PRODUCTION OF PREBIOTIC RICH EXTRACTS FROM LIGNOCELLULOSIC BIOMASS USING SUBCRITICAL WATER WITHIN THE CONTEXT OF BIOREFINING

by

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ABSTRACT

Functional food ingredients such as prebiotics are emerging as effective tools for managing the risks associated with gastrointestinal diseases and gut related dysfunctions. This work explores the production of prebiotic rich extracts from two sources of lignocellulosic biomass – energy crop Miscanthus χ giganteus, and oat (Avena sativa) husks, an agricultural by-product. Whilst most of the research to date has been focusing on the utility of cellulose, the hemicellulose and lignin fractions have been underutilised. This work expands the value of the hemicellulose fraction by extracting the prebiotic xylo-oligosaccharides (XOS) and other hemicellulose derived products using environmentally benign sub-critical water in a 0.5 L stirred batch reactor at temperatures between 120-220°C with residence times 0-77 min, and 0.5-13% (w/v) biomass loading. The extracts were analysed with HPAEC-PAD, HPLC and colorimetric assays. Almost complete hemicellulose solubilisation was achieved, and highest yields of prebiotic (DP 2-5) XOS were observed at 170°C and 35-77 min corresponding to 44-56% with little amounts of carbohydrate degradation products and low total phenolic contents. Although higher yields were achieved with oat husks, the XOS extractability was comparable between the biomass sources. Higher extraction severities resulted in further XOS depolymerisation into monosaccharides and eventual formation of furfural and 5-HMF.

Keywords:

xylooligosaccharides, xylose, prebiotics, hemicellulose, biorefining, sub-critical water

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CHAPTER 1. INTRODUCTION

1. Introduction

Prebiotics have been shown to provide health benefits to humans as well as ruminants, swine and poultry, therefore having applications within nutraceutical, and animal feed sectors. The research presented in this thesis explores the production of prebiotic rich extracts from different biomass sources using an environmentally benign technology. There are two main arguments explaining the need of this work, which are expanded on in the following subsections of this chapter. First, is the value and supply of prebiotics and second, with the shift towards bioeconomy, the extraction processes described in this work can be integrated within the existing biorefining frameworks, thus expanding the research on the value chain arising from biomass utilisation.

1.1. Prebiotics and gut health

Undoubtedly diet is an important factor in disease risk in all population groups. Moreover, driven by the advances in rRNA sequencing technology, research about the gut and its associated microbiome has seen an exponential growth in the past ten years (see Figure 1-1) (Marchesi, 2011). As a result, the research on the implications of diet and the associated health risks thereof has seen a renaissance and provided new insights into the relationship between diet, gut microbiome and the health of the host. There is now emerging evidence that imbalances in gut microbiome are linked not only with various gut related diseases, including colon cancer (Allsopp and Rowland, 2009), infectious diarrhoea (Hibberd, 2009), chronic gastrointestinal diseases (Guarner, 2009), but also type-2 diabetes, obesity, low grade inflammation (Cani and Delzenne, 2011; Kovatcheva-Datchary and Arora, 2013), non-alcoholic fatty liver disease, even anxiety, depression

(Foster and McVey Neufeld, 2013), and several allergic disorders (Candela et al., 2010; Salminen and Isolauri, 2009).

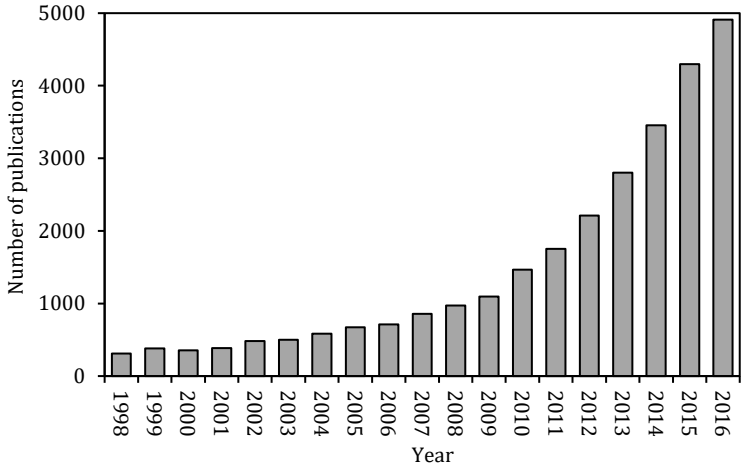

Figure 1-1: Number of publications related to gut microbiome obtained from Thompson Reuters Web of Science using the following search keywords and Boolean operators – "intestinal microbiota" OR "gut microbiota" OR "intestinal flora" OR "gut flora" OR "intestinal microflora" OR "gut microflora" OR "intestinal microbiome" OR "gut microbiome"The gut microbiome is a complex community populated with bacteria and archaea (Tremaroli and Backhed, 2012). According to the first human gut microbial gene catalogue, 99% of the genes are bacterial, corresponding to more than 1000 bacterial species, from which at least 160 are commonly shared between individuals (Qin et al., 2010). Some of the species are pathogenic, while others are beneficial to the host (Buddington, 2009). As a result, successful management of the microbiome is vital in order to achieve the positive health benefits associated with healthy gut microbiome.

Several strategies have been used to modulate the gut microbiome, each with their advantages and disadvantages. For instance, the use of antibiotics to remove the pathogenic species has been widely adapted but comes with significant drawbacks,

including the development of antibiotic resistance and destabilisation of the beneficial bacterial communities (Buddington, 2009). Fortunately, other methods, namely pro- and prebiotics that are benign to the beneficial bacterial communities have been at the forefront of the recent dietary research. Probiotics – defined as "live microorganisms which when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014), have been used as dietary supplements to populate the microbiome with the beneficial bacteria. Common bacterial species that have been recognized as probiotic are *Bifidobacterium (adolescentis, animalis, bifidum, breve and longum)* and *Lactobacillus (acidophilus, casei, fermentum, gasseri, johnsonii, paracasei, plantarum, rhamnosus and salivarius)* (Health Canada, 2009).

Arguably, a more holistic strategy to improve the health of the microbiome is using prebiotics that are defined as "selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host's wellbeing and health" (Gibson et al., 2004a). The most significant advantage of prebiotics over probiotics is that the prebiotics feed the beneficial bacteria, including *Bifidobacterium* and *Lactobacillus* that already are in the gut, which allows them to grow, and by doing so increase the availability of beneficial nutrients, and reduce the numbers of the pathogenic bacteria in the gut (Steed and Macfarlane, 2009). Most common prebiotics are fructo-oligosaccharides, galacto-oligosaccharides, xylo-oligosaccharides, soya-oligosaccharides, isomalto-oligosaccharides, inulins, pyrodextrins and lactulose (Steed and Macfarlane, 2009).

This research focuses on the production of xylooligosaccharides (XOS) from the hemicellulose fraction of different sources of lignocellulosic biomass. Although the most

studied prebiotic compounds are inulins, fructo-oligosaccharides and galacto-oligosaccharides (Macfarlane et al., 2008), XOS are an attractive alternative due to the better availability of potential sources, which range from underutilised agricultural waste, energy crops, hardwoods, and softwoods. Moreover, with the advances in second generation bioethanol research, where the biofuel is produced from the cellulose fraction of a variety of lignocellulose biomass sources, hemicellulose fraction is largely left underutilised. XOS as well as other hemicellulose derived product streams could be integrated within the existing processes, therefore helping to advance the concept of biorefinery towards complete biomass valorisation.

1.2. Bioeconomy and biorefining

Increasing global population, resource depletion and climate change have prompted a shift in approach to the production, consumption, and recycling of biological resources. This is backed by the European commission's "Europe 2020 Strategy", launched in 2012, where bioeconomy was called a key element for sustainable and green growth (European Commission, 2012). Moving from fossil based economy to bioeconomy has the potential to revitalise the declining agricultural sector, stimulate the economy, reduce the dependency on fossil fuels, and improve the overall prospects for a long term sustainable growth (see Figure 1-2). One of the core elements in this approach is the development and application of biorefining technologies, which have seen a significant growth in research interest since 2007 (see Figure 1-3).

Biomass has a complex composition, which, in theory can be fractionated into wide range of valuable products similar to petroleum refining (Fitzpatrick et al., 2010), thus the term biorefinery, analogous to petroleum refinery (Yang, 2007). However, biorefining requires

larger range of processing technologies (Carvalheiro et al., 2008) due to the complex nature of biomass. Ideally, biorefinery is a zero waste facility, which produces low volume, high value products such as nutraceuticals and platform chemicals, and high volume, low value biofuels, as well as power, and therefore maximises the value derived from the biomass (Liu et al., 2010). There are four common biorefinery platforms, primarily segregated based on the source of the biomass: (1) carbon-rich chains (oil), producing biodiesel, glycerine and fatty acids from rapeseed, canola, palm oils and animal fats; (2) syngas, producing gaseous or liquid fuels and added value chemicals from lignocellulosic biomass and rubber; (3) biogas, which produces methane, carbon dioxide from liquid effluents and manure; and (4) biochemical, producing platform chemicals, nutraceuticals, bioethanol and electricity from lignocellulosic and starch biomasses (Carvalheiro et al., 2008). The work presented here fits within the latter category, more specifically – lignocellulosic biorefining.

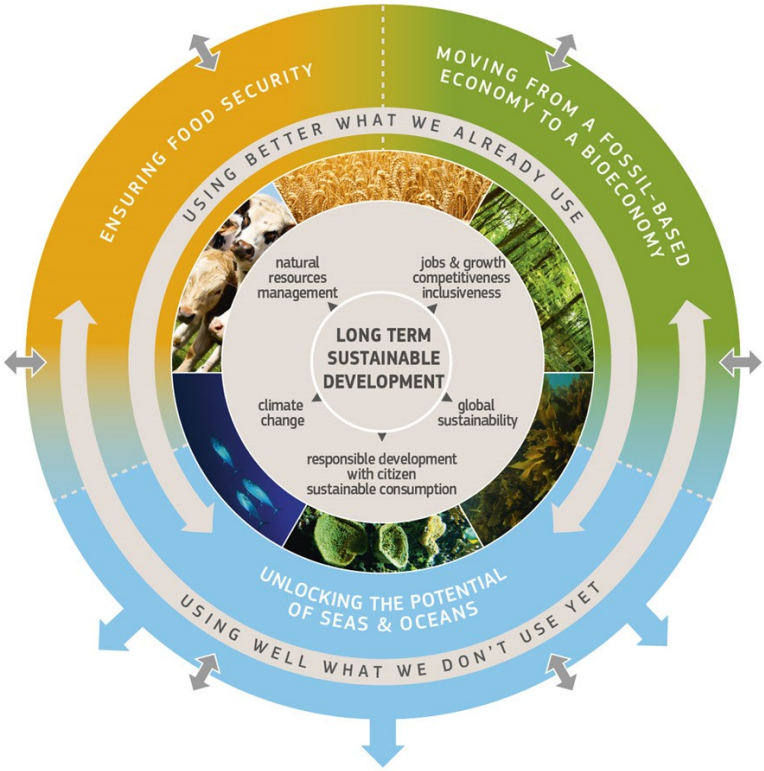

Figure 1-2: Summary of the Bioeconomy Strategy as proposed by the European Commision (European Commission, 2016)

From the aforementioned platforms, lignocellulosic biorefining is likely to see the greatest success in terms of large scale industrial application due to cheap and abundant raw materials (e.g. straw, wood, paper waste, grass, husks etc.), and good market position of the potential conversion products in both petrochemical and future biobased product markets (Kamm et al., 2007). Localised, smaller scale plants focusing on agricultural residues might also be economically feasible. However, the majority of the research to date has been focusing on the production of lignocellulosic (2nd generation) bioethanol, which is produced from the cellulose fraction of the biomass (Rubin, 2008), yet the potential for lignocellulose based product streams other than bioethanol is to be fully explored, particularly regarding the use of lignin and hemicellulose.

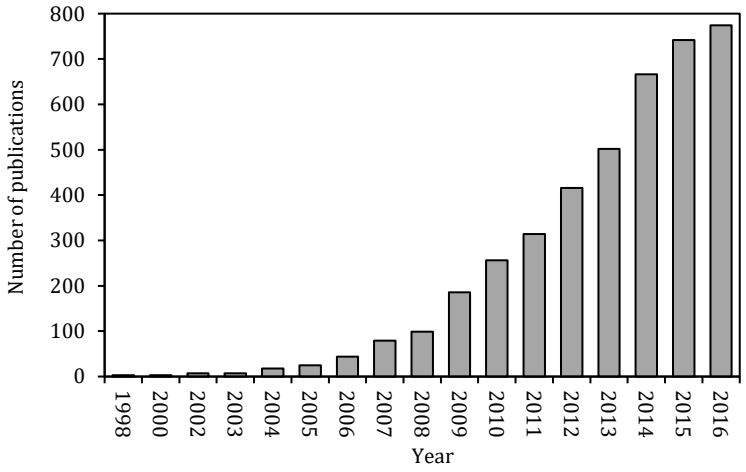

Figure 1-3: Number of publications related to biorefining obtained from Thompson Reuters Web of Science using the following search keywords and Boolean operator – "biorefining" OR "biorefinery"

One of the core principles that needs to be applied to achieve a long term economic sustainability of lignocellulosic biorefineries is the flexibility in raw material sourcing and product stream selection which can be adapted to market demand. As the lignocellulosic biomass consists of three primary polymeric fractions – cellulose, hemicellulose and lignin, each with different potential applications (see Figure 1-4), technologies that can

vary the degree of polymerisation of the final product are desirable, i.e. cellulose vs glucose vs HMF, and XOS vs xylose vs furfural etc.

Sub-critical water (subCW) based applications, as investigated in this work, hold great promise in this regard, and would allow sequential product extraction across a thermal gradient with or without modifiers (Ares-Peón et al., 2013; Lee et al., 2010; Pronyk et al., 2011; Yu et al., 2010), resulting in sequential fractions (in order) of extractives, hemicellulose, lignin and cellulose. An example of the proposed extraction sequence using subCW would be as follows. First, extractives (including non-structural components) such as lipids, waxes, and proteins would be removed at lowest extraction severity (<130 °C) (Gullón et al., 2012), followed by hydrolysis and solubilisation of hemicellulose derived products at low to medium extraction severity (<180 °C) (Ando et al., 2000). To remove lignin, which in contrast to hemicellulose, is a hydrophobic polymer (Achyuthan et al., 2010), organic solvents, such as ethanol would need to be added to water in order to mediate the lignin solubilisation (Roque et al., 2012). The remaining cellulose fraction could then be used for cellulose based applications, or depending on demand, hydrolysed to glucose with subCW at high severity (up to 240 °C) (Yu et al., 2007), and then enzymatically converted to bioethanol.

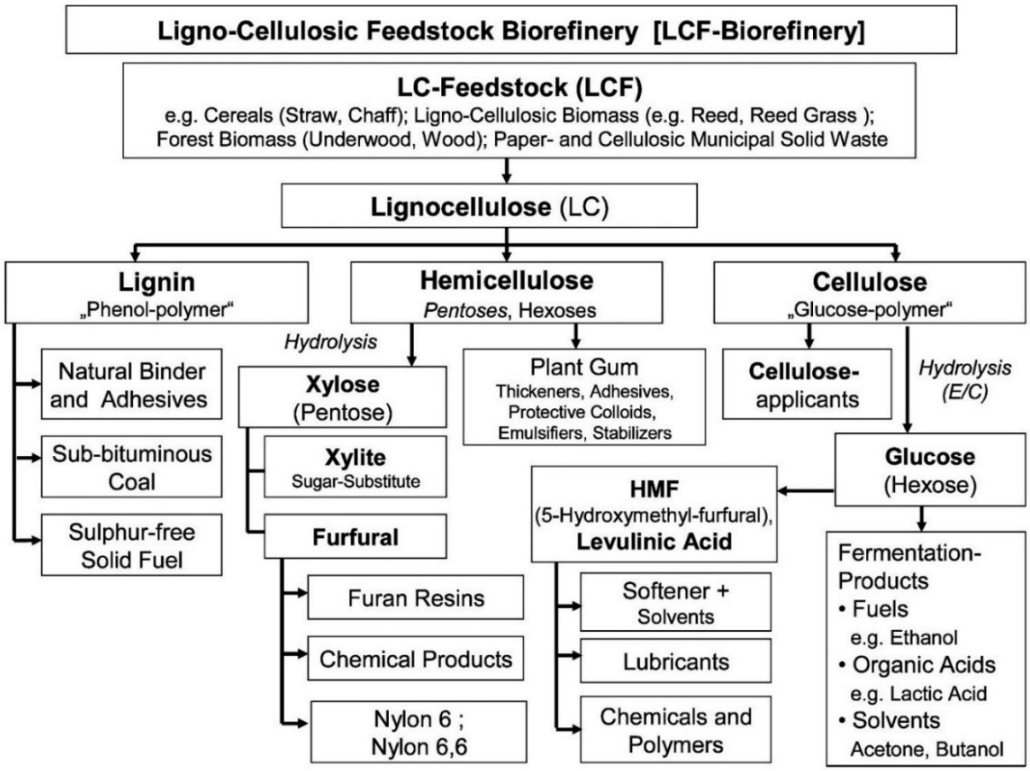

Figure 1-4: An example of potential product streams from lignocellulosic biorefineries (Kamm and Kamm, 2004)

The research presented in this thesis attempts to expand on the lignocellulosic concept as described above by exclusively focusing on the hemicellulose and hemicellulose derived prebiotic extraction using subCW from two different types of biomass – the energy crop *Miscanthus χ giganteus*, which is also known as elephant grass, and an agricultural residue, husks of *Avena sativa* (common oat).

1.3. Aims and objectives

The aim of this project is to evaluate the application of subcritical water (subCW) as environmentally benign solvent to support the following processes within the context of biorefining: (1) removal of hemicellulose from lignocellulosic biomass; and (2) production of prebiotic oligosaccharide rich extract from the recovered hemicellulose

fraction. This study can thus be broken down into the following objectives, with links identified to the later relevant thesis chapters:

1) Study the impact of process parameters on the overall efficacy of subCW as a solvent to support the extraction of high molecular weight (MW) hemicellulose polymers from *Miscanthus χ giganteus* and *Avena sativa* husks. This objective has two components:

- Assess the influence of pre-treatment and process parameters and biomass types in terms of polymer molecular weight, types and amount of substitutions on the polymer backbone (Chapters 4 and 5);
- Evaluate process routes, which support efficient recovery of hemicellulose from the aqueous phase (Chapters 4 and 5).

2) Study the feasibility and utility of subCW mediated hydrolysis of *Miscanthus χ Giganteus* and *Avena sativa* husk derived hemicellulose polymers to create xylooligosaccharides (XOS); again, this objective has two parts:

- Conduct a comparative analysis of SCW mediated hydrolysates in terms of XOS configuration and degrees of polymerisation (DP) (Chapters 4-7);
- Establish optimal process parameters for XOS production from different biomass sources (Chapters 6 and 7).

1.4. Thesis structure

To address the above stated aims and objectives, the thesis is structured into seven further chapters:

Chapter 2 – "Literature Review" provides an insight into the current understanding of hemicellulose structural features, sources, classification and chemical characteristics.

This chapter also explores the potential utility of hemicellulose derived products, and state of the art of hemicellulose extraction, purification and characterisation.

Chapter 3 – "Experimental Methodology" describes the research methodology employed in this work, including extraction procedures and conditions, characterisation protocols and apparatus, as well as statistics and calculations for data analysis.

Chapter 4 – "Scoping Investigation of Sub-Critical Water Mediated Hemicellulose Extraction from *Miscanthus χ giganteus* and *Avena sativa* Husks" investigates the effects of temperature andresidence time on the extractability of hemicellulose and xylooligosaccharides from two biomass sources.

Chapter 5 – "Compositional Differences of Sub-Critical Water Extracts from the Husks of Five *Avena sativa* varieties" investigates the extractability of hemicellulose and xylooligosaccharides between husks of different oat varieties.

Chapter 6 – "Optimal Parameters for Hemicellulose and Xylooligosaccharide Extraction Using Sub-Critical Water Mediated Hydrolysis from *Miscanthus χ giganteus* and *Avena sativa* Var. Balado and Var. Conway Husks: Design of Experiments" uses Design of Experiments to assess the optimal conditions for hemicellulose and xylooligosaccharide extraction from the two biomass types.

Chapter 7 – "Sequential Sub-Critical Water Mediated Hydrolysis of Hemicellulose Rich Extract Obtained from *Avena sativa* Var. Balado Husks and its Comparison with Arabinoxylan Standard: Design of Experiments" uses Design of Experiments to investigate whether the yields of xylooligosaccharides can be improved by sequentially exposing the extracts to sub-critical conditions.

Chapter 8 – "Conclusions and Future Work" summarizes the work of this thesis and provides short, medium and long term perspectives for future work relevant to this work.

1.5. Publications

Kalnins, R., Cox, P.W., Bowra, S. and Santos, R.C.D. (2014). *Evaluation of the Critical Fluids to Support the Production of Xylooligosaccharides from Miscanthus χ giganteus: a Step towards Integrated Biorefining.* Poster Presentation at: **14th European Meeting on Supercritical Fluids.** 18-21 May, 2014, Marseille, France.

Articles in preparation:

Kalnins, R., Bowra, S., and Simmons, M.J.H. (2017). *Compositional Differences of Virgin and Pre-treated Sub-critical Water Extracts from Miscanthus χ giganteus and the Husks of Five Avena sativa varieties.* Manuscript in preparation.

Kalnins, R., and Simmons, M.J.H. (2017). *Optimal Parameters for Xylooligosaccharide Extraction within the Context of Biorefining Using Sub-critical Water Mediated Hydrolysis from Miscanthus χ giganteus and Avena sativa Husks.* Manuscript in preparation.

CHAPTER 2. LITERATURE REVIEW

2.1. Plant cell wall

Plant cell walls are the largest carbon pool of the plant biomass (Schädel et al., 2010), and are primarily composed of natural polymers – cellulose, hemicellulose, lignin, and pectins (Cosgrove, 2005). Plant cell walls provide considerable strength and flexibility to the plants and are essential to withstand the effects of gravity and large tensile as well as compressive forces from the surrounding habitat (Burton et al., 2010). In order to adapt to these requirements, the cell wall has developed into a highly complex gel like matrix, mainly consisting of cellulose, hemicellulose, lignin and pectins (see Figure 2-1) (Achyuthan et al., 2010; Cosgrove, 2005; Ralph et al., 2004). This matrix provides the cell with flexibility and mechanical support to the cell, and allows water and other small molecules to diffuse through the wall (Burton et al., 2010).

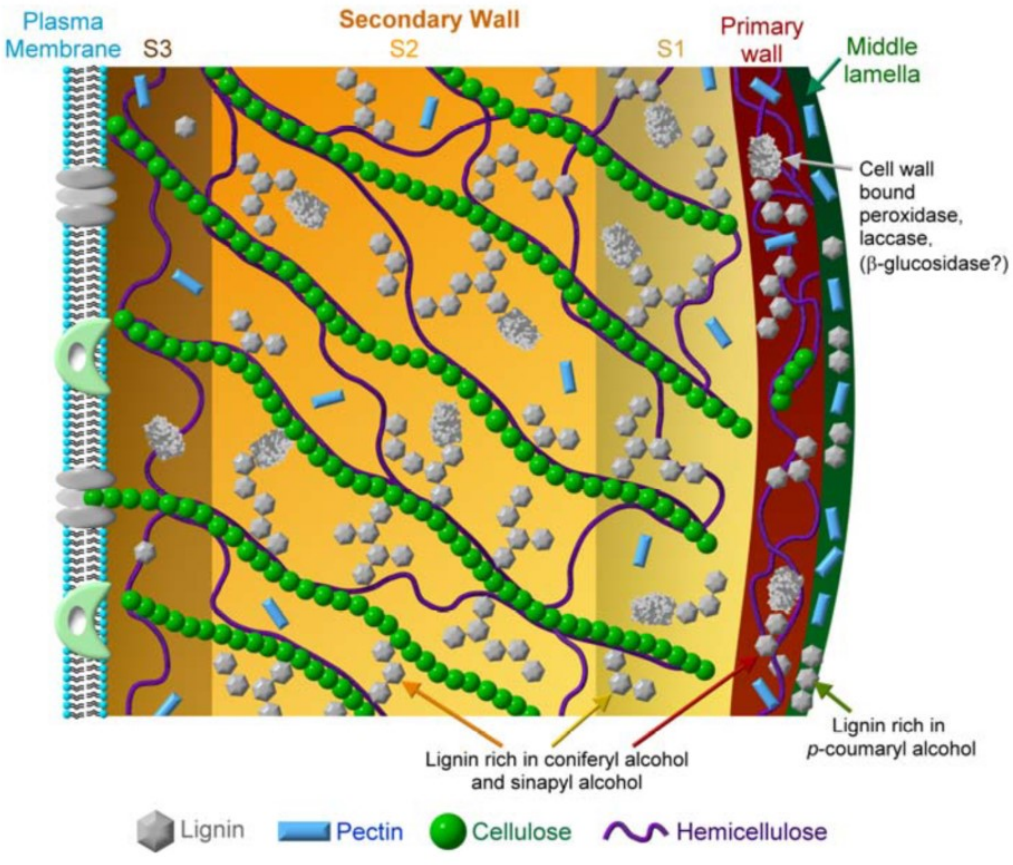

Figure 2-1: Illustration of plant cell wall (Achyuthan et al., 2010)

As can be seen from Figure 2-1, the cell wall consists of primary and secondary cell walls, which are structurally different from each other. The primary cell wall is formed while the plant cell is expanding and therefore requires to be flexible, whereas the secondary cell wall develops after the cell has stopped expanding and provides more of the structural reinforcement to the cell (Cosgrove, 2005). In the secondary cell walls, the cellulose microfibrils are laminated to each other through binding with lignin and hemicellulose (Achyuthan et al., 2010; Jeffries, 1994; Ralph et al., 2004). Lignin and cellulose levels are generally higher in secondary walls, as both are associated with mechanical strength of the wall, whereas pectins are more abundant in the primary cell walls due to their involvement in intermolecular signalling and cell extension (Burton et al., 2010; Carpita and Gibeaut, 1993). Lignin, in addition to providing the cell wall with stiffness and compressive strength, also provides resistance to insects and pathogens (Rubin, 2008). Hemicelluloses from the secondary cell walls have lower degrees of branching than in the primary cell walls, and therefore have lower solubility in aqueous media (Burton et al., 2010). The role of hemicelluloses in the plant cell walls is not clear. There are several theories suggesting that hemicellulose provides added flexibility to the cell walls, acts as a protective coating between and around cellulose and lignin, and plays an antioxidant role in the cell wall (Pristov et al., 2011; Ramos, 2003; Saha, 2003; Scheller and Ulvskov, 2010).

Cellulose is the most abundant organic polymer in the world (Xu, 2010) and is composed of unbranched (1→4)-linked β-glucan, which often form crystalline microfibrils via hydrogen bonding (see Figure 2-2), rendering the resulting structure mechanically strong, resistant to enzymatic attacks and hard to solubilize in water (Cosgrove, 2005;

Sasaki et al., 2003). Its degree of polymerisation varies from 6000-16000 (Liu and Sun, 2010).

Pectins are complex heterogeneous polysaccharides, consisting of covalently linked domains of galacturonic acid and carbohydrates such as rhamnose, xylose and arabinose (Cosgrove, 2005). Some proportion of the galacturonic acid residues can be esterified as methyl ester, and therefore possess gelling properties; furthermore, the acid residues can also be substituted with salts, most commonly calcium (Xu, 2010). Pectins are abundant in soft plant tissues such as the citrus rinds, apples and sugar beet pulp but are scarce in woody tissues and grasses (Burton and Fincher, 2014; Fincher, 2009; Xu, 2010).

Lignin is the third most abundant organic polymer in the world after cellulose and hemicellulose, and is composed of three main phenolic monomers – coniferyl, sinapyl, and *p*-coumaryl alcohols, which render the structure amorphous and highly branched (Xu, 2010). Its structure also varies between sources, and regularly repeating multiunit structures have not been found (Lu and Ralph, 2010). Lignin is hydrophobic, making it hard to solubilise in water (Achyuthan et al., 2010).

Despite being the second most abundant organic polymer in the world (Xu, 2010), hemicellulose has been the least utilised component of the lignocellulosic biomass (Gírio et al., 2010; Thompson, 2000). Like cellulose, hemicellulose also consists of (1→4)-linked backbone, but unlike cellulose, hemicellulose is mostly heterogeneous as the backbone can be decorated with side branches to varying extent depending on the source (Scheller and Ulvskov, 2010). The branching of hemicellulose prevents the formation of crystalline structures, which makes it easier to hydrolysate than cellulose (Ando et al., 2000). Depending on source, hemicellulose primarily consists of pentoses (arabinose, xylose)

and hexoses (glucose, mannose, galactose, rhamnose) but the branches can also consist of uronic and hydroxycinnamic acid esters and acetyl groups (Ebringerová, 2005; Scheller and Ulvskov, 2010). Hemicelluloses can be divided into four types: xylans, xyloglucans, mannans and mixed linkage β-glucans (Ebringerová, 2005), and have a degree of polymerisation ranging from 80-500 (Achyuthan et al., 2010; Xu, 2010).

Hemicelluloses form cross-links with pectins, cellulose and lignin through a variety of linkages. Figure 2-2 shows five mechanisms of hemicellulose interconnections with cellulose and pectins, where each letter represents a different type of linkages (Cosgrove, 2005). Hemicelluloses can be non-covalently bound to cellulose microfibrils, tethering them together (a in Figure 2-2) (Fry, 1989; Hayashi, 1989). Xyloglucans can be trapped during the formation of the ordered cellulose macrofibril (Baba et al., 1994; Hayashi et al., 1994) with the un-trapped remainder free to bind to other cellulose surfaces or matrix polymers, anchoring the microfibril to its neighbours (b in Figure 2-2) (Cosgrove, 2005). Cellulose microfibrils might simply be coated with xyloglucans through hydrogen bonds, and adhere to other polymers in the matrix without having direct linkages between the microfibrils (see c inFigure 2-2) (Talbott and Ray, 1992). Xyloglucans can be covalently attached to pectin polysaccharides and other acidic residues, forming a macromolecule that anchors the microfibrils (see d in Figure 2-2) (Keegstra et al., 1973; Thompson and Fry, 2000). Arabinoxylans might also be bound to cellulose and be crosslinked to other hemicelluloses and pectins via ferulic acid (F-A) esters (see e in Figure 2-2) (Cosgrove, 2005).

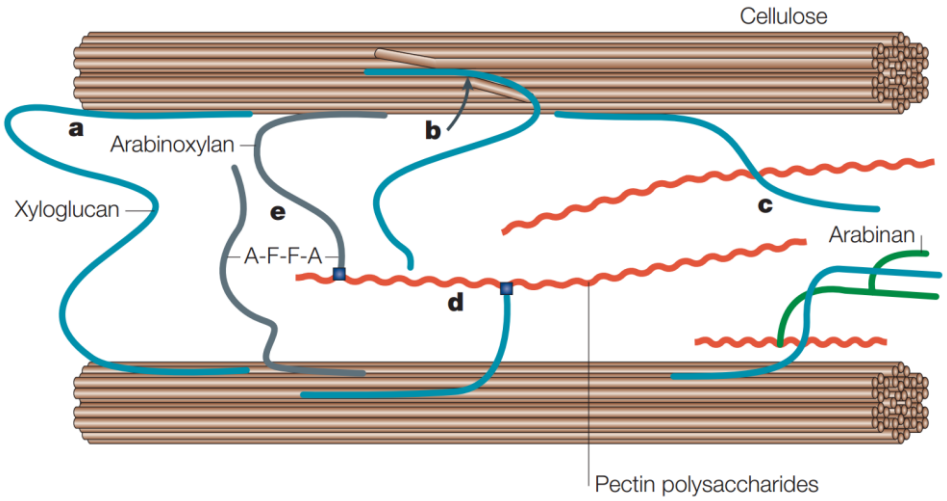

Figure 2-2: Various mechanisms of hemicellulose, cellulose and pectin interconnections; xyloglucans are shown in blue, arabinoxylans in grey, arabinan in green (all hemicelluloses), pectins in red, and cellulose in brown (Cosgrove, 2005)

Furthermore, hemicellulose is linked to lignin through covalent and non-covalent bonds, whereas cellulose is linked to lignin only non-covalently (Achyuthan et al., 2010; Acosta-Estrada et al., 2014; Westbye et al., 2007). There are four types of covalent linkages between hemicellulose and lignin – *p*-coumaric and ferulic acids in lignin can be attached to hemicellulose via benzyl ester, benzyl ether, phenyl glycoside and acetal linkages (Achyuthan et al., 2010). This lignin-hemicellulose complex surrounds the cellulose microfibrils via mostly hydrogen bonding to form a supramolecular structure that protects the cellulose and is the reason for biomass recalcitrance (Achyuthan et al., 2010).

As can be seen from Table 2-1, cellulose, hemicellulose and lignin are by far the largest components of dry plant biomass. In addition to the aforementioned pectins, plant biomass also contains extractives of non-structural nature – such as starch, simple sugars, proteins, fats, waxes, alkaloids, phenolics, mucilages, gums, glycosides, saponins, terpenes, resins (Yu et al., 2007) that serve a range of functions in the plant, including energy storage, cell signalling, waterproofing, thermoregulation, and protection from insect predation (Xu, 2010).

2.2. Occurrence of hemicellulose

Hemicellulose account for a quarter of the total global biomass (Schädel et al., 2010). It is present in monocotyledon plants (monocots), dicotyledon plants (dicots), conifers and algae (see Table 2-1). Monocots include grasses and therefore most of the agricultural biomass, including energy crops; dicots include hardwoods such as maple and oak; conifers or softwoods include pine, spruce and fir. As can be seen from Table 2-1, conifers primarily consist of mannans, whereas dicots and monocots have xylans as the main type of hemicellulose (Scheller and Ulvskov, 2010). The biomass used in this work was *Miscanthus χ giganteus* grass, which is an energy crop, and the husks of common oats (Avena sativa) that are a common agricultural residue.

2.2.1. Miscanthus χ giganteus

Miscanthus χ giganteus (MIS) is a perennial grass that holds great potential to be used as a lignocellulose feedstock (Le Ngoc Huyen et al., 2010). It is considered as an energy crop due to its potential use for second generation bioethanol production. It is high yielding sterile hybrid species, obtained from *Miscanthus sacchariflorus* and *Miscanthus sinesis*, providing yields up to 24 tonnes (t) per hectare (ha) per year in Southern Europe, and up to 16 in Northern Europe (Christian et al., 2008; Gauder et al., 2012; Murphy et al., 2013). The yields are higher than some wood and any other energy crop, including switchgrass and hemp (Godin et al., 2013; Heaton et al., 2008). As MIS is a sterile hybrid, it does not pose a threat as an invasive species, and needs to be propagated vegetatively (Lewandowski et al., 2000). MIS has high resistance to disease, is easy to harvest, resistant to relatively cold climates and has a low environmental impact (Jørgensen, 2011). Furthermore, MIS has a different, more efficient photosynthesis mechanism than,

for instance, wheat, barley and rice which results in less expensive maintenance of the crops (Fitzpatrick et al., 2010; Rubin, 2008), and grows on marginal lands (Rahman et al., 2014).

As can be seen in Table 2-1, dry MIS is typically composed of 41-53% cellulose, 23-33% hemicellulose and 7-22% of lignin (Allison et al., 2011; Hodgson et al., 2010; Murnen et al., 2007). The hemicellulose fraction of MIS is primarily composed of arabinoxylan (Hayes, 2013a), while hydroxycinnamic acid ester (mainly *p*-coumaric, ferulic and caffeic acid esters) and acetyl groups are also present in relative abundance (Le Ngoc Huyen et al., 2010; Lygin et al., 2011; Parveen et al., 2011).

2.2.2. *Avena sativa* husks

The annual worldwide production of common oat (*Avena sativa*) is approximately 25 million tonnes per year (Strychar, 2011). Up to 35% of the total weight of the oat grain constitutes of husks (Thompson et al., 2000; Welch et al., 1983). Husks or hulls enclose the groat (kernel) and protect it from the environment and pathogen attacks (Chaud et al., 2012). Industrially the husks are mechanically separated from the groats (dehulled) using a rotating drum and air aspiration (Thompson et al., 2000). Husks are largely considered as agricultural waste and often are incinerated for energy (Russ and Schnappinger, 2007).

Oat husks consist of 29-37% cellulose, 28-37% hemicellulose, 2-22% lignin (Chaud et al., 2012; Welch et al., 1983). The husk hemicellulose is primarily composed of arabinoxylan (Anderson and Krznarich, 1935). Phenolics, including hydroxycinnamic acid have also been measured in oat husks (Emmons and Peterson, 1999).

Table 2-1: Composition of selected hemicellulose sources (data aggregated from Allison et al., 2011; Deutschmann and Dekker, 2012; Garrote et al., 1999; Gírio et al., 2010; Hodgson et al., 2010; Menon and Rao, 2012; Murnen et al., 2007; Chaud et al., 2012; Welch et al., 1983); "-" indicates no data available; highlight indicates hemicellulose, and most abundant hemicellulosic sugar

	Biomass composition (% dry weight)			Hemicellulose composition (% dry weight)						
Raw material	Cellulose	Hemicellulose	Lignin	Mannose	Galactose	Xylose	Arabinose	Rhamnose	Uronic acids	Acetyl groups
Softwoods (Conifers)										
Fir	43.9	26.5	28.4	9.8-12.5	1.0-3.7	4.8-7.2	0.5-3.0	-	-	-
Pine	42-49	13-25	23-29	5.6-13.3	1.4-3.8	5.3-10.6	1.5-4.2	-	2.5-6.0	1.2-1.9
Hardwoods (Dicots)										
Eucalyptus	45-51	11-15	29	0.7-2.0	1.0-2.5	12.0-21.5	0.6-1.8	0.3-1.0	2	3.0-3.6
Maple	44.1	29.2	24	1.3-3.3	1	18.1-19.4	0.8-1.0	-	4.9	3.6-3.9
Oak	40.4	35.9	24.1	2.3-2.4	1.9-2.1	21.7	1.0-1.6	-	3	3.5
Poplar	45-51	25-28	10-21	2.2-3.5	0-1.1	16.0-21.2	0.4-2.0	-	2.3-3.7	0.5-3.9
Red maple	38.9	23.8	21.5	3.5	0.6	17.3	0.5	-	-	-
Agricultural (Monocots)										
Bamboo grass	49-50	18-20	23	0.4	-	21.6	2.9	-	-	-
Barley straw	36-43	24-33	6.3-9.8	1.3	1.7	15	4.0-8.0	-	-	-
Corn cobs	32.3-45.6	39.8	6.7-13.9	-	1.0-1.2	28.0-35.3	2.8-5.0	1	3	1.9-3.8
Corn stalks	35-39.6	16.8-35	7-18.4	0-3.0	0-2.5	13.0-25.7	2.8-4.1	-	-	-
Corn stover	35.1-39.5	20.7-24.6	11.0-19.1	0.3-0.4	0.8-2.2	14.8-25.2	2.0-3.6	-	-	1.7-1.9
Miscanthus χ giganteus	41-53	23-33	7-21.7	0.1-0.2	0.5-0.7	19-19.5	1.6-2.1	-	-	-
Oat husks	29-37	28-37	2-22	-	-	-	-	-	-	-
Rice husk	28.7-35.6	11.96-29.3	15.4-20	2.7	0.1	12.3-17.7	1.9-2.6	-	-	1.6
Rice straw	29.2-34.7	23-25.9	17-19	1.6-1.8	0.4	13.0-23.0	2.7-4.5	-	-	-
Sorghum straw	32-35	24-27	15-21	0.8	0.2	15	3	-	-	-
Sugar cane bagasse	25-45	28-32	15-25	0.5-0.6	1.6	20.5-25.6	2.3-6.3	-	-	-
Wheat bran	10.5-14.8	35.5-39.2	8.3-12.5	-	1	16	9	-	-	-
Wheat straw	35-39	22-30	12-16	0-0.8	0.7-2.4	16.9-21.0	1.6-3.8	-	-	-

2.3. Chemical characteristics of hemicellulose

The chemical structure of hemicelluloses is complex, and its variability is expressed in many structural variations such as differing side chain types, chain distribution and localisation, as well as localisation and types of glycoside linkages in the main macromolecular chain (backbone) (Ebringerová, 2005). Apart of the main building blocks of pentoses (xylose, arabinose) and hexoses (mannose, glucose, galactose) (see Figure 2-3), hemicellulose can also contain uronic, acetic, and hydroxycinnamic acid groups, as well as other sugars in small amounts such as rhamnose and fucose (Gírio et al., 2010; Saha, 2003). Distinctive characteristic of hemicelluloses is an equatorial β-(1→4)-linked backbone of glucose, mannose or xylose as the most often occurring structure (see Figure 2-4). However, β-(1→3)-linked structures are also considered as hemicelluloses, unless they are entirely consisting of β-(1→3)-linkages (Scheller and Ulvskov, 2010). Due to the differences in chemical structure and occurance in nature, hemicelluloses can be classified into four distinct types: xylans, mannans, xyloglucans and mixed chain β-glucans (Ebringerová, 2005; Scheller and Ulvskov, 2010).

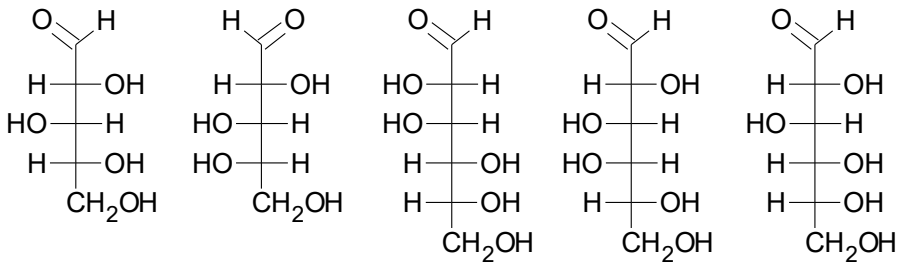

Figure 2-3: Fisher diagrams of the common hemicellulosic sugars

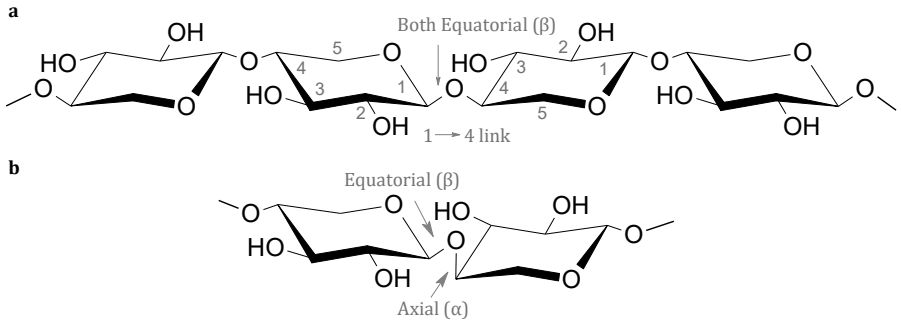

Figure 2-4: (a) β-(1→4)-linked backbone with an equatorial (β) configuration; (b) illustration of axial (α) linkage which is not a characteristic of hemicelluloses

2.3.1. Xylans

Xylans are the most abundant of the hemicellulose types and are highly branched (Thompson, 2000). Xylans usually have high degrees of polymerisation and are composed of β-(1→4)-linked D-xylose backbone, which depending on the plant can be decorated with arabinose and acetic acid residues, and less often with glucose, galactose, rhamnose and hydroxycinnamic and uronic acid residues (Aspinall, 1980; Brillouet et al., 1982; Ebringerová, 2005; Kato and Nevins, 1985; Wen et al., 2011; Wende and Fry, 1997). Due to the range of structural diversity, xylans can be divided into six subclasses: homoxylans, glucuronoxylans, (arabino)glucuronoxylans, arabinoxylans, (glucurono)arabinoxylans and heteroxylans (see Figure 2-5) (Ebringerová, 2005).

Xylans can be covalently linked to lignin, as well as with other polysaccharides via phenolic compounds (Eriksson et al., 1980; Markwalder and Neukom, 1976; Thomson, 1993). Non-covalent associations between xylan and other polysaccharides also occur, most notably adsorbtion to cellulose, and other components of hemicellulose via hydrogen bonding (Katō, 1981; McNeil and Albersheim, 1975).

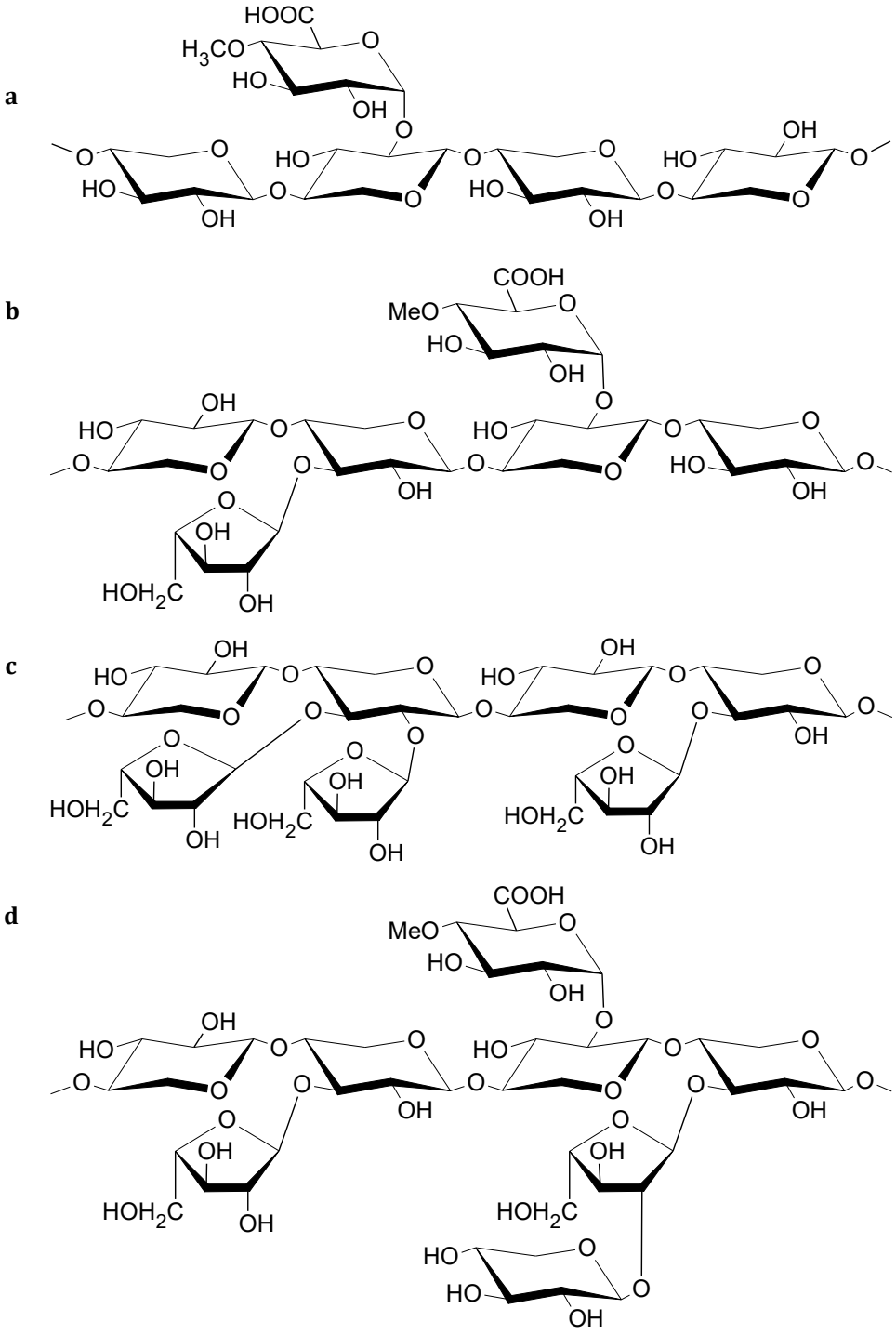

Figure 2-5: Examples of xylan (Ebringerová, 2005): (a) 4-O-methyl-D-glucurono-D-xylan; (b) (L-arabino)-4-O-methyl-D-glucurono-D-xylan; (c) L-arabino-D-xylan; (d) (D-glucurono)-L-arabino-D-xylan

Homoxylans are linear or branched hemicelluloses composed of β-(1→3)- and β-(1→4)-linked D-xylose units, and can be found in tobacco stalk, guar seed husks, esparto grass, and seaweed where they substitute cellulose as the structural polymer of the cell walls (Ebringerová, 2005; Gírio et al., 2010).

Glucuronoxylans have β-(1→4)-linked D-xylose backbone with acetyl groups attached to O-3 and to lesser extent O-2 position of xylose units, averaging to 3.5-7 acetyl groups per 10 xylose units (Alén, 2000). The xylose backbone can also be substituted with α-(1→2)-linked and 4-O-methyl uronic acid residues (see a in Figure 2-5), which are the dominating non-cellulosic polysaccharide in the secondary cell walls of dicots (Scheller and Ulvskov, 2010), including hardwoods, representing 90% of the hemicellulose fraction (Ebringerová, 2005).

(Arabino)glucuronoxylan structure is similar to glucuronoxylans but in addition to α-(1→2)-linked and 4-O-methyl uronic acid residues, L-arabinose units can also be attached at O-2 and O-3 of the xylose backbone (see b in Figure 2-5)(Ebringerová, 2005). (Arabino)glucuronoxylans account for 5-10% of hemicellulose fraction in temperate climate softwoods (Peng et al., 2012b), and up to 50% of tropical softwoods (Ebringerová, 2005).

Arabinoxylans are common in various tissues of cereals – wheat, rye, barley, oat, rice, corn, sorghum, and grasses in general (Bengtsson et al., 1992; Fincher and Stone, 1986; Gruppen et al., 1992; Hartley and Jones, 1976; Ishii, 1991). The β-(1→4)-linked D-xylose backbone has α-L-arabinose residues attached to O-2 and/or O-3 xylose units (see c in Figure 2-5); the arabinose residues in turn can be decorated with phenolic (typically ferulic and *p*-coumaric) acid residues, which are esterified to O-5 of some L-arabinose

units attached to the xylose backbone (Ebringerová, 2005; Kato and Nevins, 1985). Phenolic acids allow the hemicellulose to cross-link to other parts of hemicellulose, pectins, lignin and cell wall proteins via oxidative coupling, which allows formation of cross-linked networks of polysaccharides, which together with the non-covalent interactions render the cell walls recalcitrant to digestion (Peng et al., 2012b; Scheller and Ulvskov, 2010; Sun et al., 2004). The backbone of arabinoxylans can also contain acetyl groups (Brillouet et al., 1982; Wen et al., 2011; Wende and Fry, 1997), which accounts for up to 2% of the cell walls in grasses (Bacon et al., 1975).

(Glucurono)arabinoxylan is the main non-cellulosic component of primary cell walls, constituting up to 20% of the cell walls in grasses and cereals (Ebringerová, 2005; Scheller and Ulvskov, 2010). Similar to (arabino)glucuronoxylans, they also contain L-arabinose and uronic acid residues. However, the L-arabinose side chain can also be decorated with terminal xylose (see d in Figure 2-5) (Ebringerová, 2005; Schooneveld-Bergmans et al., 1999).

Lastly, heteroxylans are typically found in cereal bran, seeds, gums and mucilages (Ebringerová and Heinze, 2000). Heteroxylans are complex structures with β-(1→4)-linked D-xylan backbone, which is heavily decorated with a variety of mono- and oligomeric side chains, and when isolated, form highly viscous solutions (Ebringerová, 2005).

2.3.2. Mannans

Mannans are divided into galactomannans, glucomannans and (galacto)glucomannans. The backbone of mannans can consist entirely of D-mannose, as in mannans and galactomananns, or with D-mannose and D-glucose in a non-repeating pattern as in

glucomannans and (galacto)glucomannans (see Figure 2-6) (Scheller and Ulvskov, 2010). Mannans are often acetylated with 1 acetyl group per 3-4 hexose units (Alén, 2000). All mannans have β–(1→4)-linked D-mannose or combination of D-mannose and D-glucose backbone, branched from O-6 by D-galactose residues (Ebringerová, 2005). Hardwoods contain 2-5% of glucomannans, whereas up to 25% of softwoods are composed of (galacto)glucomannans, where it is the dominant hemicellulosic polysaccharide (Peng et al., 2012b; Pereira et al., 2003; Thompson, 2000).

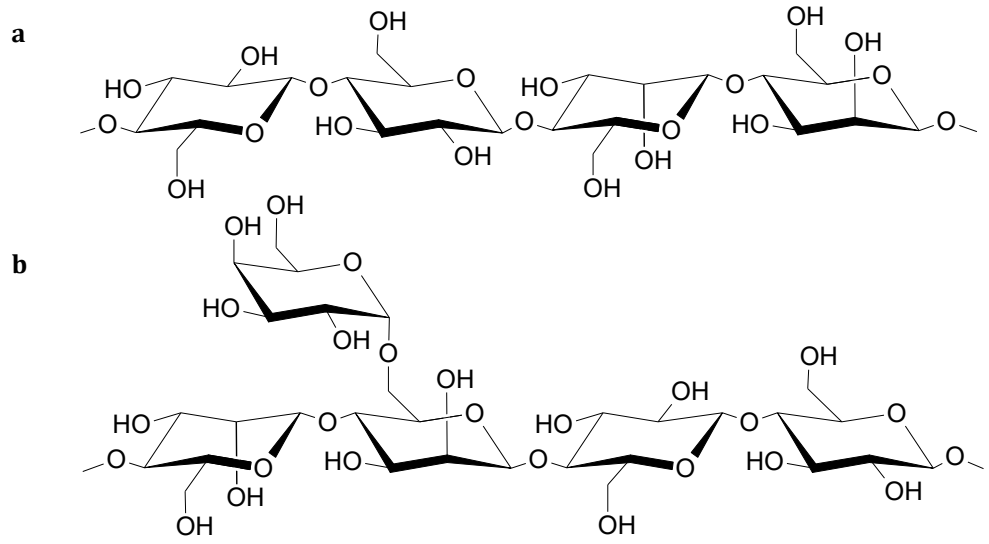

Figure 2-6: Examples of mannan: (a) D-gluco-D-mannan and (b) (D-galacto)-D-gluco-D-mannan (Ebringerová, 2005)

2.3.3. Xyloglucans

Xyloglucans have cellulosic β-(1→4)-linked D-glucose backbone with attached α-D-xylose residues (see Figure 2-7) which distribution divides the xyloglucans into types I and types II (Ebringerová, 2005). Type I or -X-X-X-G- xyloglucans consist of blocks having three xylosylated glucose units and one unsubstituted glucose unit, whereas type II or -X-X-G-G consist of blocks with two xylosylated glucose units followed by two unsubstituted glucose units (Ebringerová, 2005). The less branched xyloglucans are less soluble.

Xyloglucans are the most abundant hemicellulose in primary walls of seed producing plants except for grasses (Scheller and Ulvskov, 2010). Furthermore, together with xylan and glucomannan, xyloglucans are also present in the primary cell walls of hardwoods and softwoods (de Vries and Visser, 2001). Xyloglucan is strongly bound to cellulose via hydrogen bonds, which negatively affects its extractability (Carpita and Gibeaut, 1993; de Vries and Visser, 2001). Covalent bonds between xyloglucans, pectins and other hemicelluloses can also be formed (Carpita and Gibeaut, 1993; Ebringerová, 2005).

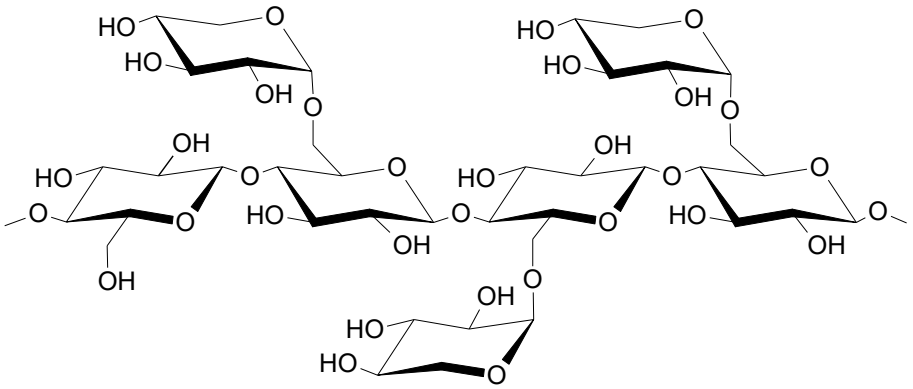

Figure 2-7: Type I or -X-X-X-G- D-xylo-D-glucan (Ebringerová, 2005)

2.3.4. Mixed linkage β-glucans

Mixed linkage β-glucans have an unbranched backbone primarily consisting of cellulosic β-(1→4)-linked D-glucans with interspersed β-(1→3)-linked D-glucans (see Figure 2-8) (Ebringerová, 2005; Izydorczyk and Dexter, 2008). Mixed linkage β-glucans mainly have three or four sequential β-(1→4) linkages for every β-(1→3) linkage, however, longer β-(1→4) linkages can also occur (Stone and Clarke, 1992). In minor contents, they are present in grasses and cereals, algae and lichens (Fincher, 2009). In contrast to cellulose, which often is crystalline and therefore insoluble in most solvents, mixed linkage β-glucans are flexible and soluble due to its angled structure (Peng et al., 2012b).

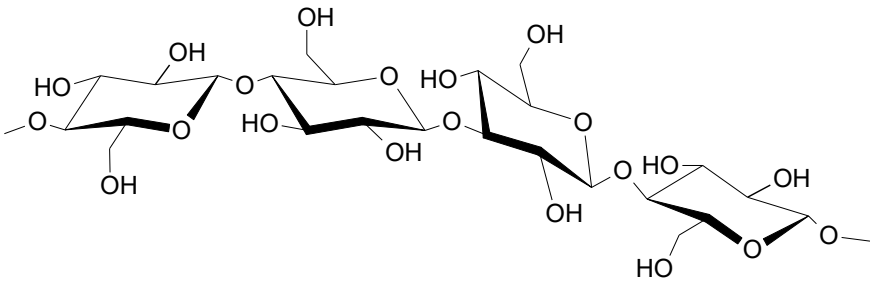

Figure 2-8: Mixed-(1→3, 1→4)-linkage β-D-glucan (Ebringerová, 2005)

2.4. Current and potential applications of hemicellulose

Hemicellulose has the potential to be the raw material for numerous applications. As hemicellulose is a natural polymer, it can be hydrolysed into different chain lengths that have different properties. Depending on the chain lengths, the hemicellulosic monosaccharides, oligosaccharides and monosaccharides have applications in packaging, food, medicine and animal feed. Moreover, by further dehydrating the hemicellulosic monosaccharides, furfurals and organic acids can be produced, which have the potential as platform chemicals to produce natural rubbers, textiles, and plastics.

2.4.1. Monosaccharides and monosaccharide derived platform chemicals

Hemicellulose can consist of xylose, mannose, galactose, glucose, mannose and rhamnose units. The most common of hemicellulosic monosaccharides is xylose. Xylose can be fermented or catalytically hydrogenated to xylitol (Li et al., 2012; Misra et al., 2012; Prakasham et al., 2009; Sirisansaneeyakul et al., 2013), which is used as a natural low-caloric sweetener with tooth decay preventing and anti-diabetic properties in chewing gums, toothpastes, and diabetic products (Gullón et al., 2012). Other sugar alcohols such as mannitol and galactitol can also be produced from mannose and galactose respectively and have similar applications to xylitol (Mäki-Arvela et al., 2011).

Hemicellulosic monosaccharides can also be fermented to other products, notably bio-hydrogen, bio-ethanol, bio-butanol, 2,3-butanediol, and 1-3-propanediol (Agbogbo et al., 2008; Chandel et al., 2009; Cheng et al., 2010b; Kurian et al., 2010; Oberling et al., 2012; Panagiotopoulos et al., 2009; Qing and Ming, 2009). Bio-ethanol production from cellulose has been widely researched (Chen and Qiu, 2010; Olsson et al., 2005; Wyman, 1996). Nevertheless, bio-ethanol production from hemicellulose is also attainable despite being more complex (Almeida et al., 2007; Chandra et al., 2007; Saha, 2003). Bio-butanol and 2,3-butanediol can be used as biofuels but have higher energy density than bio-ethanol (Menon and Rao, 2012). Furthermore, 2,3-butanediol can also be used in the production of rubber, plasticizers, fumigants and antifreeze (Celinska and Grajek, 2009). 1-3-propanediol or β-propylene glycol is widely used in the manufacture of polyesters and adhesives (Gullón et al., 2012).

Lactic acid is another bacterial fermentation product of carbohydrates including hemicellulose (Iyer et al., 2000) with wide uses in food, pharmaceuticals and cosmetics, but most notably it is a precursor to polylactic acid, which is a biodegradable polymer and a sustainable alternative to petroleum based plastics (Gullón et al., 2012).

There are other applications than fermentation where hemicellulosic monosaccharides can be used. For instance, mannose has applications in pharmaceutical industry and can act as a growth accelerator for swine (Davis et al., 2004). Galactose can be isomerised to produce tagatose, which is used as a low-calorie sweetener with prebiotic properties (Spiridon and Popa, 2008). Arabinose can be epimerised to ribose, which has applications in pharmaceuticals (Mäki-Arvela et al., 2011).

By further dehydrating the hemicellulosic pentoses and hexoses, furfural and 5-hydroxymethylfurfural (5-HMF) can be produced (Mamman et al., 2008; Rosatella et al., 2011). Furfural can be produced from both pentoses and hexoses, whereas 5-HMF can only be produced from hexoses (Yu et al., 2007). Furfural is considered as a platform chemical with wide range of applications as solvent, and insecticide; it is also used in the manufacture of 5-methylfurfural, which is a high value flavouring; furfuryl alcohol – used in the production of resins and adhesives; tetrahydrofurfuryl alcohol – environmentally friendly solvent for biocides and pesticides, dyes, coatings and curing agents; and tetrahydrofuran, a precursor to the manufacture of elastomers (Gullón et al., 2012; Mamman et al., 2008). Furfural has no synthetic production route and is exclusively produced from biomass sources (Lichtenthaler, 2006; Zeittsch, 2000). 5-HMF is also a versatile platform chemical, and can be used to manufacture biofuels such as 2,5-dimethylfuran; and 2,5-furandicarboxylic acid – building block for polyesters, polyamines, and polyurethanes (Gullón et al., 2012; Rosatella et al., 2011).

Levulinic and formic acids are generated by further decomposition of furfural and 5-HMF (Carvalheiro et al., 2004; Rosatella et al., 2011). Levulinic acid has been considered a future platform chemical as a sustainable building block for textiles, resins, coatings and plasticizers (Kamm et al., 2007; Lucia et al., 2006). Formic acid is widely used in leather tanning, textile dyeing and finishing, paper industry, in preservation of animal feed, and as an alternative to mineral acids in various other industrial applications (Gullón et al., 2012; Reutemann and Kieczka, 2000).

2.4.2. Oligosaccharides

Hemicellulose derived oligosaccharides have the potential to be used as prebiotics in animal feed, pharmaceutical, food and beverage, and nutraceutical industries (Al-Sheraji et al.; Barry et al., 2009; Collins et al., 2009; Mäkeläinen et al., 2009; Moure et al., 2006; van Laere et al., 2000). Prebiotics are non-digestible food that can selectively stimulate the growth of beneficial bacteria such as *Bifidobacteria* and *Lactobacilli.* As xylan is the most abundant hemicellulosic polymer (Ebringerová, 2005), xylooligosaccharides (XOS) derived from plant cells have the potential to capture and expand the prebiotic market as the majority of the commercially produced prebiotic compounds, including XOS, have been produced enzymatically (Casci and Rastall, 2006), and therefore are relatively expensive (Taniguchi, 2004).

XOS prebiotic efficacy has already been widely researched (Barbosa et al., 2010; Chung et al., 2007; Crittenden et al., 2002; De Boever et al., 2000; Gibson et al., 2004b; Holck et al., 2011; Kohmoto et al., 1991; Manisseri and Gudipati, 2010; Ohbuchi et al., 2009; Palframan et al., 2003; Pan et al., 2009; Rycroft et al., 2001). XOS seem to be more effective than the commercial inulin, furcto-oligosaccharides, isomalto-oligosaccharides and soybean-oligosaccharides in terms of increasing *Bifidobacteria* and *Lactobacilli* microbial numbers (Santos et al., 2006). XOS with degrees of polymerisation of 2-5 are the most effective in terms of prebiotic efficacy (Hughes et al., 2007; Moura et al., 2007; van Craeyveld et al., 2008) but branching negatively affects the XOS digestibility (Gullón et al., 2011; Ohbuchi et al., 2009).

Research has shown that consuming XOS, and therefore stimulating the growth of *Bifidobacteria* and *Lactobacillus*, can lead to immunostimulatory, anti-obesity, anti-

inflammatory, anti-microbial, anti-diabetic, anti-hyperlipidemic, anti-allergenic, anti-oxidant, and even anti-carcinogenic effects (Coconier et al., 1998; Hsu et al., 2004; Jacobsen et al., 1999; Ji et al., 2012; Kunz et al., 1999; Letllier et al., 2000; Maeda et al., 2004; Moure et al., 2006; Nabarlatz et al., 2007a; Swennen et al., 2006). Fermentation of prebiotics, allows the probiotic bacterial communities to grow in numbers, strengthen the gut-lining, and release essential nutrients such as short chain fatty acids and vitamins, which together positively affect the health and wellbeing of the host via many mechanisms (Preidis and Versalovic, 2009; Tremaroli and Backhed, 2012).

Moreover, XOS are acid resistant , heat resistant up to 100 °C, are moderately sweet with no carcinogenic effects, which together with the aforementioned health benefits, make XOS an appealing compound in functional food, pet and livestock feed, and pharmaceutical industries (Deutschmann and Dekker, 2012; Mäkeläinen et al., 2009).

2.4.3. Polysaccharides

Hemicellulose derived polysaccharides have potential applications as films, coatings, foams and gels in food, cosmetics, medicine and packaging industries (Hansen and Plackett, 2008). Although most pure xylan preparations do not form films, addition of other compounds such as lignin, glycerol and cellulose nano-fibres, allows the formation of bio-derived films (Goksu et al., 2007; Stevanic et al., 2011). Irregular (branched) hemicellulose chains are favourable for effective film formation but are likely to be soluble in water, while less substituted, more linear chains are less soluble but more crystalline (Zhang et al., 2011). Nevertheless, hydrophobic films can be made from xylan even with low degrees of substitution by xylan derivatisation with long chain succinic anhydrides (Hansen and Plackett, 2011).

Bio-derived gels and foams can have future in cosmetics, tissue engineering, drug delivery, insulation and gas storage (Aaltonen and Jauhiainen, 2009). In order to form the gels and foams, the base polymer needs to be cross-linked or be able to form a network of secondary forces (e.g. hydrogen bonds) strong enough to support the structure; and in case of aerogels even after removal of the solvent (Deutschmann and Dekker, 2012). Xylan can be mixed with other polymers such as chitosan and cellulose, to form hydrogels and aerogels (Aaltonen and Jauhiainen, 2009; Fonseca Silva et al., 2011; Salam et al., 2011). Ionic hydrogels that respond to pH by shrinking or expanding, can be produced when xylan is reacted with acrylic acid with acrylamide as a cross-linker (Peng et al., 2011).

Hemicellulosic polymers, including xylan are acid resistant and therefore can survive the digestion in the upper gastric tract, making them suitable as a vector for drug delivery applications (Deutschmann and Dekker, 2012). Furthermore, similarly to XOS, also xylans have shown some prebiotic properties (Cipriani et al., 2008; Ebringerova and Heinze, 2000; Ebringerová et al., 2008; Hromadkova et al., 2010; Pristov et al., 2011). Xylan sulfonates have generated much scientific interest in medical applications as anti-coagulant partly because of the structural similarity to heparin (Daus et al., 2011; Deutschmann and Dekker, 2012).

2.4.4. Phenolic substitutions

Hydroxycinnamic acids, particularly *p*-coumaric and ferulic acids, can be bound to hemicelluloses (Ebringerová, 2005; Scheller and Ulvskov, 2010). Phenolics, including hydroxycinnamic acids have antioxidant properties due to the reactivity of their phenol moiety via radical scavenging and radical quenching mechanisms (Shahidi et al., 1992),

which has led to research of potential health benefits of phenolics as dietary anti-oxidants (Shahidi and Ambigaipalan, 2015). Apart from the antioxidant properties, hydroxycinnamics have shown anti-microbial, anti-inflammatory, anti-carcinogenic, and anti- hyperlipidemic properties (Galati and O'brien, 2004; Machado et al., 2009; Ou and Kwok, 2004) and therefore have applications in nutraceutical, food and beverage, and cosmetics industries (Ou and Kwok, 2004Parveen, 2011 #620; Zhao and Moghadasian, 2008). Furthermore, xylans esterified with hydroxycinnamates have shown to have antioxidant properties (Wrigstedt et al., 2010). The health benefits of phenolics are more effective when they are bound, as they can be for example with hemicelluloses, and can reach the colon where they can be released by microbial enzymes (Acosta-Estrada et al., 2014; Liu, 2007).

2.5. State of the art in hemicellulose extraction

There are many potential resources from which hemicellulose can be extracted, including agricultural residues such as straw and husks, wood materials, energy crops such as miscanthus, hemp and switchgrass, as well as from the by-products of paper industry (Persson et al., 2007; Puls and Saake, 2003; Ren and Sun, 2010). Furthermore, there has been a lot of research interest in hemicellulose and lignin removal as part of a biomass pre-treatment step before cellulose hydrolysis in the second-generation ethanol production (Dias et al., 2013; Hayes, 2013b).

Because hemicelluloses are complex and have covalent linkages to themselves, lignin, and pectins, as well as strong non-covalent linkages with cellulose and other hemicelluloses (Ebringerová and Heinze, 2000), they are difficult to liberate from the lignocellulosic matrix without significantly degrading the polymer itself and extracting other

components of the cell wall (Ren and Sun, 2010). Therefore, care needs to be taken regarding the production of carbohydrate degradation products, which can negatively affect the biomass processing further downstream (Saha, 2003).

The extraction routes of hemicellulose have been extensively reviewed (Agbor et al., 2011; Alvira et al., 2010; Carvalheiro et al., 2008; Chiaramonti et al., 2012; Galbe and Zacchi, 2012; Gírio et al., 2010; Menon and Rao, 2012; Mosier et al., 2005; Peng et al., 2012b; Saha, 2003; Sathitsuksanoh et al., 2013; Sun and Cheng, 2002; Taherzadeh and Karimi, 2008; Wan and Li, 2012; Yang and Wyman, 2008), and can be divided into three classes – chemical, physiochemical, and biological. Chemical (1) routes include extractions with acid, alkali and ionic liquid media usually at conditions close to ambient; extractions performed at elevated physical parameters with or without modifiers are referred to as physiochemical (2), and include such methods as steam explosion, sub-critical water (subCW) extraction, and ammonia recycle percolation; whereas biological (3) routes usually involve enzymatic hydrolysis and fermentation. Reference tables including extraction method, conditions, biomass types, methods of characterisation, hemicellulose solubility, and hemicellulose recovery are given in Appendix D.

2.5.1. Chemical treatments

Acidic

Acids, including sulphuric (H_2SO_4), hydrochloric (HCl), nitric (HNO_3), phosphoric (H_3PO_4) and trifluoroacetic (TFA) can be used in concentrated and dilute forms for hemicellulose processing (Alvira et al., 2010; Mosier et al., 2005). Concentrated acids (40-100% depending on acid type and extraction conditions) can effectively solubilise cellulose and hemicellulose, leaving lignin rich solid residue at conditions close to ambient with little

degradation products, but at elevated conditions, the rate of polysaccharide hydrolysis is high, leading to fast formation of degradation products (Carvalheiro et al., 2008; Gírio et al., 2010). Dilute acid processes (0.5-1.5%) are typically considered for hemicellulose hydrolysis as a pre-treatment for further cellulose treatment, and are typically operated at temperatures up to 160 °C, and can provide up to 89% yield of hemicellulose derived monosaccharides (Esteghlalian et al., 1997) and 92% oligosaccharides (Otieno and Ahring, 2012b). However, the disadvantages of the acid treatments are the associated costs of acid and the necessity for a neutralisation or acid recovery step.

Alkaline

Alkaline treatments typically utilise sodium hydroxide (NaOH), potassium hydroxide (KOH), lime ($Ca(OH)_2$), and ammonium hydroxide (NH_4OH) (Menon and Rao, 2012). Alkali treatments are effective for lignin solubilisation at conditions close to ambient, leaving hemicellulose and cellulose (hollocellulose) relatively intact (Carvalheiro et al., 2008). Alkaline treatment can break the bonds between hemicellulose and lignin, hydroxycinnamates and acetyl groups (Peng et al., 2012b; Spencer and Akin, 1980), however, at highly alkaline conditions and high temperatures, hemicellulose hydrolysis also occurs (Hendriks and Zeeman, 2009). Long residence times (24-72 h) or moderate extraction temperatures (75-120 °C), can produce high yields of hemicellulose derived monosaccharides (95%) and oligosaccharides (84%) (Wan et al., 2011). Similar to acid treatments, the disadvantages of alkaline treatments are associated with the neutralisation and recovery of the alkali.

Ionic liquids

Ionic liquids, also known as 'green solvents' are salts composed of organic cations and inorganic anions with low melting points and vapour pressures, but high polarities, thermal stability (Hayes, 2009). The solvent properties of these are adjusted by varying the anion and cation composition (Alvira et al., 2010). Examples of ionic liquids are 1-butyl-3-methylimidazolium chloride and 1, 3-N-methylmorpholine-N-oxide (Menon and Rao, 2012). Ionic liquids are effective in cellulose solubilisation but are not much researched for hemicellulose solubilisation (Gírio et al., 2010). However, as with other chemical treatments, the use of ionic liquids is expensive and would require a recovery step for environmental and economic sustainability.

2.5.2. Physiochemical treatments

Sub-critical water

Hydrothermal, liquid hot water, and sub-critical water methods all are based on the phenomena of autohydrolysis, whereby at certain extraction conditions the rate of hemicellulose hydrolysis and therefore solubilisation in water is facilitated by the increasing acidity of the extraction media due to the solubilisation of easily accessible acidic compounds (acetyl and hydroxycinnamic groups) off the hemicellulosic backbone and lignin (Ando et al., 2000; Pińkowska et al., 2011; Ramos, 2003; Rasmussen et al., 2014). This is typically achieved at temperatures between 150-190 °C, short residence times (0-60 min) and pressures high enough to sustain liquid state of water (see Appendix D) (Alvira et al., 2010). Autohydrolysis is effective for hemicellulose solubilisation, leaving lignin and cellulose largely intact (Ando et al., 2000). Almost complete hemicellulose solubilisation (50-90%) is possible with relatively low carbohydrate

degradation product formation, depending on the biomass source and extraction conditions (Liu et al., 2009; Mosier et al., 2005; Vegas et al., 2008a; Yu et al., 2010). Apart from temperature, residence time and pressure, the extraction pH can also be controlled by the addition of acid or alkali modifiers in order to mitigate the generation of degradation products and maintain the rate of hemicellulose hydrolysis, (Weil et al., 1998). Largest disadvantages of sub-critical water treatment are the high capital costs of high pressure extractors.

Wet oxidation

Wet oxidation is also based on the process of autohydrolysis but with the addition of air, oxygen (O_2) or sodium carbonate (Na_2CO_3) as catalysts, usually operated at 170-200 °C, 10-12 bar for residence times up to 15 min (Olsson et al., 2005). Above 170 °C the process becomes exothermic thus reducing the energy demand (Alvira et al., 2010). Both hemicellulose and lignin solubilisation is possible with this method, and particularly high yields of hemicellulosic monosaccharides can be achieved with the alkaline catalysts (Klinke et al., 2002). As this process is based on oxidation, equipment corrosion risk is a detriment to larger scale applications.

Steam explosion

Steam explosion is another method utilising autohydrolysis, and is widely researched (Peng et al., 2012b). Like hydrothermal treatment, it can isolate the hemicellulose fraction without significantly affecting lignin and cellulose. It combines the chemical effects of autohydrolysis with mechanical forces arising when the biomass is treated with pressurised steam (20-50 bar, 160-290 °C) for short period of time, and is suddenly depressurised to atmospheric pressure via a small nozzle (Mosier et al., 2005). The

sudden water evaporation from the wet biomass aids in breaking the intra- and inter-molecular linkages in the cell walls, thus facilitating hemicellulose solubilisation in water (Carvalheiro et al., 2008; Menon and Rao, 2012). Hemicellulose solubilities up to 80% have to been reported (Hongzhang and Liying, 2007). Acidic and alkaline modifiers are also used to prevent the formation of degradation products and increase the hemicellulose solubilisation by maintaining a target pH range during the extraction (Agbor et al., 2011; Chiaramonti et al., 2012). The disadvantages associated with steam explosion are associated with the controllability of the treatment.

CO_2 explosion

Carbon dioxide explosion utilises the properties of supercritical CO_2, where the wet biomass is pressurised with CO_2 at 70-275 bar and temperatures up to 200 °C before the pressure is released as in steam explosion (Hendriks and Zeeman, 2009; Zheng et al., 1995). Pressurised CO_2 dissolves in water and forms carbonic acid, which acts as a catalyst for hemicellulose hydrolysis (Agbor et al., 2011). In addition to poor process controllability as with steam explosion, CO_2 explosion also requires higher operating pressures, and therefore are more expensive.

AFEX

Ammonia fibre explosion (AFEX) utilises the same concept as other explosion methods but instead of water or CO_2, anhydrous ammonia is used at temperatures of 60-100 °C (Agbor et al., 2011; Alvira et al., 2010). Ammonia fibre explosion is more suitable for lignin solubilisation, but some hemicellulose solubilisation is also possible (Laureano-Pérez et al., 2005). It has also been shown as effective for protein extraction (Bals et al.,

2007). After the treatment, the ammonia needs to be neutralised or recovered to be environmentally sustainable.

Ammonia recycle percolation

Another method utilizing the properties of ammonia is ammonia recycling percolation where aqueous ammonia (5-15%) flows through biomass packed column at 140-210 °C with 10-90 min residence times (Alvira et al., 2010; Kim et al., 2006; Sun and Cheng, 2002; Yoon et al., 1995). Under these conditions most of lignin and hemicellulose is solubilised (Kim et al., 2003). However, as with other chemical treatments, ammonia recovery and neutralisation is required following the treatment.

Organosolv

Organosolv processes utilise aqueous organic solvents (e.g. ethanol, methanol, acetone, ethylene glycol, tetrahydrofuryl alcohol, simethyl sulphite, ethers, ketones, phenols) with or without catalysts (HCl, H_2SO_4, oxalic, acetylsalicylic and salisylic acids) to remove the lignin and/or hemicellulose fractions (Agbor et al., 2011; Alvira et al., 2010; Gírio et al., 2010; Sun and Cheng, 2002). Water/ethanol blends with acidic modifiers such as CO_2 and H_2SO_4 have been shown to be effective for lignin removal in extraction ranges between 180-200 °C (Gírio et al., 2010; Roque et al., 2012). As with other methods, apart from sub-critical water treatments, the solvents need to be recovered after the extractions. However, the use of ethanol is promising in terms of integrated biorefinery that also produce bioethanol, as it can be sourced from the downstream process.

Extractability enhancers

Ultrasonification and microwave irradiation can be used along with some of the above described methods, including acid, alkali and hydrothermal, to improve the extractability

of hemicellulose and lower the operating conditions (Bian et al., 2014; Coelho et al., 2014; Ebringerová and Hromádková, 2010; Hromadkova et al., 2008; Lin et al., 2017).

2.5.3. Biological treatments

Biological treatments of hemicellulose can be divided into two sub-categories: (1) enzymatic hydrolysis of hemicellulosic carbohydrates (saccharification), and (2) hemicellulose fermentation to produce value added products such as ethanol, butanol, other alcohols and hydrogen (Peng et al., 2012b). Biological treatments require low energy inputs and are environmentally friendly but are slow, complex and require careful control (Menon and Rao, 2012). To fully hydrolyse hemicellulose, a variety of enzymes are necessary due to the complex hemicellulose composition. For instance, in order to hydrolyse arabinoxylan, following enzymes are necessary: endo-xylanase (hydrolyses the interior β-linkages of xylan), exo-xylanase (hydrolyses β-linkages to release xylobiose), β-xylosidase (releases xylose from short chain xylooligosaccharides), α-arabinofuranosidase (hydrolyses terminal arabinose groups off xylan backbone), α-glucuronidase (releases uronic groups), acetylxylan esterase (releases the acetyl groups), and ferulic acid and *p*-coumaric acid esterases (releases the ferulic and *p*-coumaric groups) (Gírio et al., 2010; Saha, 2003). The hydrolysed monomeric sugars can then be fermented into value added products like xylitol or bioethanol. The enzymes are produced from many sources including yeast, bacteria, fungi, algae, snails, protozoans, crustaceans, insects and seeds (Juturu and Wu, 2012; Peng et al., 2012b; Sun and Cheng, 2002).

2.6. Hemicellulose extraction in subCW

Sub-critical water (subCW) conditions correspond to temperatures and pressures below the critical point (374 °C, 220 bar) but above the boiling point (100 °C, 1 bar), where the water is kept in liquid state. At these conditions the physiochemical properties of water significantly differ from what is observed at ambient conditions (see Table 2-2). For example, at temperatures above 150 °C, the hydrogen bonding in water starts to weaken, allowing the auto-ionisation to occur, which generates hydronium (H^+) ions that act as catalysts for hemicellulose hydrolysis (Nabarlatz et al., 2004; Yu et al., 2007). Moreover, the decrease of dielectric constant and density change the water properties as a solvent, which improves the solubility of non-polar substances (Bröll et al., 1999; Kruse and Dinjus, 2007).

Table 2-2: Physiochemical properties of water at ambient, sub-critical and super-critical conditions (Bandura and Lvov, 2006; Onwudili and Williams, 2008)

	Ambient water	Sub-critical water	Super-critical water	
Temperature, T	25	250	400	400
Pressure, p (bar)	1	50	250	500
Density, ρ (g cm^{-3})	1.00	0.80	0.17	0.58
Dielectric constant, (ε)	78.5	27.1	5.9	10.5
Ionic product, pK_w	14.0	11.2	19.4	11.9
Heat capacity, c_p (kJ kg^{-1} K^{-1})	4.22	4.86	13.0	6.80
Viscosity, μ (mPa s)	0.89	0.11	0.03	0.07
Thermal conductivity, λ (mW m^{-1} K^{-1})	608	620	160	438

Hemicellulose is generally solubilised from the biomass at temperatures between 160-200 °C (Ando et al., 2000; Kabel et al., 2002) and leaves most of the lignin and cellulose relatively intact (Chen et al., 2014; Liu and Wyman, 2003; Ramos, 2003). Lignin tends to rapidly solubilise at temperatures above 220 °C (Liu and Wyman, 2003), whereas cellulose hydrolysis starts at temperatures above 230 °C, and almost complete biomass

solubilisation is achieved at temperatures close to 300 °C (Ando et al., 2000). At higher temperatures biomass gasification starts to occur (Peterson et al., 2008).

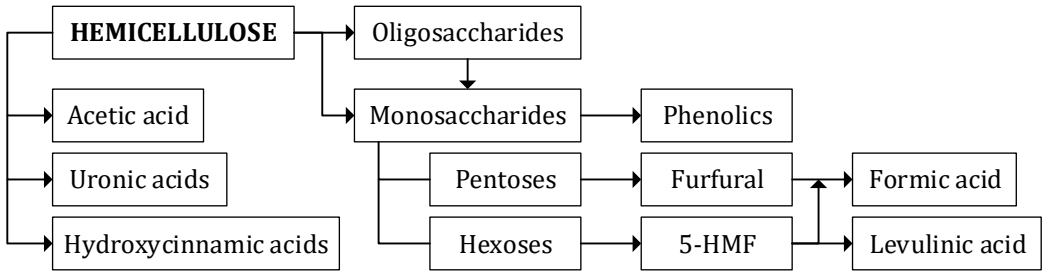

Figure 2-9: Hemicellulose degradation pathways at mild subCW

The chemical behaviour of hemicellulose in mild subCW (up to 200 °C) can be divided into two degradation pathways (see Figure 2-9): debranching and hemicellulose depolymerisation. Both reactions are catalysed by the hydronium ions (H^+) from water auto-ionisation. The debranching is essential for the hemicellulose depolymerisation because the supply of hydronium ions in the extraction medium rapidly increases with the release of acidic branches (i.e. acetyl, uronic and hydroxycinnamic groups) off the hemicellulose backbone (see Figure 2-10), which in turn rises the rate of hemicellulose hydrolysis (Ando et al., 2000; Nabarlatz et al., 2004; Parajó et al., 2004; Rasmussen et al., 2014; Yu et al., 2007). This process is called autohydrolysis. Some of the acetyl and phenolic groups (typically <1% of dry biomass) are also released from the lignin fraction at these conditions, and is generally referred to as 'acid soluble lignin' (Ando et al., 2000; Kumar et al., 2013). The release of hydroxycinnamates (*p*-coumaric and ferulic acids) helps to untether the hemicellulose from the lignocellulosic matrix because it is through the phenolic compounds how hemicellulose covalently links to itself, lignins and pectins (Eriksson et al., 1980; Markwalder and Neukom, 1976; Thomson, 1993).

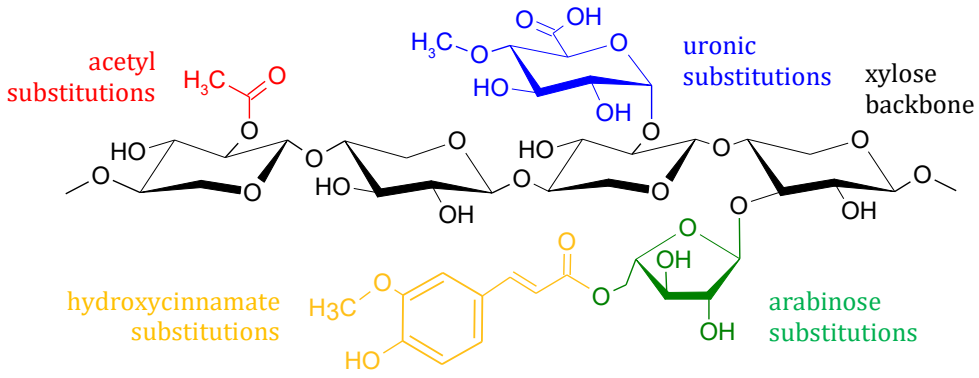

Figure 2-10: Illustration of possible backbone substitutions in arabinoxylan

It is thought that the depolymerisation of hemicellulose occurs in two, not necessarily consecutive stages that are best described as fast and slow reacting. Fast depolymerisation rates in the first stage are likely to occur because of two factors – the sudden high availability of the hydronium ions from the dissolved acidic substitutions, and easy accessibility of lateral hemicellulose chains, which are easier to break and have lower molecular weight than the more recalcitrant linear chains in the hemicellulose backbone. The more difficult to access and more recalcitrant linear chains are solubilised in the slow reacting stage, typically after the fast reacting stage, when the hydronium ion supply from the acidic substitutions has diminished, and therefore higher extraction severity is required to facilitate further hydrolysis and consequential solubilisation in water (Ando et al., 2000; Chen et al., 2014; Garrote et al., 2002; Nabarlatz et al., 2004; Ramos, 2003; Vázquez et al., 2005). This is in line with empirical results where the highly branched hemicellulose is solubilised first, at lower extraction severities with high arabinose contents relative to xylose, which decrease with extraction severity as the less substituted xylan is depolymerised (Chen et al., 2014; Garrote et al., 2002; Nabarlatz et al., 2004; Pińkowska et al., 2011).

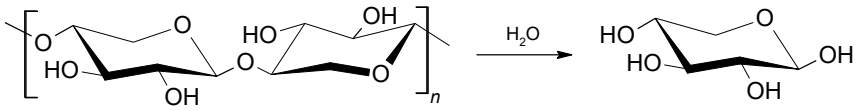

Figure 2-11: Xylan and xylooligosaccharide hydrolysis to xylose

During the hydrolysis, the hemicellulosic polysaccharides are eventually depolymerised into their corresponding oligosaccharides and monosaccharides, for instance, xylan is depolymerised to XOS and xylose (see Figure 2-11) (Chen et al., 2014; Garrote et al., 2002; Kabel et al., 2002; Nabarlatz et al., 2004; Parajó et al., 2004). The hemicellulosic monosaccharides (predominantly pentoses) can further dehydrate to furfural (from pentoses, see Figure 2-12), 5-hydroxymethyl furfural (5-HMF, from hexoses), and various phenolic compounds; and at more severe conditions furfural can decompose further to formic acid, whereas 5-HMF to formic and levulinic acids (Kumar et al., 2013; Mamman et al., 2008; Rasmussen et al., 2014; Rosatella et al., 2011; Usuki et al., 2008). At near critical conditions (close to 400 °C), hemicellulosic monosaccharides can decompose into glceraldehyde and glycoaldehyde by reretro-aldol reactions (cleavage of carbon bonds) (Sasaki, 2003).

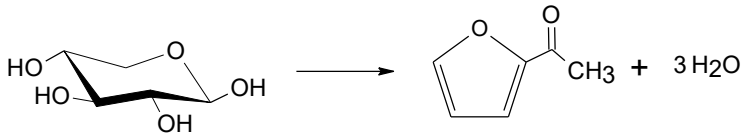

Figure 2-12: Xylose dehydration to furfural and water

2.7. Hemicellulose recovery and purification

Depending on extraction medium and conditions, the hemicellulose rich extracts can contain carbohydrates of various degrees of polymerisation, carbohydrate degradation products like organic acids and furans, phenolics from lignin, and various extractives that are of non-structural origin such as waxes, proteins and simple sugars. To recover and

refine the products with added value from the liquid fraction, extract fractionation by molecular weight is necessary. For instance, commercial purity of food-grade XOS is >75% (Gullón et al., 2009). This can be achieved with membrane based separation techniques such as ultrafiltration and nanofiltration, and/or with solvent (e.g. ethanol, propanol, acetone) precipitation. The application of these technologies for oligosaccharide refining has been widely reviewed (Abels et al., 2013; Huang et al., 2008; Jiang and Zhu, 2013; Peng et al., 2012b; Vanneste et al., 2012; Vegas et al., 2006).

2.7.1. Membrane separation

Ultrafiltration and nanofiltration have been shown to be effective for oligosaccharide purification from auto-hydrolysates (Akpinar et al., 2007; Grandison et al., 2002; Nabarlatz et al., 2007b; Sanz et al., 2005; Vegas et al., 2006). Ultrafiltration is best suited for particles with molecular weights ranging from 100-10,000 g/mol (MW_{xylose} = 150 [g/mol]) making it suitable for carbohydrates with wide range of degrees of polymerisation (DP); whereas nanofiltration is suited for molecular weights within 100-500 g/mol, and is therefore suitable for monosaccharide and short chain oligosaccharide filtration (Jiang and Zhu, 2013). Microfiltration can be used for the separation of solid and insoluble fractions of the extracts (He et al., 2012). In general, membrane separation can produce high purities (up to 91%) of carbohydrates with relatively tight molecular weight distributions (Vegas et al., 2008b). Membranes are typically made from various polymers, e.g. polysulfone, polyethersulfone, polyvinylidene fluoride, regenerated cellulose, polytetrafluoroethylene, and fluoropolymers, as well as from ceramics; and can be configured in different geometries (He et al., 2012; Persson et al., 2010). Although

membrane separation can provide high purities, it is expensive to operate, and can be prone to fouling (He et al., 2012; Persson et al., 2010).

2.7.2. Solvent precipitation

Hemicellulose can be precipitated from aqueous media by lowering its solubility with the addition of miscible organic solvents (Peng et al., 2012b). The most commonly used solvent for hemicellulose fractionation is ethanol, but other solvents such as acetone, propanol, ammonium sulphate, and ethyl acetate have also been used (Gullón et al., 2009; Izydorczyk and Biliaderis, 1992; Moure et al., 2006). Ethanol precipitation is a promising solution, as it can be integrated with potential downstream ethanol production from cellulose. Gradual ethanol precipitation by varying the concentration of ethanol (typically 15-80%) can be used to yield high purity hemicellulosic carbohydrate fractions with narrow molecular weight distribution, whereby at lower ethanol concentrations, the longer-chained, and more branched polysaccharides are precipitated, whereas the shorter-chained, more linear carbohydrates precipitate at higher ethanol concentrations (Bian et al., 2010; Peng et al., 2009a; b; Swennen et al., 2005). Although, ethanol precipitation can be appealing for the recovery of heavier hemicellulose fractions, the lighter products such as XOS require large volumes of ethanol and therefore could not be economically viable.

Aqueous hemicellulose can also be extracted from dimethylsulfoxide (DMSO) with supercritical CO_2 as antisolvent (Haimer et al., 2010; Ren and Sun, 2010). Hemicellulose fractions with narrow particle sizes can be achieved by varying the temperature, pressure and water content but similarly to ethanol precipitation require large volumes of solvent to be attractive for industrialisation (Haimer et al., 2010; Peng et al., 2012b).

2.7.3. Other methods

Passing the extracts through adsorbents (e.g. activated charcoal, diatomaceous earth, acid clay, bentonite), ion exchange resins can be effective for the removal of non-carbohydrate components, and could be used along with the solvent precipitation and membrane separation (Gullón et al., 2009; Zhu et al., 2006).

2.8. Characterisation of hemicellulosic carbohydrates

Hemicelluloses can be of complex carbohydrate structures, varying in terms of polymerisation, composition, and branching, therefore relatively complex analytical techniques are required for precise quantitative and especially qualitative characterisation. For full characterisation, several analytical methods need to be employed. Chromatography and electrophoresis are the most commonly used and can provide accurate quantitative and qualitative data, however, the latter is often limited. Therefore, spectroscopic methods are often used in conjunction to provide more detailed insight into the structural characteristics of the complex structure of hemicellulosic carbohydrates (e.g. substitutions, branch position, linkage types). Fortunately these analytical techniques have been widely researched and reviewed (Carlsson et al., 1992; Ghebregzabher et al., 1976; Mejanelle et al., 2002; Müthing, 2002; Rassi, 2002; Ruiz-Matute et al., 2011; Sanz et al., 2009; Schols et al., 2000; Soga, 2002; Stahl et al., 2002).

2.8.1. Planar chromatography

Thin-layer chromatography (TLC), high performance thin-layer chromatography (HPTLC), over pressure thin-layer chromatography (OPTLC) and ultrathin-layer chromatography (UTLC) are relatively simple characterisation techniques that can be used for monosaccharide and oligosaccharide separation (Gauch et al., 1979; Müthing,

2002; Reiffová and Nemcová, 2006; Robyt and Mukerjea, 1994; Sanz et al., 2009; Vaccari et al., 2001; Weill and Hanke, 1962; Zhang et al., 2007). They typically utilise silica-gel based sorbents coated on a glass plate as the stationary phase, and mobile phase carried out from aqueous alcohols (methanol, ethanol, isopropanol, butanol), acetonitrile, acetone and ethyl acetate. The mobile phase carrying the sample is drawn up the stationary phase by capillary action, and carbohydrate retention is achieved by the adsorption to the silica gel. Although traditionally thin-layer chromatography is carried out isocratically, modern applications can employ elution gradients by the means of automatic mode development (AMD), which can increase the carbohydrate resolution (Brandolini et al., 1995).

After the separation, the silica gel plates need to be derivatized for visualisation and quantification. This is typically achieved with spraying or dipping the plates with chromogenic reagents in strong acids, e.g. N-(1-naphythyl)ethylenediamine dihydrochloride in sulphuric acid and methanol, urea and phosphoric acid, and diphenylamine-aniline-phosphoric acid in acetone (Bounias, 1980; Reiffová and Nemcová, 2006). If the silica gel is functionalised with amino groups, visualisation in UV light can be achieved by heating the plate to 170 °C due to the *in situ* reaction of carbohydrates with the amino groups (Sanz et al., 2009). Instead of being derivatised for visualisation, the separated carbohydrates can also be isolated from the plates and analysed spectroscopically to provide better insights into their structural characteristics (Dreisewerd et al., 2006).

2.8.2. Liquid chromatography

High performance liquid chromatography (HPLC) and high performance anion exchange chromatography (HPAEC), and size exclusion chromatography (SEC) are three most commonly used liquid chromatography (LC) methods for carbohydrate analysis. In LC, the liquid mobile phase carrying the sample is flushed through a column containing the stationary phase, where the separation occurs as different carbohydrates travel through at different speeds due to the interactions with the stationary phase. There are many different types of column packings that can be used as the stationary phase to achieve carbohydrate separation. The detection of the separated carbohydrates is typically achieved with refractive index (RI) detectors, electrochemical or pulsed amperometric detectors (PAD), and evaporative light scattering detectors (ELSD). However, ultraviolet (UV) and fluorometric detectors (FD) can also be used with post-column derivatisation to include the chromogenic or fluorescent groups respectively. (Hase, 2002; Rassi, 2002; Sanz et al., 2009; Schols et al., 2000; Soga, 2002)

Traditional HPLC techniques of carbohydrate analysis can utilise different column packing materials as the stationary phase, such as alkyl- and aminoalkyl-bonded octadecyl (C_{18}) silica, cyclodextrin, and graphitized carbon phases among others (Sanz et al., 2009). Separation of carbohydrate degradation products, monosaccharides and lighter oligosaccharides are possible. However, the traditional HPLC methods are not well suited for oligosaccharide analysis due to the general lack of resolution and sensitivity (Pińkowska et al., 2011; Sluiter et al., 2008b; Usuki et al., 2008). The retention mechanism is based on the interaction of the packing with the polar materials (including carbohydrates and their degradation products), of which the most polar compounds elute

first, while the lower polarity compounds are more retained. For these columns water and acetonitrile are commonly used as the mobile phase. The detection is typically achieved with RI detection, which make the elution in gradient mode difficult but possible if solvents with the same refractive index are used. (Agblevor et al., 2007; Imanari et al., 2002; Koizumi, 2002; Ouchemoukh et al., 2010; Soga, 2002; Takahashi, 2002)

Anion exchange columns have gained popularity in recent years, and can achieve high resolutions for monosaccharide, oligosaccharide and polysaccharide separation. Carbohydrates with DP up to 80 can be effectively separated {Yang, 2013 #189; Peng, 2012 #104}(Zhang and Lee, 2002). With HPAEC, the sample is carried with highly alkaline mobile phase (typically NaOH and sodium acetate), which strips the carbohydrates into their corresponding oxyanions that can then interact with the stationary phase. Carbohydrates with differences in hydroxyl groups, anomerism, positional isomerism and the degrees of polymerization can be separated. The stationary phase is typically made of non-porous resins such as polystyrene and divinylbenzene or ethylvinylbenzene and divinylbenzene substrates agglomerated with quaternary amine functionalised latex (Sanz et al., 2009). Elution can be performed in gradient mode for increased resolution (Bowman et al., 2011; Kabel et al., 2001). The detection in HPAEC is typically achieved with pulsed amperometric detection (PAD), which can provide very high sensitivity. The mechanism of detection in PAD is based on triple-pulsed voltage through gold or platinum electrodes. The pulsed voltages partly oxidise the products from the mobile phase onto the electrodes during the voltage increase, which is cleared off the electrode with the reduction in voltage. The detection is measured by the current originating from the potential changes. The HPAEC PAD can be coupled with

spectroscopy (LC-MS). (Agopian et al., 2008; Corradini et al., 2012; Feinberg et al., 2009; Kabel et al., 2001; Li et al., 2013; Morales et al., 2008; Schols et al., 2000; Zhang et al., 2012)

With size exclusion columns and size exclusion chromatography (SEC), the separation is based on the molecular dimensions of the carbohydrates relative to the average diameter of the pores of the packing material, which is typically a cross-linked polysaccharide or polyacrylamide. The biggest drawback of size exclusion columns is its inability to separate linkage isomers. The detection of SEC is typically achieved with RI and ELSD and LLSD. Evaporative light scattering detectors (ELSD) atomise the column effluent into small droplets which are evaporated and suspended in atomising gas and diffuse light originating from mono- or polychromatic source. Liquid light scattering detectors (LLSD) respond to laser light scattered by a large molecular weight substance such as oligosaccharides and polysaccharides. (Churms, 2002; Jumel, 2002; Lafosse and Herbreteau, 2002; Pitkänen et al., 2011; Rasmussen and Meyer, 2009)

As the hemicellulose is a polymer with degrees of polymerisation up to 500 (Achyuthan et al., 2010; Xu, 2010), it is difficult to characterise directly. Most published research regarding hemicellulose characterisation use the protocols developed by the National Renewable Energy Laboratory (NREL) (Sluiter et al., 2008c) or Technical Association of the Pulp and Paper Industry (TAPPI) (Templeton et al., 2010), whereby the extracts containing hemicellulose polymers are hydrolysed to the corresponding monomers with sulphuric acid (H_2SO_4) in a two-step process with 72% and 4% H_2SO_4. The protocols suggest analysis with HPLC RI, but other analytical methods can also be used. NREL protocols have been mostly applied to carbohydrate analysis from agricultural sources, TAPPI protocols are usually applied for wood sources. NREL also provides procedures

for determination and isolation of lignin, total solids, extractives, ash and protein (Hames et al., 2008; Sluiter et al., 2008a; Sluiter et al., 2008d); these steps are suggested to be performed before the HPLC-RI analysis if non-carbohydrate content is high (Sluiter et al., 2008b).

2.8.3. Gas chromatography

Gas chromatography (GC) can also be used for monosaccharide and light molecular weight oligosaccharide separation and quantification. However, carbohydrates have relatively low volatility, therefore they need to be derivatised into more volatile compounds prior to analysis. This is achieved by transforming the carbohydrates into their corresponding methyl ethers, acetates, trifluoroacetates, trimethylsilyl ethers, trimethylsilyl oximes, alditol acetates, and aldononitriles (Adams et al., 1999; Bordiga et al., 2012; Hilz et al., 2006; Melton and Smith, 2001; Molnár-Perl, 1999; Molnár-Perl and Horváth, 1997; Sanz et al., 2002; Tisza et al., 1996). In addition to derivatisation, sample preparation steps also need to be performed to remove the insoluble lipids, proteins and other impurities from the samples. The prepared and derivatised samples are vaporised, and with the aid of carrier gas (usually mix of helium and nitrogen) as the mobile phase, injected into capillary columns, which are lined with stationary phase which is typically based on polysiloxanes. Like LC, and TLC, the separation of the compounds occurs due to their interaction with the stationary phase and the resulting difference in travelling speeds. The detection is typically achieved with flame ionisation detector (FID), which measures the ions formed during a combustion in a hydrogen flame. (Carlsson et al., 1992; Mejanelle et al., 2002; Montilla et al., 2006; Ruiz-Matute et al., 2011) The effluent from the column can also be directed to spectroscopic characterisation for more detailed

structural analysis (Fox, 2002; Molnár-Perl and Horváth, 1997; Tisza and Molnár-Perl, 1994).

2.8.4. Capillary electrophoresis

Capillary electrophoresis (CE) is emerging as potentially powerful method of carbohydrate analysis offering high resolution and polysaccharide separation up to 190 DP, as well as monosaccharide separation (Rassi, 2002; Sanz et al., 2009). In CE the separation occurs according to the ionic mobility of the compounds as the sample is carried through a capillary column by the influence of electrical field. The capillary column is typically made of fused silica, and its two ends are immersed in different electrolyte buffer reservoirs containing high voltage electrodes. Although there are many different types of CE, capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC) are commonly used for carbohydrate analysis. In CZE separation is based on the differences in charge to mass ratio, whereas separation and analysis in MEKC is achieved by the addition of micelle forming detergents added to the electrophoretic medium. The detection of the carbohydrates is achieved with RI detectors and electrochemical detectors. (Bao and Newburg, 2008; Hague et al., 2002; Hilz et al., 2006; Karamanos and Hjerpe, 2002; Rassi, 2002)

2.8.5. Spectroscopy

Spectroscopic methods particularly mass spectroscopy (MS) can be coupled with most of the above described analytical techniques because the separation of carbohydrates is not achievable with MS but structural characterisation of individual carbohydrate molecules is very effective. Oligosaccharides have been successfully analysed with electrospray ionisation mass spectroscopy (ESI MS) and matrix assisted laser desorption/ionisation

mass spectroscopy (MALDI MS) with time of flight (ToF) analyser for increased sensitivity. MS can provide information about the molecular structure of the carbohydrates, including linkage types and positions, degree of polymerisation, anomerism, and branching. (Kabel et al., 2001; Park et al., 2012; Reis et al., 2003; Sanz et al., 2009; Wang et al., 1999)

Nuclear magnetic resonance (NMR) spectroscopy can also be used for hemicellulosic carbohydrate analysis, however, its application for structural characterisation is limited compared to mass spectroscopy (Kinoshita et al., 2009). NMR relies on the magnetic properties (spin) of the atomic nuclei (Sanz et al., 2009). ^{1}H and ^{13}C spectrums are typically used for carbohydrate analysis together with different experimental techniques (de Souza et al., 2013; Gjersing et al., 2012; Petersen et al., 2014).

2.8.6. Colorimetric methods

Colorimetric methods exploit the reducing ends (aldehyde or ketone groups) of the monosaccharides, and oligosaccharides to produce compounds that are visible in UV-Vis spectrophotometer. All monosaccharides are reducing sugars, and most of the oligosaccharides have a reducing end at the terminal sugar residue of the chain (Qing et al., 2013). There are two colorimetric methods that are commonly used for reducing sugar detection. They are 3,5-dinitrosalicylic acid (DNS) assay, also known as Douglas method (Rivers et al., 1984), and phenol-sulphuric acid method (Dubois et al., 1956). Although both methods cannot provide precise accuracy and reliability, phenol-sulphuric acid method produces more reliable results than the DNS method (Chi et al., 2009; Masuko et al., 2005). Moreover, DNS method can interfere with amino acids in the samples, giving biased data (Teixeira et al., 2012).

Commercially available enzyme based colorimetric assay kits can also be used for the determination of specific monosaccharides and disaccharides (Megazyme, 2014a; b). For instance, xylose can be quantified by the increase of reduced nicotinamide-adenine dinucleotide (NADH) measured in absorbance at 340 nm, which is formed by the xylose oxidation with the nicotinamide-adenine dinucleotide (NAD+) to xylonic acid in the presence of xylose dehydrogenase (Megazyme, 2014b).

2.9. Characterisation of phenolics and furans

The degradation products of hemicellulosic carbohydrates include phenolics, furfural, 5-HMF, which can further degrade into various organic (acetic, formic, levulinic) acids. Quantification of furfural and 5-HMF is particularly important due to the toxicity of these compounds, which is important for potential applications in food, pharmaceutical and nutraceutical applications. Furthermore, furfural and 5-HMF can also act as fermentation inhibitors in downstream processing (Garda-Aparicio et al., 2006). The lethal dose of furfural that kills 50% of the population (LD_{50}) for furfural is 223 mg/kg (mice) (Castellino et al., 1963), and 1910 mg/kg (mice) for 5-HMF (Abraham et al., 2011). Unlike furfural and 5-HMF, which are exclusively generated by the dehydration of monosaccharides, the phenolics present in the extracts can be formed from different origins at different extraction severities – they can be directly solubilised from the hemicellulose polymers in the form of hydroxycinnamates (Ebringerová, 2005); from lignin in the form of acid soluble lignin (Lu and Ralph, 2010); and from monosaccharide degradation at relatively high extraction severities (Rasmussen et al., 2014).

2.9.1. High performance liquid chromatography

Phenolics from biomass sources are commonly characterised with reverse phase HPLC. Polar octyl (C_8) and octadecyl (C_{18}) silica bonded columns are typically used as the stationary phase with water and acetonitrile as the mobile phase (Lazarus et al., 1999; Marks et al., 2007; Schieber et al., 2003). Separation of furfural, 5-HMF, and different phenolics including hydroxycinnamates is possible with elution in gradient mode (Antas, 2014). The detection is usually achieved with UV/Vis detection; however, fluorescence and RI detection can also be used depending on application. Modern UV/Vis detectors with diode arrays (DAD) allows the measurement in full absorption spectra, which can provide additional information about the analysed compounds (Lee, 2000; Proestos et al., 2005). RI detectors provide lower sensitivity than the UV/Vis, whereas fluorescence detectors are more sensitive but only can be used for detection of compounds with fluorescent properties (Poppe, 1992). As with the LC methods used for carbohydrate analysis, the column effluents can also be directed to MS for detailed structural characterisation (Del Rio et al., 2004; Tomás-Barberán et al., 2001).

2.9.2. Folin-Ciocalteu reagent and colorimetric methods

Total phenolic content (TPC) is often measured with Folin-Ciocalteu reagent (FCR) method, which was originally developed for determination of uric acid (Folin and Denis, 1912), then further developed for determination of phenolic proteins (Folin and Ciocalteu, 1927), until much recently it was updated for analysis of total phenols and other oxidation substrates and antioxidants (Singleton et al., 1999), which is widely followed today (Antas, 2014). Microscale method to accommodate the modern micro-plate spectrophotometers is also developed (Waterhouse, 2001). The FCR is a mixture of

complex polymeric ions formed from phosphomolybdic and phosphotungstic heteropoly acids, which are reduced by phenolics in alkaline conditions (pH 10) to produce molybhenum-tungsten blue complex which has the maximum absorbance at 765 nm (Singleton et al., 1999). It is important that the FCR is present in large excess of the phenolics to avoid overestimation which arises from regenerative repolymerisation reactions of the phenolics at insufficient ionic supply (Singleton et al., 1999). The obtained results are typically compared to gallic acid standard, and the results are thus expressed in gallic acid equivalents (Singleton and Rossi, 1965). Unfortunately, FCR does not react with phenolics exclusively – it reacts with any oxidisable compound, e.g. reducing sugars, proteins, ascorbic acid (vitamin C), sulphites, and sulphur dioxide, which can produce biased results (Antas, 2014). Reducing sugar concentrations have to be at least 25 g/l to have a significant effect on the TPC values (Singleton et al., 1999), and protein interferences can be avoided by the use of trichloroacetic acid (TCA) to remove the phenolic proteins from the samples (Sivaraman et al., 1997). As FCR method effectively measures the reducing capacity of the samples, it can also be considered as antioxidant capacity assay (Antas, 2014).

There are other colorimetric methods for determination of TPC that are used less often that FCR method. Volumetric permanganate titration, which oxidises the phenolics by slow addition of potassium permanganate ($KMnO_4$), which renders the solution golden yellow (Smit et al., 1955). Prussian blue assay or Price and Butler method utilises the reduction of ferricyanide ions by phenolics that produces ferric ferrocyanide ($Fe_4[Fe(CN)_6]_3$), also known as Prussian blue complex (Price and Butler, 1977). Moreover, other assays for determination of antioxidant capacity can be used as a proxy

for TPC estimation. Common colorimetric antioxidant assays are oxygen radical absorbance capacity (ORAC) (Huang et al., 2002), total radical-trapping antioxidant parameter (TRAP) (Ghiselli et al., 2000), trolox equivalent antioxidant capacity (TEAC) (Re et al., 1999), ferric ion reducing antioxidant power (FRAP) (Benzie and Strain, 1996), and 2,2-diphenyl-1-pierylhydrazyl (DPPH) assays (Huang et al., 2005).

2.10. Summary of literature review findings

Prebiotic oligosaccharides are emerging as an effective tool for maintaining the health of gut microbiome and have vast potential in a variety of industries ranging from animal feed to food and nutraceuticals. However, the current production routes are mostly enzymatically based, and therefore are currently expensive, limiting the scope of applications. The extraction of prebiotic oligosaccharides from lignocellulosic biomass have the potential to reduce the production costs, because in this case the raw material – hemicellulose, is abundant in nature. Although hemicellulose extraction has been widely researched, the focus has mostly been on the hemicellulose removal to prepare the biomass for bioethanol production, often disregarding the quality of the extracted hemicellulose products. Moreover, many methods involve the use of harsh chemicals such as strong acids and alkali, which require relatively complex and expensive processes to limit the environmental impact, and which generally produce hemicellulose extracts rich in carbohydrate degradation products.

Hemicellulose extraction with subcritical water has not been fully explored, particularly in terms of optimisation for xylooligosaccharide production with careful control of extraction conditions. Furthermore, sub-critical water extraction is a simple and relatively easily scalable process with low environmental impact, which can be integrated

in the existing and future biorefineries. Furthermore, sub-critical water mediated extractions from different biomass types have been rarely compared, but are required to assess the process flexibility in terms of feedstock selection.

CHAPTER 3. MATERIALS AND METHODS

3.1. Biomass used

The biomass used in this study was *Miscanthus χ giganteus*, known as elephant grass and *Avena sativa* (common oat) husks from five different varieties – three commercial: BALADO (winter), CONWAY (spring), MASCANI (winter); and two experimental: 14355Cn and SO-I, which were bred for low lignin contents. The air-dried biomass was provided by Phytatec Ltd. (UK), and was stored in well ventilated area at room temperature. The lignocellulose and extractives composition of the biomass was determined using the methodology described in this chapter.

3.2. Raw material analysis

The raw Miscanthus and oat husk biomass was analysed for lignin, hemicellulose, cellulose and extractives contents by adapting NREL protocols (Hames et al., 2008; Sluiter et al., 2008a; Sluiter et al., 2008c). The characterisation methods used for raw biomass were different from the methods used for extract analysis as the NREL protocols are developed for characterisation of solid biomass.

3.2.1. Determination of groat count

Groat count was determined to estimate the efficiency of de-hulling of the oat husks, and was achieved by randomly selecting 50 mL of husks from well-mixed biomass, and counting the total number of husks, and husks with enclosed groats. The result was expressed as husks with groats per 100 husks. The procedure was repeated in triplicate for husks from all oat varieties.

3.2.2. Biomass particle size reduction

To obtain a uniform and small particle size distribution for effective biomass hydrolysis, the biomass was first frozen with liquid nitrogen (approx. -200 °C), and then ground with a 400 W Moulinex Vitamix Y42 blender, followed by sieving with 1400 μm mesh sieve. The process was repeated multiple times until no biomass was retained on the 1400 μm sieve.

3.2.3. Determination of extractives

Extractives, consisting of non-structural biomass contents, were determined by following a protocol from National Renewable Energy Laboratory (NREL) (Sluiter et al., 2008d). The ground biomass was placed inside a previously weighed cellulose thimble (Whatman, 26×60 mm, 1.5 mm thick) and weighed. A biomass sample was also taken to determine its dry solid weight. The thimble with biomass was then placed inside a standard Soxhlet extractor, which was operated with distilled water for 16 h to extract the water-soluble extractives from the biomass. After 16 h, the water was replaced with absolute ethanol (Sigma), and run for further 16 h to extract the ethanol soluble extractives. The Soxhlet extractor was operated with 4-5 siphoning cycles per hour. After the extraction, the thimble with biomass was dried at 55 °C for 72 h, and weighed again. The extractives contents were then calculated by using Equation 3-1,

$$\%Extr = \frac{w_{t2} - w_{te}}{(w_{t1} - w_{te})\%TS} \cdot 100\% \qquad \text{Equation 3-1}$$

where w_{t1} is the weight of the thimble with biomass before the extraction (g); w_{t2} is dry weight of the thimble with biomass after the extraction (g); w_{te} is weight of thimble (g); and %TS is total solids content of the untreated biomass (%).

3.2.4. Two-step acid hydrolysis for carbohydrate and Klason lignin analysis

The protocol for determination of carbohydrate contents was adapted from NREL (Sluiter et al., 2008c). This protocol quantifies the hemicellulose and cellulose contents of the biomass by first hydrolysing the carbohydrate polymers to monomers via a two-step acid hydrolysis, and then quantifying the monomers using analytical techniques for monosaccharide quantification. The lignin content is estimated from the acid insoluble fraction of the biomass. First, glass filtering crucibles (Pyrex, borosilicate, porosity 4) were placed in a muffle furnace at 575 °C for 4 h, then cooled to room temperature in a desiccator and weighed. 300 mg of the extractives free biomass was placed in borosilicate glass tubes, followed by 4.92 g of 72% sulphuric acid (Fluka) and mixed with borosilicate glass rod. The tubes were then placed in a water bath set at 30 °C, and incubated for 60 min with intermittent stirring every 5 min.

After the first hydrolysis step, the tubes were removed from the water bath and transferred into a 100 mL Duran bottles. The acid was diluted to 4% concentration by adding 84 mL of distilled water, the bottles were tightly capped and placed inside an oven at 120 °C for 60 min. After the second hydrolysis step, the bottles were cooled at room temperature for 30 min, and vacuum filtered in the pre-weighed glass filtering crucibles. The filtrate was stored in a freezer (-20 °C) and later used for carbohydrate analysis, while the residue left in the crucibles was washed with distilled water and dried at 105 °C for 6 h, and eventually cooled to room temperature in a desiccator before being weighed again. The crucibles were then placed in a muffle furnace at 575 °C for 4 h, cooled in a desiccator to room temperature and weighed again. The procedure was repeated at least in triplicate for each biomass type.

The acid insoluble or Klason lignin was then calculated using the Equation 3-2,

$$\%Lig = \frac{(w_{c1} - w_{ce}) - (w_{c2} - w_{ce})}{w_b \%TS_b} \cdot (100\% - \%Extr) \qquad \textit{Equation 3-2}$$

where w_{ce} is the weight of empty crucible (g); w_{c1} is weight of dry crucible and biomass after the two-step hydrolysis (g); w_{c2} is weight of ash and crucible after the muffle furnace step (g); w_b is the weight of extractives free biomass used in the analysis (g); $\%TS_b$ is the total solids content of the extractives free biomass (%); and %Extractives is the extractives content of the raw biomass (%).

Filtrates were analysed for xylose and glucose contents to estimate hemicellulose and cellulose composition of the biomass using colorimetric enzyme kits for glucose and xylose quantification (Megazyme). Enzyme kits were used instead of HPAEC due to a lack of access to the HPAEC. First, 0.475 mL of 50% sodium hydroxide (Fisher Scientific) was added to 3 mL of the filtrates, and the resulting mixture was analysed for glucose and xylose contents, following scaled-down protocols provided with the kits (Megazyme, 2014a; b), and repeated at least in triplicates. The obtained xylose and glucose concentrations were adjusted for dilution. Hemicellulose and cellulose contents of the biomass were then calculated using Equation 3-3 and Equation 3-4 respectively,

$$\%Hemicel = \frac{c_{xyl} V_f f_{xyl}}{w_b \%TS_b} \cdot (100\% - \%Extr) \qquad \textit{Equation 3-3}$$

$$\%Cel = \frac{c_{glu} V_f f_{glu}}{w_b \%TS_b} \cdot (100\% - \%Extr) \qquad \textit{Equation 3-4}$$

where c_{xyl} is xylose concentration in the filtrate (g/L); c_{glu} is the glucose concentration in the filtrate (g/L); V_f is the volume of the filtrate (L); f_{xyl} is the anhydro correction factor

for pentoses (0.88, dimensionless); f_{glu} is the anhydro correction factor for hexoses (0.90, dimensionless); w_b is the weight of extractives free biomass used in the analysis (g); $\%TS_b$ is the total solids content of the extractives free biomass (%); and %Extractives is the extractives content of the raw biomass (%).

3.3. Hemicellulose and oligosaccharide extraction from biomass

Extraction vessels of similar sizes were used during this study for different purposes. Virgin and pre-treated extractions, discussed in Chapter 4, 5 and 6 were carried out in a 0.5 L vessel, the pre-treatment was done in 5 L vessel, and sequential extract hydrolysis discussed in Chapter 7 was performed in 20 mL reactors.

3.3.1. Biomass preparation

Prior to virgin extractions with 0.5 L and pre-treatment with 5 L vessels, the necessary amount of biomass was first imbibed in a pre-determined volume of distilled water at 50 °C for 30 min. Before the imbibing biomass samples were taken for determination of total dry solids content. After the imbibing, the biomass was blended with a 400 W Moulinex Vitamix Y42 blender for total of 3 min, intermittently pausing every 30 s to rearrange the biomass and allow the blender to cool down. The biomass was blended in single batch for 0.5 L extraction, and in six batches for 5 L extraction. After the blending, the biomass was ready for the extractions. Biomass preparation as described here resulted in average particle size of 1.21±0.03 mm for Miscanthus and 0.91±0.03 mm for oat husks; particle size distributions of Miscanthus (MIS) and Balado (BAL) husks obtained by sieving are shown in Figure 3-1 (see section 3.3.2. below). For pre-treated biomass extractions with 0.5 L vessel, the pre-treated biomass was imbibed in distilled water at room temperature for 10 min before the extractions.

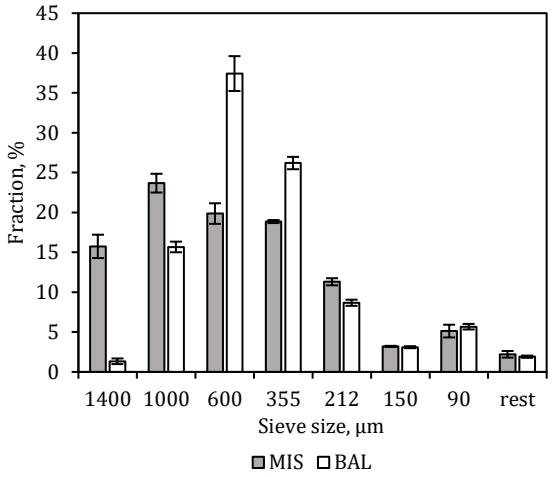

Figure 3-1: Particle size distribution of MIS and BAL husk biomass obtained by sieve analysis after imbibing and blending; error bars represent standard error of the mean based on three replicates.

3.3.2. Particle size determination

Particle size distribution was determined by first drying the imbibed and blended biomass-water mixture at 55°C for 72 h until no weight change was observed, measured with Sartorius R160P Research balance (standard deviation, ±0.02 mg), and then passing it through a set of sieves with different mesh sizes (90, 150, 212, 355, 600, 1000, 1400 µm), and weighing the retained solids in each sieve. The average particle size was determined using Equation 3-5,

$$P = \frac{\sum x_i d_i^4}{\sum x_i d_i^3} \qquad \textit{Equation 3-5}$$

where P is the average particle size (µm); x_i is mass fraction retained on sieve i (dimensionless); and d_i is the mesh size of sieve i (µm).

3.3.3. Extraction with 0.5 L vessel

A Parr 4575 high pressure stirred batch reactor with 1400 W ceramic heating jacket and Parr 4836 controller was used for this extraction. The extraction vessel volume was 0.5

L with inside diameter of 6.4 cm, and inside depth of 16.8 cm. The 200 mL of the prepared biomass mixture was placed in the 0.5 L vessel. The vessel was then sealed and heating jacket was fitted. Stirring was achieved with six-blade impeller with 3.5 cm diameter. Impeller speed was set at 360 rpm, and then N_2 gas (BOC) was used to purge the vessel for 30 s, before pressurising it to 50 bar. At this point the desired temperature was set in the controller. The heat-up profile of the reactor is shown in Figure 3-3. Extraction residence time count was started once the temperature set-point was achieved. The temperature during the extraction was controlled by adjusting the flow of coolant connected to a cooling bath set at -4 °C (see Figure 3-2 for schematic diagram of the extraction setup).

Once the desired extraction residence time was achieved, the heating jacket was removed, the coolant line was fully opened, and the reaction vessel was submerged in an ice bath until the internal temperature of the vessel had reached 50 °C. This was achieved in less than 2 min, depending on the extraction temperature. The extraction vessel was then depressurised by opening the purge line, and then opened. The extraction mixture was then passed through sieve with 45 μm mesh size. Once separated, the extract pH was measured, and a sample was taken for determination of extract mass concentration. The extract was stored in a freezer at -20 °C until further analysis. Residual biomass was then washed with distilled water, and dried at 55 °C for 72 h, after which its total dry solids content was determined.

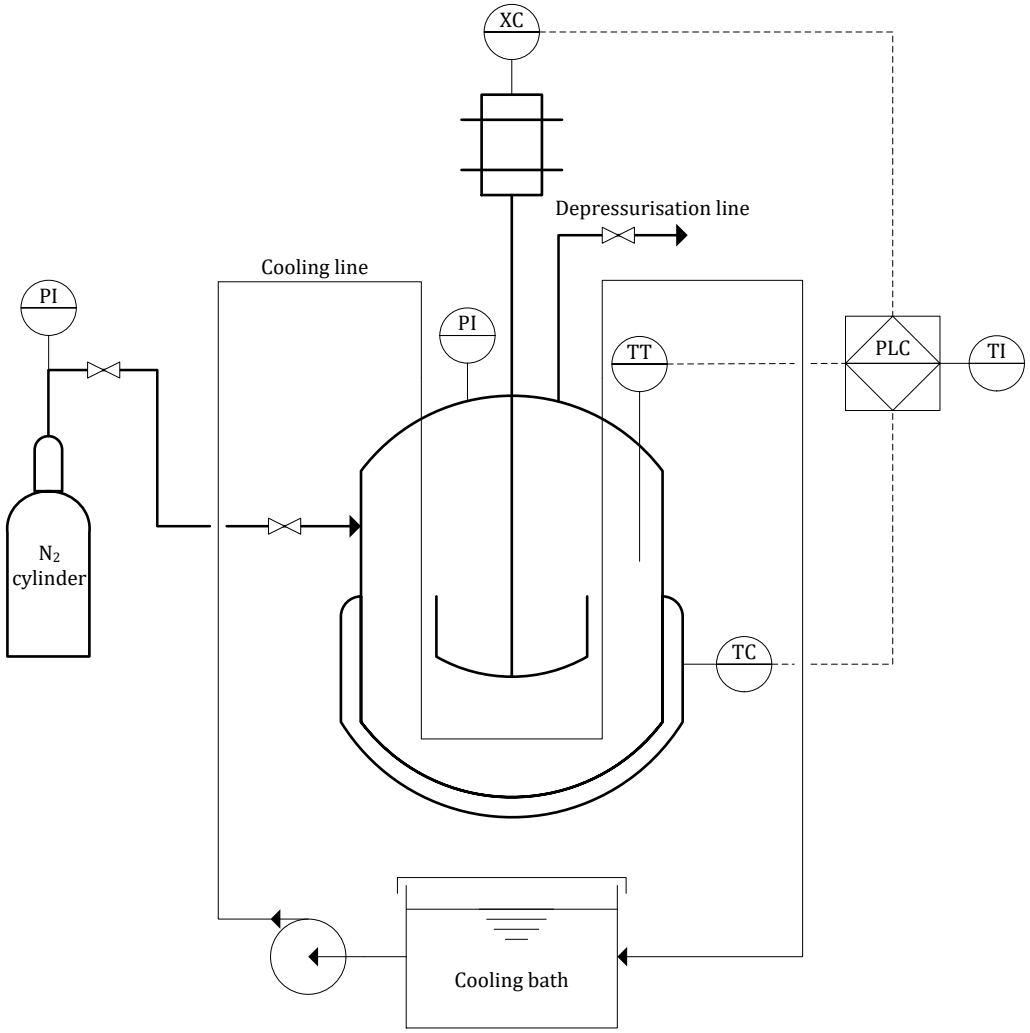

Figure 3-2: Schematic diagram of the 0.5 L and 5 L extraction setups

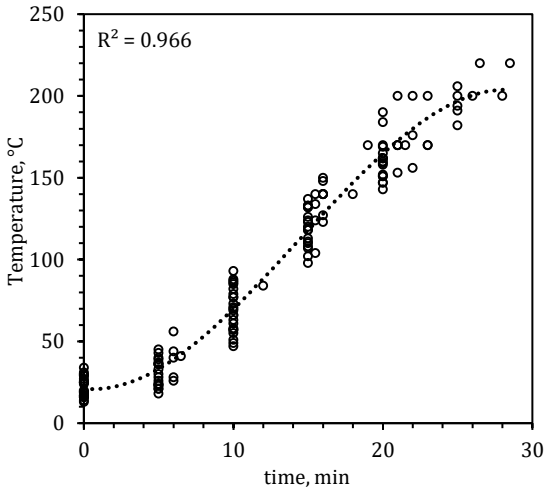

Figure 3-3: Heat-up curve of the 0.5 L vessel (dotted line represents the cubic regression fit to empirical data)

3.3.4. Pre-treatment with 5 L vessel

The pre-treatment procedure in 5 L vessel (Parr 4582) was a scaled up extraction of the 0.5 L extraction (see Figure 3-2). The inside diameter of the extraction vessel was 14 cm, with inside depth of 38.1 cm. 3 L of the prepared biomass and distilled water mixture was placed in the vessel, enclosed in a heating jacket. After sealing, the vessel was purged with N_2 (BOC) for 1 min, and was pressurised to 50 bar. Six-blade impeller (8.9 cm diameter) was used with rotation speed of 50 rpm. Temperature was set at 120 °C with Parr 4836 controller. Heat-up profile is shown in Figure 3-4. Heating was achieved through 2800 W ceramic heating jacket. After reaching the temperature set-point, the count of extraction residence time was started. The temperature of the extraction was controlled by adjusting the flow of coolant from -4 °C cooling bath. After 30 min, the cooling line was fully opened, the heating jacked turned off, and the extract was collected through sample line, which passed through a coil submerged in -4 °C coolant bath. The vessel was depressurised during the extract collection. Afterwards, the vessel was opened and taken out of the cooling jacket. The biomass was then removed from the vessel, passed through a 45 μm mesh sieve, and dried at 55 °C for 72 h, after which its total dry solids content was determined.

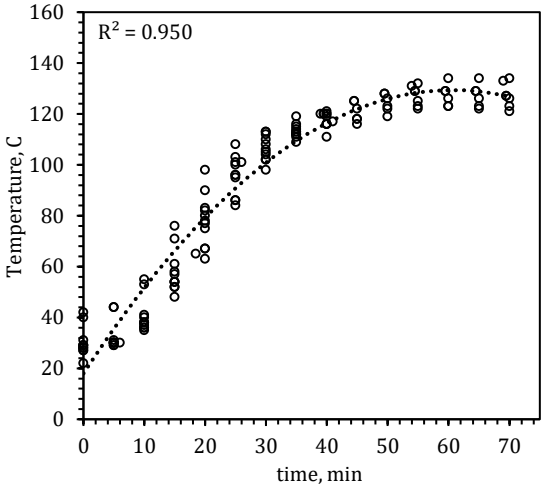

Figure 3-4: Heat-up curve of 5 L vessel (dotted line represents quadratic regression fit to the empirical data)

3.3.5. Sequential extract hydrolysis with 20 mL vessel

Sequential extract hydrolysis was performed in 20 mL tube-type reactors built from stainless steel tubes and caps (Swagelok). The dimensions of the vessels were 11.4 cm length, 1.5 cm internal diameter, 0.3 cm thickness. The extract was diluted with distilled water to 10 g/L concentration and transferred into the reactor, which was then closed and placed inside a GC oven (HP 5890 series II), together with another vessel filled with distilled water, and connected to a thermocouple. The second reactor was used for temperature control. The heat-up profile of the reactor is shown in Figure 3-5. As with other vessels, extraction residence time count was initiated once the target temperature was met. After the extraction, the vessels were placed into an ice bath for approximately one minute until internal temperature of 50 °C was reached. Extract pH was measured, and the extracts were stored in freezer (-20 °C) until further analysis.

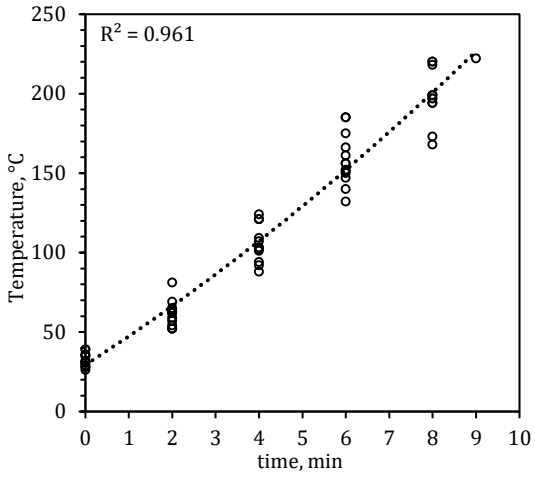

Figure 3-5: Heat-up curve of 20 mL extraction tubes (dotted line represents quadratic regression fit to the empirical data)

3.3.6. Determination of biomass total dry solids contents

Eppendorf tubes (2 mL) were placed in drying cabinet at 55 °C for at least 3 h, and cooled in humidity controlled desiccator to room temperature, before being weighed. Approximately 1.5 mL of the biomass was then placed inside the tubes, weighed, and the open Eppendorf tubes with the biomass were placed inside the drying cabinet, set at 55 °C for 72 h until no change in weight was observed. After the drying period, the tubes were cooled to room temperature in desiccator and weighed again. The procedure was repeated at least in triplicate for all biomass types. The total dry solids content (%TS) was calculated using the Equation 3-6,

$$\%TS = \left(1 - \frac{w_{ts1} - w_{ts2}}{w_{ts1} - w_{tse}}\right) \cdot 100\% \qquad \textit{Equation 3-6}$$

where w_{tse} is weight of empty Eppendorf tube (g); w_{ts1} is weight of Eppendorf tube with 'wet' biomass (g); and w_{ts2} is weight of Eppendorf tube with dry biomass (g).

3.3.7. Determination of extract mass concentration

Eppendorf tubes (2 ml) were dried at 55 °C and cooled to room temperature in a desiccator before being weighed. 1 ml of extract was pipetted into the Eppendorf tube using P1000 micropipette. The filled tubes were then placed in drying cabinet, set at 55°C for 72 h until no change in weight was observed. The tubes were then cooled to room temperature in desiccator, and weighed. The procedure was repeated at least in triplicate. The extract mass concentration (c_{extr}) was then calculated using Equation 3-7,

$$c_{extr} = \frac{w_{mc1} - w_{mce}}{V_{mc}} \qquad \textit{Equation 3-7}$$

where w_{mce} is weight of empty Eppendorf tube (g); w_{mc1} is weight of Eppendorf tube containing dried extract (g); V_{mc} is the volume pipetted into the Eppendorf tube (L).

3.3.8. Determination of biomass solubility

Solubility expresses the fraction of raw biomass solubilized in the extract. To obtain the solubility, the biomass was weighed before the extraction. After the extraction, the residual biomass was dried at 55 °C for 48 h until no weight change was observed. The dried residual biomass was then weighed, and the solubility was calculated using Equation 3-8,

$$\%\mathrm{Sol} = \frac{(\mathrm{w_{b1}}\%\mathrm{TS_{b1}} - \mathrm{w_{b2}})}{\mathrm{w_{b1}}\%\mathrm{TS_{b1}}} \cdot 100\% \qquad \textit{Equation 3-8}$$

where w_{b1} is the weight of biomass before the extraction (g); $\%TS_{b1}$ is the total solids content of the biomass used prior to extraction; and w_{b2} is the weight of residual biomass after the extraction (g).

3.4. Extract hemicellulose precipitation with ethanol

Hemicellulose was precipitated from the extracts with ethanol. The extracts were transferred to 50 ml centrifugation tubes (Fisher), and absolute ethanol (Fluka) was added to reach 60% (v/v) final ethanol concentration relative to extract volume. The tubes were closed and vigorously shaken, and left to stand at 4 °C for 60 min. The tubes containing ethanol-extract solution were then centrifuged for 10 min at 10 000 rpm using Sigma 3K30 centrifuge. After the centrifugation, the supernatant was decanted from the precipitate, and its mass concentration was determined. The supernatant was stored in a freezer at -20 °C. The precipitate was air-dried at well ventilated area for 72 h, and weighed. The process was repeated in triplicates for all tested extracts. The recovered precipitate and supernatant fractions were calculated using Equation 3-9 and Equation 3-10 respectively,

$$\%PrecRecovery = \frac{w_p}{c_{extr}V_{extr}} \cdot 100\% \qquad \textit{Equation 3-9}$$

$$\%SupRecovery = \frac{c_s f_d}{c_{extr}} \cdot 100\% \qquad \textit{Equation 3-10}$$

where w_p is weight of recovered precipitate (g); c_{extr} is the mass concentration of the extract (g/L); V_{extr} is volume of extract used for precipitation (L); c_s is mass concentration of supernatant (g/L); and f_d is the dilution factor (2.5 for 60% ethanol precipitation, dimensionless).

The effects of other ethanol concentrations (40%, 60%, and 80%) and standing temperatures (-20 °C, 4 °C, and 50 °C) were also tested (see Figure 3-6). Highest precipitate recoveries were achieved at 80% ethanol concentration and -20 °C standing temperatures. However, for economic reasons and convenience, the precipitation was

done at 60% (v/v) ethanol and 4 °C standing temperature for all extracts studied in this work.

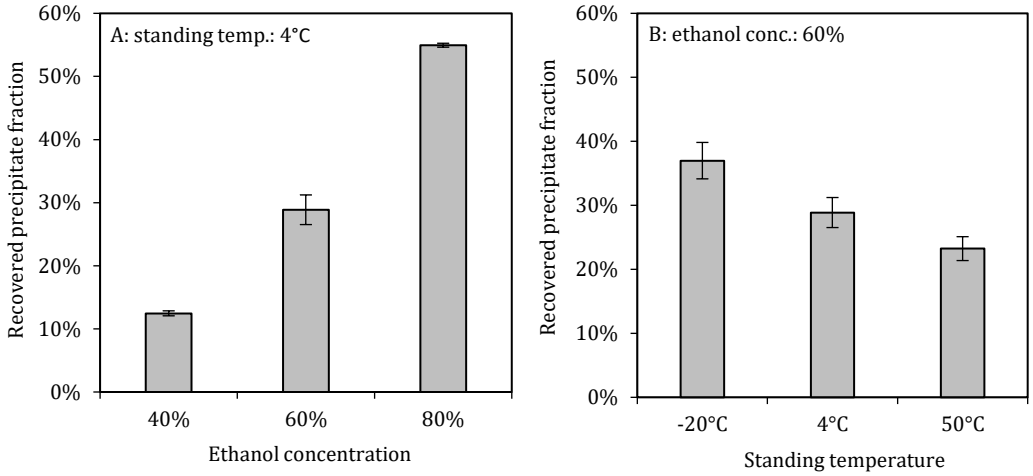

Figure 3-6: Effect of ethanol concentration (A) and standing temperature (B) before centrifugation on the precipitated fraction recovery; study based on the same extract

3.5. Characterisation methods

3.5.1. Enzyme kits for xylose and glucose quantification

Megazyme colorimetric kits for enzymatic glucose and xylose quantification were used for cellulose and hemicellulose estimation of the raw biomass by analysing the hydrolysates from the two-step acid hydrolysis of the biomass. Xylose was quantified by the increase of the reduced nicotinamide-adenine dinucleotide (NADH) measured for absorbance at 340 nm, which is formed by xylose oxidation with the nicotinamide-adenine dinucleotide (NAD^+) to xylonic acid in the presence of xylose dehydrogenase at pH 7.5 (Megazyme, 2014b). As xylose dehydrogenase also acts on glucose, hexokinase needs to be added to remove the glucose present in the samples (Megazyme, 2014b). Glucose was quantified by the increase of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) also measured at 340 nm, which is formed in two steps. First, glucose is phosphorylated with hexokinase and adenosine-5'-triphosphate (ATP) to glucose-6-

phosphate (G-6-P) with simultaneous formulation of adenosine-5'-diphosphate (ADP). Then, in the presence of G-6-P dehydrogenase, the G-6-P is oxidised by nicotine-adenine dinucleotide phosphate ($NADP^+$) to produce NADPH and gluconate-6-phosphate (Megazyme, 2014a).

The filtrates with added NaOH from the two-step acid hydrolysis were diluted, and 20 µl transferred to 850 µL UV grade plastic cuvettes (Brand) using P100 micropipette. 0.4 mL of distilled water was added to the cuvettes with P1000 micropipette. For determination of xylose, 80 µL of buffer solution, 80 µL of NAD+/ATP solution were added with P100 micropipette, followed by 4 µL of hexokinase suspension with P20 micropipette. For glucose determination, 20 µL of buffer solution and 20 µL of NADP+/ATP was added to the cuvettes with P100 micropipette. The cuvettes were capped, and vigorously shaken, and incubated for 5 min at room temperature. The absorbance of the cuvettes containing the mixtures was then measured at 340 nm with Cecil Aquarius CE7500 spectrophotometer. Then 10 µL of xylose dehydrogenase solution was added to cuvettes for xylose analysis, and 4 µL of G-6-P dehydrogenase containing suspension to cuvettes for glucose analysis with P20 micropipette. The cuvettes were again capped and shaken, and incubated at room temperature for further 6 min. The absorbance was then measured again. The same procedure was repeated for xylose and glucose standards and blanks. Absorbance was then calculated using Equation 3-11,

$$\Delta A_{xyl/glu} = (A_2 - A_1)_{xyl/glu} - (A_2 - A_1)_{blank} \qquad \textit{Equation 3-11}$$

where $\Delta A_{xyl/glu}$ is absorbance difference used for concentration calculation (AU); A_1 and A_2 are first and second absorbance measurements of the samples (AU).

Standards were used to generate standard curves (see Appendix C), which were used to determine final glucose and xylose concentration of the filtrates, calculated using Equation 3-12,

$$c_{xyl/glu} = \frac{\Delta A_{xyl/glu} f_d f_{xyl/glu}}{slope_{xyl/glu}} \qquad \textit{Equation 3-12}$$

where $\Delta A_{xyl/glu}$ is the absorbance difference between the two measurements (AU); f_d is dilution factor (dimensionless); $f_{xyl/glu}$ is anhydro correction factor (0.88 for xylose, and 0.90 for glucose, dimensionless); and $slope_{xyl/glu}$ is the slope of standard calibration curve (AU per g/L).

3.5.2. Mild acid hydrolysis for polysaccharide estimation

Single step mild acid hydrolysis (0.65 M H_2SO_4) was used to hydrolyse the polysaccharides in the extracts to the corresponding monomers in order to estimate the extract polysaccharide contents by quantifying the monosaccharides in the extract hydrolysates. For this purpose, 7 mL borosilicate glass vials with caps were used. Enough extract or arabinoxylan standard (Megazyme) was transferred to the vial to achieve 10 mg solid load per vial using P1000 micropipette; the precise volume of extract to be transferred was calculated from extract mass concentration. Then distilled water was added to reach the total volume of 4.759 mL, followed by 0.241 mL of 72% sulphuric acid (Fluka) to reach the final volume of 5 mL. The vials were capped and shaken before being placed inside a previously preheated oven, set at 100 °C, for 60 min. The vials were shaken every 15 min during the hydrolysis. After the hydrolysis, the vials were taken outside the oven and allowed to cool for 15 min at room temperature before opening them and adding 0.2 mL of 50% sodium hydroxide (Fisher Scientific). The vials were then capped again

and mixed, and analysed with HPAEC-PAD for monosaccharide contents. With every batch of hydrolysis arabinoxylan standard was also hydrolysed and used as a control to take into account incomplete hydrolysis. Hydrolysis was repeated in triplicate for every sample. Polysaccharide concentrations (c_{poly}) were then calculated using Equation 3-13,

$$c_{poly} = \frac{c_{mono} f_d f_a}{f_h} \qquad \textit{Equation 3-13}$$

where c_{mono} is the monosaccharide concentration of the hydrolysate measured by the HPAEC-PAD (g/l); f_d is the dilution factor due to the hydrolysis (dimensionless); f_a is the anhydro correction factor (0.88 for pentoses and 0.90 for hexoses, dimensionless); and f_h is the hydrolysis correction factor, which is effectively the hydrolysis yield of AX standard control (typically above 0.90, dimensionless).

Optimal residence time for the hydrolysis at 100 °C was determined by comparing the AX standard hydrolysis yields at different residence times. As can be seen from Figure 3-7, the optimal residence time was found to be 60 min, where above 95% of the original AX standard was hydrolysed.

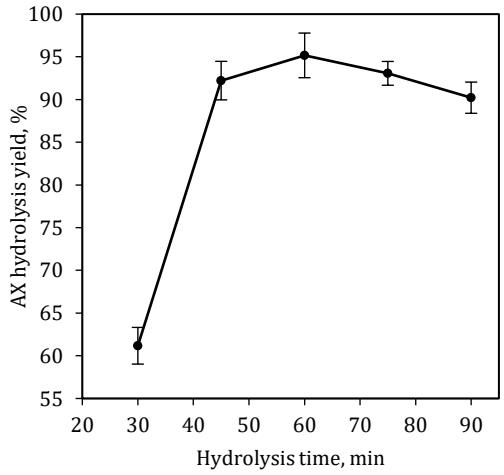

Figure 3-7: Yields of arabinoxylan (10 mg) hydrolysis with 0.65 M H_2SO_4 at 100 °C and varying residence times; error bars represent standard error of the mean of triplicates

3.5.3. HPAEC-PAD for carbohydrate analysis

High performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) was used for monosaccharide and oligosaccharide separation and quantification from the extracts and extract acid hydrolysates. ICS-5000 system (Dionex/ThermoFisher) was used, consisting of autosampler, dual gradient pump, and detector/chromatography modules. CarboPak PA1 column (4x250 mm) with CarboPak PA1 guard (4x50 mm) was used, and kept at 30 °C during the analysis. Disposable gold working electrode and Ag/AgCl reference electrode were used for the PAD, and kept at 25 °C during the analysis. The detector was set to quadruple potential waveform. Following solvents were prepared and used as eluents for the mobile phase: Milli-Q water (18.2 MΩ cm^{-1} at 25 °C); 200 mM NaOH, prepared from 50% analytical grade NaOH (Fisher Scientific), and 1 M NaOAc, prepared from anhydrous electrochemical grade sodium acetate (Thermo Scientific). All solvents were sonicated for 10 min before use. Extract samples were diluted with distilled water where necessary to reach the linear calibration range; injection volume was 10 μL.

The mobile phase flow rate was 1 mL/min, and its composition profile throughout the method was as follows. For the first 20 min method was ran isocratically with 21 mM NaOH. This concentration was found to be optimal for monosaccharide separation. At minute 20, NaOH concentration was increased from 20 mM to 80 mM and kept constant until minute 60. At the same time, NaOAc was introduced in linear gradient mode from 0 mM at minute 20 to 200 mM at min 60; during this time oligosaccharide separation occurred. At minute 60, NaOH and NaOAc concentrations were increased to 120 mM and 400 mM respectively, and were kept constant for 10 min in order to flush the column.

Lastly, the flow was set to isocratic 21 mM NaOH concentration for 25 min to re-equilibrate the column for the next injection.

Carbohydrates present in the samples were identified by the retention times (RTs) of a set of standards. The following carbohydrate standards were used – arabinose, galactose, glucose, xylose, fructose (Sigma Aldrich), and xylobiose, xylotetraose, and xylohexaose (Megazyme). RTs for xylotriose and xylopentaose were estimated by logarithmically interpolating the RTs of the oligosaccharide standards (see Figure 3-8 A); the estimated RTs agreed with the RTs of peaks between the oligosaccharide standard peaks, which were likely xylotriose and xylopentaose. Carbohydrate RTs are summarised in Table 3-1. Different concentrations of standards were used to obtain areas of the chromatogram peaks, from which linear calibration curves were obtained (shown in Appendix C.2). The calibration curve slopes of xylotriose and xylopentaose were estimated by logarithmically interpolating between the slopes of the oligosaccharide standards (see Figure 3-8 B). Extract concentrations of monosaccharides and oligosaccharides were then determined using the calibration curves, and adjusted for the dilution. Polysaccharide contents were estimated from the results of monosaccharide concentrations of extract hydrolysates, as described in section 3.5.2.

Table 3-1: Monosaccharide and oligosaccharide retention times

	RT, min		RT, min
Arabinose	9.7	Xylobiose	28.1
Galactose	12.8	Xylotriose	32.5
Glucose	13.7	Xylotetraose	34.7
Xylose	15.2	Xylopentaose	36.7
Fructose	18.3	Xylohexaose	38.5

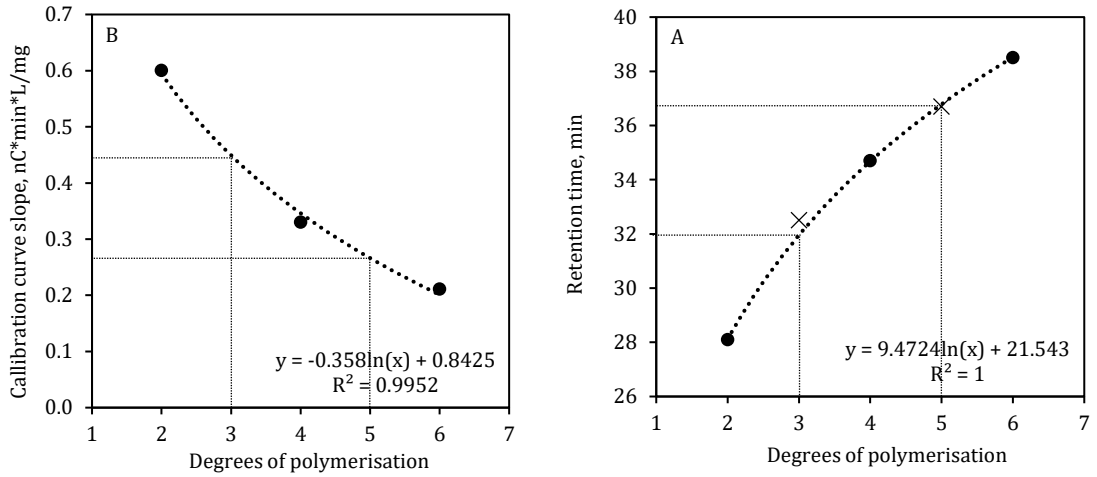

Figure 3-8: Interpolation of xylotriose and xylopentaose retention times (A) and calibration slopes (B); filled circles represent values from standards; crosses in B are retention times of peaks between the standard peaks, likely xylotriose and xylopentaose

3.5.4. HPLC for furan analysis

Qualitative and quantitative analysis of furfural and 5-HMF in the extracts was performed using reverse phase high performance liquid chromatography (HPLC). Shimadzu HPLC system was used consisting of SIL-10AD autosampler, LC10AD dual pump module, DGU-14A degasser, CTO-10AS oven, and SPD-10Avp UV detector. The UV detector was set to 280 nm wavelength. The column used for furan analysis was Phenomenex Prodigy 5 µ ODS3 100A (250x4.6 mm) with guard, which was kept at 40 °C. Solvents were prepared with water (HPLC grade, Chromasolv Plus), acetonitrile (HPLC grade, Chromasolv Plus), and acetic acid (99.8-100.5%, Fisher Scientific). Solvents used were prepared in following concentrations (v/v): (A) 2% acetic acid in water; (B) 50% water, 49.75% acetonitrile, 0.25% acetic acid; (C) acetonitrile. All solvents were sonicated for 10 min prior use to remove air from the liquid phase.

The analytical method was adopted from (Antas, 2014) but was modified for furan analysis only and was as follows: at 0-20 min linear gradient increase in solvent B from

10-55%, and corresponding decrease in A from 90-45%; followed by a linear gradient increase of B at 20-30 min from 55-100%, and corresponding decrease of A from 45-0%. After 30 min column was flushed with 100% C for 10 min; followed by reset to original conditions, i.e. 10% B and 90% A for 10 min to re-equilibrate the column for the next injection. Sample injection volume was 10 µL, and mobile phase flow rate was 1 ml/min. Extract samples were diluted with distilled water prior to injection to reach the linear calibration range. Different concentrations of furfural (RT 10.8 min) and 5-HMF (RT 7.8 min) standards (Sigma Aldrich) were analysed in order to generate the calibration curves (see Appendix C), which were used to quantify the amount of furans in the samples.

3.5.5. Estimation of total phenolic content with Folin-Ciocalteu reagent

The method for estimating total phenolic content (TPC) in the extracts was adapted from (Antas, 2014) and (Waterhouse, 2001). TPC was estimated using Folin-Ciocalteu reagent, and the results were expressed in gallic acid equivalents as the data from the extracts were compared with gallic acid (GA) standard (Sigma Aldrich). The calibration curve is shown in Appendix C. Apart from Folin-Ciocalteu reagent (Sigma Aldrich), following solutions were also used: 100% (w/v) 2,2,2-trichloroacetic acid solution (TCA), prepared from 99+% TCA (Fisher Scientific); and saturated sodium carbonate solution, prepared from anhydrous 99.95-100.05% sodium carbonate (Sigma Aldrich).

As proteins interfere with Folin-Ciocalteu reagent (Singleton et al., 1999), protein precipitation of the extracts was performed using TCA as follows. 800 µL of extract was transferred to Eppendorf tube with P1000 micropipette, followed by 120 µL of TCA with P200 micropipette. Tubes containing the extracts were capped and placed at -20 °C for 5 min, followed by further 15 min at 4 °C. Afterwards, the Eppendorf tubes were

centrifuged in Sigma 3K30 refrigerated centrifuge at 4 °C and 15000 g for 15 min. The supernatant was decanted and used in further analysis. Then the supernatants were diluted with distilled water to concentrations that produced TPC values within the linear range of the GA calibration curve. 20 µL of the diluted supernatant, GA standard or blank were transferred to 3 mL glass test tube with P100 micropipette. 1.58 mL of distilled water was then added to the test tube with P1000 micropipette, followed by 100 µL of Folin-Ciocalteu reagent with P200 micropipette. The tubes were then stirred with vortex mixer (MixiMatic Jencons) and incubated for 8 min.

Then, 300 µL of 20% (w/v) sodium carbonate solution was added to the tubes, and the tubes were stirred again using the vortex mixer, and incubated in a water bath, set at 40 °C for 30 min. After the incubation, 300 µL of the mixture were transferred to a clear 96 well microplate (Sterilin). Each sample was replicated in triplicate. The microplate containing the samples was analysed for absorbance at 750 nm in Promega Glomax microplate spectrophotometer. The final TPC of the extracts were then determined from the absorbance values of the extracts and GA calibration curves, the results were adjusted for dilution, and expressed as grams of gallic acid equivalents per litre (gGAeq/L).

Apart from the interferences with proteins, Folin-Ciocalteu reagent also interferes with reducing sugars and correction factors need to be introduced at reducing sugar concentrations above 25 g/L (Singleton et al., 1999). Reducing sugar concentration in the tested extracts was significantly below this threshold, therefore correction factors were not applied.

3.6. Other calculations

3.6.1. Contents

Carbohydrate contents were calculated from carbohydrate concentrations obtained with the HPAEC-PAD. Equation 3-14 was used for carbohydrate content calculations of virgin extractions, and Equation 3-15 for pre-treated extractions,

$$\mathrm{Cont}_{i_{virgin}} = \frac{c_i V_{extr}}{\mathrm{w_b}\%\mathrm{TS_b}} \qquad \textit{Equation 3-14}$$

$$\mathrm{Cont}_{i_{pretr}} = \frac{c_i V_{extr}\left(1 - \frac{\%Sol_{pretr}}{100}\right)}{\mathrm{w_b}\%\mathrm{TS_b}} \qquad \textit{Equation 3-15}$$

where c_i is the concentration of compound i (g/L); V_{extr} is volume of extract liquid phase (L); w_b is weight of the biomass used in extraction (g); %TS is total solids content of the biomass; $\%Sol_{pretr}$ is pre-treatment solubility. The results were therefore expressed as grams recovered per gram of raw biomass.

3.6.2. Carbohydrate yields

Carbohydrate yields were calculated from carbohydrate contents using Equation 3-16,

$$\mathrm{Yield}_{xyl/xos} = \frac{Cont_{xyl/xos} f_{xyl/xos} \cdot 100\%}{\%Hemicel} \qquad \textit{Equation 3-16}$$

where $cont_{xyl/xos}$ is xylose or xylooligosaccharide contents (g/[g of dry feed]); $f_{xyl/xos}$ is the anhydro factor for pentoses (0.88, dimensionless); and %Hemicel is the hemicellulose (xylan) content in the raw biomass.

3.7. Response surface methodology

Response surface methodology (RSM) was used to analyse data presented in Chapters 6 and 7. Analysis was performed with State-Ease Design Expert 7.0 software. Either quadratic or two factor interaction models were selected to be fitted to the data based on highest R^2 values for each model. Where necessary, power transformations were applied to the response surface models. Appropriate transformations were selected using BOX-COX plots by selecting the transformation resulting in the lowest values of residual sum of squares. Analysis of variance (ANOVA) tests were then performed for the appropriate response surface model with or without power transformations. Detailed descriptions of RSM methodology and design of experiments specific to the analysed data are described in Chapters 6 and 7, and corresponding ANOVA results and information about resulting model and transformation selection for tested data is given in Appendix A.

CHAPTER 4. SCOPING INVESTIGATION OF SUB-CRITICAL WATER MEDIATED HEMICELLULOSE EXTRACTION FROM *MISCANTHUS χ GIGANTEUS* AND *AVENA SATIVA* HUSKS

4.1. Introduction

This chapter presents the scoping results of extracting hemicellulosic components from *Miscanthus χ giganteus* and *Avena sativa* var. *Balado* husks via subCW mediated auto-hydrolysis. This is achieved using the experimental and analytical methods described in the previous chapter. The effects of extraction temperature, residence time (independent variables) are discussed in terms of extract carbohydrate distribution and total phenolic contents, as well as xylan extraction efficiency and xylooligosaccharide production (dependent variables). Furthermore, this chapter discusses the effects of pre-treatment and biomass type on extract composition.

4.2. Biomass used for extractions

Biomass used for the extractions presented in this chapter were *Miscanthus χ giganteus* (MIS) and *Avena sativa* variety Balado husks (BAL) with 93.0±0.2 % and 88.7±0.2 % total dry solids content respectively. MIS composition was measured to be the following ('±' denotes triplicate standard error of the mean): hemicellulose (xylan) 17.3±1.1 %dw, lignin 23.5±0.2 %dw, cellulose 44.5±0.7 %dw, and extractives 11.5±1.4 %dw. BAL composition was: 26.1±2.1 %dw hemicellulose (xylan), lignin 23.2±0.1 %dw, 41.4±1.2 %dw cellulose, and extractives 7.7±1.0 %dw.

4.3. Effects of temperature on virgin extract composition

Biomass preparation and following extraction procedure is described in Section 3.3. All extractions were performed with 5 %(w/v) loading and 30 min residence time, while varying the extraction temperatures between 120 °C, 140 °C and 160 °C; this temperature range was chosen to investigate if hemicellulose can be solubilised with acceptable yields at high molecular weights (MW) and low degradation products with the idea that the

obtained high MW hemicellulose fraction could be then be purified and then further hydrolysed into prebiotic oligosaccharides. Several authors have shown optimal extraction temperatures ranging from 150-160 °C with subCW for feedstocks other than MIS and BAL (Amidon and Liu, 2009; Kabel et al., 2002; Lee et al., 2010; Ligero et al., 2011; Pronyk et al., 2011). However, other authors showed higher extraction temperatures producing better yields (Carvalheiro et al., 2009; Gullon et al., 2010; Jung et al., 2013; Moniz et al., 2013; Moura et al., 2007; Vázquez et al., 2005; Xiao et al., 2013a) (Nabarlatz et al., 2004). High extraction severity could lead to hemicellulose decomposition into undesirable degradation products such as furans that are shown to negatively impact enzymatic processing of the cellulose to produce bio-ethanol (Klinke et al., 2004), which is particularly important because the scope of this research is within the biorefinery context.

The resulting extracts were analysed in terms of overall solubility, pH, mono-/oligo-/poly-saccharide composition, and total phenolic content. All experiments were performed at least in triplicate. Statistical analysis was performed in SigmaPlot 12, using Two-Way Analysis of Variance (ANOVA), and Tukey Pairwise Multiple Comparison Procedure. Error bars in the graphs represent the Standard Error of the Mean (SEM) of the samples, typically performed in triplicate.

4.3.1. Biomass solubility and extract pH

The solubilities of MIS and BAL at different extraction temperatures are given in Figure 4-1 A and solubility, extract mass concentration and pH are given in Table 4-1. Highest solubilities were observed at 160 °C (MIS: 20.2±1.0 %dw, BAL: 22.1±1.1 %dw), showing statistically significant ($P<0.05$) differences between 120 °C and 140 °C extractions for

both biomass types. Statistically insignificant (P>0.05) differences were found between 120 °C and 140 °C extractions within both BAL and MIS. The differences in solubility values between biomass types were also statistically insignificant at all extraction temperatures. Tables of P values are given in Appendix A.1.

Figure 4-1 B shows that the pH of the extracts decreases with higher extraction temperatures, with MIS pH decreasing from 5.5±0.1 to 4.5±0.1, with corresponding values from 6.2±0.2 to 4.6±0.1 for BAL. Statistically significant differences in pH values were observed between extraction temperatures for both MIS and BAL, as well as between biomass types at 120 °C and 140 °C, but not at 160 °C.

Table 4-1: Solubility, extract concentration and pH levels of virgin MIS and BAL extractions

	Temp	Solubility		Extract mass conc.		pH	
	°C	%dw	*sem*	g/L	*sem*		*sem*
V.MIS	120	11.7	*±0.5*	2.6	*±0.2*	5.5	*±0.1*
	140	13.0	*±0.3*	2.8	*±0.1*	5.0	*±0.0*
	160	20.2	*±1.0*	5.4	*±0.1*	4.5	*±0.1*
V.BAL	120	13.7	*±1.3*	3.3	*±0.3*	6.2	*±0.2*
	140	15.1	*±0.5*	4.0	*±0.6*	5.5	*±0.0*
	160	22.1	*±1.1*	7.9	*±0.5*	4.6	*±0.1*

Negative correlation between pH and solubility at increasing extraction temperatures was expected as the result of auto-hydrolysis. Auto-hydrolysis occurs when hydronium (H+) ions are generated due to the auto-ionisation of subcritical water and also when acetic and uronic groups are cleaved off from the hemicellulose backbone, which facilitates hemicellulose depolymerisation (Lee et al., 2009; Menon and Rao, 2012; Mosier et al., 2005). As a result, depolymerised hemicellulose products continue to hydrolyse until becoming soluble in water. Section 2.6 provides a detailed description of auto-hydrolysis process and hemicellulose extraction mechanism.

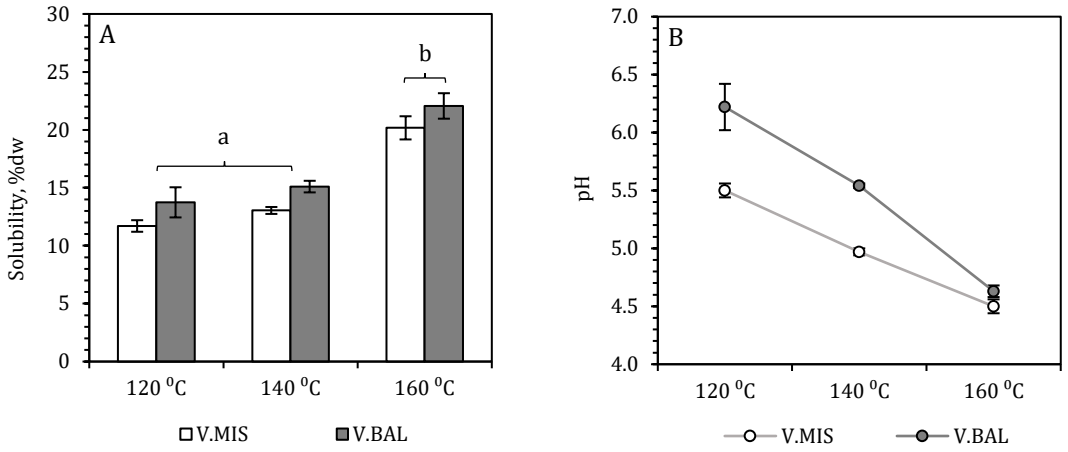

Figure 4-1: Solubility (A) and extract pH (B) of virgin Miscanthus χ giganteus (V.MIS) and virgin Avena sativa var. Balado (V.BAL) at different extraction temperatures; unmatching letters above the bars indicate statistically significant difference between the bars

4.3.2. Carbohydrate contents

Carbohydrate analysis was performed using analytical methods presented in Section 3.5.3. Qualitative and quantitative mono- and oligosaccharide analysis was performed using HPAEC-PAD directly from the diluted extract. To estimate the polysaccharide content, extracts were first subjected to acid hydrolysis. The resulting hydrolysates were then analysed for monosaccharide contents with HPAEC-PAD.

4.3.2.1. Monosaccharides and oligosaccharides

Monosaccharide and oligosaccharide standard chromatogram is shown in Figure 4-2. The monosaccharide contents of virgin MIS and BAL extracts are shown in , whereas the chromatograms of MIS and BAL 160 °C extracts are given in Figure 4-3. Calibration curves are given in Appendix C. Retention times (RTs) of XOS with DP of 3 and 5 were obtained from logarithmic interpolation between xylobiose, xylotetraose, and xylohexaose RTs.

Similarly, quantification of xylotriose and xylopentaose was done by logarithmically interpolating between the slopes of the same standard curves.

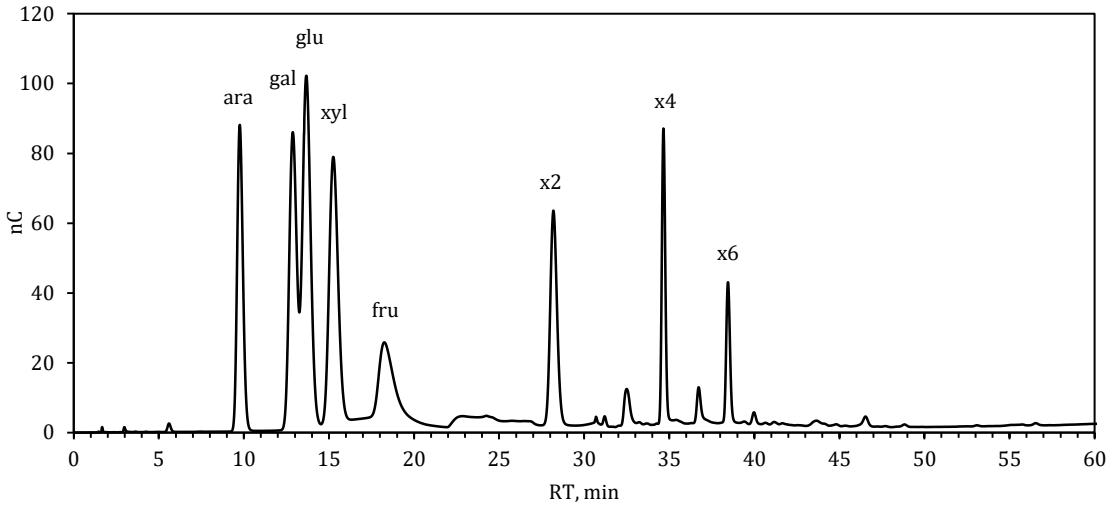

Figure 4-2: HPAEC-PAD chromatogram of standard mix; ara – arabinose; gal – galactose; glu – glucose; xyl – xylose; fru – fructose; x2 – xylobiose; x4 – xylotetraose; x6 – xylohexaose

Overall, low amounts of monosaccharides were found in the extracts. Arabinose and xylose contents increased with temperature confirming that these monomers are of structural origin – released from the lignocellulosic matrix in the cell walls. The arabinose contents at 160 °C extractions from both feedstocks were higher (MIS: 7.3±0.3 mg/[g of dry feed]; BAL: 3.3±0.6 mg/[g of dry feed]) than xylose contents (MIS: 1.5±0.1 mg/[g of dry feed]; BAL: 1.0±0.3 mg/[g of dry feed]), indicating that arabinose groups are released earlier than xylose from the arabinoxylan backbone, confirming that arabinose groups are located at the lateral chains of hemicellulose and therefore is more easily accessible for the hydronium ion attack. A small amount of galactose was observed at 160 °C but not at lower temperatures, also confirming a structural origin. In contrast, all MIS extracts contained similar amounts of glucose across all extraction temperatures, while BAL extracts showed the highest glucose contents at 120 °C and lowest at 160 °C, indicating a

non-structural origin. Most glucose was expected to be released from starch, which is more easily accessible, hydrolysed and solubilised than the structural carbohydrates, and degrades rapidly at higher temperatures. Although it was expected that fructose contents would follow similar trend to glucose as both are non-structural carbohydrates, the results were not in agreement: most fructose was extracted at 160 °C, suggesting that fructose is more difficult to access and solubilize than glucose. Furthermore, fructose might also be generated from the solubilised glucose via glucose isomerisation (Usuki et al., 2008).

Table 4-2: Monosaccharides in virgin MIS and BAL extracts between 120-160 °C

	Temp	Arabinose		Galactose		Glucose		Xylose		Fructose	
	°C	mg/g*	*sem*	mg/g*	*sem*	mg/g*	*sem*	mg/g*	*sem*	mg/g*	*sem*
V.MIS	120	0.3	*±0.1*	nd	*nd*	2.5	*±0.3*	0.4	*±0.1*	0.1	*±0.1*
	140	2.1	*±0.2*	nd	*nd*	2.6	*±0.1*	0.5	*±0.1*	0.3	*±0.3*
	160	7.3	*±0.3*	0.2	*±0.1*	2.4	*±0.1*	1.5	*±0.1*	0.7	*±0.3*
V.BAL	120	0.2	*±0.1*	nd	*nd*	1.4	*±0.4*	0.3	*±0.1*	0.6	*±0.3*
	140	0.1	*±0.1*	nd	*nd*	0.6	*±0.2*	0.7	*±0.2*	0.3	*±0.3*
	160	3.3	*±0.6*	0.3	*±0.1*	0.6	*±0.1*	1.0	*±0.3*	1.2	*±0.2*

**of dry feed*

Xylooligosaccharides (XOS) were observed only at 160 °C extracts (see Table 4-3), confirming arabinoxylan hydrolysis at this temperature. More XOS were observed in BAL than in MIS extracts, which was expected as BAL feedstock contained more xylan than MIS (26.1±2.1 %dw and 17.3±1.1 %dw respectively). However, combined XOS yield of BAL was more than twice as MIS (3.9±0.8 mg/[g of dry feed] and 1.4±0.4 g/[g of dry feed] respectively), suggesting that lignocellulosic matrix in MIS is more rigid than BAL.

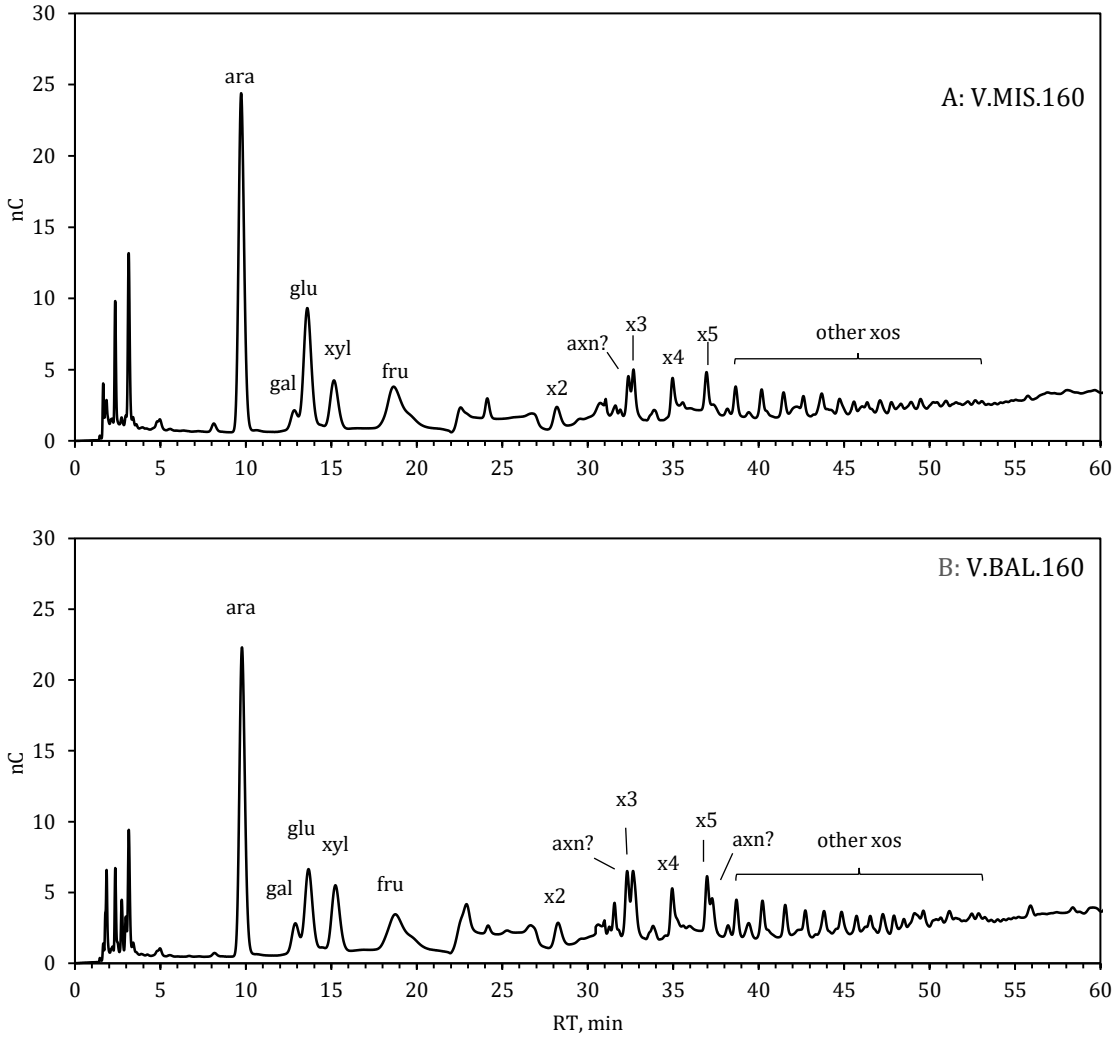

Figure 4-3: HPAEC-PAD chromatogram of virgin MIS (A) and virgin BAL (B) 160 °C extracts with the same dilution factor; axn: arabino-XOS

From chromatogram in Figure 4-3, several peaks close to XOS retention times can be observed (designated as axn) in 160 °C BAL and MIS extracts. As the hemicellulose in both MIS and BAL are shown to consist of predominantly arabinoxylan (Knudsen, 1997; Le Ngoc Huyen et al., 2010; Welch et al., 1983), and the axn peaks are in close vicinity to XOS compounds, it is possible that the axn peaks are xylose based oligosaccharides with attached arabinose residues. The same chromatogram (Figure 4-3) also show what appears to be XOS with DP more than 5.

Table 4-3: Xylooligosaccharides in virgin MIS and BAL extracts between 120-160 °C

	Temp °C	Xylobiose mg/g*	*sem*	Xylotriose mg/g*	*sem*	Xylotetraose mg/g*	*sem*	Xylopentaose mg/g*	*sem*
V.MIS	120	nd	*nd*	nd	*nd*	nd	*nd*	nd	*nd*
	140	nd	*nd*	nd	*nd*	nd	*nd*	nd	*nd*
	160	0.2	*±0.1*	0.3	*±0.1*	0.5	*±0.3*	0.3	*±0.2*
V.BAL	120	nd	*nd*	nd	*nd*	nd	*nd*	nd	*nd*
	140	nd	*nd*	nd	*nd*	nd	*nd*	nd	*nd*
	160	0.6	*±0.2*	1.5	*±0.5*	1.3	*±0.4*	1.9	*±0.4*

**of dry feed*

4.3.2.2. Polysaccharides

Polysaccharide estimation was performed as described in Section 3.5.2 by hydrolyzing the extracts with 3.5% H_2SO_4 at 100 °C for 60 min, then analysing the hydrolysate for monosaccharides. Polysaccharide contents were then calculated from monosaccharide contents as shown in Chapter 3. Polysaccharide contents of virgin MIS and BAL extracts are shown in Table 4-4. According to the literature, the predominant structural carbohydrates in monocots are (in descending order): xylose, arabinose, galactose, and mannose (Garrote et al., 1999; Gírio et al., 2010). Mannose was not observed in any extracts whilst all the former were. All structural carbohydrate contents increased with extraction temperature, reaching the highest values at 160 °C extractions. More xylan, arabinan, and galactan were extracted from BAL than MIS feedstock. The most dominant polysaccharide found at 160 °C was xylan, for BAL extracts reaching 50.1 mg/[g of dry feed] whilst for MIS: 25.7±0.4 mg/[g of dry feed], which was significantly more than was obtained at 140 °C and 120 °C. Such increase in xylan contents indicates that hemicellulose starts to solubilise between 140-160 °C. However, the observed xylan contents were low compared to the values in the literature – subCW mediated extraction from *Miscanthus χ giganteus* yielded 126 mg/g of xylan after 31 h at 130 °C, and maximum XOS yield of 60 mg/g was achieved at 150 °C after 8 h (El Hage et al., 2010).

Table 4-4: Polysaccharide contents in virgin MIS and BAL extracts between 120-160 °C

	Temp	Arabinan		Galactan		Glucan		Xylan		Fructan	
	°C	mg/g*	*sem*	mg/g*	*sem*	mg/g*	*sem*	mg/g*	*sem*	mg/g*	*sem*
V.MIS	120	1.4	*±0.1*	1.3	*±0.0*	3.2	*±0.1*	3.3	*±0.2*	0.5	*±0.1*
	140	3.0	*±0.1*	1.7	*±0.1*	3.3	*±0.1*	4.1	*±0.1*	0.6	*±0.2*
	160	7.4	*±0.1*	3.0	*±0.1*	4.0	*±0.1*	25.7	*±0.4*	1.3	*±0.1*
V.BAL	120	1.2	*±0.1*	1.1	*±0.1*	13.8	*±7.4*	2.1	*±0.1*	1.6	*±0.6*
	140	3.2	*±0.2*	1.6	*±0.2*	24.1	*±2.3*	3.7	*±0.3*	2.4	*±0.4*
	160	12.4	*±1.4*	4.2	*±0.5*	12.8	*±1.5*	50.1	*±1.2*	1.5	*±0.4*

**of dry feed*

Glucan contents increased with extraction temperature for MIS while peaking at 140 °C for BAL. At these extraction conditions, the majority of glucan is expected to originate from starch and pectins from the cell walls; this is affirmed by lower glucan contents at 160 °C for BAL, indicating that the starch has been completely solubilized and is being degraded. Small amounts of fructose were also observed in extract hydrolysates, also peaking at 140 °C for BAL and increasing with extraction temperature for MIS.

Table 4-5 summarizes the extracts with selected parameters to evaluate the extractions. Arabinose/xylose ratio peaked at 140 °C for both MIS and BAL (0.73 and 0.85 respectively), and troughed at 160 °C (0.29 and 0.22 respectively), suggesting that arabinose groups are released first from the AX in the lignocellulosic matrix. Glucose/xylose ratios were similar for 120 and 140 °C extracts but saw a significant decrease at 160 °C due to higher xylan solubility and increasing starch degradation. BAL extracts at 120 and 140 °C contained approx. 6.5 times more glucan than xylan, whilst similar amounts of glucan and xylan was extracted from MIS at the same temperatures.

Table 4-5: Carbohydrate ratios and key extraction efficacy indicators of virgin MIS and BAL extracts between 120-160 °C

	Temp °C	Ara/Xyl	Glu/Xyl	AX cont. in extr. %	Xylan yield %	XOS prod. eff. %
V.MIS	120	0.44	1.00	8.1	1.3	0.0
	140	0.73	0.80	11.7	2.3	0.0
	160	0.29	0.16	28.7	14.7	5.0
V.BAL	120	0.57	6.46	5.2	0.4	0.0
	140	0.85	6.48	7.4	0.8	0.0
	160	0.22	0.23	38.9	13.7	5.4

AX purity or AX content in extract, although relatively low, was highest at 160 °C (28.7±0.2% for MIS and 38.9±2.6% for BAL). By taking into consideration all monosaccharide and polysaccharide contents, the total weight of biomass accounted for as carbohydrates were approx. 45% for MIS and 50% for BAL. It is thought that extractable materials, other than non-structural carbohydrates (e.g. protein, lipids/waxes, and polyphenols) account for the unaccounted fractions. As shown before, extractives in MIS and BAL feedstocks accounted for 12% and 8% of dry weight, respectively. It is likely that majority of extractives are solubilised in the extracts, thus contributing to relatively low hemicellulose purity.

Xylan yields were also relatively low: 14.7±0.3% for MIS and 13.7±3.6% for BAL, indicating that the extraction conditions are too mild for complete hemicellulose solubilization. Moreover, the low XOS production efficiency shows slow rates of hemicellulose hydrolysis, also pointing to mild extraction conditions. The obtained yields were significantly lower than what was observed in the literature – Chen et al. (2014) reported XOS yields of 12% from 160 °C and 60 min subCW extractions from *Miscanthus χ giganteus*; Ligero et al. (2011) produced 65% xylan yields also from subCW extracts of *Miscanthus γ giganteus* at 160 °C at 60 min; moreover, Pronyk obtained 60% xylan yields

from 60 min extractions at 150 °C from triticale straw (Pronyk et al., 2011). The higher yields as reported in the aforementioned studies are likely due to higher extraction severities resulting from longer residence times (60 min), compared to 30 min in the extractions discussed here. Moreover, the definition of XOS was not specified in these studies, therefore the corresponding yield calculations could include XOS of heavier molecular weight distribution than this study, where only XOS with DP of 2-5 were considered.

Although low yield and low XOS production were observed, higher MW xylan can be desirable for applications other than prebiotics. For such applications, sequential hemicellulose extractions at mild conditions potentially could increase the hemicellulose yield while keeping it at high MW. This is discussed in the subsequent subsections of this chapter where the effects of a pre-treatment stage are investigated.

4.3.3. Total phenolic contents

Phenolic compounds such as ferulic acid and p-coumaric acid can be esterified to the arabinose branches of the xylan backbone (Ebringerová, 2005; Scheller and Ulvskov, 2010), and therefore could be expected to be present in the obtained extracts. Moreover, phenolics from other sources such as acid-soluble lignin, and melanoidins produced by the late stages of Maillard reactions could also be released (Gullón et al., 2009; Wang et al., 2011a). Whether in free or bound forms, phenolics have been shown to beneficially affect the host, either by being directly absorbed in the small intestine as in the case of free phenolics, or by being transformed into bio-avalable intermediates by the gut microbiota as in the case of bound phenolics (Acosta-Estrada et al., 2014; Decker, 1995; Srinivasan et al., 2007). Furthermore, oligosaccharides with phenolic substitutions

obtained from wheat bran have been shown to increase the growth of *Bifidobacterium bifidum in vitro* (Yuan et al., 2005), and increased the activity of antioxidant enzymes and blood lipid metabolism in rats (Wang et al., 2011b). It is possible that the prebiotic oligosaccharides and phenolics present in the hemicellulose extracts might synergistically affect the growth of the beneficial gut microbiota, and therefore improve the prebiotic efficacy of the extract.

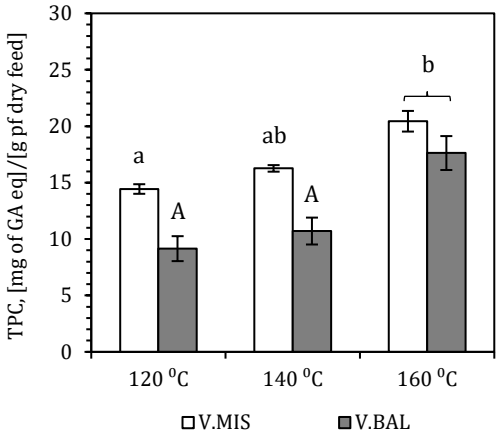

Figure 4-4: Total phenolic content of virgin MIS and BAL extracts; unmatching letters above the bars indicate statistically significant difference between the bars

As can be seen from the Figure 4-4, the total phenolic content (TPC) was statistically higher in MIS extracts at the lower temperatures, but not at 160 °C where the highest values were observed, reaching 20.5±0.9 [mg of GA eq.]/[g of dry feed] and 17.6±1.5 [mg of GA eq.]/[g of dry feed] for MIS and BAL extracts respectively. These values are considerably higher than the naturally occurring TPC in oat grains, 0.9 [mg of GA eq.]/[g of dry feed] (Alfieri and Redaelli, 2015), but comparable with TPC values found in subCW extracts obtained from rice bran, where 10 min extractions between 150 °C and 220 °C yielded 5 to 42 [mg of ferulic acid eq.]/[g of dry feed] (Pourali et al., 2010).

4.4. Pre-treatment to remove extractives

As discussed in Section 4.3, extracts from lower temperatures contained more carbohydrates of non-structural origin than carbohydrates from the lignocellulosic matrix. Furthermore, it was suggested that the unaccounted weight fraction of the extract, could include solubilized components other than carbohydrates, such as proteins, lipids/waxes, and phenolics. These components, together with non-structural carbohydrates are referred to as extractives. Thus, removing extractives via a pre-treatment step, and then using the pre-treated biomass for a sequential hemicellulose extraction would increase the AX content in the resulting extract. Furthermore, pre-treatment could also improve the hemicellulose extractability due to reduced biomass recalcitrance as the result of pre-treatment. The pre-treatment extraction presented in this section was done at 120 °C as done in the extractions discussed above, but scaled up from 0.5 L to 5 L extraction vessel. The solubilities of 5 L, 0.5 L extraction, and a sequential Soxhlet extraction, which was used to determine the extractive contents in the feedstocks, are compared in this section. Soxhlet extractions were performed with 16 h water extraction, followed with 16 h ethanol extraction. The effects of pre-treatment by using the pre-treated biomass as the feedstock for sequential hemicellulose extraction is discussed in Section 4.5. All pre-treated biomass of the same type (MIS and BAL) was pooled and mixed well before being used in the sequential hemicellulose extractions. Detailed descriptions of extraction procedures are given in Section 3.3.4.

4.4.1. Solubility comparison of 5 L, 0.5 L and Soxhlet extractions

From Figure 4-5 it can be observed that the solubilities of extractions from the 5 L vessel were significantly lower than from the 0.5 L vessel for both BAL and MIS: 9.6±0.3 %dw vs.

13.7±3.3 %dw, and 8.6±0.8 %dw vs 11.7±2.6 %dw respectively. This is likely attributable to the differences of extractor dimensions, mixing efficiencies and temperature control. The difference between the biomass types was found to be insignificant within the extractions with the same vessel volume. The solubilities of Soxhlet extraction, used for determination of extractives according to NREL (National Renewable Energy Laboratory) protocol (Sluiter et al., 2008d), did not show consistent behaviour within the same biomass type. For BAL Soxhlet extraction yielded statistically lower solubility than 5 L and 0.5 L extractions, while for MIS resulting in statistically higher solubility than 5 L extraction, and similar solubility to 0.5 L extraction. See Appendix A.2 for a statistical comparison table.

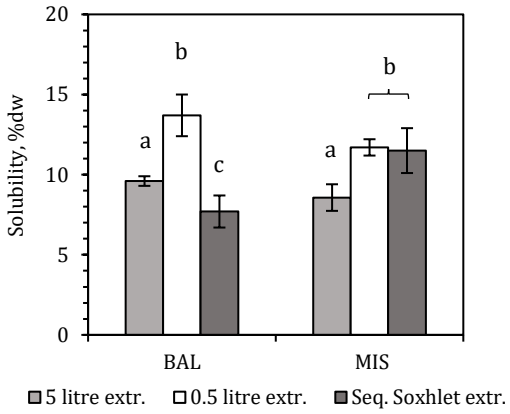

Figure 4-5: Solubility of virgin BAL extracts obtained from 5 L, 0.5 L and Soxhlet extractors; unmatching letters above the bars indicate statistically significant difference between the bars

As sequential Soxhlet extraction is used as the standardised protocol for determination of extractives, and its extraction severity is somewhat comparable with subCW extractions, lower solubility values than sequential Soxhlet extraction, would indicate under-extraction of extractives while higher solubility values would suggest that structural carbohydrates are being extracted. As can be seen from Figure 4-5, the latter appears to be true for BAL while former is for MIS, when comparing 5 L extraction with sequential

Soxhlet extraction. The subCW extraction from 0.5 L vessel yielded similar solubility to sequential Soxhlet extraction for MIS.

4.4.2. Polysaccharide contents in extracts from 5 L and 0.5 L extractions

Polysaccharide contents from 5 L and 0.5 L BAL extracts were estimated as described in Section 3.5.2, and are presented in Table 4-6. A major component in the extracts from both vessels was glucan, likely originating from starch as discussed in earlier sections. 5 L extract contained more polysaccharides, despite having lower overall solubility. Glucose and fructose based polysaccharides accounted for approx. 17 %dw of 5 L extract, whereas polysaccharides of structural origin accounted for approx. 7 %dw, suggesting that majority of extract contained extractives other than non-structural carbohydrates such as proteins and polyphenolics.

Table 4-6: Polysaccharide contents in virgin BAL 120 °C extracts

	Arabinan		Galactan		Glucan		Xylan		Fructan	
	mg/g*	*sem*	mg/g*	*sem*	mg/g*	*sem*	mg/g*	*sem*	mg/g*	*sem*
5 L extr.	1.9	*±0.1*	1.4	*±0.1*	14.0	*±2.1*	3.2	*±0.1*	2.0	*±0.4*
0.5 L extr.	1.2	*±0.1*	1.1	*±0.1*	13.8	*±7.4*	2.1	*±0.1*	1.6	*±0.6*

**of dry feed*

4.5. Effects of temperature on pre-treated extract composition

Following the conclusions from the previous subsections of this chapter, particularly regarding the contents of non-structural extractibles in the extract, a pre-treatment step before the hemicellulose extraction was added with the aim to improve the hemicellulose and hemicellulose derived product contents. Moreover, pre-treated biomass would be 'broken-in', i.e. it would have reduced structural recalcitrance, allowing for better subCW

permeability, and thus increased extractability in the following extraction stage. The effectiveness of the pre-treatment step in improving the extraction efficiency in subCW with and without modifiers has been shown in literature (Lee et al., 2010; Luterbacher et al., 2012; Vázquez et al., 2005).

Overall, the extraction procedure was similar to the virgin extractions at 120 °C with few modifications. For detailed description of the extraction procedure see Section 3.3.4. Briefly, the prepared (imbibed and blended) biomass was pre-treated in bulk using 5 L reactor at 120 °C for 30 min, 5 %(w/v) loading and then dried until approx. 90% total solid content. The dried biomass was then imbibed in water for 10 min at room temperature and extracted at 140 °C, 160 °C or 180 °C for 30 min using the 0.5 L reactor. Temperature range was increased to 140-180 °C, instead of 120-160 °C as in virgin extractions, because a peak in hemicellulose contents was not observed in the virgin extractions.

After the extraction, the reactor contents were passed through a sieve to separate the residue from the extract. The extract was then analysed for carbohydrate and total phenolic contents using HPAEC-PAD and Folin Ciocalteu assay for total phenolics. Furthermore, the extract was subjected to ethanol precipitation to fractionate and recover the heavier (higher DP) carbohydrates.

4.5.1. Biomass solubility and extract pH

Pre-treated extracts showed higher maximum solubility values than virgin extracts for both MIS and BAL at 160 °C (see Table 4-7). Highest values were observed at 180 °C with pre-treated biomass, corresponding to 27.9±0.8 %dw and 40.6±0.5 %dw for MIS and BAL respectively. However, when comparing the same temperatures (shown in Figure 4-6),

solubility was statistically lower for pre-treated extracts, except for BAL at 160 °C where the solubility of pre-treated extracts was statistically higher than virgin extracts. Note that the pre-treated biomass contained more hemicellulose per g of biomass than the biomass used in virgin extractions due to the removal of non-structural components, thus solubilising more hemicellulose. The ANOVA tables for the corresponding comparisons are shown in Appendix A.3. Lower solubility in pre-treated biomass at lower temperatures suggest that the pre-treatment of biomass was effective in removing the non-structural components from the biomass.

Table 4-7: Solubility, extract concentration and pH levels of virgin (V) and pre-treated (P) MIS and BAL temperature investigation

	Temp	Solubility		Extract mass conc.			
	°C	%dw	*sem*	g/L	*sem*	pH	*sem*
V.MIS	120	11.7	*±0.5*	2.6	*±0.2*	5.5	*±0.1*
V.MIS	140	13.0	*±0.3*	2.8	*±0.1*	5.0	*±0.0*
V.MIS	160	20.2	*±1.0*	5.4	*±0.1*	4.5	*±0.1*
P.MIS	140	7.0	*±0.6*	2.8	*±0.5*	4.8	*±0.1*
P.MIS	160	13.6	*±0.6*	7.2	*±0.3*	4.3	*±0.0*
P.MIS	180	27.8	*±0.8*	13.8	*±0.8*	3.8	*±0.0*
V.BAL	120	13.7	*±1.3*	3.3	*±0.3*	6.2	*±0.2*
V.BAL	140	15.1	*±0.5*	4.0	*±0.6*	5.5	*±0.0*
V.BAL	160	22.1	*±1.1*	7.9	*±0.5*	4.6	*±0.1*
P.BAL	140	7.4	*±0.2*	3.0	*±0.1*	4.7	*±0.0*
P.BAL	160	24.7	*±0.9*	14.4	*±0.7*	4.1	*±0.0*
P.BAL	180	40.6	*±0.5*	21.6	*±0.3*	3.6	*±0.1*

As described before, the extract pH is an indicator of the extraction severity as pH lowers with increased release of hydronium ions due to the auto-hydrolysis of hemicellulose. The pH fell to its lowest value at 180 °C in pre-treated extracts to 3.8 and 3.6 for MIS and BAL respectively; and was generally higher in virgin extracts.

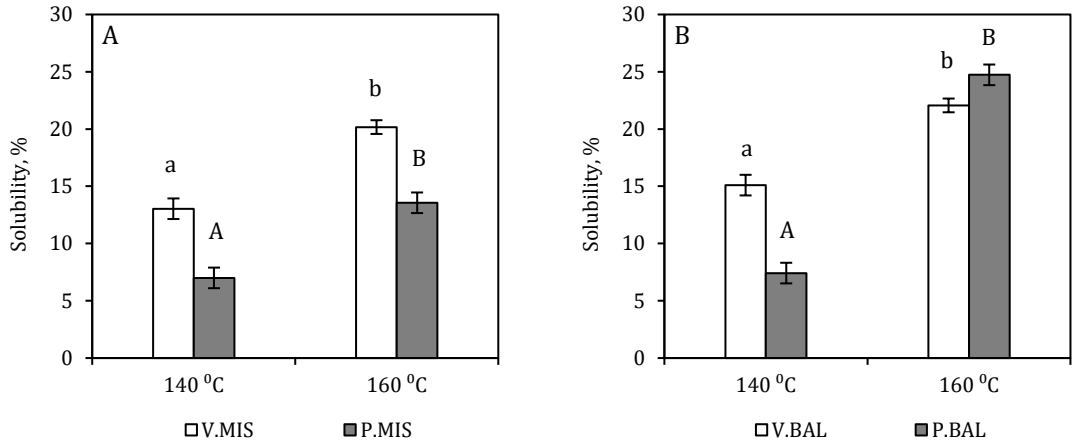

Figure 4-6: Solubility of virgin and pre-treated MIS (A) and BAL(B) at 140 °C and 160 °C; unmatching letters above the bars indicate statistically significant difference between the bars

Virgin BAL extracts had higher pH than virgin MIS but lower than MIS in pre-treated extracts. Furthermore, BAL also showed statistically significantly higher solubility values than MIS at 160 °C and 180 °C (see Figure 4-7). As shown in Section 4.3.2, hemicellulose starts solubilizing between 140 °C and 160 °C, therefore such observation can be explained as raw BAL feed contained more xylan than MIS (26.1±2.1 %dw vs 17.3±1.1 %dw respectively).

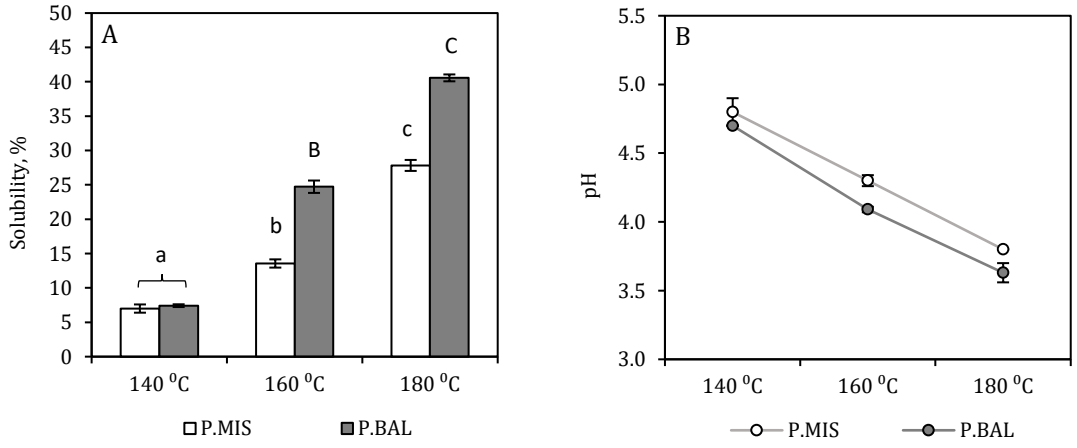

Figure 4-7: Solubility (A) and extract pH (B) of pre-treated MIS (P.MIS) and pre-treated BAL (P.BAL) at different extraction temperatures; unmatching letters above the bars indicate statistically significant difference between the bars

4.5.2. Carbohydrate contents

Carbohydrate analysis was performed using analytical methods presented in Section 3.5. Qualitative and quantitative mono- and oligosaccharide analysis was performed using HPAEC-PAD directly from the diluted extract. To estimate the polysaccharide content, extracts were first subjected to acid hydrolysis. The resulting hydrolysates were then analysed for monosaccharide contents with HPAEC-PAD. In this section the results are presented as mg per g of dry feed, the mass loss due to the solubilisation in the pre-treatment step is taken into account in the calculations.

4.5.2.1. Monosaccharides and oligosaccharides

Monosaccharide contents of the extracts are shown in Table 4-8. Extraction temperatures of 180 °C produced highest monosaccharide contents for both MIS and BAL. Most significantly, the xylose contents were found to be higher at 180 °C by an order of magnitude compared to lower temperatures; this is indicating that hemicellulose polymers are being hydrolysed at higher rate at temperatures between 160 and 180 °C. The dominant monosaccharide at 140 and 160 °C was arabinose for both biomass types, confirming that the highly branched arabinose substitutions are cleaved off the xylan backbone before xylan starts depolymerising. As expected, glucose and fructose contents decreased when comparing the pre-treated extracts with virgin extracts of the same temperatures due to the pre-treatment step where the non-structural carbohydrates were expected to be removed. Furthermore, statistically significantly more xylose was seen in pre-treated extracts than when comparing 160 °C extracts between virgin and pre-treated extractions (see Appendix A.3). This suggest that the recalcitrance of the

lignocellulosic matrix is affected by the pre-treatment step, making it more permeable, and thus facilitating the depolymerisation of hemicellulose.

Similar to monosaccharides, most prebiotic oligosaccharides (XOS with DP of 2-5) were extracted at 180 °C (see Table 4-9). Consistently with differences in solubility and monosaccharide contents between MIS and BAL, also here more XOS were extracted from BAL than MIS. From the results thus far, it is clear that there are structural differences between the biomass types. There are two potential explanations: (1) differences in structural characteristics of hemicellulose, i.e. distribution of branched vs linear hemicellulose chains, branched being more easily accessible, therefore favouring higher rates of solubilisation; and/or (2) presence of neutralising agents such as mineral salts, leading to a reduction of hydrolysis rate due to the neutralisation of the hydronium ions.

Table 4-8: Monosaccharide contents in virgin (V) and pre-treated (P) MIS and BAL extracts between 120-180 °C

	Temp	Arabinose		Galactose		Glucose		Xylose		Fructose	
	°C	mg/g*	*sem*	mg/g*	*sem*	mg/g*	*sem*	mg/g*	*sem*	mg/g*	*sem*
V.MIS	120	0.3	*±0.1*	*nd*	*nd*	2.5	*±0.3*	0.4	*±0.1*	0.1	*±0.1*
V.MIS	140	2.1	*±0.2*	*nd*	*nd*	2.6	*±0.1*	0.5	*±0.1*	0.3	*±0.3*
V.MIS	160	7.3	*±0.3*	0.2	*±0.1*	2.4	*±0.1*	1.5	*±0.1*	0.7	*±0.3*
P.MIS	140	2.7	*±0.1*	*nd*	*nd*	0.7	*±0.0*	0.3	*±0.0*	0.1	*±0.1*
P.MIS	160	6.6	*±0.3*	*nd*	*nd*	0.8	*±0.3*	1.8	*±0.1*	0.1	*±0.1*
P.MIS	180	7.9	*±0.2*	*nd*	*nd*	1.9	*±0.2*	26.2	*±2.5*	*nd*	*nd*
V.BAL	120	0.2	*±0.1*	*nd*	*nd*	1.4	*±0.4*	0.3	*±0.1*	0.6	*±0.3*
V.BAL	140	0.1	*±0.1*	*nd*	*nd*	0.6	*±0.2*	0.7	*±0.2*	0.3	*±0.3*
V.BAL	160	3.3	*±0.6*	0.3	*±0.1*	0.6	*±0.1*	1.0	*±0.3*	1.2	*±0.2*
P.BAL	140	3.0	*±0.2*	0.2	*±0.0*	0.3	*±0.0*	0.7	*±0.1*	0.3	*±0.1*
P.BAL	160	7.1	*±0.3*	0.5	*±0.1*	0.5	*±0.1*	4.5	*±0.6*	0.4	*±0.1*
P.BAL	180	9.1	*±0.6*	nd	*nd*	3.0	*±0.3*	53.4	*±10.3*	*nd*	*nd*

**of dry feed*

Compared to virgin extracts of the same extraction temperature (160 °C), the pre-treated extracts contained significantly more prebiotic XOS (see Appendix A.3 for ANOVA tables), which is consistent with the argument that pre-treatment improves the permeability of

biomass. Moreover, pre-treated BAL contained statistically more prebiotic XOS at 160 °C and 180 °C than MIS at the same extraction conditions.

Table 4-9: Oligosaccharide contents in virgin (V) and pre-treated (P) MIS and BAL extracts between 120-180 °C

	Temp	Xylobiose		Xylotriose		Xylotetraose		Xylopentaose	
	°C	mg/g*	*sem*	mg/g*	*sem*	mg/g*	*sem*	mg/g*	*sem*
V.MIS	120	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
V.MIS	140	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
V.MIS	160	0.2	*±0.1*	0.3	*±0.1*	0.5	*±0.3*	0.3	*±0.2*
P.MIS	140	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
P.MIS	160	0.8	*±0.1*	1.2	*±0.2*	1.6	*±0.2*	1.8	*±0.2*
P.MIS	180	15.8	*±2.6*	15.9	*±1.9*	20.1	*±2.2*	20.8	*±1.3*
V.BAL	120	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
V.BAL	140	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
V.BAL	160	0.6	*±0.2*	1.5	*±0.5*	1.3	*±0.4*	1.9	*±0.4*
P.BAL	140	0.2	*±0.1*	0.2	*±0.1*	0.3	*±0.1*	0.2	*±0.1*
P.BAL	160	2.4	*±0.5*	3.5	*±0.8*	4.9	*±0.8*	7.4	*±1.0*
P.BAL	180	32.4	*±3.9*	33.2	*±2.0*	34.5	*±1.6*	33.1	*±2.0*

**of dry feed*

XOS distribution in pre-treated extracts differed between different extraction temperatures. As can be seen from Table 4-9, heavier XOS (DP 4 and 5) were extracted more at 160 and 180 °C than lighter molecular weight XOS (DP 2 and 3) from both types of biomass. However, when looking at the relative ratios of XOS extracted (obtained by dividing the mass of the relevant XOS with the mass of xylopentaose at the same extraction temperature and biomass type), apparent distribution towards higher degrees of polymerisation can be seen at 160 °C, which then has the tendency to even out at 180°C, as shown in Table 4-10. The XOS distribution at 180 °C is skewed more to the heavier XOS in MIS, while in BAL the distribution is flat.

Chromatograms shown in Figure 4-8 and Figure 4-9, represent the progression of hemicellulose solubilisation. Retention times (RTs) from 9 to 20 min, represent the area of monosaccharide retention, whereas RTs from 28-38 are where the prebiotic

oligosaccharides are eluted. Chromatograms A and C in Figure 4-9 confirm the effectiveness of pre-treatment for removing the non-structural components. There are two indications supporting this: (1) virgin extract has larger glucose and fructose peaks; and (2) there are larger peaks in virgin extract with RTs up to 5 min, which represent the unknown compounds that are weakly interacting with the column packing. Moreover, the intensity of the peaks in chromatogram C are greater than in chromatogram A, despite both being 160 °C extracts, confirming that the pre-treatment effectively increases the biomass permeability thus improving the solubilisation of hemicellulose.

Table 4-10: Relative ratios of prebiotic XOS mass distribution in pre-treated MIS and BAL extracts

	Temp, °C	Xylobiose	Xylotriose	Xylotetraose	Xylopentaose
P.MIS	160	0.44	0.67	0.89	1.00
P.MIS	180	0.76	0.76	0.97	1.00
P.BAL	160	0.32	0.47	0.66	1.00
P.BAL	180	0.98	1.00	1.04	1.00

The dominant peak in Figure 4-8 is the arabinose peak, confirming that it is the first of the structural carbohydrates being released from the lignocellulosic matrix. There are several peaks just before or just after the known XOS peaks which are potentially XOS with arabinose substitutions (denoted as axn in Figure 4-8). AXN peaks appearing in chromatograms A and B are of similar size if not larger than the XOS peaks, which falls in line with the background theory whereby the highly branched substitutions are cleaved off the xylan backbone before the backbone itself starts depolymerising. Although arabinose is still the dominant peak in chromatogram C, it can be seen that the XOS peaks start to become more prevalent here, with increasing extraction severity.

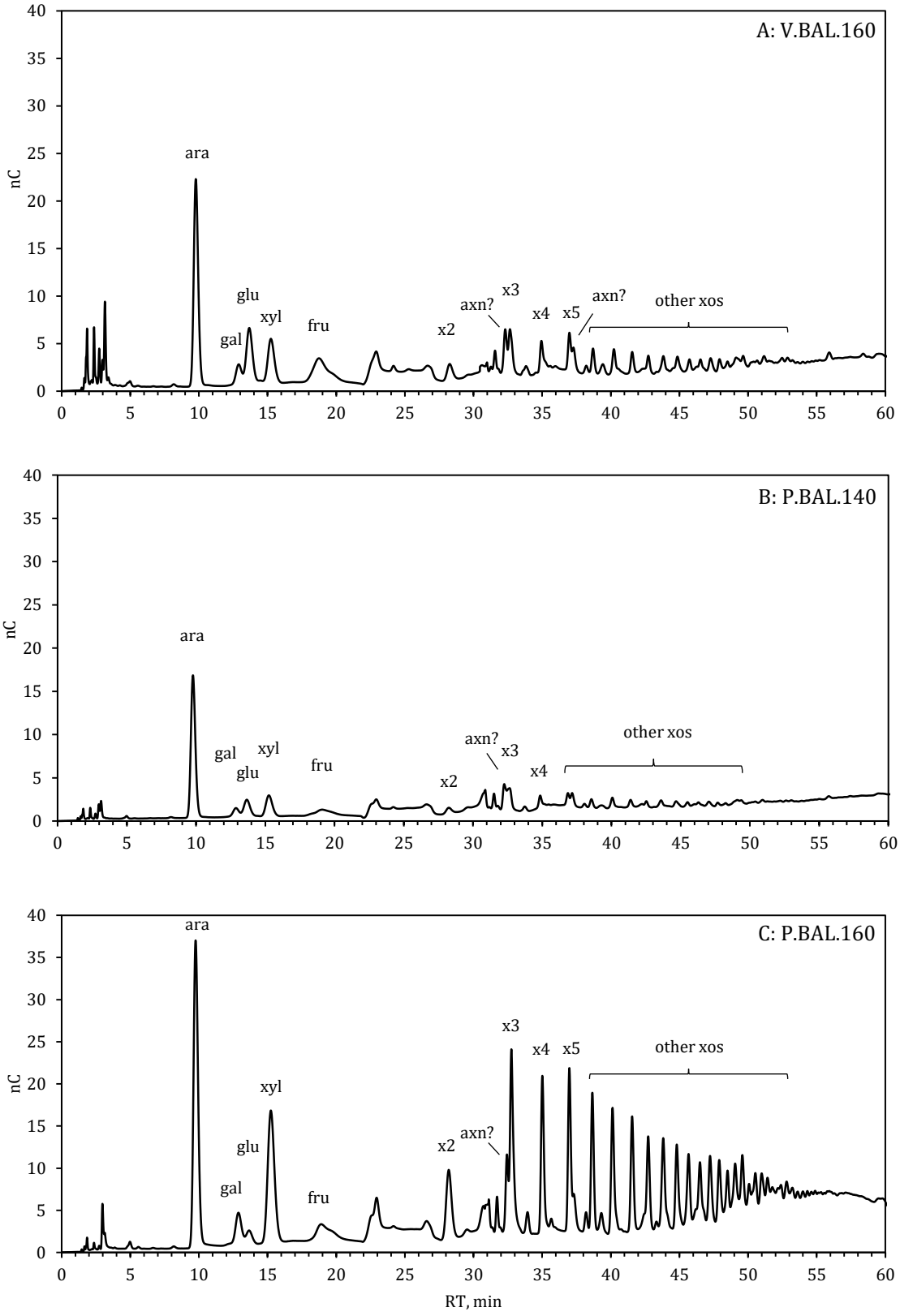

Figure 4-8: HPAEC-PAD chromatogram of virgin BAL extract from 160 °C extraction (A), pre-treated BAL, 140 °C (B), and pre-treated BAL, 160 °C; the chromatograms are quantitatively comparable as samples have consistent dilutions.

As can be seen from Figure 4-9, the axn peaks have become negligible at 180 °C. Furthermore, compared to Figure 4-8, the prebiotic XOS peaks with DP of 2-5 are more intense relative to the heavier XOS peaks. This observation is particularly evident in BAL extract (chromatogram B). In general, qualitatively speaking, MIS and BAL chromatograms were similar throughout the analysis (see Figure 4-8 and Figure 4-3), but differing quantitatively.

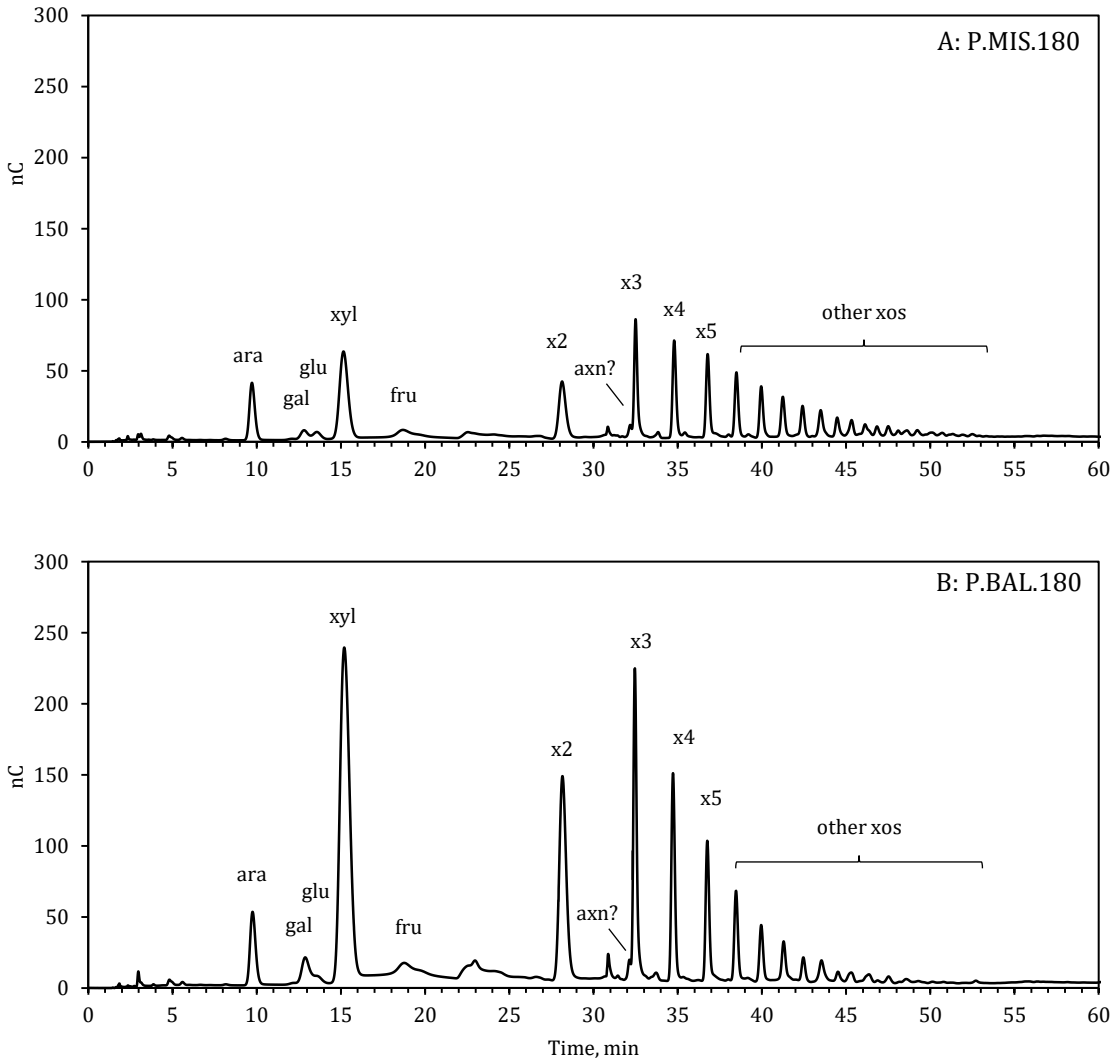

Figure 4-9: HPAEC-PAD chromatogram of pre-treated MIS (A) and pre-treated BAL (B) extracts from 180 °C with the same dilutions.

4.5.2.2. Polysaccharides

Results of extract polysaccharide contents are shown in Table 4-11. Xylan was found to be the dominant type of polysaccharide in the extracts from both biomass types, reaching the maxima at 180 °C extractions. However, considerably more xylan was extracted from BAL, peaking at 224.1±1.4 mg/[g of dry feed], compared to 133.0±4.8 mg/[g of dry feed] from MIS. Statistically significant increases in xylan values were observed when comparing 160 °C extracts from virgin and pre-treated biomass (for ANOVA tables see Appendix A.3).

The secondary hemicellulose component – arabinan also showed the highest values at 180 °C for MIS and BAL, however, the increase in values was relatively small compared to 160 °C extraction. Although galactan values in virgin extractions increased with extraction temperature for both MIS and BAL, the opposite was observed in pre-treated MIS where galactan values decreased with temperature, whereas pre-treated BAL continued to show increasing values. This suggest that the galactan in MIS is in smaller quantities, and easier accessible compared to BAL.

Furthermore, glucan contents increased with extraction temperature, in both pre-treated MIS and BAL, which is likely originating from the amorphous regions of cellulose, and not from the non-structural sources such as amylopectin and amylose. The latter forms of glucose are thought to be the prevalent source of glucan seen in virgin extractions, particularly from BAL (see Table 4-11). Also, fructan contents were lower in pre-treated extracts, and were not observed at all at 180 °C. When comparing the glucan and fructan contents between virgin and pre-treated MIS and BAL at 140 °C, it is evident that the pre-treatment was an effective step in removing the non-structural carbohydrates.

Table 4-11: Polysaccharide contents in virgin and pre-treated MIS and BAL extracts

	Temp	Arabinan		Galactan		Glucan		Xylan		Fructan	
	°C	mg/g*	*sem*	mg/g*	*sem*	mg/g*	*sem*	mg/g*	*sem*	mg/g*	*sem*
V.MIS	120	1.4	*±0.1*	1.3	*±0.0*	3.2	*±0.1*	3.3	*±0.2*	0.5	*±0.1*
V.MIS	140	3.0	*±0.1*	1.7	*±0.1*	3.3	*±0.1*	4.1	*±0.1*	0.6	*±0.2*
V.MIS	160	7.4	*±0.1*	3.0	*±0.1*	4.0	*±0.1*	25.7	*±0.4*	1.3	*±0.1*
P.MIS	140	4.4	*±0.4*	0.9	*±0.1*	2.7	*±0.3*	8.4	*±0.8*	0.1	*±0.1*
P.MIS	160	9.8	*±0.5*	0.5	*±0.5*	5.8	*±0.6*	46.4	*±4.8*	0.2	*±0.1*
P.MIS	180	9.9	*±0.2*	*nd*	*nd*	8.7	*±0.4*	133.0	*±4.8*	*nd*	*nd*
V.BAL	120	1.2	*±0.1*	1.1	*±0.1*	13.8	*±7.4*	2.1	*±0.1*	1.6	*±0.6*
V.BAL	140	3.2	*±0.2*	1.6	*±0.2*	24.1	*±2.3*	3.7	*±0.3*	2.4	*±0.4*
V.BAL	160	12.4	*±1.4*	4.2	*±0.5*	12.8	*±1.5*	50.1	*±1.2*	1.5	*±0.4*
P.BAL	140	5.4	*±0.3*	1.7	*±0.1*	2.1	*±0.2*	17.9	*±1.7*	0.4	*±0.1*
P.BAL	160	13.3	*±0.5*	5.6	*±0.4*	3.4	*±0.2*	110.7	*±8.9*	1.2	*±0.2*
P.BAL	180	14.6	*±0.2*	7.8	*±0.4*	4.0	*±0.4*	224.1	*±1.4*	*nd*	*nd*

**of dry feed*

Figure 4-10 shows the progression of xylose, pre-biotic xylooligosaccharide and xylan contents. It can be observed that between 140 °C and 160 °C xylan starts to solubilise in the liquid fraction while xylose and XOS contents are still low, suggesting that the solubilised xylan is of high molecular weight. This observation is useful if the target application downstream requires xylan of high molecular weight, such as xylan based foams, gels and films. With increasing extraction temperature (160-180 °C), the solubilised xylan starts to rapidly depolymerise into shorter chain oligosaccharides, which at 180 °C have become the dominant xylose based species. However, with increased rate of depolymerisation, the generation of xylose monomers increase, which is not desirable if the target application is prebiotic XOS. Moreover, other undesirable degradation products, such as furfural, resulting from the decomposition of monosaccharides, might also be abundant at such extraction severity. More information regarding the extraction mechanisms and generation of degradation products is given in Section 2.6.

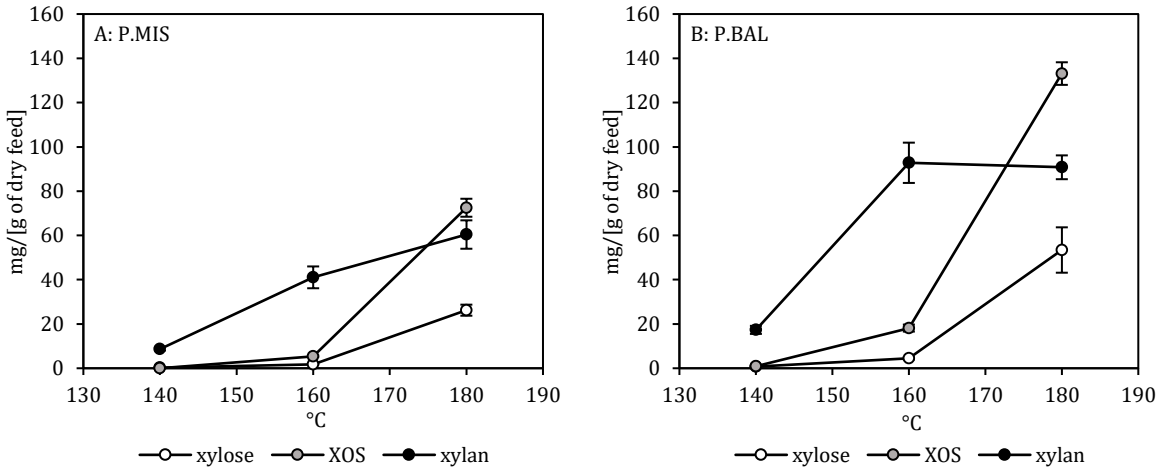

Figure 4-10: Xylose, prebiotic XOS (DP 2-5) and xylan (other than prebiotic XOS) contents in extracts obtained from pre-treated MIS and BAL at temperatures from 140-180 °C

From the results of the extract composition from different extraction temperatures and biomass types, several parameters can be derived to assess the composition of the extract and extraction efficiency; these are summarised in Table 4-12. Arabinose to xylose (Ara/Xyl) and glucose to xylose (Glu/Xyl) ratios show the fraction of arabinose and glucose relative to xylose in the extract. High values of the former suggest an early stage of hemicellulose solubilisation whereby the more accessible lateral hemicellulose chains, which are rich in arabinose, are cleaved off in a faster rate than the linear chains of xylose backbone depolymerise and thus solubilise in the liquid fraction. The results seem to follow this model as Ara/Xyl ratios decreased with increasing extraction temperature from 0.53 at 140 °C to 0.07 at 180 °C from MIS and from 0.31 to 0.06 respectively from BAL; also, higher ratios were observed in virgin extracts – with greatest values at 140 °C (0.73 and 0.85 from MIS and BAL respectively). Glucose to xylose ratio at extraction temperatures below 230 °C, at which cellulose starts to hydrolyse (Ando et al., 2000), is an indicator of non-structural carbohydrate presence in the extract. Indeed, the highest Glu/Xyl ratios were observed at virgin extracts, decreasing with temperature, reaching

the lowest values at highest temperatures from pre-treated biomass, again confirming the effectiveness of pre-treatment regarding the removal of the non-structural components.

Table 4-12: Carbohydrate ratios and key extraction efficacy indicators of virgin (V) and pre-treated (P) MIS and BAL extracts

	Temp °C	Ara/Xyl	Glu/Xyl	AX cont. in extr. %	Xylan yield %	XOS prod. eff. %
V.MIS	120	0.44	1.00	8.1	1.3	0.0
V.MIS	140	0.73	0.80	11.7	2.3	0.0
V.MIS	160	0.29	0.16	28.7	14.7	5.0
P.MIS	140	0.53	0.31	25.5	5.0	0.0
P.MIS	160	0.22	0.13	42.8	26.8	12.0
P.MIS	180	0.07	0.07	55.7	76.7	54.0
V.BAL	120	0.57	6.46	5.2	0.4	0.0
V.BAL	140	0.85	6.48	7.4	0.8	0.0
V.BAL	160	0.22	0.23	38.9	13.7	5.4
P.BAL	140	0.31	0.12	43.5	6.7	4.7
P.BAL	160	0.12	0.03	46.8	42.3	15.9
P.BAL	180	0.06	0.02	60.7	85.7	59.7

Arabinoxylan (AX) content as shown in Table 4-12, represent the arabinoxylan content of the extracts relative to the total mass of the extract and therefore can be regarded as an indicator of the hemicellulose extract purity. The highest AX content values were achieved from the 180 °C extractions, 55.7±1.0% and 60.7±1.4% for pre-treated MIS and BAL respectively. The remaining non-AX mass fraction of the extract, particularly at lower temperatures is likely to be composed of residual water soluble organic extractives (pectins, proteins, phenolics, glycosides, mucilages) and their degradation products that were not removed with the pre-treatment, while at temperatures from 160-180 °C, some lignin might also become solubilised (Ando et al., 2000). Further extract characterisation is required to assess the totality of extract components.

Xylan yield is expressed as the total xylan measured in the extract relative to the total available xylan in the biomass prior to the extraction. From the results shown in Table 4-12, xylan yield more than doubled from 160 °C to 180 °C extractions from pre-treated MIS and BAL reaching 76.7±2.9% and 85.7±0.7% respectively. Moreover, 54.0±4.0% and 59.7±3.0% of the solubilised xylan from the pre-treated MIS and BAL at 180 °C were in the prebiotic oligosaccharide form (see XOS production efficiency column in Table 4-12), which corresponded to 41.4% and 51.2% overall yields respectively.

The obtained xylan yields were generally higher than what was reported in literature. Jung (2013) obtained 75% of available xylan in subCW extracts at 180 °C and 30 min from sunflower stalks (Jung et al., 2013); Boussarsar (2009) achieved 55% xylan solubilisation from sugarcane bagasse at 170 °C and 120 min (Boussarsar et al., 2009); Carvalheiro (2009) showed xylan yields reaching 64% from wheat straw at 215 °C and 0 min residence time (the extraction was stopped when reaching the setpoint) (Carvalheiro et al., 2009); and Yu (2010) obtained 43% yield from rice straw at 180 °C and 10 min (Yu et al., 2010). These results were obtained using a single stage hydrothermal extractions. Vázquez (2005) used two stage extractions at 158 °C and 230 °C (ceasing the extractions once reaching the setpoints) to yield 66% of XOS from eucalyptus wood (Vázquez et al., 2005), which was higher than observed here.

The XOS yields from single stage extractions are presented in Table 4-13; XOS yields obtained in this work were generally similar to the literature, however, comparisons should be made cautiously due to the differences in extraction methodology and definitions. For instance, in this study XOS yields presented include the oligosaccharides with degrees of polymerisation 2-5, which are shown to produce the highest prebiotic

efficacy (Hughes et al., 2007; Moura et al., 2007). Moreover, the extraction methodology as presented here utilised two-step extraction process and was performed with subCW.

Table 4-13: XOS yields from selected literature sources and corresponding extraction conditions of single stage hydrothermal treatments of various types of biomass

Biomass	Temperature, °C	Residence time, min	XOS Yield, %	Reference
Bamboo culm	180	30	47	(Xiao et al., 2013b)
Corn cobs	190	15	58	(Nabarlatz et al., 2004)
Miscanthus giganteus	160	60	12	(Chen et al., 2014)
Wheat straw	180	30	44	(Ruiz et al., 2011)
Corn cobs	208	0	65	(Moura et al., 2007)
Corn straw	210	0	53	(Moniz et al., 2013)
Brewers' spent grain	190	5	61	(Carvalheiro et al., 2004)
Rye straw	208	0	69	(Gullon et al., 2010)
Tamarisk	190	0	20	(Xiao et al., 2013a)
Arundo donax	180	42	8	(Caparros et al., 2007)
Miscanthus giganteus	180	30	41	This study
Oat husks	180	30	51	This study

The results shown so far indicate that high molecular weight hemicellulosic polymers can be extracted at temperatures around 160 °C, while higher temperatures favour comparatively lower molecular weight oligosaccharide formation. Further investigation of hemicellulose and prebiotic XOS extraction is discussed in CHAPTER 6 by means of design of experiments and response surface methodology.

4.5.3. Total phenolic contents

Total phenolic contents (TPC) of the pre-treated extracts are shown in Figure 4-11. Overall, TPC increased with temperature for both MIS and BAL. The differences in TPC values between MIS and BAL were not statistically significant at 140 °C and 180 °C but were at 160 °C (for ANOVA tables see APPENDIX A.3), indicating that the release and/or generation of the phenolic compounds leading to the increase in TPC values at 180 °C occur at lower severity from BAL biomass.

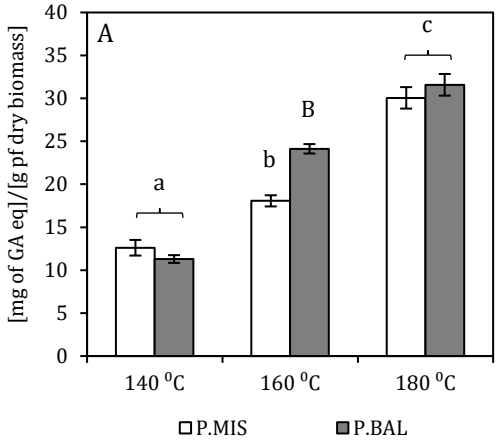

Figure 4-11: Total phenolic content of pre-treated MIS and BAL extracts; unmatching letters above the bars indicate statistically significant difference between the bars

The differences in TPC between virgin and pre-treated extracts are given in Figure 4-12. At 160 °C, TPC values were not statistically different between virgin and pre-treated extracts from MIS but were from BAL. This observation also suggests that the phenolic compounds are released at higher rates at lower extraction severity from BAL husks, while higher extraction severity was required for MIS to achieve similar TPC values.

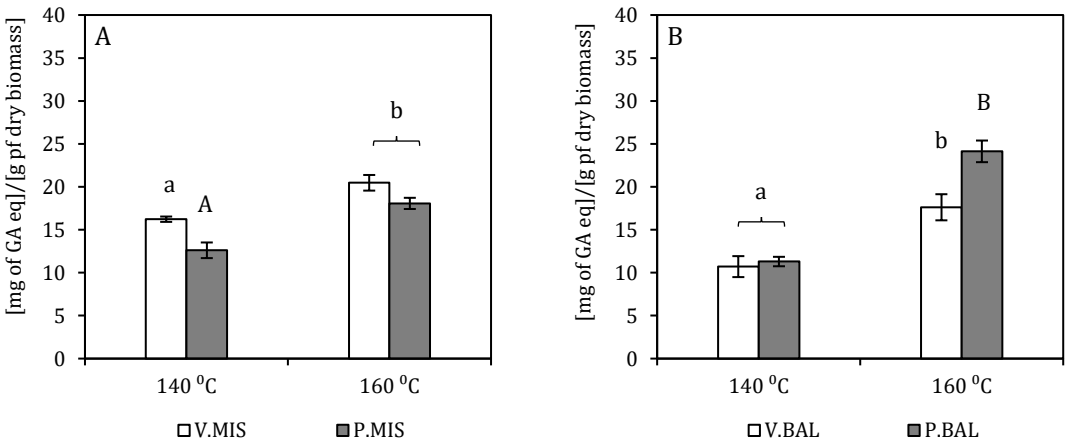

Figure 4-12: Total phenolic content of virgin and pre-treated MIS (A) and BAL (B) at 140 °C and 160 °C; unmatching letters above the bars indicate statistically significant difference between the bars

4.5.4. Fractionation using ethanol precipitation

The pre-treated extracts of the temperature investigation from MIS and BAL, as well as virgin BAL were subjected to 60%(v/v) ethanol precipitation to separate the heavier MW hemicellulose fraction from the lighter fraction. The precipitation protocol is described in Section 3.4, and the results of the precipitate and supernatant mass fractions are summarised in Figure 4-13. Lowest values of recovered precipitate fraction were obtained from 180 °C extractions, indicating that the majority of the solubilised hemicellulose at this temperature was of lighter MW and therefore retained in the supernatant. In contrast, the lower temperature extracts produced larger precipitate fraction, confirming that the larger hemicellulose polymers are indeed solubilised at these temperatures. This agrees with the observations of XOS distribution obtained with HPAEC PAD, presented in subsection above.

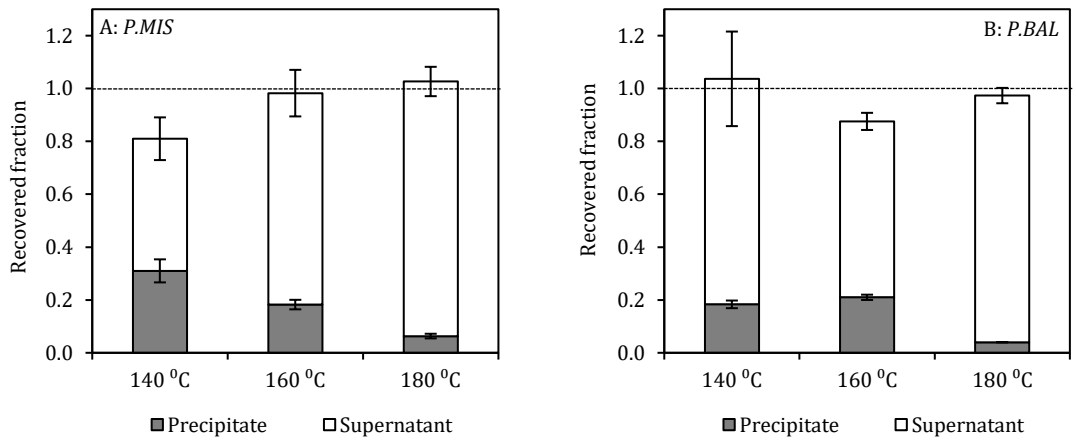

Figure 4-13: Stacked 60%(v/v) ethanol precipitate and supernatant fractions of pre-treated MIS (A) and BAL (B) extracts obtained from 140-180 °C

4.6. Effects of residence time on pre-treated extract composition

Whilst previous section focused on the effects of temperature on extract composition from pre-treated MIS and BAL by keeping the residence time constant at 30 min but

varying the extraction temperature, this section investigates the effects of residence time by subjecting the pre-treated biomass to subCW mediated hemicellulose hydrolysis. The biomass was prepared in the same way as in previous subsection. The extractions were performed at 160 °C with residence times of 0 min, 30 min and 60 min. The time measurement was initiated once the batch reached the target temperature. For full description of experimental procedure see Section 3.3.3. Temperature of 160 °C was chosen as it was the mid-point of the temperature investigation study thus allowing the results from min and max conditions (0 min and 60 min) to be compared with extracts obtained at 140 °C and 180 °C.

4.6.1. Biomass solubility and extract pH

As shown in Table 4-14, biomass solubility increased while the pH decreased with the residence time. In addition, here, BAL biomass was easier to solubilise than MIS. Although the solubility increase was significant (see ANOVA tables in APPENDIX A.4) from 30 to 60 min, reaching 19.8±0.9 %dw and 34.3±0.7 %dw for MIS and BAL respectively, it was still lower than the values obtained at 180 °C, 30 min extraction, reaching 27.8±0.8 %dw and 40.6±0.5 %dw correspondingly. Moreover, solubility at 0 min (160 °C) was lower than the results from 140 °C, 30 min extraction. These results indicate that, apart from the extraction temperature, extraction residence time is also a significant factor affecting the biomass solubility.

Table 4-14: Solubility, extract mass concentration, and pH of pre-treated MIS and BAL residence time investigation

	Residence time	Solubility		Extract mass conc.		pH	
	min	%dw	sem	g/L	sem		sem
P.MIS	0	4.8	±0.9	2.2	±0.2	5.0	±0.0
P.MIS	30	13.6	±0.6	7.2	±0.7	4.3	±0.0
P.MIS	60	19.8	±0.9	10.6	±0.7	4.0	±0.0
P.BAL	0	4.8	±0.9	1.8	±0.5	5.2	±0.2
P.BAL	30	24.7	±0.9	14.4	±0.6	4.1	±0.0
P.BAL	60	34.3	±0.7	20.1	±0.8	4.0	±0.1

4.6.2. Carbohydrate contents

The results thus far show that residence time was a significant factor in biomass solubility. This section explores the carbohydrate distribution in the corresponding extracts. Analytical methods by which the results described here were obtained are presented in Section 3.5. Direct extract analysis with HPAEC with PAD was performed for monosaccharide and oligosaccharide distribution, and two-step acid hydrolysis of the extracts was performed to estimate the polysaccharide contents.

4.6.2.1. Monosaccharides and oligosaccharides

The measured arabinose and xylose contents in the extract increased with the residence time. Arabinose reached 8.1±0.1 mg/[g of dry feed] and 10.1±0.6 mg/[g of dry feed] for pre-treated MIS and BAL respectively (see Table 4-15), which was more than was observed in the corresponding 180 °C, 30 min extractions, indicating that arabinose had been decomposing at higher rates than becoming solubilised at 180 °C and 30 min extraction. In contrast, the xylose values from 60 min extracts were comparatively low in comparison with the values from the 180 °C extracts, suggesting that still relatively large fraction of solubilised hemicellulose at 160 °C 60 min extractions were in high MW.

Table 4-15: Monosaccharide contents of pre-treated MIS and BAL extracts, residence time investigation

	Time	Arabinose		Galactose		Glucose		Xylose		Fructose	
	min	mg/g*	*sem*	mg/g*	*sem*	mg/g*	*sem*	mg/g*	*sem*	mg/g*	*sem*
P.MIS	0	1.5	*±0.1*	*nd*	*nd*	*nd*	*±0.0*	0.2	*±0.0*	0.1	*±0.1*
P.MIS	30	6.6	*±0.3*	*nd*	*nd*	0.8	*±0.3*	1.8	*±0.1*	0.1	*±0.1*
P.MIS	60	8.1	*±0.1*	*nd*	*nd*	0.7	*±0.0*	4.8	*±0.1*	*nd*	*nd*
P.BAL	0	1.6	*±0.5*	0.1	*±0.0*	0.2	*±0.1*	0.3	*±0.2*	0.1	*±0.1*
P.BAL	30	7.1	*±0.3*	0.5	*±0.1*	0.5	*±0.1*	4.5	*±0.6*	0.4	*±0.1*
P.BAL	60	10.1	*±0.6*	nd	*nd*	1.7	*±0.2*	14.4	*±1.3*	*nd*	*nd*

**of dry feed*

Similarly, more prebiotic oligosaccharides were extracted with increasing residence times (see Table 4-16) for both biomass types, however, significantly more XOS were solubilised from BAL than MIS. Prebiotic XOS distribution was still skewed more towards the heavier MW XOS from 30 and 60 min extracts, i.e. xylotetraose and xylopentaose was extracted more than xylobiose and xylotriose, while no oligosaccharides were observed from the 0 min extracts.

Table 4-16: Prebiotic xylooligosaccharide contents of pre-treated MIS and BAL extracts, residence time investigation

	Time	Xylobiose		Xylotriose		Xylotetraose		Xylopentaose	
	min	mg/g*	*sem*	mg/g*	*sem*	mg/g*	*sem*	mg/g*	*sem*
P.MIS	0	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
P.MIS	30	0.8	*±0.1*	1.2	*±0.2*	1.6	*±0.2*	1.8	*±0.2*
P.MIS	60	3.1	*±0.2*	3.6	*±0.3*	5.2	*±0.2*	6.1	*±0.1*
P.BAL	0	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
P.BAL	30	2.4	*±0.5*	3.5	*±0.8*	4.9	*±0.8*	7.4	*±1.0*
P.BAL	60	8.9	*±0.8*	11.4	*±1.0*	15.7	*±1.3*	19.4	*±1.5*

**of dry feed*

4.6.2.2. Polysaccharides

The results of the polysaccharide contents are presented in Table 4-17. Arabinan, xylan, glucan, and fructan contents of the extracts increased with the residence time of the extraction for both MIS and BAL. Similar to arabinose contents, also here, the arabinan contents from the 60 min extraction were higher than at the 180 °C extractions. The

dominant polysaccharide in all extracts from the residence time investigation was xylan. The increase in xylan values from 30 min to 60 min was also significant but did not reach the levels of the 180 °C extraction. Galactan contents increased to 9.4±1.7 mg/[g of dry feed] at 60 min for BAL while no galactan was observed from MIS at the same residence times, pointing to differences in hemicellulose structural composition between the biomass types.

Table 4-17: Polysaccharide contents of pre-treated MIS and BAL extracts, residence time investigation

	Time	Arabinan		Galactan		Glucan		Xylan		Fructan	
	min	mg/g*	*sem*	mg/g*	*sem*	mg/g*	*sem*	mg/g*	*sem*	mg/g*	*sem*
P.MIS	0	2.3	*±0.1*	0.9	*±0.0*	2.3	*±0.1*	4.4	*±0.2*	0.1	*±0.1*
P.MIS	30	9.8	*±0.5*	0.5	*±0.5*	5.8	*±0.6*	46.4	*±4.8*	0.2	*±0.1*
P.MIS	60	10.3	*±0.1*	*nd*	*nd*	8.1	*±0.3*	73.4	*±0.7*	0.3	*±0.1*
P.BAL	0	2.6	*±0.9*	1.0	*±0.3*	2.3	*±0.2*	6.9	*±3.4*	0.1	*±0.1*
P.BAL	30	13.3	*±0.5*	5.6	*±0.4*	3.4	*±0.2*	110.7	*±8.9*	1.2	*±0.2*
P.BAL	60	17.6	*±3.0*	9.4	*±1.7*	4.5	*±0.6*	163.0	*±19.8*	2.1	*±0.4*

**of dry feed*

In terms of extraction efficacy (seeTable 4-18) results followed similar trends to the temperature investigation of pre-treated biomass – Ara/Xyl and Glu/Xyl ratios decreased with extraction severity, while xylan yield and prebiotic XOS production increased. 42.7±1.0% and 62.5±7.5% of total available xylan was solubilised at 60 min from MIS and BAL respectively, from which quarter to a third was prebiotic XOS. However, AX content of the extracts, although increased from 0 to 30 min, was lower at 60 min, suggesting that during this period something other than carbohydrates became solubilised in the extract. Likely candidates for this could be phenolics originating from acid soluble lignin and carbohydrate degradation (Kumar et al., 2013; Rasmussen et al., 2014) and/or melanoidins from phenolic and reducing sugar interactions (Wang et al., 2011a; Wiboonsirikul et al., 2007). Other compounds also present in the extract but likely to a

lesser amount could be the decomposition products of monosaccharides such as 5-HMF and furfural.

Table 4-18: Carbohydrate ratios and key extraction efficacy indicators of pre-treated MIS and BAL extracts, residence time investigation

	Time min	Ara/Xyl	Glu/Xyl	AX cont. in extr. %	Xylan yield %	XOS prod. eff. %
P.MIS	0	0.53	0.53	16.3	2.7	0.0
P.MIS	30	0.22	0.13	42.8	26.8	12.0
P.MIS	60	0.14	0.11	41.7	42.7	24.3
P.BAL	0	0.44	0.47	26.3	2.7	4.3
P.BAL	30	0.12	0.03	46.8	42.3	15.9
P.BAL	60	0.09	0.03	44.1	62.5	33.5

4.6.3. Total phenolic contents

As can be seen from Figure 4-14, total phenolic contents (TPC) increased with extraction residence time for both MIS and BAL. However, BAL extracts showed statistically higher values than MIS at 30 min and 60 min (for ANOVA tables see Appendix A.4). BAL extracts at 160 °C and 60 min had higher TPC than BAL extracts from 180 °C and 30 min extractions, reaching 34.1±1.8 and 31.6±1.3 [mg GA eq]/[g of dry feed] respectively, indicating that phenolics had started to decompose at 180 °C. In contrast, TPC contents were highest at 180 °C for MIS, and peak in values was not observed.

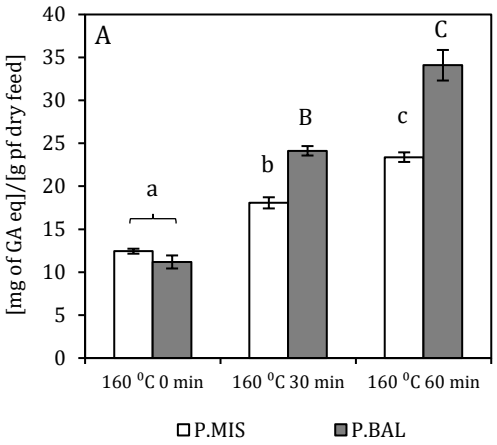

Figure 4-14: Total phenolic contents of pre-treated MIS and BAL time investigation; unmatching letters above the bars indicate statistically significant difference between the bars

4.6.4. Fractionation using ethanol precipitation

Similar to temperature investigation, 60%(v/v) ethanol precipitation was performed on the extracts obtained from the time investigation. The precipitation protocol is described in Section 3.4, and the results in terms of supernatant and precipitate mass fractions are presented in Figure 4-15. Recovered mass fraction of the precipitates decreased with extraction residence time, clearly suggesting that with increasing extraction severity the extracts contained less high MW hemicellulosic compounds. Note that due to the low concentrations of the extracts obtained from the 0 min BAL extractions, recovery of precipitates and supernatants was challenging, therefore resulting in values with high uncertainty.

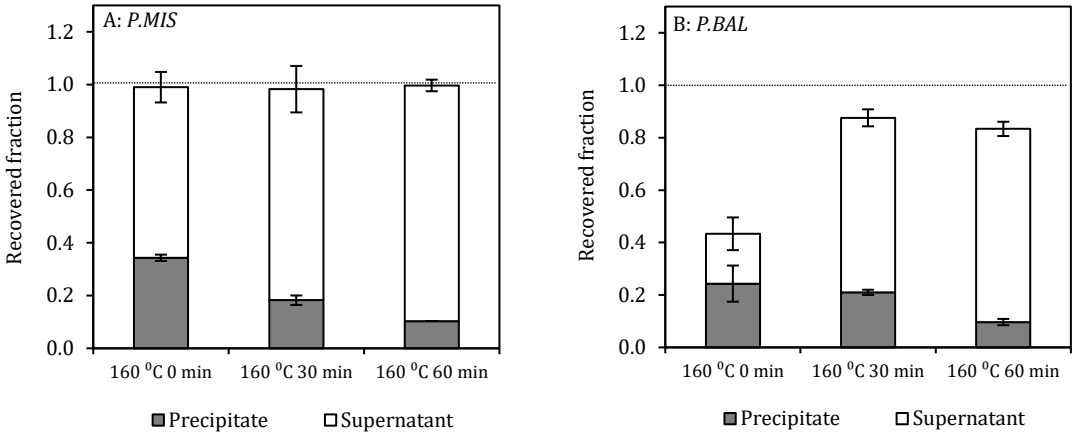

Figure 4-15: Stacked 60%(v/v) ethanol precipitate and supernatant fractions of pre-treated MIS (A) and BAL (B) extracts obtained at 0, 30 and 60 min residence times and 160 °C

4.7. Chapter conclusions

The presented results already provide an insight into a potential in utilising subCW mediated extraction to produce monosaccharides, oligosaccharides and polysaccharides derived from the hemicellulose fraction of the biomass (see Table 4-19). It was found that, although extractability was different depending on the biomass source, the differences were subtle, suggesting that segregation of extractions from different types of biomass might not be required. Furthermore, it was found that a pre-treatment step at low temperatures was beneficial for higher purity and composition of the following hemicellulose extracts. The valorisation of the extracts obtained from the pre-treatment, containing the non-structural components of the cell, should be investigated further. In terms of the hemicellulose extraction, it was shown that by manipulating the extraction severity with temperature and residence time, it was possible to target products of particular molecular weights. This opens doors to many potential applications, including xylitol, prebiotics, and biodegradable films and gels among others. Prebiotics showed to be the preferable choice as the target product, providing relatively high yields. Such flexibility in extraction selectivity could be seen as an economic advantage, allowing to

switch between the target products depending on the market demand. However, further studies need to be performed to optimise these parameters, select the most efficient and cost effective product recovery route, and assess the potential impact of the hemicellulose extraction on the residual biomass in the downstream processing, which is relevant in terms of the wider biorefinery context.

Table 4-19: Key findings of Chapter 4

Comparisons	Key observations
virgin vs. pre-treated extracts	- Higher hemicellulose purity in pre-treated extracts - Pre-treatment is effective for removal of extractives - Higher XOS and xylose yields in pre-treated extracts
MIS vs. BAL	- Significantly higher xylan yields in BAL extracts - Similar hemicellulose purity in extracts - Similar total phenolic contents in extracts
temperature vs. residence time	- Highest yields were achieved at 180 °C for both biomass types - Longer residence times (up to 60 min at 160 °C) improved hemicellulose extractability - Temperature and residence time control can be used for targeting different MW products

CHAPTER 5. COMPOSITIONAL DIFFERENCES OF SUB-CRITICAL WATER EXTRACTS FROM THE HUSKS OF FIVE *AVENA SATIVA* VARIETIES

5.1. Introduction

The previous chapter investigated the effects of temperature, residence time and pre-treatment on energy crop *Miscanthus γ giganteus* and *Avena sativa* var. Balado husks, an agricultural waste/by-product. Furthermore, it was found that the composition of the extracts from the two genera were different. This chapter explores the differences in hemicellulose and hemicellulose derivatives extraction between different varieties within the same species of *Avena sativa*. Five varieties were studied – SO-I, 14355Cn, Conway, Mascani, and the aforementioned Balado. All extractions were performed at 160 °C, 30 min residence time and 50 bar pressure with virgin and pre-treated husks. The extracts were then analysed for carbohydrate compositional and molecular weight distribution, overall solubility, furan and total phenolic contents.

5.2. Biomass used for extractions

SO-I and 14355Cn are developed for low lignin hulls and high oil contents in the groats, which make them more digestible and therefore more preferable as ruminant feed (Marshall et al., 2015; McKinnon et al., 2008). Mascani and Balado are high yielding commercial winter oat varieties. The former is the most popular oat variety in the UK, but the latter is higher yielding (Senova, 2016a; c). Conway is a commercial spring variety with excellent resistance to mildew (Senova, 2016b). Lignocellulosic composition, extractives content and dry solid weight of the husks from the varieties used are shown in Table 5-1.

Table 5-1: Composition of the raw husks from the varieties used in the extractions

Variety	Total solids		Cellulose		Hemicell.*		Lignin		Extractives	
	%dw	sem	%dw	sem	%dw	sem	%dw	sem	%dw	sem
SO-I *(low lignin)*	90.1	*±0.1*	53.6	*±3.4*	18.9	*±1.1*	11.9	*±0.4*	13.4	*±1.3*
14355Cn *(low lignin)*	89.4	*±0.2*	26.2	*±1.3*	23.9	*±1.1*	16.1	*±0.3*	31.6	*±2.8*
Conway *(spring)*	88.9	*±0.2*	33.9	*±2.3*	26.4	*±0.4*	32.8	*±0.1*	6.7	*±0.5*
Mascani *(winter)*	87.9	*±0.7*	35.5	*±2.2*	26.2	*±0.7*	30.2	*±0.2*	6.0	*±0.3*
Balado *(winter)*	88.7	*±0.2*	41.4	*±1.2*	26.1	*±2.1*	23.2	*±0.1*	7.7	*±1.0*

*measured as xylan

5.3. Pre-treatment to remove extractives

In previous chapter, pre-treatment was shown to be effective in removing the extractives from the biomass and thus improved the hemicellulose solubility. Due to the high extractives contents in the low lignin varieties (see Table 5-1 and Figure 5-1), pre-treatment step was also adapted and the results from the succeeding extractions were compared to virgin extractions. The results are shown in the following subsection. The solubilities of the extracts from the pre-treatment step were comparable to the extractives contents. The high extractives contents of the low lignin varieties (SO-I and 14355Cn) might be an indirect artefact of the low lignin contents in the husks. It was visually noticeable that the biomass from the low lignin varieties contained more groats than from other varieties. This was quantified by taking samples from the biomass and counting how many husks contained groats. Figure 5-1 shows the results. Indeed, the low lignin varieties contained more groats than other varieties suggesting a correlation with the high extractives content. It is not clear, however, whether the high groat contents observed were due to an inefficient de-hulling process as a result of poor choice of machine settings, or as a result of the husk structure due to the low lignin contents.

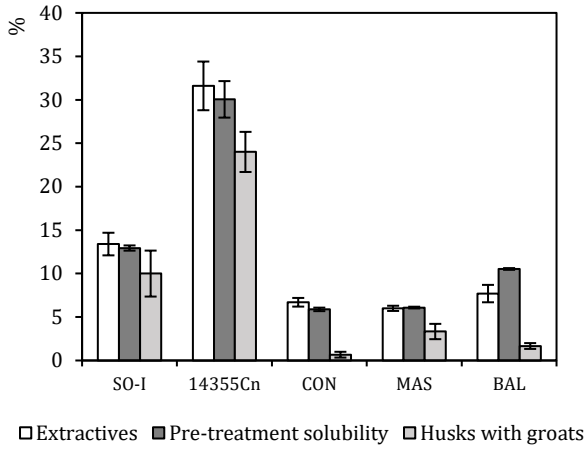

Figure 5-1: Extractives contents, pre-treatment solubility and groats found in husks from five oat varieties

5.4. Comparison of virgin and pre-treated extract composition

Virgin and pre-treated husks from five different oat varieties were subjected to subcritical water extractions at 160 °C and 50 bar for 30 min. Loading was 5 %(w/v). The obtained extracts were then analysed for mono-, oligo- and poly-saccharide contents using HPAEC-PAD, furan contents with HPLC and total phenolic contents with Folin-Ciocalteu colorimetric assay. Extracts were also subjected to ethanol precipitation in order to verify the carbohydrate molecular weight distribution obtained from the results with HPAEC-PAD. Complete extraction methodology and analytical techniques are described in Section 3.5.

5.4.1. Biomass solubility and extract pH

Solubility results are summarised in Figure 5-2, while extract mass concentration and pH is shown in Table 5-2. Significantly higher solubilities were observed for all commercial varieties, i.e. SO-I, and 14355Cn (see Appendix A for ANOVA tables). However, solubilities of low-lignin varieties were different: SO-I variety showed insignificant difference between the virgin and pre-treated biomass, while solubility of 14355Cn decreased

significantly with the pre-treatment. Increase in solubility values of the pre-treated biomass, which was evident for the varieties with low extractives contents (CON, MAS and BAL), was likely due to reduced biomass recalcitrance as the result of the pre-treatment as well as the increase in the cumulative extraction severity.

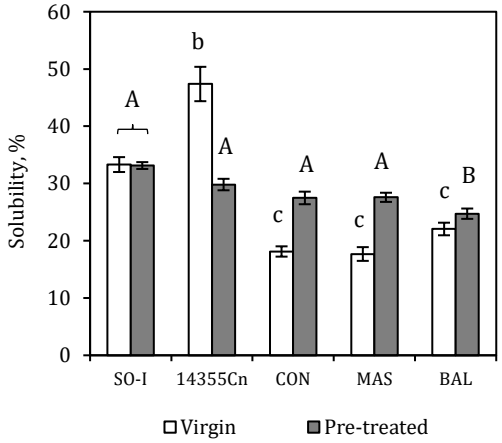

Figure 5-2: Solubility values of virgin and pre-treated oat husk varieties (160 °C, 30 min); unmatching letters above the bars indicate statistically significant difference between the bars

The outlying solubility results from the low-lignin varieties are likely due to the high extractives contents of the virgin biomass, which were then removed by the pre-treatment step, consequently resulting in lower values as in the case of 14355Cn. The solubility results across all pre-treated varieties were similar, once the extractives are removed.

Table 5-2: Solubility, extract mass concentration and pH of virgin and pre-treated oat husk varieties (160 °C, 30 min)

	Solubility		Extract mass conc.		pH	
	%dw	sem	g/l	sem		sem
V.SO-I	33.3	±1.3	15.5	±0.3	4.6	±0.0
V.14355Cn	47.4	±3.0	21.6	±1.1	4.8	±0.0
V.CON	18.1	±0.9	7.5	±0.5	4.4	±0.0
V.MAS	17.7	±1.2	7.2	±0.7	4.6	±0.1
V.BAL	22.1	±1.1	7.9	±0.8	4.6	±0.1
P.SO-I	33.1	±0.6	17.7	±0.7	4.1	±0.0
P.14355Cn	29.8	±1.0	14.9	±0.3	4.6	±0.0
P.CON	27.5	±1.1	14.5	±0.8	3.8	±0.0
P.MAS	27.6	±0.8	15.0	±0.4	3.9	±0.0
P.BAL	24.7	±0.9	14.4	±0.6	4.1	±0.0

5.4.2. Carbohydrate contents

Mono-saccharide and oligo-saccharide analysis was performed directly from diluted extracts using HPAEC-PAD. To determine the poly-saccharide contents, extracts were first hydrolysed, the resulting hydrolysates were then analysed for monosaccharide contents using the same methodology (see Section 3.5). The results from the pre-treated extracts were adjusted to take into account the losses in biomass in the pre-treatment step.

5.4.2.1. Monosaccharides and oligosaccharides

As can be observed from Table 5-3, pre-treatment generally increased the extract monosaccharide contents with exception of glucose and fructose, which were solubilised in the pre-treatment step, therefore indicating that the extracts were purer in terms of solubilised lignocellulosic components. The dominating monosaccharide at these extraction conditions were arabinose for all varieties. Overall, the monosaccharide composition was similar across all husk varieties.

Table 5-3: Monosaccharide contents in virgin (V) and pre-treated (P) husk extracts

	Arabinose		Galactose		Glucose		Xylose		Fructose	
	mg/g*	sem	mg/g*	sem	mg/g*	sem	mg/g*	sem	mg/g*	sem
V.SO-I	6.1	±0.1	0.5	±0.0	1.2	±0.1	1.3	±0.1	2.4	±0.1
V.14355Cn	5.1	±0.1	0.4	±0.0	3.7	±0.2	0.8	±0.0	4.6	±0.1
V.CON	6.7	±0.0	0.7	±0.0	0.6	±0.0	1.9	±0.1	1.6	±0.1
V.MAS	5.4	±0.2	0.6	±0.0	0.7	±0.0	1.1	±0.1	1.8	±0.1
V.BAL	3.3	±1.2	0.3	±0.1	0.6	±0.1	1.0	±0.3	1.2	±0.5
P.SO-I	7.9	±0.3	0.7	±0.0	0.2	±0.0	2.8	±0.1	1.6	±0.1
P.14355Cn	5.1	±0.1	0.4	±0.0	0.9	±0.1	1.1	±0.0	1.7	±0.1
P.CON	8.9	±0.2	1.4	±0.0	0.1	±0.0	4.7	±0.3	1.4	±0.1
P.MAS	8.3	±0.4	1.2	±0.1	0.1	±0.0	3.7	±0.3	1.3	±0.1
P.BAL	7.1	±0.3	0.5	±0.1	0.5	±0.1	4.5	±0.6	*nd*	*nd*

**of dry feed*

Similarly, prebiotic oligosaccharide contents were also higher in the pre-treated extracts (see Table 5-4). Extracts from the low lignin varieties (SO-I and 14355Cn) contained less oligosaccharides than the commercial varieties. It is possible that this could be an indication of structural differences arising from the cultivation for the low lignin trait. As a result, heavier molecular weight hemicellulose components might have developed to account for the low lignin content and provide the required structural rigidity of the cell wall. If this is true, it would require harsher extraction conditions to depolymerise and therefore solubilise hemicellulose. Table 5-4 shows that extract oligosaccharide contents are skewed to heavier molecular weight species, suggesting that hemicellulosic components are still with high molecular weights and can be further hydrolysed, hence improved prebiotic oligosaccharide yields are likely with increased extraction severity.

Table 5-4: Oligosaccharide contents in virgin (V) and pre-treated (P) husk extracts

	Xylobiose		Xylotriose		Xylotetraose		Xylopentaose	
	mg/g*	sem	mg/g*	sem	mg/g*	sem	mg/g*	sem
V.SO-I	0.6	±0.1	1.8	±0.1	1.3	±0.1	1.7	±0.1
V.14355Cn	0.5	±0.0	1.3	±0.1	1.0	±0.0	1.5	±0.0
V.CON	0.8	±0.1	2.4	±0.1	1.6	±0.1	1.7	±0.2
V.MAS	0.4	±0.0	1.7	±0.1	1.0	±0.1	1.3	±0.1
V.BAL	0.6	±0.2	1.5	±0.5	1.3	±0.4	1.9	±0.4
P.SO-I	1.6	±0.1	3.8	±0.3	3.3	±0.3	4.6	±0.2
P.14355Cn	0.6	±0.0	1.2	±0.3	1.3	±0.2	1.9	±0.2
P.CON	2.8	±0.3	4.5	±1.2	5.5	±0.5	7.4	±0.5
P.MAS	2.4	±0.2	3.6	±0.9	5.0	±0.5	6.8	±0.5
P.BAL	2.4	±0.5	3.5	±0.8	4.9	±0.8	7.4	±1.0

**of dry feed*

5.4.2.2. Polysaccharides

Highest xylan contents were observed in extracts from the pre-treated biomass (see Table 5-5 and Figure 5-3). All varieties except 14355Cn produced significantly more xylan from the pre-treated extractions (see Appendix A for ANOVA tables). Xylan contents from

virgin low-lignin varieties were significantly higher than from the virgin commercial varieties. Such observation suggests that the xylan from the low-lignin varieties solubilises at lower extraction severities than the commercial varieties, while maintaining high degrees of polymerisation as indicated by the relatively low XOS contents. This can be attributable to a lower degree of association between hemicellulose, cellulose to lignin as the result of breeding for low-lignin contents. The other major hemicellulosic carbohydrate, arabinan contents from virgin and pre-treated extracts were similar within SO-I and BAL varieties, lower in pre-treated extracts for 14355Cn, and higher in pre-treated extracts for CON and MAS. Higher galactan contents were observed in all pre-treated extracts.

Table 5-5: polysaccharide contents of virgin (V) and pre-treated (P) oat husks

	Arabinan		Galactan		Glucan		Xylan		Fructan	
	mg/g*	sem	mg/g*	sem	mg/g*	sem	mg/g*	sem	mg/g*	sem
V.SO-I	18.9	±0.3	3.3	±0.4	60.5	±10.6	112.2	±1.7	*nd*	*nd*
V.14355Cn	17.3	±1.0	1.2	±1.2	199.9	±9.2	80.9	±6.0	*nd*	*nd*
V.CON	15.4	±0.9	4.1	±0.3	4.9	±1.2	61.1	±3.5	*nd*	*nd*
V.MAS	12.8	±0.4	3.5	±0.4	13.0	±1.6	51.7	±4.1	*nd*	*nd*
V.BAL	12.4	±1.4	4.2	±0.5	12.8	±1.5	50.1	±1.2	0.9	±0.4
P.SO-I	17.8	±1.3	6.1	±0.8	4.2	±0.6	166.5	±8.0	*nd*	*nd*
P.14355Cn	11.3	±0.5	3.3	±0.2	21.9	±1.0	75.9	±3.7	*nd*	*nd*
P.CON	18.2	±0.5	9.3	±0.3	0.8	±0.5	147.9	±7.6	*nd*	*nd*
P.MAS	17.5	±0.3	8.6	±0.5	1.3	±0.3	157.9	±4.2	*nd*	*nd*
P.BAL	13.3	±0.5	5.6	±0.4	3.4	±0.2	110.7	±8.9	*nd*	*nd*

**of dry feed*

As expected, lower glucan contents were observed in the pre-treated extracts as the majority of glucan originating from the non-structural components of the biomass would be removed with the pre-treatment. In virgin extracts, however, significantly higher glucan contents were found in the low lignin varieties. As described earlier, this could be due to an inefficient de-hulling process, leaving some of the groats with the husks.

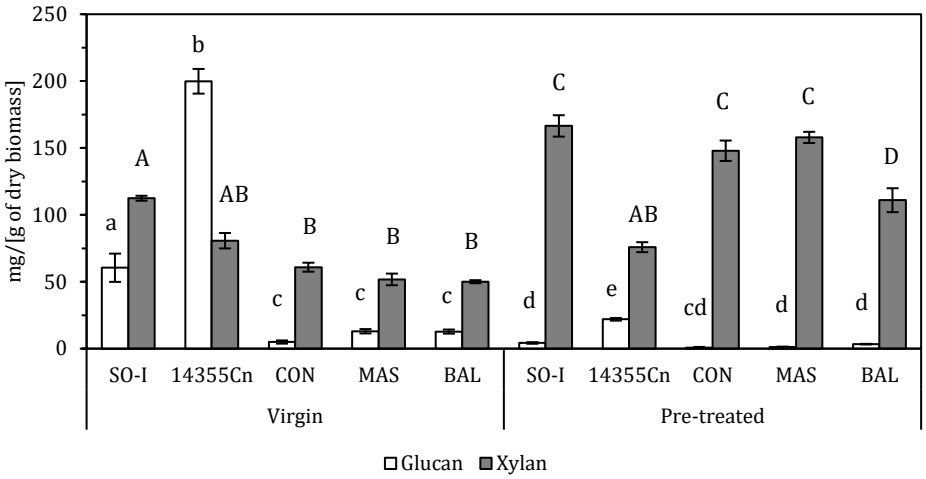

Figure 5-3: Glucan and Xylan contents in virgin and pre-treated oat husk varieties; unmatching letters above the bars indicate statistically significant difference between the bars

In terms of arabinose to xylose ratios, virgin low lignin varieties produced lower values than the commercial varieties (see Table 5-6), however the ratios were similar across all varieties for pre-treated extracts. As expected, reduction in glucose to xylose ratios was observed in the pre-treated extracts. Arabinoxylan contents in the pre-treated extracts were higher than virgin extracts, reaching close to 60% in terms of extract dry weight for SO-I, CON and MAS, but were lower for BAL and 14355Cn. The unaccounted extract fractions are likely composed of polyphenols of lignin origin as well as other non-structural cell components such as waxes, lipids and protein, as well as some carbohydrate degradation products.

Low-lignin varieties at virgin extractions produced higher xylan yields than the commercial varieties; and from the pre-treated extractions, SO-I produced the highest xylan yields overall, reaching 88%, which is in agreement with the above stated hypothesis, whereby xylan is easier solubilised from the low-lignin varieties due to lower degrees of association with lignin. However, the yield of the other pre-treated low-lignin

variety, 14355Cn, was the lowest across all pre-treated varieties. XOS extraction efficiency, which is expressed as fraction in % of pre-biotic XOS from the total xylan yield, was lower with low-lignin varieties, indicating that the xylan present in the extracts are of higher molecular weights.

Table 5-6: Carbohydrate ratios and key extraction efficacy indicators for virgin (V) and pre-treated (P) oat husk extracts

	Ara/Xyl	Glu/Xyl	AX content %	Xylan yield %	XOS prod. eff. %
V.SO-I	0.17	0.54	38.1	59.3	4.7
V.14355Cn	0.21	2.51	20.6	34.0	5.7
V.CON	0.25	0.08	45.7	23.3	11.0
V.MAS	0.25	0.25	40.0	20.0	8.3
V.BAL	0.22	0.23	38.9	13.7	5.4
P.SO-I	0.11	0.03	58.6	88.0	8.0
P.14355Cn	0.15	0.29	40.7	31.7	6.7
P.CON	0.12	0.01	59.4	56.0	13.7
P.MAS	0.11	0.01	62.2	60.3	11.3
P.BAL	0.12	0.03	46.8	42.3	15.9

5.4.3. Furan contents

Furfural and 5-HMF are the degradation products of pentoses and hexoses respectively. In general, low amounts of 5-HMF were found in the extracts which was expected as both main hemicellulosic carbohydrates in oat husks are pentoses. High contents of 5-HMF would be an indicator of either degradation of glucose and fructose of non-structural origin, and therefore present at relatively low extraction severities; or of glucan from cellulose, which would be expected to occur at high extraction severities. Indeed, low 5-HMF values were observed in the extracts as can be seen in Table 5-7. In contrast, extracts contained higher amounts of furfural, degradation product of arabinose and xylose. Furan contents were similar across all varieties, but were higher in the pre-treated extracts, possibly due to reduced biomass recalcitrance as a result of the pre-treatment.

Table 5-7: Furan contents of virgin (V) and pre-treated (P) oat husk extracts

	5-HMF		Furfural	
	mg/g*	sem	mg/g*	Sem
V.SO-I	0.06	±0.01	0.79	±0.05
V.14355Cn	0.12	±0.01	0.56	±0.01
V.CON	0.05	±0.01	0.58	±0.06
V.MAS	*nd*	±0.01	0.35	±0.02
V.BAL	0.07	±0.02	0.93	±0.33
P.SO-I	0.05	±0.01	1.80	±0.22
P.14355Cn	0.08	±0.01	0.90	±0.03
P.CON	*nd*	±0.01	1.20	±0.13
P.MAS	0.13	±0.11	0.90	±0.46
P.BAL	0.06	±0.01	1.85	±0.35

5.4.4. Total phenolic contents

Extract total phenolic contents (TPC), shown in Figure 5-4 increased for BAL, decreased for low-lignin varieties, and stayed the same for CON and MAS when comparing virgin and pre-treated extracts. Overall, TPC were similar across all varieties and between both, virgin and pre-treated extractions, with the exception of 14355Cn, which produced significantly lower values in pre-treated extracts. It is possible that the virgin extracts would contain more phenolics of non-structural origin, while the pre-treated extracts would contain more phenolics originating from lignin due to the increased extraction severity due to the pre-treatment. This argument is supported by the lower TPC contents in the low-lignin varieties after the pre-treatment because the non-structural phenolics would be washed out with the pre-treatment; moreover, lower amounts of structural phenolics would be solubilised compared to the commercial varieties due to the lower lignin availability.

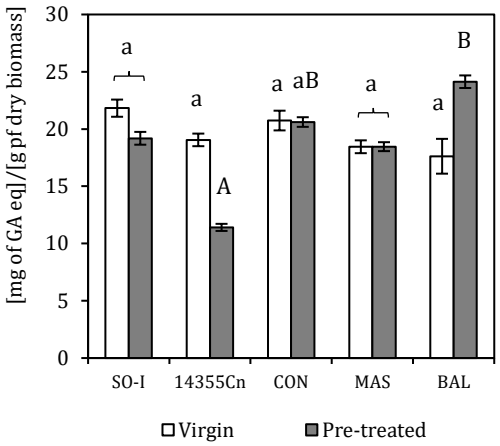

Figure 5-4: Total phenolic contents of virgin and pre-treated oat husk extracts; unmatching letters above the bars indicate statistically significant difference between the bars

5.4.5. Fractionation using ethanol precipitation

Figure 5-5 shows the precipitate and supernatant fractions of virgin and pre-treated husks after extract fractionation with ethanol (60% v/v ethanol/extract). Overall, the fraction of recovered precipitate lowered after the pre-treatment, which points to the hemicellulose depolymerisation due to higher cumulative extraction severity. As discussed above, low-lignin varieties produced less prebiotic XOS despite having high xylan content, which led to suggestion that the hemicellulose derivatives solubilised in the extracts from the low-lignin varieties are of higher molecular weight. This was confirmed with ethanol precipitation as both low-lignin varieties produced the highest precipitate fractions from both virgin and pre-treated extracts.

Some of the recovered precipitate, particularly from virgin low-lignin varieties, might also include glucan originating from the non-structural components solubilised in the extract. From the commercial varieties, contrasting the low-lignin varieties, CON produced the lowest precipitate fractions from both virgin and pre-treated extracts despite having relatively high xylan contents in the extract. This may indicate lower

molecular weight distribution compared to other varieties, which suggests that CON might be the most favourable of the varieties for prebiotic extraction.

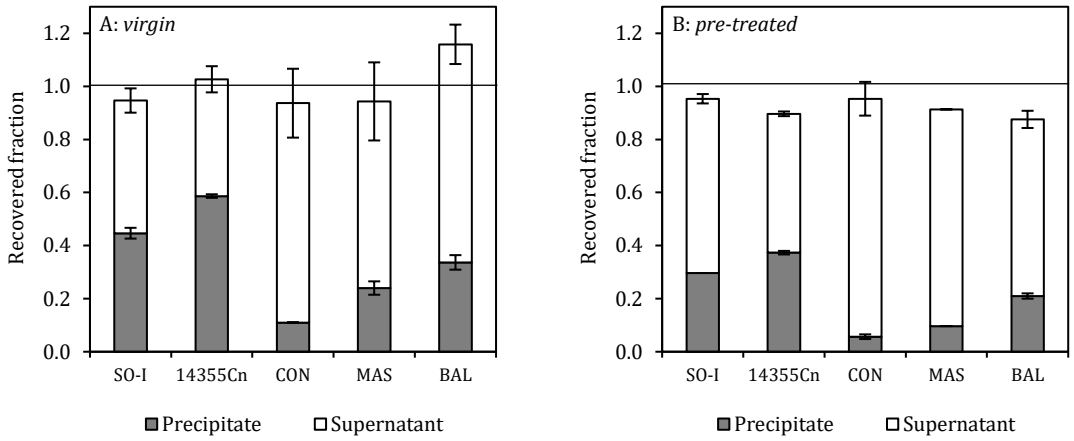

Figure 5-5: Stacked 60%(v/v) ethanol precipitate and supernatant fractions of virgin (A) and pre-treated (B) oat husk extracts

5.5. Chapter conclusions

Results discussed in this chapter confirmed the positive effect of pre-treatment towards extract composition and xylan and XOS yields. Moreover, it was observed that the oat husks can contain significant amounts of groats within the biomass but it was not clear whether it was a characteristic of the particular varieties or as a result of inefficient de-hulling. Nevertheless, the husks of the low-lignin varieties contained higher amounts of groats, resulting in higher glucan contents in virgin extract, which was mitigated by adopting the pre-treatment. Low-lignin varieties differed from the commercial varieties also in terms of xylan and XOS contents – SO-I and 14355Cn produced lower amounts of XOS at the same extraction conditions while maintaining relatively high xylan contents in the extract, indicating that the solubilised hemicellulose from these varieties is of higher molecular weights. This was confirmed by the ethanol precipitation study, where low-lignin varieties produced higher precipitate fraction. It was suggested that such

differences were due to the compositional characteristics of the varieties as a result of breeding for low lignin contents. In particular, it was hypothesized that lack of lignin would require more rigid hemicellulose and cellulose matrix.

From the commercial varieties, Conway showed the lowest amount of precipitate fraction as well as highest XOS contents, indicating that hemicellulose derivatives in the extract are of relatively low molecular weights, which suggests that this variety is likely to be suitable for XOS extraction, while the heavier low-lignin varieties for other applications such as films. In terms of xylan and XOS yields, varieties produced good values for former but low values for the latter. It is expected that higher extraction severity would be required to achieve high XOS yields, which can be achieved either by increasing residence time and temperature, or by performing a sequential XOS extraction from already heavy-xylan-rich extracts such as obtained here from SO-I and 14355Cn.

All varieties produced low amounts of furan contents in the extracts. In terms of total phenolic contents, all commercial varieties produced similar or higher values from the pre-treated extracts while low-lignin varieties produced lower values after the pre-treatment, suggesting that the origin of the phenolics in the extract particularly with higher extraction severity are originating from the lignin fraction of the biomass.

CHAPTER 6. OPTIMAL PARAMETERS FOR HEMICELLULOSE AND XYLOOLIGOSACCHARIDE EXTRACTION USING SUB-CRITICAL WATER MEDIATED HYDROLYSIS FROM *MISCANTHUS χ GIGANTEUS, AVENA SATIVA* VAR *BALADO* AND VAR *CONWAY* HUSKS: DESIGN OF EXPERIMENTS

6.1. Introduction

The objective of this chapter is to investigate the optimal conditions in terms of prebiotic xylooligosaccharides (XOS), total arabinoxylan and xylose extraction via subCW mediated autohydrolysis of three different types of biomass – husks of two different oat varieties (*Avena sativa*) and perennial grass *Miscanthus χ giganteus* (MIS). From the five oat varieties discussed in previous chapter *Balado* (BAL) and *Conway* (CON) were chosen for several reasons: firstly, both are commercially available varieties, rather than experimental as the low-lignin varieties; secondly, BAL is a winter variety while CON is spring, therefore having higher likelihood of being structurally different; and thirdly, CON showed lowest amounts of recovered precipitate in ethanol fractionation study (see previous chapter), indicating lower molecular weight distribution than BAL, which produced the highest amounts of recovered precipitate from the commercial varieties.

To obtain the optimal conditions all types of biomass were pre-treated using the conditions described in Section 3.3.4. Then, response surface methodology (RSM) was used to explore the relationships between three extraction factors – extraction temperature, residence time, and loading. The resulting extracts were then analysed for carbohydrate composition, total phenolic, and furan contents. Other responses were also measured, such as biomass solubility, extract pH and extract mass concentration. Detailed description of extraction procedures and analytical methods is given in Section 3.5.

6.2. Design of experiments and response surface methodology (RSM)

The experiments were designed using central composite design (CCD) methodology with three factors – extraction temperature, residence time and loading. CCD is the most

popular RSM design and consists of three types of design points (see Figure 6-1) – factorial to account for all factor combinations, axial to calculate orthogonality and rotatability of the design, and centre points to estimate the experimental error (Anderson and Whitcomb, 2005). Temperature and residence time amplitudes were chosen based on the understanding of the extraction conditions from the investigations discussed in previous chapters, signifying an extraction area where curvature in measured responses were expected. The chosen extraction temperature range was 140-200 °C with axial points at 120 °C and 220 °C. The amplitude for the residence time was chosen to be 10-60 min with axial points at 0 and 77 min. Loading range, on the other hand, was limited to the physical boundaries of the equipment – biomass to water loading higher than 13% (w/v) resulted in agitator failure due to insufficient power, therefore the selected range was 2-10% with axial points at 0.5% and 13%. Extractions from factorial and axial points were performed once, while the centre point was performed five times to evaluate the standard error of the design, in total accounting to twenty experiments (see Table 6-1 for summary).

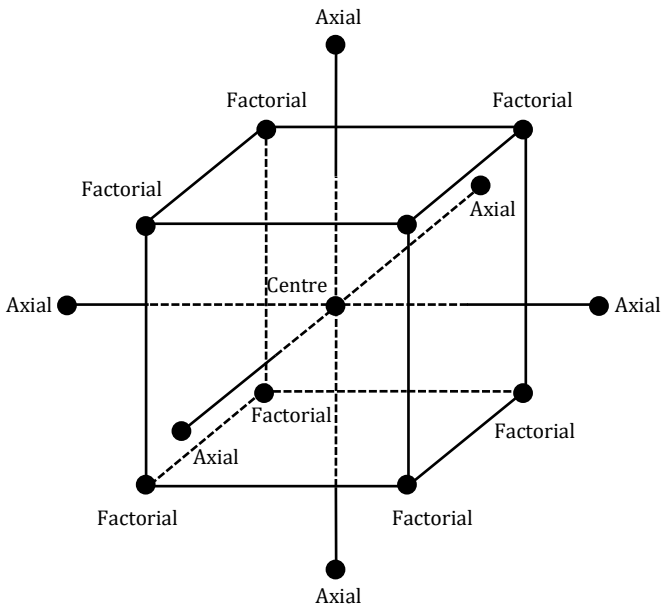

Figure 6-1: Three factor central composite design cube with factorial, axial and centre points

Apart from the three factor CCD, the resulting data were also analysed as two factor RSM design, by reducing the dimensionality via the extraction severity factor log(R_0), which combines the extraction temperature and residence time. Using RSM design with severity factor allowed visualisation of the results with 3D plots, which provide better visual insight for response analysis.

Table 6-1: Three factor (temp., res.time and loading) central composite design of experiments with corresponding severity factor and design point type

Extr. Nr.	S. factor, log(R_0)	Temperature, °C	Res. time, min	Loading, %[w/v]	Design Point
1	4.42	170	77	6	Axial
2	4.01	140	60	2	Factorial
3	5.12	200	60	2	Factorial
4	4.21	170	35	13	Axial
5	5.05	200	10	2	Factorial
6	4.21	170	35	6	Centre
7	3.88	170	0	6	Axial
8	4.01	140	60	10	Factorial
9	4.21	170	35	6	Centre
10	4.21	170	35	6	Centre
11	4.21	170	35	6	Centre
12	3.50	140	10	10	Factorial
13	5.50	220	35	6	Axial
14	5.12	200	60	10	Factorial
15	4.21	170	35	0.5	Axial
16	4.21	170	35	6	Centre
17	3.50	140	10	2	Factorial
18	3.61	120	35	6	Axial
19	4.21	170	35	6	Centre
20	5.05	200	10	10	Factorial

6.3. Severity factor

Several authors have reduced the dimensionality of the experiments by combining temperature and residence time with a severity factor (Ares-Peón et al., 2013; Carvalheiro et al., 2009; Lee et al., 2010; Vegas et al., 2008a; Xiao et al., 2013a). The severity factor takes into account the temperature rise within the vessel, and therefore is a universal

indicator of extraction conditions, and thus allows comparisons between extractions that are performed with different extraction vessels, as long as the same calculations are used. The general formula of severity factor ($logR_0$) is given below in Equation 6-1 (Abatzoglou et al., 1992; Overend et al., 1987),

$$\log R_0 = \log\left[\int_0^t \exp\left(\frac{T(t) - T_{ref}}{\omega}\right) dt\right] \qquad \textit{Equation 6-1}$$

where t is time (min); T is temperature (°C); T_{ref} and ω are reference parameters described in literature with values of 100 °C and 14.75 °C respectively.

Although the above equation describes severity factor for a single stage extraction, the extractions discussed in this chapter consist of two stages – pre-treatment in a 5 L vessel and main extraction in 0.5 L vessel. Furthermore, each stage can be described to consist of two phases – a heat-up and an extraction phase. Therefore, if the extraction kinetics remain the same, and that Equation 6-1 remains valid through all stages, the severity factor combining the pre-treatment and main extraction can be written as shown in Equation 6-2 below,

$$\begin{aligned} \log R_0 &= \log[R_{0PrHeat} + R_{0PrExtr} + R_{0Heat} + R_{0Extr}] \\ &= \log\left[\int_{t_{0PrHeat}}^{t_{fPrHeat}} \exp\left(\frac{T(t) - T_{ref}}{\omega}\right) dt \right. \\ &+ \int_{t_{0PrExtr}}^{t_{fPrExtr}} \exp\left(\frac{T'(t) - T_{ref}}{\omega}\right) dt \\ &+ \int_{t_{0Heat}}^{t_{fHeat}} \exp\left(\frac{T''(t) - T_{ref}}{\omega}\right) dt \\ &\left. + \int_{t_{0Extr}}^{t_{fExtr}} \exp\left(\frac{T'''(t) - T_{ref}}{\omega}\right) dt\right] \end{aligned} \qquad \textit{Equation 6-2}$$

where $R_{0PrHeat}$ is severity factor resulting from heat-up phase in the pre-treatment stage; $R_{0PrExtr}$ is severity factor of the pre-treatment extraction phase, initiated once the temperature set-point is reached; R_{0Heat} is the severity factor of the heat-up phase in the main extraction stage; R_{0Extr} is the severity factor of the main extraction; t_0 is the initial, and t_f is the final residence time (min) of the corresponding phase, while subscripts of t_0 and t_f follow the same nomenclature as in R_0 above; T, T', T'', and T''' represent the combined temperature profiles for each phase (°C). Relevant heat-up profiles of the pre-treatment and extraction are shown in Section 3.3.

Severity factor ($logR_0$) of the pre-treatment was calculated to be 1.71, corresponding to 120 °C extraction in a 5 L vessel with 38 min and 30 min heat-up and residence times respectively. The highest $logR_0$ value was calculated to be 5.50, corresponding to 220 °C extraction in 0.5 L vessel with 28 min heat-up time and 30 min residence time of pre-treated biomass. Calculated severity factor values corresponding to the temperature and residence time ranges used this design are shown in Figure 6-2.

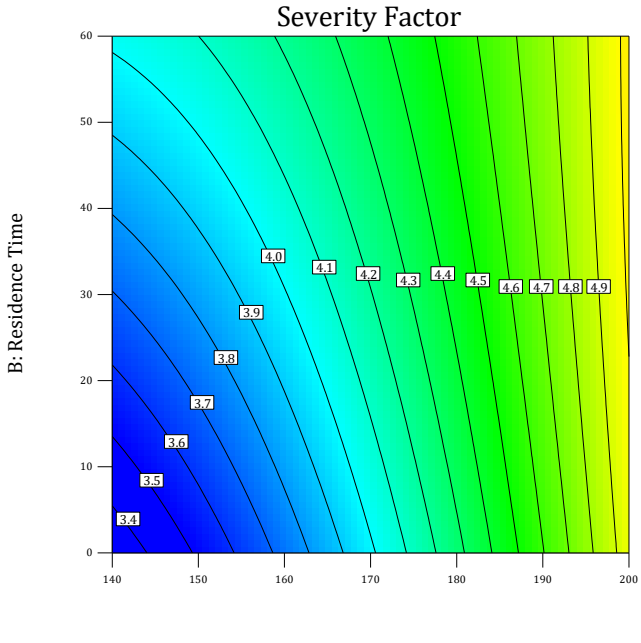

Figure 6-2: Visualisation of severity factor as a function of temperature (°C) and residence time (min)

6.4. Effects of extraction severity and loading on extract composition

Extracts obtained from the designed set of experiments were then analysed for carbohydrate composition, total phenolic contents, furan contents and pH, while total biomass solubility was calculated from the residual biomass. As described in previous chapters, monosaccharides and oligosaccharides were quantified directly from the extract using HPAEC-PAD, whereas to obtain an estimate of polysaccharide contents, extracts were first subjected to two-step acid hydrolysis, and the hydrolysates were then analysed for monosaccharide contents using the same analytical method. 5-HMF and furfural were quantified using reverse phase HPLC, and total phenolic contents of the extract were estimated using Folin-Ciocalteu colorimetric assay. All analytical methods are described in detail in Section 3.5. The relevant analytical data was then put in the RSM models as responses and analysed with Stat-Ease Design-Expert 7.0 software. The relationship of the extraction conditions (factors) and responses were calculated by quadratic polynomial equation with logarithmic, inverse, and inverse square root transformations were necessary as suggested by Box-Cox Plot. Analysis of variance (ANOVA) was used to evaluate the models. RSM model fit to the experimental data was analysed by evaluating sets of R^2, adjusted R^2, and predicted R^2 (see Table 6-2 and Table 6-3 for three factor CCD and two factor (severity) design respectively).

Whereas R^2 is a measure of variation around the mean explained by the model, adjusted R^2 is a measure of variation around the mean, adjusted for the number of terms in the model, and is generally lower than R^2 for models with many terms. Predicted R^2 is a measure of variation in the new data as explained by the model, which is calculated by systematically removing each observation from the model and determining how well the

model predicts the removed observation. (Neter et al., 1996) The difference of predicted R^2 and adjusted R^2 greater than 0.2 suggests an issue with either the model or the data (Anderson and Whitcomb, 2005). Models failing to meet this criterion were considered as uncertain.

*Table 6-2: R^2 results of three factor central composite RSM models (models meeting the Anderson criterion in **bold**; -: negative R^2 value)*

	BALADO			CONWAY			MISCANTHUS		
Response	R^2	Adj R^2	Pred R^2	R^2	Adj R^2	Pred R^2	R^2	Adj R^2	Pred R^2
Solubility	**0.90**	**0.85**	**0.71**	0.89	0.80	0.21	0.96	0.92	0.68
pH	**0.97**	**0.96**	**0.90**	**0.94**	**0.88**	**0.80**	0.95	0.90	0.60
TPC	**0.95**	**0.93**	**0.86**	**0.96**	**0.93**	**0.73**	**0.97**	**0.94**	**0.80**
Furfural	**0.93**	**0.89**	**0.78**	**0.97**	**0.95**	**0.75**	**0.95**	**0.92**	**0.72**
Xylose	0.60	0.37	-	0.63	0.31	-	0.58	0.20	-
XOS	0.61	0.41	-	0.62	0.28	-	0.57	0.18	-
AX	**0.90**	**0.84**	**0.67**	0.88	0.77	0.01	0.81	0.64	-

From Table 6-2 and Table 6-3 it can be seen that none of the xylose and XOS models achieved good fit to the experimental data, while the two factor models generally achieved better fits for solubility, pH and TPC responses. The models for furfural achieved good fits from the three factor CCDs but not from the two factor designs. ANOVA summary tables for three factor designs of solubility, pH, TPC and furfural, and for two factor designs of solubility, pH, TPC, and AX are given in Appendix A.

*Table 6-3: R^2 results of severity factor adjusted two factor RSM models (valid models in **bold**, -: negative value)*

	BALADO			CONWAY			MISCANTHUS		
Response	R^2	Adj R^2	Pred R^2	R^2	Adj R^2	Pred R^2	R^2	Adj R^2	Pred R^2
Solubility	**0.84**	**0.80**	**0.71**	**0.81**	**0.74**	**0.63**	**0.89**	**0.86**	**0.77**
pH	**0.91**	**0.89**	**0.80**	**0.89**	**0.86**	**0.76**	**0.90**	**0.87**	**0.77**
TPC	**0.90**	**0.88**	**0.81**	**0.89**	**0.85**	**0.72**	**0.91**	**0.88**	**0.79**
Furfural	0.58	0.47	0.16	0.67	0.55	0.17	0.73	0.64	0.29
Xylose	0.65	0.57	0.25	0.69	0.58	0.23	0.60	0.46	-
XOS	0.59	0.49	0.22	0.58	0.42	-	0.52	0.35	-
AX	**0.82**	**0.78**	**0.61**	0.83	0.77	0.51	0.67	0.55	0.15

6.4.1. Biomass solubility and extract pH

As can be seen from Figure 6-3, biomass solubility increased with extraction severity and slightly decreased with loading for all biomass types. Results produced similar models for BAL and CON, predicting solubility values above 50% at $logR_0$ above 4.8 and biomass loading below 5% (w/v). MIS model produced lower solubility values than husk models, reaching the highest values at $logR_0$ above 5.0 and loading below 3.5%. Higher solubility values at lower loads, can be explained by the dispersion of the solids resulting in higher surface area availability, which would not be the case with high loads, where the biomass is closely packed. As the xylan contents in the pre-treated biomass were approximately 29% for BAL and CON, and 19% for MIS, any higher solubility values are an indication of lignin and/or cellulose solubilisation.

Solubility values equal to the xylan content were observed at $logR_0$ values close to 4.0. ANOVA of the two factor solubility models showed that the most significant term was severity factor, producing probability (P-) values <0.05 for the linear terms for all tested types of biomass, and for the quadratic terms of BAL and CON, while loading and interactions between the terms resulted in P-values >0.05, and therefore were considered statistically insignificant. Unsurprisingly, from the three factor models, the linear terms of temperature and residence time were significant for BAL and CON, while all three of the linear factors were significant for MIS; quadratic term of residence time was also significant for husks but not for MIS.

The main driving force of biomass solubilisation in subCW is the process of auto-hydrolysis, which can be indirectly observed through the proxy of extract acidity. The pH is expected to decrease with extraction severity as auto-hydrolysis of the lignocellulosic

matrix unfolds, where hydronium ions (H^+) first produced by the auto-ionisation of subCW start attacking the easily accessible acetyl groups on the lateral hemicellulose chains. As a result acetic acid is produced, which further contributes to the presence of hydronium ions, consequently improving the likelihood of cleaving the harder-to-break glycosidic bonds of xylan, leading to xylan depolymerisation and consequent solubilisation (Garrote et al., 1999; Lee et al., 2009; Mosier et al., 2005). Apart from acetic acid, other acids, such as uronic, *p*-coumaric and ferulic acids, may also be released from the lateral xylan chains which also contribute to this process (Garrote et al., 1999; Otieno and Ahring, 2012a). Furthermore, at high extraction severity, formic and levulinic acids might also form from furfural and 5-HMF, the degradation products of pentoses and hexoses, contributing to increased acidity (Pińkowska et al., 2011).

From the results of extract pH shown in Figure 6-4, indeed the pH decreases with extraction severity, and in the case of BAL and MIS also with loading. ANOVA of the two factor models showed that the linear and quadratic terms of $logR_0$ were significant factors for all biomass types. For MIS linear factor of loading was also significant. Similarly, three factor models with exploded $logR_0$ into temperature and residence time, showed that the linear factors of temperature, residence time and loading were significant for BAL and MIS, with only temperature and residence time significant for CON; in quadratic terms, both temperature and residence time was significant for BAL, and only residence time for MIS. Extract acidity of all types of biomass was generally similar until $logR_0$ 4.5. However, at higher $logR_0$ values, the effect of loading became apparent in BAL and MIS extracts but not in CON.

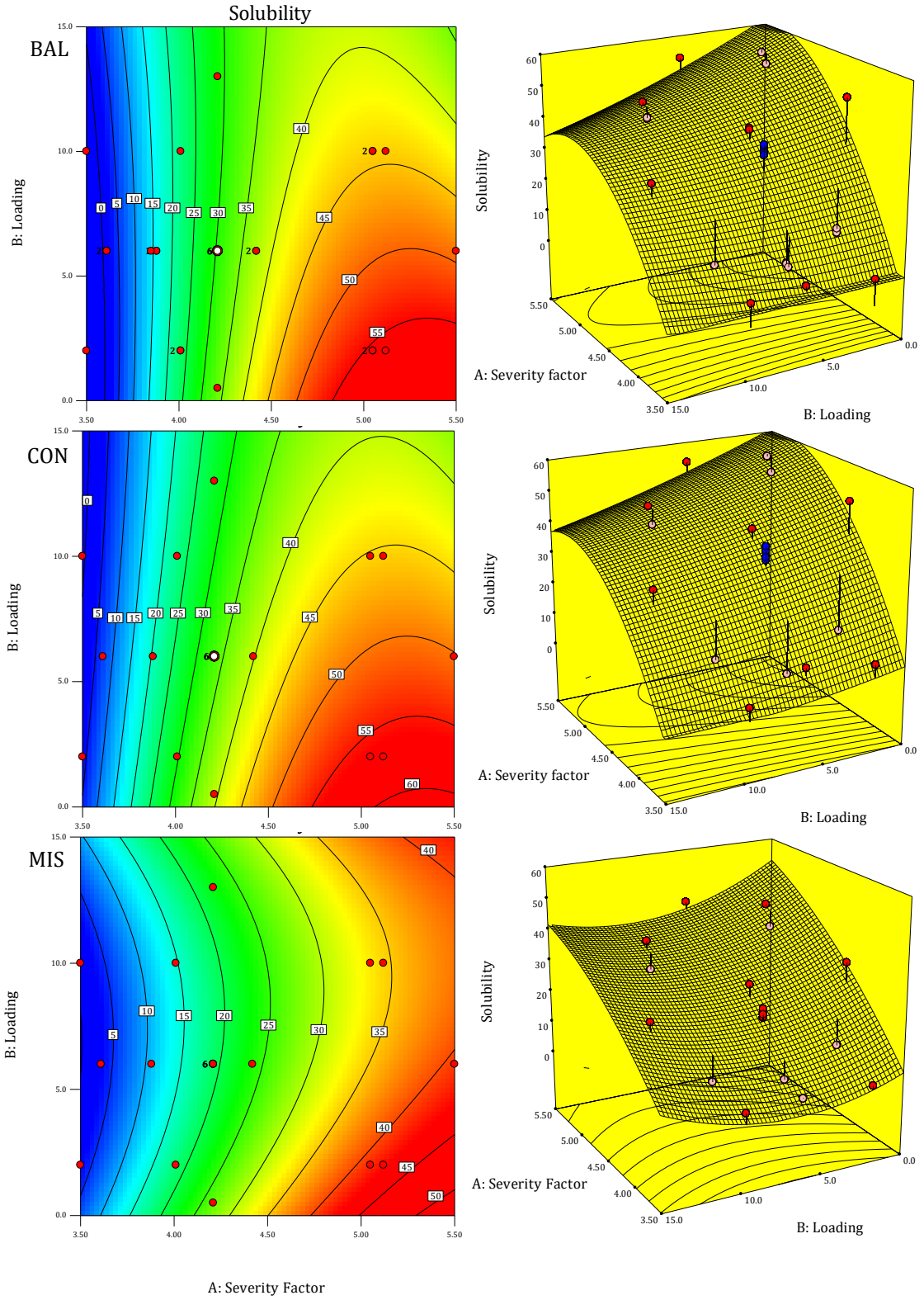

Figure 6-3: Contour and 3D surface graphs of BAL, CON, and MIS solubility (%dw) as a response of extraction severity factor and loading (%[w/v]); 3D graphs rotated for clarity of vizualisation

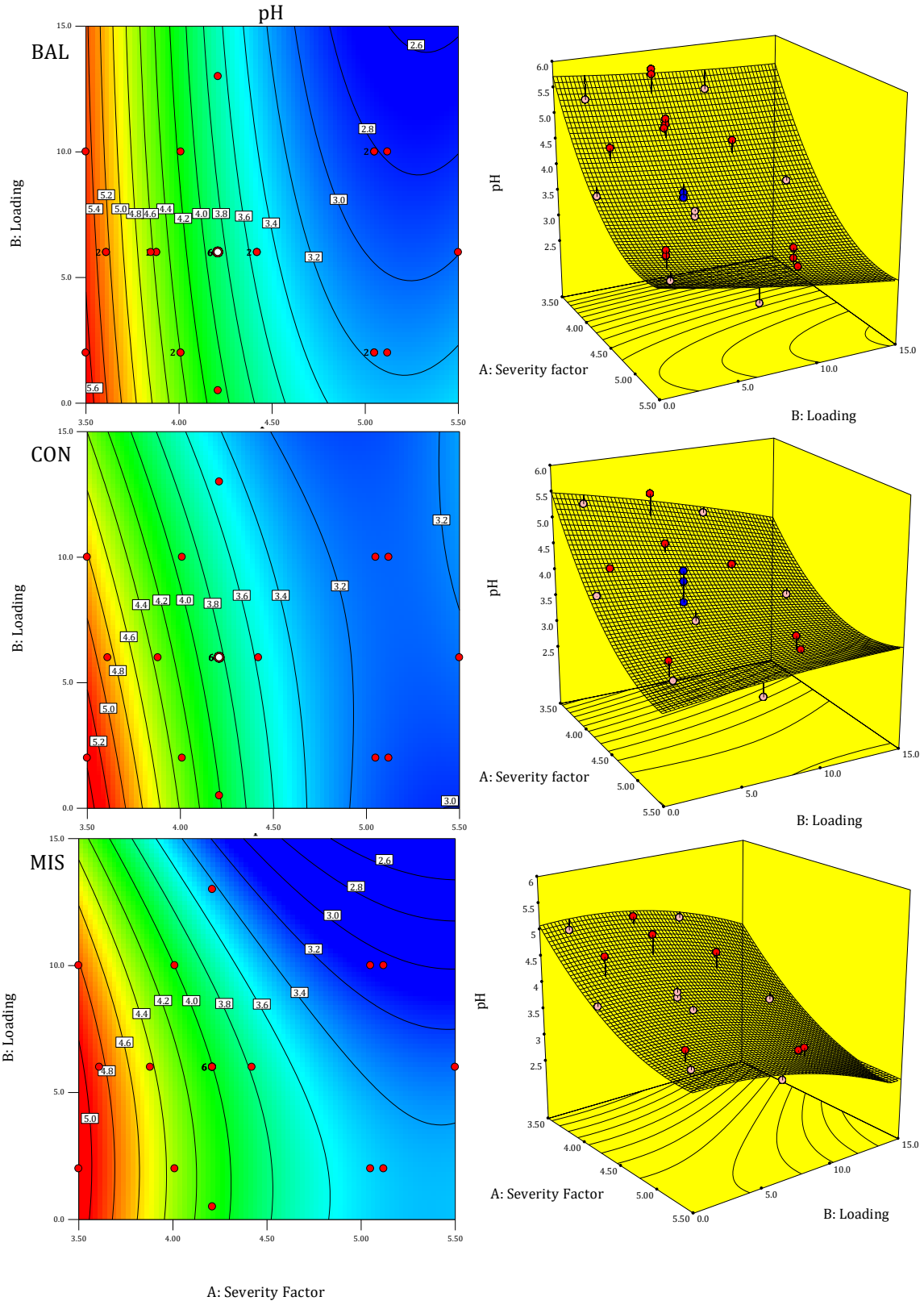

Figure 6-4: Contour and 3D surface graphs of BAL, CON, and MIS extract pH as a response of extraction severity factor and loading (%[w/v]); 3D graphs rotated for clarity of vizualisation

6.4.2. Carbohydrate composition of the extracts

In contrast to solubility and extract pH, model fitting on the carbohydrate composition data was problematic as evident by the low predicted R^2 values shown in Table 6-2 and Table 6-3. Nevertheless, the results are presented here with the traditional methods of data analysis, and will be discussed following the molecular weights of the compounds, i.e. starting from monosaccharides and finishing with polysaccharides.

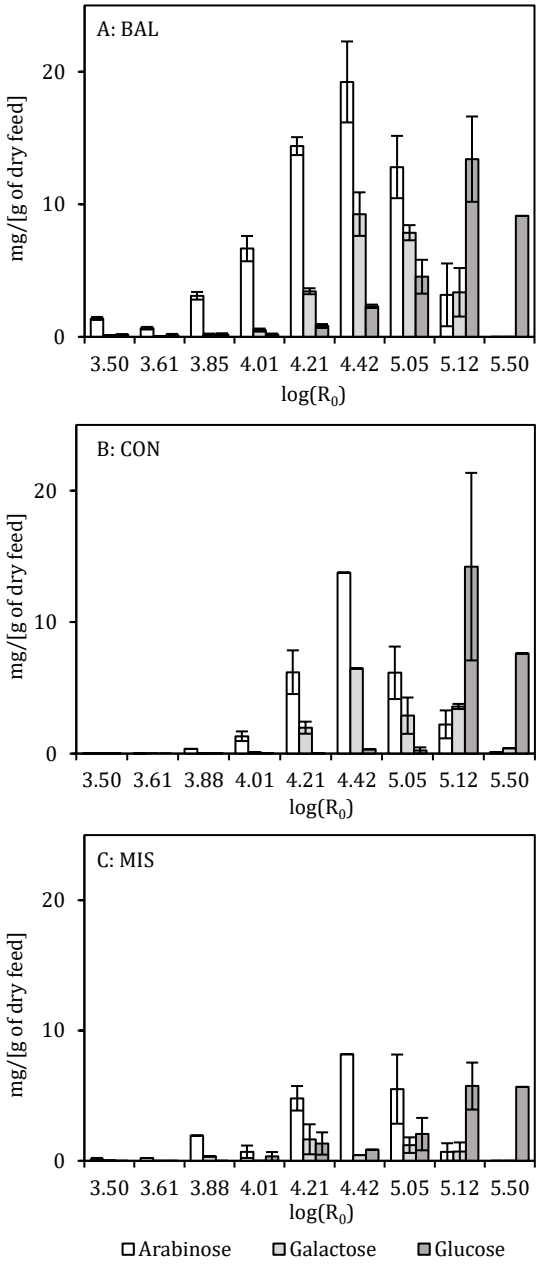

Figure 6-5: Extract contents of monosaccharides other than xylose at different extraction severities

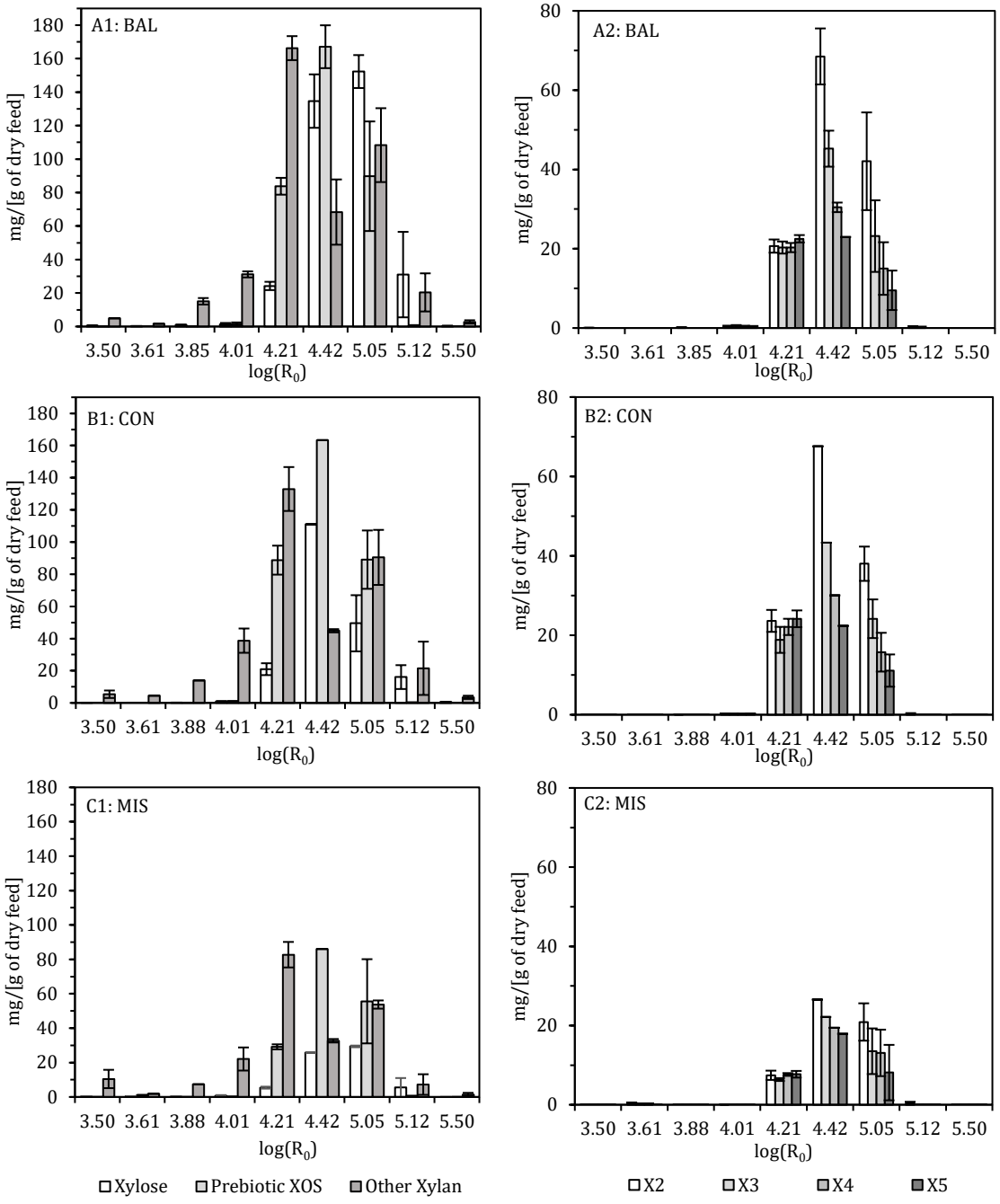

Figure 6-6: Xylose, XOS (DP2-5) and Xylan (excluding XOS with DP2-5) contents other than XOS of extracts obtained at different extraction severities (left); prebiotic XOS distribution at selected extraction severities of the same extracts (right)

From the extract composition of carbohydrate monomers other than xylan, shown in Figure 6-5, the degree of branching along the xylan backbone can be seen as evident from arabinose and galactose contents. The composition of these two monosaccharides show

that the hemicellulose branching is different between all three biomass types. Highest contents of arabinose and galactose, and therefore branching were observed at $logR_0$ of 4.42 (170 °C and 77 min residence time), for all biomass types. BAL produced the highest values, reaching 19 and 9 mg/[g of dry feed], while CON extracts measured at 14 and 6 mg/[g of dry feed] respectively, despite having virtually identical xylan contents in raw biomass (26.1 and 26.4 %dw respectively). MIS produced the lowest amounts of arabinose at 6 mg/[g of dry feed] and almost negligible amounts of galactose. As discussed before, the lateral branches of hemicellulose, including arabinose and galactose substitutions, were expected to be cleaved before the linear xylose chains of the hemicellulose backbone. This was confirmed for BAL and MIS but not for CON as the maximum xylose contents (152 mg/g for BAL, and 29 mg/g for MIS) were observed at higher extraction severities – $logR_0$ of 5.05, than arabinose and galactose, while the xylose peaked (111 mg/g) at $logR_0$ 4.42 for CON extracts (see Figure 6-6).

It has to be noted that at these extraction severities, biomass solubility already was higher than the xylan contents of the biomass, suggesting that components of lignin origin were likely to be present in the extracts. Several authors have reported peak xylose contents at similar extraction severities as CON – at $logR_0$ 4.35 from corn stover (Buruiana et al., 2014) and flowering plant *Ulex europæus* (Ares-Peón et al., 2013) and $logR_0$ 4.36 from wheat straw (Carvalheiro et al., 2009). Reduced xylose, arabinose and galactose values after their peaks indicated that the rate of hemicellulose depolymerisation into the corresponding monomers has become lower than the rate of monomer degradation into furfural in case of arabinose and xylose and 5-HMF in case of galactose.

Another shift in extract composition in terms of monosaccharides was observed at the extremes of the tested extraction severity. While remaining low up to $logR_0$ of 4.42, at higher severities glucose contents of the extracts steadily increased, peaking at 5.12, and again decreasing at $logR_0$ of 5.50. This indicates that at high extraction severities, corresponding to 200 °C and longer than 10 min residence time, cellulose starts to depolymerise, releasing glucose (see Figure 6-5), which is then quickly followed by its degradation into 5-HMF at extraction severity corresponding to 220 °C and 35 min residence time (see Figure 6-8).

In this work, XOS are defined as XOS with the degree of polymerisation (DP) of 2-5, which were found to be the most effective in terms of prebiotic efficacy (Hughes et al., 2007; Moura et al., 2007). Extract composition of prebiotic XOS and xylan other than prebiotic XOS, as well as prebiotic XOS distribution are shown in Figure 6-6, where it can be observed that the prebiotic XOS fraction peaked at 167 mg/g for BAL, 163 mg/g for CON and 86 mg/g for MIS. XOS were the dominant xylose based species in the extract at $logR_0$ of 4.42, which corresponds to extraction temperature of 170 °C and 77 min residence time.

At extraction severities between 3.85-4.21, xylan was the dominant species, whereas prebiotic XOS were more abundant at extracts from $logR_0$ of 4.42.At extraction severities above 4.42, the prebiotic XOS contents decreased as the rate of XOS degradation into xylose and furfural overtook the rate of xylan depolymerisation into XOS (see Figure 6-6, Figure 6-7, and Figure 6-8). At $logR_0$ of 4.42, the fraction of prebiotic XOS relative to xylose and other xylan was highest for MIS, but similar between BAL and CON extracts, suggesting a structural difference in terms of the degree of hemicellulose branching

between the husks and the energy crop, the latter appearing to be better suitable for prebiotic extractions. Moreover, the distribution of prebiotic XOS also was different in MIS extracts, having less distinct differences in distribution at the peak extraction severity (see the left side of Figure 6-6), while the results from husks were similar. At $logR_0$ of 4.21, extracts consisted of similar amounts of prebiotic components relative to each other, while at higher severities the distribution became skewed towards the lower weight XOS, which is a clear indication of XOS depolymerisation. This observation is important, if higher DP components should be targeted, which could be used for other applications of hemicellulose requiring higher molecular weight polysaccharides.

The obtained peak XOS contents in the extract and the corresponding yields were similar to the published results of subCW mediated extractions from different biomass sources (see Table 6-4). Extraction conditions in terms of temperature and residence times for the optimal XOS yields were also similar, however, the calculated severity factor values for the results in this study were considerably higher than the other published work. This can be explained by the additional pre-treatment step that was performed prior to the extractions, accounting for $logR_0$ 1.71, which increased the overall extraction severity values while apparently not producing significant changes in the underlying hemicellulose structure due to the relatively mild extraction conditions.

Possibly the most interesting behaviour of hemicellulose solubilisation in subCW can be observed at $logR_0$ of 5.05 corresponding to extraction temperature of 200 °C and 10 min residence time (see left hand side of Figure 6-6 and circled areas in Figure 6-7). Several authors have observed two stages of hemicellulose solubilisation – in the first stage, the heterogeneous xylan, accounting for the majority of xylan, is easily hydrolysed following

first order reaction kinetics, leaving the remaining rigid xylan fraction to be hydrolysed at a slower rate in the second stage (Garrote et al., 1999; Nabarlatz et al., 2004; Otieno and Ahring, 2012a). As can be seen from Figure 6-6, the extract contents of non-prebiotic xylan peaked at $logR_0$ of 4.21, decreased at 4.42 and then spiked again at $logR_0$ 5.05 across all three biomass types. The same can be observed in Figure 6-7, which shows the total arabinoxylan (AX) contents in the extract. The spike in xylan contents at $logR_0$ 5.05 was observed for all biomass types, and appeared to be inconsistent with the degradation trend with increasing extraction severity.

Table 6-4: Literature summary of extract XOS contents, yields and extraction conditions from various biomass sources

Biomass	XOS content, mg/g	XOS yield, %	Temp., °C	Res. time, min	Sev.f., $logR_0$	Reference
Ulex europæus	-	79	200	0	3.79	(Ares-Peón et al., 2013)
Arundo donax	177	-	180	42	-	(Caparros et al., 2007)
Olive tree prunings	60	55	180	10	-	(Cara et al., 2012)
Wheat straw	105	50	215	0	3.96	(Carvalheiro et al., 2009)
MIS	135	-	200	5	-	(Chen et al., 2014)
Rye straw	-	69	208	0	-	(Gullon et al., 2010)
MIS	90	65	160	60	-	(Ligero et al., 2011)
Corn straw	-	53	215	0	3.75	(Moniz et al., 2013)
Corn cobs	250	-	-	-	3.75	(Moura et al., 2007)
Corn cobs	-	58	190	15	-	(Nabarlatz et al., 2004)
Wheat straw	-	44	180	30	-	(Ruiz et al., 2011)
DDGS	80	-	180	20	-	(Samala et al., 2015)
Corn Flour	90	-	190	10	-	(Samala et al., 2015)
Tamarix ramosissima	93	-	190	0	2.7	(Xiao et al., 2013a)
Bamboo culm	-	47	180	30	-	(Xiao et al., 2013b)
BAL	167	56	170	77	4.42	This study
CON	163	55	170	77	4.42	This study
MIS	86	44	170	77	4.42	This study

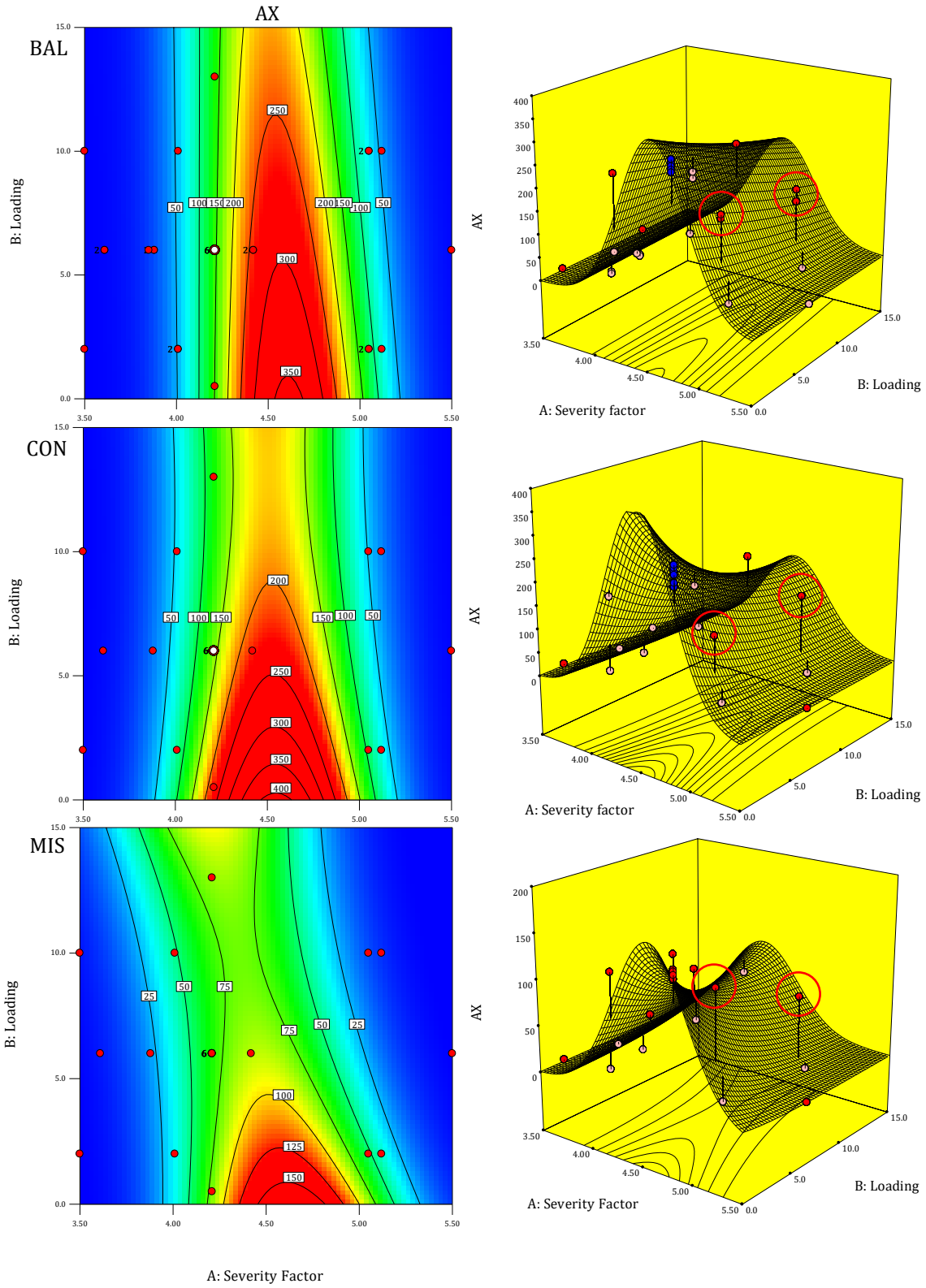

Figure 6-7: Contour and 3D surface graphs of BAL, CON, and MIS Arabinoxylan (AX) contents (mg/[g of dry feed]) as a response of extraction severity factor and loading (%[w/v]); red circles represent conditios when less reactive hemicellulose fraction is released; 3D graphs rotated for clarity of vizualisation

The concept of two extraction stages appears to be a fitting explanation of these spikes. While the majority of hemicellulose was already solubilised up to logR_0 4.50 following the first stage of solubilisation, the more recalcitrant hemicellulose fraction was solubilised at much higher extraction severity, but was readily decomposed soon after. Overall, peak extraction of AX was observed at logR_0 4.50. From the contour graphs in Figure 6-7, lower biomass loading produced higher AX contents in the extracts for all types of biomass, however, its impact appeared to be different for each type. Higher AX contents at lower biomass loadings can be explained by the better exposure of surface area to the liquid phase, which allows better permeability of the hydronium ions. Contrary to solubility and pH, the quadratic RSM models did not fit the measured data well, possibly due to the two stage extraction behaviour of xylan.

Results from BAL extractions produced acceptable R^2 values (see Table 6-2 and Table 6-3). Although the model fit was not acceptable for CON and MIS according to R^2 values, both are shown in Figure 6-7 for comparison. ANOVA results from BAL AX models showed that the linear and quadratic terms were the only significant factors in the two factor model, while for the three factor CCD model, the linear and quadratic terms of temperature and residence time, as well as their interaction was shown to be significant. Full details of ANOVA result summaries are given in Appendix A.

As mentioned, by modifying extraction severity, hemicellulose derived products of different molecular weights can be targeted. This can be seen when analysing the yields of the major components, shown in Table 6-5, where the yields of xylose, prebiotic XOS and total xylan are presented. It shows that the optimal conditions for targeting highest xylan yields corresponded to logR_0 of 4.21 (170 °C, 35 min) for the husks, yielding 96%

and 84% for BAL and CON respectively, while $logR_0$ of 4.42 (170 °C, 77 min) provided the highest yield for MIS, reaching 69% of the total available xylan in the raw biomass.

However, at these conditions, the solubilised xylan molecules already had undergone some depolymerisation, shifting the molecular weight profile towards the XOS, which is evident by the relatively high yields of XOS, particularly in the husk extracts. If high molecular weight hemicellulose were to be targeted, lower extraction severities should be used despite the lower yields. In case of targeting the prebiotic XOS (DP 2-5), optimal conditions should occur around $logR_0$ of 4.42, providing yields up to 56% in case of BAL, 55% for CON, and 44% for MIS. At the same conditions relatively high amounts of xylose were also present, accounting to 45% for BAL, 37% for CON and only 13% for MIS extracts.

Optimal conditions for highest xylose yields were not consistent between the biomass types – in case of BAL and MIS, $logR_0$ of 5.05 (200 °C, 10 min) achieved the highest yields corresponding to 51% and 15% respectively, while highest xylose yield of 37% for CON were achieved at $logR_0$ 4.42. Xylose yield discrepancy between the biomass types at the same extraction conditions suggest that the husks are likely to have more branched xylan structure than MIS with more axial (α) glycosidic bonds that are easier to cleave and solubilise than the more resistant equatorial (β) glycosidic bonds (Housecroft and Constable, 2006). Higher degrees of hemicellulose branching in husks than MIS is in agreement with higher contents of other monosaccharides, discussed above.

Table 6-5: Obtained yields of xylose, XOS and Xylan at different extraction conditions and loading

log(R_0)	Xylose yield, %			XOS yield, %			Xylan yield, %		
	BAL	CON	MIS	BAL	CON	MIS	BAL	CON	MIS
3.50	0.2±0.1	0	0.1	0	0	0	1.9	2.1±0.6	6.1±2.2
3.61	0.1	0	0.2	0	0	0.6	0.7±0.1	1.7	1.7
3.88	0.3±0.1	0.1	0	0.1±0.1	0	0	5.8±0.6	5.3	4.2
4.01	0.5±0.2	0.4±0.1	0.3±0.1	0.5±0.2	0.3±0.1	0	12.5±0.7	15.0±1.9	12.8±2.7
4.21	8.2±0.8	7.0±1.2	2.8±0.3	28.2±1.6	29.6±2.8	14.8±0.7	95.7±2.5	84.1±2.4	64.6±4.0
4.42	45.4±3.8	37.0	13.1	56.3±3.0	54.5	43.8	90.2±1.8	78.9	68.6
5.05	51.3±2.9	16.5±4.1	15.0±0.2	30.3±9.5	29.8±4.3	28.3±8.8	75.9±4.6	67.9±0.4	63.2±10.9
5.12	10.5±6.1	5.4±1.7	2.8±2.0	0.2±0.1	0.1±0.1	0.2±0.1	8.0±3.2	8.3±4.5	4.4±2.6
5.50	0.1	0.2	0	0	0	0	1.0	1.3	0.8

6.4.3. Furan contents

Furfural and 5-HMF are the degradation products of pentoses and hexoses, and therefore were expected to be present in extracts particularly from the higher extraction severities. As can be seen from Figure 6-8, furfural was the dominant of the two compounds, as the hemicellulose fraction is predominantly composed of two pentoses – arabinose and xylose. Furfural contents from the husk extracts spiked twice – first at logR_0 4.42 (170 °C, 77 min), and then at logR_0 5.12 (200 °C, 60 min), which corresponds to the release and degradation of arabinose from the lateral hemicellulose chains at the lower severity, and then the depolymerisation of hemicellulose linear chains and consequent xylose degradation. At the most severe extraction conditions, furfural contents decreased, which indicated furfural decomposition into formic acid and other volatile components. On the other hand, 5-HMF was present in the extracts only at the highest extraction severities (logR_0 5.12 and 5.50), indicating that at these conditions, cellulose depolymerisation into glucose and glucose degradation takes place.

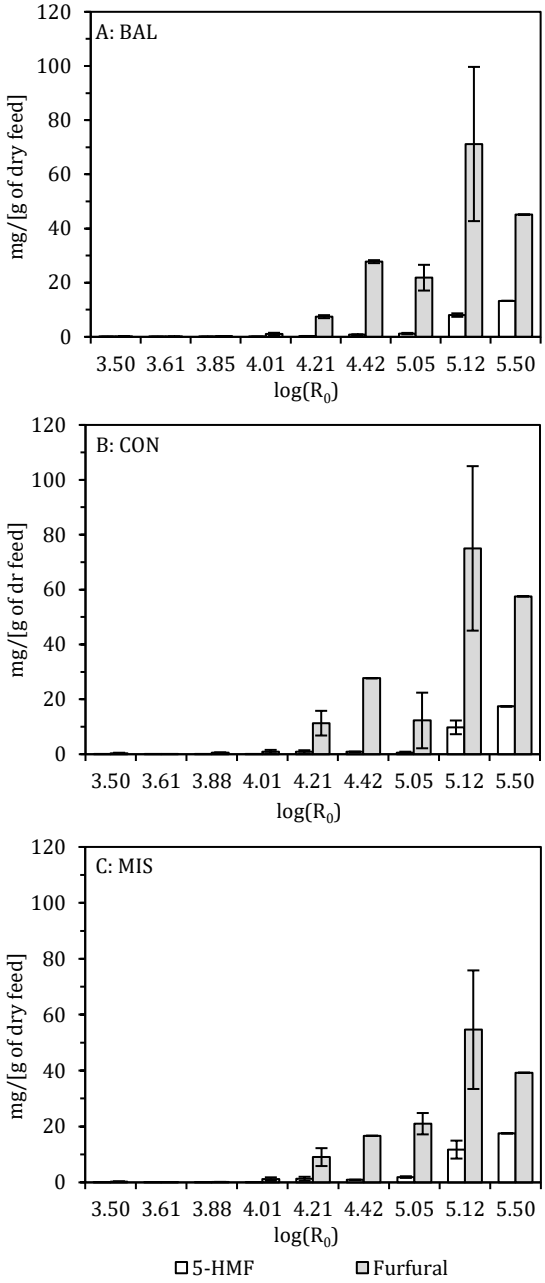

Figure 6-8: Extract furan contents at different extraction severities

6.4.4. Total phenolic contents

The results of extract total phenolic contents (TPC) are presented in Figure 6-9. Overall TPC values were similar between all biomass types, although slightly lower values were observed from MIS extracts. As can be seen from the contour graphs, both extraction severity and loading influenced the TPC response, which is supported by the ANOVA

results (see Appendix A) – both linear and quadratic terms of temperature, residence time and loading were found to be significant ($P<0.05$) for all biomass types. For CON, interactions between temperature and residence time, and temperature and loading were also significant. Similarly, the interaction between temperature and loading was significant for MIS. Accordingly, for the two factor model, linear and quadratic terms of severity and loading were also significant for all biomass types, while the interactions between these factors were not.

The optimal condition area for highest TPC values were between $logR_0$ 4.2-5.2 (equivalent to 175-210 °C, at 10 min) at biomass loadings below 6% (w/v), providing average values of 40±7, 42±8 and 34±4 [mg of gallic acid equivalent]/[g of dry feed] for BAL, CON and MIS respectively. These values were comparable with the peak TPC values of rice bran extracts, obtained at much higher extraction conditions (peaking and plateauing at 225-375 °C, 10 min) (Pourali et al., 2010), however, the extraction was performed in small steel tubes with much faster heat-up times, and without the pre-treatment step.

As discussed previously, lower biomass loadings appear to improve extractability of certain components due to higher biomass surface area exposure for hydronium ion permeability. Although TPC does not provide information about what type of phenolic compounds are in the extract, these are likely phenolics originating from the acid soluble lignin fraction, meladonins – products of Maillard reaction between carbohydrates and proteins, and hydroxycinnamic acids, esterified to the arabinose units of the hemicellulose backbone (Tekin et al., 2014; Wang et al., 2011a). As the health benefits of the aforementioned phenolics have been demonstrated, including positive effects on the gut health (Acosta-Estrada et al., 2014; Dykes and Rooney, 2007; Halliwell, 1996;

Martinez-Saez et al., 2014; Ou and Sun, 2014; Seo et al., 2015; Shahidi and Ambigaipalan, 2015; Snelders et al., 2014; Srinivasan et al., 2007; Wallace et al., 2010; Wang et al., 2011b), combining them with the prebiotic compounds such as XOS could have the potential of acting synergistically to improve the health of gut microbiome, and therefore could be formulated as an effective nutraceutical.

6.4.5. Summary of extract composition

The extract composition across different extraction severities is summarised in Figure 6-10, from which three phases of extraction can be observed. During the first phase, corresponding to $logR_0$ 3.50-4.01, small amounts of biomass was solubilised, evident by the low extract concentrations. Here, the dominant compounds were high molecular weight xylan and phenolics, as well as some arabinose and non-structural carbohydrates that failed to be removed during the pre-treatment step. During the second phase between $logR_0$ 4.01-5.05, extract concentration increased as most of hemicellulose was solubilised. Consequently, hemicellulose derived products were the dominant compounds at this phase. The optimal conditions for XOS production were at $logR_0$ 4.42, where the prebiotic XOS were the major fraction. Although AX fraction decreased between $logR_0$ 4.21-4.42, at $logR_0$ 5.05, extract contents of AX increased again as the more recalcitrant, linearly chained xylan was solubilised. The third and final phase consisted of rapid hemicellulose degradation as furfural contents became the dominant fraction and the extract concentration decreased due to the formation of volatile components.

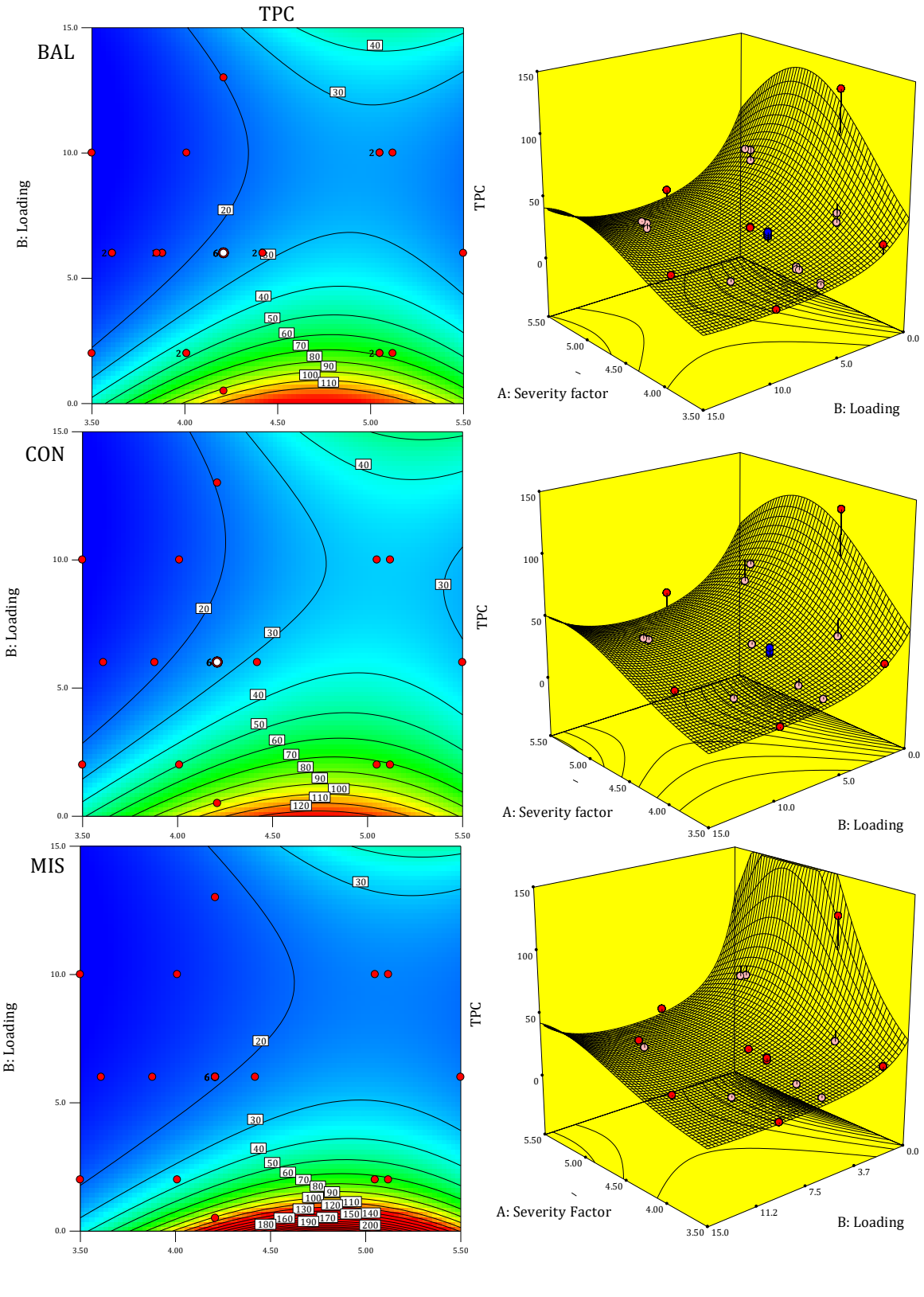

Figure 6-9: Contour and 3D surface graphs of BAL, CON, and MIS total phenolic contents (TPC, expressed as mgGAeq/[g of dry feed]) as a response of extraction severity factor and loading (%[w/v]); 3D graphs rotated for clarity of vizualisation

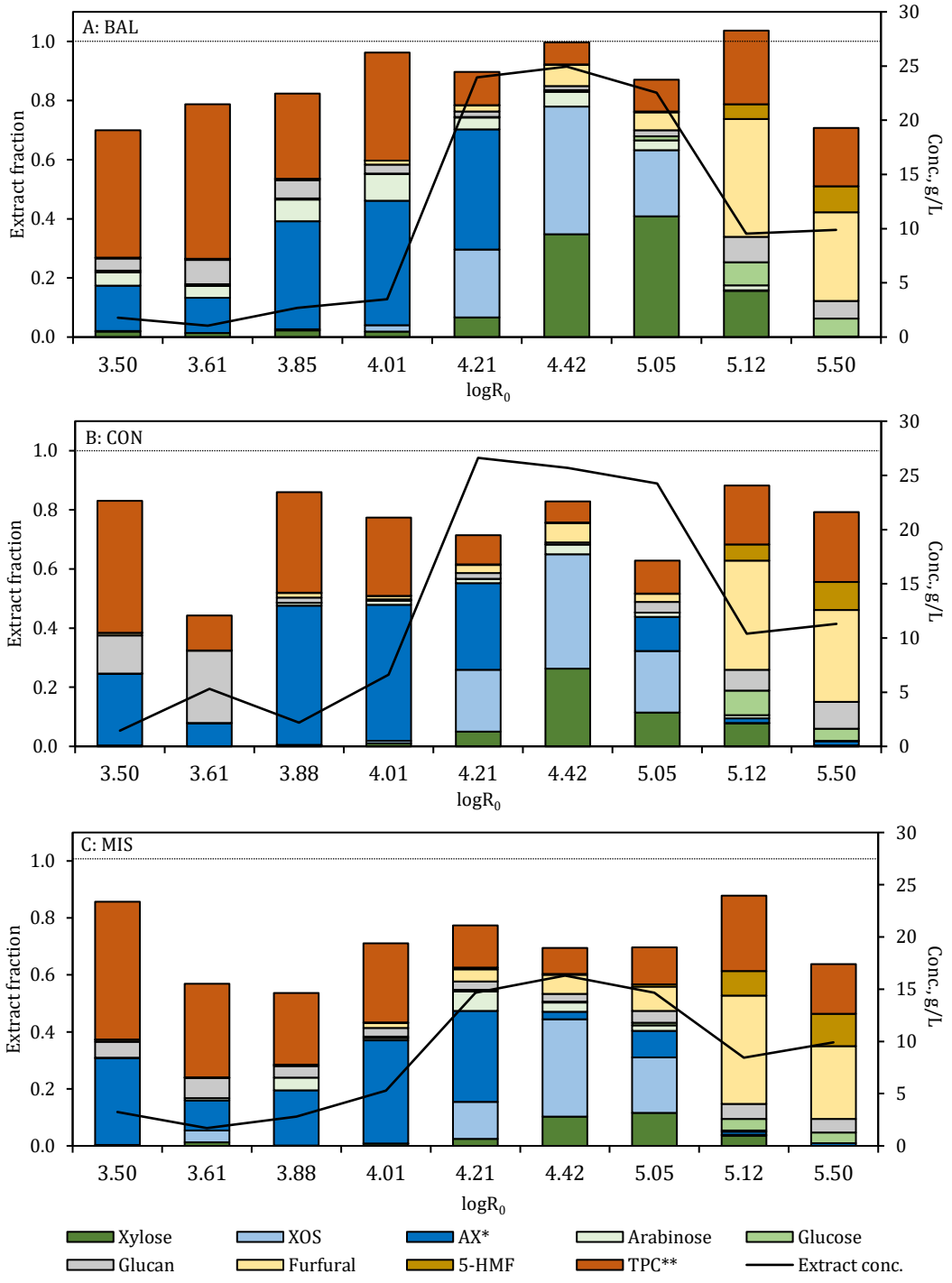

*Figure 6-10: Main components of extracts, expressed as stacked averages of extract mass fractions (left axis), and extract mass concentrations at different extraction severities (right axis); *arabinoxylan other than XOS, xylose, and arabinose; **TPC values expressed in mass of gallic acid equivalents*[1]

[1] error bars omitted for the clarity of visualisation

6.5. Chapter conclusions

The general understanding of hemicellulose solubilisation in subCW was further developed from the previous two chapters by utilising response surface methodology (RSM). The influence of extraction temperature, residence time and loading was assessed in terms of affecting extract composition. Although fitting RSM models to the data from xylose, prebiotic XOS, and AX (with exception of BAL) contents could not be established, RSM models for biomass solubility, extract pH and total phenolic contents achieved good data fits and were presented. It was found that the majority of hemicellulose was extracted at extraction severities between 4.21-5.05. Within this range, molecular weight profile of hemicellulose products in the extracts could be modified depending on the target products. For instance, to achieve the optimal high molecular weight xylan contents, extractions had to be performed at severity factor of 4.21 that corresponded to 170 °C and 35 min. For highest prebiotic XOS yields however, more severe conditions were required, corresponding to extraction severity 4.42 or 170 °C and 77 min. These conditions were found to be optimal for all tested biomass types, but, the conditions varied for the highest xylose yields. By analysing the extract composition, it was found that hemicellulose solubilisation can be described to occur in three phases: first xylan substitutions – arabinose and galactose are solubilised, followed by the lateral xylose chains, while the linear xylose chains are solubilised last. Similar to hemicellulose, highest total phenolic contents of the extract were also found between 4.21-5.05, which was assessed to be beneficial for further development of an extract derived nutraceutical, consisting of prebiotics and polyphenolics.

CHAPTER 7. SEQUENTIAL SUB-CRITICAL WATER MEDIATED HYDROLYSIS OF HEMICELLULOSE RICH EXTRACT OBTAINED FROM *AVENA SATIVA* VAR *BALADO* HUSKS AND ITS COMPARISON WITH ARABINOXYLAN STANDARD: DESIGN OF EXPERIMENTS

7.1. Introduction

In previous chapter, optimal extraction conditions were found for highest extract XOS contents. Although XOS were the dominant component, the extract also contained higher molecular weight xylan, which theoretically could be further hydrolysed to increase the yields of XOS. In order to investigate if XOS yields could be further improved, an extract consisting of XOS and higher molecular weight xylan, obtained by following the same methodology used in previous chapter, was subjected to further subCW mediated hydrolysis in a small 20 ml tube reactors at varying extraction temperature and residence times. As a reference, arabinoxylan (AX, Megazyme, wheat flour origin) standard with the same concentration as the extract was also subjected to the same extraction conditions. This chapter discusses the findings of the sequential extract hydrolysis and compares the results to the hydrolysis of AX standard.

7.2. Design of experiments and response surface methodology (RSM)

Two factor central composite design (CCD) was selected with extraction temperature and residence time as the factors. The subCW extract used for these experiments was obtained from pre-treated BAL husks that was extracted at extraction severity 4.27 which corresponded to 172 °C for 44 min with 10 %(w/v) loading in the 0.5 L reactor. At this extraction severity the extract contained 3.4% furfural, 2.3% arabinose, 10.7% xylose, 23.0% prebiotic XOS, and 52.1% xylan other than prebiotic XOS. The extract was diluted with distilled water to 10 g/l mass concentration. The AX standard was prepared with the same concentration, and contained 95% arabinoxylan with 38/62 arabinose to xylose ratio. Both the diluted extract and AX standard were then placed in 20 ml stainless steel tube reactors and subjected to extraction conditions shown in Table 7-1, ranging from

172-228 °C and 0-36 min residence time, corresponding to extraction severity 2.07-3.66, calculated using Equation 6-2. The reactors were heated in a GC oven. For more detailed description of extraction procedure see Section 3.3.5.

Table 7-1: Central composite design of experiments with the corresponding severity factor (accumulated severity in brackets for BAL extract) and design point type

Extr. Nr.	S. factor, log(R_0)	Temp., °C	Res. time, min	Design Point
1	2.78 (7.05)	200	15	Centre
2	2.14 (6.41)	172	15	Axial
3	2.78 (7.05)	200	15	Centre
4	2.87 (7.14)	200	36	Axial
5	2.07 (6.34)	180	0	Factorial
6	2.70 (6.97)	200	0	Axial
7	2.78 (7.05)	200	15	Centre
8	2.78 (7.05)	200	15	Centre
9	3.66 (7.93)	228	15	Axial
10	2.78 (7.05)	200	15	Centre
11	3.38 (7.65)	220	30	Factorial
12	2.45 (6.72)	180	30	Factorial
13	3.33 (7.60)	220	0	Factorial

7.3. Effects of severity factor on extract composition

The extracts were characterised for furan, monosaccharide, oligosaccharide and total phenolic contents (TPC), as well as extract concentration and pH with the analytical methods described before (see Section 3.5). The obtained results were then used as responses in CCD, and fitted with quadratic models with transformations where necessary. The model fit to the data was then evaluated by using R^2, adjusted R^2 and predicted R^2. From the R^2 results which are shown in Table 7-2, it can be seen that the models achieved relatively good fits, except for TPC and furfural results for BAL extract, and xylose results for AX standard. Although only few models met the Anderson criterion of having the difference between adjusted R^2 and predicted R^2 of 0.2 or lower, the majority of the models produced R^2 values above 0.5, which was considered to be an acceptable fit

considering the model dimensionality. Nevertheless, discretion needs to be applied when interpreting these models.

*Table 7-2: R^2 results of central composite RSM model (models meeting the Anderson criterion in **bold**; -: negative R^2 value)*

	BALADO Extract			AX standard		
Response	R^2	Adj R^2	Pred R^2	R^2	Adj R^2	Pred R^2
Extract conc.	0.94	0.90	0.65	**0.98**	**0.96**	**0.86**
pH	0.95	0.91	0.58	0.94	0.90	0.57
TPC	0.85	0.75	0.05	0.88	0.83	0.62
Furfural	0.66	0.41	-	**0.85**	**0.80**	**0.65**
Arabinose	0.94	0.90	0.63	**0.96**	**0.93**	**0.75**
Xylose	**0.87**	**0.77**	**0.72**	0.68	0.53	-
XOS	0.91	0.85	0.49	0.95	0.91	0.70

7.3.1. Extract concentration and pH

As can be seen from Figure 7-1, the concentration of BAL extract and AX standard decreased with increasing extraction temperature and residence time. Decrease in extract concentration with increasing extraction severity can be explained by generation of volatile components due to the degradation of carbohydrates in the extract. Moreover, the concentration of BAL extract started to rapidly decrease at lower temperatures than the AX standard, suggesting that as the result of accumulated extraction severity in previous extractions, the BAL extract contained lower molecular weight xylan than the AX standard, which was then rapidly depolymerised into the corresponding monomers, followed by further degradation into furans and volatile components. In terms of significant factors of the models, linear terms of temperature and residence time, as well as the interaction of the two factors was significant ($P<0.05$) for both BAL and AX standard extracts, and quadratic term of temperature was significant for the model of AX standard extract concentration.

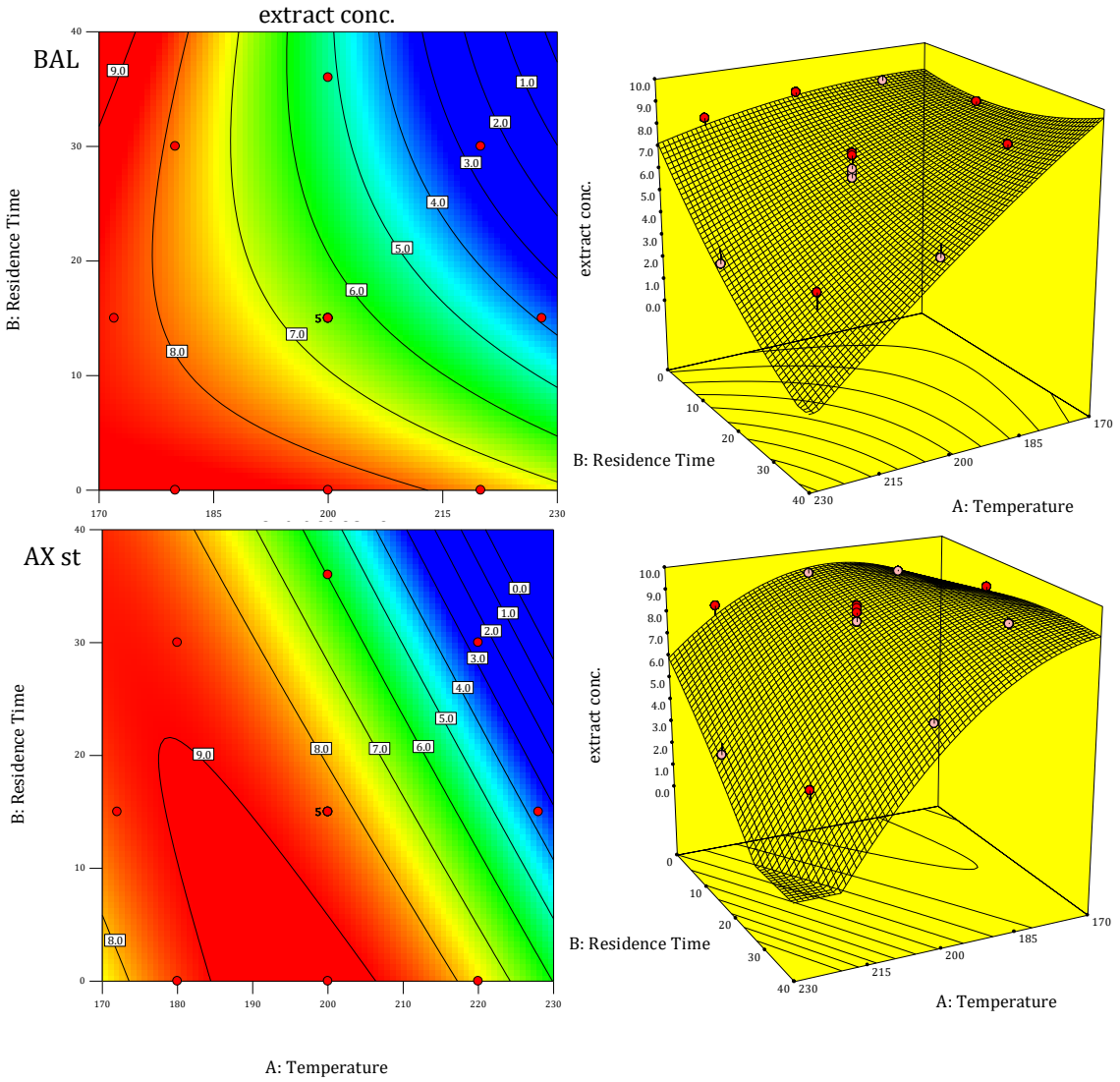

Figure 7-1: Contour and 3D surface graphs of BAL and AX standard extract mass concentration (g/L) as a response of extraction temperature (°C) and residence time (min); 3D graphs rotated for clarity of vizualisation

As can be seen in Figure 7-2, similar response pattern to extract concentration was also observed with the extract pH levels, which also decreased with extraction severity. However, the initial decrease in pH occurred at lower extraction conditions than the extract concentration, signalling the beginning of deacetylation of xylan molecules, which apparently require to reach a critical concentration of hydronium ions in the liquid phase to start the depolymerisation. ANOVA analysis of the pH models showed that the linear and quadratic terms of temperature and residence time were significant for the AX

standard model, while for BAL extract the significant terms were linear temperature, linear and quadratic residence time, and the interaction between the temperature and residence time.

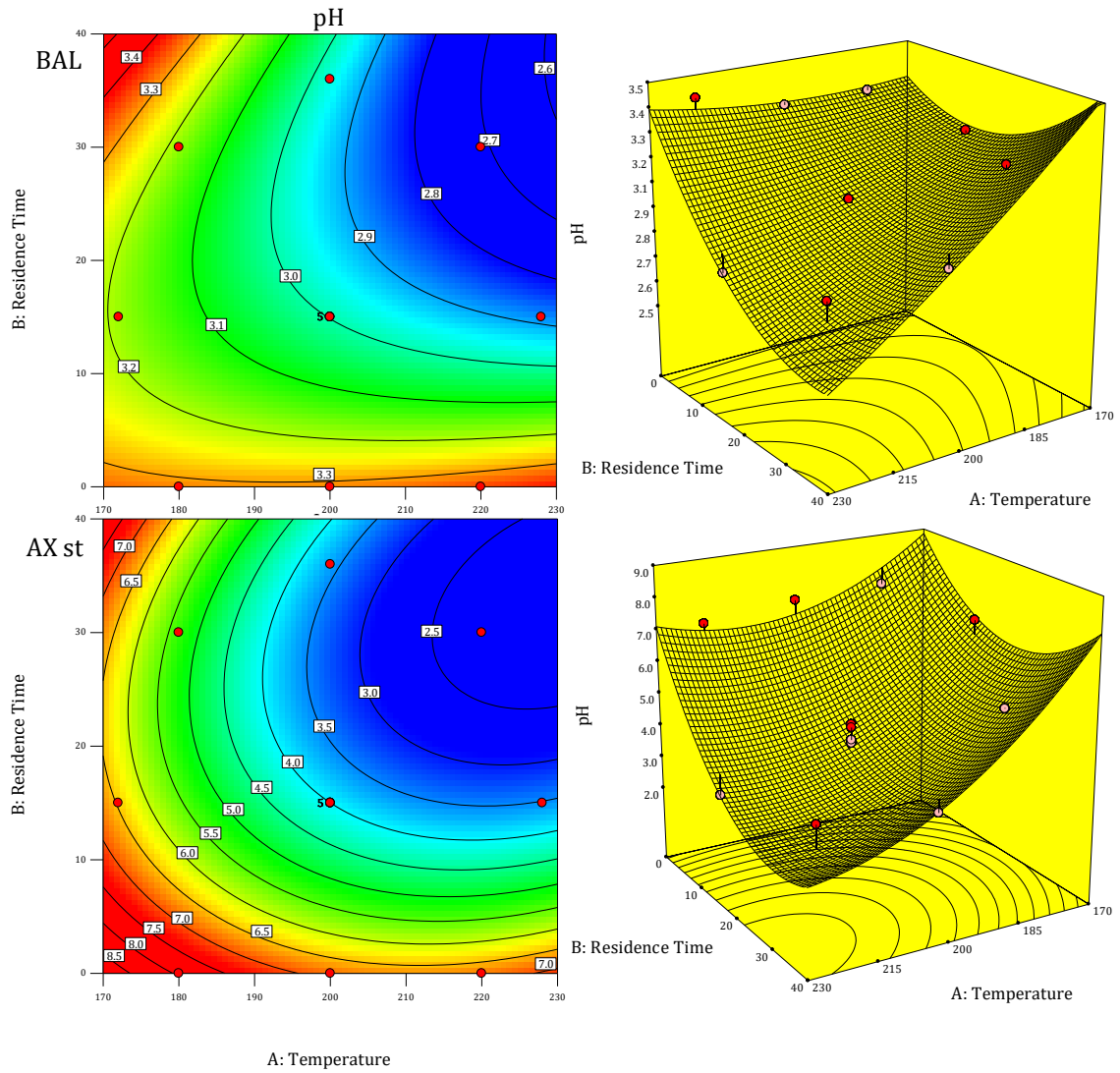

Figure 7-2: Contour and 3D surface graphs of BAL and AX standard extract pH as a response of extraction temperature (°C) and residence time (min); 3D graphs rotated for clarity of vizualisation

7.3.2. Carbohydrate concentration

The difference between the AX standard and BAL extracts in terms of xylan molecular weight distribution was confirmed by extract arabinose, xylose and XOS contents. As can

be observed from Figure 7-3, arabinose contents of BAL extracts did not see an increase from the 0.23 g/L in the original extract prior to the sequential extraction. Moreover, the arabinose contents remained close to the original levels in extracts obtained at short residence times or low temperatures, but degraded rapidly with increasing extraction severity, indicating that little or no arabinose attached to the xylan backbone was present in the original extract. The significant factors for the arabinose model of BAL extract were linear and quadratic terms of temperature, the linear term of residence time, and the interaction between residence time and temperature.

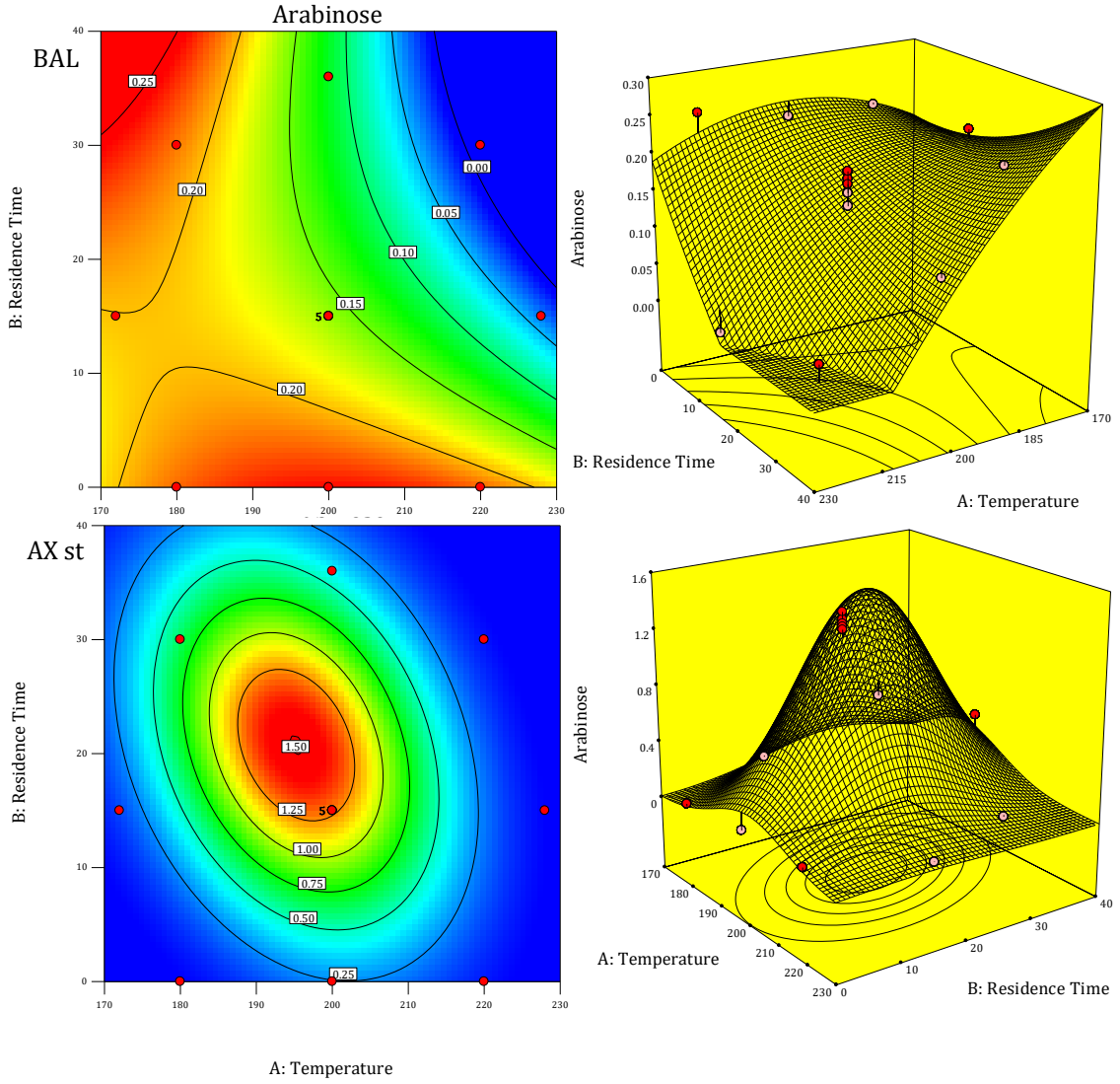

Figure 7-3: Contour and 3D surface graphs of BAL and AX standard extract arabinose concentration (g/L) as a response of extraction temperature (°C) and residence time (min); 3D graphs rotated for clarity of vizualisation

The arabinose contents in AX standard extracts, in contrast to BAL extracts, did increase with extraction temperature and residence time, reaching the maximum values of 1.37±0.02 g/L at the design centre points (severity factor 2.78), which corresponded to 33% yield of the total available arabinose. According to the RSM model, the optimal conditions for arabinose extraction were between 190-200 °C and 15-25 min residence time; higher extraction conditions resulted in lower arabinose contents the rate of arabinose degradation increased. The significant factors of the model were linear and quadratic temperature terms, quadratic residence time term and the interaction between temperature and residence time.

Contrary to arabinose (Figure 7-3), xylose concentration (Figure 7-4) for BAL extract increased from the original concentration of 1.07 g/L, reaching up to 1.52±0.14 g/L at the design centre points yielding 26% of total available xylose from xylan in the extract. According to the RSM design, the optimal conditions for highest xylose yields were achieved between 172-200°C and 15-36 min suggesting xylose was produced from the available xylan in the extract. At extraction conditions below 185 °C and below 10 min xylose concentration remained close to the original, whilst higher conditions than the stated optimal range resulted in reduced xylose concentration due to its degradation. Model's significant factors were linear and quadratic terms of temperature and temperature and residence time interaction. Although the quadratic model fit for the xylose concentration of the AX standard extract was poor, as evident by the poor R^2 values (see Table 7-2) and the response mesh shown in Figure 7-4, the data points showed highest xylose concentration at 200 °C and residence times 15-36 min reaching up to 1.34

g/L at 200 °C and 36 min (severity factor 2.87), which corresponded to 20% yield. At extraction temperatures above and below 200 °C xylose concentration was significantly lower, due to low xylose production rates at low extraction conditions, and high rates of xylose degradation at high extraction severity.

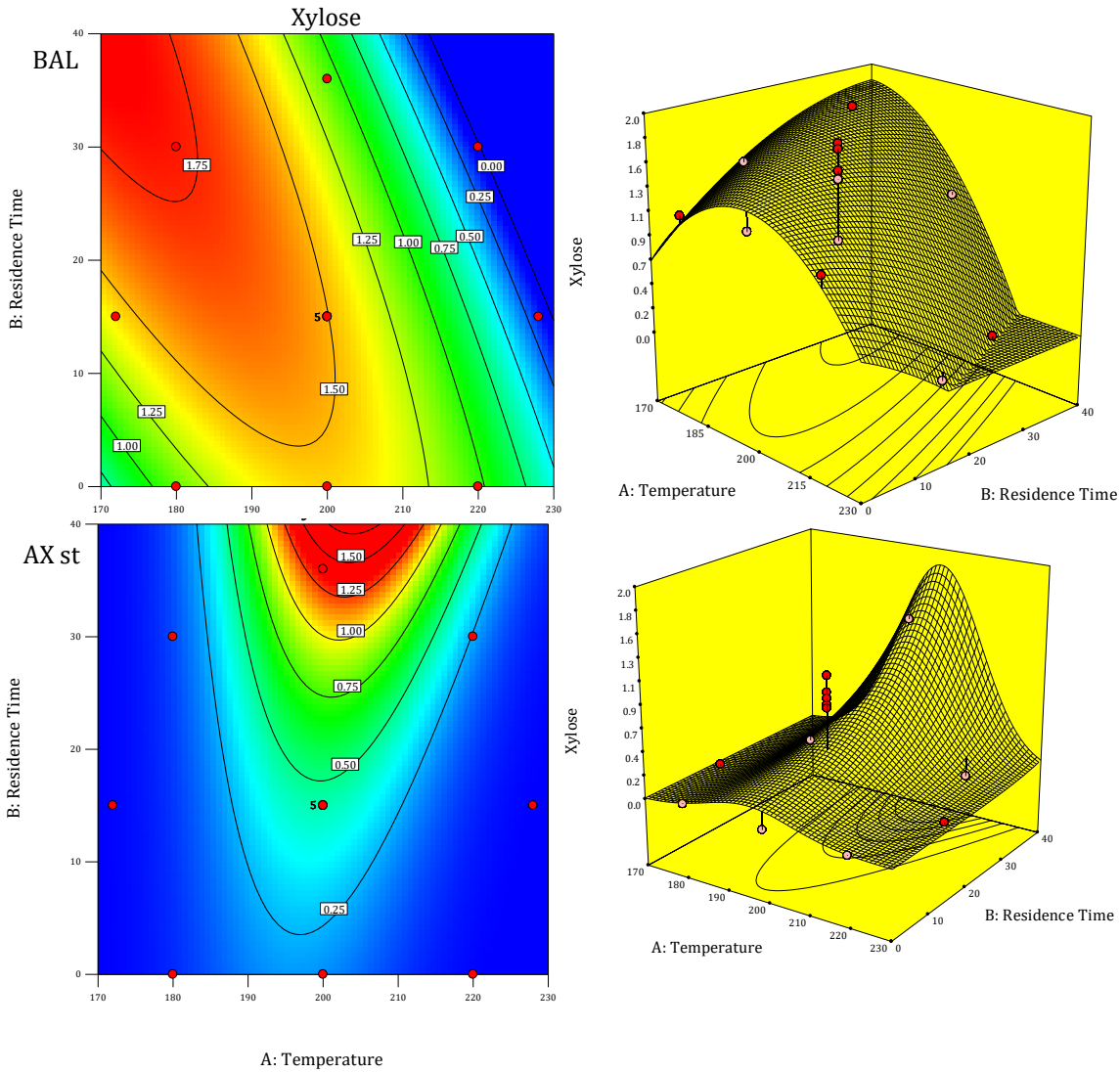

Figure 7-4: Contour and 3D surface graphs of BAL and AX standard extract xylose concentration (g/L) as a response of extraction temperature (°C) and residence time (min); 3D graphs rotated for clarity of vizualisation

The original XOS (DP 2-5) concentration of the BAL extract before the sequential extraction were 2.30 g/L, and as can be seen from Figure 7-5, did not increase during the tested extraction conditions, but remained close to the original concentration at the

lowest extraction temperatures and residence times, and decreasing with extraction severity. Such behaviour was not expected as the original extract also contained 5.21 g/L of xylan, which was thought to depolymerise into XOS during the sequential extraction. As the increase in XOS concentration was not observed, it is possible that the xylan fraction present in BAL extract was already of low molecular weight, and during the tested extraction conditions depolymerised with a rate lower than the rate of XOS degradation, resulting in overall decrease in XOS concentration. The significant terms of the BAL extract XOS model were linear and quadratic terms of temperature and residence time and interaction between the residence time and temperature.

Unsurprisingly and in contrast with the BAL extract, the XOS concentration of the AX standard extracts increased and peaked within the ranges of tested extraction conditions. The peak XOS concentration of 1.71±0.09 g/L, corresponding to 26% of the total available xylan, was achieved at the design centre points (severity factor 2.78). According to the RSM model (see Figure 7-5) the optimal extraction range for highest XOS concentrations was at 195-205 °C and 15-20 min. Extracts below and above these conditions produced lower XOS concentrations, as at lower extraction severities little or no XOS were produced, while at high severities, the XOS were depolymerised and eventually degraded into furans and volatile components.

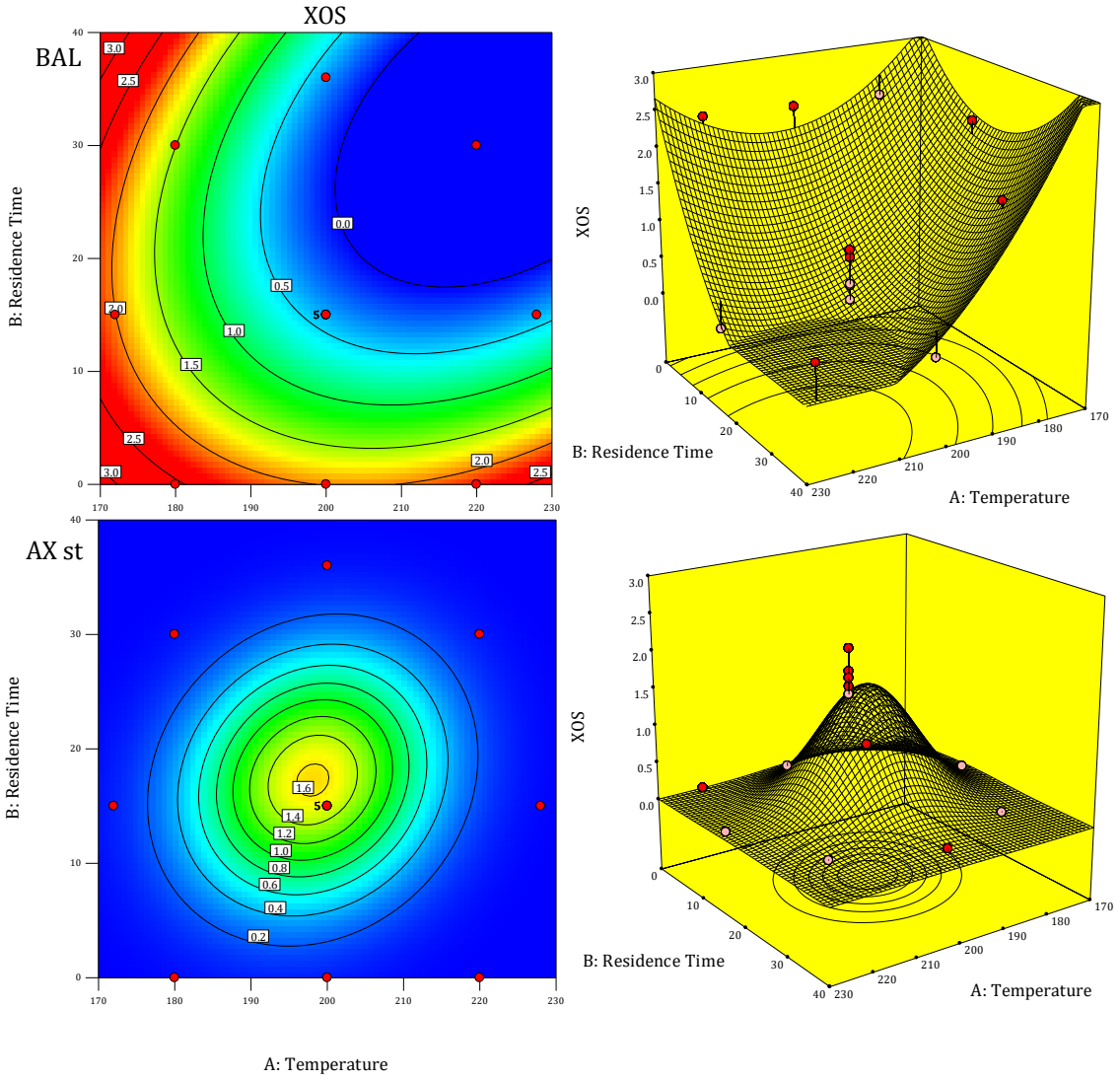

Figure 7-5: Contour and 3D surface graphs of BAL and AX standard extract prebiotic XOS concentration (g/L) as a response of extraction temperature (°C) and residence time (min); 3D graphs rotated for clarity of vizualisation

7.3.3. Furan concentration

As furfural is a degradation product of arabinose and xylose, its presence in the extracts indicate that the extraction conditions have likely been too severe. As can be observed from Figure 7-6, furfural concentration for BAL and AX standard extracts peaked at relatively severe extraction conditions. BAL extract peak furfural concentration was higher, 2.67 g/L at 200 °C and 36 min, while for AX standard, highest concentration of 2.36 g/L was achieved at 228 °C and 15 min. Moreover, the differences in furfural

concentration were the most distinct at the design centre points where BAL standard averaged 2.04±0.05 g/L and AX standard averaged 0.35±0.05 g/L. This difference was in agreement with carbohydrate concentrations, indicating that due to the accumulated extraction severity from previous extractions, xylan in BAL extract was of lower molecular weight, thus requiring lower extraction conditions to initiate the production of furfural. Another interesting difference between the two biomass types was observed at 220 °C and 30 min, where the furan contents in BAL extract dropped significantly to 0.97 g/L, compared to the nearest response points at 200 °C, 36 min and 228 °C, 15 min; such decrease was not observed from the AX standard extract, where the value remained close to the highest measured value (2.26 g/L). This observation suggests that furfural in BAL extract from 220 °C, 30 min had started to degrade into volatile components such as formic acid. As can be seen from R^2 values in Table 7-2, the model fit to the data was poor for BAL, and acceptable for AX standard extracts. The significant factors of AX standard model were the linear temperature and residence time, as well as the interaction factor.

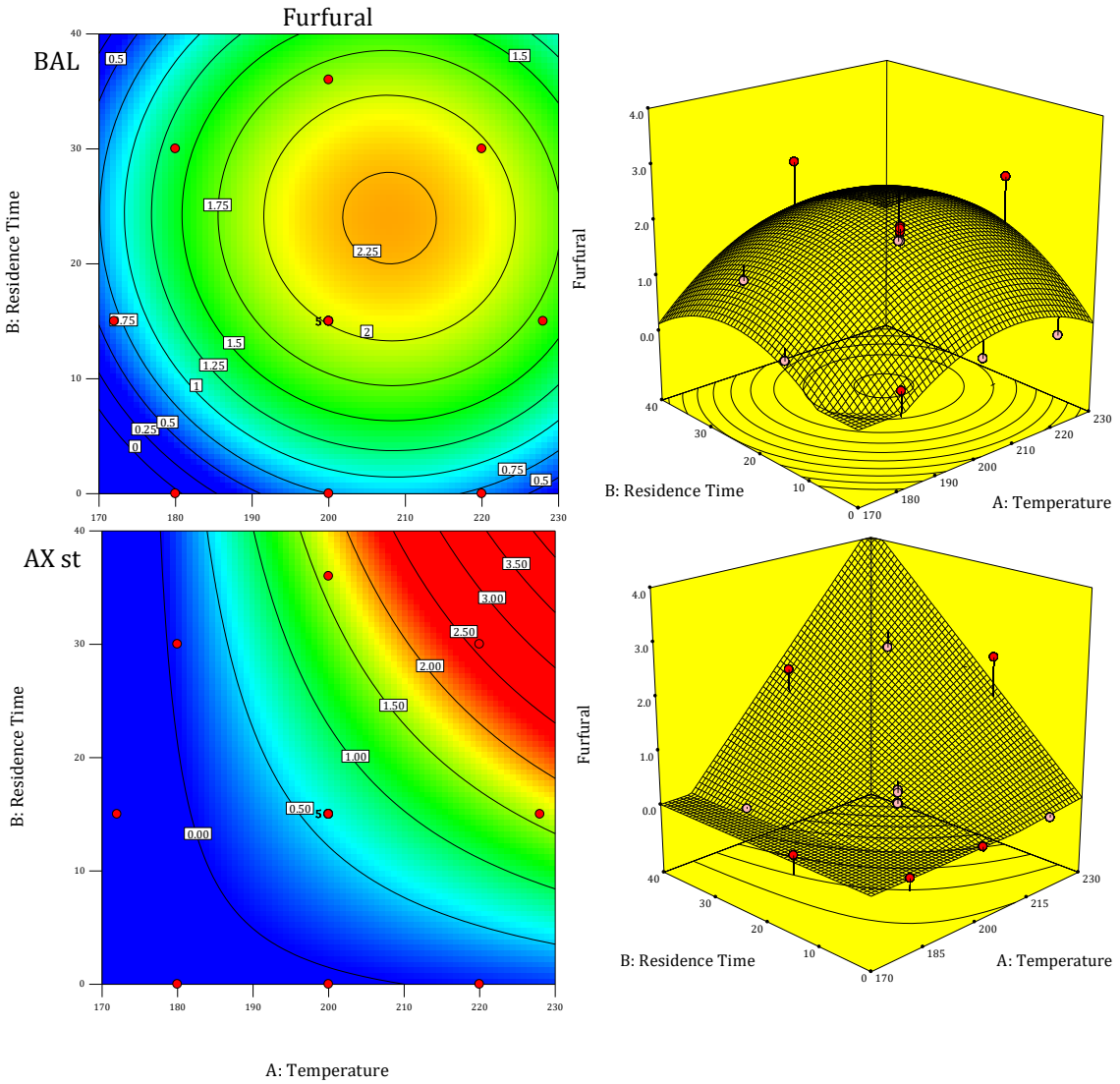

Figure 7-6: Contour and 3D surface graphs of BAL and AX standard extract furfural concentration (g/L) as a response of extraction temperature (°C) and residence time (min); 3D graphs rotated for clarity of vizualisation

7.3.4. Total phenolic concentration

Total phenolic contents (TPC) in BAL extract was higher than in the AX standard extract, which was expected as the AX standard did not contain any phenolic compounds. However, as can be seen in Figure 7-7, moderate TPC values were observed in the AX standard extract, increasing with extraction severity, and reaching the maximum of 0.79 gGAeq/L at 228 °C and 30 min. As the AX standard did not contain any phenolic compounds, likely explanation of these results is the formation of phenolics from

arabinose and xylose monomer degradation (Kumar et al., 2013; Rasmussen et al., 2014). Interaction of reducing sugars such as arabinose and xylose with the Folin-Ciocalteu reagent is also possible (Singleton et al., 1999), however, arabinose, xylose and XOS concentrations peaked at lower extraction conditions than the measured TPC values. Furthermore, to have a significant effect on the TPC values, reducing sugar concentration should be at least 25 g/L (Singleton et al., 1999), which is significantly higher than the maximum measured arabinose and xylose concentrations, which were 1.44 g/L and 1.34 g/L respectively. Moreover, the AX extract TPC values appeared to be somewhat correlated with the extract furan concentration, suggesting that a possible interaction between the Folin-Ciocalteu reagent and AX degradation products.

As with the furfural model, the significant terms of TPC model for the AX standard extracts were linear temperature and residence time, and the interaction term. Model fit for BAL extracts was poor according to R^2 values (see Table 7-2). TPC contents in BAL extract also increased with temperature, but peaked between 15-25 min, reaching the highest value of 1.43 gGAeq/L at 228 °C and 15 min from the original 0.58 gGAeq/L before the sequential extraction. In light of the results from the AX standard, the increase in TPC values for BAL extracts could also be attributable to the interaction of carbohydrate degradation products and Flin-Ciocalteu reagent.

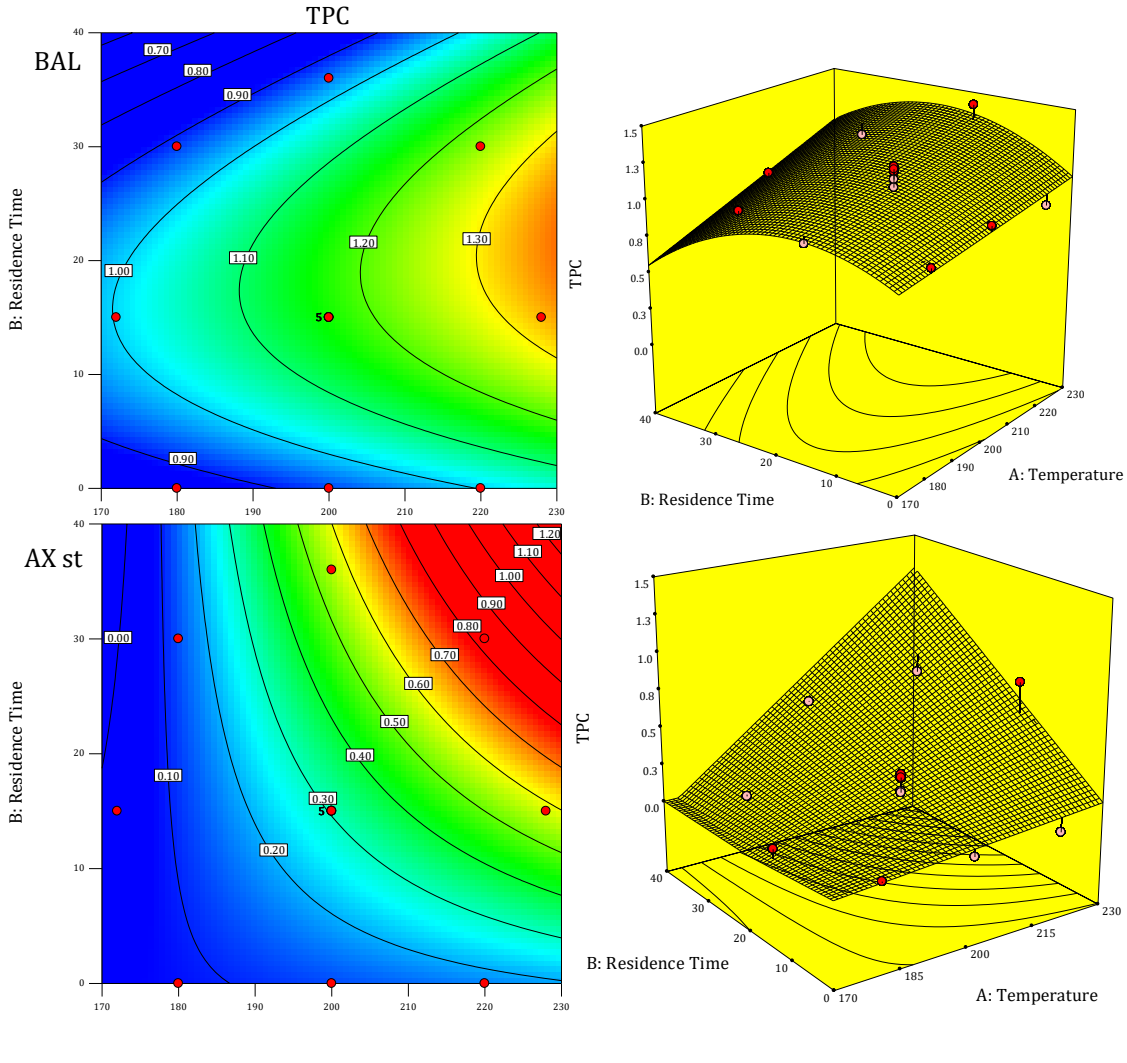

Figure 7-7: Contour and 3D surface graphs of BAL and AX standard extract total phenolic concentration (expressed as gGAeq./L) as a response of extraction temperature (°C) and residence time (min); 3D graphs rotated for clarity of vizualisation

7.4. Chapter conclusions

Results presented in this chapter showed that yields of prebiotic XOS could not be improved with a sequential subCW treatment of hemicellulose extracts. However, the results showed that the yields of xylose could be increased due to the continued depolymerisation of xylan and XOS present in the extract. AX standard was also subjected to the same extraction conditions as the hemicellulose extracts. The analysis of the AX standard extracts produced results in line with the original hemicellulose extractions

discussed in Chapter 6, but were significantly different than the sequentially treated extracts, which was a result of differences in xylan molecular weights – high MW in AX standard and relatively low MW xylan in the hemicellulose extract. Peak concentrations of arabinose, xylose and XOS were observed from the AX standard extractions, providing maximum yields of 33%, 20% and 26% respectively. Moreover, AX standard extracts showed relatively high total phenolic values. Potential interaction between Folin-Ciocalteu reagent used in the analytical assay, and the degradation products of AX, which include phenolics was suggested as the likely explanation of the TPC results.

CHAPTER 8. CONCLUSIONS AND FUTURE WORK

8.1. Conclusions

The work presented here has demonstrated the utility of sub-critical water as an environmentally benign solvent to remove hemicellulose from *Miscanthus χ giganteus* and *Avena sativa* husks, and produce extracts rich in prebiotic oligosaccharides. Furthermore, it was also demonstrated that different molecular weight hemicellulose products can be targeted by controlling extraction temperature and residence time – at lower extraction severities, extracts contained higher molecular weight polymers, whilst higher extraction severities produced extracts rich in hemicellulosic oligomers and monomers.

Water pre-treatment of the biomass at relatively low temperatures (120 °C) was effective for removing non-structural components of the biomass (extractives), which increased extract purities from the following extractions. It was shown to be particularly effective for certain varieties of *Avena sativa* husks with high groat contents, a result of inefficient dehulling. The pre-treatment also improved the yields of hemicellulosic products downstream by reducing the recalcitrance and improving the permeability of the biomass. Therefore, pre-treated biomass was used for determination of optimal extraction conditions.

The optimal conditions for hemicellulose extraction for all tested biomass types were 170°C with residence times between 35-77 min, which corresponded to severity factor ($\log R_0$) values 4.21-4.42. Prebiotic xylooligosaccharide (DP 2-5) yields between 44-56% and total hemicellulosic carbohydrate yields between 69-90% depending on biomass source were achieved with 77 min residence times. The corresponding extract purities in terms of prebiotic oligosaccharides were between 34-43%, and in terms of hemicellulosic carbohydrates 50-83%, with furfural and 5-HMF accounting for 6.8-7.4% of the extract

by weight. Although highest yields and extract purities were achieved from *Avena sativa* husks, in general, the biomass extractability was comparable between different all biomass types, therefore suggesting that the same operating conditions could be applied for many types of non-woody lignocellulosic biomass.

Husks from five different *Avena sativa* varieties were also subjected to sub-critical water treatment at 160 °C, 30 min residence time, with and without pre-treatment to investigate potential differences in hemicellulose extractability. Three commercial varieties (Balado, Mascani, Conway) and two experimental low-lignin varieties (SO-I and 14355Cn) were investigated. The extracts from low-lignin varieties consisted of less prebiotic xylooligosaccharides compared to the commercial varieties, suggesting a more rigid hemicellulose structure in the cell walls to account for the lower lignin contents. Better yields and purities were achieved in extracts from pre-treated biomass. Hemicellulosic carbohydrate contents in pre-treated extracts varied between 41-62%, whereas hemicellulosic carbohydrate yields were between 32-88% depending on variety.

By analysing extract composition, it was found that hemicellulose solubilisation in sub-critical water occurs in three steps. First, the lateral hemicellulose chains containing arabinose and galactose, as well as xylose are solubilised at relatively mild conditions, 140-170 °C. Then, the more rigid and linear chains accounting for the majority of the hemicellulose fraction are solubilised between 170-200 °C. At this second stage, the hemicellulose is rapidly depolymerised into oligosaccharides and monosaccharides, and highest extraction yields and purities are observed. Lastly, at extraction conditions above 200 °C, the most recalcitrant and difficult to access hemicellulose is solubilised, while the

monosaccharides are rapidly dehydrogenated into furfural, 5-HMF and phenolic compounds.

Hemicellulose extracts were also fractionised with 60 %(v/v) ethanol. Heavier molecular weight hemicellulosic polysaccharides were precipitated from the extracts, leaving the lighter products, including prebiotic oligosaccharides in the supernatants. It was also found that the fraction of precipitated hemicellulose can be increased by using higher ethanol concentrations and lower temperatures during the fractionation.

The extracts were also analysed for total phenolic contents using Folin-Ciocalteu reagent. It was found that the extracts obtained from mildest extraction conditions (120-140 °C) contained more phenolics than hemicellulosic carbohydrates. At the optimal conditions for prebiotic oligosaccharide extraction, the extracts contained 7.1-9.1% of phenolic compounds by dry weight depending on biomass source, but increased to 20-26% in 200°C, 60 min extracts. The same extracts obtained at high temperatures also contained 37-40% furfural and 5-9% 5-HMF, thus suggesting that phenolic compounds were generated as a result of monosaccharide degradation.

8.2. Future work

There are many future directions to advance this work further. Most significantly, more research needs to be done in terms of extract fractionation and purification. This can be achieved either by solvent precipitation or membrane separation coupled with solid/liquid separation methods such as spray-drying or freeze-drying. For instance, large molecular weight carbohydrates could be recovered as precipitates, whereas the prebiotic rich supernatants would be spray-dried into micro-pellets or freeze-dried. The

fractionation could also be achieved through micro-, ultra-, and nanofiltration, followed by spray-drying or freeze-drying of the filtrates.

The dried extracts should be investigated in terms of their prebiotic efficacy *in vitro* and *in vivo*. Relatively simple *in vitro* study can be designed using faecal microbiota of different origins exposed to prebiotics, increase in volatile metabolites such as acetic, butyric and propanoic acids could be an indicator of prebiotic efficacy. *In vivo* studies could also be carried out with rodents, poultry and swine, and upon positive results from animal studies, eventually trialled for human consumption.

Another area where more research should be carried out is the extraction of hemicellulosic compounds. More specifically, in terms of adding acidic or alkaline modifiers such as CO_2, and lime ($Ca[OH]_2$), and microwave assisted extractions. Addition of CO_2 would reduce the pH of the extraction, which could possibly lower the extraction conditions necessary to achieve optimal yields, whereas alkaline modifiers could potentially be useful for heavier molecular weight carbohydrate extractions at relatively low temperatures. Furthermore, extractions supported by microwave irradiation should also be explored, which could potentially improve the biomass permeability, therefore improving hemicellulose extractability. Continuous extraction of hemicellulosic products should also be explored using continuous stirred tank reactors (CSTR), and continuous oscillatory baffled reactors (COBR).

The hemicellulose extraction process integration into sustainable biorefineries should also be further explored. This entails analysing the residue composition after the hemicellulose removal, its suitability for sequential lignin extractions, and cellulose fibre quality for biofuel production. Moreover, economic feasibility studies and comprehensive

life cycle analysis for integrated biorefineries need to be carried out, taking into consideration different biomass sources and different product value streams.

Lastly, the sub-critical water extraction to produce xylooligosaccharides could be scaled up to a pilot scale biorefinery, based on the process flow diagram shown in Figure 8-1, to demonstrate the feasibility of large scale process. The pilot scale biorefinery would require at least a batch reactor, spray dryer, stirred tank for precipitation, bioreactor for enzymatic biofuel production, distillation column, mill for biomass comminution, equipment for extract purification (activated carbon adsorption, ultrafiltration etc.), and suitable analytical equipment for extract characterisation.

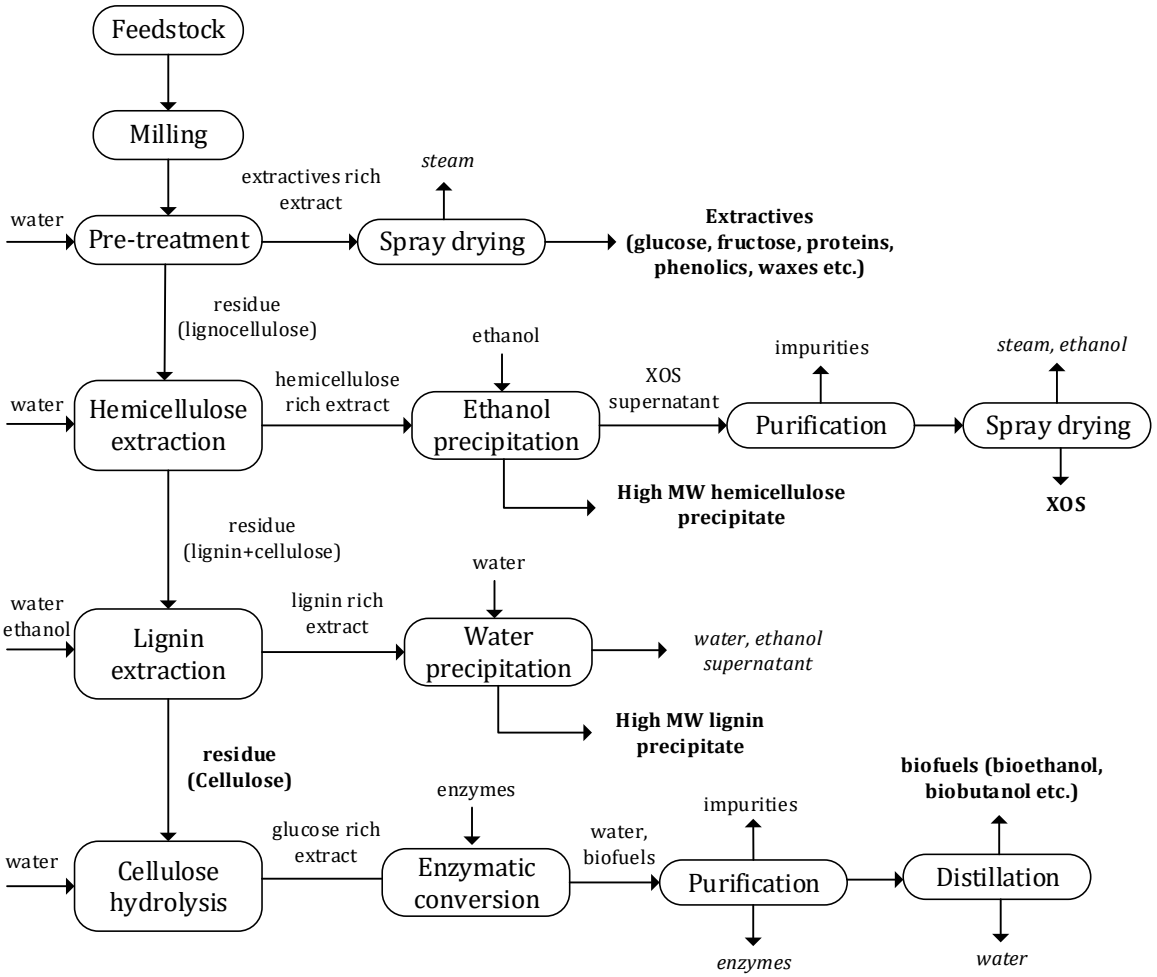

Figure 8-1: Simplified process flow diagram of sub-critical water based biorefinery (products in **bold**; recyclable streams in *italics*)

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APPENDIX A: ANOVA TABLES

A.1. Effects of temperature on virgin extract composition

*Table A.1-1: Tukey tests for **Solubility** of virgin MIS and BAL extracts*

Comparisons for factor	Comparison	Diff of Means	p	q	P	P<0.050
Biomass	BAL vs. MIS	1.997	2	2.964	0.049	Yes
Temperature	160 vs. 120	8.394	3	10.301	<0.001	Yes
	160 vs. 140	7.039	3	8.638	<0.001	Yes
	140 vs. 120	1.355	3	1.603	0.505	No
Temperature within MIS	160 vs. 120	8.475	3	6.342	<0.001	Yes
	160 vs. 140	7.127	3	5.333	0.003	Yes
	140 vs. 120	1.348	3	1.008	0.759	No
Temperature within BAL	160 vs. 120	8.314	3	8.911	<0.001	Yes
	160 vs. 140	6.952	3	7.451	<0.001	Yes
	140 vs. 120	1.362	3	1.316	0.627	No
Biomass within 120	BAL vs. MIS	2.046	2	1.712	0.240	No
Biomass within 140	BAL vs. MIS	2.06	2	1.724	0.236	No
Biomass within 160	BAL vs. MIS	1.885	2	1.701	0.242	No

*Table A.1-2: Tukey tests for **pH** of virgin MIS and BAL extracts*

Comparisons for factor	Comparison	Diff of Means	p	q	P	P<0.050
Biomass	BAL vs. MIS	0.473	2	7.437	<0.001	Yes
Temperature	120 vs. 160	1.297	3	16.874	<0.001	Yes
	120 vs. 140	0.607	3	7.607	<0.001	Yes
	140 vs. 160	0.691	3	8.984	<0.001	Yes
Temperature within MIS	120 vs. 160	1.000	3	7.931	<0.001	Yes
	120 vs. 140	0.533	3	4.230	0.018	Yes
	140 vs. 160	0.467	3	3.701	0.041	Ye
Temperature within BAL	120 vs. 160	1.595	3	18.117	<0.001	Yes
	120 vs. 140	0.680	3	6.962	<0.001	Yes
	140 vs. 160	0.915	3	10.393	<0.001	Yes
Biomass within 120	BAL vs. MIS	0.720	2	6.384	<0.001	Yes
Biomass within 140	BAL vs. MIS	0.573	2	5.084	0.002	Yes
Biomass within 160	BAL vs. MIS	0.125	2	1.196	0.408	No

*Table A.1-3: Tukey tests for **Xylan contents** in virgin MIS and BAL extracts*

Comparisons for factor	Comparison	Diff of Means	p	q	P	P<0.050
Biomass	BAL vs. MIS	7.53	2	13.212	<0.001	Yes
Temperature	120 vs. 160	35.106	3	52.101	<0.001	Yes
	120 vs. 140	33.927	3	50.350	<0.001	Yes
	140 vs. 160	1.179	3	1.585	0.52	No
Temperature within MIS	120 vs. 160	22.164	3	23.548	<0.001	Yes
	120 vs. 140	21.437	3	20.371	<0.001	Yes
	140 vs. 160	0.727	3	0.691	0.878	No
Temperature within BAL	120 vs. 160	48.049	3	49.819	<0.001	Yes
	120 vs. 140	46.417	3	55.136	<0.001	Yes
	140 vs. 160	1.632	3	1.551	0.534	No
Biomass within 120	BAL vs. MIS	1.4	2	1.331	0.366	No
Biomass within 140	BAL vs. MIS	0.495	2	0.470	0.745	No
Biomass within 160	BAL vs. MIS	24.485	2	29.084	<0.001	Yes

*Table A.1-4: Tukey tests for **Total phenolic contents** in virgin MIS and BAL extracts*

Comparisons for factor	Comparison	Diff of Means	p	q	P	P<0.050
Biomass	BAL vs. MIS	4.639	2	5.814	<0.001	Yes
Temperature	120 vs. 160	6.807	3	6.99	<0.001	Yes
	120 vs. 140	4.711	3	5.024	0.007	Yes
	140 vs. 160	2.096	3	2.058	0.337	No
Temperature within MIS	120 vs. 160	6.005	3	4.037	0.028	Yes
	120 vs. 140	4.179	3	2.809	0.146	No
	140 vs. 160	1.826	3	1.228	0.667	No
Temperature within BAL	120 vs. 160	7.609	3	6.052	0.002	Yes
	120 vs. 140	5.243	3	4.591	0.013	Yes
	140 vs. 160	2.365	3	1.700	0.468	No
Biomass within 120	BAL vs. MIS	5.354	2	3.599	0.021	Yes
Biomass within 140	BAL vs. MIS	4.814	2	3.460	0.026	Yes
Biomass within 160	BAL vs. MIS	3.75	2	2.983	0.050	No

A.2. Pre-treatment to remove extractives

*Table A.2-1: Tukey tests for **Solubility** of virgin MIS and BAL extraction from 5 L, 0.5 L and Soxhlet extractors*

Comparisons for factor	Comparison	Diff of Means	p	q	P	P<0.050
Extractor	0.5 L vs. 5 L	2.724	3	6.818	0.001	Yes
	0.5 L vs. Sox.	2.235	3	5.766	0.004	Yes
	Sox. vs. 5 L	0.489	3	1.224	0.671	No
Biomass	BAL vs. MIS	0.815	2	2.524	0.1	No
Biomass within 5 l extr.	BAL vs. MIS	1.076	2	1.85	0.215	No
Biomass within 0.5 l extr.	BAL vs. MIS	0.279	2	0.508	0.726	No
Biomass within Soxhlet extr.	BAL vs. MIS	3.8	2	6.932	<0.001	Yes
Extractor within MIS	0.5 L vs. 5 L	3.123	3	5.095	0.01	Yes
	0.5 L vs. Sox.	0.196	3	0.357	0.966	No
	Sox. vs. 5 L	2.927	3	4.775	0.014	Yes
Extractor within BAL	0.5 L vs. 5 L	2.326	3	4.536	0.019	Yes
	0.5 L vs. Sox.	4.275	3	7.797	<0.001	Yes
	Sox. vs. 5 L	1.949	3	3.8	0.048	Yes

A.3. Effects of temperature on pre-treated extract composition

*Table A.3-1: Tukey tests for **Solubility** of pre-treated MIS and BAL extracts*

Comparisons for factor	Comparison	Diff of Means	p	q	P	P<0.050
Biomass	BAL vs. MIS	8.110	2	13.630	<0.001	Yes
Temperature	180 vs. 140	26.993	3	34.096	<0.001	Yes
	180 vs. 160	15.056	3	21.661	<0.001	Yes
	160 vs. 140	11.937	3	17.174	<0.001	Yes
Temperature within MIS	180 vs. 140	20.832	3	18.607	<0.001	Yes
	180 vs. 160	14.268	3	13.624	<0.001	Yes
	160 vs. 140	6.564	3	6.268	<0.001	Yes
Temperature within BAL	180 vs. 140	33.154	3	29.612	<0.001	Yes
	180 vs. 160	15.844	3	17.332	<0.001	Yes
	160 vs. 140	17.310	3	18.935	<0.001	Yes
Biomass within 140	BAL vs. MIS	0.421	2	0.376	0.794	No
Biomass within 160	BAL vs. MIS	11.166	2	13.551	<0.001	Yes
Biomass within 180	BAL vs. MIS	12.743	2	11.381	<0.001	Yes

*Table A.3-2: Tukey tests for **Solubility** of virgin and pre-treated MIS*

Comparisons for factor	Comparison	Diff of Means	p	q	P	P<0.050
V/P	V vs. P	6.324	2	13.357	<0.001	Yes
Temperature	160 vs. 140	6.846	2	14.458	<0.001	Yes
Temperature within V	160 vs. 140	7.127	2	10.306	<0.001	Yes
Temperature within P	160 vs. 140	6.564	2	10.147	<0.001	Yes
V/P within 140	V vs. P	6.043	2	8.738	<0.001	Yes
V/P within 160	V vs. P	6.606	2	10.212	<0.001	Yes

*Table A.3-3: Tukey tests for **Solubility** of virgin and pre-treated BAL*

Comparisons for factor	Comparison	Diff of Means	p	q	P	P<0.050
V/P	V vs. P	2.504	2	3.216	0.034	Yes
Temperature	160 vs. 140	12.131	2	15.583	<0.001	Yes
Temperature within V	160 vs. 140	6.952	2	6.870	<0.001	Yes
Temperature within P	160 vs. 140	17.310	2	14.628	<0.001	Yes
V/P within 140	V vs. P	7.683	2	5.927	<0.001	Yes
V/P within 160	V vs. P	2.675	2	3.102	0.040	Yes

*Table A.3-4: Tukey tests for **Xylose** of virgin and pre-treated BAL and MIS*

Comparisons for factor	Comparison	Diff of Means	p	q	P	P<0.050
Temperature	160 vs. 140	1.629	2	6.443	<0.001	Yes
Biomass within V	MIS vs.BAL	0.167	2	0.453	0.751	No
Biomass within P	BAL vs. MIS	1.559	2	4.505	0.004	Yes
V/P within MIS	P vs. V	0.0443	2	0.116	0.935	No
V/P within BAL	P vs. V	1.770	2	5.337	<0.001	Yes
Temperature within V	160 vs. 140	0.626	2	1.697	0.241	No
Temperature within P	160 vs. 140	2.632	2	7.607	<0.001	Yes
V/P within 140	P vs. V	0.0960	2	0.244	0.865	No
V/P within 160	P vs. V	1.911	2	6.032	<0.001	Yes

*Table A.3-5: Tukey tests for **Xylooligosaccharides** of virgin and pre-treated BAL and MIS*

Comparisons for factor	Comparison	Diff of Means	p	q	P	P<0.050
V/P	P vs. V	4.812	2	3.742	0.013	Yes
Biomass	BAL vs. MIS	4.041	2	3.142	0.034	Yes
Temperature	160 vs. 140	6.977	2	5.426	<0.001	Yes
Temperature within V	160 vs. 140	2.613	2	1.450	0.314	No
Temperature within P	160 vs. 140	11.342	2	6.182	<0.001	Yes
V/P within 140	P vs. V	0.447	2	0.226	0.874	No
V/P within 160	P vs. V	9.176	2	5.601	<0.001	Yes

*Table A.3-6: Tukey tests for **Xylan** of virgin and pre-treated BAL and MIS*

Comparisons for factor	Comparison	Diff of Means	p	q	P	P<0.050
V/P	P vs. V	21.305	2	3.709	0.015	Yes
Biomass	BAL vs. MIS	28.138	2	4.899	0.002	Yes
Temperature	160 vs. 140	53.357	2	9.290	<0.001	Yes
Temperature within MIS	160 vs. 140	29.771	2	3.434	0.023	Yes
Temperature within BAL	160 vs. 140	76.942	2	10.210	<0.001	Yes
Biomass within BAL	BAL vs. MIS	4.553	2	0.508	0.722	No
Biomass within 160	BAL vs. MIS	51.724	2	7.188	<0.001	Yes

*Table A.3-7: Tukey tests for **Xylose** of pre-treated BAL and MIS*

Comparisons for factor	Comparison	Diff of Means	p	q	P	P<0.050
Biomass	BAL vs. MIS	10.103	2	5.072	0.002	Yes
Temperature	180 vs. 140	39.334	3	14.839	<0.001	Yes
	180 vs. 160	36.701	3	15.771	<0.001	Yes
	160 vs. 140	2.632	3	1.131	0.708	No
Temperature within MIS	180 vs. 140	25.976	3	6.929	<0.001	Yes
	180 vs. 160	24.427	3	6.966	<0.001	Yes
	160 vs. 140	1.548	3	0.442	0.948	No
Temperature within BAL	180 vs. 140	52.692	3	14.056	<0.001	Yes
	180 vs. 160	48.976	3	16.001	<0.001	Yes
	160 vs. 140	3.716	3	1.214	0.672	No
Biomass within 140	BAL vs. MIS	0.475	2	0.127	0.930	No
Biomass within 160	BAL vs. MIS	2.643	2	0.958	0.506	No
Biomass within 180	BAL vs. MIS	27.191	2	7.254	<0.001	Yes

*Table A.3-8: Tukey tests for **Xylooligosaccharides** of pre-treated BAL and MIS*

Comparisons for factor	Comparison	Diff of Means	p	q	P	P<0.050
Biomass	BAL vs. MIS	24.755	2	9.564	<0.001	Yes
Temperature	180 vs. 140	102.375	3	29.726	<0.001	Yes
	180 vs. 160	91.034	3	30.107	<0.001	Yes
	160 vs. 140	11.342	3	3.751	0.040	Yes
Temperature within MIS	180 vs. 140	72.505	3	14.886	<0.001	Yes
	180 vs. 160	67.083	3	14.724	<0.001	Yes
	160 vs. 140	5.421	3	1.190	0.683	No
Temperature within BAL	180 vs. 140	132.246	3	27.152	<0.001	Yes
	180 vs. 160	114.984	3	28.914	<0.001	Yes
	160 vs. 140	17.262	3	4.341	0.017	Yes
Biomass within 140	BAL vs. MIS	0.895	2	0.184	0.898	No
Biomass within 160	BAL vs. MIS	12.735	2	3.553	0.021	Yes
Biomass within 180	BAL vs. MIS	60.636	2	12.450	<0.001	Yes

*Table A.3-9: Tukey tests for **Xylan** of pre-treated BAL and MIS*

Comparisons for factor	Comparison	Diff of Means	p	q	P	P<0.050
Biomass	BAL vs. MIS	54.998	2	9.953	<0.001	Yes
Temperature	180 vs. 140	165.398	3	22.495	<0.001	Yes
	180 vs. 160	99.998	3	15.491	<0.001	Yes
	160 vs. 140	65.400	3	10.131	<0.001	Yes
Temperature within MIS	180 vs. 140	124.548	3	11.978	<0.001	Yes
	180 vs. 160	86.537	3	8.897	<0.001	Yes
	160 vs. 140	38.011	3	3.908	0.032	Yes
Temperature within BAL	180 vs. 140	206.247	3	19.835	<0.001	Yes
	180 vs. 160	113.459	3	13.364	<0.001	Yes
	160 vs. 140	92.788	3	10.929	<0.001	Yes
Biomass within 140	BAL vs. MIS	9.506	2	0.914	0.526	No
Biomass within 160	BAL vs. MIS	64.284	2	8.400	<0.001	Yes
Biomass within 180	BAL vs. MIS	91.205	2	8.771	<0.001	Yes

*Table A.3-10: Tukey tests for **Total phenolic content** of pre-treated BAL and MIS*

Comparisons for factor	Comparison	Diff of Means	p	q	P	P<0.050
Biomass	BAL vs. MIS	2.090	2	3.953	0.012	Yes
Temperature	180 vs. 140	18.845	3	27.434	<0.001	Yes
	180 vs. 160	9.686	3	15.446	<0.001	Yes
	160 vs. 140	9.159	3	14.606	<0.001	Yes
Temperature within MIS	180 vs. 140	17.440	3	17.953	<0.001	Yes
	180 vs. 160	11.929	3	12.279	<0.001	Yes
	160 vs. 140	5.512	3	5.674	0.002	Yes
Temperature within BAL	180 vs. 140	20.249	3	20.845	<0.001	Yes
	180 vs. 160	7.443	3	9.384	<0.001	Yes
	160 vs. 140	12.806	3	16.146	<0.001	Yes
Biomass within 140	BAL vs. MIS	1.278	2	1.315	0.365	No
Biomass within 160	BAL vs. MIS	6.017	2	7.586	<0.001	Yes
Biomass within 180	BAL vs. MIS	1.531	2	1.576	0.280	No

*Table A.3-11: Tukey tests for **Total phenolic content** of virgin and pre-treated MIS*

Comparisons for factor	Comparison	Diff of Means	p	q	P	P<0.050
V/P	V vs. P	2.993	2	5.622	0.004	Yes
Temperature	160 vs. 140	4.845	2	9.102	<0.001	Yes
Temperature within V	160 vs. 140	4.179	2	5.551	0.005	Yes
Temperature within P	160 vs. 140	5.512	2	7.321	0.001	Yes
V/P within 140	V vs. P	3.659	2	4.860	0.009	Yes
V/P within 160	V vs. P	2.327	2	3.091	0.061	No

*Table A.3-12: Tukey tests for **Total phenolic content** of virgin and pre-treated BAL*

Comparisons for factor	Comparison	Diff of Means	p	q	P	P<0.050
V/P	V vs. P	3.562	2	3.935	0.011	Yes
Temperature	160 vs. 140	9.860	2	10.893	<0.001	Yes
Temperature within V	160 vs. 140	6.914	2	5.877	<0.001	Yes
Temperature within P	160 vs. 140	12.806	2	9.307	<0.001	Yes
V/P within 140	V vs. P	0.616	2	0.408	0.776	No
V/P within 160	V vs. P	6.507	2	6.489	<0.001	Yes

A.4. Effects of residence time on pre-treated extract composition

*Table A.4-1: Tukey tests for **Solubility** of pre-treated BAL and MIS*

Comparisons for factor	Comparison	Diff of Means	p	q	P	P<0.050
Biomass	BAL vs. MIS	8.520	2	13.490	<0.001	Yes
Residence Time	60 vs. 0	22.221	3	26.443	<0.001	Yes
	60 vs. 30	7.874	3	10.672	<0.001	Yes
	30 vs. 0	14.347	3	19.446	<0.001	Yes
Residence Time within MIS	60 vs. 0	14.935	3	12.568	<0.001	Yes
	60 vs. 30	6.216	3	5.592	0.002	Yes
	30 vs. 0	8.719	3	7.844	<0.001	Yes
Residence Time within BAL	60 vs. 0	29.507	3	24.829	<0.001	Yes
	60 vs. 30	9.532	3	9.823	<0.001	Yes
	30 vs. 0	19.975	3	20.586	<0.001	Yes
Biomass within 30 min	BAL vs. MIS	11.166	2	12.767	<0.001	Yes
Biomass within 0 min	BAL vs. MIS	0.0897	2	0.0755	0.958	No
Biomass within 60 min	BAL vs. MIS	14.482	2	12.186	<0.001	Yes

*Table A.4-2: Tukey tests for **Xylose** of pre-treated BAL and MIS*

Comparisons for factor	Comparison	Diff of Means	p	q	P	P<0.050
Biomass	BAL vs. MIS	4.130	2	10.477	<0.001	Yes
Residence Time	60 vs. 0	9.363	3	17.852	<0.001	Yes
	60 vs. 30	6.497	3	14.110	<0.001	Yes
	30 vs. 0	2.866	3	6.223	<0.001	Yes
Residence Time within MIS	60 vs. 0	4.641	3	6.257	<0.001	Yes
	60 vs. 30	3.021	3	4.354	0.016	Yes
	30 vs. 0	1.620	3	2.335	0.250	No
Residence Time within BAL	60 vs. 0	14.085	3	18.990	<0.001	Yes
	60 vs. 30	9.974	3	16.468	<0.001	Yes
	30 vs. 0	4.112	3	6.789	<0.001	Yes
Biomass within 30 min	BAL vs. MIS	2.643	2	4.841	0.003	Yes
Biomass within 0 min	BAL vs. MIS	0.151	2	0.204	0.887	No
Biomass within 60 min	BAL vs. MIS	9.595	2	12.936	<0.001	Yes

*Table A.4-3: Tukey tests for **Xylooligosaccharides** of pre-treated BAL and MIS*

Comparisons for factor	Comparison	Diff of Means	p	q	P	P<0.050
Biomass	BAL vs. MIS	16.738	2	8.484	<0.001	Yes
Residence Time	60 vs. 0	36.597	3	13.942	<0.001	Yes
	60 vs. 30	24.872	3	10.792	<0.001	Yes
	30 vs. 0	11.726	3	5.088	0.005	Yes
Residence Time within MIS	60 vs. 0	17.985	3	4.845	0.008	Yes
	60 vs. 30	12.564	3	3.618	0.048	Yes
	30 vs. 0	5.421	3	1.561	0.523	No
Residence Time within BAL	60 vs. 0	55.210	3	14.872	<0.001	Yes
	60 vs. 30	37.180	3	12.266	<0.001	Yes
	30 vs. 0	18.030	3	5.948	0.001	Yes
Biomass within 30 min	BAL vs. MIS	12.735	2	4.661	0.004	Yes
Biomass within 0 min	BAL vs. MIS	0.127	2	0.0341	0.981	No
Biomass within 60 min	BAL vs. MIS	37.351	2	10.061	<0.001	Yes

*Table A.4-4: Tukey tests for **Xylan** of pre-treated BAL and MIS*

Comparisons for factor	Comparison	Diff of Means	p	q	P	P<0.050
Biomass	BAL vs. MIS	60.581	2	8.330	<0.001	Yes
Residence Time	60 vs. 0	125.183	3	12.936	<0.001	Yes
	60 vs. 30	52.273	3	6.152	0.001	Yes
	30 vs. 0	72.910	3	8.581	<0.001	Yes
Residence Time within MIS	60 vs. 0	68.976	3	5.040	0.006	Yes
	60 vs. 30	26.946	3	2.105	0.319	No
	30 vs. 0	42.030	3	3.283	0.077	No
Residence Time within BAL	60 vs. 0	181.390	3	13.254	<0.001	Yes
	60 vs. 30	77.599	3	6.944	<0.001	Yes
	30 vs. 0	103.791	3	9.288	<0.001	Yes
Biomass within 30 min	BAL vs. MIS	64.284	2	6.382	<0.001	Yes
Biomass within 0 min	BAL vs. MIS	2.524	2	0.184	0.898	No
Biomass within 60 min	BAL vs. MIS	114.937	2	8.398	<0.001	Yes

*Table A.4-4: Tukey tests for **Total phenolic content** of pre-treated BAL and MIS*

Comparisons for factor	Comparison	Diff of Means	p	q	P	P<0.050
Biomass	BAL vs. MIS	5.128	2	9.770	<0.001	Yes
Residence Time	60 vs. 0	16.905	3	24.795	<0.001	Yes
	60 vs. 30	7.615	3	12.235	<0.001	Yes
	30 vs. 0	9.290	3	14.926	<0.001	Yes
Residence Time within MIS	60 vs. 0	10.916	3	11.321	<0.001	Yes
	60 vs. 30	5.287	3	5.484	0.003	Yes
	30 vs. 0	5.628	3	5.837	0.002	Yes
Residence Time within BAL	60 vs. 0	22.894	3	23.744	<0.001	Yes
	60 vs. 30	9.943	3	12.629	<0.001	Yes
	30 vs. 0	12.951	3	16.450	<0.001	Yes
Biomass within 30 min	BAL vs. MIS	6.017	2	7.643	<0.001	Yes
Biomass within 0 min	BAL vs. MIS	1.306	2	1.354	0.351	No
Biomass within 60 min	BAL vs. MIS	10.672	2	11.068	<0.001	Yes

A.5. Composition of extracts from various oat husk varieties

*Table A.5-1: Tukey tests for **Solubility** of virgin (V) and pre-treated (P) husk varieties*

Comparisons for factor	Comparison	Diff of Means	p	q	P	P<0.050
V/P	V vs. P	0.839	2	1.299	0.366	No
Biomass within V	14355Cn vs. MAS	29.698	5	19.176	<0.001	Yes
	14355Cn vs. CON	29.257	5	18.891	<0.001	Yes
	14355Cn vs. BAL	25.327	5	19.724	<0.001	Yes
	14355Cn vs. SO-I	14.070	5	9.085	<0.001	Yes
	SO-I vs. MAS	15.628	5	10.091	<0.001	Yes
	SO-I vs. CON	15.187	5	9.806	<0.001	Yes
	SO-I vs. BAL	11.257	5	8.767	<0.001	Yes
	BAL vs. MAS	4.371	5	3.404	0.140	No
	BAL vs. CON	3.930	5	3.060	0.220	No
	CON vs. MAS	0.441	5	0.285	1.000	No
Biomass within P	SO-I vs. BAL	8.413	5	6.653	<0.001	Yes
	SO-I vs. CON	5.665	5	3.658	0.098	No
	SO-I vs. MAS	5.557	5	3.588	0.108	No
	SO-I vs. 14355Cn	3.326	5	2.148	0.559	No
	14355Cn vs. BAL	5.087	5	4.023	0.056	No
	14355Cn vs. CON	2.339	5	1.510	0.821	No
	14355Cn vs. MAS	2.231	5	1.441	0.845	No
	MAS vs. BAL	2.856	5	2.259	0.510	No
	MAS vs. CON	0.108	5	0.0698	1.000	No
	CON vs. BAL	2.748	5	2.173	0.548	No
V/P within BAL	P vs. V	2.675	2	2.903	0.049	Yes
V/P within SO-I	V vs. P	0.169	2	0.109	0.939	No
V/P within 14355Cn	V vs. P	17.565	2	11.342	<0.001	Yes
V/P within CON	P vs. V	9.353	2	6.039	<0.001	Yes
V/P within MAS	P vs. V	9.902	2	6.394	<0.001	Yes

*Table A.5-2: Tukey tests for **Total phenolic contents** of extracts from virgin (V) and pre-treated (P) husk varieties*

Comparisons for factor	Comparison	Diff of Means	p	q	P	P<0.050
V/P	V vs. P	0.775	2	1.396	0.331	No
Biomass within V	SO-I vs. BAL	4.189	5	3.800	0.079	No
	SO-I vs. MAS	3.427	5	2.578	0.379	No
	SO-I vs. 14355Cn	2.735	5	2.057	0.598	No
	SO-I vs. CON	1.116	5	0.840	0.975	No
	CON vs. BAL	3.073	5	2.787	0.303	No
	CON vs. MAS	2.311	5	1.738	0.735	No
	CON vs. 14355Cn	1.619	5	1.218	0.909	No
	14355Cn vs. BAL	1.454	5	1.319	0.882	No
	14355Cn vs. MAS	0.692	5	0.520	0.996	No
	MAS vs. BAL	0.762	5	0.691	0.988	No
Biomass within P	BAL vs. 14355Cn	12.745	5	11.740	<0.001	Yes
	BAL vs. MAS	5.691	5	5.242	0.007	Yes
	BAL vs. SO-I	4.958	5	4.567	0.023	Yes
	BAL vs. CON	3.541	5	3.262	0.170	No
	CON vs. 14355Cn	9.204	5	6.923	<0.001	Yes
	CON vs. MAS	2.150	5	1.617	0.782	No
	CON vs. SO-I	1.417	5	1.066	0.942	No
	SO-I vs. 14355Cn	7.787	5	5.857	0.002	Yes
	SO-I vs. MAS	0.733	5	0.551	0.995	No
	MAS vs. 14355Cn	7.054	5	5.306	0.006	Yes
V/P within BAL	P vs. V	6.507	2	8.225	<0.001	Yes
V/P within SO-I	V vs. P	2.639	2	1.985	0.171	No
V/P within 14355Cn	V vs. P	7.691	2	5.785	<0.001	Yes
V/P within CON	V vs. P	0.106	2	0.0796	0.956	No
V/P within MAS	P vs. V	0.0549	2	0.0413	0.977	No

*Table A.5-3: Tukey tests for **Glucan contents** of extracts from virgin (V) and pre-treated (P) husk varieties*

Comparisons for factor	Comparison	Diff of Means	p	q	P	P<0.050
V/P	V vs. P	51.593	2	31.922	<0.001	Yes
Biomass within V	14355Cn vs. CON	194.938	5	50.981	<0.001	Yes
	14355Cn vs. BAL	188.536	5	55.127	<0.001	Yes
	14355Cn vs. MAS	186.903	5	48.880	<0.001	Yes
	14355Cn vs. SO-I	139.416	5	36.461	<0.001	Yes
	SO-I vs. CON	55.521	5	14.520	<0.001	Yes
	SO-I vs. BAL	49.120	5	14.362	<0.001	Yes
	SO-I vs. MAS	47.486	5	12.419	<0.001	Yes
	MAS vs. CON	8.035	5	2.101	0.580	No
	MAS vs. BAL	1.633	5	0.478	0.997	No
	BAL vs. CON	6.402	5	1.872	0.679	No
Biomass within P	14355Cn vs. CON	21.066	5	5.509	0.005	Yes
	14355Cn vs. MAS	20.622	5	5.393	0.006	Yes
	14355Cn vs. BAL	18.537	5	5.937	0.002	Yes
	14355Cn vs. SO-I	17.663	5	4.619	0.022	Yes
	SO-I vs. CON	3.403	5	0.890	0.969	No
	SO-I vs. MAS	2.959	5	0.774	0.981	No
	SO-I vs. BAL	0.874	5	0.280	1.000	No
	BAL vs. CON	2.529	5	0.810	0.978	No
	BAL vs. MAS	2.085	5	0.668	0.989	No
	MAS vs. CON	0.444	5	0.116	1.000	No
V/P within BAL	V vs. P	7.975	2	3.053	0.040	Yes
V/P within SO-I	V vs. P	56.221	2	14.703	<0.001	Yes
V/P within 14355Cn	V vs. P	177.974	2	46.545	<0.001	Yes
V/P within CON	V vs. P	4.102	2	1.073	0.455	No
V/P within MAS	V vs. P	11.693	2	3.058	0.039	Yes

*Table A.5-4: Tukey tests for **Xylan contents** of extracts from virgin (V) and pre-treated (P) husk varieties*

Comparisons for factor	Comparison	Diff of Means	p	q	P	P<0.050
V/P	P vs. V	64.627	2	14.663	<0.001	Yes
Biomass within V	SO-I vs. BAL	62.389	5	6.705	<0.001	Yes
	SO-I vs. MAS	60.681	5	5.833	0.003	Yes
	SO-I vs. CON	51.553	5	4.956	0.013	Yes
	SO-I vs. 14355Cn	31.745	5	3.052	0.226	No
	14355Cn vs. BAL	30.644	5	3.293	0.167	No
	14355Cn vs. MAS	28.936	5	2.781	0.309	No
	14355Cn vs. CON	19.808	5	1.904	0.666	No
	CON vs. BAL	10.836	5	1.165	0.921	No
	CON vs. MAS	9.128	5	0.877	0.971	No
	MAS vs. BAL	1.708	5	0.184	1.000	No
Biomass within P	SO-I vs. 14355Cn	90.640	5	8.713	<0.001	Yes
	SO-I vs. BAL	35.812	5	4.152	0.048	Yes
	SO-I vs. CON	18.564	5	1.785	0.716	No
	SO-I vs. MAS	8.574	5	0.824	0.977	No
	MAS vs. 14355Cn	82.066	5	7.889	<0.001	Yes
	MAS vs. BAL	27.238	5	3.158	0.198	No
	MAS vs. CON	9.990	5	0.960	0.959	No
	CON vs. 14355Cn	72.076	5	6.928	<0.001	Yes
	CON vs. BAL	17.248	5	2.000	0.624	No
	BAL vs. 14355Cn	54.828	5	6.356	0.001	Yes
V/P within BAL	P vs. V	80.648	2	11.103	<0.001	Yes
V/P within SO-I	P vs. V	54.072	2	5.198	0.001	Yes
V/P within 14355Cn	V vs. P	4.823	2	0.464	0.746	No
V/P within CON	P vs. V	87.060	2	8.369	<0.001	Yes
V/P within MAS	P vs. V	106.179	2	10.207	<0.001	Yes

A.6. RSM summary tables for BAL CON and MIS

*Table A.6-1: RSM summary table of three factor RSM model for **BALADO Solubility***

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	8565.3	9	951.70	17.18	< 0.0001
A-Temperature	7105.9	1	7105.88	128.26	< 0.0001
B-Residence Time	977.3	1	977.34	17.64	0.0006
C-Loading	140.7	1	140.71	2.54	0.1294
AB	6.5	1	6.47	0.12	0.7367
AC	105.0	1	105.02	1.90	0.1864
BC	0.6	1	0.57	0.01	0.9205
A^2	129.2	1	129.20	2.33	0.1451
B^2	470.7	1	470.72	8.50	0.0097
C^2	19.3	1	19.28	0.35	0.5630
Residual	941.9	17	55.40		
Lack of Fit	933.4	6	155.57	203.61	< 0.0001
Pure Error	8.4	11	0.76		
Cor Total	9507.1	26			

Std. Dev.	7.4	R-Squared	0.90
Mean	28.8	Adj R-Squared	0.85
C.V. %	25.8	Pred R-Squared	0.71
PRESS	2787.1	Adeq Precision	14.43

Final Equation in Terms of Coded Factors:

Solubility = 35.38 + 19.61*A + 7.14*B – 3.04*C – 0.80*A*B – 3.24*A*C + 0.24*B*C – 2.67*A^2 – 5.26*B^2 + 1.18*C^2

*Table A.6-2: RSM summary table of three factor RSM model for **CONWAY Solubility***

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	5243.0	9	582.6	9.4	0.0008
A-Temperature	3998.1	1	3998.1	64.6	< 0.0001
B-Residence Time	607.2	1	607.2	9.8	0.0106
C-Loading	228.2	1	228.2	3.7	0.0837
AB	0.2	1	0.2	0.0	0.9580
AC	31.2	1	31.2	0.5	0.4938
BC	2.0	1	2.0	0.0	0.8609
A^2	99.1	1	99.1	1.6	0.2344
B^2	437.5	1	437.5	7.1	0.0239
C^2	7.7	1	7.7	0.1	0.7317
Residual	618.6	10	61.9		
Lack of Fit	604.5	5	120.9	43.0	0.0004
Pure Error	14.1	5	2.8		
Cor Total	5861.6	19			

Std. Dev.	7.9	R-Squared	0.89
Mean	32.3	Adj R-Squared	0.80
C.V. %	24.4	Pred R-Squared	0.21
PRESS	4617.0	Adeq Precision	10.37

Final Equation in Terms of Coded Factors:

Solubility = 37.52 + 17.17*A + 6.97*B – 4.28*C – 0.15*A*B – 1.98*A*C + 0.50*B*C – 2.65*A^2 – 6.21*B^2 + 0.80*C^2

*Table A.6-3: RSM summary table of three factor RSM model for **MISCANTHUS Solubility***

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	3026.5	9	336.3	25.0	< 0.0001
A-Temperature	2542.1	1	2542.1	189.1	< 0.0001
B-Residence Time	284.6	1	284.6	21.2	0.0010
C-Loading	86.7	1	86.7	6.5	0.0294
AB	7.4	1	7.4	0.6	0.4749
AC	13.8	1	13.8	1.0	0.3352
BC	0.6	1	0.6	0.0	0.8436
A^2	1.9	1	1.9	0.1	0.7127
B^2	58.4	1	58.4	4.3	0.0638
C^2	97.0	1	97.0	7.2	0.0229
Residual	134.5	10	13.4		
Lack of Fit	129.4	5	25.9	25.5	0.0014
Pure Error	5.1	5	1.0		
Cor Total	3160.9	19			

Std. Dev.	3.7	R-Squared	0.96
Mean	21.6	Adj R-Squared	0.92
C.V. %	17.0	Pred R-Squared	0.68
PRESS	1007.4	Adeq Precision	17.89

Final Equation in Terms of Coded Factors:

Solubility = 20.96 + 13.69*A + 4.77*B – 2.64*C + 0.96*A*B – 1.31*A*C – 0.26*B*C + 0.37*A^2 – 2.27*B^2 + 2.84*C^2

*Table A.6-4: RSM summary table of three factor RSM model for **BALADO pH***

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	18.651	9	2.072	65.192	< 0.0001
A-Temperature	15.389	1	15.389	484.095	< 0.0001
B-Residence Time	2.506	1	2.506	78.825	< 0.0001
C-Loading	0.219	1	0.219	6.879	0.0178
AB	0.101	1	0.101	3.188	0.0921
AC	0.019	1	0.019	0.585	0.4549
BC	0.003	1	0.003	0.103	0.7521
A^2	0.378	1	0.378	11.888	0.0031
B^2	0.502	1	0.502	15.785	0.0010
C^2	0.001	1	0.001	0.041	0.8415
Residual	0.540	17	0.032		
Lack of Fit	0.485	6	0.081	16.180	< 0.0001
Pure Error	0.055	11	0.005		
Cor Total	19.192	26			

Std. Dev.	0.18	R-Squared	0.97
Mean	3.97	Adj R-Squared	0.96
C.V. %	4.49	Pred R-Squared	0.90
PRESS	1.99	Adeq Precision	28.04

Final Equation in Terms of Coded Factors:

pH = 3.69 – 0.91*A – 0.36*B – 0.12*C + 0.10*A*B – 0.04*A*C + 0.02*B*C + 0.14*A^2 + 0.17*B^2 – 0.01*C^2

*Table A.6-5: RSM summary table of three factor RSM model for **CONWAY pH***

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	0.032	9	0.004	16.080	< 0.0001
A-Temperature	0.026	1	0.026	118.848	< 0.0001
B-Residence Time	0.004	1	0.004	20.102	0.0012
C-Loading	0.001	1	0.001	2.836	0.1231
AB	0.000	1	0.000	0.532	0.4824
AC	0.000	1	0.000	1.305	0.2798
BC	0.000	1	0.000	0.097	0.7615
A^2	0.000	1	0.000	0.002	0.9613
B^2	0.000	1	0.000	0.770	0.4008
C^2	0.000	1	0.000	0.394	0.5445
Residual	0.002	10	0.000		
Lack of Fit	0.001	5	0.000	0.388	0.8388
Pure Error	0.002	5	0.000		
Cor Total	0.034	19			

Std. Dev.	0.015	R-Squared	0.94
Mean	0.265	Adj R-Squared	0.88
C.V. %	5.598	Pred R-Squared	0.80
PRESS	0.007	Adeq Precision	14.87

Final Equation in Terms of Coded Factors:

$$1.0/(pH) = 0.265 + 0.044*A + 0.019*B + 0.007*C - 0.004*A*B - 0.006*A*C - 0.02*B*C + 0.01*A^2 - 0.004*B^2 + 0.003*C^2$$

*Table A.6-6: RSM summary table of three factor RSM model for **MISCANTHUS pH***

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	6.344	9	0.705	19.477	< 0.0001
A-Temperature	4.960	1	4.960	137.066	< 0.0001
B-Residence Time	0.540	1	0.540	14.928	0.0031
C-Loading	0.402	1	0.402	11.102	0.0076
AB	0.020	1	0.020	0.553	0.4743
AC	0.005	1	0.005	0.138	0.7179
BC	0.005	1	0.005	0.138	0.7179
A^2	0.064	1	0.064	1.769	0.2130
B^2	0.194	1	0.194	5.361	0.0431
C^2	0.107	1	0.107	2.966	0.1157
Residual	0.362	10	0.036		
Lack of Fit	0.354	5	0.071	42.427	0.0004
Pure Error	0.008	5	0.002		
Cor Total	6.706	19			

Std. Dev.	0.190	R-Squared	0.95
Mean	3.965	Adj R-Squared	0.90
C.V. %	4.798	Pred R-Squared	0.60
PRESS	2.675	Adeq Precision	15.12

Final Equation in Terms of Coded Factors:

$$pH = 3.903 - 0.605*A - 0.208*B - 0.180*C + 0.050*A*B - 0.025*A*C + 0.025*B*C + 0.067*A^2 + 0.131*B^2 - 0.094*C^2$$

*Table A.6-7: RSM summary table of three factor RSM model for **BALADO** extract **Total Phenolic Contents***

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	2.164	9	0.240	39.224	< 0.0001
A-Temperature	0.660	1	0.660	107.692	< 0.0001
B-Residence Time	0.144	1	0.144	23.473	0.0002
C-Loading	0.981	1	0.981	160.011	< 0.0001
AB	0.009	1	0.009	1.446	0.2456
AC	0.017	1	0.017	2.730	0.1169
BC	0.003	1	0.003	0.466	0.5041
A^2	0.144	1	0.144	23.474	0.0002
B^2	0.085	1	0.085	13.823	0.0017
C^2	0.206	1	0.206	33.575	< 0.0001
Residual	0.104	17	0.006		
Lack of Fit	0.086	6	0.014	8.418	0.0014
Pure Error	0.019	11	0.002		
Cor Total	2.268	26			

Std. Dev.	0.078	R-Squared	0.95
Mean	1.395	Adj R-Squared	0.93
C.V. %	5.611	Pred R-Squared	0.86
PRESS	0.306	Adeq Precision	25.89

Final Equation in Terms of Coded Factors:

$$\text{Log10(TPC)} = 1.446 + 0.189*A + 0.087*B - 0.254*C - 0.030*A*B + 0.041*A*C + 0.017*B*C - 0.089*A^2 - 0.071*B^2 + 0.122*C^2$$

*Table A.6-8: RSM summary table of three factor RSM model for **CONWAY** extract **Total Phenolic Contents***

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	0.083	9	0.009	29.821	< 0.0001
A-Temperature	0.031	1	0.031	99.963	< 0.0001
B-Residence Time	0.006	1	0.006	18.575	0.0015
C-Loading	0.031	1	0.031	99.234	< 0.0001
AB	0.002	1	0.002	6.806	0.0261
AC	0.004	1	0.004	13.107	0.0047
BC	0.001	1	0.001	2.572	0.1398
A^2	0.005	1	0.005	15.637	0.0027
B^2	0.005	1	0.005	17.498	0.0019
C^2	0.004	1	0.004	11.336	0.0072
Residual	0.003	10	0.000		
Lack of Fit	0.003	5	0.001	20.279	0.0025
Pure Error	0.000	5	0.000		
Cor Total	0.087	19			

Std. Dev.	0.018	R-Squared	0.96
Mean	0.198	Adj R-Squared	0.93
C.V. %	8.887	Pred R-Squared	0.73
PRESS	0.023	Adeq Precision	23.43

Final Equation in Terms of Coded Factors:

1.0/Sqrt(TPC) = 0.182 – 0.048*A – 0.022*B + 0.050*C + 0.016*A*B – 0.023*A*B – 0.010*B*C + 0.019*A^2 + 0.022*B^2 – 0.017*C^2

*Table A.6-9: RSM summary table of three factor RSM model for **MISCANTHUS** extract **Total Phenolic Contents***

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	0.073	9	0.008	35.080	< 0.0001
A-Temperature	0.025	1	0.025	106.492	< 0.0001
B-Residence Time	0.004	1	0.004	18.245	0.0016
C-Loading	0.035	1	0.035	152.137	< 0.0001
AB	0.000	1	0.000	0.977	0.3463
AC	0.001	1	0.001	3.384	0.0957
BC	0.000	1	0.000	0.838	0.3814
A^2	0.004	1	0.004	15.669	0.0027
B^2	0.002	1	0.002	8.503	0.0154
C^2	0.008	1	0.008	36.409	0.0001
Residual	0.002	10	0.000		
Lack of Fit	0.002	5	0.000	19.120	0.0028
Pure Error	0.000	5	0.000		
Cor Total	0.075	19			

Std. Dev.	0.015	R-Squared	0.97
Mean	0.221	Adj R-Squared	0.94
C.V. %	6.879	Pred R-Squared	0.80
PRESS	0.015	Adeq Precision	24.20

Final Equation in Terms of Coded Factors:

1.0/Sqrt(TPC) = 0.218 – 0.043*A – 0.018*B + 0.053*C + 0.005*A*B – 0.010*A*C – 0.005*B*C + 0.016*A^2 + 0.013*B^2 – 0.026*C^2

*Table A.6-10: RSM summary table of three factor RSM model for **Furfural** contents in **BALADO** extracts*

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	26.29	9	2.92	23.43	< 0.0001
A-Temperature	20.41	1	20.41	163.72	< 0.0001
B-Residence Time	4.86	1	4.86	38.94	< 0.0001
C-Loading	0.09	1	0.09	0.74	0.4025
AB	0.06	1	0.06	0.48	0.4985
AC	0.16	1	0.16	1.29	0.2709
BC	0.04	1	0.04	0.36	0.5579
A^2	0.71	1	0.71	5.72	0.0286
B^2	0.38	1	0.38	3.01	0.1007
C^2	0.15	1	0.15	1.21	0.2869
Residual	2.12	17	0.12		
Lack of Fit	1.47	6	0.24	4.12	0.0206
Pure Error	0.65	11	0.06		
Cor Total	28.41	26			

Std. Dev.	0.35	R-Squared	0.93
Mean	0.49	Adj R-Squared	0.89
C.V. %	72.76	Pred R-Squared	0.78
PRESS	6.25	Adeq Precision	16.55

Final Equation in Terms of Coded Factors:

Log10(Furfural) = 0.74 + 1.05*A + 0.50*B – 0.08*C – 0.08*A*B + 0.13*A*C – 0.07*B*C – 0.20*A^2 – 0.15*B^2 + 0.10*C^2

*Table A.6-11: RSM summary table of three factor RSM model for **Furfural** contents in **CONWAY** extracts*

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	15.43	9	1.71	38.24	< 0.0001
A-Temperature	10.46	1	10.46	233.31	< 0.0001
B-Residence Time	2.44	1	2.44	54.50	< 0.0001
C-Loading	1.11	1	1.11	24.84	0.0006
AB	0.17	1	0.17	3.75	0.0816
AC	0.00	1	0.00	0.01	0.9067
BC	0.04	1	0.04	0.86	0.3758
A^2	0.87	1	0.87	19.44	0.0013
B^2	0.25	1	0.25	5.62	0.0392
C^2	0.49	1	0.49	10.98	0.0078
Residual	0.45	10	0.04		
Lack of Fit	0.44	5	0.09	109.17	< 0.0001
Pure Error	0.00	5	0.00		
Cor Total	15.87	19			

Std. Dev.	0.21	R-Squared	0.97
Mean	0.65	Adj R-Squared	0.95
C.V. %	32.67	Pred R-Squared	0.75
PRESS	4.00	Adeq Precision	24.46

Final Equation in Terms of Coded Factors:

Log10(Furfural) = 0.78 + 0.88*A + 0.44*B – 0.30*C + 0.14*A*B – 0.01*A*C + 0.07*B*C – 0.25*A^2 – 0.15*B^2 + 0.20*C^2

*Table A.6-12: RSM summary table of three factor RSM model for **Furfural** contents in **MISCANTHUS** extracts*

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	16.81	9	1.87	25.91	< 0.0001
A-Temperature	12.26	1	12.26	170.11	< 0.0001
B-Residence Time	2.00	1	2.00	27.73	0.0004
C-Loading	0.14	1	0.14	1.88	0.2003
AB	0.02	1	0.02	0.23	0.6434
AC	0.02	1	0.02	0.27	0.6158
BC	0.11	1	0.11	1.57	0.2394
A^2	0.80	1	0.80	11.16	0.0075
B^2	0.16	1	0.16	2.17	0.1717
C^2	1.45	1	1.45	20.12	0.0012
Residual	0.72	10	0.07		
Lack of Fit	0.71	5	0.14	53.80	0.0002
Pure Error	0.01	5	0.00		
Cor Total	17.53	19			

Std. Dev.	0.27	R-Squared	0.96
Mean	0.58	Adj R-Squared	0.92
C.V. %	46.40	Pred R-Squared	0.72
PRESS	4.88	Adeq Precision	19.59

Final Equation in Terms of Coded Factors:

Log10(Furfural) = 0.59 + 0.95*A + 0.40*B – 0.10*C – 0.05*A*B + 0.05*A*B – 0.12*B*C – 0.24*A^2 – 0.12*B^2 + 0.35*C^2

*Table A.6-13: RSM summary table of two factor severity RSM model for **BALADO Solubility***

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	7953.7	5	1590.7	21.5	< 0.0001
A-Severity factor	5768.4	1	5768.4	78.0	< 0.0001
B-Loading	146.2	1	146.2	2.0	0.1744
AB	108.9	1	108.9	1.5	0.2385
A^2	790.3	1	790.3	10.7	0.0037
B^2	3.6	1	3.6	0.0	0.8275
Residual	1553.4	21	74.0		
Lack of Fit	1545.0	10	154.5	202.2	< 0.0001
Pure Error	8.4	11	0.8		
Cor Total	9507.1	26			

Std. Dev.	8.6	R-Squared	0.84
Mean	28.8	Adj R-Squared	0.80
C.V. %	29.9	Pred R-Squared	0.71
PRESS	2795.1	Adeq Precision	15.51

Final Equation in Terms of Coded Factors:

Solubility = 39.9 + 27.3*A – 3.1*B – 4.7*A*B – 18.0*A^2 + 0.5*B^2

*Table A.6-14: RSM summary table of two factor severity RSM model for **CONWAY Solubility***

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	4755.7	5	951.1	12.0	0.0001
A-Severity factor	3621.0	1	3621.0	45.8	< 0.0001
B-Loading	226.2	1	226.2	2.9	0.1127
AB	18.8	1	18.8	0.2	0.6332
A^2	561.1	1	561.1	7.1	0.0185
B^2	1.7	1	1.7	0.0	0.8871
Residual	1105.9	14	79.0		
Lack of Fit	1091.9	9	121.3	43.1	0.0003
Pure Error	14.1	5	2.8		
Cor Total	5861.6	19			

Std. Dev.	8.9	R-Squared	0.81
Mean	32.3	Adj R-Squared	0.74
C.V. %	27.5	Pred R-Squared	0.63
PRESS	2185.0	Adeq Precision	11.86

Final Equation in Terms of Coded Factors:

Solubility = 41.9 + 24.5*A – 4.4*B – 2.2*A*B – 16.2*A^2 + 0.4*B^2

*Table A.6-15: RSM summary table of two factor severity RSM model for **MISCANTHUS Solubility***

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	2825.4	5	565.1	23.6	< 0.0001
A-Severity factor	2495.7	1	2495.7	104.1	< 0.0001
B-Loading	89.3	1	89.3	3.7	0.0741
AB	13.7	1	13.7	0.6	0.4618
A^2	60.2	1	60.2	2.5	0.1352
B^2	65.0	1	65.0	2.7	0.1218
Residual	335.5	14	24.0		
Lack of Fit	330.5	9	36.7	36.1	0.0005
Pure Error	5.1	5	1.0		
Cor Total	3160.9	19			

Std. Dev.	4.9	R-Squared	0.89
Mean	21.6	Adj R-Squared	0.86
C.V. %	22.6	Pred R-Squared	0.77
PRESS	739.6	Adeq Precision	15.33

Final Equation in Terms of Coded Factors:

Solubility = 25.5 + 20.4*A – 2.8*B – 1.9*A*B – 5.3*A^2 + 2.3*B^2

*Table A.6-16: RSM summary table of two factor severity RSM model for **BALADO pH***

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	17.43	5	3.49	41.50	< 0.0001
A-Severity factor	12.79	1	12.79	152.27	< 0.0001
B-Loading	0.34	1	0.34	3.99	0.0588
AB	0.02	1	0.02	0.23	0.6354
A^2	1.76	1	1.76	20.94	0.0002
B^2	0.00	1	0.00	0.03	0.8732
Residual	1.76	21	0.08		
Lack of Fit	1.71	10	0.17	34.17	< 0.0001
Pure Error	0.06	11	0.01		
Cor Total	19.19	26			

Std. Dev.	0.29	R-Squared	0.91
Mean	3.97	Adj R-Squared	0.89
C.V. %	7.29	Pred R-Squared	0.80
PRESS	3.85	Adeq Precision	21.06

Final Equation in Terms of Coded Factors:

pH = 3.43 – 1.28*A – 0.15*B – 0.06*A*B + 0.85*A^2 + 0.01*B^2

*Table A.6-17: RSM summary table of two factor severity RSM model for **CONWAY pH***

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	7.51	5	1.50	23.54	< 0.0001
A-Severity factor	5.71	1	5.71	89.45	< 0.0001
B-Loading	0.11	1	0.11	1.72	0.2109
AB	0.13	1	0.13	2.05	0.1740
A^2	0.89	1	0.89	13.93	0.0022
B^2	0.00	1	0.00	0.05	0.8218
Residual	0.89	14	0.06		
Lack of Fit	0.54	9	0.06	0.85	0.6096
Pure Error	0.35	5	0.07		
Cor Total	8.40	19			

Std. Dev.	0.25	R-Squared	0.89
Mean	3.87	Adj R-Squared	0.86
C.V. %	6.53	Pred R-Squared	0.76
PRESS	2.04	Adeq Precision	16.41

Final Equation in Terms of Coded Factors:

$$pH = 3.50 - 0.97*A - 0.10*B + 0.18*A*B + 0.64*A^2 - 0.02*B^2$$

*Table A.6-18: RSM summary table of two factor severity RSM model for **MISCANTHUS pH***

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	6.06	5	1.21	26.16	< 0.0001
A-Severity factor	4.59	1	4.59	99.24	< 0.0001
B-Loading	0.43	1	0.43	9.19	0.0090
AB	0.01	1	0.01	0.13	0.7273
A^2	0.46	1	0.46	9.90	0.0071
B^2	0.08	1	0.08	1.67	0.2175
Residual	0.65	14	0.05		
Lack of Fit	0.64	9	0.07	42.66	0.0003
Pure Error	0.01	5	0.00		
Cor Total	6.71	19			

Std. Dev.	0.22	R-Squared	0.90
Mean	3.97	Adj R-Squared	0.87
C.V. %	5.43	Pred R-Squared	0.77
PRESS	1.56	Adeq Precision	17.56

Final Equation in Terms of Coded Factors:

$$pH = 3.71 - 0.87*A - 0.19*B - 0.04*A*B + 0.46*A^2 - 0.08*B^2$$

*Table A.6-19: RSM summary table of two factor severity RSM model for **BALADO** extract **Total Phenolic Contents***

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	2.05	5	0.41	38.97	< 0.0001
A-Severity factor	0.45	1	0.45	42.71	< 0.0001
B-Loading	0.89	1	0.89	84.28	< 0.0001
AB	0.02	1	0.02	2.00	0.1720
A^2	0.26	1	0.26	25.21	< 0.0001
B^2	0.22	1	0.22	20.86	0.0002
Residual	0.22	21	0.01		
Lack of Fit	0.20	10	0.02	11.92	0.0002
Pure Error	0.02	11	0.00		
Cor Total	2.27	26			

Std. Dev.	0.10	R-Squared	0.90
Mean	1.40	Adj R-Squared	0.88
C.V. %	7.35	Pred R-Squared	0.81
PRESS	0.44	Adeq Precision	25.83

Final Equation in Terms of Coded Factors:

$$\log10(TPC) = 1.47 + 0.24*A - 0.24*B + 0.07*A*B - 0.33*A^2 + 0.13*B^2$$

*Table A.6-20: RSM summary table of two factor severity RSM model for **CONWAY** extract **Total Phenolic Contents***

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	1.37	5	0.27	22.97	< 0.0001
A-Severity factor	0.39	1	0.39	32.44	< 0.0001
B-Loading	0.57	1	0.57	48.17	< 0.0001
AB	0.03	1	0.03	2.27	0.1543
A^2	0.16	1	0.16	13.51	0.0025
B^2	0.13	1	0.13	10.77	0.0055
Residual	0.17	14	0.01		
Lack of Fit	0.16	9	0.02	26.62	0.0011
Pure Error	0.00	5	0.00		
Cor Total	1.53	19			

Std. Dev.	0.11	R-Squared	0.89
Mean	1.45	Adj R-Squared	0.85
C.V. %	7.53	Pred R-Squared	0.72
PRESS	0.43	Adeq Precision	19.53

Final Equation in Terms of Coded Factors:

$$\log10(TPC) = 1.52 + 0.25*A - 0.22*B + 0.08*A*B - 0.27*A^2 + 0.10*B^2$$

*Table A.6-21: RSM summary table of two factor severity RSM model for **MISCANTHUS** extract **Total Phenolic Contents***

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	0.07	5	0.01	29.91	< 0.0001
A-Severity factor	0.02	1	0.02	43.01	< 0.0001
B-Loading	0.03	1	0.03	63.67	< 0.0001
AB	0.00	1	0.00	2.08	0.1715
A^2	0.01	1	0.01	15.42	0.0015
B^2	0.01	1	0.01	18.30	0.0008
Residual	0.01	14	0.00		
Lack of Fit	0.01	9	0.00	30.61	0.0008
Pure Error	0.00	5	0.00		
Cor Total	0.08	19			

Std. Dev.	0.02	R-Squared	0.91
Mean	0.22	Adj R-Squared	0.88
C.V. %	9.71	Pred R-Squared	0.79
PRESS	0.02	Adeq Precision	21.98

Final Equation in Terms of Coded Factors:

$$1.0/\text{sqrt(TPC)} = 0.21 - 0.06*A + 0.05*B - 0.02*A*B + 0.06*A^2 - 0.03*B^2$$

*Table A.6-22: RSM summary table of two factor severity RSM model for **Arabinoxylan** contents in **BALADO** extracts*

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	16.18	5	3.24	19.58	< 0.0001
A-Severity factor	0.80	1	0.80	4.85	0.0390
B-Loading	0.06	1	0.06	0.34	0.5649
AB	0.06	1	0.06	0.38	0.5442
A^2	13.15	1	13.15	79.55	< 0.0001
B^2	0.18	1	0.18	1.07	0.3124
Residual	3.47	21	0.17		
Lack of Fit	3.44	10	0.34	108.14	< 0.0001
Pure Error	0.03	11	0.00		
Cor Total	19.65	26			

Std. Dev.	0.41	R-Squared	0.82
Mean	1.60	Adj R-Squared	0.78
C.V. %	25.39	Pred R-Squared	0.61
PRESS	7.66	Adeq Precision	13.53

Final Equation in Terms of Coded Factors:

$$\text{Log10(AX)} = 2.39 + 0.32*A - 0.06*B - 0.11*A*B - 2.33*A^2 + 0.11*B^2$$

*Table A.6-23: RSM summary table of two factor severity RSM model for **Arabinoxylan** contents in **CONWAY** extracts*

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	10.18	5	2.04	13.43	< 0.0001
A-Severity factor	0.10	1	0.10	0.65	0.4342
B-Loading	0.16	1	0.16	1.04	0.3247
AB	0.01	1	0.01	0.06	0.8088
A^2	9.64	1	9.64	63.57	< 0.0001
B^2	0.01	1	0.01	0.08	0.7850
Residual	2.12	14	0.15		
Lack of Fit	2.12	9	0.24	141.96	< 0.0001
Pure Error	0.01	5	0.00		
Cor Total	12.31	19			

Std. Dev.	0.39	R-Squared	0.83
Mean	1.65	Adj R-Squared	0.77
C.V. %	23.58	Pred R-Squared	0.51
PRESS	6.00	Adeq Precision	10.63

Final Equation in Terms of Coded Factors:

$$Log10(AX) = 2.37 + 0.13*A - 0.12*B - 0.05*A*B - 2.12*A^2 + 0.03*B^2$$

*Table A.6-24: RSM summary table of two factor severity RSM model for **Arabinoxylan** contents in **MISCANTHUS** extracts*

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	7.28	5	1.46	5.67	0.0046
A-Severity factor	0.19	1	0.19	0.75	0.3996
B-Loading	0.14	1	0.14	0.53	0.4793
AB	0.60	1	0.60	2.35	0.1479
A^2	6.61	1	6.61	25.75	0.0002
B^2	0.01	1	0.01	0.05	0.8236
Residual	3.59	14	0.26		
Lack of Fit	3.59	9	0.40	225.27	< 0.0001
Pure Error	8.8E-003	5	1.8E-003		
Cor Total	10.87	19			

Std. Dev.	0.51	R-Squared	0.67
Mean	1.40	Adj R-Squared	0.55
C.V. %	36.25	Pred R-Squared	0.15
PRESS	9.25	Adeq Precision	6.76

Final Equation in Terms of Coded Factors:

$$Log10(AX) = 1.92 - 0.18*A - 0.17*B - 0.61*A*B - 1.75*A^2 + 0.081*B^2$$

A.7. RSM summary tables for BAL extract and AX standard hydrolysis

*Table A.7-1: RSM summary table of two factor model for **BALADO** extract **concentration***

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	39.10	5	7.82	21.61	0.0004
A-Temperature	23.40	1	23.40	64.66	< 0.0001
B-Residence Time	12.05	1	12.05	33.30	0.0007
AB	4.41	1	4.41	12.19	0.0101
A^2	0.28	1	0.28	0.76	0.4121
B^2	0.80	1	0.80	2.20	0.1815
Residual	2.53	7	0.36		
Lack of Fit	1.61	3	0.54	2.34	0.2149
Pure Error	0.92	4	0.23		
Cor Total	41.63	12			

Std. Dev.	0.60	R-Squared	0.94
Mean	6.56	Adj R-Squared	0.90
C.V. %	9.17	Pred R-Squared	0.65
PRESS	14.56	Adeq Precision	15.11

Final Equation in Terms of Coded Factors:

Extract conc. = 6.22 – 2.50*A – 1.62*B – 1.76*A*B – 0.39*A^2 + 0.59*B^2

*Table A.7-2: RSM summary table of two factor model for **AX standard** extract **concentration***

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	52.83	5	10.57	59.84	< 0.0001
A-Temperature	31.24	1	31.24	176.87	< 0.0001
B-Residence Time	12.56	1	12.56	71.14	< 0.0001
AB	6.63	1	6.63	37.55	0.0005
A^2	8.64	1	8.64	48.91	0.0002
B^2	0.51	1	0.51	2.90	0.1323
Residual	1.24	7	0.18		
Lack of Fit	0.80	3	0.27	2.48	0.2003
Pure Error	0.43	4	0.11		
Cor Total	54.07	12			

Std. Dev.	0.42	R-Squared	0.98
Mean	7.46	Adj R-Squared	0.96
C.V. %	5.63	Pred R-Squared	0.86
PRESS	7.42	Adeq Precision	23.50

Final Equation in Terms of Coded Factors:

Extract conc. = 8.10 – 2.89*A – 1.65*B – 2.16*A*B – 2.20*A^2 – 0.47*B^2

*Table A.7-3: RSM summary table of two factor model for **BALADO** extract **pH***

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	0.425	5	0.08	26.17	0.0002
A-Temperature	0.131	1	0.13	40.33	0.0004
B-Residence Time	0.140	1	0.14	43.15	0.0003
AB	0.063	1	0.06	19.24	0.0032
A^2	0.003	1	0.00	0.80	0.4007
B^2	0.071	1	0.07	21.87	0.0023
Residual	0.023	7	0.00		
Lack of Fit	0.023	3	0.01		
Pure Error	0.000	4	0.00		
Cor Total	0.448	12			

Std. Dev.	0.06	R-Squared	0.95
Mean	3.07	Adj R-Squared	0.91
C.V. %	1.86	Pred R-Squared	0.58
PRESS	0.19	Adeq Precision	16.49

Final Equation in Terms of Coded Factors:

pH = 2.96 – 2.96 – 0.19*A – 0.17*B – 0.21*A*B + 0.04*A^2 + 0.18*B^2

*Table A.7-4: RSM summary table of two factor model for **AX standard** extract **pH***

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	33.62	5	6.72	22.34	0.0004
A-Temperature	9.59	1	9.59	31.86	0.0008
B-Residence Time	12.09	1	12.09	40.18	0.0004
AB	0.90	1	0.90	3.00	0.1269
A^2	1.75	1	1.75	5.83	0.0465
B^2	5.51	1	5.51	18.31	0.0037
Residual	2.11	7	0.30		
Lack of Fit	1.81	3	0.60	8.29	0.0343
Pure Error	0.29	4	0.07		
Cor Total	35.73	12			

Std. Dev.	0.55	R-Squared	0.94
Mean	4.83	Adj R-Squared	0.90
C.V. %	11.36	Pred R-Squared	0.57
PRESS	15.19	Adeq Precision	15.18

Final Equation in Terms of Coded Factors:

pH = 3.69 – 1.60*A – 1.62*B – 0.80*A*B + 0.99*A^2 +1.55*B^2

*Table A.7-5: RSM summary table of two factor model for **BALADO** extract **Arabinose** concentration*

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	0.067	5	0.013	22.23	0.0004
A-Temperature	0.036	1	0.036	59.92	0.0001
B-Residence Time	0.018	1	0.018	29.61	0.0010
AB	0.013	1	0.013	22.21	0.0022
A^2	0.003	1	0.003	5.63	0.0494
B^2	0.002	1	0.002	3.31	0.1116
Residual	0.004	7	0.001		
Lack of Fit	0.003	3	0.001	2.99	0.1590
Pure Error	0.001	4	0.000		
Cor Total	0.071	12			

Std. Dev.	0.02	R-Squared	0.94
Mean	0.16	Adj R-Squared	0.90
C.V. %	15.61	Pred R-Squared	0.63
PRESS	0.03	Adeq Precision	15.52

Final Equation in Terms of Coded Factors:

Arabinose = 0.15 – 0.10*A – 0.06*B – 0.10*A*B – 0.04*A^2 + 0.03*B^2

*Table A.7-6: RSM summary table of two factor model for **AX standard** extract **Arabinose** concentration*

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	4.08	5	0.82	32.56	0.0001
A-Temperature	0.38	1	0.38	15.12	0.0060
B-Residence Time	0.06	1	0.06	2.30	0.1729
AB	0.35	1	0.35	13.86	0.0074
A^2	2.39	1	2.39	95.32	< 0.0001
B^2	0.95	1	0.95	37.96	0.0005
Residual	0.18	7	0.03		
Lack of Fit	0.17	3	0.06	274.61	< 0.0001
Pure Error	0.00	4	0.00		
Cor Total	4.25	12			

Std. Dev.	0.16	R-Squared	0.96
Mean	-0.48	Adj R-Squared	0.93
C.V. %	32.67	Pred R-Squared	0.75
PRESS	1.04	Adeq Precision	13.35

Final Equation in Terms of Coded Factors:

log10(Arabinose) = 0.14 – 0.32*A + 0.11*B – 0.49*A*B – 1.16*A^2 – 0.64*B^2

*Table A.7-7: RSM summary table of two factor model for **BALADO** extract **Xylose** concentration*

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	3.48	5	0.70	9.20	0.0055
A-Temperature	2.17	1	2.17	28.74	0.0011
B-Residence Time	0.15	1	0.15	1.93	0.2073
AB	0.70	1	0.70	9.23	0.0189
A^2	0.93	1	0.93	12.35	0.0098
B^2	0.10	1	0.10	1.38	0.2781
Residual	0.53	7	0.08		
Lack of Fit	0.06	3	0.02	0.17	0.9130
Pure Error	0.47	4	0.12		
Cor Total	4.01	12			

Std. Dev.	0.27	R-Squared	0.87
Mean	1.20	Adj R-Squared	0.77
C.V. %	22.96	Pred R-Squared	0.72
PRESS	1.11	Adeq Precision	9.40

Final Equation in Terms of Coded Factors:

Xylose = 1.48 – 0.76*A – 0.18*B – 0.70*A*B – 0.72*A^2 – 0.21*B^2

*Table A.7-8: RSM summary table of two factor model for **BALADO** extract **XOS** concentration*

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	11.03	5	2.21	14.39	0.0014
A-Temperature	3.21	1	3.21	20.95	0.0026
B-Residence Time	3.09	1	3.09	20.13	0.0028
AB	0.69	1	0.69	4.53	0.0709
A^2	1.15	1	1.15	7.52	0.0289
B^2	2.03	1	2.03	13.20	0.0084
Residual	1.07	7	0.15		
Lack of Fit	0.73	3	0.24	2.89	0.1661
Pure Error	0.34	4	0.08		
Cor Total	12.11	12			

Std. Dev.	0.39	R-Squared	0.91
Mean	0.96	Adj R-Squared	0.85
C.V. %	40.71	Pred R-Squared	0.49
PRESS	6.23	Adeq Precision	11.44

Final Equation in Terms of Coded Factors:

XOS = 0.23 – 0.93*A – 0.82*B – 0.70*A*B + 0.80*A^2 + 0.94*B^2

*Table A.7-9: RSM summary table of two factor model for **AX standard** extract **XOS** concentration*

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	9.45	5	1.89	26.38	0.0002
A-Temperature	0.14	1	0.14	1.98	0.2021
B-Residence Time	0.03	1	0.03	0.47	0.5172
AB	0.27	1	0.27	3.73	0.0946
A^2	4.32	1	4.32	60.29	0.0001
B^2	4.73	1	4.73	66.04	< 0.0001
Residual	0.50	7	0.07		
Lack of Fit	0.49	3	0.16	49.06	0.0013
Pure Error	0.01	4	0.00		
Cor Total	9.95	12			

Std. Dev.	0.27	R-Squared	0.95
Mean	-0.83	Adj R-Squared	0.91
C.V. %	32.33	Pred R-Squared	0.70
PRESS	2.95	Adeq Precision	14.12

Final Equation in Terms of Coded Factors:

log10(XOS) = 0.20 – 0.19*A – 0.09*B + 0.43*A*B – 1.56*A^2 – 1.43*B^2

*Table A.7-10: RSM summary table of two factor model for **AX standard** extract **Furfural** concentration*

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	8.49	3	2.83	16.95	0.0005
A-Temperature	4.76	1	4.76	28.52	0.0005
B-Residence Time	3.44	1	3.44	20.58	0.0014
AB	1.24	1	1.24	7.42	0.0235
Residual	1.50	9	0.17		
Lack of Fit	1.44	5	0.29	17.62	0.0079
Pure Error	0.07	4	0.02		
Cor Total	10.00	12			

Std. Dev.	0.41	R-Squared	0.85
Mean	0.65	Adj R-Squared	0.80
C.V. %	62.40	Pred R-Squared	0.65
PRESS	3.47	Adeq Precision	12.91

Final Equation in Terms of Coded Factors:

Furfural = 0.77 + 1.13*A + 0.84*B + 0.93*A*B

*Table A.7-11: RSM summary table of two factor model for **AX standard** extract **TPC** concentration*

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	0.61	3	0.20	21.17	0.0002
A-Temperature	0.37	1	0.37	38.80	0.0002
B-Residence Time	0.22	1	0.22	22.56	0.0010
AB	0.09	1	0.09	9.62	0.0127
Residual	0.09	9	0.01		
Lack of Fit	0.07	5	0.01	4.66	0.0805
Pure Error	0.01	4	0.00		
Cor Total	0.70	12			

Std. Dev.	0.10	R-Squared	0.88
Mean	0.31	Adj R-Squared	0.83
C.V. %	31.55	Pred R-Squared	0.62
PRESS	0.27	Adeq Precision	14.65

Final Equation in Terms of Coded Factors:

TPC = 0.34 + 0.32*A + 0.21*B +0.26*A*B

APPENDIX B: RESULTS TABLES

B.1. MIS and BAL

Table B.1-1: Various results of extractions and extract precipitation

	Temp	Residence time	Solubility		Extract mass conc.		pH		Precipitate fraction		Supernatant fraction	
	°C	min	%dw	sem	g/L	sem		sem	%	sem	%	sem
V.MIS	120	30	11.7	±0.5	2.6	±0.2	5.5	±0.1	*nd*	*nd*	*nd*	*nd*
V.MIS	140	30	13.0	±0.3	2.8	±0.1	5.0	±0.0	*nd*	*nd*	*nd*	*nd*
V.MIS	160	30	20.2	±1.0	5.4	±0.1	4.5	±0.1	*nd*	*nd*	*nd*	*nd*
P.MIS	**140**	**30**	**7.0**	**±0.6**	**2.8**	**±0.5**	**4.8**	**±0.1**	**31.0**	**±6.9**	**50.0**	**±8.1**
P.MIS	**160**	**30**	**13.6**	**±0.6**	**7.2**	**±0.3**	**4.3**	**±0.0**	**18.3**	**±7.1**	**80.0**	**±8.8**
P.MIS	**180**	**30**	**27.8**	**±0.8**	**13.8**	**±0.8**	**3.8**	**±0.0**	**6.3**	**±2.7**	**96.3**	**±5.6**
P.MIS	*160*	*0*	*4.8*	*±0.9*	*2.2*	*±0.2*	*5.0*	*±0.0*	*34.3*	*±4.4*	*64.7*	*±5.8*
P.MIS	*160*	*30*	*13.6*	*±0.6*	*7.2*	*±0.7*	*4.3*	*±0.0*	*18.3*	*±1.8*	*80.0*	*±8.8*
P.MIS	*160*	*60*	*19.8*	*±0.9*	*10.6*	*±0.7*	*4.0*	*±0.0*	*10.3*	*±0.9*	*89.3*	*±2.2*
V.BAL	120	30	13.7	±1.3	3.3	±0.3	6.2	±0.2	62.3	±1.5	48.3	±18.2
V.BAL	140	30	15.1	±0.5	4.0	±0.6	5.5	±0.0	50.0	±1.0	62.7	±5.0
V.BAL	160	30	22.1	±1.1	7.9	±0.5	4.6	±0.1	33.7	±0.0	82.2	±7.4
P.BAL	**140**	**30**	**7.4**	**±0.2**	**3.0**	**±0.1**	**4.7**	**±0.0**	**18.3**	**±1.2**	**85.3**	**±17.9**
P.BAL	**160**	**30**	**24.7**	**±0.9**	**14.4**	**±0.7**	**4.1**	**±0.0**	**21.0**	**±1.8**	**66.6**	**±3.2**
P.BAL	**180**	**30**	**40.6**	**±0.5**	**21.6**	**±0.3**	**3.6**	**±0.1**	**4.0**	**±0.0**	**93.3**	**±2.9**
P.BAL	*160*	*0*	*4.8*	*±0.9*	*1.8*	*±0.5*	*5.2*	*±0.2*	*24.3*	*±6.9*	*19.0*	*±6.2*
P.BAL	*160*	*30*	*24.7*	*±0.9*	*14.4*	*±0.6*	*4.1*	*±0.0*	*21.0*	*±1.0*	*66.6*	*±3.2*
P.BAL	*160*	*60*	*34.3*	*±0.7*	*20.1*	*±0.8*	*4.0*	*±0.1*	*9.7*	*±1.2*	*73.7*	*±2.7*

*Table B.1-2: Extract **monosaccharide contents***

	Temp	time	Arabinose		Galactose		Glucose		Xylose		Fructose	
	°C	min	mg/g*	sem	mg/g*	sem	mg/g*	sem	mg/g*	sem	mg/g*	sem
V.MIS	120	30	0.3	±0.1	*nd*	*nd*	2.5	±0.3	0.4	±0.1	*nd*	*nd*
V.MIS	140	30	2.1	±0.2	*nd*	*nd*	2.6	±0.1	0.5	±0.1	0.3	±0.3
V.MIS	160	30	7.3	±0.3	0.2	±0.1	2.4	±0.1	1.5	±0.1	0.7	±0.3
P.MIS	**140**	**30**	**2.7**	**±0.1**	***nd***	***nd***	**0.7**	**±0.0**	**0.3**	**±0.0**	***nd***	***nd***
P.MIS	**160**	**30**	**6.6**	**±0.3**	***nd***	***nd***	**0.8**	**±0.3**	**1.8**	**±0.1**	***nd***	***nd***
P.MIS	**180**	**30**	**7.9**	**±0.2**	***nd***	***nd***	**1.9**	**±0.2**	**26.2**	**±2.5**	***nd***	***nd***
P.MIS	*160*	*0*	*1.5*	*±0.1*	*nd*	*nd*	*nd*	*±0.0*	*0.2*	*±0.0*	*nd*	*nd*
P.MIS	*160*	*30*	*6.6*	*±0.3*	*nd*	*nd*	*0.8*	*±0.3*	*1.8*	*±0.1*	*nd*	*nd*
P.MIS	*160*	*60*	*8.1*	*±0.1*	*nd*	*nd*	*0.7*	*±0.0*	*4.8*	*±0.1*	*nd*	*nd*
V.BAL	120	30	0.2	±0.1	*nd*	*nd*	1.4	±0.4	0.3	±0.1	0.6	±0.3
V.BAL	140	30	0.1	±0.1	*nd*	*nd*	0.6	±0.2	0.7	±0.2	0.3	±0.3
V.BAL	160	30	3.3	±0.6	0.3	±0.1	0.6	±0.1	1.0	±0.3	1.2	±0.2
P.BAL	**140**	**30**	**3.0**	**±0.2**	**0.2**	**±0.0**	**0.3**	**±0.0**	**0.7**	**±0.1**	***nd***	***nd***
P.BAL	**160**	**30**	**7.1**	**±0.3**	**0.5**	**±0.1**	**0.5**	**±0.1**	**4.5**	**±0.6**	***nd***	***nd***
P.BAL	**180**	**30**	**9.1**	**±0.6**	**nd**	**nd**	**3.0**	**±0.3**	**53.4**	**±10.3**	***nd***	***nd***
P.BAL	*160*	*0*	*1.6*	*±0.5*	*0.1*	*±0.0*	*0.2*	*±0.1*	*0.3*	*±0.2*	*nd*	*nd*
P.BAL	*160*	*30*	*7.1*	*±0.3*	*0.5*	*±0.1*	*0.5*	*±0.1*	*4.5*	*±0.6*	*nd*	*nd*
P.BAL	*160*	*60*	*10.1*	*±0.6*	*nd*	*nd*	*1.7*	*±0.2*	*14.4*	*±1.3*	*nd*	*nd*

**of dry feed*

*Table B.1-3: Extract **oligosaccharide contents***

	Temp	time	Xylobiose		Xylotriose		Xylotetraose		Xylopentaose	
	°C	min	mg/g*	sem	mg/g*	sem	mg/g*	sem	mg/g*	sem
V.MIS	120	30	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
V.MIS	140	30	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
V.MIS	160	30	0.2	±0.1	0.3	±0.1	0.5	±0.3	0.3	±0.2
P.MIS	**140**	**30**	***nd***	***nd***	***nd***	***nd***	***nd***	***nd***	***nd***	***nd***
P.MIS	**160**	**30**	**0.8**	**±0.1**	**1.2**	**±0.2**	**1.6**	**±0.2**	**1.8**	**±0.2**
P.MIS	**180**	**30**	**15.8**	**±2.6**	**15.9**	**±1.9**	**20.1**	**±2.2**	**20.8**	**±1.3**
P.MIS	*160*	*0*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
P.MIS	*160*	*30*	*0.8*	*±0.1*	*1.2*	*±0.2*	*1.6*	*±0.2*	*1.8*	*±0.2*
P.MIS	*160*	*60*	*3.1*	*±0.2*	*3.6*	*±0.3*	*5.2*	*±0.2*	*6.1*	*±0.1*
V.BAL	120	30	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
V.BAL	140	30	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
V.BAL	160	30	0.6	±0.2	1.5	±0.5	1.3	±0.4	1.9	±0.4
P.BAL	**140**	**30**	**0.2**	**±0.1**	**0.2**	**±0.1**	**0.3**	**±0.1**	**0.2**	**±0.1**
P.BAL	**160**	**30**	**2.4**	**±0.5**	**3.5**	**±0.8**	**4.9**	**±0.8**	**7.4**	**±1.0**
P.BAL	**180**	**30**	**32.4**	**±3.9**	**33.2**	**±2.0**	**34.5**	**±1.6**	**33.1**	**±2.0**
P.BAL	*160*	*0*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
P.BAL	*160*	*30*	*2.4*	*±0.5*	*3.5*	*±0.8*	*4.9*	*±0.8*	*7.4*	*±1.0*
P.BAL	*160*	*60*	*8.9*	*±0.8*	*11.4*	*±1.0*	*15.7*	*±1.3*	*19.4*	*±1.5*

**of dry feed*

*Table B.1-4: Extract **polysaccharide contents***

	Temp	time	Arabinan		Galactan		Glucan		Xylan		Fructan	
	°C	min	mg/g*	sem	mg/g*	sem	mg/g*	sem	mg/g*	sem	mg/g*	sem
V.MIS	120	30	1.4	±0.1	1.3	±0.0	3.2	±0.1	3.3	±0.2	0.5	±0.1
V.MIS	140	30	3.0	±0.1	1.7	±0.1	3.3	±0.1	4.1	±0.1	0.6	±0.2
V.MIS	160	30	7.4	±0.1	3.0	±0.1	4.0	±0.1	25.7	±0.4	1.3	±0.1
P.MIS	**140**	**30**	**4.4**	**±0.4**	**0.9**	**±0.1**	**2.7**	**±0.3**	**8.4**	**±0.8**	***nd***	***nd***
P.MIS	**160**	**30**	**9.8**	**±0.5**	**0.5**	**±0.5**	**5.8**	**±0.6**	**46.4**	**±4.8**	***nd***	***nd***
P.MIS	**180**	**30**	**9.9**	**±0.2**	***nd***	***nd***	**8.7**	**±0.4**	**133.0**	**±4.8**	***nd***	***nd***
P.MIS	*160*	*0*	*2.3*	*±0.1*	*0.9*	*±0.0*	*2.3*	*±0.1*	*4.4*	*±0.2*	nd	nd
P.MIS	*160*	*30*	*9.8*	*±0.5*	*0.5*	*±0.5*	*5.8*	*±0.6*	*46.4*	*±4.8*	nd	nd
P.MIS	*160*	*60*	*10.3*	*±0.1*	nd	nd	*8.1*	*±0.3*	*73.4*	*±0.7*	nd	nd
V.BAL	120	30	1.2	±0.1	1.1	±0.1	13.8	±7.4	2.1	±0.1	1.6	±0.6
V.BAL	140	30	3.2	±0.2	1.6	±0.2	24.1	±2.3	3.7	±0.3	2.4	±0.4
V.BAL	160	30	12.4	±1.4	4.2	±0.5	12.8	±1.5	50.1	±1.2	0.9	±0.4
P.BAL	**140**	**30**	**5.4**	**±0.3**	**1.7**	**±0.1**	**2.1**	**±0.2**	**17.9**	**±1.7**	***nd***	***nd***
P.BAL	**160**	**30**	**13.3**	**±0.5**	**5.6**	**±0.4**	**3.4**	**±0.2**	**110.7**	**±8.9**	***nd***	***nd***
P.BAL	**180**	**30**	**14.6**	**±0.2**	**7.8**	**±0.4**	**4.0**	**±0.4**	**224.1**	**±1.4**	***nd***	***nd***
P.BAL	*160*	*0*	*2.6*	*±0.9*	*1.0*	*±0.3*	*2.3*	*±0.2*	*6.9*	*±3.4*	nd	nd
P.BAL	*160*	*30*	*13.3*	*±0.5*	*5.6*	*±0.4*	*3.4*	*±0.2*	*110.7*	*±8.9*	nd	nd
P.BAL	*160*	*60*	*17.6*	*±3.0*	*9.4*	*±1.7*	*4.5*	*±0.6*	*163.0*	*±19.8*	nd	nd

**of dry feed*

Table B.1-5: Extract quality and efficiency indicators

	Temp	time	Ara/Xyl		Glu/Xyl		AX content		Xylan yield		XOS prod. eff.	
	°C	min		sem		sem	%	sem	%	sem	%	sem
V.MIS	120	30	0.44	±0.02	1.00	±0.04	8.1	±1.0	1.3	±0.7	0.0	±0.0
V.MIS	140	30	0.73	±0.01	0.80	±0.02	11.7	±0.1	2.3	±0.3	0.0	±0.0
V.MIS	160	30	0.29	±0.01	0.16	±0.02	28.7	±0.2	14.7	±0.3	5.0	±2.2
P.MIS	**140**	**30**	**0.53**	**±0.01**	**0.31**	**±0.01**	**25.5**	**±3.4**	**5.0**	**±0.6**	**0.0**	**±0.0**
P.MIS	**160**	**30**	**0.22**	**±0.02**	**0.13**	**±0.01**	**42.8**	**±4.5**	**26.8**	**±2.6**	**12.0**	**±0.4**
P.MIS	**180**	**30**	**0.07**	**±0.02**	**0.07**	**±0.02**	**55.7**	**±1.0**	**76.7**	**±2.9**	**54.0**	**±4.0**
P.MIS	160	0	0.53	±0.04	0.53	±0.03	16.3	±1.0	2.7	±0.3	0.0	±0.0
P.MIS	160	30	0.22	±0.02	0.13	±0.01	42.8	±4.5	26.8	±2.6	12.0	±0.4
P.MIS	160	60	0.14	±0.02	0.11	±0.01	41.7	±1.0	42.7	±0.3	24.3	±0.3
V.BAL	*120*	*30*	*0.57*	*±0.02*	*6.46*	*±3.16*	*5.2*	*±0.4*	*0.4*	*±0.2*	*0.0*	*±0.0*
V.BAL	*140*	*30*	*0.85*	*±0.01*	*6.48*	*±0.19*	*7.4*	*±0.6*	*0.8*	*±0.4*	*0.0*	*±0.0*
V.BAL	*160*	*30*	*0.22*	*±0.01*	*0.23*	*±0.02*	*38.9*	*±2.6*	*13.7*	*±3.6*	*5.4*	*±1.7*
P.BAL	**140**	**30**	**0.31**	**±0.02**	**0.12**	**±0.02**	**43.5**	**±2.6**	**6.7**	**±0.7**	**4.7**	**±2.3**
P.BAL	**160**	**30**	**0.12**	**±0.01**	**0.03**	**±0.01**	**46.8**	**±2.6**	**42.3**	**±3.4**	**15.9**	**±1.7**
P.BAL	**180**	**30**	**0.06**	**±0.02**	**0.02**	**±0.01**	**60.7**	**±1.4**	**85.7**	**±0.7**	**59.7**	**±3.0**
P.BAL	*160*	*0*	*0.44*	*±0.06*	*0.47*	*±0.15*	*26.3*	*±5.0*	*2.7*	*±1.2*	*4.3*	*±4.3*
P.BAL	*160*	*30*	*0.12*	*±0.01*	*0.03*	*±0.01*	*46.8*	*±2.6*	*42.3*	*±3.4*	*15.9*	*±1.7*
P.BAL	*160*	*60*	*0.09*	*±0.02*	*0.03*	*±0.01*	*44.1*	*±1.3*	*62.5*	*±7.5*	*33.5*	*±0.5*

*Table B.1-6: Extract **Total phenolic contents***

	Temp °C	time min	Total phenolic content [mg GA eq.]/g*	sem
V.MIS	120	30	14.4	±0.4
V.MIS	140	30	16.2	±0.3
V.MIS	160	30	20.5	±0.9
P.MIS	**140**	**30**	**12.6**	**±0.9**
P.MIS	**160**	**30**	**18.1**	**±0.7**
P.MIS	**180**	**30**	**30.1**	**±1.3**
P.MIS	*160*	*0*	*12.4*	*±0.3*
P.MIS	*160*	*30*	*18.1*	*±0.7*
P.MIS	*160*	*60*	*23.4*	*±0.6*
V.BAL	120	30	9.1	±1.1
V.BAL	140	30	10.7	±1.2
V.BAL	160	30	17.6	±1.5
P.BAL	**140**	**30**	**11.3**	**±0.5**
P.BAL	**160**	**30**	**24.1**	**±0.6**
P.BAL	**180**	**30**	**31.6**	**±1.3**
P.BAL	*160*	*0*	*11.2*	*±0.8*
P.BAL	*160*	*30*	*24.1*	*±0.6*
P.BAL	*160*	*60*	*34.1*	*±1.8*

**of dry feed*

B.2. Oat husk varieties

Table B.2-1: Various results of extractions and extract precipitation

	Solubility		Extract mass conc.		pH		Precipitate fraction		Supernatant fraction	
	%dw	sem	g/L	sem		sem	%	sem	%	sem
V.SO-I	33.3	±1.3	15.5	±0.3	4.6	±0.0	44.7	±2.0	50.0	±4.6
V.14355	47.4	±3.0	21.6	±1.1	4.8	±0.0	58.7	±0.7	44.0	±4.9
V.CON	18.1	±0.9	7.5	±0.5	4.4	±0.0	11.0	±0.0	82.7	±13.0
V.MAS	17.7	±1.2	7.2	±0.7	4.6	±0.1	24.0	±2.5	70.3	±14.7
V.BAL	22.1	±1.1	7.9	±0.8	4.6	±0.1	33.7	±2.7	82.2	±7.4
P.SO-I	**33.1**	**±0.6**	**17.7**	**±0.7**	**4.1**	**±0.0**	**29.7**	**±0.0**	**65.7**	**±1.8**
P.14355	**29.8**	**±1.0**	**14.9**	**±0.3**	**4.6**	**±0.0**	**37.3**	**±0.7**	**52.3**	**±0.9**
P.CON	**27.5**	**±1.1**	**14.5**	**±0.8**	**3.8**	**±0.0**	**5.7**	**±0.9**	**89.7**	**±6.4**
P.MAS	**27.6**	**±0.8**	**15.0**	**±0.4**	**3.9**	**±0.0**	**9.7**	**±0.0**	**81.7**	**±0.5**
P.BAL	**24.7**	**±0.9**	**14.4**	**±0.6**	**4.1**	**±0.0**	**21.0**	**±1.0**	**66.6**	**±3.2**

*Table B.2-2: Extract **monosaccharide contents***

	Arabinose		Galactose		Glucose		Xylose		Fructose	
	mg/g*	sem	mg/g*	sem	mg/g*	sem	mg/g*	sem	mg/g*	sem
V.SO-I	6.1	±0.1	0.5	±0.0	1.2	±0.1	1.3	±0.1	2.4	±0.1
V.14355	5.1	±0.1	0.4	±0.0	3.7	±0.2	0.8	±0.0	4.6	±0.1
V.CON	6.7	±0.0	0.7	±0.0	0.6	±0.0	1.9	±0.1	1.6	±0.1
V.MAS	5.4	±0.2	0.6	±0.0	0.7	±0.0	1.1	±0.1	1.8	±0.1
V.BAL	3.3	±1.2	0.3	±0.1	0.6	±0.1	1.0	±0.3	1.2	±0.5
P.SO-I	**7.9**	**±0.3**	**0.7**	**±0.0**	**0.2**	**±0.0**	**2.8**	**±0.1**	**1.6**	**±0.1**
P.14355	**5.1**	**±0.1**	**0.4**	**±0.0**	**0.9**	**±0.1**	**1.1**	**±0.0**	**1.7**	**±0.1**
P.CON	**8.9**	**±0.2**	**1.4**	**±0.0**	**0.1**	**±0.0**	**4.7**	**±0.3**	**1.4**	**±0.1**
P.MAS	**8.3**	**±0.4**	**1.2**	**±0.1**	**0.1**	**±0.0**	**3.7**	**±0.3**	**1.3**	**±0.1**
P.BAL	**7.1**	**±0.3**	**0.5**	**±0.1**	**0.5**	**±0.1**	**4.5**	**±0.6**	***nd***	**±0.0**

**of dry feed*

*Table B.2-3: Extract **oligosaccharide contents***

	Xylobiose		Xylotriose		Xylotetraose		Xylopentaose	
	mg/g*	sem	mg/g*	sem	mg/g*	sem	mg/g*	sem
V.SO-I	0.6	±0.1	1.8	±0.1	1.3	±0.1	1.7	±0.1
V.14355	0.5	±0.0	1.3	±0.1	1.0	±0.0	1.5	±0.0
V.CON	0.8	±0.1	2.4	±0.1	1.6	±0.1	1.7	±0.2
V.MAS	0.4	±0.0	1.7	±0.1	1.0	±0.1	1.3	±0.1
V.BAL	0.6	±0.2	1.5	±0.5	1.3	±0.4	1.9	±0.4
P.SO-I	**1.6**	**±0.1**	**3.8**	**±0.3**	**3.3**	**±0.3**	**4.6**	**±0.2**
P.14355	**0.6**	**±0.0**	**1.2**	**±0.3**	**1.3**	**±0.2**	**1.9**	**±0.2**
P.CON	**2.8**	**±0.3**	**4.5**	**±1.2**	**5.5**	**±0.5**	**7.4**	**±0.5**
P.MAS	**2.4**	**±0.2**	**3.6**	**±0.9**	**5.0**	**±0.5**	**6.8**	**±0.5**
P.BAL	**2.4**	**±0.5**	**3.5**	**±0.8**	**4.9**	**±0.8**	**7.4**	**±1.0**

**of dry feed*

*Table B.2-4: Extract **polysaccharide contents***

	Arabinan		Galactan		Glucan		Xylan		Fructan	
	mg/g*	sem	mg/g*	sem	mg/g*	sem	mg/g*	sem	mg/g*	sem
V.SO-I	18.9	±0.3	3.3	±0.4	60.5	±10.6	112.2	±1.7	*nd*	*nd*
V.14355	17.3	±1.0	1.2	±1.2	199.9	±9.2	80.9	±6.0	*nd*	*nd*
V.CON	15.4	±0.9	4.1	±0.3	4.9	±1.2	61.1	±3.5	*nd*	*nd*
V.MAS	12.8	±0.4	3.5	±0.4	13.0	±1.6	51.7	±4.1	*nd*	*nd*
V.BAL	12.4	±1.4	4.2	±0.5	12.8	±1.5	50.1	±1.2	0.9	±0.4
P.SO-I	**17.8**	**±1.3**	**6.1**	**±0.8**	**4.2**	**±0.6**	**166.5**	**±8.0**	***nd***	***nd***
P.14355	**11.3**	**±0.5**	**3.3**	**±0.2**	**21.9**	**±1.0**	**75.9**	**±3.7**	***nd***	***nd***
P.CON	**18.2**	**±0.5**	**9.3**	**±0.3**	**0.8**	**±0.5**	**147.9**	**±7.6**	***nd***	***nd***
P.MAS	**17.5**	**±0.3**	**8.6**	**±0.5**	**1.3**	**±0.3**	**157.9**	**±4.2**	***nd***	***nd***
P.BAL	**13.3**	**±0.5**	**5.6**	**±0.4**	**3.4**	**±0.2**	**110.7**	**±8.9**	***nd***	***nd***

**of dry feed*

Table B.2-5: Extract quality and efficiency indicators

	Ara/Xyl		Glu/Xyl		AX content		Xylan yield		XOS prod. eff.	
		sem		sem	%	sem	%	sem	%	sem
V.SO-I	0.17	±0.02	0.54	±0.09	38.1	±0.1	59.3	±0.7	4.7	±0.3
V.14355	0.21	±0.01	2.51	±0.26	20.6	±2.5	34.0	±2.5	5.7	±0.3
V.CON	0.25	±0.01	0.08	±0.02	45.7	±3.3	23.3	±1.2	11.0	±1.0
V.MAS	0.25	±0.01	0.25	±0.01	40.0	±0.8	20.0	±1.5	8.3	±0.9
V.BAL	0.22	±0.01	0.23	±0.02	38.9	±2.6	13.7	±3.6	5.4	±1.7
P.SO-I	**0.11**	**±0.02**	**0.03**	**±0.01**	**58.6**	**±3.3**	**88.0**	**±4.0**	**8.0**	**±0.6**
P.14355	**0.15**	**±0.02**	**0.29**	**±0.03**	**40.7**	**±1.6**	**31.7**	**±1.8**	**6.7**	**±0.7**
P.CON	**0.12**	**±0.01**	**0.01**	**±0**	**59.4**	**±0.5**	**56.0**	**±2.9**	**13.7**	**±0.9**
P.MAS	**0.11**	**±0.02**	**0.01**	**±0**	**62.2**	**±0.7**	**60.3**	**±1.5**	**11.3**	**±0.3**
P.BAL	**0.12**	**±0.01**	**0.03**	**±0**	**46.8**	**±2.6**	**42.3**	**±3.4**	**15.9**	**±1.7**

*Table B.2-6: Extract **Total phenolic contents** and **hydroxycinnamate contents***

	Total phenolic content [mg GA eq.]/g*	sem	Caffeic acid mg/g*	sem	P-coumaric acid mg/g*	sem	Ferulic acid mg/g*	sem
V.SO-I	21.8	±0.8	1.38	±0.19	2.50	±0.28	1.19	±0.22
V.14355	19.1	±0.6	0.68	±0.07	1.25	±0.12	0.82	±0.11
V.CON	20.7	±0.9	1.12	±0.08	2.92	±0.03	0.27	±0.03
V.MAS	18.5	±0.6	0.89	±0.02	2.47	±0.03	0.28	±0.01
V.BAL	17.6	±1.5	0.85	±0.08	2.45	±0.17	0.37	±0.04
P.SO-I	**19.2**	**±0.6**	**0.95**	**±0.20**	**4.55**	**±0.25**	**1.17**	**±0.06**
P.14355	**11.4**	**±0.3**	**0.51**	**±0.02**	**1.74**	**±0.14**	**0.92**	**±0.14**
P.CON	**20.6**	**±0.4**	**0.90**	**±0.04**	**3.70**	**±0.30**	**0.32**	**±0.05**
P.MAS	**18.5**	**±0.4**	**0.40**	**±0.22**	**2.12**	**±1.06**	**0.21**	**±0.10**
P.BAL	**24.1**	**±0.6**	**0.58**	**±0.07**	**2.85**	**±0.05**	**0.37**	**±0.02**

**of dry feed*

*Table B.2-7: Extract **furan contents***

	5-HMF mg/g*	sem	Furfural mg/g*	sem
V.SO-I	0.06	±0.01	0.79	±0.05
V.14355	0.12	±0.01	0.56	±0.01
V.CON	0.05	±0.01	0.58	±0.06
V.MAS	*nd*	±0.01	0.35	±0.02
V.BAL	0.07	±0.02	0.93	±0.33
P.SO-I	**0.05**	**±0.01**	**1.80**	**±0.22**
P.14355	**0.08**	**±0.01**	**0.90**	**±0.03**
P.CON	***nd***	**±0.01**	**1.20**	**±0.13**
P.MAS	**0.13**	**±0.11**	**0.90**	**±0.46**
P.BAL	**0.06**	**±0.01**	**1.85**	**±0.35**

**of dry feed*

B.3. RSM BAL MIS CON

*B.3-1: **Solubility** and **pH** of BAL CON and MIS extracts*

log(R_0)	T	t	Loading	Solubility %			Ph		
	°C	min	%[w/v]	BAL	CON	MIS	BAL	CON	MIS
3.5	140	10	10	3.7	3.4	4.6	5.1	4.7	4.7
3.5	140	10	2	1.8	8.1	4.4	5.2	5.2	4.9
3.61	120	35	6	2.7	9.7	3.0	5.7	5.3	5.0
3.61	120	35	6	2.6	-	-	5.6	-	-
3.85	140	38	6	5.0	-	-	4.7	-	-
3.88	170	0	6	5.8	3.8	4.9	4.8	4.5	4.8
3.88	170	0	6	5.6	-	-	4.9	-	-
4.01	140	60	2	9.4	11.9	10.0	4.6	4.3	4.7
4.01	140	60	10	7.7	10.6	6.4	4.4	4.0	4.3
4.01	140	60	2	10.7	-	-	4.6	-	-
4.21	170	35	13	32.9	32.3	25.0	3.6	3.4	3.3
4.21	170	35	6	38.3	39.6	20.9	3.6	3.6	3.9
4.21	170	35	6	36.8	38.5	19.9	3.6	3.6	3.9
4.21	170	35	6	36.9	39.0	20.1	3.6	3.6	3.9
4.21	170	35	6	37.9	38.1	21.4	3.7	3.6	3.9
4.21	170	35	0.5	48.4	49.4	32.7	3.9	4.0	4.0
4.21	170	35	6	35.8	35.3	21.0	3.7	4.0	3.9
4.21	170	35	6	35.1	36.2	22.7	3.7	4.2	3.8
4.42	170	77	6	41.1	42.7	27.8	3.4	3.4	3.7
4.42	170	77	6	40.6	-	-	3.5	-	-
5.05	200	10	2	52.2	51.6	36.3	3.4	3.4	3.7
5.05	200	10	10	40.6	40.4	28.5	2.9	3.4	3.1
5.05	200	10	2	52.1	-	-	3.5	-	-
5.05	200	10	10	40.9	-	-	3.1	-	-
5.12	200	60	2	55.2	56.2	43.0	3.0	3.1	3.4
5.12	200	60	10	45.1	45.6	36.9	2.8	3.2	3.2
5.5	220	35	6	52.8	53.5	42.8	2.7	2.9	3.2

*B.3-2: Extract **Total Phenolic Contents** of BAL CON and MIS*

log(R_0)	T	t	Loading	Total phenolics mgGAeq/[g of dry biomass]		
	°C	min	%[w/v]	BAL	CON	MIS
3.5	140	10	10	7	7	9
3.5	140	10	2	29	28	23
3.61	120	35	6	9	10	9
3.61	120	35	6	7	-	-
3.85	140	38	6	12	-	-
3.88	170	0	6	13	12	11
3.88	170	0	6	11	-	-
4.01	140	60	2	40	35	28
4.01	140	60	10	11	12	11
4.01	140	60	2	32	-	-
4.21	170	35	13	21	23	18
4.21	170	35	6	31	33	22
4.21	170	35	6	28	31	22
4.21	170	35	6	29	30	20
4.21	170	35	6	30	33	22
4.21	170	35	0.5	131	129	120
4.21	170	35	6	28	28	21
4.21	170	35	6	29	30	20
4.42	170	77	6	29	30	23
4.42	170	77	6	28	-	-
5.05	200	10	2	65	68	55
5.05	200	10	10	26	28	19
5.05	200	10	2	56	-	-
5.05	200	10	10	22	-	-
5.12	200	60	2	64	52	53
5.12	200	60	10	26	27	23
5.5	220	35	6	30	44	27

*B.3-3: **Monosaccharide** contents of **BAL** extracts*

	log(R_0)	T °C	t min	Loading %[w/v]	Ara	Gal	Glu	Xyl	Fru
					mg/[g of dry biomass]				
BAL	3.5	140	10	10	1	0	0	1	0
BAL	3.5	140	10	2	1	0	0	0	0
BAL	3.61	120	35	6	1	0	0	0	0
BAL	3.61	120	35	6	1	0	0	0	0
BAL	3.85	140	38	6	4	0	0	1	0
BAL	3.88	170	0	6	3	0	0	1	0
BAL	3.88	170	0	6	3	0	0	1	0
BAL	4.01	140	60	2	9	1	0	3	0
BAL	4.01	140	60	10	5	0	0	1	0
BAL	4.01	140	60	2	6	0	0	0	0
BAL	4.21	170	35	13	17	5	1	35	2
BAL	4.21	170	35	6	18	4	0	35	2
BAL	4.21	170	35	6	14	3	1	24	2
BAL	4.21	170	35	6	13	3	1	22	2
BAL	4.21	170	35	6	13	3	1	20	2
BAL	4.21	170	35	0.5	15	3	1	23	2
BAL	4.21	170	35	6	12	3	1	17	2
BAL	4.21	170	35	6	13	3	2	18	1
BAL	4.42	170	77	6	22	11	2	151	0
BAL	4.42	170	77	6	16	8	2	119	0
BAL	5.05	200	10	2	19	10	2	179	5
BAL	5.05	200	10	10	9	8	7	145	0
BAL	5.05	200	10	2	14	7	3	133	4
BAL	5.05	200	10	10	9	8	6	153	0
BAL	5.12	200	60	2	6	5	17	57	2
BAL	5.12	200	60	10	1	2	10	5	0
BAL	5.5	220	35	6	0	0	9	0	0

*B.3-4: **Monosaccharide** contents of **CON** extracts*

	log(R_0)	T °C	t min	Loading %[w/v]	Ara	Gal	Glu	Xyl	Fru
					mg/[g of dry biomass]				
CON	3.5	140	10	10	0	0	0	0	0
CON	3.5	140	10	2	0	0	0	0	0
CON	3.61	120	35	6	0	0	0	0	0
CON	3.88	170	0	6	0	0	0	0	0
CON	4.01	140	60	2	2	0	0	1	0
CON	4.01	140	60	10	1	0	0	1	0
CON	4.21	170	35	13	14	4	0	38	0
CON	4.21	170	35	6	6	2	0	21	0
CON	4.21	170	35	6	4	2	0	21	1
CON	4.21	170	35	6	6	2	0	21	0
CON	4.21	170	35	6	9	3	0	28	0
CON	4.21	170	35	1	0	0	0	1	0
CON	4.21	170	35	6	10	2	0	19	0
CON	4.21	170	35	6	0	0	0	18	0
CON	4.42	170	77	6	14	6	0	111	3
CON	5.05	200	10	2	8	4	0	67	0
CON	5.05	200	10	10	4	2	0	32	0
CON	5.12	200	60	2	3	3	7	23	0
CON	5.12	200	60	10	1	4	21	9	0
CON	5.5	220	35	6	0	0	8	1	0

*B.3-5: **Monosaccharide** contents of **MIS** extracts*

	log(R_0)	T °C	t min	Loading %[w/v]	Ara	Gal	Glu	Xyl	Fru
					mg/[g of dry biomass]				
MIS	3.5	140	10	10	0	0	0	0	0
MIS	3.5	140	10	2	0	0	0	0	0
MIS	3.61	120	35	6	0	0	0	0	0
MIS	3.88	170	0	6	2	0	0	0	0
MIS	4.01	140	60	2	0	0	1	0	0
MIS	4.01	140	60	10	1	0	0	1	1
MIS	4.21	170	35	13	4	3	7	5	0
MIS	4.21	170	35	6	5	0	0	6	2
MIS	4.21	170	35	6	6	0	0	4	1
MIS	4.21	170	35	6	7	1	0	3	2
MIS	4.21	170	35	6	4	0	0	4	0
MIS	4.21	170	35	0.5	9	9	3	5	0
MIS	4.21	170	35	6	0	0	0	8	0
MIS	4.21	170	35	6	4	0	1	7	0
MIS	4.42	170	77	6	8	0	1	26	0
MIS	5.05	200	10	2	8	1	1	29	0
MIS	5.05	200	10	10	3	2	3	30	0
MIS	5.12	200	60	2	1	1	8	11	0
MIS	5.12	200	60	10	0	0	4	0	0
MIS	5.5	220	35	6	0	0	6	0	0

*B.3-6: **Xylooligosaccharide** contents of **BAL** extracts*

	log(R_0)	T °C	t min	Loading %[w/v]	X2	X3	X4	X5	X6	Total XOS (X2-X5)
					mg/[g of dry biomass]					
BAL	3.5	140	10	10	0	0	0	0	0	0
BAL	3.5	140	10	2	0	0	0	0	0	0
BAL	3.61	120	35	6	0	0	0	0	0	0
BAL	3.61	120	35	6	0	0	0	0	0	0
BAL	3.85	140	38	6	0	0	0	0	0	0
BAL	3.88	170	0	6	0	0	0	0	0	0
BAL	3.88	170	0	6	0	0	0	0	0	0
BAL	4.01	140	60	2	1	1	1	1	1	3
BAL	4.01	140	60	10	0	1	0	0	1	2
BAL	4.01	140	60	2	0	0	0	0	0	0
BAL	4.21	170	35	13	27	26	24	22	24	98
BAL	4.21	170	35	6	27	26	26	25	24	104
BAL	4.21	170	35	6	22	21	20	25	26	88
BAL	4.21	170	35	6	20	19	19	22	24	80
BAL	4.21	170	35	6	20	20	20	24	25	84
BAL	4.21	170	35	0.5	21	20	21	25	26	87
BAL	4.21	170	35	6	14	15	15	19	19	63
BAL	4.21	170	35	6	15	15	17	19	20	66
BAL	4.42	170	77	6	76	50	32	23	17	180
BAL	4.42	170	77	6	61	41	29	23	17	154
BAL	5.05	200	10	2	69	42	25	17	11	153
BAL	5.05	200	10	10	18	6	3	0	0	27
BAL	5.05	200	10	2	57	35	27	19	13	139
BAL	5.05	200	10	10	24	10	4	2	0	40
BAL	5.12	200	60	2	0	0	0	0	0	1
BAL	5.12	200	60	10	0	0	0	0	0	0
BAL	5.5	220	35	6	0	0	0	0	0	0

*B.3-7: **Xylooligosaccharide** contents of **CON** extracts*

	log(R_0)	T °C	t min	Loading %[w/v]	X2	X3	X4	X5	X6	Total XOS (X2-X5)
					mg/[g of dry biomass]					
CON	3.5	140	10	10	0	0	0	0	0	0
CON	3.5	140	10	2	0	0	0	0	0	0
CON	3.61	120	35	6	0	0	0	0	0	0
CON	3.88	170	0	6	0	0	0	0	0	0
CON	4.01	140	60	2	0	0	0	0	1	1
CON	4.01	140	60	10	0	0	0	0	0	0
CON	4.21	170	35	13	39	33	31	31	30	134
CON	4.21	170	35	6	23	21	23	24	25	92
CON	4.21	170	35	6	23	21	23	26	26	93
CON	4.21	170	35	6	24	22	23	26	26	95
CON	4.21	170	35	6	26	2	24	27	27	79
CON	4.21	170	35	1	11	9	10	11	14	40
CON	4.21	170	35	6	20	20	21	23	25	85
CON	4.21	170	35	6	23	21	22	25	27	91
CON	4.42	170	77	6	68	43	30	22	16	163
CON	5.05	200	10	2	42	29	21	15	11	107
CON	5.05	200	10	10	34	19	11	7	4	71
CON	5.12	200	60	2	0	0	0	0	0	0
CON	5.12	200	60	10	0	0	0	0	0	0
CON	5.5	220	35	6	0	0	0	0	0	0

*B.3-8: **Xylooligosaccharide** contents of **MIS** extracts*

	log(R_0)	T °C	t min	Loading %[w/v]	X2	X3	X4	X5	X6	Total XOS (X2-X5)
					mg/[g of dry biomass]					
MIS	3.5	140	10	10	0	0	0	0	0	0
MIS	3.5	140	10	2	0	0	0	0	0	0
MIS	3.61	120	35	6	0	0	0	0	0	1
MIS	3.88	170	0	6	0	0	0	0	0	0
MIS	4.01	140	60	2	0	0	0	0	0	0
MIS	4.01	140	60	10	0	0	0	0	0	0
MIS	4.21	170	35	13	15	7	7	2	2	32
MIS	4.21	170	35	6	7	7	8	9	10	32
MIS	4.21	170	35	6	6	7	7	9	10	29
MIS	4.21	170	35	6	5	5	7	7	9	23
MIS	4.21	170	35	6	6	6	7	8	9	27
MIS	4.21	170	35	0.5	6	6	8	8	8	27
MIS	4.21	170	35	6	6	6	7	7	7	26
MIS	4.21	170	35	6	8	8	10	10	10	36
MIS	4.42	170	77	6	27	22	19	18	16	86
MIS	5.05	200	10	2	26	19	19	16	14	80
MIS	5.05	200	10	10	16	8	7	0	0	31
MIS	5.12	200	60	2	1	0	0	0	0	1
MIS	5.12	200	60	10	0	0	0	0	0	0
MIS	5.5	220	35	6	0	0	0	0	0	0

B.3-9: ***Polysaccharide*** *contents of* ***BAL*** *extracts*

	log(R_0)	T °C	t min	Loading %[w/v]	ARA	GAL	GLU	XYL	FRU	ARA/XYL
					mg/[g of dry biomass]					
BAL	3.5	140	10	10	2	1	1	5	0	0.41
BAL	3.5	140	10	2	2	0	1	5	0	0.40
BAL	3.61	120	35	6	1	0	1	2	0	0.67
BAL	3.61	120	35	6	1	0	1	2	0	0.55
BAL	3.85	140	38	6	6	2	3	18	0	0.33
BAL	3.88	170	0	6	5	0	4	16	0	0.29
BAL	3.88	170	0	6	3	1	1	11	0	0.30
BAL	4.01	140	60	2	9	3	2	37	0	0.25
BAL	4.01	140	60	10	7	2	2	30	0	0.25
BAL	4.01	140	60	2	8	2	3	32	0	0.25
BAL	4.21	170	35	13	17	9	4	232	0	0.07
BAL	4.21	170	35	6	20	0	14	265	0	0.08
BAL	4.21	170	35	6	19	9	4	256	0	0.08
BAL	4.21	170	35	6	19	9	3	248	0	0.08
BAL	4.21	170	35	6	17	8	2	233	0	0.07
BAL	4.21	170	35	0.5	22	9	2	290	0	0.08
BAL	4.21	170	35	6	18	0	11	241	0	0.08
BAL	4.21	170	35	6	19	0	12	236	0	0.08
BAL	4.42	170	77	6	15	9	5	229	0	0.07
BAL	4.42	170	77	6	14	9	6	242	0	0.06
BAL	5.05	200	10	2	12	2	9	215	0	0.05
BAL	5.05	200	10	10	9	4	11	190	0	0.05
BAL	5.05	200	10	2	13	7	6	225	0	0.06
BAL	5.05	200	10	10	8	8	6	163	0	0.05
BAL	5.12	200	60	2	3	3	14	33	7	0.09
BAL	5.12	200	60	10	1	0	15	9	4	0.09
BAL	5.5	220	35	6	0	0	9	3	5	0.14

B.3-10: ***Polysaccharide*** *contents of* ***CON*** *extracts*

	log(R_0)	T °C	t min	Loading %[w/v]	ARA	GAL	GLU	XYL	FRU	ARA/XYL
					mg/[g of dry biomass]					
CON	3.5	140	10	10	2	0	0	3	0	0.62
CON	3.5	140	10	2	3	0	16	8	0	0.38
CON	3.61	120	35	6	2	0	21	5	0	0.51
CON	3.88	170	0	6	3	1	1	14	0	0.25
CON	4.01	140	60	2	8	3	1	32	0	0.26
CON	4.01	140	60	10	10	5	0	47	0	0.21
CON	4.21	170	35	13	15	10	2	198	0	0.08
CON	4.21	170	35	6	18	13	0	223	0	0.08
CON	4.21	170	35	6	18	12	0	232	0	0.08
CON	4.21	170	35	6	20	13	0	240	0	0.08
CON	4.21	170	35	6	20	13	0	246	0	0.08
CON	4.21	170	35	1	21	9	17	233	0	0.09
CON	4.21	170	35	6	18	6	18	196	0	0.09
CON	4.21	170	35	6	18	0	27	206	0	0.09
CON	4.42	170	77	6	15	10	3	208	0	0.07
CON	5.05	200	10	2	12	10	1	181	0	0.07
CON	5.05	200	10	10	9	0	26	178	0	0.05
CON	5.12	200	60	2	3	3	13	39	0	0.08
CON	5.12	200	60	10	0	0	13	5	1	0.00
CON	5.5	220	35	6	0	0	17	3	1	0.00

*B.3-11: **Polysaccharide** contents of **MIS** extracts*

	log(R_0)	T °C	t min	Loading %[w/v]	ARA	GAL	GLU	XYL	FRU	ARA/XYL
					mg/[g of dry biomass]					
MIS	3.5	140	10	10	4	1	2	16	0	0.24
MIS	3.5	140	10	2	2	0	2	5	0	0.43
MIS	3.61	120	35	6	1	0	2	3	0	0.49
MIS	3.88	170	0	6	3	1	2	7	0	0.42
MIS	4.01	140	60	2	6	1	2	15	0	0.40
MIS	4.01	140	60	10	8	2	3	29	0	0.27
MIS	4.21	170	35	13	3	2	11	72	0	0.05
MIS	4.21	170	35	6	11	4	5	110	0	0.10
MIS	4.21	170	35	6	12	4	5	116	0	0.10
MIS	4.21	170	35	6	11	4	5	104	0	0.11
MIS	4.21	170	35	6	12	3	5	105	0	0.11
MIS	4.21	170	35	0.5	13	4	7	141	0	0.09
MIS	4.21	170	35	6	12	3	5	114	0	0.10
MIS	4.21	170	35	6	12	0	9	133	0	0.09
MIS	4.42	170	77	6	8	3	7	119	0	0.07
MIS	5.05	200	10	2	8	5	7	136	0	0.06
MIS	5.05	200	10	10	3	2	13	82	0	0.04
MIS	5.12	200	60	2	1	1	10	14	0	0.07
MIS	5.12	200	60	10	0	0	5	1	1	0.07
MIS	5.5	220	35	6	0	0	7	1	1	0.07

*B.3-12: **Furan** contents in BAL CON and MIS extracts*

log(R_0)	T	t	Loading	5-HMF mg/[g of dry biomass]			Furfural mg/[g of dry biomass]		
	°C	min	%[w/v]	BAL	CON	MIS	BAL	CON	MIS
3.5	140	10	10	0.0	0.0	0.0	0.1	0.1	0.2
3.5	140	10	2	0.0	0.1	0.1	0.2	0.5	0.3
3.61	120	35	6	0.0	0.0	0.0	0.1	0.0	0.0
3.61	120	35	6	0.0	-	-	0.0	-	-
3.85	140	38	6	0.0	-	-	0.1	-	-
3.88	170	0	6	0.0	0.0	0.0	0.1	0.6	0.2
3.88	170	0	6	0.0	-	-	0.4	-	-
4.01	140	60	2	0.1	0.1	0.1	1.9	1.6	1.8
4.01	140	60	10	0.0	0.0	0.1	0.3	0.3	0.6
4.01	140	60	2	0.0	-	-	0.9	-	-
4.21	170	35	13	0.3	0.3	2.7	10.9	12.3	25.3
4.21	170	35	6	0.3	0.2	0.3	8.4	6.2	4.4
4.21	170	35	6	0.2	0.2	0.3	6.9	6.2	4.2
4.21	170	35	6	0.3	0.2	0.2	7.2	6.3	3.5
4.21	170	35	6	0.5	0.2	0.3	6.8	6.3	3.8
4.21	170	35	0.5	0.2	5.0	5.8	6.9	42.3	22.0
4.21	170	35	6	0.3	0.5	0.4	6.7	5.6	4.3
4.21	170	35	6	0.2	0.5	0.4	5.7	5.4	5.0
4.42	170	77	6	0.8	0.9	0.9	28.3	27.7	16.7
4.42	170	77	6	0.8	-	-	27.2	-	-
5.05	200	10	2	0.7	0.9	1.6	9.6	22.4	17.2
5.05	200	10	10	1.9	0.2	2.2	32.8	2.2	24.8
5.05	200	10	2	0.9	-	-	21.9	-	-
5.05	200	10	10	1.3	-	-	23.1	-	-
5.12	200	60	2	7.4	7.3	8.5	99.7	105.0	75.8
5.12	200	60	10	8.7	12.3	15.0	42.7	45.0	33.4
5.5	220	35	6	13.3	17.4	17.6	45.1	57.5	39.2

B.4. RSM BAL extract and AX standard

*B.4-1: Extract mass concentration, pH, furfural and arabinose concentrations of sequential **BALADO** extracts*

log(R_0) S.Factor	°C Temp.	min Res. t	g/L Extr.conc.	pH	gGAeq/L TPC	g/L Furfural	g/L Arabinose	g/L Xylose
6.34	180	0	8.5	3.3	0.88	0.39	0.22	1.20
6.41	172	15	8.5	3.2	0.95	0.44	0.21	1.39
6.72	180	30	8.1	3.2	0.95	1.08	0.21	1.79
6.97	200	0	8.5	3.3	0.96	0.43	0.22	1.27
7.05	200	15	6.9	3.0	1.13	2.07	0.17	1.77
7.05	200	15	7.1	3.0	1.18	1.93	0.17	1.81
7.05	200	15	6.2	3.0	1.22	2.16	0.18	0.96
7.05	200	15	6.3	3.0	1.08	1.90	0.14	1.50
7.05	200	15	5.9	3.0	1.20	2.13	0.15	1.58
7.14	200	36	4.6	2.9	0.99	2.67	0.11	1.06
7.60	220	0	8.0	3.4	0.92	0.35	0.25	1.14
7.65	220	30	3.4	2.8	1.16	0.97	0.01	0.06
7.93	228	15	3.2	2.8	1.43	2.54	0.00	0.06
Prior treatment			10	3.4	0.58	0.34	0.23	1.07

*B.4-2: Extract mass concentration, pH, furfural and arabinose concentrations of sequential **AX standard** extracts*

log(R_0) S.Factor	°C Temp.	min Res. t	g/L Extr.conc.	pH	gGAeq/L TPC	g/L Furfural	g/L Arabinose	g/L Xylose
2.07	180	0	8.6	7.4	0.09	0.02	0.07	0.06
2.14	172	15	8.8	6.9	0.09	0.01	0.14	0.03
2.45	180	30	8.7	5.3	0.12	0.04	0.52	0.03
2.70	200	0	9.0	7.3	0.08	0.01	0.12	0.02
2.78	200	15	8.7	4.2	0.24	0.21	1.44	0.85
2.78	200	15	8.8	4.3	0.25	0.22	1.39	0.88
2.78	200	15	8.1	3.8	0.34	0.45	1.37	1.00
2.78	200	15	8.4	3.8	0.34	0.41	1.32	0.94
2.78	200	15	8.1	3.7	0.36	0.47	1.34	1.16
2.87	200	36	5.8	3.2	0.55	2.05	0.51	1.34
3.33	220	0	8.1	7.0	0.07	0.01	0.12	0.01
3.38	220	30	3.0	3.0	0.71	2.26	0.06	0.05
3.66	228	15	3.1	2.9	0.79	2.36	0.05	0.03

*B.4-3: Xylooligosaccharide concentrations of sequential **BALADO** extracts*

log(R_0) S.Factor	°C Temp.	min Res. t	g/L X2	g/L X3	g/L X4	g/L X5	g/L X6	g/L XOS*
6.34	180	0	0.69	0.70	0.54	0.34	0.27	2.26
6.41	172	15	0.72	0.72	0.47	0.28	0.22	2.19
6.72	180	30	0.71	0.49	0.25	0.15	0.08	1.60
6.97	200	0	0.73	0.70	0.52	0.33	0.27	2.28
7.05	200	15	0.48	0.18	0.06	0.00	0.00	0.72
7.05	200	15	0.38	0.17	0.07	0.00	0.00	0.61
7.05	200	15	0.15	0.03	0.04	0.00	0.00	0.23
7.05	200	15	0.19	0.06	0.00	0.00	0.00	0.24
7.05	200	15	0.02	0.00	0.00	0.00	0.00	0.02
7.14	200	36	0.00	0.00	0.00	0.00	0.00	0.00
7.60	220	0	0.72	0.75	0.50	0.37	0.28	2.33
7.65	220	30	0.00	0.00	0.00	0.00	0.00	0.00
7.93	228	15	0.01	0.00	0.00	0.00	0.00	0.01
Prior treatment			0.68	0.77	0.53	0.33	0.27	2.30

*X2-X5

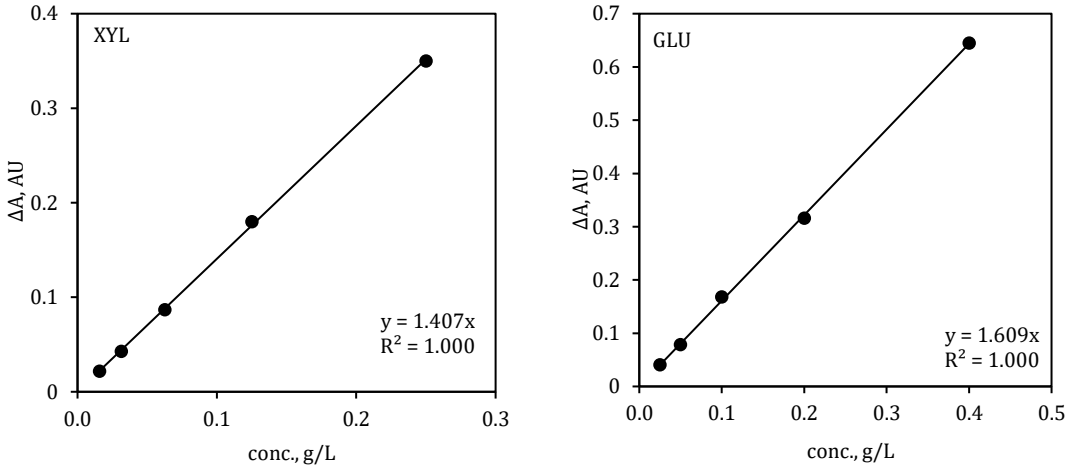
*B.4-4: Xylooligosaccharide concentrations of sequential **AX standard** extracts*

log(R_0) S.Factor	°C Temp.	min Res. t	g/L X2	g/L X3	g/L X4	g/L X5	g/L X6	g/L XOS*
6.34	180	0	0.02	0.01	0.01	0.01	0.01	0.05
6.41	172	15	0.02	0.02	0.02	0.01	0.01	0.07
6.72	180	30	0.03	0.00	0.00	0.00	0.00	0.03
6.97	200	0	0.01	0.01	0.00	0.00	0.00	0.02
7.05	200	15	0.59	0.39	0.32	0.27	0.28	1.57
7.05	200	15	0.51	0.35	0.29	0.32	0.29	1.47
7.05	200	15	0.60	0.49	0.36	0.32	0.31	1.77
7.05	200	15	0.59	0.44	0.34	0.31	0.29	1.69
7.05	200	15	0.77	0.65	0.35	0.30	0.23	2.08
7.14	200	36	0.07	0.00	0.00	0.00	0.00	0.07
7.60	220	0	0.01	0.00	0.00	0.00	0.00	0.01
7.65	220	30	0.03	0.02	0.00	0.00	0.00	0.04
7.93	228	15	0.02	0.00	0.00	0.00	0.00	0.02

*X2-X5

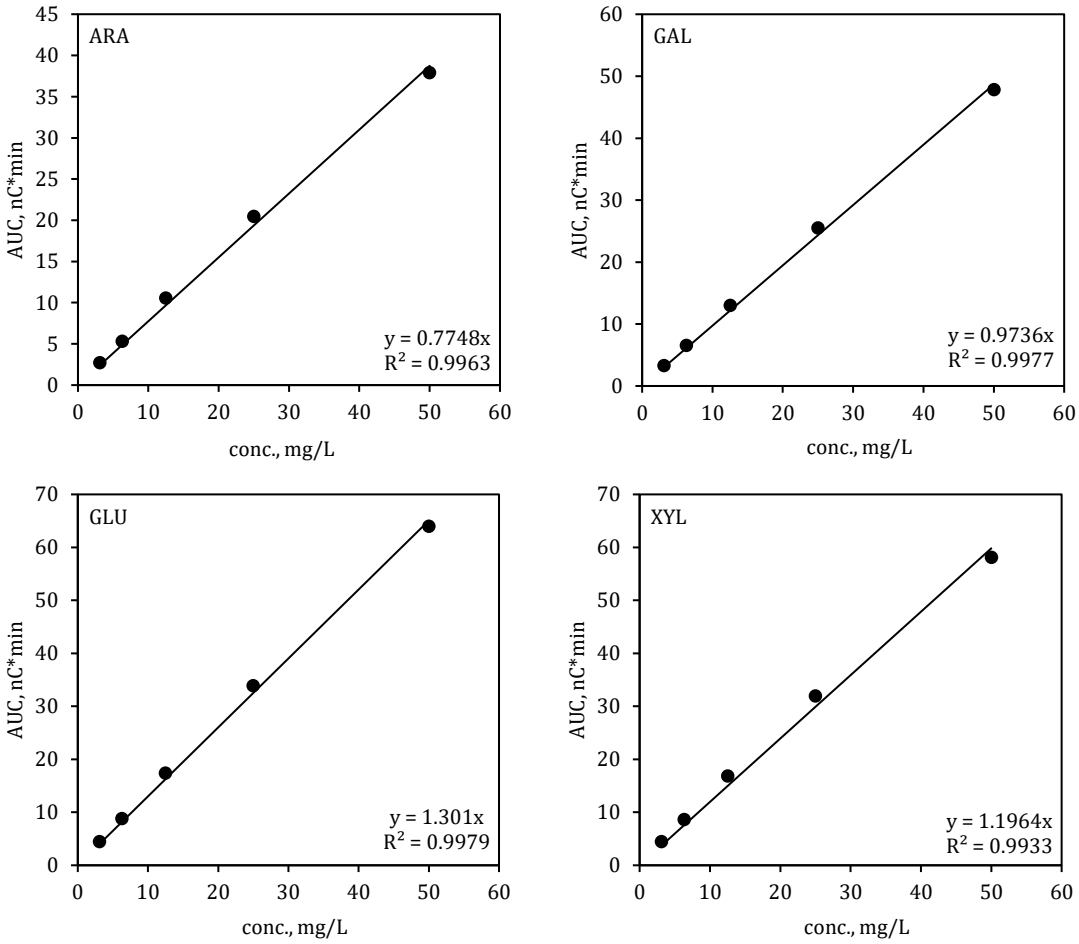
APPENDIX C: CALIBRATION CURVES

C.1. Enzyme kits

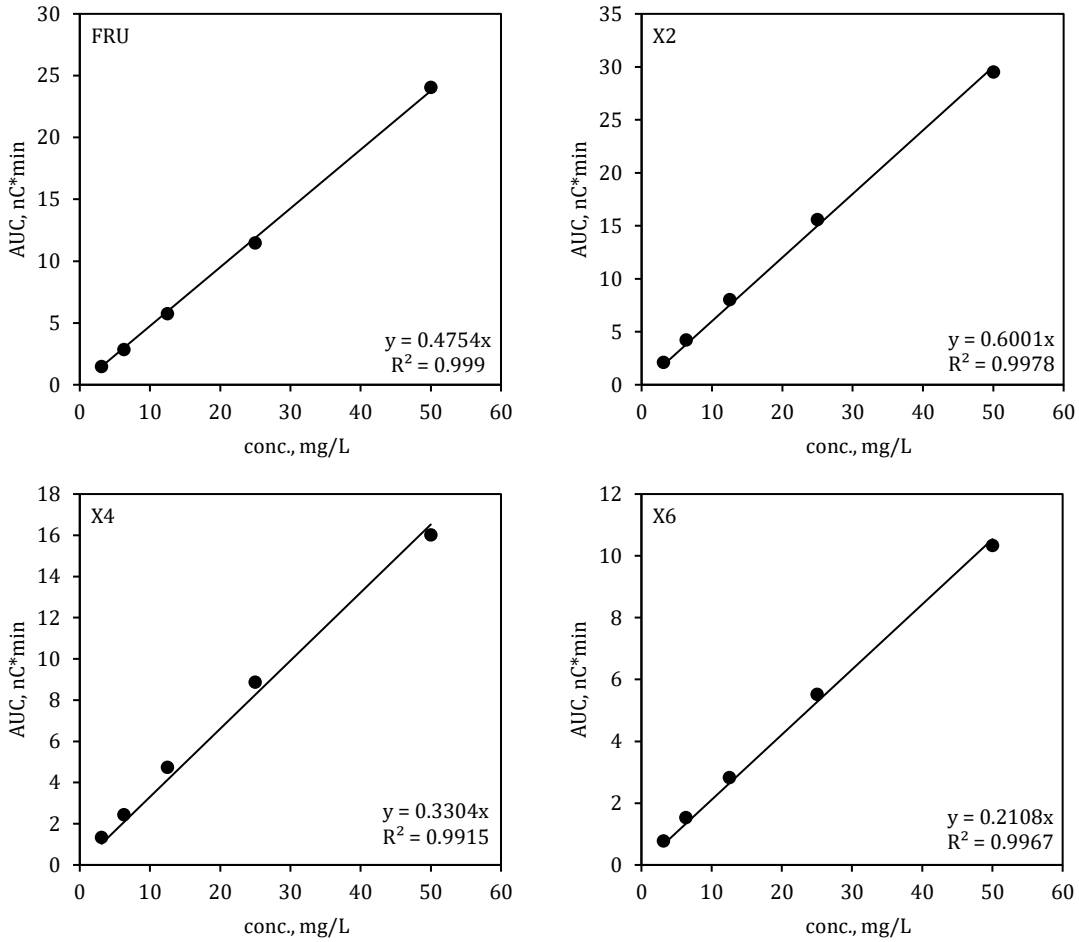


C.1-1: Enzyme kit calibration curves of xylose (XYL) and glucose (GLU)

C.2. HPAEC-PAD PA1 column

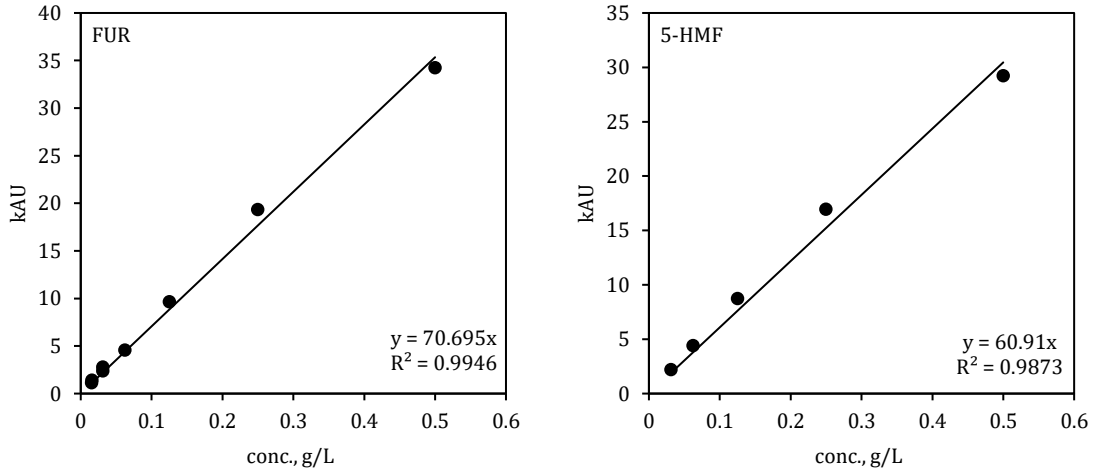


C.2-1: PA1 calibration curves of arabinose (ARA), galactose (GAL), glucose (GLU), and xylose (XYL)



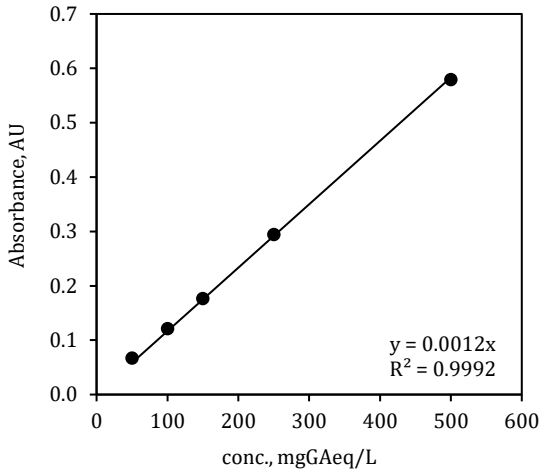
C.2-2: PA1 column calibration curves of fructose (FRU), xylobiose (X2), xylotetraose (X4), and xylohexaose (X6)

C.3. HPLC PRODIGY column



C.3-1: Prodigy column calibration curves of furfural (FUR) and 5-HMF

C.4. Folin-Ciocalteu total phenolics



C.4-1: TPC calibration curve

APPENDIX D: REFERENCE TABLES

D.1-1: Summary of hemicellulose extraction methods (in alphabetical order), method conditions and results; "-" indicate "no data available"

Method	*Temp., °C*	*Pressure, bar*	*Res. time, min*	*Loading, w/w*	*Feedstock*	*Hemicellulose solubilisation*	*Hemicellulose recovery*	*DP/molar mass*	*Methods of characterisation*	*Reference*
Acid, H_2SO_4, 0.1% at 60 °C for 12 h, followed by steam treatment, batch	145	-	60	10%	*Miscanthus sinensis*	-	65% XOS	-	NREL, HPLC PRI, IC	(Otieno and Ahring, 2012b)
					Panicum virgatum		84% XOS			
					Calamagrostis acutiflora		88% XOS			
					Bagasse		92% XOS			
Acid, H_2SO_4, 0.125 mol/l, batch	220	-	1.8	8%	Olive stones	-	80% pentoses	-	TAPPI, HPLC RI	(Montané et al., 2002)
Acid, H_2SO_4, 1.2%, batch	180	-	0.5	10%	Switchgrass	-	87% xylose	-	TAPPI, HPLC RI	(Esteghlalian et al., 1997)
					Poplar		89% xylose			
					Corn stover		83% xylose			
Acid, H_2SO_4, 1-2%, batch	150-170	-	May-15	6%	Empty fruit bunches	-	80%	-	NREL, HPLC RI	(Hong et al., 2013)
Acid, H_2SO_4, 2.5%, batch	180	-	15	9%	Wheat straw	75% xylan	45% xylan	-	H2SO4 hydrolysis, GLC, HPSEC RI, HPAEC PAD, MALDI ToF MS	(Kabel et al., 2007)
AFEX, ammonia, batch	180	48	30	50%	Poplar wood	9% xylan	-	-	NREL, HPLC RI, MS	(Balan et al., 2009)
	90	21	5	50%	Corn stover	14% xylan				
Alkali extraction, 24% KOH, followed by dilute acid hydrolysis, 0.25M H_2SO_4, batch	35 (KOH extraction), 100 (H2SO4 hydrolysis)	-	180 (KOH extraction), 30 (H2SO4 hydrolysis)	2%	Tobacco stalk	-	27% xylose and XOS	DP 1-6	HPLC RI,	(Akpinar et al., 2009)
					Cotton stalk		14% xylose and XOS	DP 1-7		
					Sunflower stalk		23% xylose and XOS	DP 1-8		
					Wheat straw		20% xylose and XOS	DP 1-9		
Alkali, 0.5M KOH with ultrasonication	35	-	150	2%	Wheat straw	-	65%	10730-22890 g/mol	GC FID, FTIR, NMR	(Sun and Tomkinson, 2002)
Alkali, 10% KOH, followed by graded ethanol precipitation, batch	25	-	600	4%	*Caragana korshinskii*	-	-	14890-58810 g/mol	HPAEC PAD, GPC, FTIR, NMR	(Bian et al., 2010)
Alkali, 4% NaOH, batch	24	-	1440	9%	Soybean straw	-	~95 xylose	-	NREL, HPLC RI	(Wan et al., 2011)
Alkali, $Ca(OH)_2$, batch	180	22	240	6%	Poplar wood	>80% xylan	-	-	NRLEL, HPLC RI	(Sierra et al., 2009)

Method	*Temp., °C*	*Pressure, bar*	*Res. time, min*	*Loading, w/w*	*Feedstock*	*Hemicellulose solubilisation*	*Hemicellulose recovery*	*DP/molar mass*	*Methods of characterisation*	*Reference*
Alkali, KOH, 24% and H_3BO_3, 2%, batch	20	-	120	1.20%	Wheat straw	-	34%	-	NREL, HPLC RI	(Lawther et al., 1996)
Alkali, NaOH, 0.2 M, batch	-	-	60	1%	Psyllium seed husk	-	77% (arabino)xylan	>1000 Da	TFA hydrolysis, GC FID	(van Craeyveld et al., 2009)
Alkali, NaOH, 10%, batch	75	-	120	17%	Corn stover	-	90% xylan	-	NREL and TAPPI, HPLC ED	(Cheng et al., 2010a)
Alkali, NaOH, 12%, overnight, followed by steam treatment, batch	121	-	45	-	Corn cobs	-	84% XOS	-	H2SO4 hydrolysis, HPLC RI, TLC, total reducing sugars, FT IR	(Samanta et al., 2012)
Alkali, NaOH, 2%	120	2	60	10%	Corn fibre	-	88%	-	H2SO4 hydrolysis, HPLC RI	(Gaspar et al., 2007)
Alkali, NaOH, 3%, batch	121	-	60	15%	Sugarcane tops	45% xylan	-	-	NREL, HPLC RI	(Sindhu et al., 2014)
Alkali, NaOH, 5.7%, batch	100	-	35	33%	Aspen wood chips	-	23%	-	PAPTAC, IC	(Liu et al., 2011)
Alkali, sequential, KOH, 0.5, 1.0 and 2.0 mol/l, batch	25	-	600	4%	Peashrub	-	92%	38810-83460 g/mol	H2SO4 hydrolysis, HPAEC PAD, GPC, FT IR, MS, NMR	(Peng et al., 2012a)
Alkali, using 1M of various alkalis, batch	50	-	180	4%	Bamboo	-	-	8200-53070 g/mol	HPAEC PAD, GPC, FTIR, NMR,	(Wen et al., 2011)
Ammonia recycled percolation (ARP), 15%, flowthrough	170	23	10	-	Corn stover	87% xylan	59% xylan	-	NREL, HPLC RI, FT IR	(Kim et al., 2003)
Aqueous ammonia,10%, batch	100	May-15	Oct-20	9%	Miscanthus	-	77%	-	NREL, HPLC RI, NMR, FTIR	(Liu et al., 2013)
Dilute acid (0.2-1.6% H_2SO_4), batch	170-210	30	10	16%	Olive tree pruning	Up to 100% xylose	-	-	NREL, HPLC RI	(Cara et al., 2008)
Dilute acid (1% H_2SO_4) followed by hot water, batch	60 (H_2SO_4)	-	720 (H_2SO_4)	-	Corn cobs	-	-	-	DNS, HPAEC PED	(Yang et al., 2005)
Dilute acid (1% H_2SO_4) followed by hot water, batch	150 (hot water)	-	25-30 (hot water)	-	Corn cobs	-	-	-	DNS, HPAEC PED	(Yang et al., 2005)
Dilute acid (4%H_2SO_4), batch	130	-	120	20%	Rice husk	96%	72% xylose	-	HPLC DR	(Zhang et al., 2010)
Dilute acid, 1-1.5%H_2SO_4, batch	121	-	60	10%	Switchgrass	>80%	-	-	NREL, HPLC RI,	(Yang et al., 2009)

Method	*Temp., °C*	*Pressure, bar*	*Res. time, min*	*Loading, w/w*	*Feedstock*	*Hemicellulose solubilisation*	*Hemicellulose recovery*	*DP/molar mass*	*Methods of characterisation*	*Reference*
Dilute acid, 2% -10% oxalic acid, batch	150-190	-	15-40	20%	Giant reed, Arundo donax	-	55% xylose	-	HPAEC PAD, HPLC RI	(Scordia et al., 2011)
Dilute acid, 2%H_2SO_4, 0.5% Tween 20 assisted, batch	121	-	90	9%	Wheat straw	-	~78% xylose	-	NREL, HPLC RI	(Qi et al., 2010)
Dilute acid, 3%H_2SO_4, batch	121	-	90	<1%	Culm of Sasa Kurilensis	-	87% xylose	-	HPLC RI	(Miura et al., 2010)
Dilute acid, 89 mM maleic acid, batch	170	-	10	10%	Wheat straw	86% xylose	81% xylose	-	NREL, TAPPI, HPLC RI, HPAEC PAD	(Kootstra et al., 2009)
Dilute acid, H_2SO_4, 0.5%, batch	190	-	10	9%	Rice hulls	-	68% xylan	-	NREL, HPLC RI	(Lopez et al., 2010)
Dilute acid, H_2SO_4, 1%, batch	121	-	27	9%	Rice straw	-	77% xylose	-	NREL, HPLC RI	(Roberto et al., 2003)
Dilute acid, H_2SO_4, 1.2%, batch	140	-	30	9%	Coastal Bermuda grass	-	83% xylose	-	NREL, HPLC RI	(Redding et al., 2011)
Dilute alkali, 0.75% NaOH, batch	121	-	15	9%	Bermuda grass	-	-	-	NREL, HPLC RI	(Wang et al., 2010)
DMSO and alkali, KOH (10%-24%) and sodium borate (0.05%), batch	20	-	960	-	Wheat straw	-	-	6000-30000 g/mol	GC FID, GPC	(Lawther et al., 1995)
Ethanol/benzene extraction, batch	-	118	-	4%	Corn stalk	-	~90% pentoses	-	ASTM, GC FID	(Rubio et al., 1998)
Hot water + carbon dioxide, batch	170	450	60	5%	Switchgrass	-	13% xylose	1000-5000 Da	SEC RI; NREL, HPLC RI	(King et al., 2012)
		300	90		Corn cobs		19% xylose	500-2000 Da		
Hot water with $CuCl_2$, flowthrough	184	50	8	18%	Sweet sorghum bagasse	-	78% xylose	-	NREL, HPLC RI, GC FID, MS EI	(Yu et al., 2011)
Hot water, batch	160	-	120	20%	Maple	73% xylan	58% xylan	-	H2SO4 hydrolysis, followed by 1H NMR	(Amidon and Liu, 2009)
Hot water, batch	180	-	30	5%	Sunflower stalks	69%	65%	-	NREL, HPLC RI	(Jung et al., 2013)
Hot water, batch	190	-	5	11%	Brewery's spent grain	90% xylan	61% xylan	DP >7	H2SO4 hydrolysis, HPLC RI	(Carvalheiro et al., 2004)

Method	*Temp., °C*	*Pressure, bar*	*Res. time, min*	*Loading, w/w*	*Feedstock*	*Hemicellulose solubilisation*	*Hemicellulose recovery*	*DP/molar mass*	*Methods of characterisation*	*Reference*
Hot water, batch	200	-	-	11%	Ulex Europaeus		71% xylan		TAPPI, HPLC RI	(Ares-Peón et al., 2013)
Hot water, batch	202	-	-	11%	Corn cobs	81% xylan	62% XOS	-	TAPPI, HPLC RI	(Garrote et al., 2008)
Hot water, batch	180	-	20	5%	Paper pulp by-product, press-lye	-	58% XOS	DP 1-8	H2SO4 hydrolysis; HPAEC-PAD; MALDI ToF MS; SEC RI; FT-IR; 13C CP/MAS NMR	(Griebl et al., 2005)
Hot water, batch	205	-	-	11%	Rye straw	53% xylan	69% XOS	DP >6	H2SO4 hydrolysis, HPLC RI; GPC RI	(Gullon et al., 2010)
Hot water, batch	155	-	60	9%	Wheat bran	69% xylan	27% xylan	-	Two step H2SO4 hydrolysis, HPAEC PAD, SEC, MALDI ToF MS	(Kabel et al., 2002)
	150		120	11%	Brewery's spent grain	62% xylan	49% xylan			
	160		75	11%	Corn cobs	65% xylan	61% xylan			
	160		60	11%	Eucalyptus wood	61% xylan	64% xylan			
Hot water, batch	170	-	60	17%	Eucalyptus globulus	-	76% xylan	DP 6	H2SO4 hydrolysis, HPAEC PAD, GC FID, SEC RI, TOC	(Leschinsky et al., 2009)
Hot water, batch	170	-	120	20%	Hardwood	-	62% xylan	-	H2SO4 hydrolysis, HPLC RI	(Pu et al., 2011)
Hot water, batch	180	-	30	9%	Wheat straw	-	44% xylan	-	H2SO4 hydrolysis, HPLC RI	(Ruiz et al., 2011)
Hot water, batch	190	-	10	10%	Switchgrass	-	64% xylan	-	NREL, HPLC RI	(Suryawati et al., 2009)
Hot water, batch	180	-	40	11%	Rice husks	85% xylan	60% XOS	DP <25	TAPPI, HPAEC PAD, HPSEC, MALDI ToF MS	(Vegas et al., 2008a)
Hot water, batch	180	20	10	10%	Rice straw	92% xylan	43% xylan	-	NREL, HPLC RI	(Yu et al., 2010)

Method	*Temp., °C*	*Pressure, bar*	*Res. time, min*	*Loading, w/w*	*Feedstock*	*Hemicellulose solubilisation*	*Hemicellulose recovery*	*DP/molar mass*	*Methods of characterisation*	*Reference*
Hot water, batch	180	-	42	11%	Arundo donax	-	78% XOS	-	TAPPI, HPLC RI	(Caparros et al., 2007)
Hot water, batch	160	-	60	5%	*Miscanthus giganteus*	-	65% XOS	-	TAPPI, HPAEC PAD	(Ligero et al., 2011)
Hot water, batch	180	-	-	10%	Corn fibre	-	63% XOS	-	NREL, HPLC RI	(Samala et al., 2012)
Hot water, batch	202	-	-	11%	Corn cobs	-	79% XOS	-	TAPPI. HPLC	(Vázquez et al., 2006)
Hot water, batch	180	-	30	9%	Bamboo	60% xylan	47% XOS	DP >6	NREL, HPAEC PAD, GPC	(Xiao et al., 2013b)
Hot water, batch	215	-	-	9%	Wheat straw	83% (arabino)xylan	64% (arabino)xylan	-	Two step H2SO4 hydrolysis, HPLC RI	(Carvalheiro et al., 2009)
Hot water, batch	-	118	-	4%	Corn stalk	-	~80% pentoses	-	ASTM, GC FID	(Rubio et al., 1998)
Hot water, batch	215	-	2	5%	Corn fibre	70% pentoses	82% pentoses recovered	-	NREL, HPLC RI	(Allen et al., 2001)
Hot water, batch	190	12.1	10	9%	Soybean straw	-	~40% xylose	-	NREL, HPLC RI	(Wan et al., 2011)
Hot water, batch	170	-	240	5%	Sugarcane bagasse	-	55% xylose	-	HPAEC PAD, TLC, HPSEC RI	(Boussarsar et al., 2009)
Hot water, batch	180	-	10	9%	Wheat straw	-	-	-	GC FI	(Holopainen-Mantila et al., 2013)
Hot water, batch; Gel Filtration Chromatography for XOS purification	180	-	10	17%	Olive tree pruning	-	55% xylan	DP 7-25	SEC RI; H2SO4 hydrolysis, HPLC RI	(Cara et al., 2012)
Hot water, batch; purification: GFC RI	208	-	27	11%	Corn cobs	-	65% xylan	DP 2-6	H2SO4 hydrolysis, HPLC RI, SEC RI	(Moura et al., 2007)
Hot water, batch; purification: high pressure membrane filtration	179	-	23	14%	Almond shells	63%	58% XOS	100-70000 Da	Two step H2SO4 hydrolysis, HPLC RI, GPC UV/RI	(Nabarlatz et al., 2007b)
Hot water, continuous; purification: two step membrane filtration (UF and NF) followed by activated carbon treatment and ion exchange demineralisation	200	18	11	14%	Corn cobs	-	82% xylan	DP 1-20	Two step H2SO4 hydrolysis, HPLC RI, MALDI ToF MS	(Makishima et al., 2009)
Hot water, flowthrough	210	-	10	-	Corn stover	-	65% xylan	-	NREL, HPLC RI	(Yoo et al., 2011)

Method	*Temp., °C*	*Pressure, bar*	*Res. time, min*	*Loading, w/w*	*Feedstock*	*Hemicellulose solubilisation*	*Hemicellulose recovery*	*DP/molar mass*	*Methods of characterisation*	*Reference*
Hot water, flowthrough	150	110	60	2%	Triticale straw	-	60% xylose and XOS	-	NREL, HPLC RI, HPLC DAD	(Pronyk et al., 2011)
Inorganic salts, $FeCl_3$, 0.1 mol/l, batch	140	-	20	9%	Corn stover	-	89% XOS + xylose	-	NREL, HPLC RI	(Liu et al., 2009)
Ionic liquid [Amim]Cl, followed by alkali, NaOH, 0.5 M, batch	100 ([Amim]Cl)	-	720 ([Amim]Cl)	5% ([Amim]Cl)	Bamboo	-	-	16110 g/mol	HPAEC PAD, GPC, 13C NMR, 2D NMR	(Yang et al., 2013)
Ionic liquid, 1-ethyl-3-methylimidazolium acetate, 49.5%, batch	158	-	216	5%	Wheat straw	-	71.4% fermentable sugars	-	NREL, HPLC RI	(Fu and Mazza, 2011)
Lime pretreatment, 1 g $Ca(OH)_2$/g biomass, batch	100	3.5	60	6%	Switchgrass	-	93% xylan	-	NREL, HPLC RI	(Falls and Holtzapple, 2011)
Microwave assisted alkali, NaOH, 5%, batch	140	-	10	10%	Wheat straw	80% xylan	73% xylan	-	NREL, HPLC RI	(Janker-Obermeier et al., 2012)
Microwave assisted aqueous ethanol, 30%, batch	180	-	10	4%	Flax shives	40%	-	DP 4	NREL, HPLC RI, IMP Chromatography, HPSEC RI, FT IR	(Buranov and Mazza, 2010)
Microwave assisted water, batch	180		10	4%	Flax shives	19%		DP 3		
Mild acid hydrolysis, HCl, 0.2 M, batch	90	-	1440	0.20%	Psyllium seed husk	-	97% (arabino)xylan	DP 31	TFA hydrolysis, GC FID	(van Craeyveld et al., 2009)
Organosolv, ethanol, 50%, H_2SO_4 0.16%, batch	170	-	60	13%	Barley straw	-	92% xylan	-	NREL, HPLC RI	(Kim et al., 2011)
Organosolv, ethanol:water: 60:40, batch	175	-	60	17%	Wheat straw	57% xylan	44% xylose + XOS	-	NREL, HPAEC PAD	(Huijgen et al., 2012)
Pressurised aqueous ethanol (PAE), 30%, flowthrough	180	52	117	4%	Flax shives	80%	-	DP 3	NREL, HPLC RI, IMP Chromatography, HPSEC RI, FT IR	(Buranov and Mazza, 2010)
Pressurised low polarity water (PLPW), flowthrough					Flax shives	90%		DP 3		
Sequential treatments using 1.5% NaOH, 0.25% ammonium oxalate, acetic acid and sodium chlorite, 24% KOH and 2% boric acid, batch	-	-	-	-	Wheat straw	50%	-	8400-15000 g/mol	GC FID, GPC, FTIR	(Lawther and Sun, 1996)

Method	*Temp., °C*	*Pressure, bar*	*Res. time, min*	*Loading, w/w*	*Feedstock*	*Hemicellulose solubilisation*	*Hemicellulose recovery*	*DP/molar mass*	*Methods of characterisation*	*Reference*
Sequential treatments using 90% dioxane, 80% dioxane with 0.05 M HCl, DMSO, 8% NaOH, batch	-	-	-	-	Barley straw	87%	32%	12600-28800 g/mol	GC FID, GPC, FTIR, NMR	(Sun et al., 2011)
Sequential treatments using NaOH, $NaBH_4$, ethanol, acetone, followed by dilute acid treatment trifluoroacetic acid, batch	-	-	-	-	Wheat bran	-	-	DP 1-5	TLC, HPLC DR, GFC, GC FID, NMR	(Brillouet et al., 1982)
Soaking in 20% ethanol and 15% aqueous ammonia, batch	60	-	1440	10%	Corn stover	10% xylan	-	-	NREL, HPLC R	(Kim et al., 2009)
Steam and 0.5% H_2SO_4, batch	190	-	4	52%	*Salix schwerinii* x *Salix viminalis*	74% XOS and xylose	-	-	HPLC RI	(Sassner et al., 2008)
Steam explosion, batch	210	41	5	-	Sunflower stalks	67% xylose	28% xylose	-	NREL, HPLC RI	(Ruiz et al., 2008)
Steam explosion, batch	205	40	10	-	Sugarcane Bagasse	-	40% xylan	-	Two step H2SO4 hydrolysis, HPLC RI	(Martín et al., 2008)
Steam, batch	215	-	2	70%	Corn fibre	76% pentoses	40% pentoses recovered	-	NREL, HPLC RI	(Allen et al., 2001)
Two stage hot water, batch	160/170	-	30/60	14%	Coastal bermuda grass	94%	37% xylose	-	NREL, AEC ED	(Lee et al., 2010)
Wet oxidation, hot water + Na_2CO_3 + O_2, batch	195	12	15	6%	Sugarcane bagasse	-	18% xylan	-	Two step H2SO4 hydrolysis, HPLC RI	(Martín et al., 2008)