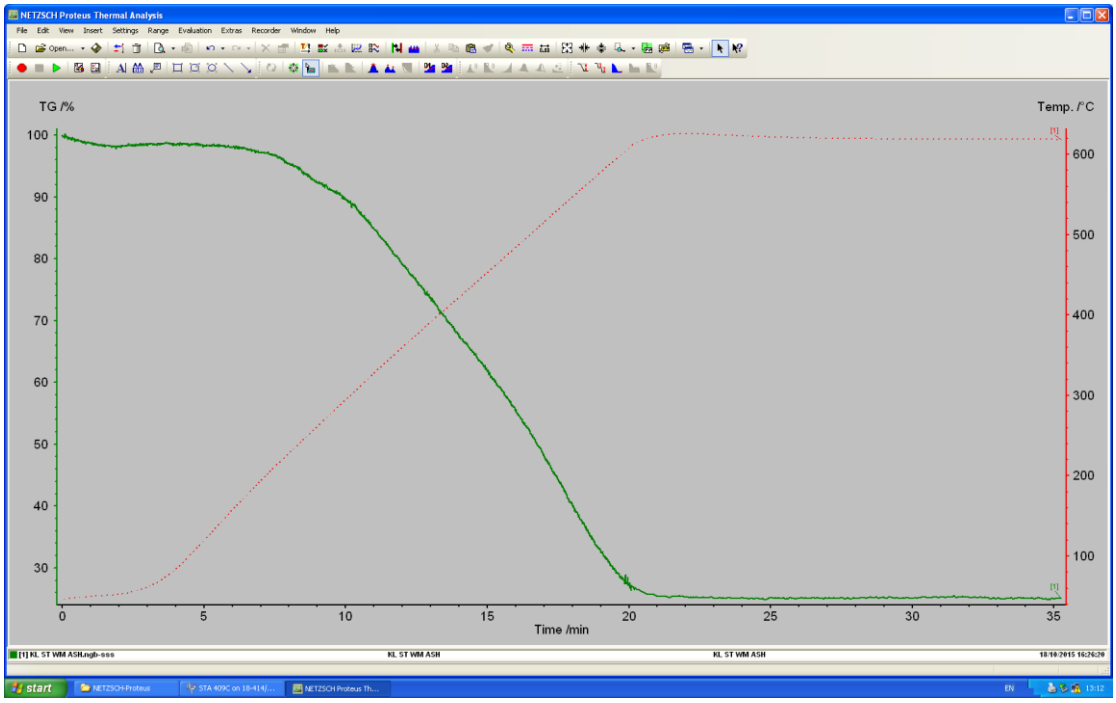


Figure 6-8f Ash content determination (using TG analysis) of Klason lignin of microwave isolated residue from WS, isolation condition: 190 ºC, 10min, 0.2 M sulfuric acid, 0.2 g feedstock in 15 ml solution.

6.2.2.5 FTIR analysis

The method and apparatus of FTIR analysis are same to those in 6.1.2.5. The spectra are offered in Figure 3-4, Figure 6-3 (unpresented spectra of MSP lignin in Chapter 3) and Figure 6-9 (unpresented spectra of WE and WS lignin in Chapter 3).

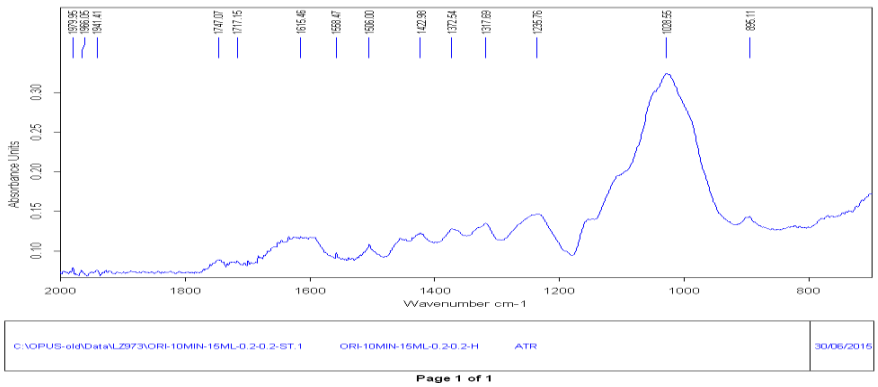


Figure 6-9a FTIR spectrum of WE

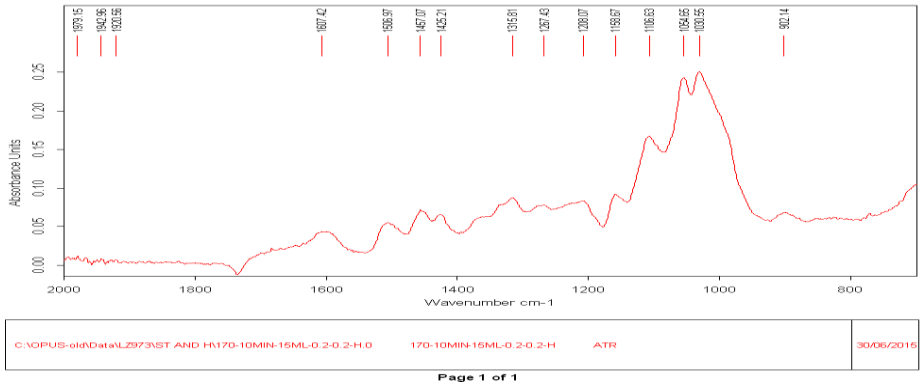


Figure 6-9b FTIR spectra of microwave residual lignin from WE, isolation condition: 170 ºC, 10min, 0.2 M sulfuric acid, 0.5 g feedstock in 15 ml solution.

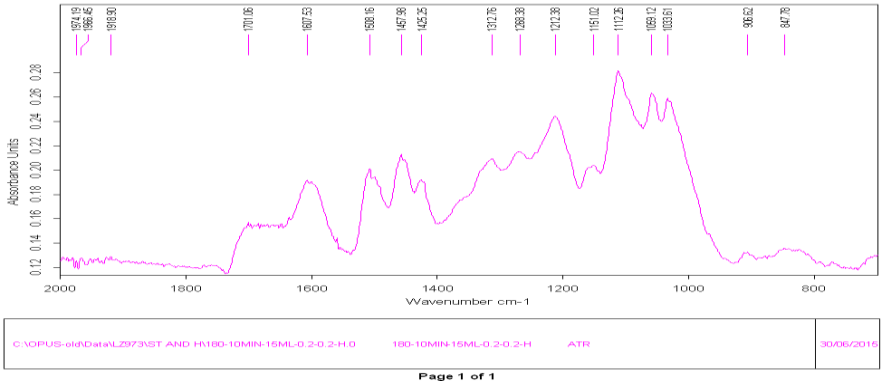


Figure 6-9c FTIR spectra of microwave residual lignin from WE, isolation condition: 180 ºC, 10min, 0.2 M sulfuric acid, 0.5 g feedstock in 15 ml solution.

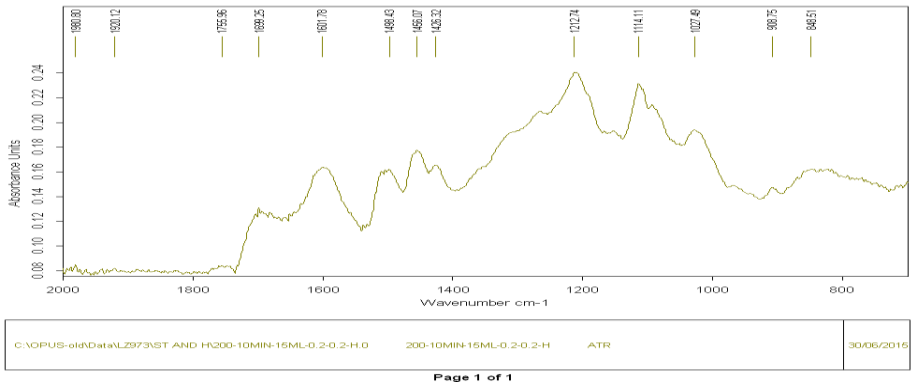


Figure 6-9d FTIR spectra of microwave residual lignin from WE, isolation condition: 200 ºC, 10min, 0.2 M sulfuric acid, 0.5 g feedstock in 15 ml solution.

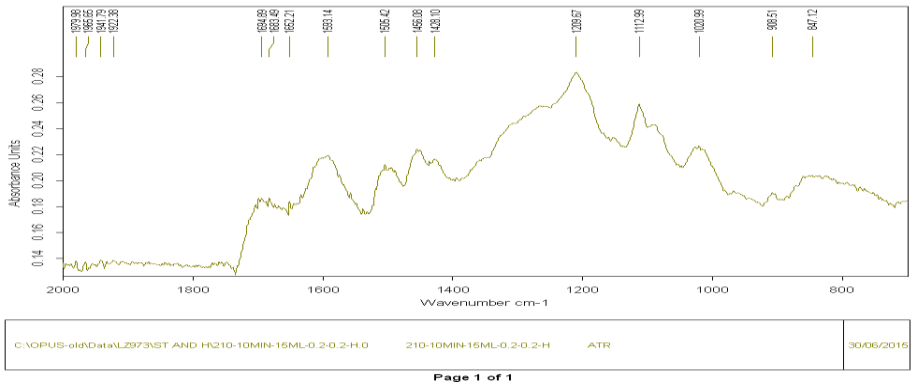


Figure 6-9e FTIR spectra of microwave residual lignin from WE, isolation condition: 210 ºC, 10min, 0.2 M sulfuric acid, 0.5 g feedstock in 15 ml solution.

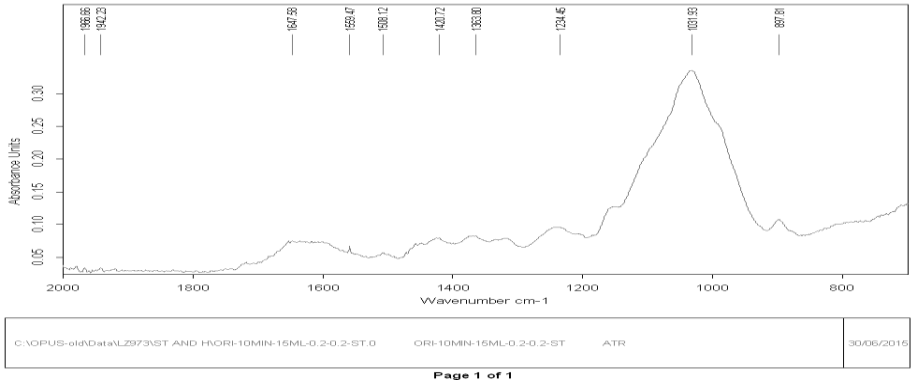


Figure 6-9f FTIR spectra WS.

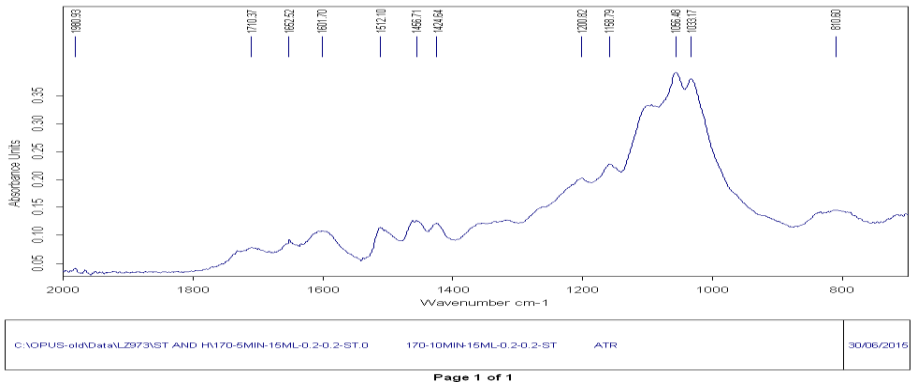


Figure 6-9g FTIR spectra of microwave residual lignin from WS, isolation condition: 170 ºC, 10min, 0.2 M sulfuric acid, 0.5 g feedstock in 15 ml solution.

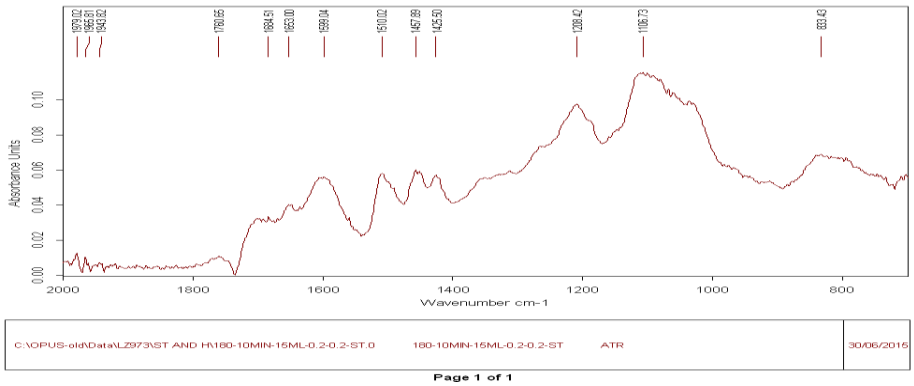


Figure 6-9h FTIR spectra of microwave residual lignin from WS, isolation condition: 180 ºC, 10min, 0.2 M sulfuric acid, 0.5 g feedstock in 15 ml solution.

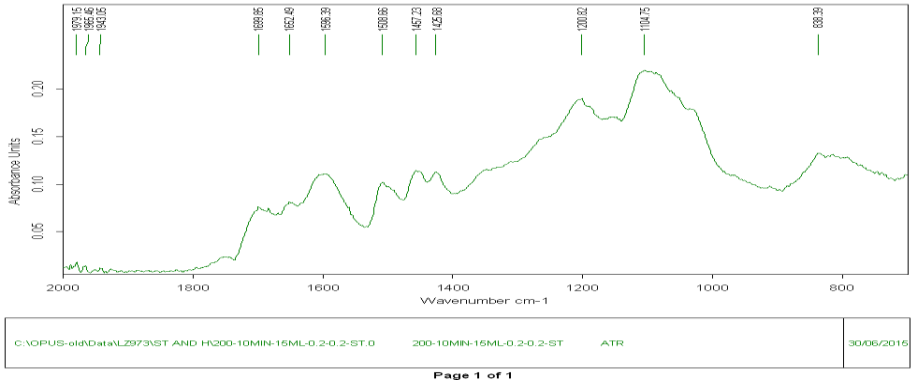


Figure 6-9i FTIR spectra of microwave residual lignin from WS, isolation condition: 200 ºC, 10min, 0.2 M sulfuric acid, 0.5 g feedstock in 15 ml solution.

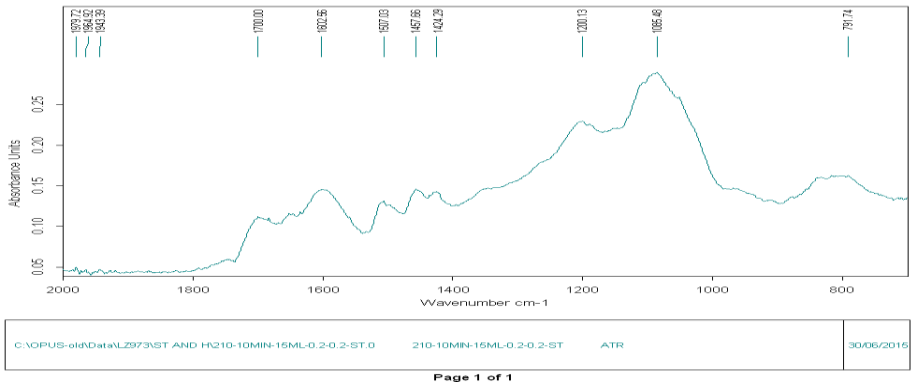


Figure 6-9j FTIR spectra of microwave residual lignin from WS, isolation condition: 210 ºC, 10min, 0.2 M sulfuric acid, 0.5 g feedstock in 15 ml solution.

6.2.2.6 Solid-state ^{13}C NMR spectroscopy analysis.

The method and apparatus are same to those in 6.1.2.6. The SSNMR spectra are shown in Figure 3-6.

6.2.2.7 Pyrolysis-GC/MS analysis

The method and apparatus of Py-GC/MS are same to those in 6.0.2.7. The results are given in Figure 3-7, Figure 6-4, Figure 6-10, Table 6-3 and Table 6-6.

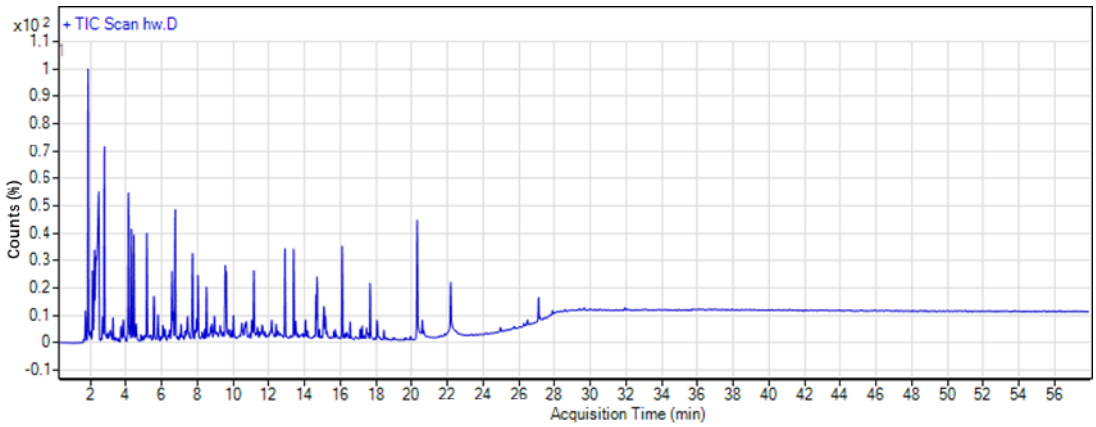


Figure 6-10a The GC spectrum of WE in Py-GC/MS

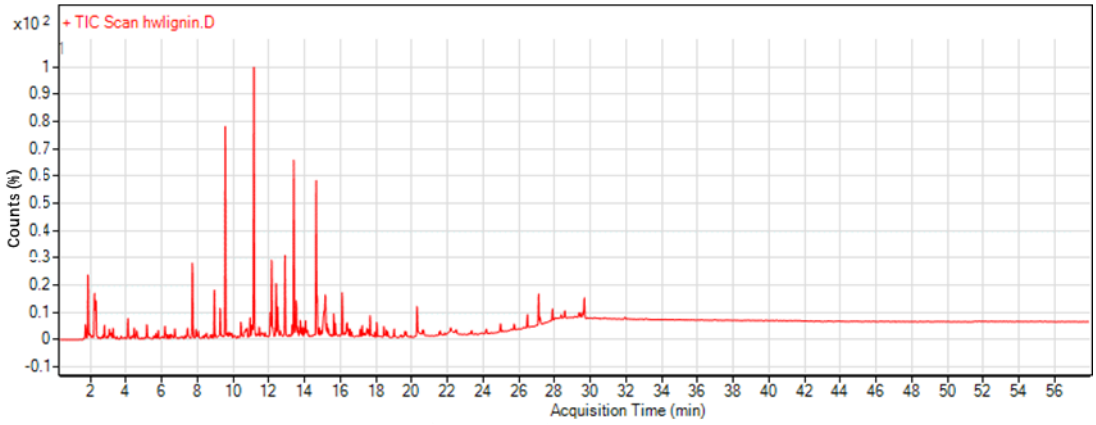


Figure 6-10b The GC spectrum of microwave isolated lignin from WE in Py-GC/MS, isolation conditions: 190 ºC, 10 min, 0.2 g feedstock in 15 ml 0.2 M H_2SO_4, in CEM discover microwave reactor

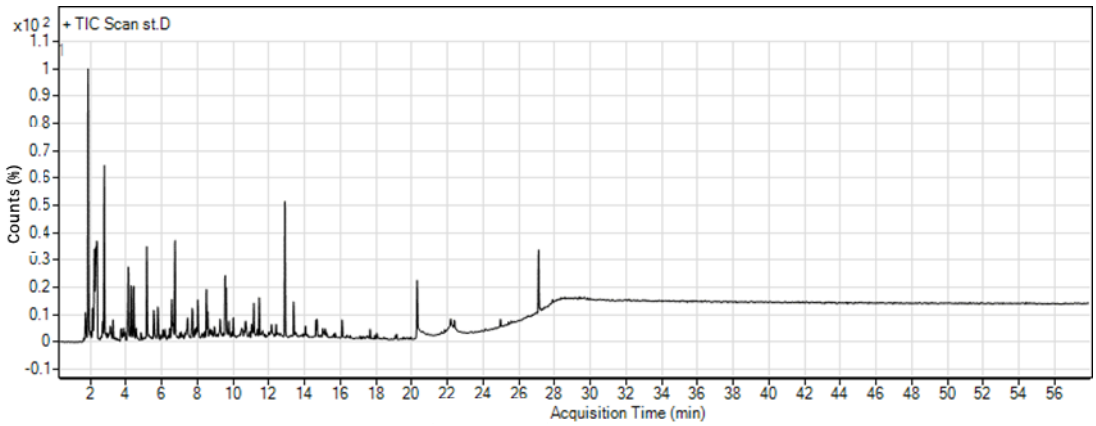


Figure 6-10c The GC spectrum of WS in Py-GC/MS

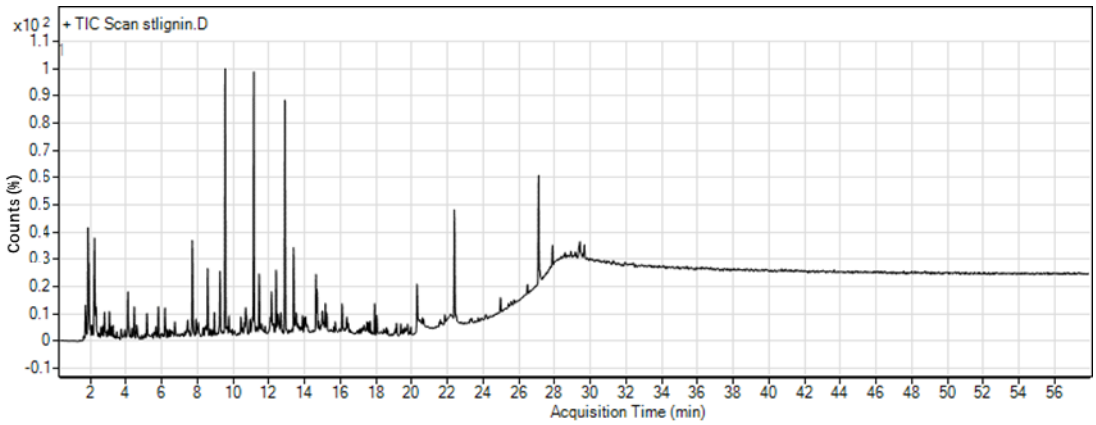


Figure 6-10d The GC spectrum of microwave isolated lignin from Ws in Py-GC/MS, isolation conditions: 190 ºC, 10 min, 0.2 g feedstock in 15 ml 0.2 M H_2SO_4, in CEM discover microwave reactor

Table 6-6a Compounds list of Py-GC/MS of WE

Name	Peak Area%	RT min
Methyl glyoxal	6.2	1.864
2-Amino-1,3-propanediol	1.6	2.116
Pyruvic acid	2.4	2.218
Acetic acid	12.5	2.458
Methacrolein	0.7	2.676
1-hydroxy-2-Propanone	4.4	2.779
3,4-dihydro-2H-Pyran	0.8	3.842
Acetylglycolic acid	3.6	4.127
Succindialdehyde	2.6	4.287
Propanoic acid, 2-oxo-, methyl ester	2.4	4.424
Furfural	2.5	5.156
2-Furanmethanol	1.4	5.556
5-Hexen-2-one	0.8	5.773
2(5H)-Furanone	1.8	6.562
Cyclohexanone	0.7	6.619
1,2-Cyclopentanedione	3.5	6.745
Phenol	2.4	7.716
4-methyl-Cyclohexanone	0.8	7.945
3,4-dihydro-2-methoxy-2H-Pyran	1.6	8.013
2-hydroxy-3-methyl-2-Cyclopenten-1-one	1.3	8.493
2-[2-(5-Norbornenyl)oxy]-tetrahydropyran	1.2	8.939
3-Nonyn-2-ol	0.7	9.259
2-methoxy-Phenol	1.5	9.545
6-Oxa-bicyclo[3.1.0]hexan-3-ol	1.8	9.591
4-Piperidinemethanamine	0.6	10.014
Creosol	1.8	11.145
4,4-diethyl-3-methylene-1-Oxetan-2-one	0.9	12.151
2-Methoxy-4-vinylphenol	2.1	12.894
2,6-dimethoxy-Phenol	2.0	13.374
1,2,4-Trimethoxybenzene	0.9	14.631
trans-Ieoeugenol	1.4	14.688
Levoglucosan	1.2	15.077
3',5'-Dimethoxyacetophenone	2.0	16.094
(E)-2,6-Dimethoxy-4-(prop-1-en-1-yl)phenol	1.3	17.66
1-(4-hydroxy-3,5-dimethoxyphenyl)-Ethanone,	0.8	18.049
n-Hexadecanoic acid	3.8	20.3
Octadecanoic acid	2.9	22.175

Table 6-6b Compounds list of Py-GC/MS of 190 ºC residual lignin from WE

Name	Peak area%	RT min
Butane	3.0	1.853
Pyruvic acid	1.8	2.218
Acetic acid	2.9	2.298
1,3-Hexadien-5-yne	0.7	2.778
3-methyl-1-Hexanol	0.6	3.053
2,5-dimethyl-Furan	0.5	3.27
1,3,5-Cycloheptatriene	0.8	4.093
1,5-dimethyl-1H-Pyrazole	0.6	5.156
Spiro[2.4]heptan-4-one	0.5	7.43
Phenol	2.9	7.705
2-methyl-Phenol	1.7	8.939
3-methyl-Phenol	1.3	9.259
2-methoxy-Phenol	7.6	9.545
2,6-dimethyl-Phenol	0.7	10.425
2-Methoxy-6-methylphenol	0.7	10.939
Creosol	10.7	11.145
3-methoxy-1,2-Benzenediol	3.5	12.139
4-ethyl-2-methoxy-Phenol	2.1	12.391
3-methyl-1,2-Benzenediol	1.5	12.471
2-Methoxy-4-vinylphenol	3.3	12.894
2,6-dimethoxy-Phenol	6.6	13.385
trans-m-Propenyl guaiacol	1.9	13.522
2,3-Dimethoxybenzyl alcohol	0.8	13.751
Vanillin	0.8	14.037
trans-Ieoeugenol	7.1	14.642
Levoglucosan	4.2	15.145
R-Limonene	0.5	15.237
5-tert-Butylpyrogallol	0.7	15.625
1-(3,4-dimethoxyphenyl)-Ethanone,	1.6	16.094
2,6-dimethoxy-4-(2-propenyl)-Phenol	0.9	17.66
1-(4-hydroxy-3,5-dimethoxyphenyl)-Ethanone	0.6	18.037
trans-Sinapyl alcohol	0.5	18.437
n-Hexadecanoic acid	1.9	20.289
Octadecanoic acid	1.3	22.175

Table 6-6c Compounds list of Py-GC/MS of WS

Name	Peak Area%	RT min
Methyl glyoxal	9.8	1.864
Pyruvic acid	3.7	2.218
Acetic acid	9.3	2.356
3-hydroxy-Butanal	0.8	2.676
1-hydroxy-2-Propanone	6.0	2.767
2,3-Epoxyhexanol	1.0	3.11
Ethyl-1-propenyl ether	0.7	3.259
1,2-Epoxy-3-propyl acetate	3.1	4.116
Succindialdehyde	1.9	4.276
Methyl pyruvate	2.0	4.424
Furfural	3.4	5.156
2-Furanmethanol	1.4	5.544
3-methyl-4-Penten-2-one	1.5	5.773
cis-2-Hexene	2.3	6.55
1,2-Cyclopentanedione	3.6	6.733
Phenol	1.4	7.705
3,4-dihydro-2-methoxy-2H-Pyran	1.6	8.013
Corylon	1.6	8.493
D-Limonene	0.8	8.562
3-methyl-Phenol	1.0	9.259
2-methoxy-Phenol	3.8	9.545
Creosol	1.2	11.145
Coumaran	1.3	11.442
4-ethyl-2-methoxy-Phenol	0.6	12.391
2-Methoxy-4-vinylphenol	4.5	12.882
2,6-dimethoxy-Phenol	1.1	13.374
trans-m-Propenyl guaiacol	0.6	14.688
d-Mannose	0.6	15.008
3-tert-Butyl-4-hydroxyanisole	0.6	16.094
1-(4-hydroxy-3,5-dimethoxyphenyl)-Ethanone,	0.2	18.037
n-Hexadecanoic acid	3.4	20.3
Octadecanoic acid	1.3	22.38

Table 6-6d Compounds list of Py-GC/MS of 190 ºC residual lignin from WS

Name	Peak Area%	RT min
Methyl glyoxal	5.6	1.853
5-Methylhexan-5-olide	4.5	2.219
1,3-Hexadien-5-yne	0.6	2.779
1-Heptene	0.6	3.053
1,5-Heptadien-3-yne	1.5	4.093
1-ethynyl-1-cyclohexene	0.9	5.796
Phenol	3.1	7.705
Limonene	1.7	8.562
3-methyl-Phenol	2.1	9.259
2-methoxy-Phenol	7.9	9.545
Creosol	7.7	11.145
2-methyl-Benzaldehyde	1.9	11.442
4-ethyl-2-methoxy-Phenol	1.8	12.391
Ethyl 2-[(4-methylphenyl)amino]propanoate	0.7	12.665
2-Methoxy-4-vinylphenol	7.2	12.883
2,6-dimethoxy-Phenol	2.5	13.374
2,5-Octadecadiynoic acid, methyl ester	1.4	13.523
4-methoxy-3-(methoxymethyl)-Phenol	1.8	14.631
trans-m-Propenyl guaiacol	1.2	14.688
Arachidonic acid methyl ester	1.6	14.986
3-tert-Butyl-4-hydroxyanisole	1.1	16.094
1-(4-hydroxy-3,5-dimethoxyphenyl)-Ethanone,	0.6	18.037
n-Hexadecanoic acid	4.3	20.289
4,4'-(1-methylethylidene)bis-Phenol	4.4	22.381

6.2.2.8 Yeast cultures

Inoculum cultures for both yeasts were prepared by culturing in 10 mL of SPME media (soy peptone: 30 g L^{-1}; malt extract: 25 g L^{-1}) and 10 mL of YPD media (yeast extract: 10 g L^{-1}; peptone: 20 g L^{-1}; glucose: 20 g L^{-1}) and were incubated at 20 °C and 25 °C for *M. pulcherrima* and *C. curvatus*, respectively, with an agitation of 180 rpm for 24 h. The hydrolysates prepared from the microwave processing stage were filtered, adjusted to pH 4 with 2 molar NaOH solution, then diluted to a 1 : 2 ratio with a salt solution. For *M. pulcherrima*, the salt solution was composed of the following: KH_2PO_4 (2 g L^{-1}),

$MgSO_4 \cdot 7H_2O$ (0.376 g L^{-1}), $MgCl_2 \cdot 6H_2O$ (2.16 g L^{-1}), $ZnSO_4 \cdot 7H_2O$ (0.04 g L^{-1}), $(NH_4)_2SO_4$ (0.126 g L^{-1}), NH_4Cl (0.708 g L^{-1}), yeast extract (2.00 g L^{-1}), $CaCl_2 \cdot 2H_2O$ (0.300 g L^{-1}) and tartaric acid (30.0 g L^{-1}). The pH was adjusted to 4 before diluting. For *C. curvatus*, the concentrated salt solution was composed of the following: KH_2PO_4 (14.0 g L^{-1}), Na_2HPO_4 (5.00 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (3.00 g L^{-1}), $FeCl_3 \cdot 6H_2O$ (0.300 g L^{-1}), $ZnSO_4 \cdot 7H_2O$ (0.040 g L^{-1}), $MnSO_4 \cdot 1H_2O$ (0.120 g L^{-1}), $CaCl_2 \cdot 2H_2O$ (0.300 g L^{-1}), $(NH_4)_2SO_4$(1.00 g L^{-1}) and yeast extract (2.00 g L^{-1}). The pH was adjusted to 6.5 before diluting.

1 mL cultures were inoculated with 25 μL of inoculum in 24-well plates and incubated at 180 rpm at 20 °C and 25 °C for *M. pulcherrima* and *C. curvatus*, respectively, for 168 h. Throughout the fermentation process the growth of the cultures was estimated by analysis of the absorbance at 600 nm and the biomass was recovered by centrifugation in 1.5 Eppendorf tubes (10 000 rpm, 5 min). The supernatant and the pellets were stored separately at −20 °C prior to further analysis.

For the characterization of the fatty acid profile, an adapted literature method was used,[273] whereby the biomass pellets were suspended in methanol/H_2SO_4 1% v/v and heated at 90 °C for 3 h in sealed pressure tubes. The resulting fatty acid methyl esters were extracted with hexane, washed with water and analysed using GC-MS.

The DP1 and DP2 saccharides were analysed using an Agilent 1260 Infinity HPLC system (USA) equipped with an Agilent Hi-Plex H (300 × 7.7 mm, 8 μm particle size) column. For levoglucosan, glucose, fructose, xylose, cellobiose, rhamnose and the organic acids (lactic, formic and acetic acids), an isocratic (no gradient) mobile phase of

0.005 M H_2S0_4, a flow-rate of 0.4 mL min^{-1}, a column temperature of 60 °C, a refractive index detector at 55 °C, a total run time of 35 minutes, and an injection volume of 5 µL were used. For inhibitor analysis (furfural, levoglucosenone and 5-HMF), the following parameters were used: an ACE C18 column (250 × 4.6 mm, 5 µm particle size), an isocratic (no gradient) mobile phase of acetonitrile : water (25/75), a flow-rate of 0.8 mL min^{-1}, a column temperature of 30 °C, a 220 nm DAD detector, a total run time of 22 minutes, and an injection volume of 5 µL. For both experiments, five samples of mixed standard chemicals (0.5, 0.75, 1.0, 1.5 and 2.0 mg mL^{-1}) were used for the calibration.

The samples also had their oligosaccharide profiles analysed using a Dionex ICS-5000 HPLC system (Thermo Scientific, USA) with a Dionex CarboPac SA10 column (4 × 250 mm) and guard column with a 1.5 mL min^{-1} flow rate using pulsed amperometric detection. The separation was carried out at 25 °C for 10 min using gradient elution with 1 mM sodium hydroxide (NaOH) at 1.5 mL min^{-1} and a 10 µL injection volume. The samples were appropriately diluted in Milli-Q water and filtered through a 0.22 µm syringe filter prior to analysis.

The yeast cultures results are shown in Figure 3-8, Figure 3-9 and Figure 3-10. The HPLC results are shown in Table 6-7. The microwave treatment conditions of each trial:

Trial 1, feedstock/solution ratio 13.3g L^{-1}, 170 ºC;

Trial 2, feedstock/solution ratio 13.3g L^{-1}, 190 ºC;

Trial 3, feedstock/solution ratio 33 g L^{-1}, 190 ºC;

Trial 4, feedstock/solution ratio 66 g L^{-1}, 190 ºC;

Trial 5, feedstock/solution ratio 100 g L^{-1}, 190 ºC.

For all 5 trials, the holding time and solution are 10 min and 0.2 M H_2SO_4, respectively.

Table 6-7a The concentration of mono-/di-sugars, organic acids and sugar dehydration products in hydrolysate before fermentation (mg/mL)

TRIALS	1	2	3	4	5
Cellobiose	0.0015	0.0000	0	0.073754	0.069923
Glucose	0.9234	0.8777	1.011303	3.048679	3.197313
Xylose & fructose	0.5034	0.0968	0.109956	0.226181	0.245211
Rhamnose	0.0891	0.0171	0.047127	0.06187	0.068964
Levoglucosan	0.0915	0.0853	0.101712	0.226719	0.375763
Lactic Acid	0.0140	0.0264	0.089916	0.226719	0.375763
Formic Acid	0.1849	0.7329	1.700197	3.237685	4.837249
Acetic Acid	0.2569	0.2982	0.742105	1.190291	1.420105
HMF	0.1516	0.0881	0.461813	0.665673	0.933645
Levoglucosenone	0	0.0067	0.068451	0.152468	0.256873
Furfural	0.3349	0.3679	0.704702	1.208777	1.343329

Table 6-7b The concentration of mono-/di-sugars, organic acids and sugar dehydration products in hydrolysate after fermentation by *C. curvatus* (mg/mL)

TRIAL	1	2	3	4	5
Cellobiose	0.0038	<0.001	<0.001	<0.001	<0.001
Glucose	0.3231	0.2319	0.569796	1.229616	1.581205
Xylose and fructose	0.3421	<0.001	<0.001	0.010155	0.026539
Rhamnose	0.013	<0.001	<0.001	<0.001	<0.001
Levoglucosan	<0.001	<0.001	<0.001	<0.001	<0.001
Lactic acid	0.0097	0.0174	0.088376	0.097643	0.052715
Formic acid	0.119	0.481	1.068898	1.577447	1.991716
Acetic acid	0.1677	0.1861	0.683147	0.892445	0.946714
HMF	0.09126	0.06916	0.170523	0.277707	0.324714
Levoglucosenone	<0.001	<0.001	0.112928	0.086011	0.085566
Furfural	<0.001	<0.001	<0.001	<0.001	<0.001

Table 6-7c The concentration of mono-/di-sugars, organic acids and sugar dehydration products in hydrolysate after fermentation by *M. pulcherrima* (mg/mL)

TRIALS	1	2	3	4	5
Cellobiose	0.0024	<0.001	0.012479	0.006681	0.019958

Glucose	0.2824	0.2008	0.325796	0.637624	0.958172
Xylose and fructose	0.3257	0.0014	<0.001	<0.001	0.013949
Rhamnose	0.007	<0.001	<0.001	<0.001	<0.001
Levoglucosan	<0.001	<0.001	<0.001	<0.001	<0.001
Lactic acid	0.0084	0.0159	0.079563	0.09512	0.083387
Formic acid	0.096	0.516	1.020142	1.436043	1.876194
Acetic acid	0.1744	0.1811	0.630598	0.804813	0.951135
HMF	0.09945	0.0423	0.250573	0.306492	0.326895
Levoglucosenone	0.0027	<0.001	0.125912	0.143876	0.099484
Furfural	<0.001	0.0063	<0.001	<0.001	<0.001

6.3 Chapter 4

6.3.1 Materials

The bilberry presscake was provided by The Swedish University of Agricultural Sciences. The raw material was placed in an oven at 70 °C for 3 days until a constant weight was obtained (70% mass loss through removal of water). The dried bilberry presscake (DBP) contained bilberry fruit (average diameter 1 cm), leaves, stems, and bilberry seeds. No further processing was performed on all materials employed in lab scale trials. For pilot scale conversion, bilberry presscake was processed as received without drying (wet bilberry presscake, WBP) but with maceration using a Robot Coupe Blixer 4vv instrument to form a slurry with a maximum particle size of 4 mm diameter.

Glucose, fructose, rhamnose, formic acid, and furfural were purchased from Sigma-Aldrich. Levoglucosan and 5-hydroxymethylfurfural (HMF) were purchased from Carbosynth. Xylose was purchased from VWR. Cellobiose was purchased from Fluorochem. Lactic acid was purchased from Wardle. Acetic acid was purchased from Alfa Aesar. Levoglucosenone was purchased from Dextra.

Acetone, ethanol, and heptane were purchased from VWR chemicals. Deionized water was obtained from an internal source in the lab.

6.3.2 Experimental methods and Data

6.3.2.1 Hydrolysis of dried bilberry presscake

A CEM Mars 6 microwave reactor (USA) was used for the experiments. Dried bilberry presscake (DBP) was combined with deionised water (60 mL) at different ratios in microwave vessels prior to microwave hydrolysis. The experimental conditions are outlined in Table 4-5. (Trials 9r−12r are repeat experiments.) Samples were heated to their target temperature, with a set ramping time of 5 min (variable ramping rate). Following each trial, the samples were filtered to obtain the solid residue and liquid phase for further analysis. The solid residue was weighed (after drying at 105 °C) to calculate the conversion as follows:

$$M_r = m_1/m_0 \times 100\%$$

$$M_c = 100\% - M_r$$

M_r, *residue mass, wt %*

M_c, *conversion, wt %*

m_1, *residue mass, g*

m_0, *mass of the original feedstock, g*

The optimum conditions for microwave hydrolysis of bilberry waste presscake to produce a high yield of hydrolysate were investigated according to a Box−Behnken design with 15 runs (3 center points) as Tables 4-1 and Table 4-2. The variance analysis based Box-

Benhken design are shown in Table 4-3 and Table 6-8 (optimised variance analysis).

Table 6-8 Optimised variance analysis by excluding AB and A^2.

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F
Model	952.51	7	136.07	70.27	< 0.0001
A	258.90	1	258.90	133.70	< 0.0001
B	85.54	1	85.54	44.18	0.0003
C	491.57	1	491.57	253.86	< 0.0001
AC	21.86	1	21.86	11.29	0.0121
BC	39.82	1	39.82	20.56	0.0027
B^2	17.16	1	17.16	8.86	0.0206
C^2	33.94	1	33.94	17.53	0.0041
Residual	13.55	7	1.94		
Lack of Fit	11.23	5	2.25	1.93	0.3755
Pure Error	2.33	2	1.16		
Cor Total	966.06	14			

Soxhlet extraction was carried out using a 250 mL round-bottom flask (RBF) heated with a heating plate set at 20 °C above the solvent boiling point. The Soxhlet apparatus was directly connected to the RBF and a water-cooled condenser (Figure 6-11). A 6 g portion of the DBP was loaded in a cellulose thimble and carefully placed in the Soxhlet extraction chamber. A 150 mL portion of the selected solvent was charged in the RBF with a magnetic stirrer. The system was then assembled and heated for 4 h (corresponding to roughly 4−5 cycles depending on the solvent). In the case of the water extraction the system was left for 24 h. The samples were filtered to obtain the solid residue and liquid phase for further analysis.

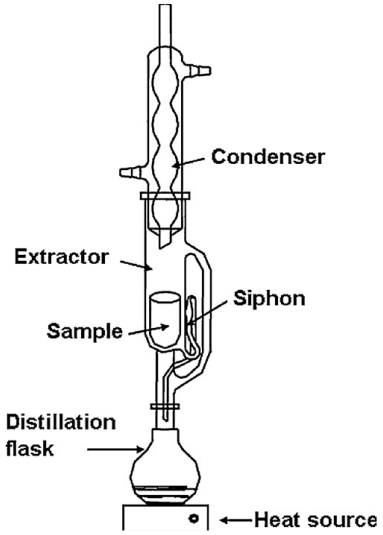


Figure 6-11 Soxhlet extraction diagram

The pilot scale trials were carried out using a modified pyrolysis microwave (Sairem Labotron Pyro, 60K Pyro). The modifications (Figure 6-12) included removal of the existing feed hopper and auger and disconnection of the char collector followed by the installation of a nonmetallic double diaphragm pump, stainless steel separation vessel, and new hopper and hoses. For each trial, 5 kg of bilberry presscake (the equivalent of 1.5 kg of dried bilberry presscake per run) was defrosted, macerated in a Robot Coupe Blixer 4vv instrument, and mixed with a total of 12 L of deionized water to form a slurry with a maximum particle size of 4 mm diameter (chosen due to the solids handling capability of the pump). The slurry was charged in to the microwave and then recirculated for 10 min, at 230 L min^{-1} to ensure a homogeneous mixture. The trials were carried out in triplicate. Microwave power was applied, initially at 1 kW for microwave leakage tests to be carried out and then, following safety testing, at 6 kW until the target temperature of 95 °C was reached. The bilberry presscake/water slurry was recirculated at 95 °C for 60 min. Then, 400 mL samples were taken at 30 and 60 min time points from the three trials (Trials P1-30, P1-60, P2-30, P2-60, P3-30, and P4-60, respectively). The conversion

ratios are shown in Table 6-9.

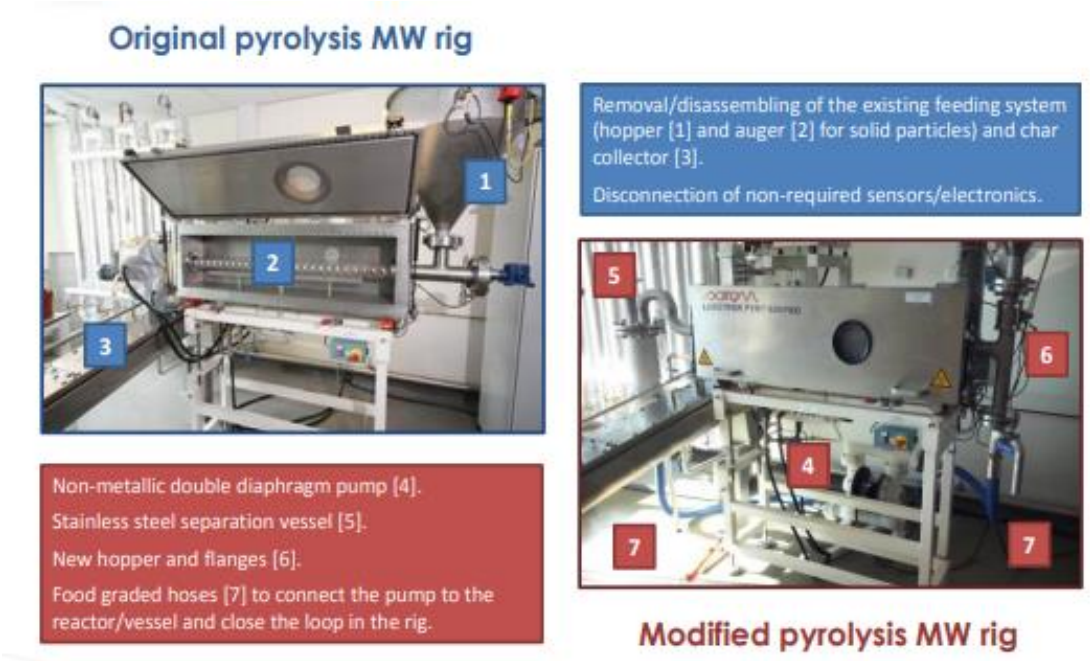


Figure 6-12 Modified microwave pyrolysis rig used for pilot-scale trials

Table 6-9 Conversion for Trials 1, 2 and 3 at pilot scale

Sample	Conversion (%)
Trial P1-30 minutes	53.1
Trial P1-60 minutes	56.1
Trial P2-30 minutes	52.3
Trial P2-60 minutes	52.8
Trial P3-30 minutes	68.3
Trial P3-60 minutes	69.6

6.3.2.2 Elemental analysis

The elemental analysis method and apparatus are same to those in 6.1.2.3. The results are given in Table 4-6.

6.3.2.3 Thermal gravimetric analysis

The thermal gravimetric analysis method and apparatus are same to those in 6.1.2.4. The results are given in Figure 4-2 and Figure 6-13.

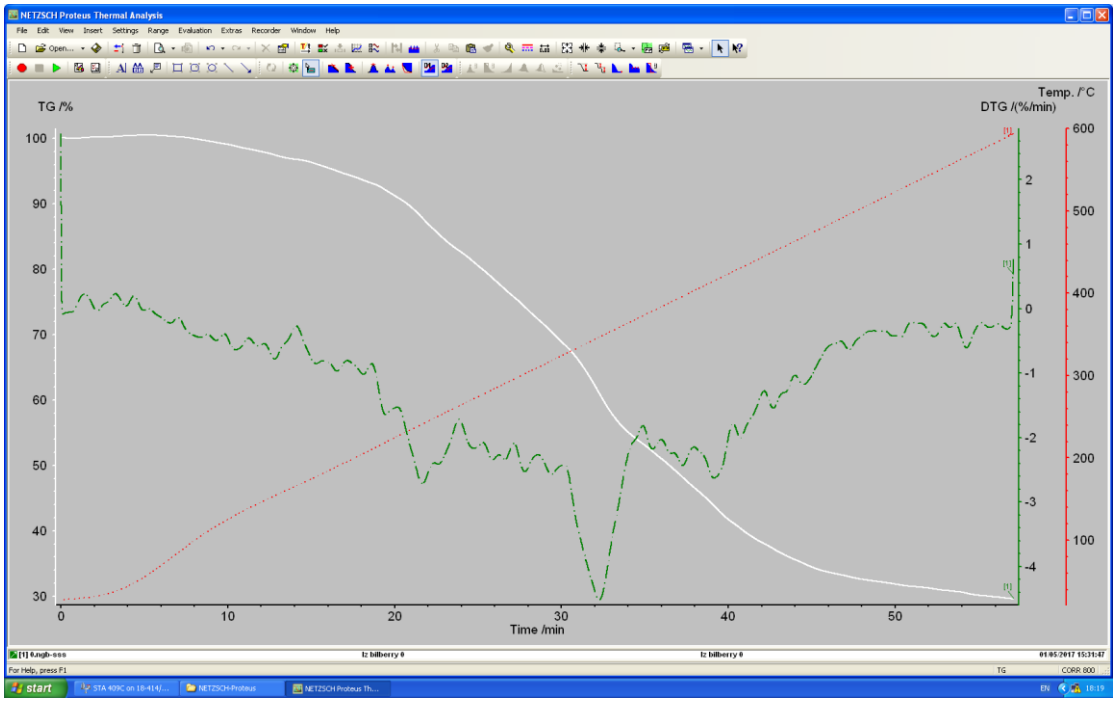


Figure 6-13a) TG curves of DBP (The white curve is TG curve, the green curve is DTG curve, the red curve is temperature curve)

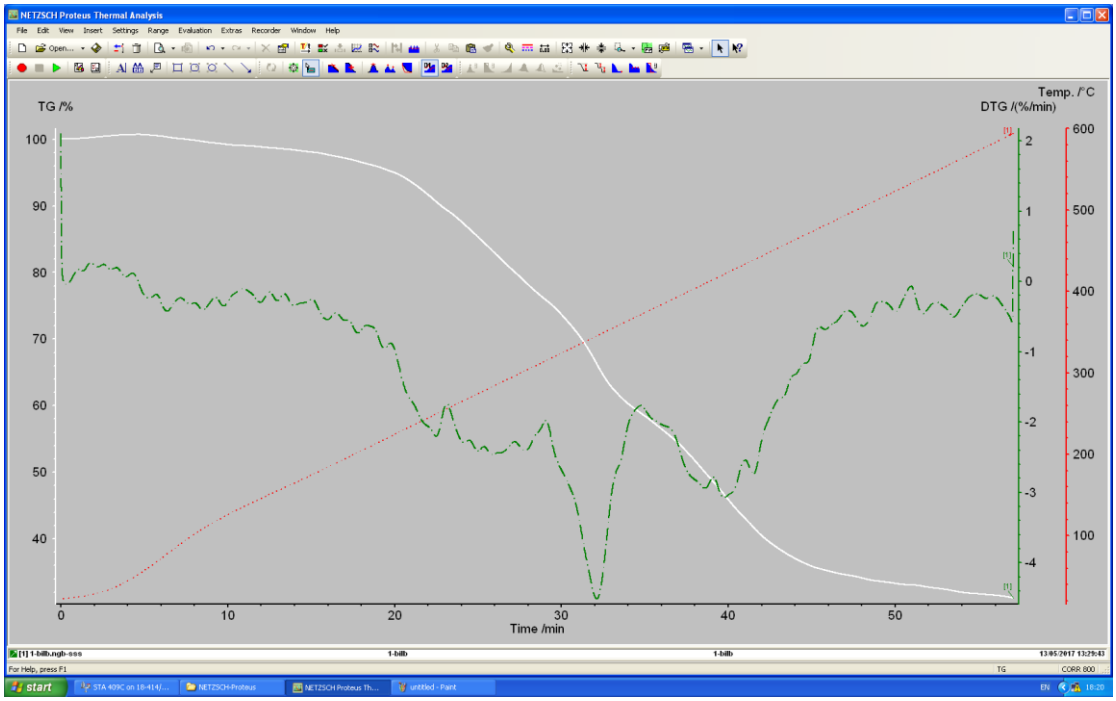


Figure 6-13b) TG curves of residue of Trial 1 (condition in Table 4-5)

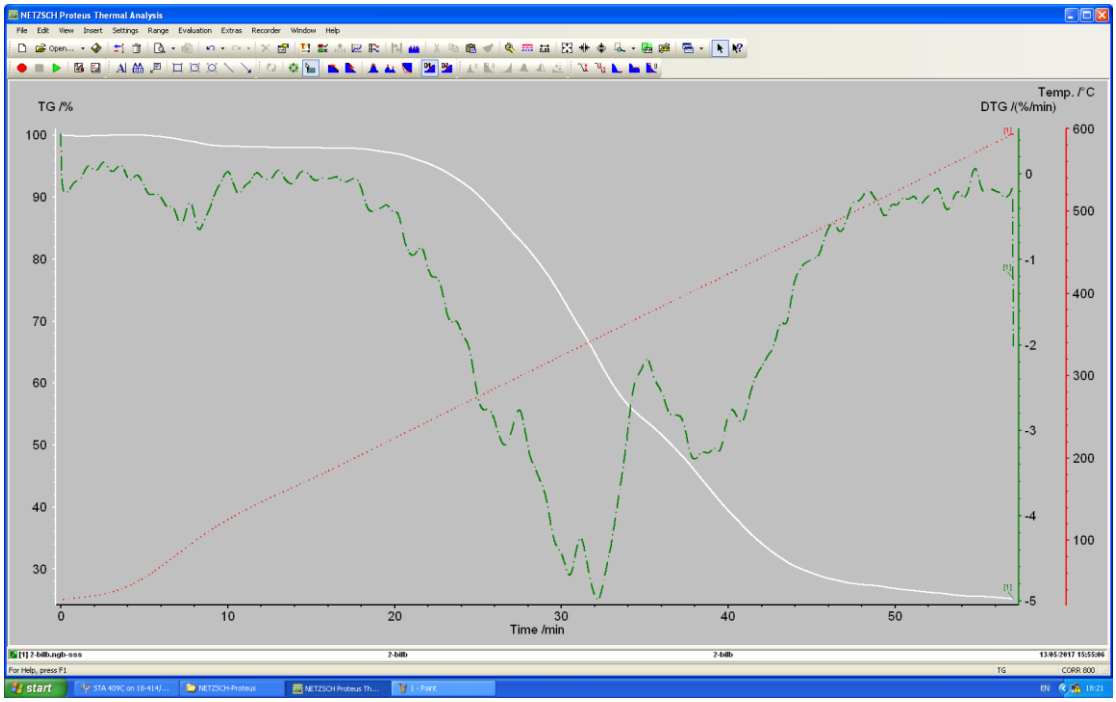


Figure 6-13c) TG curves of residue of Trial 2 (condition in Table 4-5)

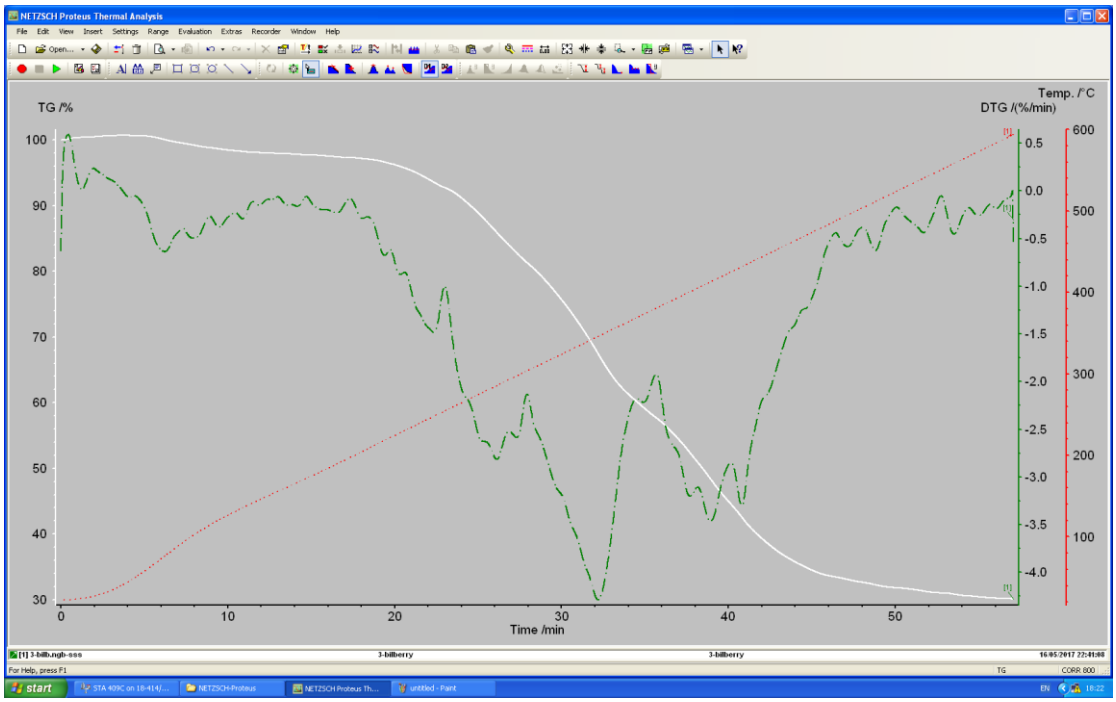


Figure 6-13d) TG curves of residue of Trial 3 (condition in Table 4-5)

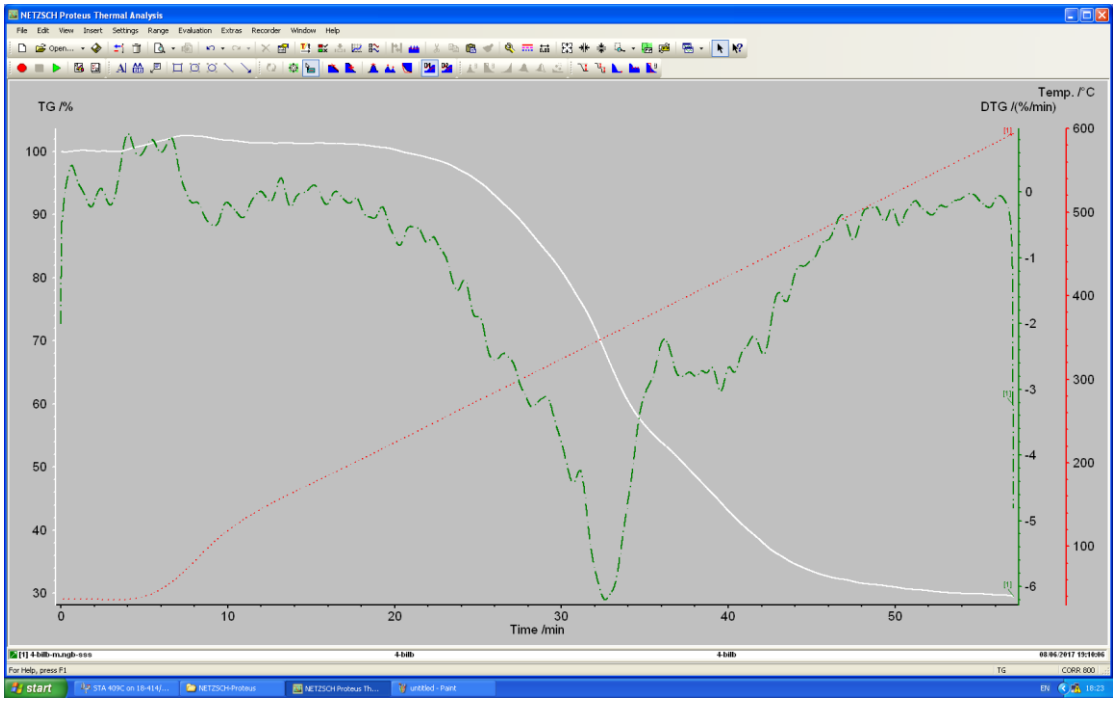


Figure 6-13e) TG curves of residue of Trial 4 (condition in Table 4-5)

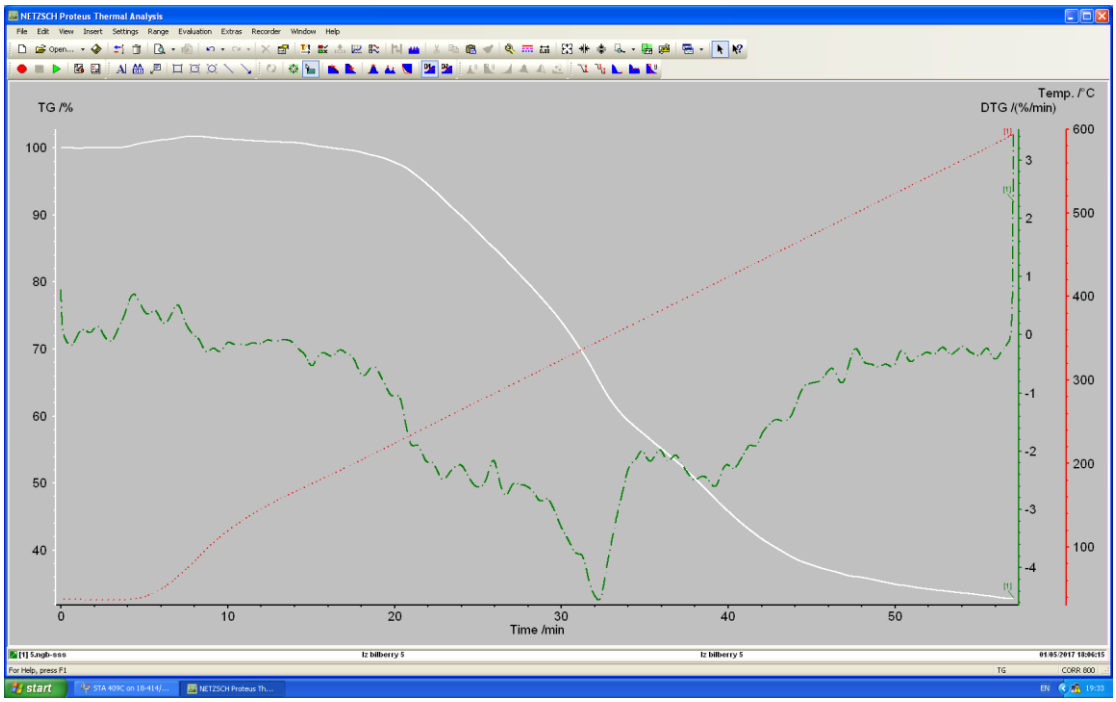


Figure 6-13f) TG curves of residue of Trial 5 (condition in Table 4-5)

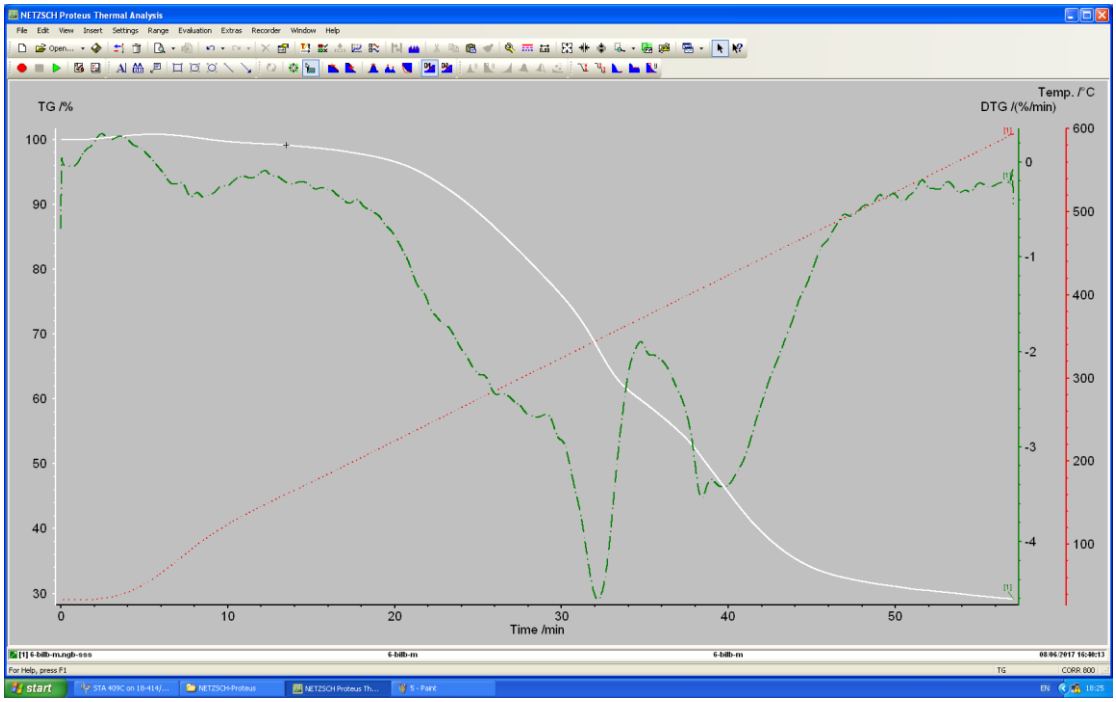


Figure 6-13g) TG curves of residue of Trial 6 (condition in Table 4-5)

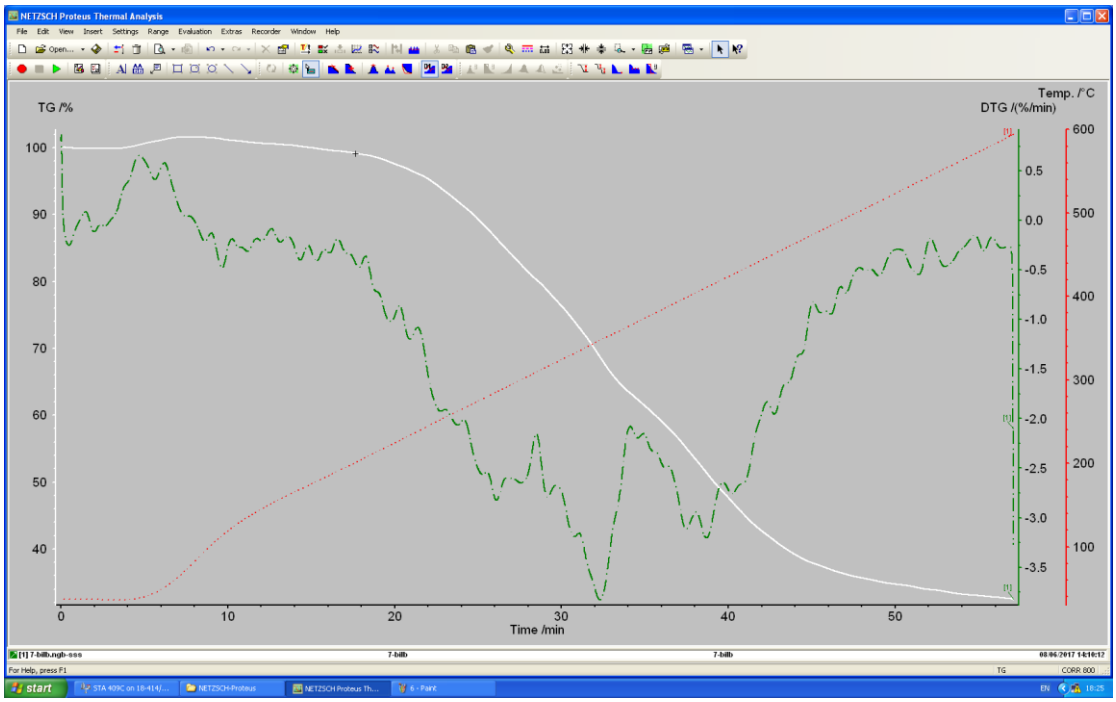


Figure 6-13h) TG curves of residue of Trial 7 (condition in Table 4-5)

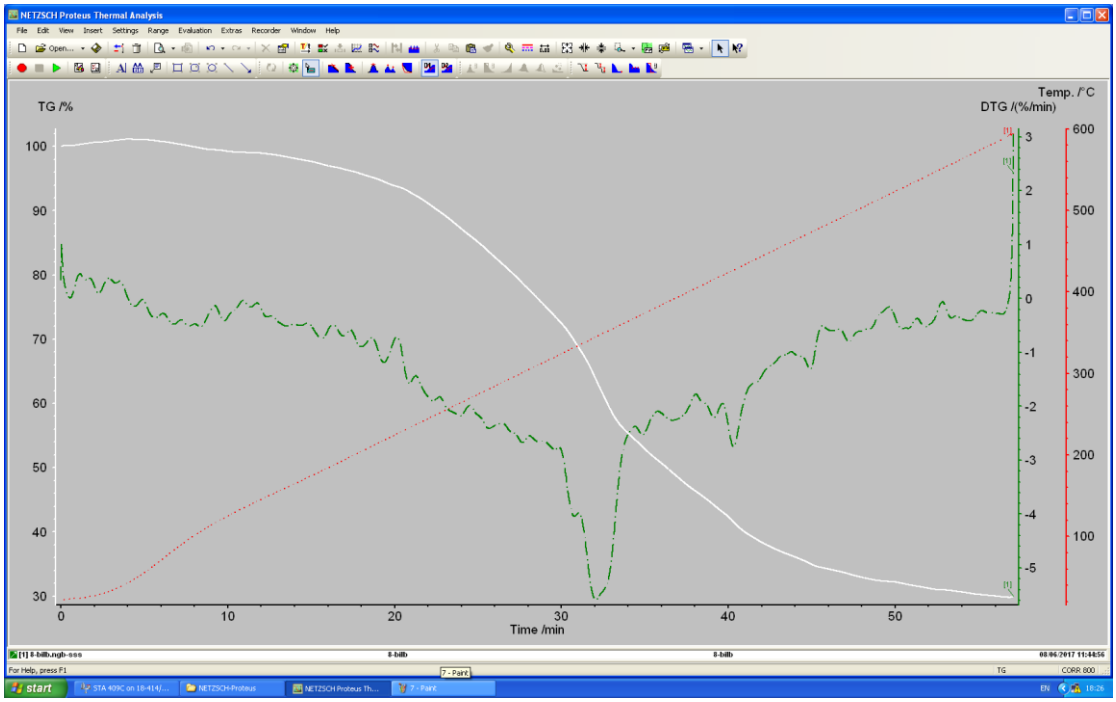


Figure 6-13i) TG curves of residue of Trial 8 (condition in Table 4-5)

6.3.2.4 FTIR Spectroscopy analysis

The method and apparatus of FTIR analysis are same to those in 6.1.2.5. The spectra are offered in Figure 4-3 and Figure 6-14.

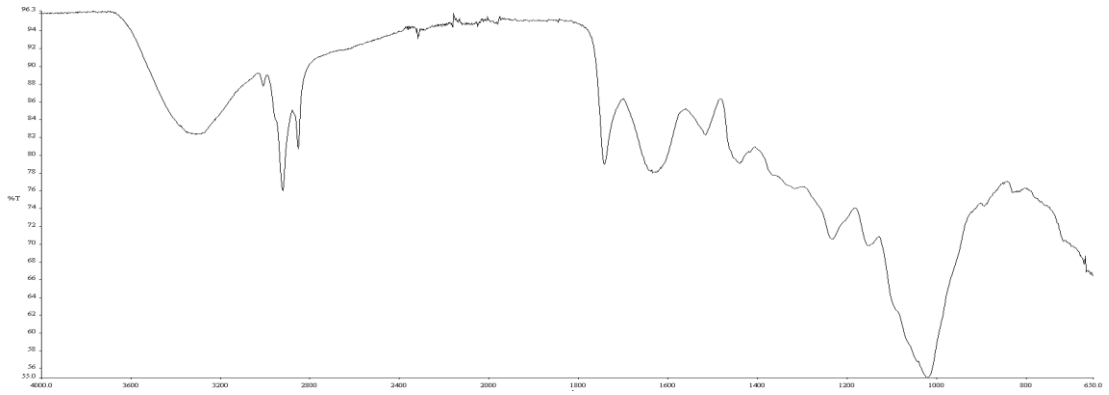


Figure 6-14a) FTIR spectrum of hydrolysis residue of Trial 5 (conditions in Table 4-5)

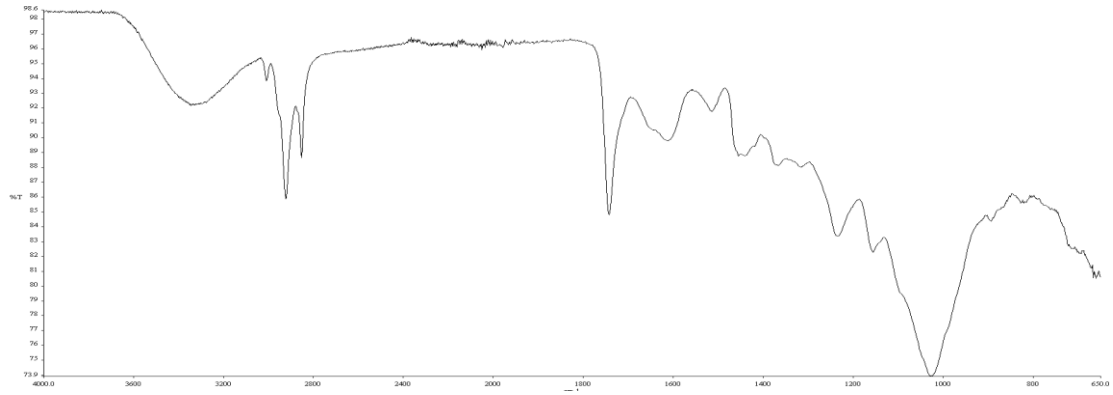


Figure 6-14b) FTIR spectrum of hydrolysis residue of Trial 6 (conditions in Table 4-5)

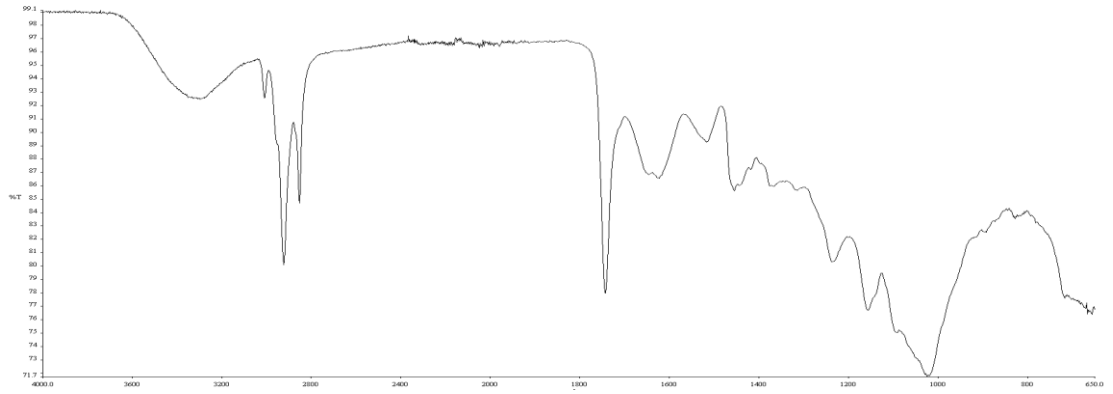


Figure 6-14c) FTIR spectrum of hydrolysis residue of Trial 7 (conditions in Table 4-5)

6.2.2.5 UV-vis spectrophotometer analysis

A Jasco V-550 UV−vis spectrophotometer was used for anthocyanins content analysis. It is reported[274–276] that the absorbance of solution at 530 nm is directly in proportion to the concentration of anthocyanins. Therefore, in this research the absorbance at 530 nm is used to roughly reflect the anthocyanin content. The results are shown in Figure 4-4 and Figure 6-15.

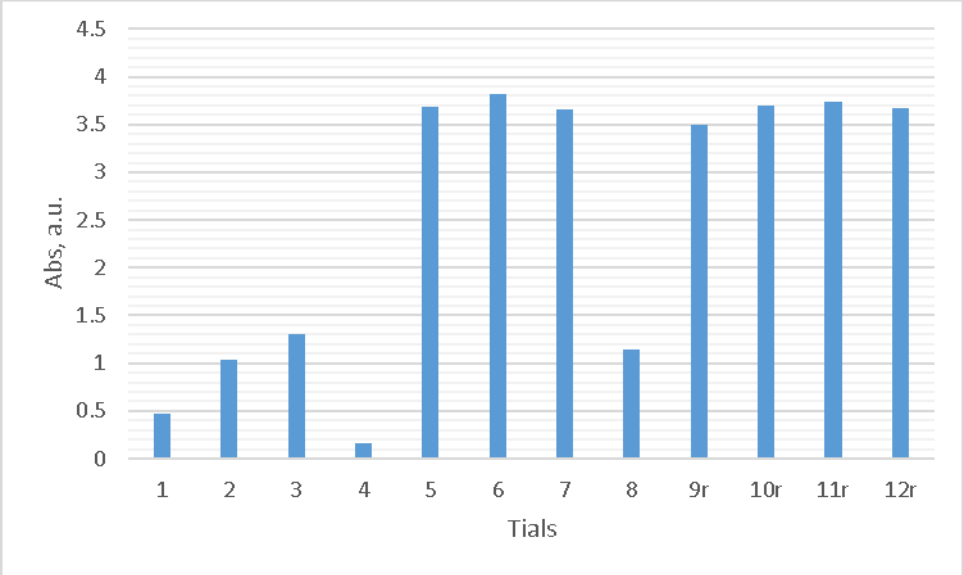


Figure 6-15 Absorbance of hydrolysate at 530 nm of UV−vis spectroscopy of different trials. (conditions in Table 4-5)

6.3.2.6 Liquid state ^{13}C NMR spectroscopy analysis

The method and apparatus of liquid state ^{13}C NMR was same to those in 6.1.2.8. The result is shown in Figure 4-7.

6.3.2.7 Pectin yield determination

To determine the pectin content in hydrolysate of pilot scale trial (Trial P2-60), 100 mL of processed mixture was taken to record the mass. After filtration, the filtrate was taken and twice the volume of ethanol added to precipitate pectin. The mixture was kept at room temperature for 24 h. Then, the solid was filtered, washed with hot ethanol, and dried. This residue was weighed to calculate pectin yield. The yield of pectin is 6.3 %.

6.3.2.8 GC/MS and GC-FID analysis

5 ml of the hydrolysate was transferred into a vial and the solvent (water) was removed under vacuum using a rotary evaporator. The residue that remained (the ‘hydrolysate’)

was weighed to obtain the hydrolysate yield and then redissolved in2 ml of methanol and transferred to a sealed crimp-top vial for analysis by GC-MS and GC-FID.

An Agilent 6890N GC-FID following parameters were used: Zebron ZB5HT Infemo column, carrier gas He, flow-rate 2.0 ml min^{-1}, initial temperature at 50 °C, ramp rate at 10 °C min^{-1} until 290 °C and holding for 10 min, split ratio with 5:1, and injector temperature at 290 °C.

The GC-MS results were obtained using a PerkinElmer Clarus 500 GC/ MS (USA). All the parameters were identical to those used for the GC-FID analysis. The identities of the compounds were determined by comparing the mass spectra with NIST lab database. The spectra are shown in Figure 6-16 and Figure 6-17.

As mono-, di-saccharides have limited solubility in methanol and this was the solvent used preparing the GC sample, their relative peaks were of very low intensity. The major peaks could be attributed to furfural (5.26 min), furan (7.56 min) and HMF (12.80 min), which had been proven abundant in aqueous phase by HPLC. Besides the sugar dehydration products, there are also some other chemicals present with significant peak area. The peak at 18.04 min was assigned as oxalic acid, 2-ethylhexyl isohexyl ester.

The GC-FID spectra offered similar information to the GC-MS with one minor difference. With increased temperature and longer hydrolysis time, the areas of all peaks increased significantly, indicating higher yields of the individual compounds could be achieved.

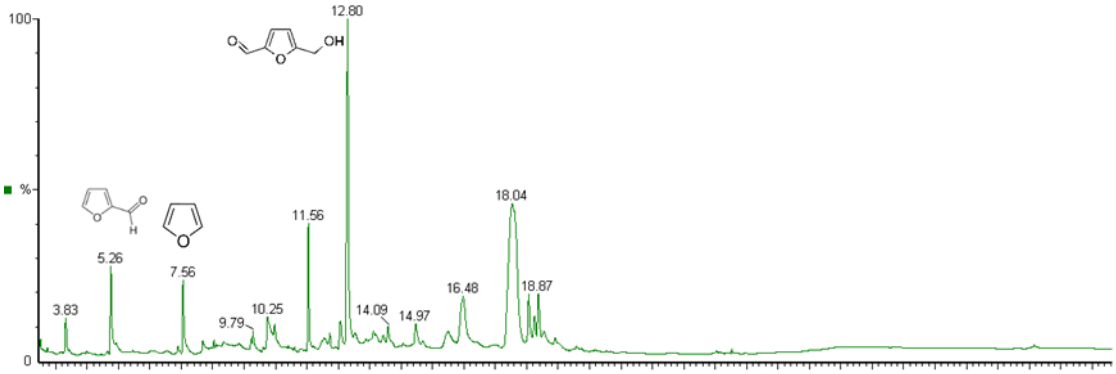


Figure 6-16 GC/MS spectrum of hydrolysate of bilberry in Trial 10r (condition in Table 4-5)

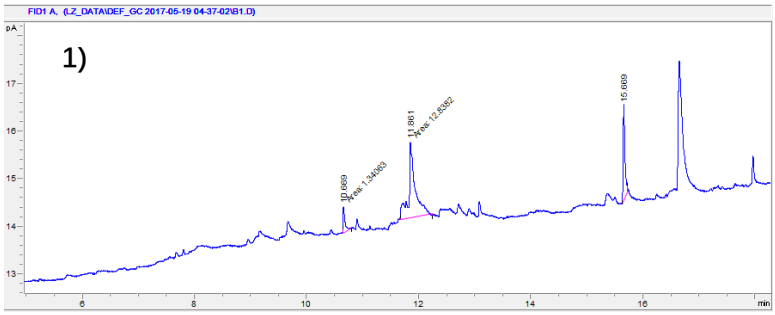


Figure 6-17a GC-FID spectra of DBP hydrolysate of Trial 1

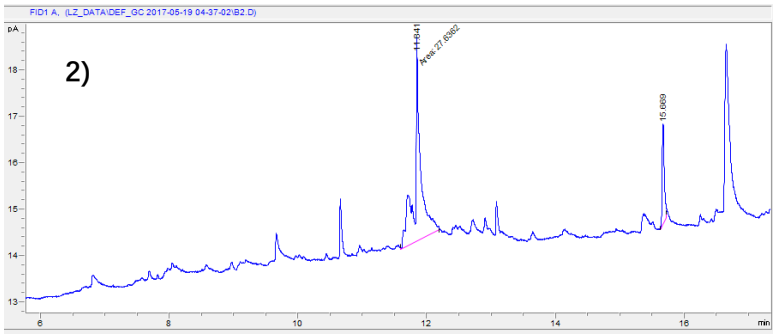


Figure 6-17b GC-FID spectra of DBP hydrolysate of Trial 2

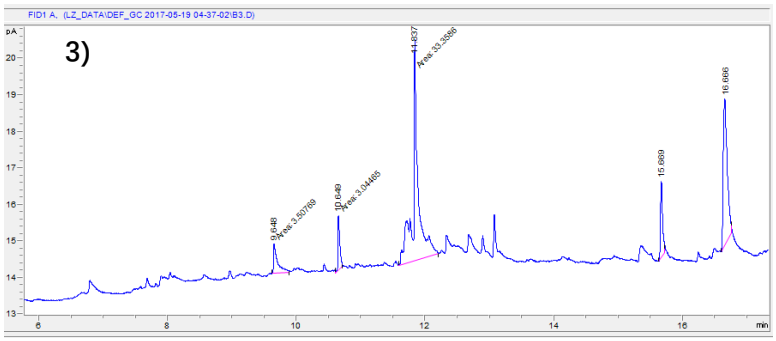


Figure 6-17c GC-FID spectra of DBP hydrolysate of Trial 3

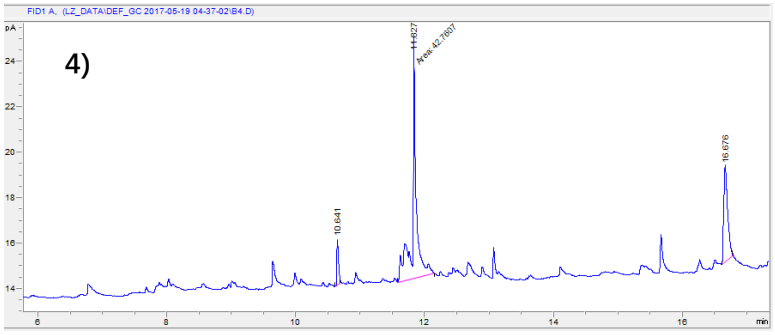


Figure 6-17d GC-FID spectra of DBP hydrolysate of Trial 4

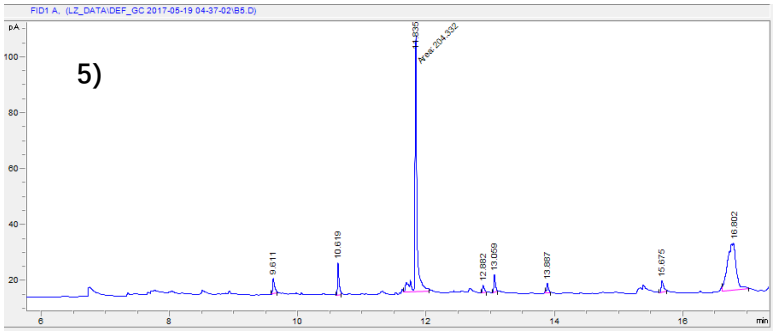


Figure 6-17e GC-FID spectra of DBP hydrolysate of Trial 5

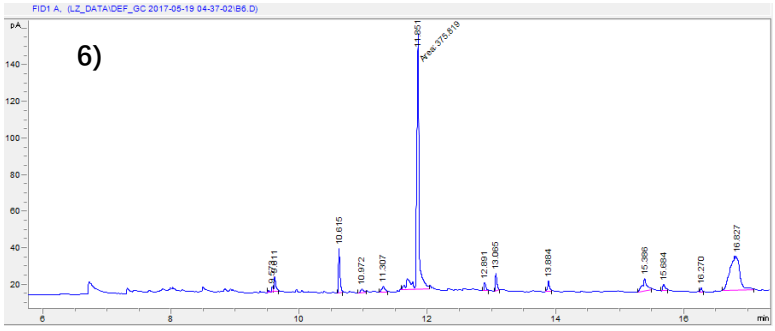


Figure 6-17f GC-FID spectra of DBP hydrolysate of Trial 6

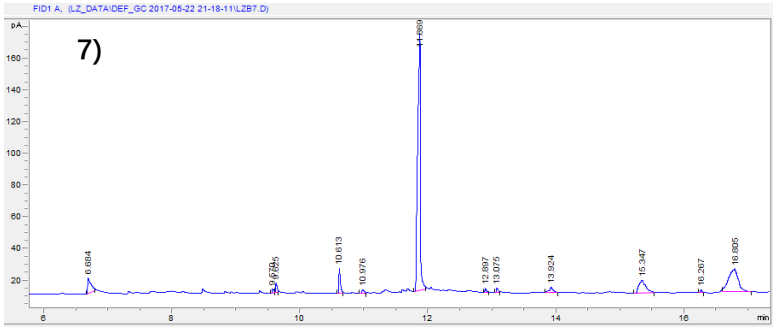


Figure 6-17g GC-FID spectra of DBP hydrolysate of Trial 7

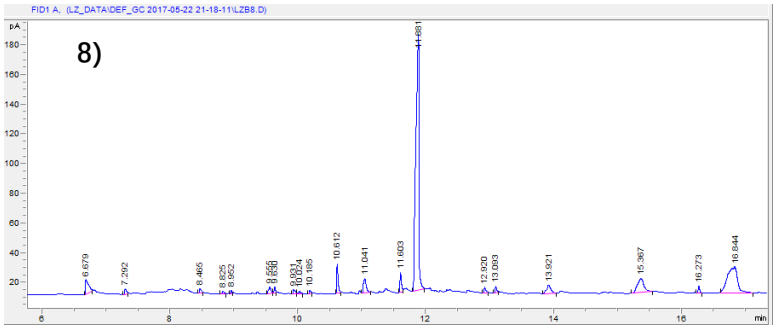


Figure 6-17h GC-FID spectra of DBP hydrolysate of Trial 8

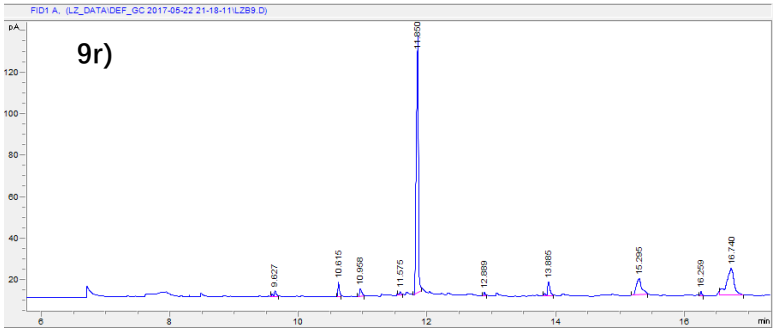


Figure 6-17i GC-FID spectra of DBP hydrolysate of Trial 9r

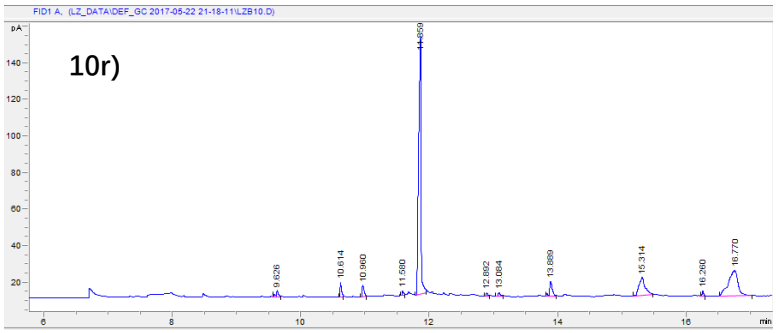


Figure 6-17j GC-FID spectra of DBP hydrolysate of Trial 10r

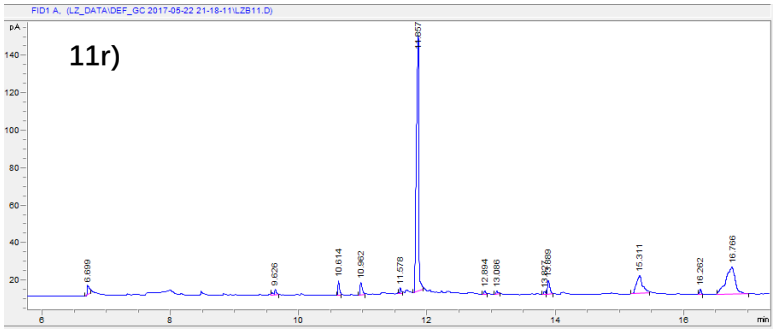


Figure 6-17k GC-FID spectra of DBP hydrolysate of Trial 11r

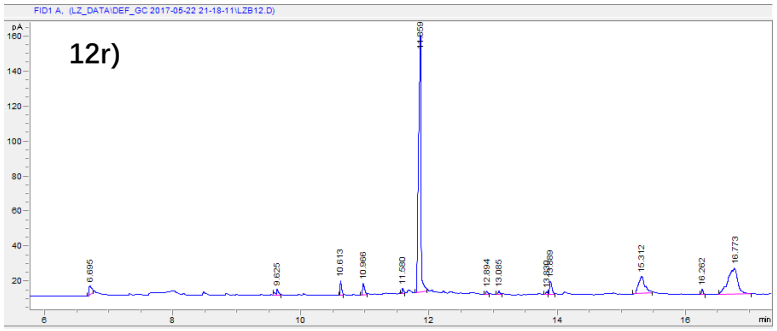


Figure 6-17l GC-FID spectra of DBP hydrolysate of Trial 12r

6.3.2.9 HPLC analysis

The mono- and disaccharides and organic acids of the aqueous phase were analysed using an Agilent 1260 Infinity HPLC instrument equipped with an Agilent Hi-Plex H (300 × 7.7 mm, 8 µm particle size) column. For levoglucosan, glucose, fructose, xylose, cellobiose, rhamnose, and organic acids (lactic, formic, acetic acids), the mobile phase of 0.005 M H_2S0_4, isocratic (no gradient), flow-rate of 0.4 mL min^{-1}, column temperature of 60 °C, refractive index detector at 55 °C, total run time of 35 min, and injection volume of 5 µL were used. For analysis of furfural, levoglucosenone, and 5-HMF, the following parameters were used: ACE C18 (250 × 4.6 mm, 5 µm particle size) column, mobile phase of acetonitrile/water (25:75), isocratic (no gradient), flow-rate of 0.8 mL min^{-1}, column temperature of 30 °C, DAD detector at 220 nm, total run time of 22 min, and injection

volume of 5 μL. External standards were prepared for both methods at five concentrations (0.5, 0.75, 1.0, 1.5, 2.0 mg mL^{-1}). The results are shown in Figure 4-5, Figure 4-11 Table 6-10 and Table 6-11.

Table 6-10 The concentration and yield of compounds in hydrolysate of lab-scale trials (conc, mg/ml; yield, %)

Trial	Cellbiose		glucose		fructose and xylose		rhamnose		levglucosan		Sum of sugar yield	latic acid		formic acid		acetic acid		levulinic acid		Sum of organic acid yield	Hmf		levoglucosanone		Sum of sugar dehydration products
	Conc	yield	conc	yield	conc	yield	conc	yield	conc	yield		conc	yield	conc	yield	conc	yield	conc	yield		conc	yield	conc	yield	
1	0.34	3.42	0.28	2.81	0.33	3.26	0.58	5.80	0.24	2.44	17.73	0.04	0.42	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.42	n.a.	n.a.	0.23	2.26	2.26
2	0.32	3.23	0.29	2.86	0.38	3.78	0.92	9.16	0.24	2.43	21.45	0.07	0.72	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.72	n.a.	n.a.	0.56	5.65	5.65
3	0.38	3.82	0.29	2.86	0.38	3.85	0.94	9.43	0.25	2.48	22.45	0.07	0.68	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.68	n.a.	n.a.	0.45	4.49	4.49
4	0.40	4.00	0.31	3.12	0.45	4.47	1.08	10.77	0.25	2.51	24.87	0.08	0.79	0.64	6.42	0.75	7.52	n.a.	n.a.	14.73	0.023	0.233	0.54	5.37	5.61
5	0.86	0.86	0.34	0.34	1.04	1.04	4.74	4.74	0.27	0.27	7.27	0.37	0.37	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.37	0.193	0.193	1.33	1.33	1.52
6	0.42	0.42	0.36	0.36	1.51	1.51	6.71	6.71	0.27	0.27	9.28	0.50	0.50	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.50	0.690	0.690	2.35	2.35	3.04
7	0.41	0.41	0.38	0.38	1.57	1.57	7.03	7.03	0.29	0.29	9.67	0.49	0.49	n.a.	n.a.	0.79	0.79	0.47	0.47	1.74	0.472	0.472	1.79	1.79	2.26
8	0.51	0.51	0.49	0.49	1.76	1.76	7.27	7.27	0.29	0.29	10.31	0.60	0.60	1.07	1.07	1.13	1.13	0.25	0.25	3.05	1.741	1.741	2.83	2.83	4.57
9r	0.38	0.68	0.33	0.60	0.94	1.71	3.79	6.89	0.27	0.49	10.37	0.30	0.54	n.a.	n.a.	0.62	1.12	0.36	0.66	2.32	0.505	0.918	1.83	3.33	4.25
10r	0.39	0.71	0.33	0.59	1.10	2.00	4.33	7.87	0.27	0.49	11.67	0.31	0.56	n.a.	n.a.	0.59	1.07	0.23	0.42	2.05	0.484	0.879	1.87	3.39	4.27
11r	0.39	0.71	0.33	0.61	0.97	1.76	3.91	7.11	0.27	0.49	10.68	0.34	0.61	n.a.	n.a.	0.53	0.96	0.24	0.43	2.01	0.416	0.756	1.97	3.57	4.33
12r	0.38	0.68	0.34	0.62	0.99	1.79	3.97	7.21	0.27	0.49	10.80	0.29	0.53	n.a.	n.a.	0.59	1.06	0.20	0.37	1.96	0.510	0.928	1.86	3.38	4.31
SW	0.47	1.17	0.91	2.28	0.85	2.12	0.38	0.95	0.24	0.61	7.12	0.23	0.57	0.52	1.30	0.49	1.22	0.13	0.31	3.39	n.a.	n.a.	n.a.	n.a.	0.00

n.a. , not available due to low concentration (peak area<200 in HPLC analysis)

Table 6-11 The concentration and yield of compounds in hydrolysate of pilot-scale trials (conc, mg/ml;

Trial	Cellobiose		Glucose		Fructose and Xylose		Rhamnose		Levoglucosan		Lactic acid		Formic acid		Acetic acid		Levulinic acid		HMF		Levogluco-sanone	
	conc	yield	Conc	Yield	Conc	Yield	Conc	Yield	Conc	Yield	Conc	Yield	conc	yield	conc	Yield	conc	Yield	conc	yield	conc	yield
P1-30	0.85	0.73	4.10	3.50	3.85	3.29	1.35	1.15	0.06	0.05	0.09	0.08	0.02	0.02	0.05	0.05	-	-	1.18	1.0	0.07	0.06
P1-60	0.05	0.04	4.34	3.71	4.06	3.47	1.43	1.22	0.03	0.03	0.10	0.09	0.04	0.03	0.05	0.05	-	-	0.70	0.6	0.51	0.44
P2-30	0.05	0.04	5.20	4.45	4.81	4.11	1.69	1.44	0.03	0.03	0.06	0.05	0.02	0.02	0.07	0.06	0.13	0.11	2.07	1.8	0.07	0.06
P2-60	0.07	0.06	5.13	4.38	4.72	4.03	1.72	1.47	0.03	0.03	0.06	0.05	0.05	0.05	0.05	0.05	-	-	2.95	2.5	0.04	0.03
P3-30	0.11	0.09	3.98	3.40	3.69	3.15	1.29	1.10	0.02	0.02	0.05	0.04	0.04	0.03	0.09	0.08	-	-	0.46	0.4	0.07	0.06
P3-60	0.08	0.07	3.77	3.22	3.46	2.96	1.24	1.06	0.02	0.02	0.05	0.04	0.03	0.03	0.15	0.13	-	-	1.17	1	0.12	0.10

Abbreviation

H-unit	Hydroxyphenyl monomer
G-unit	Guaiacyl monomer
S-unit	Sinapyl monomer
β-O-4	Aryglycerol-β-ether dimer interlinkage
β-β′	Resinols interlinkage
β-5	Phenylcoumaran interlinkage
β-1	Pirodienone interlinkage
KL	Klason lignin
MWL	Milled wood lignin
EMAL	Enzymatic mild acidolysis lignin
CEL	Cellulolytic enzyme lignin
PH	Primary hydrolysis of Klason protocol
SH	Second hydrolysis of Klason protocol
L-C	Lignin-carbohydrate
LCC	Lignin carbohydrate complex
HR	Hydrolysis residue
Py-GC/MS	Pyrolysis Gas Chromatography Mass Spectrometry
C/H	Carbon and hydrogen element
MRL	Microwave-assisted hydrolysis residue lignin, only refers to lignin Isolated at conditions of 10min holding time, 0.2M H_2SO_4, reactor as

	CEM DISCOVER (detailed condition in experimental)
MSP	Mixed softwood pellet
AL	Conventional heating acidolysis residue lignin, refers to lignin isolated at conditions of 190 ºC, 10min holding time, 0.2M H_2SO_4, reactor as Anton PAAR, Monowave 50 (detailed condition in experimental)
GC/MS	Gas Chromatography Mass Spectrometry
liquid ^{13}C NMR	Liquid carbon-13 nuclear magnetic resonance
TG	Thermal gravimetric
DTG	Derivative thermo-gravimetric
FTIR	Fourier-transform infrared spectroscopy
SSNMR	Solid state carbon-13 nuclear magnetic resonance
CP	Cross Polarisation
CPNQS	Cross Polarisation Non Quarternary suppression
WE	Willow edging
WS	Wheat straw
R^2	Coefficient of determination
DP1	Monosaccharide sugars
DP2	Disaccharide sugars
DP3–DP5	Lower molecular weight oligosaccharides
DP6+	Larger oligosaccharides
S. cerevisiae	*Saccharomyces cerevisiae*
C. curvatus (Cc)	*Cryptococcus curvatus*

M. pulcherrima (Mp)	*Metschnikowia pulcherrima*
5-HMF	5-(Hydroxymethyl)furfural
P value	Probability value
DBP	Dried bilberry presscake
Trial SW	The trial of Soxhlet extraction of DBP using deionised water
HPLC	High-performance liquid chromatography
UV−Vis	Ultraviolet–visible spectroscopy
Abs	Absorbance
RG-I	Rhamnogalacturonan I pectin
RG-II	Rhamnogalacturonan II pectin
CapEx	Capital expenditure
BDC	Biorenewables Development Center
TAPPI	Technical Association of the Pulp and Paper Industry
ICP	Inductively coupled plasma mass spectrometry
SPME media	Soy peptone: 30 g L^{-1}; malt extract: 25 g L^{-1}
YPD media	Yeast extract: 10 g L^{-1}; peptone: 20 g L^{-1}; glucose: 20 g L^{-1}
DAD	Diode-array detectors
WBP	Wet bilberry presscake
RBF	Round-bottom flask
GC-FID	Gas Chromatography – Flame Ionisation Detector

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