Forest residues as a potential feedstock for a biorefinery: material balance and pretreatment strategies

by

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Abstract

Forest residues represent an abundant and potentially sustainable source of biomass which could be used as a feedstock for a biomass-to-chemicals-and-fuels process (biorefinery). However, due to the heterogeneity of forest residues, one of the expected challenges will be to obtain an accurate material balance of both the starting and pretreated material. As current compositional analysis methods have been developed to quantify more homogenous feedstocks such as whitewood and agricultural crops, it is likely that they will have difficulty in providing a complete material balance for these more diverse substrates. The research work initially assessed the robustness of established methods to quantify a variety of forest residues (bark, hog fuel, forest thinnings, logging residue, disturbance wood) before and after steam pretreatment. It was anticipated that the diverse chemistry and heterogeneity of forest residues would make it difficult to obtain an accurate material balance. Although the NREL recommended methods provided a reasonable estimate of carbohydrate components of the various feedstocks, method revision was necessary to accurately quantify the non-carbohydrate components and thus obtain an acceptable summative mass closure. This was particularly evident for high-extractive containing residues such as bark. After steam pretreatment, the incomplete removal of extractives from the pretreated material proved to be more problematic. The refined material balance methods were subsequently used to evaluate the potential of using pretreated forest residues as a biorefinery feedstock. Acid catalysed steam pretreatment was not as effective on forest residues and poor sugar yields were obtained despite using high enzyme loadings. It was likely that, in the acidic medium resulting from SO_2 catalysed steam pretreatment, the extractives reacted with the lignin and consequently restricted enzyme accessibility to the cellulose. In contrast, an alkaline pretreatment effectively removed most of the extractives and lignin from cellulosic components of the bark. The resulting cellulose-rich, water insoluble component could be almost completely hydrolyzed. It was apparent that established analytical methods will have to be modified to obtain a representative material balance of both the starting and pretreated material and that, even with “tailoring” pretreatment/fractionation strategies, forest residues will prove to be challenging feedstocks for any potential bioconversion process.

Preface

All of the work presented henceforth was conducted in the Forest Products Biotechnology and Biofuels Laboratories at the University of British Columbia, Point Grey campus.

A version of Chapter 3.1 has been published in the *Biotechnology for Biofuels* journal [Bukhardt S, Kumar L, Chandra R, Saddler J: How effective are traditional methods of compositional analysis in providing an accurate material balance for a range of softwood derived residues? *Biotechnology for Biofuels* 2013 6:90]. I was the responsible for all major areas of data collection and initial manuscript composition, while receiving guidance from J Saddler, R Chandra, and L Kumar during the concept formation, data analysis, and manuscript revision stages. J Saddler was the supervisory author on this project and was involved throughout the project in concept formation and manuscript edits.

In Chapter 3.2, I was responsible for all major areas of data collection and analysis, as well as the majority of thesis manuscript composition. L Kumar and J Saddler were involved throughout the stages of concept formation and contributed to manuscript edits. L Kumar additionally contributed to data analysis. J Saddler was the supervisory author on this project and was involved throughout the project in concept formation and manuscript edits.

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List of Abbreviations

AACC	American Association of Cereal Chemists
AHP	alkaline hydrogen peroxide
AFEX	ammonia fiber expansion
AOAC	Association of Analytical Communities
BCA	bicinchoninic acid
BK-LPP	beetle-killed lodgepole pine white wood
Ctec2	Cellic Ctec2 (Novozymes)
DNS	dinitrosalicytic acid
FPU	filter paper units
GC	gas chromatography
HLPC	high performance liquid chromatography
H_2O_2	hydrogen peroxide
HOG I&II	hog fuels
HTP	high-throughput (analysis technique)
IFS	interface fire slash
LAP	laboratory analytical procedure (NREL)
LR	logging residue
MBTH	3-methyl-2-benzothaizolinonehydrazone
MC	moisture content
MSW	municipal solid waste
NaOH	sodium hydroxide
(N)IR	(near) infrared
NMR	nuclear magnetic resonance
NREL	National Renewable Energy Laboratory
SO_2	sulfur dioxide
TAPPI	Technical Association of the Pulp and Paper Industry
USDA	United States Department of Agriculture
USFDA	United States Food and Drug Administration
UV	ultra violet
WW	white wood (Douglas fir)

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1. Introduction

1.1 Background

Climate change and energy security concerns, due to the continued use of fossil fuels, have created a global interest in using clean and renewable resources to try to meet some of our future energy and commodity chemical demands. Over the last two decades, some parts of the world, such as Brazil and the US, have used bioethanol as a partial substitute for gasoline. As a consequence, global production of fuel ethanol is expected to double since 2006 reaching 115 billion liters in 2013 (OECD/FAO, 2013). However, in order to make a complete transition from a 'fossil fuel economy' to a 'bioeconomy', the whole barrel of oil needs to be replaced by biomass-derived counterparts. The so called "Biorefinery" concept integrates biomass conversion and fractionation processes to produce different types of fuels and chemicals in an analogous fashion to how oil now gives us the fuel and thousands of chemicals, we use in our daily life.

Most of the current fuel ethanol is produced from starch and sugar-based feedstocks. However, the use of energy and water intensive food crops for the production of fuels has led to skepticism over the long term sustainability of starch/sugar cane to ethanol processes (Shapouri et al., 2002). Owing to both physical and political limitations on the expansion of biofuel production on arable land, new production capacity will most likely be based on more abundantly available and likely, less expensive cellulosic biomass such as agricultural and forest residues. However, being a "highly heterogeneous" substrate, lignocellulosic materials pose several challenges as a biorefinery feedstock when compared to "more uniform" sugar or starch-based feedstocks.

The physical structure and chemical composition of lignocellulosic materials varies from the ultra-structural level (such as different locations of the plant cell wall) to the microscopic level (such as the different cell types, microfibril organization, etc.). The degree of heterogeneity is even greater when comparing the macro-level structures (such as different anatomical fractions of the plants or different species and types of plants such as

angiosperms to gymnosperms to grasses) (Hon & Shiraishi, 1991). In addition to lignin, extractives and ash, lignocelluloses typically contain different types of carbohydrate polymers. The content and structure of these cell wall polymers also change considerably depending on the anatomical region and the type of biomass (Sjostrom, 1993).

In the past, bioconversion research has mostly utilized relatively "clean" material such as white wood chips and agricultural residues. These feedstocks are much more homogenous compared to "forest residues" such as bark or hog fuel that will be likely be used, where the degree of complexity and heterogeneity is much higher. However, with increasing feedstock competition and the requirements to meet large scale biomass demand, future biorefineries will invariably have to use biomass resources such as forest residues as one of their options. For example, in order to meet the US mandate of replacing 30% of traditional fuels with biofuels by 2030, an estimated one billion dry tons of biomass per year is expected to be required (Perlack et al., 2005). The USDA has reported that this amount of biomass can only be obtained using strategic planning (Perlack et al., 2005; U.S., 2011) where effective utilization of different biomass resources including both agricultural and forest biomass is required. A major challenge will be modifying and monitoring the feedstock and product streams so that the heterogeneity of substrates such as forest residues can be tailored to the various biorefinery unit operations. As will be described in more detail within the thesis, one of the goals of the work was to assess the suitability of using forest residues as a biorefinery feedstock compared to white wood chips. Six different forest residues were used in the study including logging residues, interface fire slash, bark, hog fuel and disturbance wood.

Similar to an oil refinery, one of the prerequisites of being able to optimise the different unit operations of an integrated biorefinery process is the accurate quantification of the different biomass components and the ability to establish a material balance throughout the overall process. The heterogeneity of lignocellulosic materials is a critical barrier to this objective. A reasonable mass balance can only be derived from a combination of several analytical procedures, ranging from the accurate determination of moisture

content to the more complex structural analysis (including quantification) of natural polymers such as cellulose and lignin. The current methods used to analyse different biomass components have been historically derived from primarily the pulp and paper (Sluiter et al., 2010) and agriculture sectors (Hames, 2010). These sectors are typically only interested in semi-quantitative analyses of a few of the biomass components which, for example, would indirectly reflect the properties of a specific characteristic such as the brightness of a pulp, pulp yield or animal digestibility. The detailed quantification of the total starting material and each biomass component through a multi-step process was not a major focus of either the forest or agricultural based sectors. However, the oil crisis of the late 1970's changed this perspective by precipitating an interest in the potential of producing fuels and chemicals from biomass.

The National Renewable Energy Laboratory (NREL) in Golden, Colorado, has developed a comprehensive set of laboratory analytical procedures for characterising and quantifying different biomass components (NREL, 2011). These methods have been cited extensively in the bioconversion literature (Kumar et al., 2010; Li et al., 2010; Xu et al., 2010). Despite some reported limitations with the recommended methods, the NREL Laboratory Analytical Procedures (LAP) provide a comprehensive set of protocols. It has been shown that these methods can quantify the majority of the constituents present in a "typical" cellulosic biomass, while achieving a good summative mass closure with a maximum ±5% variation reported between different labs (Agblevor et al., 1993; Templeton et al., 2010). However, these methods have been largely optimized for relatively clean and homogenous lignocellulosic feedstocks such as white wood and agricultural biomass. In contrast, forest residues are typically very heterogeneous and they exhibit several differences in chemical and structural composition (Afzal et al., 2010; Pettersson & Nordfjell, 2007; Robinson et al., 2002). For example, compared to white wood, forest residues can contain significantly higher amounts of ash, extractives, lignin, and other 'difficult-to-extract' components such as suberin (Huang et al., 2011; Sjostrom, 1993). The type and complexity of the extractives in forest residues (bark in particular) are significantly different from those in agricultural residues. The composition of forest residues, such as

bark or logging residue, varies significantly based on age, location, the time of year they were harvested, storage, and handling practices (Athanassiadis, 2011; Fuller, 1985; Robinson et al., 2002; Slaven et al., 2011). Due to their heterogeneity and inherent complexity, it was anticipated that it would be more difficult to accurately quantify the lignocellulosic components of forest residues than either white wood or agricultural residues using the existing analytical methods. One major goal of this thesis was to assess whether the currently established analytical methods for white wood chips and agricultural residues are robust enough to quantify the chemical composition of forest residues and obtain a good summative mass closure and material balance both before and after a pretreatment.

As well as obtaining a good material balance, equally important is developing appropriate preprocessing/pretreatment strategies for the bioconversion of forest residues in a biorefinery. Ideally, a pretreatment should enable the recovery of all of the valuable biomass components in a reactive form. In addition, an effective pretreatment should allow us to obtain a reasonable material balance while generating a cellulosic component to be easily hydrolysed in a subsequent enzymatic hydrolysis. Due to the differences in the structure and chemical composition, we expected that the types of pretreatment process and the conditions, which are typically employed for processing white wood chips, would be different from that of forest residues.

1.2 Biomass resources in BC

As the demand for lignocellulose will increase, all types of biomass including the residues from both the agricultural and forestry industries will be needed as feedstocks for future biorefineries. On the northwest coast of the US and Canada, most of the residual biomass originates from the forest, which is dominantly softwood-derived. The major biogeoclimatic zones of forest present in British Columbia are Coastal Douglas Fir, Coastal/Interior Western Hemlock, and Sub-Boreal/Boreal Spruce (Pojar et al., 1987). Although, these forests cover about two-thirds of the land area in British Columbia (COFI, 2012), less than one percent of the forest is harvested in a given year. BC has a higher

annual cut than other provinces, and overall a larger quantity of tree residues, the majority of which are available for use (Bradley, 2010).

It has been estimated that 13-16 million dry metric tons per year of woody biomass could be available for biofuel production in British Columbia (Bradley, 2010). The vigorous spread of the mountain pine beetle epidemic in the recent years has increased the availability of wood and forest residues. It was estimated that 3 million tonnes of mountain pine beetle derived wood could be harvested annually and potentially used as a feedstock for a biorefinery over the next 20 years (Table 1). British Columbia also accumulates woody biomass residues from urban developments, logging sites, and sawmills. Some of these residues are fairly easily accessible and include debarking residues, hog fuel, sawmill residues, and woody urban waste. There are also less accessible residues including logging residue left onsite or at the roadside and forest thinnings or juvenile wood. The availability of forest residues will be highly dependent on the ease of harvesting and transportation costs. The use of conventional harvesting systems over specialized equipment or decreasing the distance needed for transportation might create a more available feedstock (Rummer, 2007).

Sawmill residues represent the most accessible biomass residues in the province. Due to reduced transportation and harvesting costs, sawmill residues receive demand from other industries which include particle board, animal bedding and wood pellets. Currently, the wood pellet industry consumes the largest quantity of what could have been, until recently, considered “available” sawmill residues. Wood pellets in BC (~2 million tonnes) are currently exported to Europe and used in combustion and cogeneration applications (Ackom et al., 2010). Currently, Canada has 42 wood pellet plants and 3 million tonnes of wood pellet production capacity. In addition to sawdust, large piles of bark debris are also generated in sawmills as a result of debarking operation at the sawmill (Walker, 2006). Some of this biomass is sold as hog fuel for biomass boilers as mandated by the provincial government (Bradley, 2010; Environment, 2011). Recently, there has also been increased interest in bark as a raw material for the production of pellets (Lehtikangas, 2001).

Urban biomass waste represents another potential source of biomass that is fairly easily accessible. Urban waste composed of lignocellulosic biomass consisting of municipal solid waste (MSW), yard trimmings, and demolition waste. Within British Columbia, this is estimated to account for 1.3 million dry metric tons every year (Bradley, 2010). Most of the urban waste can be derived from densely populated areas and is likely to be inexpensive due to shorter transportation costs and a decreased urban propensity for waste (Ralevic & Layzell, 2006).

Although less accessible, compared to saw mill residues and urban wastes, logging slash left onsite or at the roadside and forest thinnings represent another potential and perhaps one of the most inexpensive biomass feedstocks for biofuels/biorefinery. The forest residues left on logging sites are currently burned or left to decay. Due to the differences in the dimensions, these residues may require removal by specialized equipment compared to traditional wood logs (Lindroos et al., 2010; Rummer, 2007). In addition, once harvested, the climatic conditions and the method of storage can influence the moisture content of the residues. A higher moisture content and lower bulk density of the residues can increase its transportation costs (Hamelinck et al., 2005; Pettersson & Nordfjell, 2007). However, integrating the operations of conventional logging and slash removal could reduce the price of delivered biomass (Björheden, 2000; U.S., 2011). Bundling of loose forest slash into compact bundles or chipping at the roadside has been suggested as one option to try to improve the harvesting and shipping costs (Lindroos et al., 2010; Spinelli et al., 2007; Visser et al., 2009).

Due to the recent increase in the magnitude of forest fires in North America, there was an increased interest in removing the forest thinnings, which are recognized to be potential fire hazards (Kocoloski et al., 2011). Since 2006, the number of wildfires across the US has increased by an average of 110% (Center, 2012). Although ecologically indispensable, removal of large amounts of fire slash is an expensive practice. According to a study by the USDA Forest Service, the cost of removing forest thinnings in the western states can be as low as $10 per oven dried ton (odt) of roadside biomass and as high as

$500/odt from the some logging sites in western Washington and Oregon (Skog et al., 2008). At a roadside price of $10/odt, 6.3 million odt of forest biomass were estimated to be available, and at $40/odt this amount would increase to 11 million odt (Cook & O'Laughlin, 2011). However, creating a value for fire slash by using this material as a feedstock for biorefinery applications might compensate for the cost of removal of these residues from the forest.

Disturbance wood includes prematurely killed trees due to disease, infestation, fire, or drought. Mountain pine beetle killed trees represent a potentially available disturbance wood in British Columbia. The spread of beetle-affected wood has increased from less than 1 million hectares in 2001 to nearly 18 million hectares in 2010 (Forests, 2012). Although the growth rate of the MPB epidemic has declined from 2008 onward, the pine beetles have adapted to other forest species hosts such as Jack pine, which has allowed the beetles to spread to the boreal forests of British Columbia and Alberta (Canada, 2012). After harvesting, the primary intention for the wood is to be produced into value-added or "specialty" products (such as denim pine) to recoup much of the lost value of the wood (McGarrity & Hoberg, 2005). However, the excess wood together with the residues from these processes are expected to be a valuable feedstock for biorefinery applications (Byrne et al., 2005). The conventional methods and equipment used by the logging industry for traditional lumber can be used to harvest infected trees. As mountain pine beetle wood is a relatively cleaner feedstock compared to urban biomass wastes or hog fuels, they will have competitive uses. For example, if the wood pellet industry continues to expand, beetle-killed wood residues may be an appealing source for future wood pellet production.

Stumps and roots which are left after harvesting the trees, also represent an unexploited source of biomass to feed a biorefinery. Despite their abundant availability, they are generally not included in the estimate of potentially available logging residues since these residues require specialized equipment due to their unusual shape and dimensions. Additionally, removing roots is also believed to disturb the nutrient dynamics in the soil and destroy the local ecosystem (Walmsley & Godbold, 2010). Therefore, the

natural decomposition of tree stumps has been considered vital to the regeneration of the forest, necessary for the prevention of erosion, and important for the maintenance of an overall healthy ecosystem (Dyck & Mees, 1990; Mann & Tolbert, 2000). In most logging sites, stump harvesting is prohibited for these reasons. However, more research is required to determine the impact of stump harvesting on future productivity, forest carbon stores, and soil nutrients (Walmsley & Godbold, 2010). For example, in Sweden, the harvesting of stumps is permitted, but well regulated, and is limited to no more than 10% of the logging site to minimise the environmental impact (Stendahl, 2011). A re-evaluation of the impacts of stump harvesting will soon to be conducted by the Swedish Forestry Agency. Their preliminary research and economic studies have also indicated that shredding extracted stumps prior to transportation can reduce transportation costs and can remove some of the soil, thereby reducing the problems associated with high ash content in the processing facility (Athanassiadis, 2011).

Table 1: Potential forest residues available for bioenergy/biorefining applications in British Columbia

Source	Amount (million dmt/y)*	Notes	Reference
Sawmill residue	Limited availability	Relatively cleaner and homogenous, competition from other uses, dry and ground to a particle size of >3 mm	(Bradley, 2010; Environment, 1993; Spelter & Toth, 2009)
Woody urban waste	1.3	Low cost, high white wood content Often contaminated with dirt/other municipal solid waste	(Bradley, 2010)
Logging residue	9.9	Wide availability, mostly bark, tree tops, limbs. Often contaminated with dirt. High moisture content and high transportation cost	(Bradley, 2010; Hamelinck et al., 2005; Pettersson & Nordfjell, 2007)
Forest Thinnings	1.2	High cost of removal, government incentives to remove them to minimise forest fires, high needle/ bark content	(Ralevic & Layzell, 2006; Rummer, 2008)
Disturbance wood (mostly beetle killed wood)	2.4 (tentative 20-year supply)	High cost of removal, competition from other uses such as wood pellets	(Byrne et al., 2005; Ralevic & Layzell, 2006)
Stump/roots	N/A	Potentially available, however removal could disrupt forest ecosystem, requires further study on the removal practices.	(Dyck & Mees, 1990; Walmsley & Godbold, 2010)
* dmt – dry metric tons			

As is indicated in the Table 1 and was mentioned earlier, the forest residues available in BC are abundant, but diverse depending on their origin and harvesting practices. Therefore, the physical properties (moisture content, particle size distribution etc.) and chemical composition would apparently vary between these feedstocks. In the

next section, the chemical components of biomass which are important in a biorefinery concept are discussed as are the challenges associated with quantifying these constituents in forest residues.

1.3 Biomass components important in a biorefinery concept

Lignocellulosic biomass is primarily comprised of three polymeric components, lignin, hemicellulose, and cellulose (Hon & Shiraishi, 1991). However, depending on the type of lignocellulose, these substituents can vary in their content and structure. In addition to these polymers, extractives and ash can have a significant contribution to the overall chemical composition depending on the sources of biomass (Table 2).

Hemicellulose is a heteropolysaccharide composed of different sugars including glucose, xylose, galactose, mannose, and arabinose (Sjostrom, 1993). Hemicellulose has a lower degree of polymerisation (DP) than cellulose, of the order of 150-200 depending on the type of backbone and branching units. The amount and structure of hemicellulose can vary significantly between softwood, hardwood and non-wood feedstocks (Table 2). The dominant hemicellulosic component in softwood is O-acetyl 4-O-methyl galactoglucomannan (15-25%), followed by arabino 4-O-methyl glucoronoxylan (5-10%) and in some cases arabinogalactan (0.5-3%) (Shimizu, 1991; Sjostrom, 1993). The main component of hardwood hemicellulose is O-acetyl arabinoglucoronoxylan (15-30%) and 3-5% glucomannan. In agricultural residues, hemicelluloses are primarily composed of O-acetyl arabinoxylans (20-28%) (Shimizu, 1991; Soltes, 1983).

Cellulose is relatively more homogeneous compared to hemicellulose and consists of approximately 10000 glucose units linked together by a beta-1-4 glycosidic bonds to form a linear molecule (Sjostrom, 1993). Despite being a homo polysaccharide, cellulose can exists in both crystalline and amorphous forms with natural cellulose being highly crystalline. Both intra molecular and inter-molecular hydrogen bonding together with Van der Waals forces lead to a tightly packed structure for cellulose and therefore, cellulose is difficult to breakdown compared to hemicellulose. Lignin is a complex three-dimensional

aromatic polymer, formed primarily from three phenyl propane unit precursors by several different linkages such as α-O-4, β-O-4, β-β, β-5, 5-5 and α-O-5 (Sjostrom, 1993). The type and extent of these linkages are also dependent on the sources of biomass. Softwood lignin is almost entirely composed of coniferyl alcohols moieties, while hardwoods contain both coniferyl and sinapyl units, and grasses contain all three lignin units including those based on ρ-coumaryl alcohols (10-25%,) (McCarthy & Islam, 2000; Sakakibara, 1991). Part of the lignin is also connected to carbohydrate components such as hemicellulose via covalent bonds such as esters, ethers and glycosides (Biermann, 1988; Sjostrom, 1993).

Table 2: Typical chemical composition of some of the lignocellulosic biomass

Biomass	Carbohydrates (%)	Lignin (%)	Extractives (%)	Ash (%)	Reference
Agricultural Residues	55-65	12-24	6-20	4-10	(Bura et al., 2009; Hames et al., 2003; Templeton et al., 2010)
Softwood	63-72	23-33	2-5	< 1.5	(Hames et al., 2003; Templeton et al., 2010)
Forest Residues	33-60	32-47	5-40	1-7	(Kemppainen et al., 2012; Kim et al., 2005; Robinson et al., 2002; Schmitt et al., 2012; Zhang et al., 2012)

In addition to carbohydrate polymers and lignin, biomass also contains extractives and ash. Although these components are generally present in minor amounts in white

wood, they constitute a significant proportion in agricultural residues. For example, ash and extractives together contribute <5% of softwood (Biermann, 1996) whereas ash alone can be present up to 15% in some of the agricultural residues such as rice husk (Sjostrom, 1993; Templeton et al., 2010). Extractives are a broad category that includes most of the remainder of biomass that could not be characterized as structural carbohydrates, lignin or ash and are low molecular weight and soluble in water and/or organic solvents. In softwood, these include fats, waxes, terpenes, resin acids, flavonoids and other polyphenols (Kai, 1991; Kurth, 1944; Sjostrom, 1993). Extractive components in agricultural residues include some soluble carbohydrates (starch, sucrose and pectins) and gums, fats, and proteins (Hames, 2010). Different anatomical fractions of biomass contain varying levels of extractives. For example in white wood, heartwood fractions contains significantly higher amount of extractives compared to sap wood (Biermann, 1996). In addition, when comparing the xylem and phloem fractions of wood, bark contains substantially higher amount of extractives compared to white wood (Browning, 1967; Laks, 1991). In the bark, extractives acts as a protective barrier to insect and microbial attack (Kurth, 1947). Extractives in bark are diverse and include terpenes, diterpenes, phenolics, fatty acids, aliphatic alcohols, sterols, tannins, pectins, gums, starches, minerals, proteins, phlobaphenes, rosin acids, and uronic acids or simple sugars, depending on the species and solvents used for extraction (Browning, 1967; Harkin & Rowe, 1971; Kurth, 1947; Seca & Silva, 2008). Another high molecular weight extractive component that is difficult to extract is Suberin (Browning, 1967). Suberin is an aliphatic-aromatic cross-linked polymer present in the outer layer of bark where it functions as a barrier to the external environment for the tree (Gandini et al., 2006). Due to the diversity and complex chemistry of various extractive components in bark, it is difficult to remove all of the extractives using a single solvent. The age, growth site, location of the bark examined (inner or outer bark), and other environmental conditions can all affect the chemical composition of bark (Harkin & Rowe, 1971). The variations in the fiber structure and chemical constituents of bark can ultimately affect the robustness of the analytical methods to accurately quantify the different biomass components.

While the content and chemistry of these components will vary significantly with the type of the tissue or the biomass species, the recovery of most of these components are extremely important in a biorefinery. Sugars can be considered as an important component of the feedstock as these sugars can be subsequently fermented or chemically converted to a range of fuels and chemicals. Lignin is also a promising component to derive materials as well as phenol based chemicals. Lignosulfonates, produced by the sulfite pulping industry, are used as dispersants, binders, emulsifiers, etc. (Lebo et al., 2000). Furthermore, lignosulfonates already have markets for use in cement, as a feedstock for vanillin, animal feed, stabilizer, dye, brick, and emulsifier industries, as well as expanders for lead-acid batteries (Lebo et al., 2000; Lora & Glasser, 2002; Száva, 1989). Lignin from alkaline pulping processes can also be used in industrial applications. Alkali lignin is sulfur-free and of low molecular weight, and therefore easily extractable (Li et al., 2009; Lora & Glasser, 2002). Alkali lignin has been shown to have high potential to use in epoxy, phenolic or isocyanate resins (Li & Sarkanen, 2002; Simionescu et al., 1993), or as bio dispersants (Lora & Glasser, 2002). Therefore, the use of lignin for high value applications will be highly dependent on properties such as polydispersity index, purity, viscoelasticity, availability of different functional groups (Gellerstedt et al., 2010; Glasser et al., 1983). These characteristics are mainly dependant on the species and the type of processes from which the lignin is derived (Glasser et al., 1983). Extractives also have great potential to provide various products such as antimicrobial formulations, as well as many high value chemicals and specialised pharmaceutical products (Table 3).

In order to enable the optimisation of different unit operations to ensure the recovery all biomass components in reactive form, the first step is to develop robust analytical methods to accurately track each component through the entire process scheme.

Table 3: Examples of valuable co-products that can be created from different wood components

Category	Biomass Product	Feedstock source
Fuel	Bioethanol	Carbohydrates (Chandra et al., 2007; Ewanick et al., 2007)
Chemical	Pharmaceutics (antioxidant, anti-inflammatory, antibacterial)	Extractives (Bark) (Li et al., 2008; Pietarinen et al., 2006; Yang et al., 2004)
Material	Polyester, polyurethane	Extractives (suberin) (Cordeiro et al., 1998; Gandini et al., 2006)
Material	PLA, PHB (bioplastic)	Carbohydrates (Kumar & Prabakaran, 2006; Madhavan Nampoothiri et al., 2010)
Fuel	Wood pellets	Milled wood (Spelter & Toth, 2009)
Chemical	Phenols (adhesives)	Extractives (Bark) (García-Pérez et al., 2012; Yazaki & Collins, 1994)
Material	Lignin Carbon Fibers	Lignin (Gellerstedt et al., 2010; Kadla et al., 2002)
Material	Lead-acid battery expanders	Lignin (Száva, 1989)

1.4 Development of biomass analytical methods

1.4.1 Importance of a "material balance" in a biorefinery

The "mass balance", also called "material balance" is the application of the law of conservation of biomass to analyse a process system by accounting for the mass of each reactant/product entering or leaving a process unit. Similar to an oil refinery, an accurate material balance will be critical for the design and optimisation of bioconversion/biorefinery processes. In an oil refinery, the high cost of oil requires all fractions of the starting material to be accurately quantified and used. Biorefineries will undoubtedly employ the same approach for processing biomass. In order to ensure a reliable material balance, having robust analytical methods to quantify each biomass component entering and leaving the system is extremely important. As was mentioned previously, the methods used to quantify different biomass components have mostly been developed by workers in the pulp and paper and agriculture sectors (Figure 1).

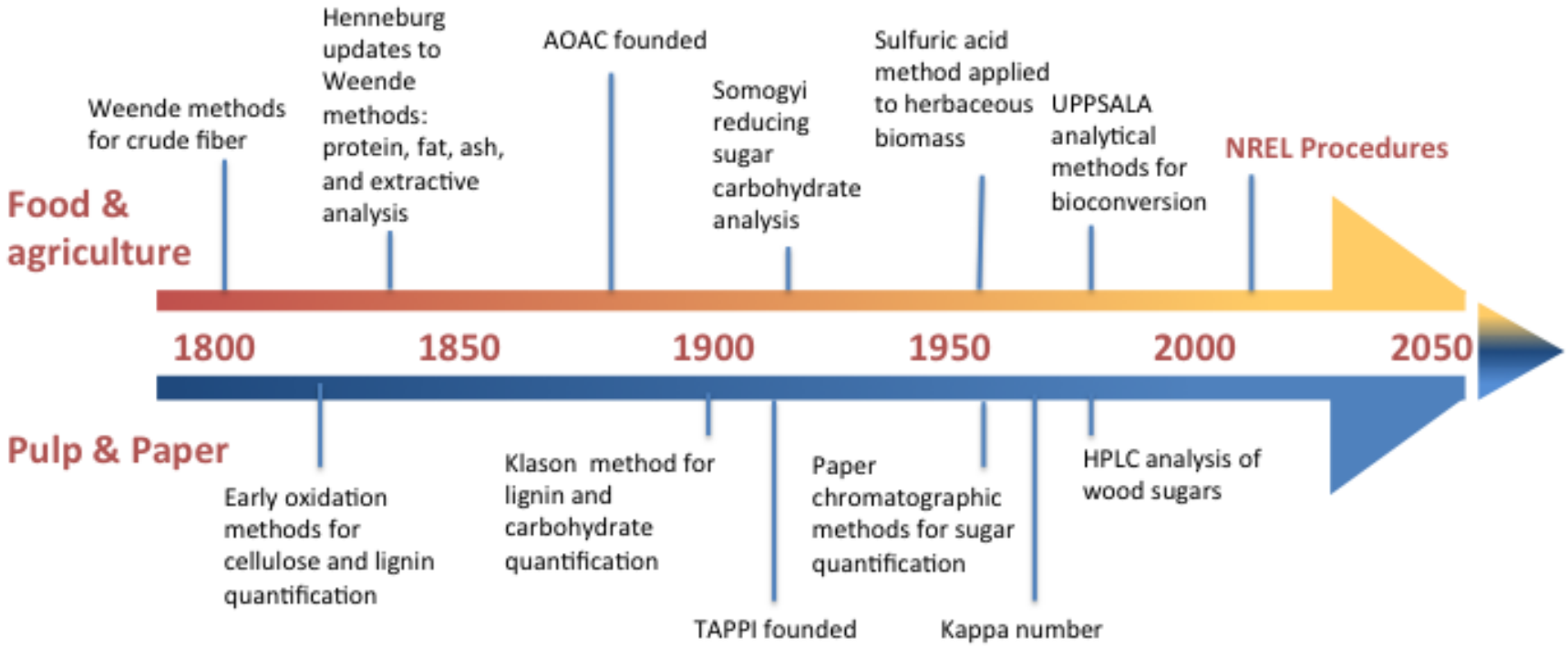

Figure 1: History of the development of biomass analytical methods.
(Freeman, 1944a; McCarthy & Islam, 2000; Phillips, 1944; Sluiter et al., 2010)

1.4.2 Pulp and paper based methods

Many of the existing wood/pulp analytical methods were originally conceptualised and refined by The Technical Association of Pulp and Paper Industries (TAPPI) over the last 50-60 years (TAPPI, 2013). The primary goal of the TAPPI methods was to aid papermakers in assessing pulp and paper characteristics such as, the bleachability, extent of delignification of the pulp, reduce pitch deposits caused by extractives, changes in pulp yield, etc. (Fatehi et al., 2009; TAPPI, 1993; TAPPI, 1997). It should be noted that a detailed material balance of individual biomass components was not a primary concern since, although one goal was to obtain good pulp yields, achieving the desired pulp properties was considered to be even more important.

During the early development of the wet chemical analysis of wood, cellulose and holocellulose were among the two components that were quantified. As early as 1850s, wood chemists Shultze and Henneberg, conducted α-cellulose analyses using aqueous potassium chlorate and nitric acid (Freeman, 1944a; Phillips, 1944). The goal of this method was to oxidise the non-cellulosic components and gravimetrically determine the remaining cellulose present in the wood sample. However, the oxidants used were not selective enough to prevent cellulose degradation and the method therefore resulted in low yields and an under estimation of cellulose. This method was further fine-tuned by Lifschutz in 1891 (Freeman, 1944a) by employing a combination of nitric and sulfuric acid. Later, Cross and Bevan in 1918 (Cross & Bevan, 1922) used nitric acid and potassium permanganate. Cross and Bevan also tried to improve the method of chlorination as a more selective alternative which was originally conceptualised by French chemists Fremy and Terreil in 1868 (Freeman, 1944a). Further improvements to the chlorination methods were developed into the 1930s, yet the methods were still arbitrary and proximate and hence not widely accepted as a standard method for the quantification of cellulose.

Holocellulose represent both the cellulose and hemicellulose components of biomass and thus can distinguish between the carbohydrate and non-carbohydrate components of biomass. Schmidt and Graumann first developed a more specific method in 1921 and initially

the holocellulose were termed as “Skelettsubstanz” meaning skeletal substance (Freeman, 1944b). Their method used alternate treatments with chlorine dioxide and sodium sulfite solution which resulted in more selective removal of lignin and an enriched carbohydrate residue. However, this method was quite time consuming, taking approximately 1 month to complete the mild oxidation without degrading the carbohydrates. In 1933, Ritter and Kurth devised a faster method that used chlorine and alcohol-pyridine solution in exchange for Schmidt’s reagents and reduced the time of the procedure to just over 10 hours (Ritter & Kurth, 1933). The purpose was to chlorinate the lignin and remove the chlorolignin by using an organic solvent. It was Ritter and Kurth, who first proposed the term “holocellulose” for the whole carbohydrate fraction of the wood sample (Freeman, 1944b).

Parallel to the development of methods for the quantification of cellulose, some indirect methods were also developed for lignin quantification. Consumption of oxidising agents during the oxidation of lignin was one of the early approaches including the Schulze’s method using potassium chlorate and nitric acid. Again the challenge was the non-selectiveness of the oxidation methods (Phillips, 1944). Waentig and Gierisch advanced the indirect lignin analysis methods in 1920 by using excess chlorine gas as the reagent and while measuring the percent increase in weight as the “chlorine number” (Phillips, 1944). This method prompted the pulp and paper industry to suggest “kappa numbers” be used as a standard for comparing the relative lignin content of the samples. By 1960, TAPPI had established a more selective oxidation procedure for indirect lignin quantification based on the “kappa number”, a measurement based on the amount of oxidant consumed when titrated into a solution containing excess potassium permanganate combined with pulp (TAPPI, 1993). In addition to providing information on the relative lignin content, such oxidative procedures were more useful in providing information about the bleaching requirements for the pulp to reach a given level of brightness (Hatfield & Fukushima, 2005). The Kappa procedure subsequently became the standard procedure for assessing the bleachability requirements in the pulp and paper industry (TAPPI, 1993; Tasman & Berzins, 1957).

In the early twentieth century, Peter Johan Klason proposed a direct method for lignin quantification. The procedure used concentrated sulfuric acid to dissolve the carbohydrate fraction which generated a lignin residue that could be directly measured (McCarthy & Islam, 2000; Pettersen, 1984; Phillips, 1944). Although this gravimetric measurement of lignin remains, so far, one of the most reliable methods of quantifying lignin (Hatfield & Fukushima, 2005), the ability to accurately quantity different types of lignin with varying physical and chemical properties present in different types of biomass is still problematic. For example, some researchers recognized that some of the biomass components may have been "double-counted" during lignin analysis (Mahood & Cable, 1922; Ritter et al., 1933). In the 1930s, wood scientists began to explore the impact of different pre-extraction methods prior to sulfuric acid analysis to improve the accuracy of lignin quantification (Ritter & Barbour, 1935; Ritter et al., 1932). Without proper extraction techniques, the lignin content would likely to be overestimated due to carbohydrates or extractives condensing with the lignin (Sherrard & Harris, 1932). However, lignin could also be underestimated due to the solubilisation of some of the lignin fractions (Harris & Mitchell, 1939). This later formed the basis for quantifying the acid soluble lignin by the UV absorption measurement proposed by TAPPI (TAPPI, 1985). However, there are still challenges associated with using the same extinction coefficient for different types of lignin samples (Dence, 1992). In addition, the interference from the sugar degradation products such as furfural and hydroxymethylfurfural made it challenging to obtain a selective absorption for lignin at 280nm wavelength. The 205nm, which is now used for the measurement of acid soluble lignin is also prone to some interference from the carbohydrate monomers (Hatfield & Fukushima, 2005).

1.4.3 Agriculture based methods

In parallel to the development of TAPPI methods for characterising the wood and pulp, methods were also developed by the agriculture industries to analyze agricultural residues such as rice straw, wheat straw, corn stover, etc. Since agricultural residues were primarily used as an animal feed, the objectives of most agricultural methods were to assess the animal nutrition and forage digestibility of the biomass feedstock.

The Weende methods were the first set of methods which provided semi-quantitative estimates of the nutritional qualities of feedstuffs (Faithfull, 2002). These methods were developed in 1806 by German scientist Heinrich Einhof, but further revised in 1859 by Henneburg to not only estimate the indigestible matter, but to determine the approximate amount of moisture, protein, fat/oil, fibre, ash, and nitrogen-free extract of the biomass (Faithfull, 2002; Van Soest & McQueen, 1973). The Henneburg, methods were further standardized by the AOAC (Association of Official Agricultural Chemists). Improvements in the crude Weende methods led to the development of the currently used acid detergent fiber (ADF), neutral detergent fiber (NDF), and acid detergent lignin (ADL) analytical methods (International et al., 2006; Raffrenato & Van Amburgh, 2011). These standard procedures provide sufficiently accurate methods such that the nutritional traits and digestibility of agricultural feedstocks can be determined. They remain as some of today's standard methods used to characterise agricultural residues for animal feed (Dong & Rasco, 1987; Faithfull, 2002).

Several methods have been proposed for the determination of lignin, including direct methods (Klason and acid detergent lignin), indirect methods (Kappa number, as described in pulp and paper), solubilisation methods (acetyl bromide, Thioglycolate), and non-invasive methods (NIR, NMR, UV). However, these methods often do not result in the same value when applied to the same feedstocks (Hatfield & Fukushima, 2005). In a study comparing the acetyl bromide and acid detergent methods for determining lignin content, Klason lignin, and potassium permanganate lignin, no agreement was found among the methods (Lacerda et al., 2006). Standard errors for the solubilisation methods were substantially higher than the other methods such as the gravimetric methods (Hatfield & Fukushima, 2005; Lacerda et al., 2006). For examples acetyl bromide and Klason yielded the highest lignin values, while acid detergent lignin yielded the lowest numbers (Lacerda et al., 2006).

Despite significant advances in biomass analytical methods from both the pulp and paper and agricultural sectors, the objective of these methods has not been specifically to obtain a complete material balance. These more "quantitative methods" have in fact been further advanced by bioconversion researchers since the oil crisis of the late 1970s, to enable

the quantification of all of the biomass components entering and leaving a unit process with the goal of establishing a detailed material balance.

1.4.4 Bioconversion analytical methods

The Klason sulfuric acid method has been modified to enable the determination of cellulose and hemicellulosic components with the simultaneous quantification of lignin (Sluiter et al., 2010). The concept was to standardise the sulfuric acid hydrolysis conditions to quantitatively hydrolyse all of the polysaccharides (both cellulose and hemicelluloses) to monomeric sugars. The accurate analysis of the released monomeric sugars allows the back calculation of how much cellulose and hemicelluloses were present in the biomass (Dunning & Lathrop, 1945; Peterson et al., 1932). The insoluble residue from the analysis was gravimetrically determined as lignin.

For the analysis of sugars in the acid hydrolysate, earlier methods were mostly colorimetric assays (Moore & Johnson, 1967; Saeman et al., 1954). One of the first sugar quantification methods was the Shaffer-Somogyi reducing sugar method, which was soon modified by Saeman et al using a quantitative paper chromatography in 1954 (Saeman et al., 1945; Saeman et al., 1954; Shaffer & Somogyi, 1933). The Shaffer-Somogyi method was later updated by Nelson (1944) to more accurately quantify glucose during blood sugar analysis which was later adapted for wood hydrolysates (Nelson, 1944; Saeman et al., 1954; Shaffer & Somogyi, 1933). In 1954, dinitrosalicylic acid was introduced as a reagent for determining reducing sugars (Miller, 1959). However, the enzyme assays and incubation conditions were found to interfere with colour development thus the method ended being mostly semi-quantitative (Bailey, 1988; Breuil & Saddler, 1985). With the development of gas-liquid chromatography and HPLC analyses in 1980s, quantification of individual sugar components became easier (Slavin & Marlett, 1983; Sloneker, 1971). Researches subsequently compared colorimetric, HPLC and GC methods for sugar analysis, and found HPLC to be the more robust and quantitative method, with less tedious sample preparation (Irick et al., 1988; Pettersen et al., 1984; Schwald et al., 1988). After mid-1980, GC and HPLC methods became the commonly accepted procedures for determining the individual sugars present in biomass acid hydrolysates

(Irick et al., 1988; Pettersen et al., 1984; Schwald et al., 1988; Slavin & Marlett, 1983; Sloneker, 1971).

Although the standardisation of acid hydrolysis step allowed a near-accurate quantification of carbohydrate components, the determination of lignin still suffered from the interference from the extractives (Norman & Jenkins, 1934; Ritter & Barbour, 1935; Ritter et al., 1932). The amplitude of the errors was found to be much higher with agricultural residues due to the higher amount of extractives present in those substrates (Norman, 1937). Researchers subsequently recommended the selective removal of extractives from the biomass prior to the lignin determination. Unlike lignin and carbohydrates, extractives represent a much broader category of compounds. These range from the hydrophobic fats and waxes to moderately water soluble polyphenols to highly polar compounds such as sugars (Figure 2). Extractives found in biomass can be volatile in the presence of steam, soluble in water, soluble in organic solvents (such as dichloromethane, benzene, toluene, etc), soluble in polar solvents (such as ethanol, acetone, methanol, etc), or soluble in an alkali solution (such as 1% NaOH) (Browning, 1967). Since quantification of individual components present in these extractives is extremely complex, researchers have proposed using a combination of solvents to achieve the near-complete and simultaneous removal of the various extractive components to minimise their interference with subsequent quantification of the carbohydrates and lignin (Ajuong & Breese, 1998; Browning, 1967; Challinor, 1996; Fernandez et al., 2001; Kai, 1991). All of the non-lignin and non-carbohydrate components removed from this solvent extraction step are collectively called “extractives” (Figure 2).

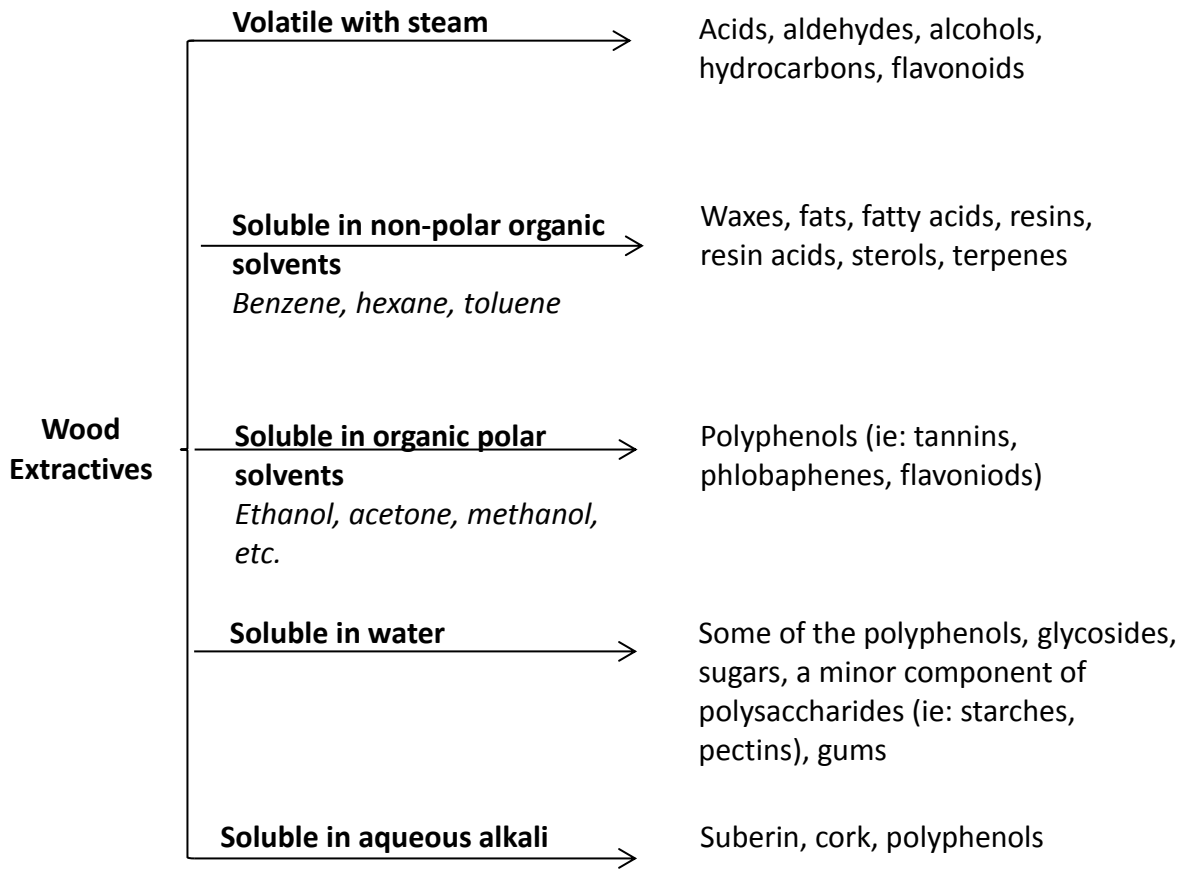

Figure 2: Solubility of Wood Extractives in Different Solvents (Browning, 1967; Laks, 1991)

1.4.5 NREL laboratory analytical procedures (NREL-LAP)

At the present time, the most comprehensive set of laboratory analytical procedures for determining the composition of lignocellulosic biomass has been developed by the National Renewable Energy Laboratory (NREL) in Golden, CO (NREL, 2011). In summary, NREL has combined the TAPPI Klason methods with the methods that were proposed by the Swedish Agrobioenergy Project in 1985 (UPPSALA methods) (Theander, 1985). The UPPSALA methods were mainly developed for cereal crops, beets, willow, forage crops, and agricultural residues such as wheat straw whereas the TAPPI Klason method was primarily developed for white wood. NREL has successfully integrated all of the different steps of carbohydrate and lignin analysis into one single step and standardised the entire set of procedures to quantify most components of white wood and agricultural residues within a single procedure (NREL, 2011).

The NREL-LAP protocols are currently most appropriate for analysing white wood and agricultural residues. After drying, milling, and carrying out appropriate extractions, depending on whether agricultural residues or white wood, the procedure uses a 72% sulfuric acid solution in a primary hydrolysis at 30°C, followed by dilution with water and a secondary high temperature hydrolysis (NREL, 2008a). During this two-stage analytical procedure, the polysaccharides are almost quantitatively hydrolysed to soluble monosaccharides, leaving behind most of the lignin as an 'insoluble residue' that is washed, vacuum filtered and measured gravimetrically. A set of synthetic sugars are run in parallel with the liquid samples during the dilute-acid hydrolysis to account for any degradation. The dissolved monosaccharides are then measured using chromatography (HPLC) techniques and the corresponding carbohydrate polymers are back calculated (NREL, 2008a).

Subsequently, NREL conducted a round robin study in 1993, involving 19 laboratories in 12 nations primarily to assess the robustness of the standardised protocols. The same sample of poplar, radiata pine, wheat straw and bagasse were given to each team (Agblevor et al., 1993). While the analysis of most of the components in biomass showed fairly good reproducibility, the extractive values generated the largest discrepancy between different labs. However, the only solvent used in the extraction procedure in the study was 95% ethanol. It was soon realised that all of the extractives present in in agricultural residues, wood, bark or other woody biomass cannot be extracted by a single solvent (Miranda et al., 2012; TAPPI, 1997). Subsequently, NREL standardised a two-step extraction protocol involving both water followed by an ethanol extraction particularly for agricultural residues. Based on the structure of agricultural biomass, which contains more starches and non-structural carbohydrates, a water extraction would be more important than a non-polar solvent for many of these extractives. A recent round robin study involving the most updated set of protocols showed that the NREL-LAP method can account for the majority of constituents of a 'typical' cellulosic biomass, while achieving a fairly good summative mass closure (maximum ±5% variation reported between different labs) (Agblevor et al., 1993; Templeton et al., 2010). However, these extraction methods may not be appropriate for materials like bark, which have a very

high amount of diverse extractives, including polyphenols and fats/waxes. Multiple solvents from both polar and non-polar categories may be required for exhaustive extractive removal.

Although the NREL methods appear to be the best set of wet chemistry procedures developed so far, there are still some notable limitations with these methods. The sulfuric acid analysis procedure is ideally most suited for a biomass sample where only cellulose and lignin are present. Hemicellulose or pectin constituents such as xylan or uronic acids, can degrade in concentrated sulfuric acid due to decarboxylation and dehydration reactions (Biermann, 1988; De Ruiter et al., 1992). Although the modified method tried to correct this problem by running a set of synthetic sugar standards in parallel with the samples, the extent to which they degrade will vary with their concentrations in the biomass (Bertaud et al., 2002; Sundberg et al., 1996). In addition, the NREL-LAP protocols have been standardised for typical lignocellulosic material such as agricultural residues and pulp chips (white wood) (Hames, 2010; Li et al., 2010; Xu et al., 2010). The suitability of these methods for analysing more heterogeneous materials such as forest residues needs to be assessed.

1.4.6 The influence of pretreatment and fractionation on the material balance

Pretreatment, the first step in the bioconversion process is designed to disrupt and fractionate the lignocellulosic biomass matrix. One of the objectives of pretreatment is to make the cellulose more accessible to the enzymes while facilitating the recovery of all of the biomass components in a reactive form. This usually involves the partial or full removal of the hemicellulose and lignin, which can enrich the cellulose content of the water insoluble component. Obtaining a good material balance is crucial in evaluating different pretreatment processes and conditions for a given feedstock. However, the current analytical methods for measuring the biomass components have been largely standardized for the untreated, “raw” material. Pretreatment introduces additional complexity to the compositional analysis methods, due to the different extent of reactions of the biomass components including; the depolymerisation of lignin, formation of pseudo-lignin, formation of monomeric sugars which are more susceptible to degradation compared to a polysaccharide component.

Depending on the pretreatment process used, lignin can undergo a series of reactions. One such typical reaction is the condensation of extractives with lignin in an acidic medium. (Hemmingson, 1987). This can make the separation of extractives difficult and the analysis of the lignin more challenging, further leading to an overestimation of lignin during the Klason analysis (NREL, 2008a; Sluiter et al., 2010). Pseudo-lignins, can also be formed from the degradation products of carbohydrates, polymerizing with the lignin during pretreatment and subsequently detected as lignin during the Klason analysis (Hu et al., 2012; Li et al., 2005; Sannigrahi et al., 2011). It is also difficult to selectively remove the extractives from pretreated biomass due to the partial solubilisation of depolymerised lignin fragments. Consequently, an extraction step is not typically carried out for most of the pretreated substrates (Hames, 2010), leading to further inaccuracies in the material balance. The water soluble hemicellulose sugars remaining in the solid material after a pretreatment may also degrade when being exposed to 72% sulfuric acid (Biermann, 1988; Hames, 2010). Therefore, a thorough washing of the solids before Klason analysis is recommended to remove the soluble hemicellulosic sugars that were released during pretreatment.

Analyses of the water soluble stream after pretreatment has also been standardized by NREL (NREL, 2006) for sugars and degradation products. Since the liquid fraction can be directly subjected to HPLC analysis, determination of monomeric sugars is straightforward. However, the determination of oligomeric sugars is more complicated. Shevchenko et al (2000) standardised an acid hydrolysis step to nearly quantitatively convert all oligomers to monomeric sugars. This procedure is now revised and recommended by NREL for the quantification of total oligomeric sugars (NREL, 2006). The total carbohydrate yield for a pretreatment process includes the sum of the sugar content of the water soluble and insoluble streams. Partial solubilisation and degradation of lignin and extractives during pretreatment make it difficult to accurately trace them in solution (NREL, 2011). Although UV spectroscopy has been used to measure acid soluble lignin (ASL), non-lignin components, such as HMF/furfural and some of the extractive can also absorb in this UV region, compromising the accuracy of this procedure (Sluiter et al., 2010). These interferences and additional variables, together with the limitations of the current methods themselves, all contribute to making the

summative mass closure being significantly more challenging after the raw material has undergone pretreatment.

1.4.7 Rapid biomass analysis

Despite being reasonably accurate for establishing a material balance, wet chemistry methods are quite time-consuming and labour-intensive. The entire set of NREL-LAP protocols requires a minimum of half-a-week to complete, even when done by a highly trained professional. In addition, the cost of carrying out a complete set of wet chemistry methods is between $800-2000 per sample (Hames et al., 2003). However, a biorefinery at commercial scale should have the ability to alter the process conditions within minutes during their operation, based on the changes in composition of the incoming feedstocks and changes in the output (Hames, 2010). Thus, online monitoring requires inexpensive and rapid analysis of the process streams to enable the industrial screening of the feedstocks and process control. Researchers have investigated a number of different rapid analytical methods over years and these methods are summarised in Table 4. Although these methods are not able to establish an accurate and complete material balance, they have the potential to be used as semi quantitative methods to provide online monitoring and process control.

Table 4: Current Methods for Sugar Analysis

Method	Examples	Advantages	Disadvantages
Spectroscopic	NIR Raman	Measures all components at a rapid rate, inexpensive (Hames et al., 2003)	Requires numerous calibrations, needs homogenized, clean or dry feedstocks (Adapa et al., 2009b)
Colorimetric	DNS BCA Nelson-Somogyi MBTH	Rapid method, inexpensive (Decker et al., 2009)	Measures only some sugars, prone to interferences from oligomers or enzymes (Anthon & Barrett, 2002; Walker, 1996)
Amperometric	Capillary electrophoresis Peroxidase membrane	Can measure oligomers and is not prone to enzyme interferences (Hou et al., 1998)	Potential for long sampling times, generally can only measure one to a type of sugar at once (Decker et al., 2009)
Instrumental	HPLC, GC (UPLC)	Very specific, measures all sugars at once (Hames, 2010)	Slow, requires significant preparation for each sample (Hames, 2010)

1.4.7.1 Infrared (IR) and Raman Spectroscopy

Infrared (IR) and Raman are both spectroscopic methods which measure the interaction of electromagnetic radiations with matter. However, the methods differ with regard to the type of interactions being measured (Adapa et al., 2009a). While IR spectra emanate from the absorption of radiation by vibrating and rotating molecules, Raman spectra measures the scattering of radiation. Despite some overlap between the two methods, the two spectra are complementary, as there are distinct advantages and limitations of both of the systems.

Of the two methods, Near IR, (NIR) is most commonly reported an effective tool for measuring the compositional analysis of biomass (Adapa et al., 2009a; Hames et al., 2003; Urtubia et al., 2004). NIR has high signal-to-noise ratio, which results in shorter sampling times (Sohn et al., 2004). High accuracy and sensitivity allows the analysis of samples with a high

degree of impurity. However, samples should be completely dry prior to IR analysis since water can cause interferences due to strong O-H bond vibrations (Adapa et al., 2009a). In addition, the samples for IR must be pre-ground for a representative measurement since IR spectroscopy cannot analyze the material below the surface (Adapa et al., 2009a; Philip Ye et al., 2008).

Unlike IR, Raman spectroscopy uses a beam of monochromatic radiation (Archibald et al., 1998a). Raman's monochromatic radiation makes it more selective than IR for measuring non-polar bond vibrations, which means wet or dry material can be measured and the O-H bonds creates only minimal interference (Archibald et al., 1998b). The Raman method is also capable of analyzing deeper into the sample surface compared to NIR, thereby offering more information about the sample being measured (Sohn et al., 2004). However, the use of monochromatic radiation generates a weaker-intensity spectrum when compared to IR and therefore requires longer sampling times (Adapa et al., 2009a). Pure substances are preferred for analysis due to the smaller signal-to-noise-ratio. Raman can also heat a sample due to laser energy output. Therefore, shorter sampling times are desirable to avoid altering the sample during analysis. Additionally, biomass sample fluorescence can suppress the weaker Raman scatter interfering with the analysis. However, recent developments on the enhancement of probe tip design and automated fluorescence subtraction have increased the accuracy of Raman (Lieber & Mahadevan-Jansen, 2003; Yeo et al., 2009).

There is widespread potential of using Infrared and Raman spectroscopy as a rapid analytical tool for biomass process streams. NIR methods are estimated to cost about $10/sample and have the potential to generate data, which could be compatible with a process control interface (Hames et al., 2003). NREL has been developing NIR for the analysis of a range of corn stover feedstocks (Hames et al., 2003). However, the heterogeneity and diversity of lignocellulosic biomass complicates NIR/Raman's ability to create a robust calibration standards (Adapa et al., 2009a). Challenges not only exist between different species of feedstock (for example, wheat straw vs. pine), but also when analyzing the different components within the same feedstock. While cellulose, non-structural carbohydrates, and proteins have been more successfully analyzed by NIR, inconsistent structures of hemicellulose, lignin and extractives,

makes their calibration and measurement complicated across feedstocks and different pretreatments (Schimleck & Yazaki, 2003; Schulz & Baranska, 2007) (Tamaki & Mazza, 2011). However, since most inorganic compounds do not generate vibrations in the spectra, NIR has a higher efficiency to indirectly measure minor components such as ash (Lestander & Rhen, 2005). One of the disadvantages of the spectroscopic methods is the limitations of generalized calibrations. Once created, a calibration in NIR will be primarily be applicable to the unique types of biomass or a given type of pretreatment processes (Urtubia et al., 2004).

1.4.7.2 High-throughput techniques

High-through put techniques were developed primarily for measuring the carbohydrate content of biomass and is useful in the fast screening thousands of biomass samples for their potential for bioconversion. High throughput techniques are widely used in the genetic screening and evaluation of biomass for biofuel production. The technique is also useful in evaluating different types of pretreatment processes and conditions in releasing sugars as well in determining the enzyme loading for releasing a targeted sugar yield. Researchers have widely used spectrophotometric methods using dinitrosalicylic acid (DNS), bicinchoninic acid (BCA), Nelson-Somogyi and 3-methyl-2-benzothaizolinonehydrazone (MBTH) for detecting the released sugars. For more selective measurements, enzyme electrodes and instrumental methods such as gas chromatography, HPLC and ultra-pressure liquid chromatography (UPLC) (Decker et al., 2009) were also clubbed with the high throughput screening.

These methods have an advantage of being robust, low cost, fairly fast (5-20 minutes). Since the analysis is conducted at a smaller scale, the samples may not be truly representative to make a fair evaluation. Moreover, the methods generally lack the ability to analyze multiple sugars (Decker et al., 2009). Despite having the shortest sample time of the above methods, 3,5-dinitrosalicylic acid (DNS) has the least degree of sugar sensitivity, and creates a hazardous waste (Bailey, 1988; Miller, 1959). 2-2’ bicinchoninic acid (BCA) and Nelson-Somogyi are more sensitive than the DNS method, since they measure the reduction of Cu^{2+} to Cu^{1+} and can suffer from the interference from proteins (Nelson, 1944; Walker, 1996). 3-methyl-2-benzothaizolinonehydrazone (MBTH) is the most recent of the spectrophotometric methods,

and has been recognized for having a high specificity for sugars with minimal interference from enzymes (Anthon & Barrett, 2002; Gomez et al., 2010). In this method, oligomers generate a higher response than monomers; therefore this method will have issues in accurately comparing different pretreatment conditions or enzyme formulations.

In addition to colorimetric methods, amperometric methods can measure glucose, cellobiose or xylose sugars (Decker et al., 2009). In amperometric methods, electron transfer is detected by an electrode by which an enzyme is embedded in a gel-polymer to facilitate the reaction and electron transfer. For example, peroxide formed by glucose oxidase can be detected by a peroxidase membrane (Hou et al., 1998). The disadvantages of enzyme electrodes can include long sampling times and potential deactivation of the enzyme from the inhibitory compounds released during pretreatment or during the formation of the polymer (Decker et al., 2009).

Instrument methods represent another category of HTP lignocellulosic sugar analysis. Apart from HPLC (as mentioned earlier), GC is another instrumental analysis that can be used to measure sugars by derivatization with reagents such as alditol acetate, hexamethyldisilazane, or N-Trimethylsilylimadazole (Fox et al., 1989; García-Raso et al., 1987; Kakehi & Honda, 1989). The run time of the samples in HPLC can be reduced to less than a few minutes by using UPLC systems. However, these instrumental methods generally require tedious sample preparation making them less feasible for instantaneous online measurements. However, new instrumental techniques that combine the speed with more specific and detailed analysis are currently under development. One example is NREL's HTP analytical pyrolysis which was recently reported to have the potential to carry out detailed compositional analyses at a rate of hundreds of samples per day (Scanlon, 2013).

1.5 Bioconversion of forest residues

Previous studies on the bioconversion of forest residues have mainly focussed on using "relatively clean" feedstocks such as saw mill residues, beetle killed wood chips and forest thinnings. This work has mainly focussed on using similar pretreatment and hydrolysis

conditions as used for white wood feedstocks (Berlin et al., 2007; Del Rio et al., 2010; Ewanick et al., 2007; Kim, 2005; Luo et al., 2010; Nguyen et al., 2000; Pan et al., 2008) (Table 5). This seems to be primarily because these workers used feedstocks whose chemical composition and physical characteristics were closer to white wood chips. However, some work has shown that the same pretreatment conditions that are effective on softwood chips are not effective on bark and other heterogeneous residues such as hog fuel, yard waste, etc. (Kim et al., 2005; Robinson et al., 2002). This earlier work reported low sugar yields during enzymatic hydrolysis and poor fermentation performance (Boussaid et al., 2001; Kemppainen et al., 2012; Kim et al., 2005; Schmitt et al., 2012; Zhang et al., 2012). Forest residues such as bark have a high extractive content, but low glucan content. Therefore, pretreatment strategies which do not remove the extractives before enzymatic hydrolysis and fermentation could be expected to have downstream problems, including enzymatic/yeast inhibition and low sugar concentrations (Robinson et al., 2002; Ximenes et al., 2010).

It should be noted that in forest residues such as bark, the extractives and lignin together can contribute more than 60% of the original feedstock while carbohydrates represent a lesser portion of the overall composition (Robinson et al., 2002). Therefore the theoretical yield and titre of sugars or fermentation products such as ethanol will consequently be lower (Huang et al., 2011; Robinson et al., 2002). However, there is an opportunity use both lignin and extractive components for the production of high-value chemicals or polymers. For example, condensed tannins, found in softwood bark, have the potential to be used as pharmaceutical antioxidants and anti-inflammatories (Pietarinen et al., 2006), while other bark extractives such as polyphenols could be used as phenolic adhesives (Yazaki & Collins, 1994). Suberin has also been reported as a potential source of monomers for polymers including polyurethane and polyester (Cordeiro et al., 1998; Gandini et al., 2006). Suberin's waxy-like characteristics also render it a good additive in other products such as ink formulations for waterless painting (Gandini et al., 2006). In addition to the extractive components, lignin has the potential to be used in a wide range of products, from carbon fibers (Kadla et al., 2002) to polyurethane foams to dispersants and resins (Lora & Glasser, 2002). Despite the possibility of using extractives and lignin components for value added co-products to improve the overall

process economics, the scope of the previous work was mainly producing sugars or ethanol from forest residues. Therefore, further research is required to tailor the pretreatment processes and conditions to enable the recovery of all biomass components in a reactive form in high yield and concentrations (Kemppainen et al., 2012; Lissens et al., 2004; Vázquez et al., 1987; Zhang et al., 2012).

Table 5: A brief summary of the previous work using forest residues as a feedstock for bioconversion

Reference	Feedstock	Preparation of feedstock/Pretreatment	Hydrolysis	Fermentation
Robinson et al 2002	Douglas Fir Bark	Chipped, SO_2 impregnated, Steam pretreatment	-	WSF (*S. cerevisiae*)
Nguyen et al 1999a	White fir, Ponderosa Pine Juvenile Wood	Milled, Dilute acid impregnation, Steam explosion reactor	2-stage Dilute Acid	*S. cerevisiae*
Nguyen et al 2000	White fir, Ponderosa Pine Juvenile Wood	Milled, Dilute Acid Pretreatment (Single and 2-stage)	Enzymatic (2 and 10% C, 60FPU/g cellulose)	SSF (*S. cerevisiae*, 15 and 25 FPU/g cellulose, 10% C)
Kim et al 2005	Hemlock Hog Fuel	Milled, mixed	2-stage Dilute Acid	-
Nguyen et al 1999b	MSW – lumber waste, almond tree prunings, wheat straw, office paper, newsprint	Milled, 0.4% H_2SO_4 acid soaked, Steam pretreatment	Enzymatic (6% C, 66FPU/g cellulose)	*S. cerevisiae*
Ewanick et al 2007	Lodgepole pine Disturbance Wood	Chipped, SO_2 impregnated, Steam pretreatment	Enzymatic (2%C, 20FPU/g cellulose)	SHF, SSF (*S. cerevisiae*, 40FPU/g cellulose, 5% C)
Schmitt et al 2012	MSW – yard waste (mixed waste, paper waste)	SO_2 impregnated, Steam pretreatment (Dilute acid)	Enzymatic (2%C, 20FPU/g cellulose)	*R. mucilaginosa*

Reference	Feedstock	Preparation of feedstock/Pretreatment	Hydrolysis	Fermentation
Ferreira et al 2010	Forest underbrush	Milled, Dilute acid	Enzymatic (5%C, 40FPU/g cellulose)	*P. stipitis*
Berlin et al 2007	Lodgepole pine Disturbance Wood	Organosolv	Enzymatic (2%C, 40FPU/g cellulose)	*S. cerevisiae*
Zhang et al 2012	Douglas fir logging residue	Chipped, SPORL pretreatment	Enzymatic (2%C, 20FPU/g cellulose)	-
Kemppainen et al 2012	Spruce Bark	Steam pretreatment (with and w/o 0.5% H_2SO_4), Hot water extraction	Enzymatic (1%, 10 and 25FPU/g cellulose)	*S. cerevisiae*
Lissens et al 2004	MSW – yard waste	Milled, Wet oxidation	Enzymatic (2%C, 25FPU/g cellulose)	SSF (*S. cerevisiae*, 5, 10, 20 FPU/g cellulose, 10%C)

1.6 Rationale and objectives of the thesis

From earlier work, it is highly likely that current analytical methods, while standardised for typical lignocelluloses such as agricultural residues and white wood chips, are inadequate for accurately quantifying the diverse components present in forest residues. This is primarily due to their high level of heterogeneity and significant differences in their chemical/structural properties. Previous studies have not fully addressed the complexity of these types of biomass when completing a compositional analysis and material balance (Hames, 2010; Sluiter et al., 2010). The work described in the first part of the thesis (Chapter 3.1) assessed the response of six different heterogeneous softwood residues to the analytical methods currently established for white wood and agricultural residues (Sluiter et al., 2010) (Chapter 3.1). Softwood-derived hog fuel (HOG I & II), logging residue (LR), forest thinnings/interface fire slash (IFS), beetle-killed white wood (BK-LPP), and lodgepole pine bark (BARK) were used to represent the wide spectrum of available forest residues in British Columbia. Different extraction strategies were evaluated with the objective of improving the robustness of the current methods when applied to a more complex feedstock such as bark or hog fuel. Subsequently, we looked at how a typical

pretreatment such as steam pretreatment might influence the ability of the existing methods to obtain a good material balance. We also examined how the heterogeneous distribution of physical properties such as moisture content and particle size might influence the efficiency of steam pretreatment by comparing the homogenized and non-homogenized substrates.

After evaluating possible strategies for improving the material balance, we carried out a preliminary assessment of the enzymatic hydrolysis of the steam pretreated forest residues (Chapter 3.2). For softwood chips, it was found that an acid catalysed steam pretreatment in combination with a subsequent lignin modifying or removing post treatment could result in the fast and complete hydrolysis of the cellulosic component at low enzyme loadings while facilitating the recovery of most of the hemicellulosic sugars. Therefore, the suitability of such an approach in processing forest residues was investigated in Chapter 3.2. Due to the chemical and structural differences in forest residues, we anticipate that the pretreatment conditions that were optimised for white wood might not be effective for forest residues. Some of the forest residues such as bark are extractive rich (~50% by weight) and acid catalyzed pretreatments are less likely to remove the extractive components. Therefore, the efficiency of alkaline pretreatment strategies in solubilising most of the extractive components and producing a carbohydrate enriched water insoluble component was also investigated. The cellulosic component was subsequently subjected to the enzymatic hydrolysis at different enzyme loadings to assess their amenability to enzymatic hydrolysis.

2. Materials and methods

2.1 Biomass samples

Six different forest residues were used in this study (Figure 3, Table 6). Two softwood derived hog fuels (Hog I, Hog II) received from Nippon Paper USA, Port Angeles, WA were sampled from two different shipments of hog fuel arrived at the industry gate. The samples varied in their content of Western Hemlock (*Tsuga heterophylla)* debarking debris and woody urban waste (delivered from Rainier Urban and Hermann Local) (Figure 3a, 3b). Logging Residue (LR) was chipped onsite and collected by "Pioneer Biomass" from approximately 100 km east of Williams Lake, BC (Figure 3c). Forest thinnings (Interface Fire Slash-IFS) were chipped fresh onsite at Williams Lake and consist primarily of Douglas fir, Pine, and some Aspen (Figure 3d). Beetle-killed lodgepole pine white wood (BK-LPP) chips were received from Tolko Industries Ltd Vernon, BC (average age 101 ± 20 years) (Figure 3f). Lodgepole pine bark was obtained by debarking the freshly cut wood logs in the process development unit of our lab (Figure 3e). The control white wood sample used in the study was derived from 27-year-old Douglas-fir (*Pseudotsuga menziesii*) trees, which had been grown in the coastal regions of British Columbia. In order to avoid any potential decay during storage, all samples were frozen, soon after received at -20°C till further use.

In addition to different origins, the forest residues vary significantly in their physical characteristics. The moisture content of the biomass samples (as received) varied from 7–60% and particle size was highly heterogeneous (Table 6). In order to make a realistic comparison between the samples, all of biomass substrates were conditioned for uniform particle size and moisture content prior to steam pretreatment. The samples were first air dried and subsequently ground to 2 mm size, and rewet to moisture content of 50%.

Figure 3: Forest residues used in the work. a) Hog fuel I (HOG I), b) Hog fuel II (HOG II), c) Logging residue (LR), d) Interface fire slash (IFS), e) Lodgepole pine bark (BARK), f) Beetle-killed lodgepole pine white wood (BK-LPP)

Table 6: The physical characteristics and other properties of different forest residues used in the study

	Source*	Moisture (% wt/wt) **	Average Size (mm×mm×mm)	Notes
Hog fuel I (HOG I)	Olympic peninsula debarking debris, mostly Western Hemlock	62	40×5×2	Appeared to have a higher bark content. Expected to be challenging to processes the bark and contaminated feedstocks.
Hog fuel II (HOG II)	Olympic peninsula debarking debris, woody urban waste, Western Hemlock	58	55×10×5	Primarily woody urban waste, which is extremely variable and reported to have higher ash content (Nguyen et al., 1999)
Logging Residues (LR)	Williams Lake, mostly Lodgepole pine	42	80×25×10	Contained branches with higher ratio of compression wood. This will likely contribute to higher lignin content when compared to white wood. Will likely have more collapsed cell walls (Bamber, 2001; Blanchette et al., 1994)
Interface fire slash (IFS)	Williams Lake, some aspen, mostly Douglas-fir and Pine	28	85×50×15	Juvenile wood contains thinner cell walls, shorter fiber length and higher lignin content (Myers et al., 1996; Yeh et al., 2005; Zobel, 1984)
Lodgepole pine white wood (BK-LPP)	Beetle-killed	7	25×25×5	Disturbance wood. Possibly lower extractive content as this is first utilised in the fungal colonisation of dead trees. Overall expected to be similar to white wood (Ewanick et al., 2007)
Bark	Lodgepole pine, freshly debarked	33	150×30×2	Reported to be high in extractives, high in lignin, low in carbohydrates, and higher in ash compared to white wood (Robinson et al., 2002; Sjostrom, 1993)
*All of these materials are mostly softwood derived ** Microbial growth might occur in forest residues during storage with resulting sugar and extractive losses (Pettersson & Nordfjell, 2007)				

2.2 Pretreatments

2.2.1 Steam pretreatment

Prior to steam pretreatment, the biomass samples were impregnated by adding a specified amount of SO_2 (4% wt/wt of the substrate (Ewanick et al., 2007)) to sealable plastic bags containing 150 g dry weight of the biomass. After impregnation, the bags were immediately sealed and left for 1 h. The bags were subsequently opened for approximately 30 minutes to let the residual SO_2 escape in the fume hood. SO_2 retention by the biomass samples was determined by weighing the plastic bags again before the steam pretreatment was carried out.

Steam pretreatment was conducted in a 2 L StakeTech steam gun at 200 and 180˚C for 5 min. After the pretreatment, the whole slurry was removed and the water soluble and insoluble fractions were separated by vacuum filtration. The solid fraction of the slurry was washed extensively with water and vacuum filtered to a final moisture content >60%. A small, representative fraction of this sample was subjected to chemical compositional analysis including the determination of lignin and carbohydrate contents. The liquid fraction was analyzed for the carbohydrate content (both monomeric and oligomeric sugars) solubilised during steam pretreatment.

2.2.2 Alkali pretreatment

Alkali pretreatment was carried out in a 200 mL capacity Parr reactor heated by oil bath. The reaction was conducted at a 10% solids loading and sodium hydroxide loading was varied from 1 – 2%. This range of loading was chosen based on the conditions previously used in the literature for efficient removal of extractives and lignin (McIntosh & Vancov, 2011; Sills & Gossett, 2012; Wang & Henriksson, 2011; Zanuttini et al., 1999). In order to assess the near optimal pretreatment conditions for enhanced sugar recovery and enzymatic hydrolysis, the influence of different reaction times (in the range of 1- 3 hours) and temperatures (in the range 120 – 160˚C) were evaluated.

2.2.3 Alkaline peroxide post treatment

Alkaline hydrogen peroxide treatment was carried out according to Yang et al (2002). This post treatment was carried out on the steam pretreated hog fuel I, bark and white wood. Hydrogen peroxide (Sigma Aldrich 30%) in H_2O was used as a reagent in the reaction. The hydrogen peroxide loading for the reaction was 1% (wt/wt) of the total reaction mixture (equivalent to 50% wt/wt of the oven dry sample) and the pH before starting the treatment was adjusted to 11.5 using 1M NaOH. The reaction was conducted at a final substrate consistency (% wt of dry matter in the total reaction mixture) of 2%. The temperature and reaction time were 80°C and 45 minutes respectively. After the treatment, the resulting slurry was separated into liquid and solid fractions by filtration through a Buchner funnel and the solid fraction was washed extensively with water until the pH of the filtrate dropped to ~7.0.

2.3 Enzymatic hydrolysis

The water insoluble component after the steam pretreatment, post treatment and alkali pretreatment were enzymatically hydrolysed at 2% (w/wt) consistency in acetate buffer (50 mM, pH 4.8) at 50 ˚C and 150 rpm. The commercial enzyme preparation, Cellic Ctec2 received from Novozymes North America Incorporated (Franklinton, NC, U.S.) was used for all enzymatic hydrolysis experiments and all hydrolysis were done in duplicates. The filter paper activity of the cellulase preparation was 100.6 FPU/ml (209.8 mg/ml). The reaction mixture containing the substrate and buffer were incubated at 50°C for 30 minutes and the required amount of enzymes was immediately added to the buffered reaction mixture to initiate the hydrolysis reaction. Sampling of 500 µl supernatant was completed in intervals at every 12, 24, 48 and 72 hours. After sampling, the enzyme activity was stopped by incubating the aliquots on a hot plate at 100 ˚C for 10 minutes and the samples were subsequently stored at 4°C till further use. The monomeric sugars released during hydrolysis were quantified by HPLC as detailed below.

2.4 Analytical methods

All chemical compositional analyses was done according to the NREL LAP protocol unless and otherwise specified (NREL, 2008b). Moisture contents were determined by drying to a constant weight at 105 °C in a convection oven.

2.4.1 Extractives

Extractive content of the raw materials was quantified according to NREL's LAP protocol (NREL, 2005b). Briefly, ten grams of Wiley milled (40 mesh) and dried biomass samples were subjected to Soxhlet extraction with water for 24h at approximately six siphon cycles/h. subsequent to extraction, the extract in the round-bottomed flask was dried in the oven at 105 ˚C for 24h to determine the amount of water soluble extractives. The water extracted biomass was subsequently subjected to a second extraction step using ethanol by the same procedure. The ethanol extract was first evaporated to dryness in the fumehood and subsequently placed in the oven at 105 ˚C for overnight. Alkali extraction was completed in a 1:20 ratio of solid : liquid, with 5 grams in 1% NaOH in water in a reflux condenser for 2 hours (Kofujita et al., 1999; Pettersen, 1984). The remaining insoluble solids were weighed to determine the amount of extractives soluble in alkali.

2.4.2 Ash

Determination of ash was carried out in a muffle furnace at 550˚C for 5h according to the NREL LAP protocol (NREL, 2005a). Ash determination was done both prior to and after completing water and ethanol extractions of the biomass samples and completed in duplicate.

2.4.3 Lignin and carbohydrates

The determination of the lignin content and structural carbohydrates of the raw material and pretreated substrates were determined according to the NREL LAP (NREL, 2008a). All analyses were completed in triplicate. As specified in the NREL LAP, a two stage sulfuric acid method was used to hydrolyze the carbohydrate component while measuring the insoluble residue as acid insoluble lignin (AIL). The first stage of the sulfuric acid

hydrolysis was conducted at room temperature and applied 3mL of 72% sulfuric acid to a 0.2g dry prepared sample as specified by NREL (NREL, 2008b). The acid sample was then stirred for 2h before being diluted to 4% acid concentration (addition of 112ml of water) and transferred to an autoclave set at 121°C for 1 hour for the second stage hydrolysis. A batch of sugar standards was also autoclaved in parallel with the samples to correct for the hydrolysis loss factor. Acid-insoluble lignin was measured by filtration and subtracting the oven-dried lignin filter weight from the oven-dried lignin filter+lignin weight. Acid-soluble lignin was determined by UV absorption at 205 nm using an extinction coefficient of 110 $l\ g^{-1}\ cm^{-1}$ (Dence, 1992).

Monomeric sugars were determined by a DX-3000 high-performance liquid chromatography (HPLC) (Dionex, Sunnyvale, CA), using an anion exchange column (Dionex CarboPac PA1). The eluent was deionized water at a flow rate of 1 ml/min. After filtering the sample through a 0.45-µm nylon syringe filter (Chromatographic Specialties Inc., Brockville, ON, Canada), aliquots of 20µl sample were injected to the column. Baseline stability and detector sensitivity were optimized by post column addition of 0.2 M NaOH at a flow rate of 0.5 ml/min using a Dionex AXP pump. The column was reconditioned using 1 M NaOH after each analysis. Standard sugar dilutions were prepared using analytical-grade sugars: L-arabinose, D-galactose, D-glucose, D-xylose and D-mannose (Sigma) and all of the standard dilutions were also run in parallel with the samples during the post hydrolysis step to correct for any hydrolysis loss factor. L-fucose (Sigma) was used as an internal standard for the normalisation of the HPLC response.

In order to quantify the fraction of oligomeric sugars present in the pretreatment liquid, post-hydrolysis were performed according to Shevchenko et al (2000). Duplicate samples containing the water soluble fraction were post-hydrolysed after adding concentrated sulphuric acid to achieve a final concentration of 3% acid. The post-hydrolysis was performed by heating the solution at 121°C for 1 hour in an autoclave. A batch of sugar standards was also autoclaved under the same conditions to correct for the hydrolysis loss factor. The monomeric sugars were quantified by HPLC as described above and the fraction

of oligomeric sugars was calculated by subtracting the amount of monomeric sugars present in the pretreatment liquid from the total amount of monomeric sugars present after the post hydrolysis of the same sample.

3 Results and discussion

3.1 The "robustness" of current analytical methods to determine the "Material balance" of processing forest residues

3.1.1 Introduction

Despite being abundantly available and supposedly inexpensive, forest residues exhibit numerous challenges as a feedstock for bioconversion applications. Due to their complex chemistry and heterogeneous nature, one of the expected challenges in processing forest residues is the difficulty in obtaining an accurate material balance. As mentioned in the introduction, the National Renewable Energy Laboratory (NREL)'s developed a comprehensive set of laboratory analytical procedures to determine the material balance of cleaner and relatively homogeneous feedstocks such as agricultural residues and white wood. An assessment of the robustness of these methods for determining the chemical composition of a range of heterogeneous forest residues was one of the major objectives of the research reported here.

Compared to white wood and agricultural residues, there are significant variations in the type, amount and complexity of various chemical components present in the forest residues. For example, forest residues can contain significantly higher amounts of ash, extractives, lignin, and other 'difficult-to-extract' components such as Suberin (Huang et al., 2011; Sjostrom, 1993). The amount and complexity of the extractives in forest residues (bark in particular) are substantially different from those of white wood and agricultural residues. The extractives content of white wood is generally lower and mostly comprised of lipophilic materials such as fats and waxes as well as resins, terpenoids and steroids (Laks, 1991). Due to this heterogeneity and inherent complexity, the composition of extractives is largely defined by the solvent used to extract them such as ethanol-benzene, acetone, ethanol or water. Thus, it is difficult to use one extraction protocol to completely remove all of the polar and non-polar components present in an extractive-rich biomass such as bark. In previous studies where more heterogeneous feedstock's were used, (Kim et al., 2005; Nguyen et al., 1998; Robinson et al., 2002), the authors reported the difficulty in achieving a

good material balance and in trying to quantify the individual components present in both the original biomass sample and after the pretreatment and fractionation.

Therefore, the objective of the described work was to assess how reproducible or robust the NREL-LAP methods might be in accurately quantifying the various chemical components of a range of potential forest residues and in providing good summative mass closures for each of the substrates. Six different softwood residues (hog fuel I & II, logging residue (LR), forest thinnings/interface fire slash (IFS), beetle-killed lodgepole pine wood chips (BK-LPP), and bark) were collected and the NREL recommended methods (Sluiter et al., 2010) were assessed for their ability to provide a good material balance and to quantify the major biomass components. We also investigated how a "typical" pretreatment such as steam pretreatment might influence the reproducibility and accuracy of the respective summative mass closure and the recovery of the various biomass components after pretreatment. Different extraction strategies were subsequently evaluated to see if they could enhance the accuracy of established methods when a more heterogeneous feedstock such as bark or hog fuel was used as a bioconversion feedstock.

3.1.2 Results and discussion

3.1.2.1 Compositional analysis of the raw material

Initially, each of the six biomass samples were analysed using the NREL LAP recommended compositional analysis methods (Agblevor et al., 1993; Templeton et al., 2010) without any prior extraction. The klason protocol was directly applied to the biomass substrates without first removing the extractives and the resulting chemical composition is described in the first 10 columns of Table 7. It was apparent that the total carbohydrates, lignin and ash together contributed 89 – 97% of the total dry weight of the starting materials depending on the source of the biomass (Table 7). Residues such as bark, which were anticipated to have a higher extractive content, gave the poorest mass closure (Table 7) indicating that some of the components particularly the extractives were not accounted for in this preliminary analysis.

Table 7: Chemical composition of the raw materials before steam pretreatment (% dry weight)*

	Arabinan	Galactan	Glucan	Xylan	Mannan	Lignin		Ash***	Sum (without accounting for extractives)	Extractives as determined by a separate extraction	Overall sum
						AIL	ASL				
HOG I	1.8 (0.1)**	1.8 (0.1)	29.6 (0.3)	4.5 (0.0)	6.0 (0.2)	42.8 (0.1)	1.2 (0.1)	6.9 (0.0)	94.6 (0.4)	6.2 (0.2)	101
HOG II	1.2 (0.0)	1.9 (0.0)	37.5 (0.7)	4.6 (0.1)	7.9 (0.2)	39.7 (0.2)	1.0 (0.1)	2.9 (0.1)	96.7 (0.7)	5.7 (0.3)	102
LR	2.4 (0.3)	3.0 (0.1)	33.6 (1.0)	5.5 (0.2)	7.8 (0.2)	38.6 (0.7)	1.1 (0.1)	0.4 (0.2)	92.4 (1.3)	9.4 (0.0)	102
IFS	1.9 (0.0)	5.6 (0.1)	36.1 (0.8)	6.6 (0.1)	9.9 (0.2)	33.3 (0.4)	0.6 (0.0)	0.1 (0.0)	94.1 (0.9)	6.3 (1.1)	100
BKLPP	1.6 (0.2)	2.7 (0.2)	42.4 (0.3)	5.9 (0.6)	11.4 (0.1)	28.2 (0.8)	0.4 (0.0)	0.1 (0.0)	92.7 (1.1)	3.8 (0.2)	97
BARK	6.4 (0.0)	3.5 (0.0)	19.2 (0.0)	3.1 (0.0)	1.5 (0.0)	52.7 (0.5)	1.2 (0.0)	2.0 (0.2)	89.4 (0.5)	19.0 (1.5)	109

HOG I & II are two different types of hog fuels collected from Nippon Paper. Hog I is more bark intensive.
LR refers to Logging Residue
IFS is Interface Fire Slash (or Forest Thinnings)
LPP refers to Beetle-killed lodge pole pine white wood
Bark samples are sourced from lodge pole pine wood
* Carbohydrates and lignin analysis were completed prior to extraction. The values are expressed as g per 100 g of original material
**The values in the bracket represent the deviations from the average of triplicate analysis
***Ash values reported are from biomass samples prior to extraction

This initial analysis was followed by the comprehensive NREL LAP method where the extractives were first quantified by a standard “water followed by ethanol” extraction prior to acid hydrolysis. These extractive values are also given in the second to last column of Table 7. This type of pre-extraction procedure is typically used to determine the extractive content of agricultural feedstocks (McIntosh & Vancov, 2011; NREL, 2005b). Since the extractives in forest residues such as bark are predominantly polar in nature, this extraction strategy was expected to remove the majority of the extractive components (Miranda et al., 2012; Sjostrom, 1993). When the resulting extractive values were combined with the carbohydrate, lignin and ash values taken prior to extraction, an improved summative mass closure of 97 – 109% was obtained (Table 7, last column). However, some of the values were significantly higher than 100%! It was likely that the extractives within the biomass if not removed, will interfere with the analysis, leading to an overestimation of certain components. Earlier work had shown that lignin is likely to be overestimated when high concentrations of extractives were present in the biomass material that had been quantified by the Klason technique (Hatfield & Fukushima, 2005; Hemmingson, 1987). The beetle killed lodgepole pine sample, which resembles a typical “white wood” containing lower levels of extractives, showed minimum interference with the lignin determination. In contrast, the most excessive value for mass closure was obtained with the bark sample, which contained the highest amount of extractives and therefore had the greatest probability of extractive precipitation with lignin during lignin quantification (Table 7). Previous work had also shown that, in addition to overestimating the amount of lignin present, the extractives and ash can also influence the carbohydrate analyses (Hames, 2010). However, all of the forest derived residues contained little ash (less than 7% ash) and little or no influence on the carbohydrate was anticipated. However, we did anticipate there would be some interference by the extractives present in each of the biomass substrates (particularly the bark) during the carbohydrate analysis.

Table 8: Chemical composition of the raw materials (% dry weight).

	Arabinan	Galactan	Glucan	Xylan	Mannan	Lignin		Extractives	Ash**	Sum
						AIL	ASL			
HOG I	1.0 (0.0)*	1.8 (0.0)	31.5 (0.2)	4.5 (0.0)	7.1 (0.1)	36.1 (0.2)	0.6 (0.1)	6.2 (0.2)	6.9 (0.0)	96
HOG II	0.8 (0.0)	1.8 (0.1)	36.7 (1.6)	4.5 (0.3)	9.0 (0.5)	33.8 (0.2)	0.5 (0.0)	5.7 (0.3)	2.9 (0.1)	96
LR	1.5 (0.0)	2.7 (0.0)	35.5 (0.9)	5.2 (0.1)	10.8 (0.3)	25.8 (1.2)	0.3 (0.1)	9.4 (0.0)	0.4 (0.2)	92
IFS	1.4 (0.0)	2.3 (0.0)	37.6 (1.0)	6.3 (0.2)	10.3 (0.3)	23.5 (0.7)	0.2 (0.0)	6.3 (1.1)	0.1 (0.0)	88
BKLPP	1.3 (0.0)	2.2 (0.0)	42.3 (0.7)	5.5 (0.1)	13.0 (0.2)	24.9 (1.2)	0.2 (0.0)	3.8 (0.2)	0.1 (0.0)	93
BARK	4.7 (0.1)	3.0 (0.0)	19.0 (0.3)	3.1 (0.0)	1.7 (0.1)	34.5 (0.7)	0.7 (0.0)	19.0 (1.5)	2.0 (0.2)	88

The carbohydrate and lignin analyses was a carried out after previous extraction of the substrates with water and ethanol. The values are expressed as g per 100 g of original material

HOG I & II are two different types of hog fuels collected from Nippon Paper. Hog I is more bark intensive.

LR refers to Logging Residue

IFS is Interface Fire Slash (or Forest Thinnings)

LPP refers to Beetle-killed lodge pole pine white wood

Bark samples are sourced from lodge pole pine wood

*The values in the bracket represent the deviations from the average of three replicate analysis **Ash values reported are from biomass samples prior to extraction

Table 9: The influence of an extraction step on the lignin and carbohydrate content of the original biomass (expressed as g per 100 g of original material)

	Extractives		**Acid Insoluble Lignin**			**Hemicellulose**		**Glucan**	
Extraction	Water	Water + Ethanol	Unextracted	After water extraction	After water followed by ethanol extraction	Unextracted	After water followed by ethanol extraction	Unextracted	After water followed by ethanol extraction
HOG I	3.6 (0.1)	6.2 (0.2)	42.8 (0.1)	39.8 (0.5)	36.1 (0.2)	14.1 (0.3)	14.4 (0.1)	29.6 (0.3)	31.5 (0.2)
HOG II	3.5 (0.7)	5.7 (0.3)	39.7 (0.2)	35.4 (1.8)	33.8 (0.2)	15.6 (0.2)	16.1 (0.6)	37.5 (0.7)	36.7 (1.6)
LR	5.2 (0.3)	9.4 (0.1)	38.6 (0.7)	28.2 (0.1)	25.8 (1.2)	18.7 (0.4)	20.2 (0.3)	33.6 (1.0)	35.5 (0.9)
IFS	4.1 (0.3)	6.3 (1.1)	33.3 (0.4)	26.7 (0.3)	23.5 (0.7)	24.0 (0.2)	20.3 0.4)	36.1 (0.8)	37.6 (1.0)
BKLPP	2.6 (0.2)	3.8 (0.2)	28.2 (0.8)	25.0 (0.8)	24.9 (1.2)	21.6 (0.6)	22.0 (0.2)	42.4 (0.3)	42.3 (0.7)
BARK	13.6 (1.2)	19.0 (1.5)	52.7 (0.5)	39.4 (1.1)	34.5 (0.7)	14.5 (0.1)	12.5 (0.1)	19.2 (0.0)	19.0 (0.3)

To try to better determine the extent of extractive interference in achieving an accurate material balance in each of the residue samples, a complete compositional analysis was carried out on the material, which was obtained after a two stage water-ethanol extraction process (likely to be extractive-free). It should be noted that, in this case, the klason procedure was applied to the extractive-free material obtained after a two stage water-ethanol extraction (Table 8). In the earlier analysis (Table 7), the acid hydrolysis was carried out directly on the biomass substrates which had not had prior extraction (Table 7). As was anticipated, prior removal of the extractives had a substantial effect on the compositional analysis of the forest residues with the lignin values decreasing significantly (3 – 18%) (Table 8, Table 9). As was also expected, the interference due to extractives was considerably higher for the bark and logging residues since these substrates contained greater amounts of extractives.

It was apparent that prior water-ethanol extraction followed by the compositional analysis of the "extractive free" material resulted in a summative mass closure of 88 – 96% (Table 8). The lowest summative value was observed with the bark, forest thinnings and logging residues. This was likely due to some missing components which were not accounted for in the analysis. One of the components that may not have been picked up is pectin which would be released as uronic acid (Agblevor et al., 1993; Kurth, 1947). However, the uronic acid content was not analyzed in this study. As mentioned previously, the interface fire slash contained a blend of juvenile wood samples from both softwoods and hardwoods. Hardwood hemicellulose is generally more acetylated and therefore should have some acetyl groups, which were also not quantified. The bark sample gave the lowest mass closure, possibly be due to residual extractives which were still present in the substrate even after a water-ethanol extraction step (Table 8). Some of these residual extractives may have been solubilized in the concentrated acid and thus not accounted for during a normal Klason analysis.

Our attempts to achieve a good material balance from a high extractive containing forest biomass sample without a prior extraction step resulted in an overestimation of the

lignin but only minor variations in the carbohydrate content (Table 9). The largest variation in carbohydrate was observed with the hemicellulose content of the bark and IFS samples, where a respective 2 and 4% loss seem to have resulted from the two stage extraction (Table 9). This loss was likely due to the solubilisation of the neutral sugars present in the pectin component of these materials, as they can be relatively easily removed by hot water hydrolysis (Hames, 2010; Sjostrom, 1993).

As noted earlier, the water-ethanol procedure recommended in the NREL LAP method was primarily developed with agricultural residues in mind (NREL, 2005b). In contrast, probable forest residue feedstocks such as bark or hog fuel, are known to contain high molecular weight extractive components, which cannot be entirely solubilized by a simple water-ethanol extraction (Pietarinen et al., 2006; TAPPI, 1997). Even for agricultural and whitewood feedstocks, large variations in extractive content have been reported between different laboratories (Agblevor et al., 1993). For the forest residues studied here, it was possible that, even after a water-ethanol extraction, the residual extractives might be hydrolysed in the concentrated acid medium or have precipitated and interfered with the lignin quantification. It has also been shown that significant amounts of both polar and non-polar extractable components are present in these types of biomass (Ajuong & Breese, 1998; Pettersen, 1984; Sjostrom, 1993; TAPPI, 1997). For example, poly flavonoids, terpenes, resin acids, fats, and suberin are all found in bark due to the protective, anti-fungal/insect properties they provide the tree. Due to the diversity and quantity of extractives in bark a broad spectrum of methods have been developed to remove and characterize the different types of extractives (Huang et al., 2011; Miranda et al., 2012; Valentín et al., 2010; Yazaki & Collins, 1994). Mild alkali has been reported to be one of the most effective methods for the removal of most of the extractives with minimal influence on subsequent assessment of the carbohydrate content (Kofujita et al., 1999; Vázquez et al., 1987). A 1.0% NaOH solution in reflux has often been used for maximum extractive removal from bark and to provide more a realistic estimation of the lignin content (Browning, 1967). Therefore, we next applied an alkali extraction to the untreated bark and hog fuel samples to determine if this approach might improve the summative mass closure.

Table 10: Chemical composition of bark and hog fuel based on alkali extraction prior to compositional analysis (% dry weight)

(Carbohydrates and lignin analysis completed after extraction with 1% NaOH and subsequently the values are expressed as g per 100 g of original material)

	Bark	Hog fuel I
Arabinan	3.6 (0.0)	1.2 (0.0)
Galactan	2.1 (0.0)	1.5 (0.0)
Glucan	19.2 (0.3)	29.4 (0.2)
Xylan	3.2 (0.0)	4.1 (0.1)
Mannan	1.5 (0.0)	4.5 (0.2)
Lignin (AIL)	20.1 (0.7)	27.6 (0.7)
Lignin (ASL)	0.3 (0.0)	0.3 (0.0)
Ash	2.0 (0.2)	6.9 (0.0)
Extractives	42.9 (0.9)	23.5 (0.7)
Mass closure	95.8	99.4
*The values in the bracket represent the deviations from the average of triplicate analysis **Ash values reported are from biomass samples prior to extraction		

The bark and hog fuel samples were shown to contain 43 and 24% alkali-soluble extractives respectively (Table 10) with the bark values similar to those found previously with pine bark (Miranda et al., 2012; Yazaki & Collins, 1994). The alkali extraction further reduced the lignin content of the original material to 21 and 28% respectively for the bark and hog fuel samples. This was equivalent to a 14% and 8% further reduction in lignin content when compared to the lignin values determined after water-ethanol extraction. These results indicated that the alkali extraction effectively solubilised most of the extractives and resulted in a better summative mass closure of 96 and 99% respectively for both the bark and hog fuel substrates. It was also likely that the hydrolysis of extractive components such as suberin and long chain fatty acids and their subsequent dissolution in the alkaline solution, was representative of the efficiency of removal of the majority of the extractive compounds (Kofujita et al., 1999). However, the alkali extraction did result in the loss of some of the hemicellulosic sugars, particularly arabinose and galactose (Table 10). About 3 and 2% arabinan and galactan appeared to have been extracted in alkali, which

could be attributed to the more efficient extraction of pectins in an alkaline medium (Phatak et al., 1988). Overall, alkali extraction resulted in a significantly better summative mass closure of the bark and hog fuel biomass residues.

3.1.2.2 Influence of steam pretreatment in obtaining a material balance

As was discussed previously, the forest residues used in this work had a broad range of particle sizes and moisture content. Previous studies had demonstrated that particle size and moisture content could significantly influence the efficiency of steam pretreatment (Cullis et al., 2004). Thus, we first dried and ground the biomass to a 1-2 mm particle size and subsequently conditioned this material to a uniform moisture content. Subsequently, the biomass samples were impregnated with 4% SO_2 and steam pretreated at two sets of conditions; 180°C for 5 minutes, and 200°C for 5 minutes. A compositional analysis was subsequently carried out for both the water insoluble and soluble fractions.

As earlier work had shown that the pretreatment could influence our ability to achieve a good mass closure, due factors such as degradation reactions producing materials such as pseudo-lignin (Hemmingson, 1987), we next assessed whether steam pretreatment of the forest residue substrates might influence the robustness of the compositional analysis and our ability to achieve a reasonable material balance. After steam pretreatment, the water insoluble fraction was subjected to a chemical compositional analysis (Table 11, Table 12). However, unlike the starting material, the water insoluble, cellulosic rich component was not be subjected to a prior extraction procedure. Depending on the severity of the applied conditions, steam pretreatment has been reported to depolymerise the lignin into smaller molecular weight fractions, which would solubilise during an extraction using an organic solvent or alkali (Kumar et al., 2011; Li & Gellerstedt, 2008). Although the compositional analysis of the water insoluble component was carried out without any prior extraction, the summative mass closure obtained was reasonably good, ranging from 96 – 101% for the low severity (Table 11) and 89 – 100% for the high severity conditions (Table 12).

Table 11: Chemical composition of the water insoluble component after the steam pretreatment at 180°C, 5 minutes 4% SO_2 (% dry weight)

(The raw material was ground to a particle size of ~2mm and moisture content was adjusted to ~50% by wet weight of the sample).

	Arabinan	Galactan	Glucan	Xylan	Mannan	Lignin	Ash	Sum
HOG I	0.3 (0.0)	0.8 (0.0)	36.8 (0.5)	3.2 (0.0)	3.6 (0.0)	51.0 (0.8)	3.8 (0.2)	99.5 (0.8)
HOG II	0.2 (0.0)	1.0 (0.0)	42.4 (0.4)	3.8 (0.1)	5.9 (0.1)	45.1 (0.3)	2.5 (0.7)	100.9 (1.9)
LR	0.7 (0.0)	1.3 (0.0)	43.6 (0.5)	3.4 (0.0)	4.3 (0.1)	41.7 (0.7)	0.6 (0.0)	95.6 (0.9)
IFS	0.8 (0.0)	1.5 (0.0)	44.5 (0.4)	5.2 (0.0)	5.3 (0.0)	38.2 (0.2)	0.5 (0.3)	96.0 (1.4)
BKLPP	0.4 (0.1)	0.8 (0.1)	49.6 (0.1)	3.5 (0.2)	4.6 (1.0)	38.2 (0.9)	0.1 (0.1)	97.2 (0.6)
BARK	3.6 (0.0)	2.4 (0.0)	22.2 (0.5)	3.2 (0.0)	1.4 (0.0)	64.6 (1.8)	2.0 (0.4)	99.4 (1.9)
*The values in the bracket represent the deviations from the average of triplicate analysis								

Table 12: Chemical composition of the water insoluble component after the steam pretreatment at 200°C, 5 minutes 4% SO_2 (% dry weight)

(The raw material was ground to a particle size of ~2mm and moisture content was adjusted to ~50% by wet weight of the sample).

	Arabinan	Galactan	Glucan	Xylan	Mannan	Lignin	Ash	Sum
HOG I	0.3 (0.0)	0.6 (0.1)	31.1 (0.9)	1.9 (0.1)	2.1 (0.1)	50.9 (1.1)	5.0 (0.0)	100.1 (0.9)
HOG II	0.1 (0.0)	0.4 (0.0)	42.7 (0.5)	1.7 (0.1)	2.6 (0.1)	42.5 (1.3)	1.5 (0.1)	99.7 (0.5)
LR	0.3 (0.0)	0.7 (0.0)	42.0 (0.5)	1.2 (0.0)	1.8 (0.0)	45.4 (1.4)	0.6 (0.2)	95.4 (0.5)
IFS	0.4 (0.0)	0.7 (0.0)	45.2 (0.3)	2.3 (0.0)	2.3 (0.1)	39.8 (1.8)	0.5 (0.7)	93.9 (0.3)
BKLPP	0.3 (0.0)	0.4 (0.1)	51.7 (1.0)	1.0 (0.1)	1.7 (0.2)	39.8 (0.5)	0.3 (0.1)	95.2 (0.8)
BARK	2.6 (0.2)	1.4 (0.1)	22.4 (0.8)	1.4 (0.1)	0.9 (0.1)	58.5 (1.6)	1.9 (0.4)	89.1 (1.0)
*The values in the bracket represent the deviations from the average of triplicate analysis								

The reasonable mass balance closure obtained (Table 11,Table 12) implied that most of the extractives had been volatilised or solubilised during steam pretreatment and any remaining extractives were quantitatively precipitated with the lignin during analysis. In general, the amount of lignin that was detected in the water insoluble component after steam pretreatment was slightly higher than what was measured in the original material (Figure 4). This was likely due to the reaction of extractives with the lignin during steam pretreatment, leading to higher lignin values being detected. Earlier work had shown that some of the extractives had condensed with the lignin during steam pretreatment as well as during a subsequent Klason analysis. Thus, increasing the reported lignin values (Hemmingson, 1987). However, it is likely that the solubility of the extractives and their incorporation to lignin will also be influenced by the severity of steam pretreatment conditions used. It appears that pretreatment at 180°C did not sufficiently fragment and solubilise the extractives, leaving most of them in their native form and allowing their precipitation with the lignin during Klason analysis. The more severe steam pretreatment at higher temperatures likely depolymerised the extractives, leading to their dissolution and reducing their presence in the insoluble component. Thus, they did not contribute significantly to the lignin quantification.

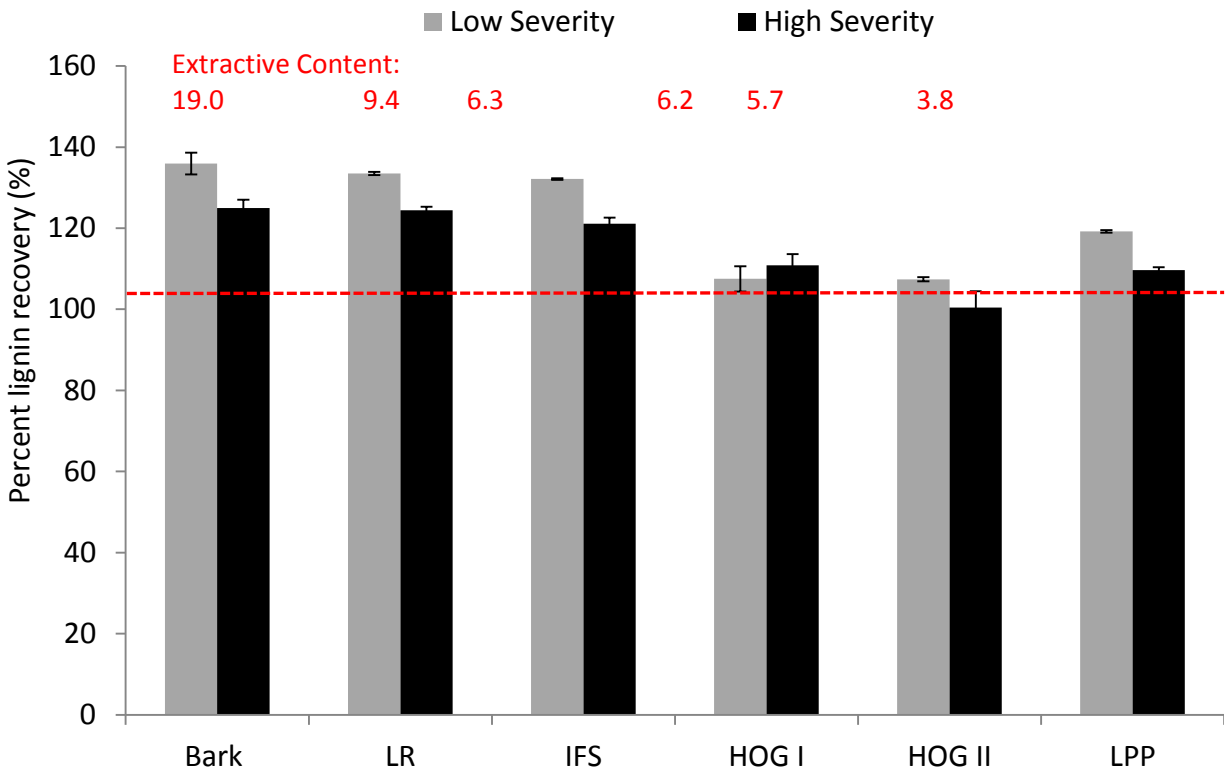

Figure 4: Influence of extractives on the recovery of lignin in the water insoluble component after the steam pretreatment of the homogenized forest residues at 180 and 200°C.

3.1.2.3 Sugar recovery during steam pretreatment and the suitability of the materials for bioconversion

Unlike the problems encountered with the lignin quantification, good reproducibility and mass balance was generally obtained with the carbohydrate values. The sugar recoveries were calculated based on the overall recovery measured after pretreatment (Appendix B). Both the pretreatment severities that were assessed resulted in near complete glucan recovery (>90%) in the combined water soluble and insoluble fractions (Table 13). The total hemicellulose recovery at the lower severity was 85-100%, while the recovery decreased to 68-77% at the high severity, although most of the hemicellulose was recovered in a monomeric form (>55%) (Table 14). This should allow their ready fermentation without the need to further hydrolyse the oligomeric sugars. In addition, it is

likely that the solubilisation of most of the hemicellulose will enhance the accessibility of the cellulase enzymes to the cellulose in a subsequent enzymatic hydrolysis of the cellulosic component (Bura et al., 2009; Várnai et al., 2010).

Table 13: Recovery of original glucan after the steam pretreatment of the forest residues (expressed as g per 100g of starting biomass).

Sample name	Original glucan per 100 g starting material (g)	Recovered after 180˚C pretreatment (g)		Recovered after 200˚C pretreatment (g)	
		WS*	WI**	WS*	WI**
HOG I	31.5 (0.2)	1.4 (0.1)	27.1 (0.0)	1.3 (0.1)	24.5 (0.0)
HOG II	36.7 (1.6)	1.0 (0.0)	34.8 (0.2)	1.2 (0.0)	34.1 (0.4)
IR	35.5 (0.9)	2.1 (0.0)	32.3 (0.3)	2.2 (0.0)	29.7 (0.4)
IFS	37.6 (1.0)	1.5 (0.1)	37.0 (0.3)	2.0 (0.1)	32.3 (0.2)
BKLPP	42.3 (0.7)	2.1 (0.2)	38.6 (0.5)	2.5 (0.2)	32.7 (0.6)
BARK	19.0 (0.3)	0.7 (0.5)	19.2 (0.2)	1.7 (0.5)	16.5 (0.6)

***** Water soluble component after the pretreatment
** Water insoluble cellulosic component after the steam pretreatment
The glucan obtained in the water soluble fraction in 180˚C and 200˚C samples had 80-90% and 0-45% proportion respectively in the oligomeric and monomeric form.

Table 14: Recovery of original hemicellulosic sugars after the steam pretreatment of forest residues (expressed as g per 100g of starting biomass)

Sample name	Original hemicellulose per 100 g starting material (g)	Recovered after 180˚C pretreatment (g)		Recovered after 200˚C pretreatment (g)	
		WS*	WI**	WS*	WI**
HOG I	14.4 (0.3)	6.8 (0.1)	6.4 (0.0)	6.0 (0.1)	3.8 (0.0)
HOG II	16.1 (0.2)	5.1 (0.1)	9.2 (0.2)	6.7 (0.1)	3.9 (0.3)
IR	20.2 (0.4)	11.6 (0.1)	7.3 (0.3)	9.9 (0.2)	2.8 (0.4)
IFS	20.3 (0.2)	9.9 (0.4)	10.6 (0.2)	13.2 (0.3)	4.0 (0.2)
BKLPP	22.0 (0.6)	11.8 (0.5)	7.3 (0.4)	12.9 (0.1)	2.3 (0.6)
BARK	12.5 (0.1)	5.7 (0.4)	9.2 (0.2)	6.5 (0.1)	4.6 (0.6)

***** Water soluble component after the pretreatment
** Water insoluble cellulosic component after the steam pretreatment
The hemicellulosic sugars obtained in the water soluble fraction in 180˚C and 200˚C samples had 53-80% and 5-45% proportion respectively in the oligomeric and monomeric form.

When evaluating the potential of forest residues as candidate substrates for a biomass-to-ethanol process the theoretical sugar/ethanol yield that can be anticipated will likely be significantly lower than what could be expected using white wood. With the exception of the bark sample, which showed a significantly lower carbohydrate composition, all of the other residues still contained 43-64% polysaccharides. In addition to a lower theoretical sugar/ethanol yields, the enzymes and yeast used in the conversion can be significantly inhibited by the high level of extractives and lignin present in bark (Nakagame et al., 2010; Robinson et al., 2002). However, extractives such as tannins, have the potential to be used as a high value co-products such as phenolic resins and pharmaceutical/nutraceutical feedstocks (Baysal et al., 2007; Kofujita et al., 1999; Pietarinen et al., 2006). Lignin can also be used to make phenolic and epoxy resins, carbon fibers and several other valuable products (Huang et al., 2011; Kadla et al., 2002). Therefore the selective fractionation and removal of extractives and lignin might not only aid in achieving a better material balance for pretreated forest residues, it might also help us derive higher-value co-products while providing a "carbohydrate enriched" fraction that could be used as the sugar feedstock for fuel and chemicals.

3.1.3 Conclusion

Despite the challenges resulting from the heterogeneity of the six different forest residue biomass substrates, a reasonable summative mass closure could be obtained before and after steam pretreatment. However, method revision and optimisation was required, particularly the effective removal of extractives to ensure that representative and reproducible values for the major lignin and carbohydrate components could be derived. With the increasing realisation that the extractive components of biomass are in themselves potentially valuable chemical feedstocks, further improvements in the solvents and extractive procedures used to characterize them should help achieve both improved mass balance closure and better characterisation of the individual components within the extractives fraction.

3.2 Pretreatment and enzymatic hydrolysis strategies to enhance overall sugar yield from forest residues

3.2.1 Introduction

In the earlier work (Chapter 3.1), it was noted that forest residues generally contained a lower amount of carbohydrates when compared to typical white wood or agricultural residues. As a result, the theoretical yield of sugars or ethanol for a given amount of forest residues will typically be lower. In addition, the low carbohydrate content together with the higher amount of inhibitory extractives that will probably be present in forest residues will likely make it challenging to obtain good overall sugar recovery and use. However, from a biorefinery perspective, there is an opportunity to use the non-carbohydrate components such as lignin and extractive components for the production of high-value chemicals or polymers while using the polysaccharide fraction for the production of sugars. Therefore, the effective utilisation of forest residues in a biorefinery approach requires the recovery of most biomass components including the carbohydrates, extractives and lignin in a useable form and their subsequent valorisation to fuel, materials and/or chemicals. However, the appropriate pretreatment and fractionation strategies to recover forest residue components in a "manageable" form have not yet been developed. In the work described in this chapter, a preliminary assessment of pretreatment strategies to enhance the enzymatic hydrolysis of cellulose and the overall sugar yield while maximizing the recovery of the non-carbohydrate components such as extractives and lignin was carried out. With the complex physical characteristics and chemistry of some of the forest residues, the pretreatment strategies and conditions that are typically uses for "more homogenous" feedstocks such as white wood, will likely have to be modified.

Previous work has shown that the nature of the substrate is heavily influenced by the effectiveness of various pretreatment processes and the optimised process conditions (Chandra et al., 2007; Kabel et al., 2007). For example, agricultural residues and hardwoods are generally considered to be more easily treated while softwoods are typically more recalcitrant (Liu, 2010). This means, the pretreatment process needs to be tailored for the

type of biomass available for processing. For example, AFEX (Ammonia Fibre Explosion) was quite effective on agricultural residues but is unlikely to be used to pretreat softwoods (Holtzapple et al., 1991), although it can provide reasonable pretreatment for hardwoods (Balan et al., 2009). Among the different pretreatment methods developed by various research groups, acid catalysed (SO_2 or H_2SO_4) steam pretreatment/dilute acid pretreatment has been shown to be robust in processing a range of lignocellulosic feedstocks including agricultural residues, hardwoods and softwoods (Hendriks & Zeeman, 2009). However, the efficiency of acid catalysed steam pretreatment in processing forest residues, particularly from a biorefinery perspective, has not yet been assessed.

We first assessed the response of six different softwood derived forest residues to SO_2 catalysed steam pretreatment in terms of sugar recovery, efficient fractionation and subsequent ease of enzymatic hydrolysis of the cellulosic component. Previously, as shown in chapter 3.1, most of the carbohydrates could be recovered in the combined water soluble and insoluble fractions using typical steam pretreatment conditions employed for softwoods. However, we had not investigated how responsive the resulting cellulosic component would be to enzymatic hydrolysis. Steam pretreatment could solubilise most of the hemicellulosic sugars present in forest residues which should ideally enhance the enzymatic accessibility to the cellulosic component (Mansfield et al., 1999). However, it is possible that the high extractive content together with lignin, still remaining on the steam pretreated forest residues could restrict the accessibility of the enzymes to the cellulose (Li et al., 2007; Mansfield et al., 1999). In addition, it has been shown that some of the extractive components can potentially inhibit and deactivate the enzymes (Ximenes et al., 2011; Ximenes et al., 2010). Therefore, it is possible that steam pretreated forest residues, which are rich in lignin and extractives, may not be as efficiently hydrolysed as steam pretreated white wood when subjected to the same set of pretreatment and enzymatic hydrolysis conditions.

Alkaline pretreatment methods also hold great potential as a pretreatment strategy for processing forest residues (Jansson et al., 2010; Vázquez et al., 1987; Zanuttini et al.,

1999). Unlike acid based pretreatments, alkaline processes have been previously shown to remove the majority of the extractives present in the forest residues, resulting in a carbohydrate-enriched water insoluble component (Kofujita et al., 1999). Alkaline pretreatments were also reported to be effective in partially removing lignin due to increased lignin depolymerisation and subsequent dissolution in alkali (McCarthy & Islam, 2000). It should be noted that extractives and lignin can together contribute up to 60% of the pine bark weight (Kofujita et al., 1999; Miranda et al., 2012; Vázquez et al., 1987) and their effective removal is likely to enhance the enzymatic accessibility to the resulting cellulose enriched water insoluble component. Therefore, pretreatment in an alkaline medium should provide cleaner fractionation while enhancing the enzymatic hydrolysis of the cellulosic component. However, the alkaline environment is generally less efficient in solubilizing the hemicellulosic component compared to acidic pretreatments (Carvalheiro et al., 2008; Kofujita et al., 1999) and the residual hemicellulose therefore can potentially restrict the ease of enzymatic hydrolysis (Mansfield et al., 1999). In order to assess which mechanism is more influential in enhancing cellulose hydrolysis of extractive rich forest residues, the efficiency of alkaline pretreatment was compared to that of acid catalysed steam pretreatment. In order to perform a fair comparison, we first evaluated different alkaline pretreatment conditions in terms of their efficiency to solubilise extractives and lignin and assessed their subsequent effect on enzymatic hydrolysis. Subsequently, the efficiency of the best set of alkaline pretreatment conditions was compared to that of SO_2 catalysed steam pretreatment for their ability to enhance the enzymatic hydrolysis as well as obtaining an overall sugar yield.

3.2.2 Enzymatic hydrolysis of steam pretreated forest residues

As was reported in Chapter 3.1, SO_2 catalysed steam pretreatment resulted in the dissolution of majority of the hemicellulosic sugars resulting in a water insoluble component mostly comprised of lignin and cellulose. However, from the mass balance data, most of the extractives appeared to have precipitated with lignin during SO_2 catalysed pretreatment (Chapter 3.1). In order to determine how these changes in the chemical

composition influenced their susceptibility to enzymatic hydrolysis, an enzymatic hydrolysis of steam pretreated forest residues at an enzyme loading of 25 FPU/g cellulose was carried out (Figure 5).

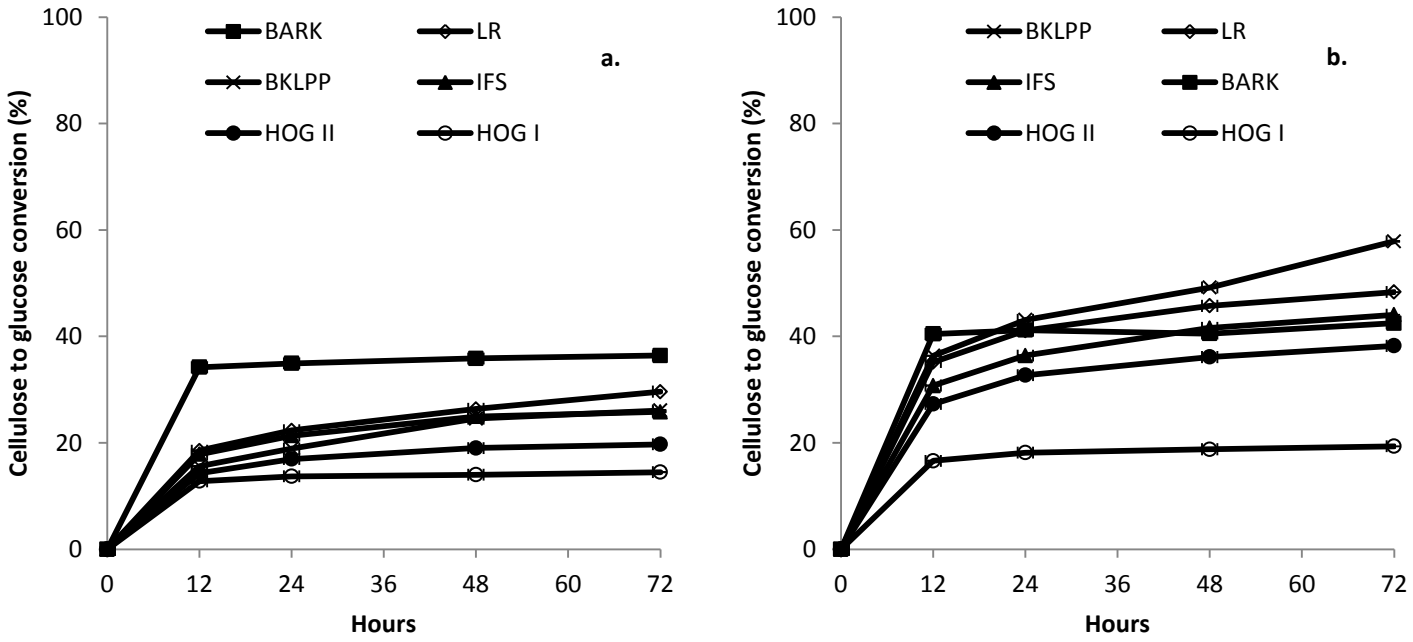

Figure 5: Enzymatic hydrolysis of the water insoluble cellulosic component after the steam pretreatment of forest residues

a) 180°C, 5min, 4% SO2 b) 200°C, 5min, 4% SO2. Enzyme loading: 50 mg/g cellulose (~25FPU Ctec 2/g cellulose) *The error bars represent the standard deviations from the average

As was anticipated, poor hydrolysis yield (less than 60%) were obtained on all of the steam pretreated forest residues (Figure 5a&b). An increase in the pretreatment severity resulted in a 3-17% improvement in cellulose hydrolysis. This could likely be attributed to the higher extent of hemicellulose removal and the consequent increase in cellulose accessibility. However, the extent of improvement with increase in pretreatment severity was highly dependent on the type of forest residues that was used (Figure 5a&b). Interestingly, increasing the pretreatment severity had only a minor effect on the cellulose hydrolysis of bark (Figure 5a&b). Despite a greater removal of hemicellulose at higher severity, the sustained recalcitrance of bark suggested that lignin, together with the residual extractives, might play a critical role in limiting cellulose hydrolysis (Figure 5a&b) (Ewanick et al., 2007; Kumar et al., 2010). It should be noted that lignin and extractives together contribute 60% of the weight of the high severity treated bark and the enrichment of these non-carbohydrate components have likely restricted the accessibility of the enzymes to the cellulosic component. Previous work has shown that extractives can condense with the lignin at high severity steam pretreatment conditions (Hemmingson, 1987). The high recovery of “lignin” obtained after steam pretreatment of bark compared to other samples also supported this possible mechanism (Chapter 3.1). In comparison, the BKLPP sample which had a lower extractive content and closely resembles white wood, an increase in pretreatment severity resulted in a significant increase in cellulose hydrolysis. It appears that enhanced hemicellulose removal improves the enzymatic hydrolysis of low extractive-containing forest residues. However, for the case of high extractive-containing residues such as bark, the consequential enrichment of extractives and lignin appears to mask any improvement that might be obtained with the greater removal of hemicellulose (Figure 5a&b).

Since the enzymes used for the cellulose hydrolysis contained substantial levels of hemicellulase activity, we next assessed the extent of the release of hemicellulosic components during the enzymatic hydrolysis of steam pretreated forest residues. It should be noted that despite a significant dissolution of hemicellulose during pretreatment, the

water insoluble solids of some of the forest residues (particularly those treated at lower severity) still contained a significant amount of residual hemicellulose (Chapter 3.1, Table 14). It was apparent that beetle-killed lodgepole pine, logging residue and bark had the highest degree of hemicellulose removal (70 – 90%) during enzymatic hydrolysis when compared to hog fuel and IFS (Figure 6). This implied that the extent of hemicellulose removal did not necessarily correlate to the extent of cellulose hydrolysis (Figure 5 & Figure 6). Thus it was likely that other substrate factors, such as residual lignin and extractives, were also restricting cellulose hydrolysis.

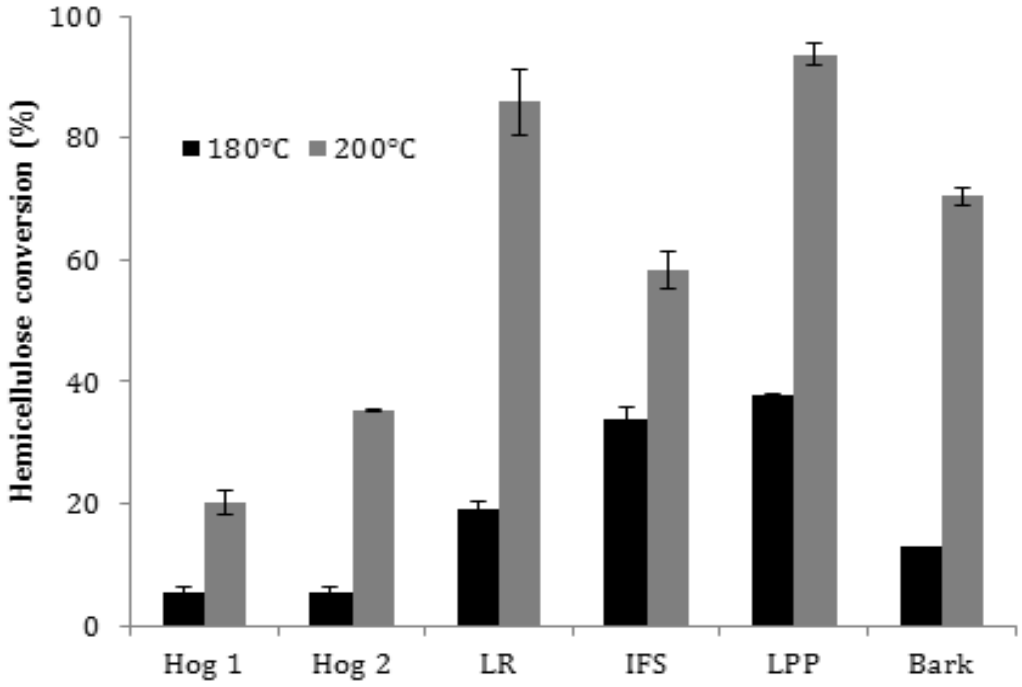

Figure 6: Conversion of hemicellulose to monomeric sugars after the 72 hour enzymatic hydrolysis of the water insoluble component of steam pretreated forest residues.

Enzyme loading: 50 mg/g cellulose (~25FPU Ctec 2/g cellulose). *The error bars represent the standard deviations from the average.

Since poor sugar yields were obtained after the enzymatic hydrolysis of steam pretreated forest residues at an enzyme loading of 25FPU/g cellulose, the influence of increasing the enzyme loading on the hydrolysis profile of the selected substrates (Figure 7) was next assessed. Lodgepole pine bark (BARK) and beetle-killed lodgepole pine white

wood samples (BK-LPP) which had been pretreated at high severity were selected for this comparison, representing high extractive containing and low extractive containing forest residues respectively. At all enzyme loadings (low, medium and high), the pretreated white wood performed consistently better than bark. It should be noted that, although the 12 hour hydrolysis yields were almost similar for both bark and white wood samples

Figure 7), after 12 hours, the hydrolysis of bark appeared to have leveled-off, while the hydrolysis of BKLPP continued to increase. The difference in 72 hour hydrolysis yields was most apparent at the highest enzyme loading (70 mg/g cellulose) where BKLPP reached about a 25% higher cellulose conversion than bark. The results appear to indicate that it could be the higher extractive content of the bark, which caused the poor hydrolysis yield even at significantly higher enzyme loadings.

Incomplete hydrolysis yields at high enzyme loadings for both steam pretreated bark and BK-LPP indicated the high recalcitrance of softwood derived forest residues. This was similar to what had been reported earlier with the white wood substrates from several softwood species such as Douglas-fir, Lodgepole pine, Pinus radiata, Spruce, etc. (Ewanick et al., 2007; Kumar et al., 2010; Pan et al., 2004). In this previous work, a post treatment was found to be necessary to obtain a fast and complete enzymatic hydrolysis of steam pretreated softwoods at low enzyme loadings (Pan et al., 2004; Yang et al., 2002). This type of post treatment had a primary goal of removing or modifying lignin to enhance cellulose accessibility (Kumar et al., 2011). With the anticipation that a post treatment would likely enhance the hydrolysis yield to completion regardless of the original extractive content of the substrates, an alkaline hydrogen peroxide post treatment was applied to these substrates and the influence on enzymatic hydrolysis was assessed.

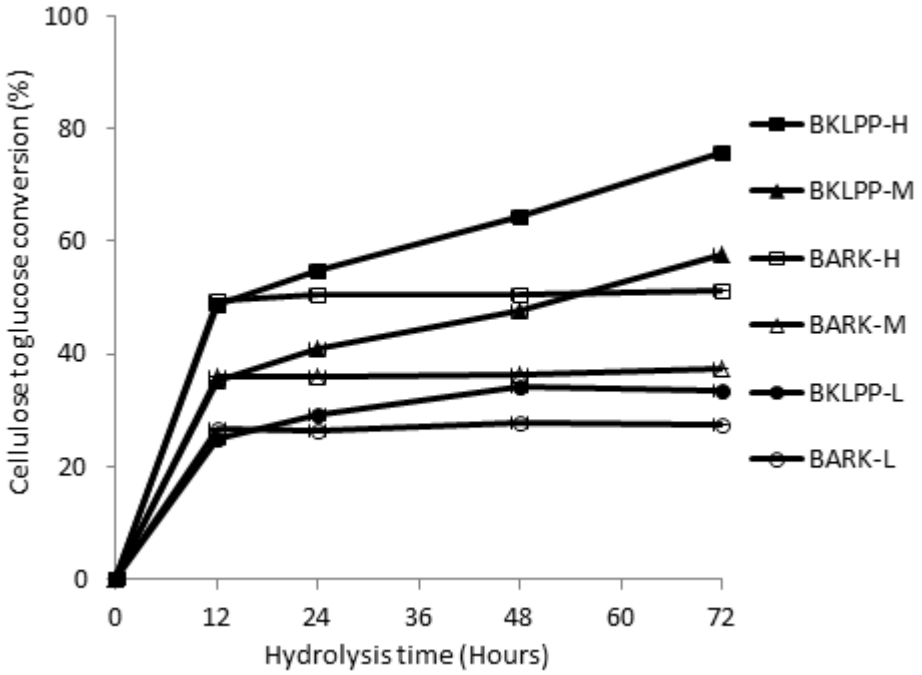

Figure 7: Influence of enzyme loading on the hydrolysis profile of steam pretreated beetle-killed white wood (BKLPP) and bark.

Steam pretreatment condition: 200°C, 5 minutes and 4% SO2. Enzyme loadings: L: 30, M: 50, and H: 70 mg/g cellulose (15, 25, 35 FPU Ctec 2/g cellulose). *The error bars represent the standard deviations from the average.

3.2.3 Influence of a post-treatment on the enzymatic hydrolysis of steam pretreated white wood, hog fuel, and bark

Many post-treatments have been studied for their efficiency on steam pretreated softwoods including partial delignification (such as sodium chlorite, alkaline hydrogen peroxide, alkali, alkali-oxygen etc.) and lignin modification processes (such as sulfonation or carboxylation) (Kumar et al., 2011; Nakagame et al., 2011; Pan et al., 2005; Pan et al., 2004; Yang et al., 2002). Alkali treatment has been shown to solubilize lignin and consequently improve cellulose hydrolysis (Feist et al., 1970). While this method has been shown to be effective on hardwoods, the delignification of softwood substrates was poor and there was little improvement in the efficiency of enzymatic hydrolysis (Ramos et al., 1992). However,

the addition of hydrogen peroxide to the alkaline conditions was shown to result in substantial levels of lignin depolymerisation and dissolution (Yang et al., 2002). In subsequent studies the alkaline hydrogen peroxide (AHP) addition was optimised and shown to be one of the most effective post-treatment methods for obtaining a fast and complete hydrolysis of steam pretreated softwoods (Kumar et al., 2011; Pan et al., 2004; Yang et al., 2002).

This same alkaline hydrogen peroxide treatment was applied to the high severity steam pretreated white wood and bark and the degree of delignification was determined (Table 15). As was anticipated, the AHP post treatment significantly reduced the lignin content of the steam pretreated substrates. However, despite the reductions in the lignin content, the residual lignin present in the peroxide treated bark was still quite high compared to that of white wood. This likely indicated that condensation reactions occurred between the lignin/extractives and between different lignin moieties during the steam pretreatment of bark. This made it difficult to remove in subsequent post treatments (Li et al., 2007). As was expected, the enzymatic hydrolysis of the steam pretreated and subsequently post treated white wood resulted in faster and more complete cellulose hydrolysis (Figure 8). Thus, peroxide post treatment of steam pretreated bark also resulted in high sugar yields after enzymatic hydrolysis despite their higher residual lignin content. Although the process did not result in substantial delignification of steam pretreated bark, it is possible that alkaline hydrogen peroxides might have removed that fraction of lignin which significantly restricts the accessibility of the cellulase enzymes to cellulose. In addition, alkaline hydrogen peroxide treatment has been reported to oxidise the side chain structures of lignin making it less hydrophobic (Pan et al., 2005; Yang et al., 2002). This can also increase cellulose accessibility and minimise the unproductive hydrophobic interaction of the cellulase enzymes with lignin (Yang et al., 2002).

Table 15: Influence of alkaline peroxide post treatment on the chemical composition of steam pretreated bark and hog fuel

	Arabinan	Galactan	Glucan	Xylan	Mannan	Lignin	Ash	Sum
SP-WW	0.3 (0.0)	0.4 (0.1)	51.7 (1.0)	1.0 (0.1)	1.7 (0.2)	39.8 (0.5)	0.3 (0.1)	95.2 (0.8)
SP-BARK	2.6 (0.2)	1.4 (0.1)	22.4 (0.8)	1.4 (0.1)	0.9 (0.1)	58.5 (1.6)	1.9 (0.4)	89.1 (1.0)
SP-BARK-AHP	0.4 (0.2)*	0.8 (0.2)	41.2 (0.4)	2.1 (0.1)	1.1 (0.1)	45.2 (0.2)	2.0 (0.0)	93 (0.5)
SP-WW-AHP	BDL	BDL	76.4 (0.7)	0.2 (0.0)	0.4 (0.1)	19.7 (0.1)	BDL	96.7 (0.8)
*The values in the bracket represent the deviations from the average of triplicate analysis.								

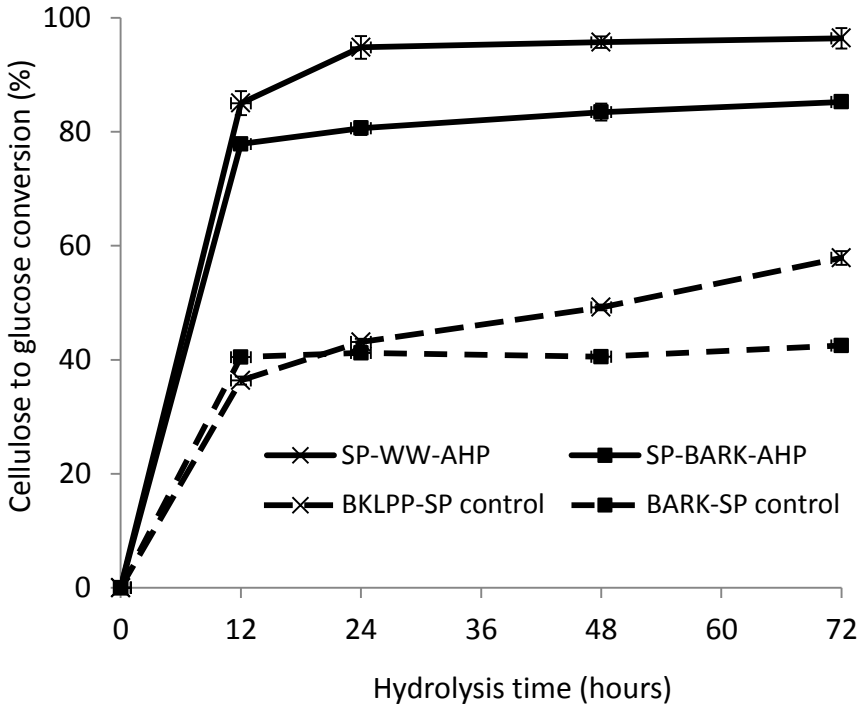

Figure 8: Influence of alkaline hydrogen peroxide post treatment on the enzymatic hydrolysis of steam pretreated white wood (LPP) and bark

(Steam pretreated at 200˚C, 5 minutes and 4% SO2). Enzyme loading: 50 mg/g cellulose (~25FPU Ctec 2/g cellulose). AHP corresponds to alkaline hydrogen peroxide post-treatment. The control samples refer to the hydrolysis yields of the steam pretreated samples. *The error bars represent the standard deviations from the average.

3.2.4 Alkaline pretreatment as a pretreatment strategy for extractive-rich forest residues

In earlier work (Chapter 3.1), it was found that extraction in an alkaline medium could remove most of the extractives present in the bark, resulting in a carbohydrate-enriched substrate. This indicated that, unlike an acid based pretreatment, pretreatment in an alkaline medium might remove most of the extractives from bark while achieving partial delignification, generating substrates more susceptible to subsequent enzymatic hydrolysis. As a first step, a range of alkaline pretreatment conditions were assessed using sodium hydroxide to remove lignin and extractives and to, hopefully, enhance the ease of enzymatic hydrolysis.

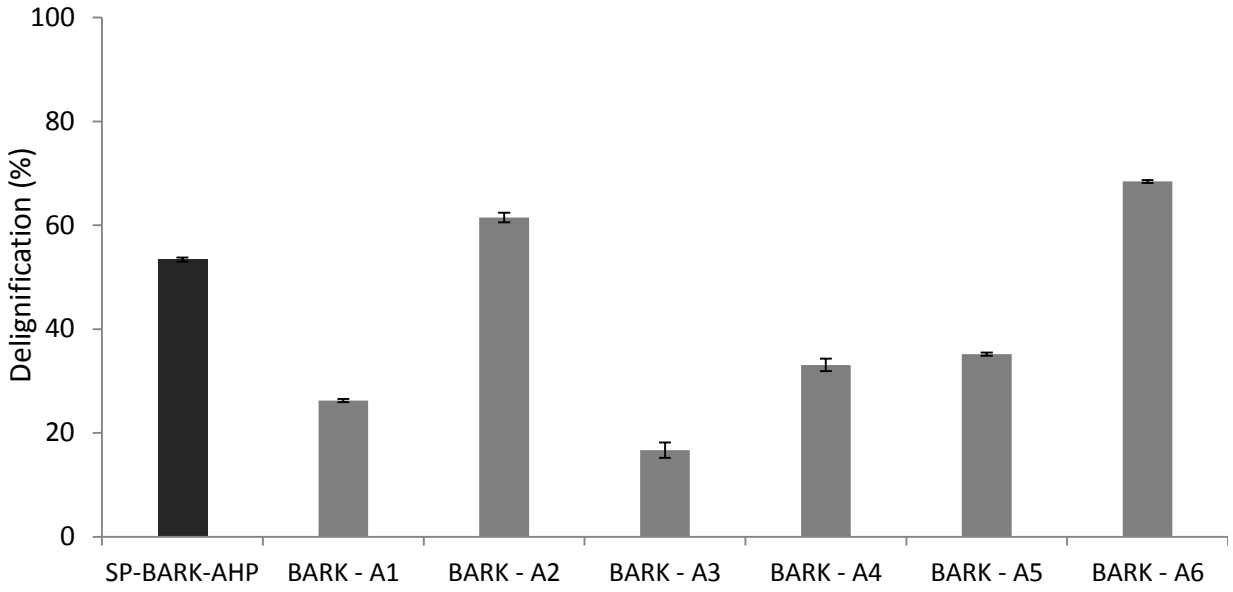

Figure 9: Comparison of different alkaline pretreatment conditions in delignifying bark.

*Alkali pretreatment condition 1(A1) corresponds to 160°C, 1h and 1% NaOH. Condition 2(A2) corresponds to 120°C, 3h and 2% NaOH. Condition 3 (A3) corresponds to 160°C, 3h and 1% NaOH, condition 4 (A4) corresponds to 120°C, 3h and 1% NaOH. Condition 5(A5) corresponds to 120°C, 1h and 1% NaOH. Condition 6 (A6) corresponds to 120°C, 1h and 2% NaOH.**The values in the bracket represent the deviations from the average.

Table 16: Influence of alkaline pretreatments on the chemical composition of beetle-killed lodgepole pine white wood and bark *

	Arabinan	Galactan	Glucan	Xylan	Mannan	Lignin	Ash	Sum
BKLPP A1	1.5 (0.1)**	1.5 (0.1)	50.7 (1.8)	6.5 (0.3)	5.3 (0.2)	30.1 (0.2)	0.1 (0.0)	96 (1.8)
BARK A1	4.4 (0.1)	3.5 (0.0)	29.9 (0.2)	2.6 (0.2)	2.6 (0.2)	44.8 (0.9)	2.1 (0.2)	94 (0.6)
BARK A2	5.1 (0.0)	2.3 (0.0)	43.0 (1.0)	6.9 (0.0)	1.9 (0.0)	32.8 (1.8)	2.1 (0.2)	94 (1.0)
BARK A3	2.1 (0.3)	1.9 (0.4)	34.3 (1.5)	4.9 (0.3)	1.9 (0.1)	51.6 (1.2)	2.1 (0.2)	99 (2.6)
BARK A4	5.4 (0.1)	3.1 (0.2)	29.1 (1.4)	6.9 (0.0)	1.9 (0.2)	44.3 (0.3)	2.1 (0.2)	93 (1.5)
BARK A5	5.7 (0.2)	3.1 (0.1)	30.7 (1.5)	6.8 (0.4)	1.9 (0.1)	43.5 (0.5)	2.1 (0.2)	94 (1.5)
BARK A6	4.3 (0.2)	2.2 (0.1)	44.5 (0.4)	10.6 (0.2)	1.7 (0.0)	29.2 (0.9)	2.1 (0.2)	95 (0.6)
SP BARK	2.6 (0.2)	1.4 (0.1)	22.4 (0.8)	1.4 (0.1)	0.9 (0.1)	58.5 (1.6)	1.9(0.4)	89.1(1.0)

*Alkali pretreatment condition 1(A1) corresponds to 160°C, 1h and 1% NaOH. Condition 2(A2) corresponds to 120°C, 3h and 2% NaOH. Condition 3 (A3) corresponds to 160°C, 3h and 1% NaOH, condition 4(A4) corresponds to 120°C, 3h and 1% NaOH. Condition 5(A5) corresponds to 120°C, 1h and 1% NaOH. Condition 6 (A6) corresponds to 120°C, 1h and 2% NaOH**.** SP BARK corresponds to the control sample, bark steam pretreated at 200˚C.

****The values in the bracket represent the deviations from the average.

The chemical compositional analysis of the alkaline pretreated bark (using NaOH) indicated that substantial levels of delignification had occurred when compared to steam pretreated substrates (Table 16). The lignin content of some of the alkaline pretreated bark samples reduced by almost 50% (Table 16) when compared to that of steam pretreated bark. Surprisingly, the extent of delignification achieved after some of the single stage alkali treatment conditions for bark was even higher than what was obtained with applying a "combination of steam pretreatment and AHP post-treatment" (Figure 9). In addition, the glucan content of the alkali treated samples was also higher than that of the steam pretreated substrates (Table 16). This was likely due to the near complete dissolution of extractives during the alkaline pretreatment of bark. This was in contrast to steam pretreatment where the extractives likely precipitated with the lignin and were consequently retained in the water insoluble cellulosic component (Hemmingson, 1987; Li et al., 2007).

Changing the conditions of the alkaline pretreatment had a significant impact on the chemical composition of the bark. Surprisingly, the milder alkaline pretreatment conditions (A2 and A6) resulted in greater levels of delignification while still providing good overall sugar recovery when compared to when high severity pretreatment conditions were used (Table 16, Figure 9). The lignin content of the higher severity alkaline pretreated substrates (A3, A1) appeared to be higher. The possible reasons behind the poor delignification efficiency of the high severity alkaline pretreatments could be due to condensation within the lignin moieties as well as condensation between the extractive components and lignin, particularly at advanced stages of alkaline cooking (Chakar & Ragauskas, 2004; Gustafson et al., 1983). It should be noted that lignin condensation has been reported during alkaline cooking conditions especially at harsher conditions (Biermann, 1996). Interestingly, the alkali loading seemed to have a more pronounced effect on the delignification of bark when compared to other variables such as temperature and reaction time (Table 16, Figure 9).

When the enzymatic hydrolysis yields of the alkali pretreated substrates were compared, the sugar yields generally correlated with the extent of delignification achieved (Figure 10). Milder alkali pretreatment conditions used with the bark resulted in a substantial increase in enzymatic hydrolysis when compared to steam pretreated bark (Figure 9). The bark sample pretreated at 120°C, 1h and 2% NaOH reached near complete hydrolysis (>94%), which was greater than the yields obtained with acid-catalyzed steam pretreatment or when applying a combination of "steam pretreatment and post treatment" (Figure 11). When the total glucose recovery was subsequently compared, it was found that a single stage alkali treatment with subsequent enzymatic hydrolysis of bark released almost similar amounts of sugar when compared to the total sugars obtained after steam pretreatment, post treatment and enzymatic hydrolysis. This seemed to indicate that a single stage alkali treatment might be a more appropriate pretreatment strategy for bark when compared to acidic pretreatments both in terms of recovering the various biomass components in a reactive form and enhancing the enzymatic accessibility of the cellulosic component. However, it should be noted that alkaline pretreatment was not an effective pretreatment method for beetle killed white wood. The alkaline pretreated BKLPP was poorly hydrolysed, even after 72 hours of hydrolysis, when compared to alkaline pretreated bark, leading to an overall lower sugar yield (Figure 10). This suggested that the type and conditions of pretreatment need to be tailored to suit the specific feedstock. From these preliminary results, alkaline pretreatment strategies are more effective for high-extractive containing forest residues while acid catalysed steam pretreatment is effective on lower-extractive containing substrates which more closely resembles white wood.

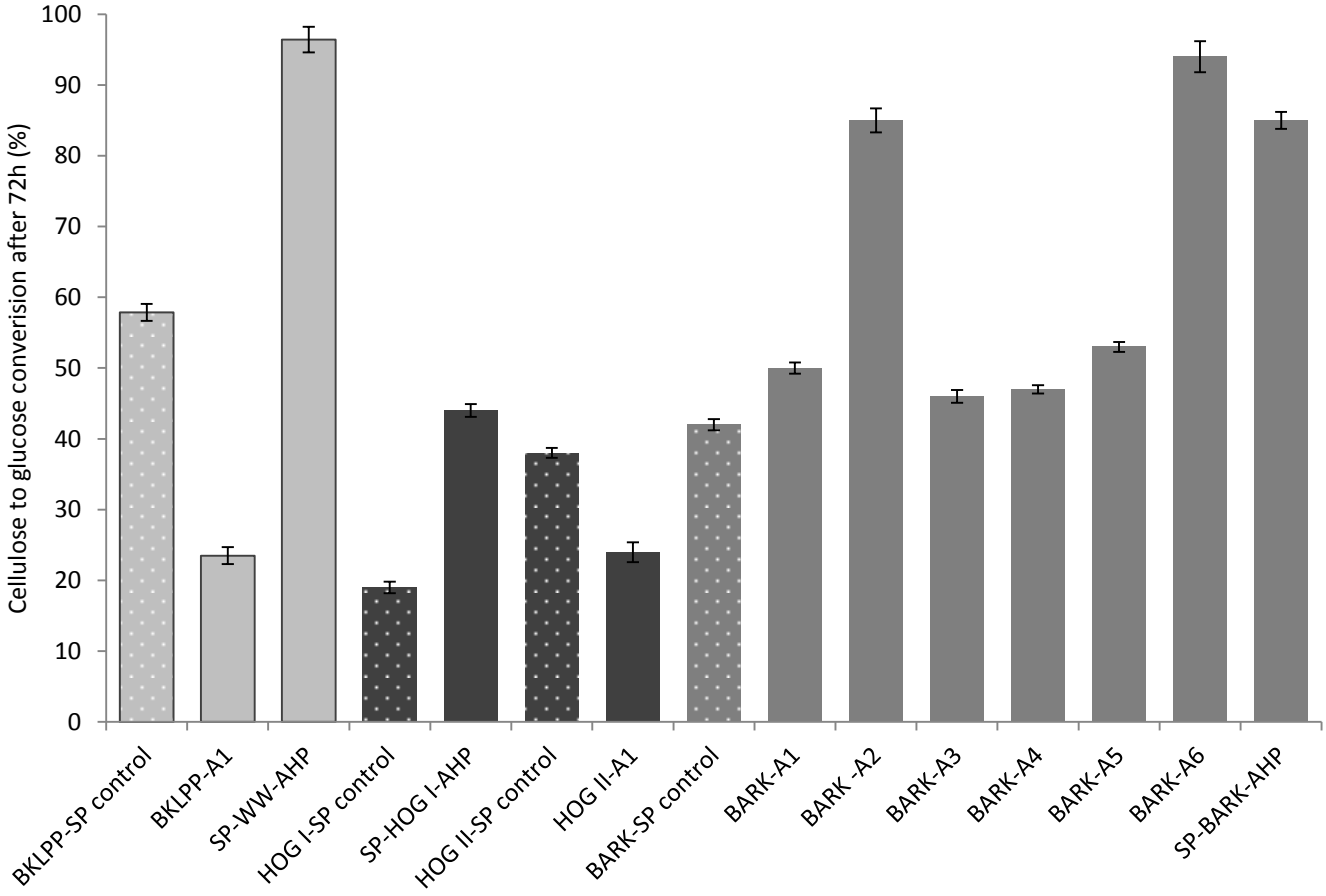

Figure 10: Influence of alkaline pretreatment on the 72 hour enzymatic hydrolysis yields of bark and white wood.

Enzyme loading: 50 mg/g cellulose (~25FPU Ctec 2/g cellulose).*Alkali pretreatment condition 1(A1) corresponds to 160°C, 1h and 1% NaOH. Condition 2(A2) corresponds to 120°C, 3h and 2% NaOH. Condition 3 (A3) corresponds to 160°C, 3h and 1% NaOH, condition 4(A4) corresponds to 120°C, 3h and 1% NaOH. Condition 5(A5) corresponds to 120°C, 1h and 1% NaOH. Condition 6 (A6) corresponds to 120°C, 1h and 2% NaOH. AHP is alkaline peroxide post-treatment carried out on the steam pretreated substrate and steam pretreatment was carried out at 200˚C for 5min with 4% SO_2 catalyst loading.**The values in the bracket represent the deviations from the average.

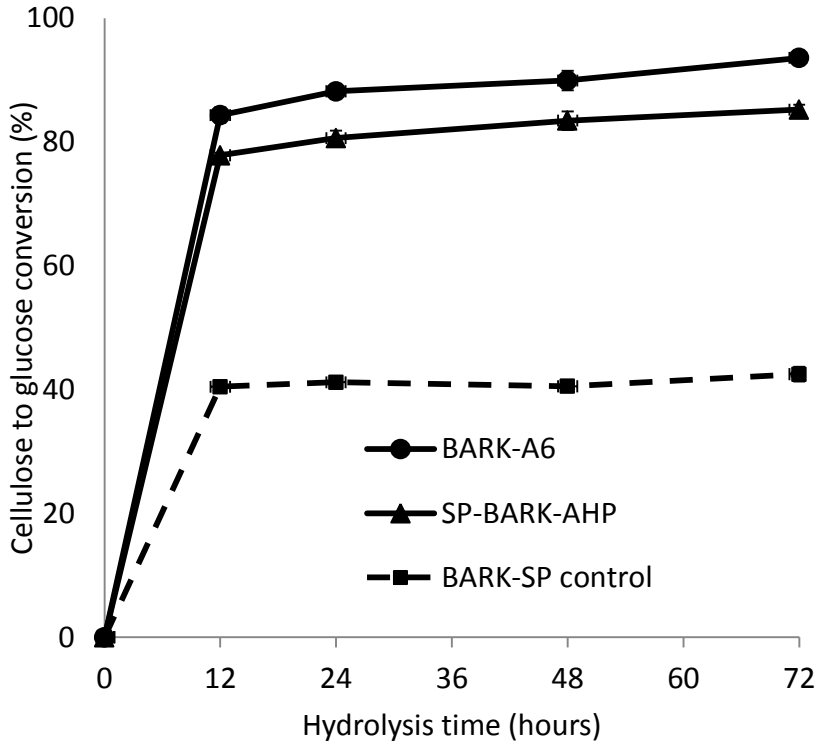

Figure 11: Enzymatic hydrolysis profiles of alkali pretreated bark, steam pretreated bark, steam pretreated and subsequently post treated bark.

Enzyme loading: 50 mg/g cellulose (~25FPU Ctec 2/g cellulose).*Alkali pretreatment condition 1(A1) corresponds to 160°C, 1h and 1% NaOH. Condition 6 (A6) corresponds to 120°C, 1h and 2% NaOH. AHP is alkaline peroxide post-treatment done on the steam pretreated substrate, and steam pretreatment was carried out at 200˚C for 5min with 4% SO_2 catalyst loading.**The values in the bracket represent the deviations from the average.

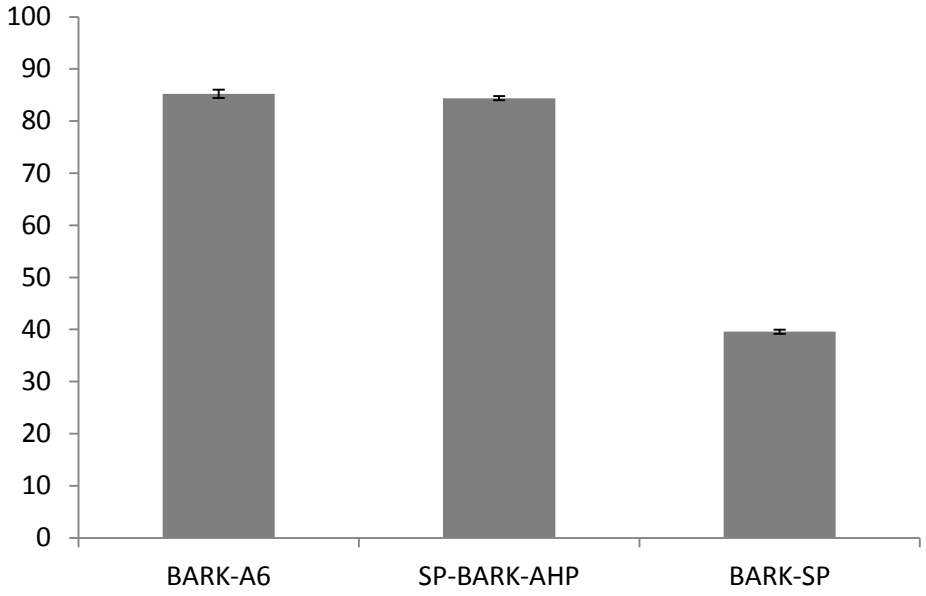

Figure 12: Influence of different pretreatment and fractionation strategies on the overall glucose yield after the pretreatment and subsequent enzymatic hydrolysis.

(A6 correspond to alkali pretreatment (to 120°C, 1h and 2% NaOH), SP-AHP refer to a combination of both steam pretreatment and post treatment, and SP refer to steam pretreatment alone (200˚C for 5min with 4% SO_2 catalyst loading). Enzyme loading: 50 mg/g cellulose (~25FPU Ctec 2/g cellulose). **The error bars represent deviations from the mean.

3.2.5 Conclusions

Although acid-catalyzed steam pretreatment is effective on softwood pulp chips, acid pretreatments seem to have limitations in processing high extractive containing forest residues such as bark. When using acid catalysed steam pretreatment to process bark, condensation reactions occur between the extractives and lignin, which not only limits efficient fractionation, it also makes the cellulosic component difficult to hydrolyse with cellulase enzymes. In addition, these reactions can further limit the recovery of lignin and extractives as value added co-products. An alkaline pretreatment approach can remove some of the lignin and the majority of extractives in a reactive form from the bark, while generating a carbohydrate-enriched water insoluble component, which can be readily hydrolysed by cellulases. It is likely that extractive-rich forest residues will require a different pretreatment strategy when compared to white wood. However, “disturbance wood” such as beetle killed lodgepole pine, could be processed as effectively as white wood chips using SO_2 catalysed steam pretreatment.

Conclusions

Due to the heterogeneity and complex chemistry of forest residues we expected that it would be challenging to both achieve an accurate material balance and to convert these materials using "traditional" methods and processes that are typically employed for more homogenous and cleaner feedstocks such as white wood/ pulp chips. One of the prerequisites for using any feedstock in a "biorefinery concept" is to obtain an accurate material balance. Thus, we first assessed the accuracy of these methods on a range of softwood derived forest residues and also evaluated how a typical pretreatment process such as steam pretreatment might influence the robustness of these methods to establish an accurate, reproducible material balance from six, representative, forest residues.

These softwood forest residues were sourced from Washington State and British Columbia (hog fuels, logging residue, interface fire slash, beetle-killed white wood, and bark). Despite the challenges resulting from the heterogeneity of the initial biomass substrates, a reasonable summative mass closure was obtained for all the substrates. It was apparent that the extractive content of the original biomass was most influential in limiting the accuracy of the material balance determination. This was particularly evident when trying to quantify the lignin content, due to the incomplete removal of extractives even after a two stage water-ethanol extraction. It was likely that residual extractives precipitated with the acid insoluble lignin during analysis, contributing to an overestimation of the lignin content. Despite the minor dissolution of the hemicellulosic components, extraction with mild alkali removed most of the extractives from the bark, significantly improving the raw material mass closure. Surprisingly, regardless of the extent of extractive removal, the currently established analytical methods provided relatively accurate and reproducible values for most of the carbohydrate components.

Obtaining a good material balance and summative mass closure was even more difficult after a steam pretreatment as the selective removal of extractives and their quantification was far more challenging after the pretreatment process. The extent of extractive removal and their precipitation with lignin was heavily dependent on the pretreatment conditions that were employed. It was apparent that that ability to establish a good material balance varied

depending on the pretreatment conditions that were used. The quantification of some of the generally minor biomass components, such as acetic acid, uronic acids, etc., may also be needed when quantifying forest residues, to enable us to better close the material balance. Although the current NREL biomass analytical procedures provided a good set of protocols to establish a fairly accurate material balance, these methods are labour intensive, time consuming and prone to inaccuracies depending on the feedstock chemistry and pretreatment processes employed. Therefore, method revision and optimisation was required, particularly for the effective removal of extractives, to ensure that representative and reproducible values were obtained for all of the biomass components as well as a more accurate mass closure.

As was anticipated, it was much more difficult to achieve an accurate material balance for the pretreated forest residues as well as achieve effective enzymatic hydrolysis of the cellulosic component. The typical pretreatment strategies used for processing “more homogenous” cellulosic biomass (such as white wood), were not very effective in processing some of the ``high-extractive`` containing forest residues such as bark. In acid-based pretreatments, the extractive components were randomly and inefficiently fractionated to water soluble and insoluble components. Some of the extractive components precipitated with the lignin, making their recovery and further valorisation difficult. In addition, these residual extractives together with the lignin remaining in the water insoluble cellulosic component likely restricted the accessibility of the enzymes to the cellulose. The removal of extractives and lignin from the steam pretreated bark could not be achieved even after a post treatment such as alkaline hydrogen peroxide treatment. Thus, for high extractive containing forest residues such as bark, a pretreatment strategy that is efficient in solubilising and fractionating extractives from the carbohydrate components, was found to more appropriate to produce a carbohydrate-enriched water insoluble component that could be readily enzymatically hydrolysed. A single stage alkali pretreatment was found to be more effective than a two-stage steam pretreatment-post treatment combination for processing “high extractive” containing forest residues such as bark. Higher alkali concentrations (>1%) and milder temperatures (~120°C) seems to favour the removal of extractives and some of the lignin while generating a cellulosic component that could be readily hydrolysed.

It was apparent that several key aspects of the process need to be refined and optimised prior to using “complex and heterogeneous” feedstock’s such as forest residues in bioconversion processes. First, rapid and reliable compositional analysis methods that provide an accurate material balance are needed. In order to generate the multiple products that will likely be required to make a biorefinery process economically attractive the unit operations will need to be optimised to ensure maximum recovery each of the cellulose, hemicellulose, lignin and extractive components in high yield and concentration. Even with good recovery the subsequent valorisation of each of the components in a biorefinery process will face considerable challenges with regard to their separation and subsequent functionalization.

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Appendix

Steam pretreatment yield of the homogenized and non-homogenized substrates

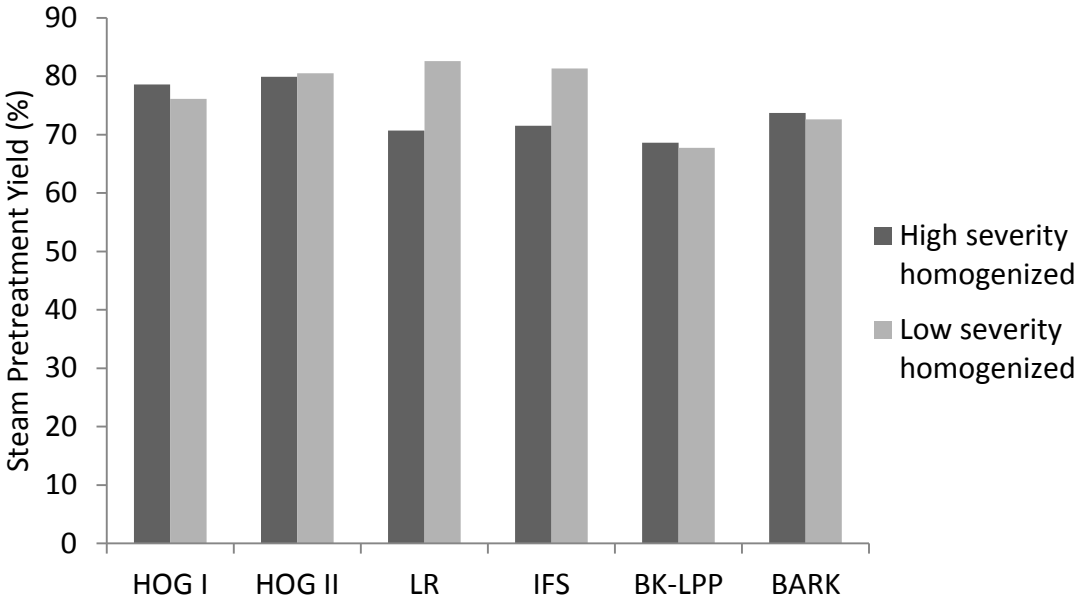

Figure 13: Forest residue overall recovery after steam pretreatment of homogenized forest residues at two severities

Low severity steam pretreatment conditions were 5minutes, 180˚C, and 4% SO_2 acid catalyst loading. High severity steam pretreatment conditions were 5minutes, 200˚C, and 4% SO_2 acid catalyst loading.