

Bachelor's Programme in Chemical engineering

Methods for analysing lignocellulose biomass porosity

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Abstract

Lignocellulosic biomass is the most abundant carbon source on Earth and thus offers an alternative biomaterial for fossil fuel-based materials. However, the bioconversion of raw lignocellulosic biomass generally involves steps of pretreatment and enzymatic hydrolysis which are hindered by the natural recalcitrance of biomass. Factors affecting this recalcitrance can be divided into structural factors and chemical factors. Structural factors include porosity which can be measured in terms of specific surface area, pore sizes, pore volume and pore size and volume distribution. Several methods have been developed for analysing porosity, however, no method to date is proven absolute and quantitative.

Thus, the aim of this thesis was to review five methods for analysing lignocellulose biomass porosity and their effectiveness and suitability. Their mechanism, advantages and disadvantages are evaluated, and their possible combinations and usefulness are reviewed. The methods chosen for this thesis were gas adsorption, mercury intrusion porosimetry (MIP), nuclear magnetic resonance (NMR) relaxometry and cryoporometry, solute exclusion and Simons' stain based on past research and their suitability to analyse wood substrates. The goal of an effective method is to analyse porosity quantitatively and accurately and obtain information of multiple porosity parameters. These methods differ greatly in their degree of results ranging from qualitative to quantitative, states of substrates and the parameters and scale of porosity they can analyse (micro- to macropores).

In addition, this thesis contained experimental work using modified Simons' stain to analyse porosity of wood chips. Simons' staining has been modified over the years, but the basis of the mechanism relies on the size and affinity of dye molecules to measure accessible surface area. In past research, Direct Orange dye 15 (DO15) and Direct Blue dye 1 (DB1) has been used, but since DO15 has been discontinued, it has been replaced by Direct Yellow dye 11 (DY11). In this experiment, bound DY11 and DB1 in softwood and hardwood chips were calculated. The aim of this work was to test the suitability of Simons' staining across a variety of substrates as it has been previously used on wood pulp. If proven effective, this semi-quantitative, quick and inexpensive method could be a valuable assay to analyse porosity of wet-state samples.

The conclusions of this thesis were that no method can analyse porosity quantitatively and with absolute precision due to the inaccuracies with any one method. In addition, future research is required for modified Simons' staining, as the results of the experimental work were inconclusive. If a quantitative and accurate analysis technique was found, it would greatly improve the utilization of biomass and thus decrease dependency on fossil fuels.

Keywords Lignocellulose biomass porosity, wood porosity, Simons' stain

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Tiivistelmä

Lignoselluloosabiomassa on suurin hiililähde maapallolla ja sitä käytetään yhä enemmän korvaamaan fossiilisia polttoaineita materiaalina. Lignoselluloosabiomassan biokonversiota tuotteiksi kuitenkin vaikeuttaa sen monimutkainen rakenne. Lignoselluloosabiomassa koostuu kolmesta biopolymeeristä, ligniinistä, hemiselluloosista ja selluloosasta, ja biomassan biokonversiossa tätä rakennetta yleisesti halutaan muokata entsyymihydrolyysillä ja esikäsittelyllä. Näihin prosesseihin vaikuttaa lignoselluloosan huokoisuus, jota voidaan mitata huokosten pinta-alana, huokosten koolla ja tilavuudella ja huokosten jakaumalla. Monia menetelmiä on kehitetty mittaamaan biomassan huokoisuutta, mutta kvantitatiivista ja tarkkaa menetelmää ei ole vielä löydetty.

Tämä kandidaatintyö tutki viittä menetelmää, joilla voidaan analysoida lignoselluloosabiomassan huokoisuutta kirjallisuuskatsauksen muodossa. Nämä viisi metodia ovat kaasuadsorptio, elohopeaporosimetria, ydinmagneettinen resonanssi relaksometria ja kryoporometria, liunneen aineen poissulkeminen (engl. *solute exclusion*) sekä Simonsin värjäys (engl. *Simons' stain*). Kirjallisuustutkimuksessa tutkittiin menetelmien mekanismeja, tulosten luotettavuutta ja menetelmien helppoutta. Menetelmien tavoitteena on mitata huokoisuutta luotettavasti, nopeasti ja kvantitatiivisesti.

Tämä kandidaatintyö sisältää myös kokeellisen osion, missä tutkittiin lehtipuun ja havupuun huokoisuutta käyttäen muokattua Simonsin värjäysmenetelmää. Simonsin värjäyksessä on aikaisemmissa tutkimuksissa käytetty suoraa oranssia väriä, mutta sen tuotanto lopetettiin. Menetelmää on käytetty sen jälkeen uudestaan suoralla keltaisella värillä puukuituihin ja todettiin, että keltainen väri toimii osittain paremmin kuin oranssi väri. Tämän perusteella menetelmää haluttiin kokeilla tässä kandidaatintyössä puulastuihin ja selvittää sen soveltuvuus analysoida puulastujen huokoisuutta. Simonsin värjäys on nopea, helppo ja osittain kvantitatiivinen menetelmä, mikä tekee siitä arvokkaan menetelmän tutkimaan huokoisuutta. Simonsin värjäyksessä keltainen väri imeytyy isoihin huokosiin ja sininen väri pieniin huokosiin, jolloin imeytynyt värin määrä kertoo adsorptiopinta-alasta ja huokosten koosta. Vapaan värin absorbaatio mitattiin UV-spektrofotometrillä ja absorbaatioista laskettiin lastuihin adsorboituneen värin konsentraatio.

Tämän kandidaatintyön kirjallisuustutkimuksessa todettiin, että huokoisuuden analysointimenetelmät eroavat toisistaan suuresti esikäsittelyn, helpouden, tulosten luotettavuuden ja mekanismien osalta, eikä mikään niistä sovellu analysoimaan huokoisuutta täysin luotettavasti ja kvantitatiivisesti. Kandidaatintyön kokeellisen työ paljasti epätarkkuuksia muokatusta Simonsin värjäysmetodista ja todettiin, että se vaatii jatkotutkimuksia.

Kandidaatintutkielman johtopäätöksenä todettiin, että menetelmät analysoimaan biomassan huokoisuutta ovat epätarkkoja ja vaativat lisätutkimuksia. Nämä menetelmät yhdistelminä pystyvät analysoimaan huokoisuutta tarkemmin ja laajemmin, mutta ei siltikään täysin kvantitatiivisesti. Tarkan huokoisuusanalyysimenetelmän kehittäminen auttaisi suuresti opimoimaan lignoselluloosabiomassan biokonversion vaihtoehtoisiksi materiaaleiksi fossiililille polttoaineille.

Avainsanat Lignoselluloosabiomassan huokoisuus, puun huokoisuus, Simonsin värjäys

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Symbols and abbreviations

Symbols

A	Absorbance
C	Concentration
ϵ	Extinction coefficient

Abbreviations

BET	Brunauer-Emmett-Teller
BJT	Barrett-Joyner-Halenda
CPMG	Carr-Purcell-Meiboom-Gill
DB1	Direct Blue 1
DO15	Direct Orange 15
DY11	Direct Yellow 11
HMW	High molecular weight
HW	Hardwood
LMW	Low molecular weight
NMR	Nuclear magnetic resonance
MIP	Mercury intrusion porosimetry
SW	Softwood

1 Introduction

The shift from fossil fuel-based materials to bio-alternatives has increased the research of lignocellulose biomass and its physical and microstructural properties. Lignocellulose biomass porosity has been identified as one of the critical determinants of how to process, modify, and utilize biomass. It links the microstructural properties of plant matter with its performance in industrial and environmental applications (Zoghiami & Paès, 2019). These lignocellulose biopolymers can be converted into bioenergy, chemicals, and materials, such as biofuel. With the escalating environmental crisis and energy shortage, utilization of lignocellulosic biomass has become ever so more important (Chen et al. 2021). Processing lignocellulose biomass into materials does not only reduce the dependency of fossil fuels but also recycles the Earth's resources thus reducing waste and decreasing pollution. However, lignocellulose biomass has a complex structure which is not understood completely in terms of its microstructure and physical properties, including understanding and quantifying its porosity. The determination of this microstructural property would greatly assist in its bioconversion into much needed materials (Crowley et al. 2022). Multiple methods have been developed for analysing porosity, however, no method to date has been proven effective.

Thus, the goal of this thesis is to review five methods for analysing lignocellulose biomass porosity, evaluate their advantages and disadvantages and some of their combinations. This thesis includes a literature review of five methods: gas adsorption, mercury intrusion porosimetry (MIP), nuclear magnetic resonance (NMR) techniques relaxometry and cryoporometry, solute exclusion and Simons' stain. These methods were chosen based on past reviews (Meng & Ragauskas, 2014) and their ability to analyse wood porosity.

In addition, this thesis contains experimental work which applies modified Simons' staining from Kwok et al. (2017) on hardwood and softwood chips. The purpose of this was to test its suitability to analyse a variety of substrates as Kwok et al. applied it to wood pulp. In this method a high molecular fraction (HMW) of Direct Yellow dye 11 (DY11) is used instead of a discontinued Direct Orange dye 15 (DO15). The amounts of adsorbed dyes, DY11 and Direct Blue 1, are calculated from absorbances of free dyes in the supernatants using a modified Beer-Lambert's law. Thus, the masses of adsorbed dye in wood pores indicates accessible surface area and pore size distribution.

2 Literature review

This literature part of the thesis introduces the background of lignocellulosic biomass structure and porosity in Section 2.1. The five methods are reviewed in Sections 2.2-2.6. Section 2.7 evaluates their degree of results and ease of performance and some of their combinations.

2.1 Background

Lignocellulosic biomass is the most abundant renewable carbon source on Earth (Kwok et al. 2017). However, its microstructure and porosity are not fully understood to date. Thus, the structure of lignocellulosic biomass is described in Section 2.1.1 and its porosity in Section 2.1.2.

2.1.1 Lignocellulosic biomass

Lignocellulosic biomass, essentially plant biomass that is not used for food or feed, is the most abundant renewable carbon source on Earth and its production is estimated to be over 1000 tonnes per year (Smith, 2019). Thus, lignocellulosic materials offer an alternative material for fossil fuels as a renewable source of fuels, bioplastics and energy (Kwok et al. 2017). Lignocellulosic biomass has a complex structure that is specific to the plant species. It is composed of polysaccharides, cellulose and hemicelluloses, and an aromatic polymer lignin (Zoghلامي & Paës, 2019). Together they form a multilayer, intricately linked, complex but stable structure (Ishizawa, 2007). However, this structure is not yet fully understood which has limited its performance as lignocellulosic-derived fuels and materials (Smith, 2019).

Zoghلامي and Paës (2019) concluded that one of the major limitations to lignocellulosic biomass utilization is its recalcitrance to enzymatic hydrolysis caused by its heterogeneous multiscale structure of plant cell walls. This has been reported in multiple studies, including in (Meng & Ragauskas, 2014) research of bioconversion of lignocellulosic biomass to biofuel. Meng and Ragauskas (2014) emphasized the importance of understanding this recalcitrance as it poses a significant challenge during steps of bioconversion. Zoghلامي and Paës (2019) reported that the factors affecting this recalcitrance can be divided into structural factors and chemical factors. Structural factors include cellulose specific surface area, cellulose crystallinity, degree of polymerization, pore size and volume. Chemical factors include composition and content of lignin, hemicelluloses and acetyl groups. They concluded that overall, this combination of relevant factors of structural and chemical information advances the efficient bioconversion of lignocellulosic biomass into bioproducts.

Lignocellulosic biomass is composed of cellulose, hemicelluloses and lignin, which together form a complex assembly of polymers that naturally

recalcitrance enzymatic conversion Zoghlami and Paës (2019). Bioconversion process generally involves mandatory steps of pretreatment and enzymatic hydrolysis (Meng & Ragauskas, 2014). The purpose of these steps is to make cellulose more accessible by changing the physical and chemical structure by facilitating the conversion of polysaccharides into fermentable sugars (Zoghlami & Paës, 2019). To overcome this recalcitrance, it is crucial to understand the structure of lignocellulosic biomass.

Zoghlami and Paës reported that cellulose is the most abundant polymer in lignocellulosic biomass, representing 40-60% of its weight. It consists of D-glucopyranose units linked via β -(1,4) glycosidic bonds. These D-glucose units are arranged in long chains that form microfibrils. When packed together, they form cellulose fibrils. They stated that cellulose fibrils are embedded in a lignocellulosic matrix that makes it resistant to enzymatic hydrolysis. In addition, the degree of polymerization, number of glucose units in the polymer, has been reported to affect the recalcitrance of biomass. They also stated that it has been implied in previous research that the long cellulose chains contain a higher amount of hydrogen bonds and thus are more difficult to hydrolyse compared to shorter chains.

Hemicelluloses are heterogeneous groups of biopolymers that represent 20-35% of the weight (Zoghlami & Paës, 2019). Zoghlami and Paës noted that they contain various monosaccharide subunits that have lower degree of polymerization than cellulose. Hemicelluloses are readily hydrolysed by dilute acids and bases and hemicellulose enzymes. They also reported that the removal of hemicelluloses during pretreatment could increase cellulose conversion by improving the accessibility of enzymes to cellulose. In addition, removal of hemicelluloses improved the fibres porosity thus increasing the surface area available for enzymes.

The third polymer in biomass structure is lignin. Zoghlami and Paës stated that lignin represents 15-40% of dry biomass weight. They described it as a complex amorphous heteropolymer of phenylpropanoid building units. Lignin is responsible for hydrophobicity and structural rigidity as it binds hemicelluloses and cellulose in the cell wall. They concluded that lignin affects negatively the conversion of cellulose. Its effect varies with lignin content, composition and structure. It plays a role as physical barriers that blocks the access of enzymes to cellulose and can also inversely adsorb enzymes due to its hydrophobicity. They also stated that removal of lignin has been reported to increase porosity and reduce non-productive adsorption sites for enzymes.

Together these polymers form an intricately linked structure. Cellulose and hemicellulose are linked by hydrogen bonds while lignin and hemicellulose are covalently bonded (Zoghlami & Paës, 2019). It has been implied that these interactions between the microfibrils of cellulose and hemicelluloses as well as the lignin-carbohydrate complex play a significant part in wood structure specifically, and the enzymatic hydrolysis of lignocellulosic biomass (Zoghlami & Paës, 2019).

2.1.2 Lignocellulose biomass porosity

In lignocellulosic biomass structure, porosity can be measured in terms of accessible surface area, pore sizes, pore volume and pore volume distribution. Accessible surface area is the accessible internal or external surface area of the pores. The sizes are divided to macro-, meso- and micropores by IUPAC. Pore volume is the total amount of empty space within biomass, while pore size and volume distribution describes the range of pore sizes present and their abundance.

Accessible surface area of lignocellulosic biomass is related to the porosity structure properties, specific surface area and pore volume. Zoghلامي and Paës (2019) reported that reduction of particle sizes or an increase in pore volume increases the accessible surface area. Furthermore, they stated that it has been shown that the enzymatic conversion of wood increases with accessible surface area. In addition, they noted that accessible surface area could enhance the fibre accessibility to hydrolytic enzymes. Specific surface area is often measured as the area accessible to enzymes, for example using solute exclusion or Simons' staining techniques.

Zoghلامي and Paës (2019) reported that even though porosity has been previously studied extensively and several methods for analysing porosity have been developed, no method to date is able to study porosity quantitatively and without underlying assumptions and inaccuracies. Zoghلامي and Paës stated that previous studies showed contradicting results between techniques and reported great differences in type of substrates, degree of results from qualitative and quantitative. In addition, there is a difference in the variety of results. Some methods can only analyse accessible surface areas, internal or external, and some can obtain information of pore sizes and pore distribution. Five of these methods were chosen for the literature review of this thesis. These methods were chosen as they can analyse wood porosity which is the focus of the experimental work. In addition, these methods have been the focus on past reviews (Meng & Ragauskas, 2014). The chosen methods were gas adsorption, mercury intrusion porosimetry (MIP), nuclear magnetic resonance (NMR) cryoporometry and relaxometry, solute exclusion and Simons' stain.

2.2 Gas adsorption

Adsorptive characterization with gas adsorption, most popular being nitrogen at 77 K, is one of the most widely used techniques to assess porous solids, providing information of pore volume, specific surface area and pore size distributions (De Lange et al. 2014). The popularity of nitrogen adsorption among other porosity analysis methods is due to its ability to assess a wide range of pore sizes from 0.35 nm to > 100 nm which includes micro- meso-

and macropores (Lowell, 2004). In addition, gas adsorption method can be used in combinations with other methods (Lowell, 2004).

Meng and Ragauskas (2014) described the mechanism as follows. Gas adsorption uses dried samples that are placed in a chamber at low temperature (77 K for nitrogen). The gas is allowed to probe the pores and the amount of gas adsorbed is recorded at different pressures. They stated that the volume of adsorbed gas against pressure can be then used to create the adsorption–desorption isotherm and the surface area can be calculated using the Brunauer-Emmett-Teller (BET) equation. While the pore volume and specific surface area can be determined using BET, the pore size distribution can be determined by Barrett-Joyner-Halenda (BJH) model (De Lange et al. 2014).

In addition to nitrogen, different adsorptives, such as CO₂, Ar, He, CH₄ and benzene or nonane can be used using the same mechanism (Lozano-Castelló et al. 2003). Lozano-Castelló et al. (2003) reported that CO₂ adsorption at 273 K can be used as an alternative for nitrogen adsorption. They noted that nitrogen adsorption may not be suitable for samples with very narrow microporosity as the narrow micropores are not accessible for nitrogen at 77 K. They reported that CO₂ molecules' dimension is similar to nitrogen and the higher temperature used for CO₂ results in larger kinetic energy which enables the molecules to probe the narrower pores. In addition, the adsorption temperature is high enough to avoid diffusion problems.

The disadvantages of nitrogen adsorption include diffusional problems of the molecules inside the narrow porosity range occur (Lozano-Castelló et al. 2003). In addition, it assumes that the pores were filled with the liquid adsorbate (Yin et al. 2015). Furthermore, De Lange et al. (2014) reported that the main sources of error in nitrogen adsorption isotherms are measurement uncertainties, experimental artefacts and human influence. They highlighted that the variability in pressure measurement accuracy and data fitting can lead to uncertainties at the extremes of the isotherm. In addition, they reported that using too small a sample or a large cell volume can artificially enlarge hysteresis affecting pore size distribution analysis with the BJH method. They noted that artificial hysteresis can be caused by experimental conditions and can be mistaken for real pore features, especially in desorption data. Nitrogen can also condense in inter-particle spaces at high relative pressures, which can lead to overestimation in pore volume. The most inaccuracies are however due to the drying of the substrate as pretreatment as water removal causes partial irreversible collapses in pores (Meng & Ragauskas, 2014). Thus, methods that require drying of the substrates are not ideal for biomass porosity analysis.

2.3 Mercury intrusion porosimetry

Mercury intrusion porosimetry (MIP) is a popular method used to analyse dry lignocellulosic biomass substrates. MIP samples are introduced to a chamber surrounded by mercury, in which the volume of mercury that has entered the pores can be calculated by increasing pressure gradually (Meng & Ragauskas, 2014). The analysis time varies between half an hour to an hour, and provides results of the pore size distribution, the total pore volume, the skeletal and apparent density and the specific surface area of a sample (Giesche, 2006).

Giesche (2006) reviewed MIP in his practical overview as an effective and quick technique for porosity analysis. He noted that it allows the investigation of pore sizes between 500 μm and 3.5 nm. Regardless of the wide variety of information, Giesche highlighted its limitations and assumptions. He stated that this method measures the largest entrance towards a pore and not the inner size of the pore and thus does not account for closed pores. In addition, it assumes pore geometry. MIP assumes a cylindrical pore geometry as it uses a modified Young-Laplace equation (Washburn equation) which inversely equates pressure to pore diameter. This equation relates the pressure difference across the curved mercury interface to the corresponding pore size using the surface tension of mercury and the contact angle between the solid and mercury. Giesche (2006) concluded that this assumption of the geometry can lead to inaccuracies in results.

Giesche (2006) reported the method as follows. Substrates are dried and water and chemicals which porous substances tend to absorb are removed. Giesche reported that MIP has no way of noticing impurities in samples resulting in inaccurate sample weight. He stated that with nearly non-porous samples a source of inaccuracies is the creation of artificial pores due to packing of the sample inside the penetrometer cell. During this pretreatment the sample is evacuated to remove air and moisture after which the sample cells are filled with mercury under reduced pressure. The pressure is increased slowly which allows mercury to penetrate the largest pores in the sample and the height of the mercury column is recorded. This method initiates with a low-pressure part. At the end of the low-pressure part, the weight of the penetrometer, mercury and the sample, is determined and the bulk density of the sample can thus be calculated. The volume of intruded mercury is measured through changes in the capacitance of the column of mercury. Lastly, intrusion pressure values are then converted into corresponding pore sizes by using the Washburn equation.

Giesche (2006) concluded that results from MIP are useful in comparative studies of porosity. However, this method relies on assumptions and results can vary depending on experimental factors. He concluded that this method

determines the largest entrance to a pore and thus not the actual pore size. The smallest pore size that can be analysed by this method is determined by the maximum pressure that the instrument can achieve. He also reported that the largest pore size is limited by the sample height. Further inaccuracies can arise from changes within the sample. For example, loose powder can become more compacted due to the pressure before mercury has penetrated the pores which then might incorrectly be interpreted as porosity. Similarly, structural changes can occur in highly porous samples. Despite these inaccuracies, Giesche (2006) concluded that MIP is an effective method for analysing lignocellulose biomass porosity, specifically due to its ability to measure a wide range of pore diameters.

2.4 NMR techniques

Nuclear Magnetic Resonance (NMR) relaxometry and cryoporometry provide can be used to estimate pore sizes and distribution and indicate water dynamics in lignocellulose biomass pores (Bränvall et al. 2021). Cryoporometry provides information of the size and shape distribution of pores in biomass (Meng & Ragauskas, 2014). NMR relaxometry, specifically 2D relaxometry, provides insight into porosity indirectly in terms of accessible pore volumes and water dynamics (Jeoh et al. 2017). However, both are expensive methods that require a complicated setup and a long experimental time (Meng & Ragauskas, 2014).

2.4.1 NMR cryoporometry

Östlund et al. (2009) studied porosity changes in water-state wood pulp in using NMR cryoporometry. They stated that the porosity of wood pulp is described by surface area and pore size distribution. In pulp fibres pore sizes vary from nanometres to micrometres with infinite set of geometries. They also stated that with softer materials, such as wood, samples should be analysed in wet states. Thus, they noted that NMR techniques are valuable porosity analysis assays as they analyse porosity parameters using water molecules.

In NMR cryoporometry, water molecules act as probing molecules to characterize pore size distribution. Östlund et al (2009) reported that by freezing water-saturated samples and analysing their melting behaviour, the melting point depression relative to bulk water can be used to calculate pore size distribution using the Gibbs-Thomson equation. This equates the deviation in melting temperature from the bulk liquid melting temperature, depressed pore size dependant temperature and the specific pore radius. Östlund et al. reported that NMR detects a signal which corresponds to the fraction liquid water of the sample. The NMR signal intensity corresponds to the quantity of ^1H in the liquid state of the sample (amount of melted solvent molecules).

Due to the difference of T₂-relaxation times differ for liquids and solids, the signal from the frozen fraction is suppressed by an appropriate relaxation delay time in the NMR pulse sequence called the CPMG (Carr-Purcell-Meiboom-Gill) pulse sequence. These signals indicate the relative water content in the pores as the signal intensity is proportional to the total amount of water melted with temperature. This allows the NMR signal to be converted to the dependency of pore radius.

Östlund et al. (2009) concluded that NMR cryoporometry is a suitable technique for characterizing pore sizes and distribution water-swollen substrates. However, the geometry of the pores was assumed to be spherical and the pore radii up to 100 nm, when in reality, biomass contains elongated pores. In addition, as the goal of the methods are to analyse porosity quantitatively, NMR techniques are more valuable assays for analysing porosity when used in combinations with other methods.

2.4.2 NMR relaxometry

Jeoh et al. (2017) proposed NMR relaxometry as an alternative approach to investigate biomass porosity by examining the state of water within the material. They hypothesised that localized biomass-water interactions provide insight into the biomass microstructure. They stated that previously used 1D NMR relaxation requires additional information due to its uncertainties with interpretation of water populations. They reported that pore and particle sizes, and the surface chemistry of solids and liquids affect the relaxation times of water associated ¹H. In addition, 1D T₂ relaxation only recognises one cell wall water pool in wood due to its inability to further resolve this pool. Thus, Jeoh et al. (2017) proposed the use of multidimensional NMR relaxation instead.

2D T₁T₂ ¹H NMR relaxation records a matrix of correlated spin-spin (T₂) and spin-lattice (T₁) relaxation times. Therefore, they facilitate resolution of physical and chemical composition influence on cellular and subcellular populations in biomass. This method measures T₁ and T₂ relaxation simultaneously in water associated biomass. In low-field NMR only ¹H from water is detected and the relaxation measures reflect the physical mobility and chemical environment of the water pools. Jeoh et al. (2017) reported that T₁ relaxation depends on the matching of molecular tumbling rates and the Larmor frequency of the spectrometer indicating either free water (longer T₁) and confined water in pores (shorter T₁). T₂ is influenced by magnetic fields from surrounding chemicals which then indicates the presence of lignin or hemicellulose. Water molecules behave differently depending on the pore size. They noted that in large pores water is more mobile, leading to longer T₁ and T₂ times, and in small or restricted pores water is less mobile leading to shorter T₁ and T₂ relaxations.

Jeoh et al. (2017) concluded that 2D T_1T_2 1H NMR relaxation of lignocellulosic biomass obtains information of water environments that 1D NMR relaxation cannot. It provides insight into the relative influence of microstructure and chemical composition within the water pools that can be used to optimize and study the effect of pretreatment, water content and behaviour of pores. However, they stated that the disadvantages include the need for supplementary porosity and structural analysis and complexity in data interpretation. In addition, NMR analysis equipment is expensive and rarely available (Meng & Ragauskas, 2014).

2.5 Solute exclusion

Solute exclusion uses probing molecules to assess the internal accessible surface area, pore sizes and volume distribution of wet substrates (Beecher et al. 2009). The method introduces a known volume and concentration of probing molecules to a substrate submerged in water. The solution is mixed and diluted by the water within the sample. Thus, all pores that are accessible to the probing molecules will contribute to the dilution. As a result, the final solute concentration reflects the volume of water in accessible pores. They reported that this application of probing molecules with different sizes then indicates the accessible surface area and pore sizes. Volume distribution can be calculated using derived formulas with initial and final solute concentrations, molecular weights, and the proportions of accessible versus inaccessible water.

Beecher et al. (2009) highlighted that solute exclusion contains multiple assumptions and disadvantages. They reported that the assumptions include assuming that the concentration of solute molecules in the accessible pores is the same as in the solution surrounding the sample and spherical pore geometry. Another assumption is that the solute molecules are able to fully penetrate the pores as closed pores are not accessible to probing molecules. This results in underestimations of accessibility. Beecher et al. reported that the major disadvantage of solute exclusion is the “ink-bottle” effect. This effect describes the exclusion of pores with narrow opening and wide inner spaces. In addition, due to osmotic pressure ionized probing molecules and non-ionic solute molecules are excluded from pores resulting in solute-free water in the pores. They suggested that this can be avoided by using molecules that interact and adsorb on fibres which then can be forced to enter the pores.

Due to these assumptions Beecher et al. suggested that solute exclusion indicates only the accessible volume distribution and surface area. Thus, results from solute exclusion are semi-quantitative and determines the total pore volume accessible to a solute molecule of different sizes as well as estimating internal enzyme accessibility (Beecher et al. 2009). However, this method is laborious to perform, indicates only the internal accessible surface area and cannot be used for absolute determination of pore size and volume distribution (Meng & Ragauskas, 2014).

2.6 Simons' stain

Simons' staining has been reported to be a quick, semi-quantitative method for analysing lignocellulose biomass porosity (Chandra & Saddler, 2012). However, originally the method was used to analyse unbeaten and beaten fibres (Xiaochun et al. 1995). It has since been modified to analyse accessible surface area and pore sizes. Section 2.6.1 describes the original basis of the method and Section 2.6.2 describes the modified Simons' staining that was used in this thesis' experimental work.

2.6.1 Original Simons' stain

Simons' staining assay was developed in 1995 by Simons, in which two colour differential stain, orange and blue, was used to investigate the mechanical damage of beaten fibres (Xiaochun et al. 1995). Simons discovered that unbeaten fibres stained blue and beaten fibre debris stained orange (Xiaochun et al. 1995). Xiaochun et al. (1995) reported the basis of the mechanism as follows. The blue dye has lower affinity for cellulose and a smaller particle size than the orange stain, which results in the blue dye particles penetrating smaller capillaries. After beating, the fibre wall was made more accessible and thus the orange dye could displace the blue dye due to its higher affinity.

However, some modifications to the method have been made to improve its mechanism. Xiaochun et al. (1995) reported that Simons' staining mechanism employs a specific set of azo-stilbene set of dyes, Direct Orange 15 (DO15) and Direct Bue 1 (DB1) in which specifically the high molecular weight fraction (HMW) of DO15 is responsible for the differential results. They reported that due to this, HMW fraction of the orange dye should be separated and used in experiments rather than the low molecular weight (LMW) fraction. They reported that the staining mechanism of original Simons' staining remains the same. The HMW orange has a higher affinity towards fibres than the blue dye and greater molecular size and is thus less capable to penetrate the fibre interior. As the blue dye molecules are smaller, smaller capillaries adsorb blue dye and stain blue. Due to the higher affinity of DO15, it displaces the blue dye in pores that it can penetrate staining them orange. They stated that fibres that stain green have both small and large pores. The adsorbance of Direct dyes such as DO and DB to cellulose is due to their large planar molecules. Thus, they become fixed to cellulose via hydrogen bonding between the dye molecules' polar groups and cellulose's hydroxyl groups (Chandra & Saddler, 2012).

Xiaochun et al. (1995) also discussed the pore size that DO15 can penetrate. They note that HMW orange stain has a hydrodynamic size larger than 5 nm and thus can probe pores with a width larger than 5 nm. Smaller pores will then stain blue. For example, they stated that as Spruce contains pores of approximately 2 nm and therefore unbeaten wood will stain almost entirely

blue. They also noted that as the action of Simons' stain is independent of the kind of fibres due to HMW orange having a higher affinity to different fibres whether they are high or low lignin content. Xiaochun et al. (1995) thus concluded that Simons' stain will show differential results between fibres with different accessibility regardless of the kind of fibres. They also concluded that Simons' stain can be used to provide information similar to solute exclusion, but in a more localized and quantitative manner.

2.6.2 Modified Simons' stain

Previously described Simons' staining has been developed even further to assess internal and external surface area and pore sizes in wet state lignocellulosic substrates using the same base mechanism. Chandra and Saddler (2012) used DO15 to analyse accessible surface area of pretreated wood pulp. They used modified Simons' staining method using HMW DO15 and reported that it is relatively fast, simple and provides a good estimation of the surface area of a cellulosic substrate and estimates how well the substrate would response to enzymatic hydrolysis. They stated that one of its attractions includes its ability to measure both internal and external surface area in comparison with solute exclusion that use larger probing molecules to measure only internal surface area. They concluded that the distribution of DO dye indicates accessibility for cellulase enzymes due to the similar size profile of dye molecules and enzymes. In addition, the ratio of bound DO15 and DB1 indicates pore size distribution. They stated that a greater ratio of DO to DB indicates that the substrate is more accessible to cellulases.

Chandra and Saddler (2012) concluded that while Simons' staining can be used to estimate the specific surface area, great fluctuations in the absolute value of the dye ratios have been observed. They found fluctuations in adsorption ratios and absorbances of free dyes and stated that this could be due to the differences in the homogeneity of the DO dye. They stated that this can be due to the undefined molecular weight of DO from the manufacturer. As DB had a more defined molecular weight, the variations is most probably due to the insufficient ultrafiltration of the HMW fraction of DO. They stated that the observed differences can be due to the variations in DO dye and its HMW and LMW isolations. Thus, they highlighted the importance of refined isolation. They concluded that Simons' staining has shown to be one of the most useful assays for assessing cellulose accessibility. However, the relative specificity of DO and DB dyes for cellulose in the presence of lignin and hemicelluloses has not been fully resolved. In addition, they state that due to the variations in the absolute absorbances of free dyes, further refinements are required to ensure isolation of HMW DO dye.

However, DO15 has been since discontinued and thus Kwok et al. (2017) proposed the use of Direct Yellow 11 (DY11) as a replacement. They noted an improvement in the results with HMW DY11 using an adapted method from (Chandra & Saddler, 2012). Kwok et al. (2017) reported that DY11 is a similar

azo-stilbene dye and correlates with enzymatic hydrolysis over a variety of substrates. They stated that both dyes are condensation products of 5-nitro-o-toluenesulfonic acid and thus their structure should have the same functionality. They experimented with both dyes and determined their adsorptions to lignin, cellulosic and lignocellulosic substrates through fractionation procedure and adsorption spectra. Similarly to the original Simons' staining mechanism, they employed a set of specific dyes that have a similar size profile to cellulase enzymes which then selectively adsorb to cellulose and applied them to wet substrates. Specifically, the HMW fraction that has been retained behind a 100 kDa membrane has a high affinity for cellulase and a hydrodynamic diameter similar to cellulase. They noted that due to this, HMW fraction of yellow or orange dyes can be used as a surrogate for cellulase that selectively binds to cellulose fibres over lignin.

Kwok et al. (2017) used steam exploded samples that were composed of glucan and acid-insoluble lignin with negligible hemicellulose content. They measured 10 mg of the substrate into tubes and mixed the samples with water-phosphate buffer (0.3 M). After impregnation, increasing concentrations of dyes were added and the samples were placed in an incubator for 6 h at 70 C. The absorbances of free dyes were measured with UV, from which the amounts of adsorbed dye in wood pulp were calculated. Their results demonstrated a positive correlation between the maximum adsorption of dye and the enzymatic hydrolysis yields. Both dyes demonstrated similar bound dye values and accessibility with respect to glucose production. Kwok et al. concluded that the adsorption of the dyes provided evidence that the bound dye may be considered as accessible cellulose. They note that size similarities to cellulase enzymes, the ease and stability of the assay and the potential for measuring both interior and exterior surface area are essential characteristics of an effective porosity probe.

Kwok et al. (2017) concluded that DY11 may replace DO15 for modified Simons' staining assay. The dyes bind to cellulase over lignin and can predict the initial rate of cellulose conversion in lignocellulosic substrates. However, they stated that with both dyes the isolation of HMW fraction results in inconsistent absolute adsorption values between samples. In addition, dilution for the spectrophotometer measurements compounds the issue. Neither dye provided an absolute measure of cellulose fibre accessibility to cellulases in lignocellulosic substrates. However, this method provides a quick, semi-quantitative assessment of cellulose accessibility and indication of pore size distribution.

2.7 Evaluation of methods

An ideal porosity analysis method would obtain quantitative information of multiple parameters of porosity (accessible surface area, pore volume...). As the goal of these methods is to obtain a wide range of information quantitatively, be relatively easy to perform and use a wet-state substrate, the five methods reviewed in this thesis were positioned in a 2-axis evaluation matrix in Figure 1. This illustration positions the methods from quantitative to qualitative and from difficult to perform to easy and inexpensive to perform. The ideal methods that are both quantitative and quick, easy and inexpensive would be positioned on the right top quadrant which is illustrated with a light green circle. This is where the most suitable and favourable methods for porosity analysis would be located.

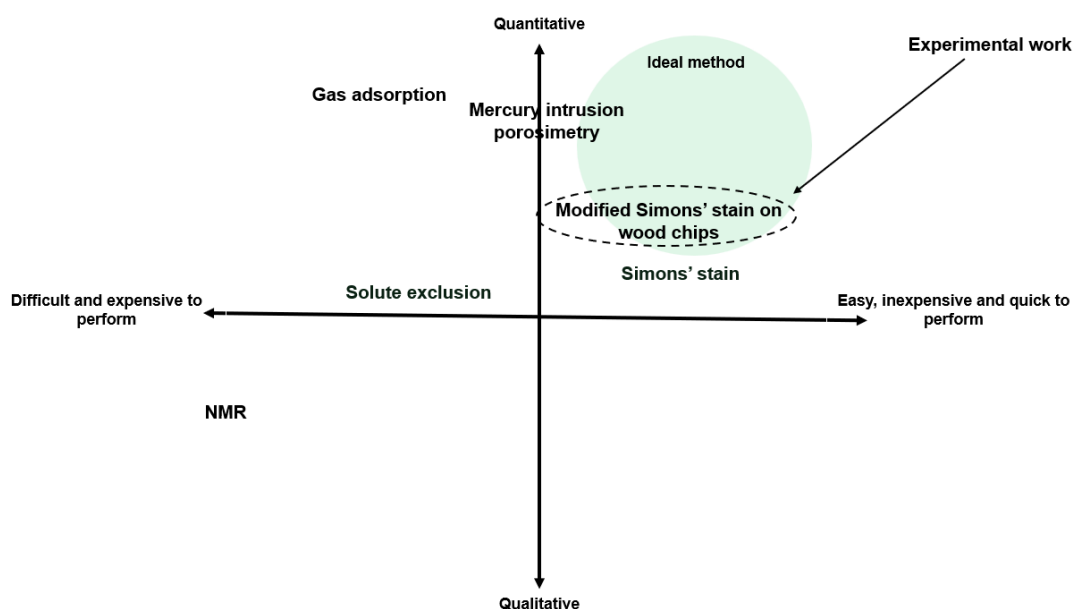


Figure 1. Illustration of the methods, and where they fit on a 2-axis evaluation matrix from qualitative to quantitative and ease of performance. The light green area represents the region where the most suitable methods are positioned. Dashed modified Simons' stain on wood chips is the method used in this thesis' experimental work.

As can be seen from Figure 1. Most methods are positioned away from the green circle, meaning that they are not ideal in analysing lignocellulose biomass porosity. NMR relaxometry and cryoporometry are both expensive and difficult to perform and they are used to obtain qualitative information about porosity. Cryoporometry can be used to estimate pore sizes and distribution and relaxometry indicates pore sizes and assists in understanding of the chemical composition and water behaviour of pores (Bränvall et al. 20120) (Jeoh et al. 2017). NMR methods are not considered absolutely quantitative

for analysing lignocellulose biomass and are thus positioned on the bottom left quadrant. MIP and gas adsorption are positioned on the top left and right quadrant as they are both quantitative analysis methods. However, as they both require drying of the substrates, they are not ideal methods for porosity analysis in lignocellulosic substrates. Gas adsorption is positioned to be more difficult to perform as the experimental time is higher than for MIP. Solute exclusion and Simons' staining mechanism are both semi-quantitative methods and are positioned in between qualitative and quantitative. Solute exclusion is more difficult to perform as it is more laborious, unspecific to cellulose and does not account for the external surface area (Meng & Ragauskas, 2014). In addition, it is less quantitative than Simons' stain as it only accounts for the external surface area (Beecher et al. 2009). Simons' staining accounts for both external and internal surface area and is easier and quicker to execute (Meng & Ragauskas, 2014). Thus, it is positioned on the top right quadrant in Figure 1.

As can be seen from Figure 1, the modified Simons' staining from Kwok et al. (2017) would be positioned slightly higher than the original Simons' staining mechanism. This is due to the use of HMW Direct yellow 11, as Kwok et al. (2017) reported that it performed better in some experiments than Direct orange dye 15. However, Kwok et al. conducted their experimental work using wood pulp as the substrate. In contrast, the experimental work in this thesis applied the modified Simons' stain method to wood chips. The goal was to evaluate the method's performance on a different lignocellulosic material. If proven effective on wood chips, this would demonstrate the method's applicability across a wider range of substrates. Thus, this versatility combined with the wet-state analysis would enhance its value. Furthermore, this would position Simons' staining even higher on the comparison framework. Successful application to wood chips would not only reinforce its ease of use but also improve its level of quantitiveness in measuring porosity.

In addition, most of these methods are used as combinations with each other to obtain a wider range of information. As shown in Figure 1, the methods differ in their degree of results ranging from qualitative to quantitative and type of substrate they require. Meng and Ragauskas (2014) reported that it is not straightforward to analyse porosity in biomass due to the behaviour of plant cells and lignocellulosic structure. They noted that one property that makes biomass difficult to analyse is the "ink-bottle" effect that most methods fail to account for, including Simons' stain and solute exclusion. In addition, considerable variation in results arises from the differing measurement principles across techniques. Thus, it is beneficial to use a combination of methods to analyse a single lignocellulosic substrate. Both to minimize variability between techniques, and to enable a more comprehensive analysis, combining methods allows for the assessment of multiple porosity parameters. While some techniques provide information on pore size distribution,

others offer insight into pore accessibility or behaviour under specific conditions.

Some popular combinations include Simons' stain and NMR, X-ray and NMR, MIP and nitrogen adsorption and Simons' staining and BET. Past studies of using these combinations are listed below in Table 1. Table 1. Also includes the advantages of using these combinations as stated in the research.

Table 1. Summary of four technique combinations used in past research and their advantages.

Combination	Research	Advantages
NMR and Simons' stain	Meng et al. (2013) studied pore size distributions and specific surface area of lignocellulosic substrates using NMR and Simons' stain. By combining Simons' stain with NMR relaxometry and diffusometry, they more effectively tracked pore expansion during pretreatment.	<ul style="list-style-type: none"> - NMR compensates for Simons' stain limitations of pore entrance restriction - Enables broader interpretation of pore structure evolution by combining accessible porosity with water dynamics and structural resolution
NMR and X-ray	Bränvall et al. (2021) combined NMR and small- and wide-angle X-ray scattering to study supramolecular changes in wood cell walls during chemical pretreatment. NMR was used to model fibril aggregate size and specific surface area in the wet state, while X-ray scattering characterized fine pore structure. Fiber saturation point measurements supported this by quantifying water-accessible pore volume.	<ul style="list-style-type: none"> - Allows pore size estimation without assuming geometry - Provides insight into both fibril structure and accessible pore space - Maintains wet-state analysis, avoiding pore collapse from drying
MIP and nitrogen adsorption	Sun et al. (2022) evaluated wood pore characteristics using MIP and nitrogen adsorption. MIP assessed macropores, while nitrogen adsorption analysed micro- and mesopores. MIP was noted to under detect nanopores, which nitrogen adsorption compensated for.	<ul style="list-style-type: none"> - Expands measurable pore size range from nanometres to micrometres - Combines pore volume (MIP) with surface area and fine pore structure (nitrogen adsorption)
Simons' stain and BET	Wang et al. (2020) analysed nanostructure and porosity in wood using BET on dried powder and Simons' stain on wet-state samples. BET provided specific surface area, while Simons' stain gave insight into enzyme-accessible porosity in more realistic conditions.	<ul style="list-style-type: none"> - Combined dry-state and wet-state porosity - Simulates wet-state industrial conditions - Provides combined insight into cell wall accessibility and structural surface area

As can be seen from Table 1, combining multiple porosity analysis methods rather than relying on a single technique provides a broader and more comprehensive understanding of lignocellulosic biomass porosity. Each method is based on different physical principles and probes specific porosity parameters, such as surface area, pore size distribution, or accessibility in dry or wet states. By integrating complementary techniques information of a wider range of pore sizes, or analysis of both structural and functional porosity can be obtained. In addition, this reduces the uncertainty introduced by the limitations of any one method. Furthermore, combining methods minimizes the inaccuracies of mechanisms leading to more robust and reliable interpretations of biomass porosity.

To conclude this literature review and study on different methods for analysing lignocellulose biomass porosity, there are great differences between methods due to the fundamental difference of mechanisms. The results range from qualitative to quantitative and the results differ in the range of information obtained. Thus, a combination is often used, which reduces the limitations of any one technique. As illustrated in Figure 1. which positions porosity analysis methods based on their degree of results and practicality, the following experimental work aims to evaluate the performance of modified Simons' stain on wood chips. This helps determine whether the method is effective for this specific substrate and thus where it should be positioned within the evaluation matrix. Furthermore, the modified Simons' staining could be a valuable complementary method used alongside techniques, such as BET, NMR or enzymatic hydrolysis.

3 Experimental work

As the experimental work uses wood as substrate, some previous studies on hardwood and softwood porosities are reviewed in Section 3.1, providing insight into wood porosity specifically. The methods and materials of the experimental work are shown in Section 3.2 followed by results in Section 3.3. Analysis of results is provided in Section 3.4.

3.1 Wood porosity

Jiangping et al. (2015) reported that pores in wood can be divided into three categories according to IUPAC classifications: macropores include pore sizes over 50 nm, mesopores with pore size 2 nm to 50 nm and micropores with pore size smaller than 2 nm. The diameter of the larger pores varies between 15-400 μm which are connected by pits. Pits contain pores with a diameter of 0.4-30 μm . They also stated that in wood liquid will diffuse through the cell walls of the lumen of the fibre. These pores have a diameter of a few nanometres due to the partial filling of space between the cellulose microfibrils by lignin and hemicellulose. They noted that these cell wall pores play an essential role in wood behaviour, such as mechanical properties, stabilization, fibre shrinkage and thus affect wood drying, preservation, modification, chemical pulping and bleaching. Therefore, they concluded that research of wood porosity plays an important role for tree cultivation, processing and material development.

Porosity of wood varies greatly between hardwoods and softwoods. Plötze and Niemz (2011) determined the densities and porosity parameters of various hardwoods and softwoods with MIP. They reported that according to pore size distribution, four size classes could be identified. Macropores with a radius of 58–2 μm and 2–0.5 μm , mesopores 500–80 nm, and micropores 80–1.8 nm. In addition, they concluded that hardwoods showed a higher ratio of micropores representing the cell wall capillaries with diameters of 0.3–60 nm. They also found a high cumulative pore volume due to the higher number of micropores with poorer accessibility.

Plötze and Niemz (2011) defined wood porosity as one minus the solid volume fraction, which can also be calculated from normal bulk density (oven-dry density) and the specific cell wall density of solids. They stated, that according to previous studies, the ring-porous hardwoods mostly had pores with a radius of 0.1–5 μm and a higher ratio of small pores <0.1 μm than large pores >5 μm and that the average pore radius is much smaller (0.05–0.35 μm) than softwoods. Plötze and Niemz noted that hardwoods have a more complicated structure and that they contain large vessels with diameters ranging from 50–400 μm (early spring wood) and 20–50 μm (latewood). Hardwoods also contain pits that are much smaller than the pits of softwoods.

Plötze and Niemz (2011) determined the normal bulk density and the specific cell wall density of hardwoods and softwoods and reported an approximately 30% lower value for softwoods. They also found a larger variation of bulk density with hardwoods. They reported that the softwood Norway spruce (*Picea abies*) had total porosity of 73.68% and porosity of 68.39% measured with MIP. For hardwood Common birch (*Betula pendula*) the total porosity of 60.45% and porosity of 57.12%.

In addition, Plötze and Niemz (2011) determined the pore volume and total specific surface area for hardwoods and softwoods and reported that hardwoods have on average 60% of the pore volume and total specific surface area of softwoods. In addition to this, they determined the relative pore volume of macro-, meso- and micropores and the most frequent pore radius in different wood species. They identified two classes of macropores 58-2 μm and 2-05 μm and reported both relative pore volumes in addition to meso-, and micropore. They reported that for Norway Spruce the relative pore volumes were 26.01% and 19.93% (macropores 58-2 μm and 2-05 μm), 49.16% (mesopores) and 4.90% (micropores) and that the most frequent pore radius was 148.2 nm. The specific surface area was measured to be 117.97 m^2/g for Norwegian spruce. For common birch the relative pore volumes were 44.88% and 41.98% (macropores 58-2 μm and 2-05 μm), 8.77% (mesopores) and 4.37% (micropores) and that the most frequent pore radius was 6399.1 nm. The total specific surface area for common birch was measured to be 63.01 m^2/g . Plötze and Niemz (2011) concluded that porosity and density are important parameters that influence the properties of wood and that they showed significant variations between species and that a difference between hardwoods and softwoods could be clearly identified. They also stated that nearly no variations were found in the cell wall density, which was determined to be 1.5 g/cm^3 .

As reported in both (Plötze & Niemz, 2011) and (Jiangping et al. 2015) wood porosity is an important characteristic and gives valuable information for wood processing. There is a significant difference in pore sizes, pore size distributions, surface areas and ratios of mesopores, macropores and micropores between Spruce and Birch. Spruce (softwood) exhibits a higher porosity, higher volume of mesopores and a significantly lower most frequent pore radius. The porosity for Birch (hardwood) was measured to be lower than spruce but had a greater most frequent pore radius and much higher volume of macropores. In the following experiment, these two wood species were probed using modified Simons' staining to assess the accessible surface area and pore sizes. In the light of this information a lower porosity but higher number of bigger pores for hardwood than softwood can be expected.

3.2 Materials and method

This method was adapted from Kwok et al. (2017) and modified for wood chips by increasing the incubation time from 6 hours to 24 hours. A solution of Direct Yellow 11 (Pylam Products, Garden City, NY) at a concentration of 10 mg/mL was ultrafiltrated with an Amicon® Ultra-15 centrifugal device (100 kDa MWCO, Merck/Sigma-Aldrich, Finland). The sample was centrifuged at $4,000 \times g$ for 40 minutes at room temperature to separate HMW components from LMW ones. This procedure was repeated twice to ensure effective separation. The material retained above the membrane was collected and freeze-dried. The resulting solid was redissolved to prepare a 10 mg/mL solution of HMW DY11 for further use. Untreated Spruce and Birch chips from Metsä were cut into 0.2 cm width pieces in the direction of fibres and then halved. Three samples of these hardwood and softwood chips were placed in the oven to calculate the average dry weight. 100 mg of dry weight wood pieces were weighted for 5 trials of hardwood and 5 trials of softwood. 4 chips in each trial were added to control the adsorption surface area. 1 ml of deionized water and phosphate (0.3 M, pH of 6) buffer was measured for each trial tube and left for impregnation overnight. Increasing concentrations of Direct Blue 1 (Sigma-Aldrich, Finland) and Direct Yellow 11 dyes were measured in tubes which were then left in the incubator for 24 hours at 70 C. Samples were centrifuged for 5 minutes and supernatants were separated and diluted for spectrophotometer analysis. Diluted samples were analysed with UV (Shimadzu UV-2550 spectrophotometer) and absorbances were recorded.

3.3 Results

Data processing for the experimental work is included in Section 3.3.1. and results are shown in Sections 3.3.2.

3.3.1 Data processing

The dry matter content (DMC, %) of softwood (SW) and hardwood (HW) was determined to be 43.63% for SW and 59.04% for HW. This was measured by oven-drying wood chips and recording their wet and dry weights. To obtain 100 mg of dry wood per sample, the required wet weights were calculated as 0.23 g for SW and 0.17 g for HW. For the experiment, five trials were prepared, each consisting of four HW and four SW chips, weighed into tubes at the corresponding wet weights.

The following initial concentrations of Direct Yellow (DY) and Direct Blue (DB) in Table 2 were measured into trial tubes.

Table 2. Initial concentrations (mg/ml) of DY and DB in wood trials.

	HW/SW1	HW/SW2	HW/SW3	HW/SW4	HW/SW5
DY/DB (mg/ml)	0.25	0.5	0.75	1.0	1.5

After collecting the samples from the incubator after 24 hours, centrifuging the samples and collecting supernatants, the supernatants were diluted by a factor of 50 and absorbances were recorded. The concentrations of dyes were calculated using modified Beer-Lambert law. Kwok et al. (2017) stated in their article that for characterizing the dual dye spectra, the Beer Lambert law may be extended as follows for DY11 and DB1:

$$\begin{aligned} A_{412nm} &= \varepsilon_{Y/412}LC_Y + \varepsilon_{B/412}LC_B \\ A_{620nm} &= \varepsilon_{Y/620}LC_Y + \varepsilon_{B/620}LC_B \end{aligned} \quad (1)$$

In which A_{412nm} and A_{620nm} are the recorded absorbances of DY11 and DB1 at wavelengths of 412 nm and 620 nm, $\varepsilon_{Y/620}$, $\varepsilon_{Y/412}$, $\varepsilon_{B/412}$ and $\varepsilon_{B/620}$ are the determined extinction coefficients for DY11 and DB1 from standardized calibration curves. L is the cuvette length, which was 1 cm in all experiments and C_Y and C_B are the concentrations of DY11 and DB1. The concentrations of DY11 and DB1 were calculated using extended Beer-Lambert law equation using matrixes. The Beer-Lamberts law simplified to a matrix form as follows:

$$\begin{bmatrix} \varepsilon_{Y/412} & \varepsilon_{B/412} \\ \varepsilon_{Y/620} & \varepsilon_{B/620} \end{bmatrix} \cdot \begin{bmatrix} C_Y \\ C_B \end{bmatrix} = \begin{bmatrix} A_{412nm} \\ A_{620nm} \end{bmatrix} \quad (2)$$

Which can be inverted to the following form

$$\begin{bmatrix} C_Y \\ C_B \end{bmatrix} = \begin{bmatrix} \varepsilon_{Y/412} & \varepsilon_{B/412} \\ \varepsilon_{Y/620} & \varepsilon_{B/620} \end{bmatrix}^{-1} \cdot \begin{bmatrix} A_{412nm} \\ A_{620nm} \end{bmatrix} \quad (3)$$

Calibration curves were determined for DY11 and DB1 by measuring absorbances of known concentrations of dyes. Extinction coefficients were calculated for dyes first using 1:10 phosphate buffer (0.3 M) and deionized water as baseline with the UV recordings. Calculated extinction coefficients were recorded in Table 3.

Table 3. Extinction coefficients for DY and DB.

Wavelength (nm)	Extinction coefficients	
	DY	DB
412	52.5	6.7
620	0.02	65.7

Concentrations of DY11 and DB1 in the supernatants were calculated using Equation 3. The concentration of bound dye in wood chips was calculated by subtracting the supernatant dye concentrations from the initial concentrations of dye in Table 2 and divided by measured dry wood masses. Calculated bound dye (mg/mg) can be seen in Tables 4 and 5.

Table 4. Bound dye (mg) per mg of hardwood (mg/mg)

	HW1	HW2	HW3	HW4	HW5
DY (mg/mg)	-0.000991	-0.001119	0.001438	0.007466	0.006797
DB (mg/mg)	0.001187	0.001243	0.002322	0.009339	0.003087

Table 5. Bound dye (mg) per mg of softwood (mg/mg)

	SW1	SW2	SW3	SW4	SW5
DY (mg/mg)	-0.000796	-0.000480	0.001829	0.003140	0.006301
DB (mg/mg)	0.000563	0.000689	0.001119	0.003001	0.002661

3.3.2 Results

Bound dye (mg/mg) were for HW in Figure 2 and for SW in Figure 3 were determined.

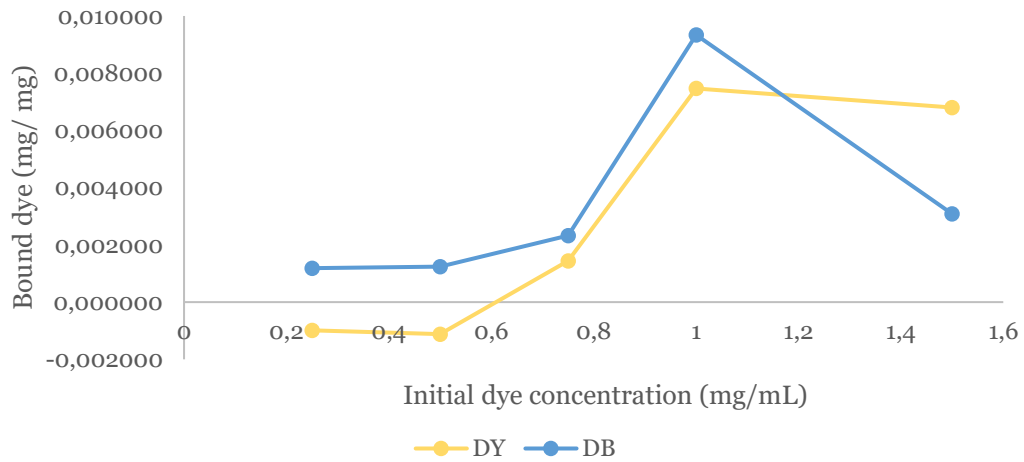


Figure 2. Bound Yellow and Blue dyes (mg/mg) of hardwood against increasing initial dye concentrations.

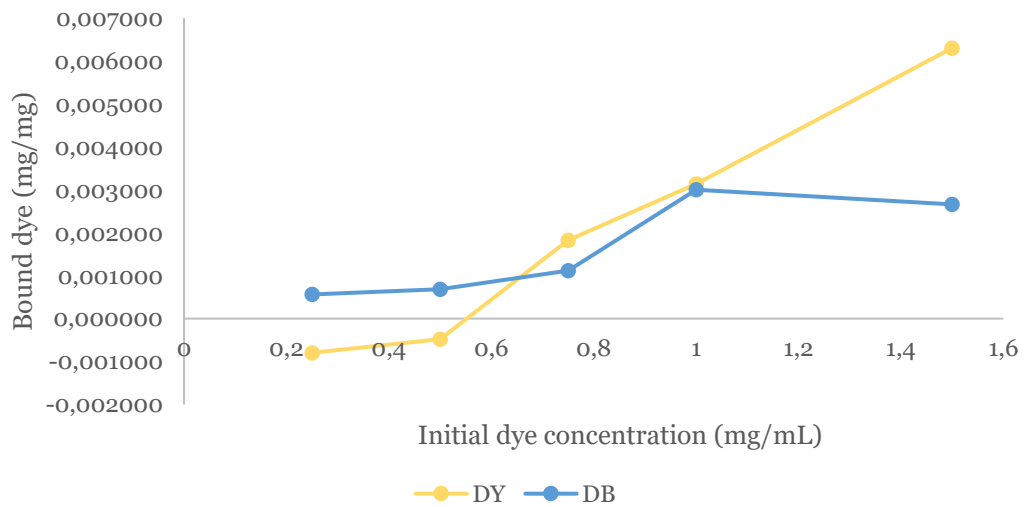


Figure 3. Bound Yellow and Blue dyes (mg/mg) of softwood against increasing initial dye concentrations.

3.4 Result analysis

The results of amounts of bound dyes for hardwood and softwood were not as expected. As can be seen in Figures 2 and 3, the masses of bound DY11 were negative for both SW and HW samples 1 and 2, which was unexpected as it indicated that concentrations of their supernatants were higher than the initial concentrations of DY11 in the samples. For both softwood and hardwood, the amount of bound dye increased with increasing initial concentration (samples 1-5) indicating that the dyes were able to penetrate the wood pores with an increasing trend as more dye molecules became available. Additionally, the results showed higher amounts of bound DB than DY for most samples suggesting that softwood and hardwood contained more smaller pores than larger pores. A plateau in bound dye curves were expected with higher initial concentrations of dyes. This would indicate that all available wood pores would be saturated with dyes. However, in Figures 2 and 3 this has not been reached. Additional samples with higher initial dye concentrations would have been required to reach the maximum absorbance of dyes and thus indicating maximum accessible cellulose. However, this was not possible within the timeline of this thesis. In Figure 2, the bound dye for HW sample 4 was unexpectedly high compared to HW sample 5, which indicated an error in the calculations or measurements. A possible error factor includes the dilution of the samples for the UV measurements, as the dilution factor was 50. A small error in measuring solutions during dilution would affect greatly the absorbance and thus the amount of bound dye. Furthermore, the sudden decrease in bound DB for softwood sample 5 indicated an error in measurements as the trend was expected to be continuously increasing.

The experiments were repeated multiple times during the experimental work. Initially, the extinction coefficients were measured using deionized water as baseline with the UV spectrophotometer and when measuring the supernatant absorbances. This however, resulted in negative values for bound DY for all SW and HW samples. The extinction coefficients and free dye absorbances were recorded again using 1:10 ratios of phosphate buffer (0.3 M) as baseline and in dilutions. It was discovered that using phosphate buffer as baseline with UV measurements lowered and shifted the absorbance curves, which then increased the concentrations of bound dye. However, some samples still resulted in negative bound dye values. A blank sample of dyes without wood was conducted (0.25 mg/ml), which showed slightly lower concentrations of DY and DB than initial concentration. This indicated that the phosphate buffer potentially reacted with the dyes or blocked their absorbance. The exact reason for this was not identified. In addition, reference samples of SW and HW without dyes were conducted and their absorbances were recorded. Their absorbances showed peaks in the UV yellow wavelength area, which indicated that the wood chips released extractives in the solutions that have some absorbance. The HW reference sample showed higher absorbance

than SW. These reference absorbances were subtracted from absorbances of supernatants in results shown in Tables 4 and 5. Ideally, the supernatants absorbances would have been recorded again using reference solutions and buffer as baseline but this was not possible within the timeline. Thus, these subtracted results are not as reliable.

The reason for these inconclusive results and discoveries of wood released extractives or the effect of the phosphate buffer on absorbances could not be identified. Averages of absorbances of multiple trials with each sample was calculated to ensure that an error was not made with absorbance recordings. In addition, reference absorbances were measured multiple times and an average absorbance was calculated before calculations. A negligible variation between trials was noted. However, due to discoveries during the experiment with the effects of phosphate and wood extractives, some supernatants were analysed over long periods of time. Time could have affected the absorbances and calculations. In addition, error factors include dilutions error, instrumental errors, and (Kwok et al. 2017) reported that there is a significant variation between batches of yellow dye. In addition, Chandra and Saddler (2012) reported variability of around 10% in absorbance recordings. Both Kwok et al. (2017) and Chandra and Saddler (2012) reported that refined isolation of HMW DY fraction is difficult to ensure as the molecular weight of DY is unknown. It was concluded that modified Simons' staining assays requires future research.

Future research should first investigate the differences in results obtained with multiple substrates as the presence of lignin and hemicelluloses has been reported to affect cellulose accessibility measurements. Previous studies (Kwok et al. 2017) and (Chandra & Saddler, 2012) have primarily used pretreated wood pulp, whereas untreated wood pulp or wood chips have been less explored. Since modified Simons' staining has so far been applied mainly on wood pulp and the results with wood chips remain inconclusive, conducting experiments simultaneously on wood pulp and chips, untreated and treated, would provide valuable information. Furthermore, this could clarify the reason as of why wood pulp yielded consistent results while wood chips have not. In addition, this could improve understanding the presence of lignin and hemicelluloses and behaviour of the dyes influence accessibility measurements.

In addition, a higher concentration of Dy could be used to reach full saturation of dyes. The substrates could also be pressurised to reach full saturation of dyes in pores. From Figures 2 and 3 can be seen that full saturation of pores has not been reached. This would minimize inaccuracies in cellulose accessibility and dye ratios. In addition, the behaviour of DY with phosphate buffer with UV measurement was unexpected. There has been some research of the effect of pH on DY absorbances, and it has been shown that pH affects

DY absorbances significantly (Kwok et al. 2017) and (Khezrianjoo, 2019). While the behavior of DY is still relatively unknown, Khezrianjoo reported significant changes in absorbances of DY with changing pH. The pH of the samples during experiments were recorded and initial pH was measured to be 6.44 and after incubation 6.34 for both HW and SW. Therefore, it was concluded that this difference in pH was not the reason for such behaviour. However, future research should also include analysis of DY as it is yet relatively unknown and rarely used in Simons' staining.

To conclude, the results indicated a somewhat increasing trend in bound dye values and thus could potentially be used to analyse cellulose accessibility and pore sizes. The results showed that HW and SW contain a higher abundance of smaller pores and that HW contain a higher number of smaller pores than SW. However, the results from this experimental work were inconclusive as fluctuation in absorbances and bound dye values were recorded. Future research is required to determine the suitability of this method on variety of lignocellulosic substrates, and first steps could include analysis of wood pulp compared to wood chips.

4 Summary and conclusions

As stated in Section 2.1, lignocellulosic biomass has immense potential in replacing fossil fuel derived materials if its bioconversion processes could be fully optimized. The bioconversion includes steps of pretreatment and enzymatic hydrolysis which are greatly affected by the complex structure of lignocellulosic biomass and its natural recalcitrance. The structure of lignocellulosic biomass is composed of lignin, hemicelluloses and cellulose which together form an intricately linked structure. This structure has a natural recalcitrance to enzymatic hydrolysis due to its heterogeneous multi scale structure of plant cell walls (Zoghلامي and Paës, 2019). Factors affecting its recalcitrance can be divided into structural factors and chemical factors. The structural factors include porosity which can be measured in terms of surface area, pore size and volume and pore volume distribution.

Porosity parameters can be analysed with multiple different methods which differ in their type of results and mechanism. No method to date can analyse porosity of lignocellulose biomass fully quantitatively and accurately. Among the methods reviewed in this thesis, most rely on assumptions and may affect the accuracy of analysis. Five of these methods were evaluated and analysed in this thesis. The aim of this thesis was to review their mechanisms, advantages and disadvantages and four of their useful combinations. The two most quantitative methods gas adsorption and MIP require a drying of the substrates. Water removal from the substrates leads to collapses of the pores resulting in inaccurate values of porosity. Thus, these two methods are not ideal. It was concluded that porosity analysis methods require future research as no method can analyse porosity absolutely and be easy to perform.

The goal of the experimental work in this thesis was to test the suitability of modified Simons' mechanism from Kwok et al. (2017) on wood chips instead of wood pulp. However, the results showed inconsistencies and fluctuation. Thus, future research of this method is required to determine its suitability for a variety of substrates. Possible factors for the results included fluctuation of absorbance analysis with UV, effect of time, wood released extractives, instrumental errors and behaviour of biomass and dyes. In addition, concentrations of free dye suggested that the wood pores were not fully saturated by the dye molecules. Future research could include pressurising of the wood-dye solution samples to ensure that all free dye is bound. Discoveries were made as well about the behaviour of the dyes with phosphate and water buffer and the release of extractives from wood in blank samples. More accurate results could be obtained by using the extractive solution as a reference in UV measurements. In addition, more reliable results could be obtained by using fresh samples to minimize the effect of time on degradation of dyes and wood extractives. First steps of future research would be to conduct the same

method for wood pulp to conclude the differences in results between substrates.

To conclude this thesis, the reviewed methods for analysing lignocellulose biomass porosity have their advantages and disadvantages. Combinations of methods can be used to obtain a wider range of information and to minimize the inaccuracies with any one method. Use of combinations in previous research has been proven effective. This is due to the differences in mechanisms, pretreatment of substrates and degree of results. Modified Simons' staining is a promising method for analysing lignocellulosic biomass but requires future research. If a fully quantitative method or a combination for analysing lignocellulose biomass porosity was one day found, it would greatly assist in optimizing bioconversion of biomass into alternative for fossil fuel-based products. Future research is required on this complex and multi-layered topic.

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