Oligosaccharides production and purification from barley bran using sequential supercritical CO_2 extraction, subcritical water hydrolysis and membrane filtration

by

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Abstract

Barley bran is a by-product of the food industry, and a good source of lipid, protein and fiber. In this thesis research, fractionation of barley bran was carried out to remove lipid, starch and protein to obtain a sample enriched in fiber, specially arabinoxylan. Fiber concentrate was used for further hydrolysis, targeting the production of xylooligosaccharides (XOS) with degree of polymerization 2-4. Lipid was extracted using supercritical CO_2, followed by enzymatic hydrolysis to remove starch. Defatted bran with 0.3% lipid was obtained. Defatted-destarched bran had 1% starch and 26.3% db arabinoxylan. Subcritical water (SCW) was used as an environmentally friendly approach to hydrolyse defatted-destarched bran. Temperature had a significant effect on the XOS production. The highest XOS content was produced at 180°C, where 112.5 mg of total XOS was obtained within 30 min, with no significant difference after 60 min hydrolysis. Deproteinized bran with 42.2% db arabinoxylan was hydrolyzed using SCW at 180°C/50 bar/30 min. Amounts of 100.9, 120.6, 112.4 and 334 mg of xylobiose, xylotriose, xylotetraose and total XOS were obtained in the hydrolysate. Deproteinized bran was also treated using enzymatic hydrolysis with endo-xylanase. Maximum amount of total XOS was 21.11 mg obtained using 10 U of enzyme at 40°C, pH of 4.5 after 4 h incubation. The recovery of total XOS from initial xylan of deproteinized bran was 78.4 and 45.1% for SCW and enzymatic hydrolysis, respectively. Purification of deproteinized bran SCW hydrolysate was performed using ultrafiltration with 1 kDa membrane to remove compounds with high molecular weight. In total, 68% of initial total XOS was recovered after passing through 1 kDa membrane. This permeate was treated by activated carbon adsorption (10% w/w) to remove monomers (arabinose and xylose) from XOS. Activated carbon was washed with aqueous ethanol solutions (15 and 30% v/v) to liberate the adsorbed XOS. Finally, 55% of xylose and 51% of arabinose were removed and 52% of total XOS was recovered in the ethanol fraction.

The results suggest that SCW hydrolysis is a promising method to produce XOS from barley bran in a short time with higher recovery than the enzymatic hydrolysis. The obtained XOS has potential use in the functional food products as prebiotics.

Keywords: Arabinoxylan, Barley bran, Nano/ultrafiltration, Subcritical water, Supercritical CO_2, Xylooligosaccharides.

Preface

Financial support for this research was provided by Alberta Pulse Growers, Natural Sciences and Engineering Research Council of Canada (Dr. Saldaña's NSERC Discovery Grant) and Alberta Innovates. This project originated with the idea of Dr. Saldaña to add value to waste or by-products from the food industry using green technologies.

This thesis is an original work by Azadeh Aghashahi and part of the chapters have been presented in conferences. Chapter 3 of this thesis has been presented as: "Aghashahi, A. and Saldaña, M.D.A. (2019) Lipid extraction from barley bran using supercritical CO_2 at the ALES GSA Research Symposium, University of Alberta, Edmonton". I was responsible for the experimental design, performing experiments, data collection and analysis, and drafting the poster. The experimental design and data obtained were discussed with my supervisor Dr. Saldaña. She revised the poster. Also, Chapter 3 has been presented as: "Aghashahi, A. and Saldaña, M.D.A. (2019) Bioactive lipids and protein removal by fractionation of barley bran at the 16th Annual Conference of the Natural Health Product Research Society (NHPRS) of Canada: Growing NHPs for the Future, Edmonton". I was responsible for the experimental design, performing experiments, data collection and analysis, and drafting the poster. Dr. Saldaña helped with the experimental design, data discussion, and revised the poster.

Chapter 3 will be submitted as: “Aghashahi, A. and Saldaña, M.D.A. (2020). Oligosaccharides production from barley bran using sequential supercritical CO_2 extraction and subcritical water hydrolysis to the Journal of Supercritical Fluids”. I was responsible for the experimental design, performing experiments, data collection and analysis. Dr. Saldaña helped me with the experimental design and data discussion.

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NOMENCLATURE

Symbols and abbreviations

ANOVA: Analysis of variance

AXOS: Arabinoxylo-oligosaccharides

AX: Arabinoxylan

DF-DS: Defatted-destarched

DNS: Dinitrosalicylic acid

DP: Degree of polymerisation

EDTA: Ethylenediamine tetra acetic acid

FOS: Fructo-oligosaccharides

HCl: Hydrochloric acid

HMF: Hydroxyl methyl furfural

KOH: Potassium hydroxide

LCM: Lignocellulosic material

MWCO: Molecular weight cut off

NaOH: Sodium hydroxide

NREL: National Renewable Energy Laboratory

OS: Oligosaccharides

POS: Pectic-oligosaccharides

P: Pressure

SCW: Subcritical water

SC-CO_2: Supercritical CO_2

T: Temperature

TFF: Tangential flow filtration

XOS: Xylo-oligosaccharides

X2: Xylobiose

X3: Xylotriose

X4: Xylotetraose

X6: Xylohexaose

Chapter 1: Introduction

1.1. Rationale

Barley *(Hordeum Vulgare L)* is one of the most important crops grown in large amounts worldwide. It is mainly used as animal feed or as a raw material for malt production (Baik and Ullrich, 2008). However, it is considered as a major food source in some regions of Asia and northern Africa (Newman and Newman, 2006). In recent years, barley has grown as an alternative to most commonly used cereals in the food industry. There is a growing interest in barley research due to the existence of non-starch polysaccharides such as mixed linkage (1-3), (1-4)-beta-D-glucans and arabinoxylans (Izydorczyk and Dexter, 2008). In addition, barley contains other valuable dietary fiber components such as cellulose, fructans, galactomannans, and arabinogalactans (MacGregor and Fincher, 1993).

Native barley kernel consists of an embryo and endosperm, which are surrounded by outer tissue layers including aleurone, testa, pericarp and hull (Jadhav et al., 1998). Pearling is a primary process in which pericarp, testa, aleurone, subaleurone layers and germ are removed as bran or pearling flour fraction and the remaining part is called pearled barley. Therefore, barley bran has a complex lignocellulosic–starch structure (Gómez et al., 2005). Barley endosperm is rich in starch, protein and beta-glucan while most arabinoxylans are present in the husk and bran (Kunze, 2010; Yeung and Vasanthan, 2001). Wang (1992) investigated the pearling of waxy and hulless

barley and reported 6-12% lipids in barley bran formed by outer layers of grain. Sullivan et al. (2010) investigated the chemical composition of whole grain barley flour and bran. These authors reported higher total starch content in flour (76.80%) than in the bran fraction (36.80%). Also, in this study, flour had 3.5% total fiber (1.9% soluble and 1.6% insoluble fiber), while bran had 37.6% total fiber (11% soluble and 26.6% insoluble fiber). The flour had lower protein content (5.41%) than the bran (10.47%). Barley grain has 8-13% (w/w) protein, but different protein types are present in barley grain tissues (Pomeranz and Shands, 1974). Barley endosperm contains mainly hordein (35–45%) and glutelin (35–45%) proteins, while cytoplasmic proteins (mainly albumin and globulin) are mostly present in the bran and germ (Lâsztity, 1984).

Currently, barley bran rich in protein, carbohydrates and lipids is a by-product and has been used for the extraction of lipids. Solvent extraction of lipids from different by products using hexane or petroleum ether as a traditional method has been reported in the literature (Devittori et al., 2000; Kok and Dowd, 1998). Supercritical CO_2 (SC-CO_2) is an alternative green extraction method, in which carbon dioxide is used as a solvent above its critical temperature (31.1°C) and pressure (73.8 bar). CO_2 is non-toxic, recyclable, cheap, non-flammable and can easily be separated from the extracts. Extraction of cocoa butter from Brazilian cocoa beans using supercritical CO_2 and ethane was reported by Saldaña et al. (2002). Also, extraction of lipids from different cereal products and oilseeds including millet bran (Devittori et al., 2000), rice bran (Sparks et al., 2006), and barley pearling flour (Temelli et al., 2013) was reported.

The other component in barley bran is arabinoxylans (AX), which are polymers composed of pentose sugars, known as pentosans. They are the major hemicellulosic component in the cell wall of cereal plants, such as wheat, rice and barley. Wheat, rye, barley and oat bran has 9.0–18%, 12.1–14.8%, 4.8–9.8% and 4.0–13.0% db of total arabinoxylan, respectively (Andersson et al., 2008). Arabinoxylans are often water-unextractable in the bran due to the strong interactions with other cell wall components such as proteins and lignin and only a small part of them (6%) is water-extractable (Maes and Delcour, 2002). Arabinoxylans are composed of a linear backbone of (1→4)-linked β-D-xylopyranose units, with attached residues of α-L-arabinofuranose to either O(2) or O(3) of xylose or to both O(2) and O(3). Phenolic compounds, mainly ferulic acid (FA), can further substitute the arabinose residues in the C-5 position (Rattan et al., 1994; Izydorczyk and Biliaderis, 1995).

Arabinoxylans hemicellulose has a potential value as a food ingredient, either as a dietary fiber or converted into different value-added biomolecules such as prebiotic xylo-oligosaccharides (XOS) and arabinoxylo-oligosaccharides (AXOS) (Broekaert et al., 2011). The XOS and AXOS can be produced from polymeric AX using acid hydrolysis, hydrothermal treatment, extensive dry ball milling or enzymatic hydrolysis (Sun et al., 2002; Falck et al., 2014). Endo-1,4-β-xylanase is the most commonly used enzyme in order to breakdown β-1,4-linked xylan. Therefore, water-unextractable arabinoxylans can be solubilized, and further released as solubilised AX. The enzyme breaks the AX to XOS and AXOS with different lengths and structures (Courtin et al.,

1999). Traditionally, extraction of hemicelluloses from cereal and other plant biomass is carried out using alkaline treatment. But alkaline treatment leads to the removal of acetyl, uronic acid and phenolic substitutions, which are valuable compounds (Egüés et al., 2014). Other methods have also been used for the extraction and purification of AX from cereal by-products, including acid pretreatment of barley husk with the AX yield of 26.1% (Höije et al., 2005), ultrasound assisted extraction from corn cob with the yield of 28.8% (Ebringerová et al., 1998) and steam pretreatment of barley husk with the yield of 25% (Persson et al., 2009). Different approaches have been used to produce XOS from xylan-rich substrates including: i) enzymatic degradation of xylan, and ii) physical, physico-chemical or chemical degradation of xylan. Zhao and Dong (2016) extracted xylan from wheat bran using alkaline treatment at pH 9 and further hydrolyzed it with endo-xylanase, obtaining 57.55% XOS with degree of polymerization (DP) 2–4 after membrane filtration using 20 and 1 kDa cutoff membranes sequentially.

An alternative method for the production of XOS is subcritical water (SCW) processing, also known as high temperature water, superheated water or hot liquid water. This technology is considered as a promising green hydrothermal treatment in which water is used at temperatures between its boiling and critical points (100 and 374°C, respectively). Enough pressure below the critical point (221 bar) is also needed to maintain water in the liquid phase (Moran and Shapiro, 2006). In SCW technology, water polarity decreases by increasing the temperature under pressure, therefore it can extract selectively polar and non-polar organic compounds of different biomass

matrices (Alvarez et al., 2014). Moreover, fast, homogeneous and efficient reactions can take place in SCW due to the formation of ionic products and decrease in the dielectric constant, viscosity, and density of water (Kruse and Dinjus, 2007). Subcritical water hydrolysis showed promising hydrolysis of hemicellulose polymers from biomass with low cost and no use of chemicals. Also, this method allows extraction of hemicelluloses as oligomers rather than monomeric sugars (Saha, 2003). SCW hydrolysis of hemicellulose to oligomers from wheat bran (Ruthes et al., 2017), triticale, wheat, barley, oat, canola, and mustard straws (Pronyk and Mazza, 2012), corn cob (Makishima et al., 2009), and bagasse (Sukhbaatar et al., 2014) was reported. Ciftci and Saldaña (2015) investigated the hydrolysis of sweet blue lupin hull to obtain hemicellulose sugars using SCW. They reported 25.4% of hemicellulose (12.8, 6.7, 4.1, and 1.8% of xylose, galactose, arabinose and mannose, respectively) in the raw material. In that study, maximum hemicellulose sugar recovery was 85.5% in the extracts obtained at 180°C, 50 bar, flow rate of 5 mL/min, and pH 6.2. Lu et al. (2009) studied the decomposition of Japanese beech by semi-continuous hot-compressed water at 200–230°C/100 bar and reported the hydrolysis of 55.1% of initial hemicellulose and obtaining XOS with 14.1% in the water soluble portion.

The oligosaccharides obtained after hydrolysis is a mixture of different compounds. Therefore, further purification is needed to obtain the crude oligosaccharides. Vacuum evaporation is a suitable method that removes acetic acid and flavours from the product, increasing the concentration of oligomers (Eden et al., 1998). Membrane techniques have been studied to remove

oligosaccharides with undesired DP range and non-saccharide compounds. Zhao and Dong (2016) used ultrafiltration (20 kDa) and nanofiltration (1 kDa) to purify xylo-oligosaccharides with DP 2-4 obtained from wheat bran. Rico et al. (2018) investigated the application of nanofiltration using a 0.3 kDa membrane for the XOS purification with DP 2-17 from autohydrolysis of peanut shells to remove low molecular weight compounds such as monosaccharides, acetic acid, and some non-saccharide compounds.

To date, there are no studies using barley bran to obtain XOS with this green technology. Therefore, barley bran rich in xylan and arabinan will be used to produce XOS as value added products.

1.2. Hypothesis

It is hypothesized that fractionation of barley bran can be performed using SC-CO_2 for lipid extraction followed by enzymatic and alkaline treatments for starch and protein removal, respectively. It is also hypothesized that SCW can be used as an alternative technology for the hydrolysis of defatted, destarched and deproteinized bran to obtain XOS. It is hypothesized that membrane separation can be effective to purify SCW hydrolysates and obtain XOS with less impurities.

1.3. Objectives

The main objective of this study was to hydrolyse the hemicellulose fraction of barley bran to obtain oligosaccharides using SCW and separate oligomers with desired DP range. To achieve this main objective, some specific objectives were to:

- Evaluate of process parameters including time, pressure and temperature on lipid extraction yield from raw barley bran using supercritical CO_2 (Chapter 3).
- Remove starch from defatted bran using enzymatic hydrolysis by incubation with alpha-amylase for 40 min at 98-100°C followed by amyloglucosidase at 60°C for 30 min (Chapter 3).
- Remove protein from defatted-destarched bran using alkaline treatment with NaOH solution (Chapter 3).
- Study the effect of parameters including temperature and time on the generation of XOS from defatted-destarched bran using SCW technology (Chapter 3).
- Study the ability of endo-1,4-β-xylanase to hydrolyze defatted, destarched and deproteinized bran to produce XOS with two concentrations of enzyme 2.5 and 10 U for different incubation times 1, 4, 8 and 16 h (Chapter 3).
- Compare SCW hydrolysis with traditional enzymatic approach based on the recovery of XOS obtained (Chapter 3).

- Purify XOS obtained from SCW hydrolysis using ultrafiltration with 3 and 1 kDa cutoff and quantification (Chapter 4).
- Purify XOS obtained from SCW hydrolysis using nanofiltration and activated carbon adsorption (Chapter 4).

Chapter 2: Literature review

2.1. Barley grain

The most important cereal crops produced in Canada are wheat, canola, soy bean, corn, oat and barley with 8.4 million tonnes of barley harvested in 2018 (Statistics Canada, 2018).

2.1.1. Classification and uses

Barley (*Hordeum vulgare L.*), a cereal grain with a huge production worldwide (Baik and Ullrich, 2008), is mostly used in malting and the feed industry (Newman and Newman, 2006). Barley grain is also converted to pot barley by abrasive action and may be further processed to grits, flakes and flour (Chatterjee and Abrol, 1977). Whole, pearled, flaked and ground barley are used in breakfast cereals, stews, soups, porridge, bakery flour blends and baby foods (Bhatty, 1993). In addition, pearled barley flour can be combined with wheat based products such as bread, cakes, cookies, noodles and extruded snack foods (Newman and Newman, 1991).

Barley is classified into different groups including spring or winter, two rowed or six rowed and hulled or hull-less, depending on the presence or absence of hull attached to the grain. Also, depending on the grain composition, barley is classified into normal, waxy or high amylose starch, high beta-glucan and proanthocyanidin free types (Baik and Ullrich, 2008).

2.1.2. Structure and chemical composition

The structure of barley grain is similar to other cereals like wheat. It is composed of endosperm, the embryo and outer covering layers. The endosperm is made up of starchy endosperm and a surrounding aleurone layer. The starchy endosperm is the largest morphological part of the barley kernel accounting for 75% of the whole grain weight (Evers and Millar, 2002). The outer layers of barley including testa, pericarp and hull embrace the endosperm (Yeung and Vasanthan, 2001). Hull is composed of two distinct overlapping structures named lemma and palea, which are tightly attached to the pericarp layer by a cementing layer (Olkku et al., 2005). Fig. 2.1 illustrates the anatomy of barley kernel. Whole barley grain contains about 65–68% starch, 10–17% protein, 4 9% beta-glucan, 2–3% lipids and 1.5–2.5% mineral (Czuchajowska et al., 1998; Izydorczyk et al., 2000; Quinde et al., 2004). Total dietary fiber content ranges from 11 to 34% and soluble dietary fiber content from 3 to 20% (Fastnaught, 2001). The endosperm contains starch granules embedded in a protein matrix (Fincher and Stone, 1986).

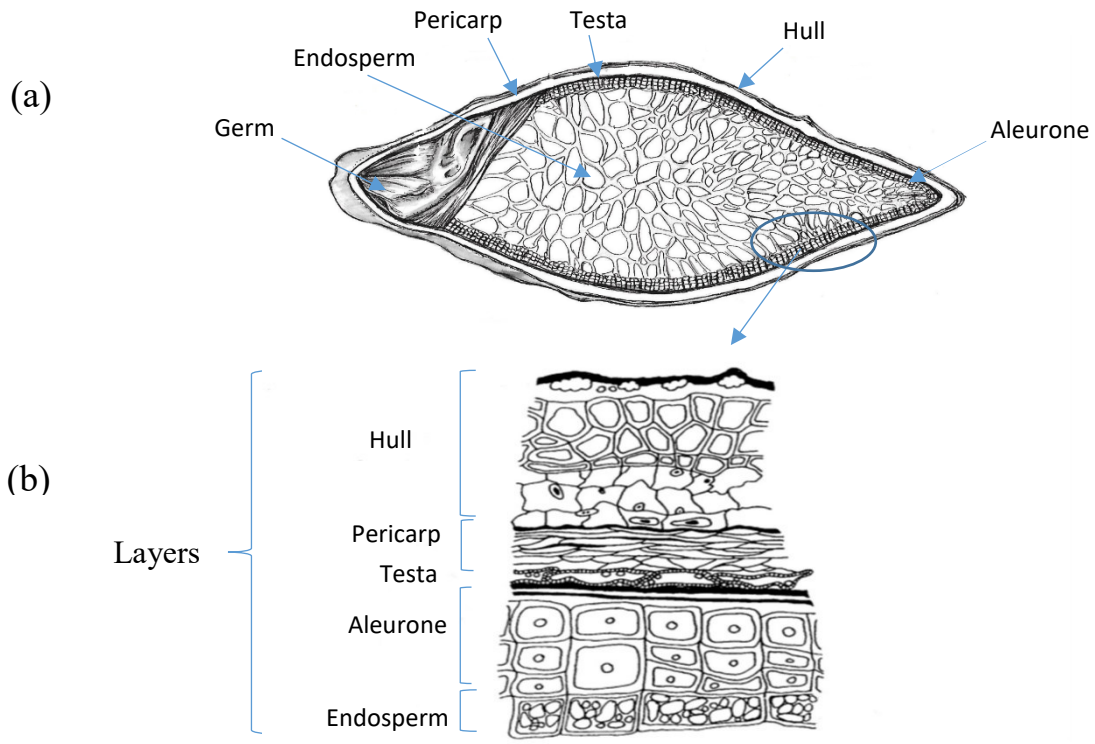


Fig. 2.1. Anatomy of barley grain: (a) whole grain and (b) layers (Adapted from Kent, 1983).

2.2. Barley bran

Bran, composed of the outer tissues of barley, including pericarp, testa, aleurone, subaleurone layers and germ are removed from the whole kernel by the pearling process. The remaining part is called pearled grain (Yeung and Vasanthan, 2001). Pearling is usually carried out after dehulling in which the hull is separated from the grain.

2.2.1. Structure

Barley bran is composed of pericarp, testa, aleurone, subaleurone layers and germ. The pericarp covering the testa consists of different cell layers including crushed cells, cross cell layers and a tube cell layer. The pericarp is divided into outer and inner layers. Mostly, the outer layer consists

of remnants of thin-walled cells and the inner layer is made up of intermediate cells, cross cells, and tube cells. The seed coat is the testa attached to the cuticle of the epidermis. It is one layer in barley responsible for the impermeability of the grain to water over its surface. The thickness of seed coat is about 5 to 8 μm (Evers and Millar, 2002). On the other hand, the aleurone layer is the outer layer of the endosperm that surrounds both starchy endosperm and the germ (embryo). The aleurone cells consist of a nucleus and aleurone granules rich in ash, protein, minerals, ferulic acid and lipid. Barley has three layers of aleurone cells, while other cereals like wheat and maize have just one layer (Pomeranz, 1973; Fincher, 1976).

2.2.2. Chemical composition

The main components of barley bran are non-starch polysaccharides, protein, lipid and lignin. It has also been considered as a lignocellulosic material (LCM). LCM is composed of three types of polymers: lignin (with phenolic nature), cellulose (a linear polymer made of glucose units linked by β,1-4 glycosidic bonds) and hemicellulose (branched hetero-polysaccharides that consist of various monosaccharides such as xylose, arabinose, mannose, glucose and rhamnose) (Garrote et al., 1999). Depending on the nature of the LCM, polymers of xylose (xylan), arabinose (arabinan), mannose (mannan) and glucose (glucan) are the main hemicellulosic components, which can be substituted via ether or ester bonds (Ebringerova and Heinze, 2000). Different types of hemicelluloses originating from various plant materials, including glucuronoxylan (Spiridon and

Popa, 2008), glucomannan (Teleman et al., 2003; Laine, 2005), xyloglucan (Ebringerova et al., 2005), arabinoglucuronoxylan (Shi et al., 2011), and arabinoxylan (Persson et al., 2009) have been reported in the literature. Arabinoglucuronoxylan, which is composed of a backbone of β-(1-4) linked xylopyranose units attached with different substitutions like 4-*O*-methyl-D-glucuronic acid and α-L-arabinofuranose units were found in the cereals. Also, arabinoxylan with a backbone of β-(1-4) linked xylopyranose units attached to α-D-glucopyranosyl uronic units, α-L-arabinofuranose and *O*-acetyl substitutes has been reported as the major component of the cell walls of cereals (Persson et al., 2009; Peng et al., 2009). Xylans of many plant materials are heteropolysaccharides composed of xylose backbone with different branching substitutions including L-arabinose, D-glucose, D-galactose, D-mannose, D-glucuronic acid, 4- O-methyl glucuronic acid, D-galacturonic acid, ferulic acid, and acetic acid residues, and smaller amounts of L-rhamnose, L-fucose, and various O-methylated neutral sugars (Sun et al., 2000).

Cruz et al. (2000) reported 23% cellulose, 27% hemicellulose, 21% lignin and 29% others (oven-dry basis) for the composition of barley bran. According to Izydorczyk and Dexter (2008), arabinoxylan and mixed linkage beta-glucan are the major non-starch polysaccharides in various tissues of barley. They reported the neutral monosaccharide composition as 3, 2.8, 33.7, 30.1, and 30.4% of total polysaccharides for galactose, mannose, arabinose, xylose and glucose and major polysaccharide components as cellulose 25.8%, beta-glucan plus arabinoxylan 63.8% in the

pericarp of barley (AC Metcalfe). However, there was less amount of arabinoxylan (11.8%) and cellulose (2%), and a higher amount of beta-glucan (62.1%) in the starchy endosperm.

The chemical composition of bran relies on the degree of pearling, which is defined as the amount of kernel removed through the pearling process. Klamczynski et al. (1998) found that starch and beta-glucan contents increased in the pearled grain after pearling. Bhatty and Rossnagel (1998) reported a reduction in total dietary fiber, ash, and protein contents and an increase in the starch and soluble fiber contents after 50% pearling of Canadian and Japanese barley. Significant amounts of protein and minerals (ash) are reported in the outer layers removed from barley grain (Klamczynski et al., 1998). Yeung and Vasanthan (2001) investigated the impact of pearling process on the composition of removed outer grain layers and the remaining fractions from Regular (Phoenix) and Waxy (Candle) hull-less barley. Samples were pearled to 10-80% (w/w) degree of pearling using a testing mill fitted with an abrasive roller. These authors reported that, regardless of the degree of pearling starch content decreased in the outer grain layers fraction compared to the whole grain and remaining fraction, meanwhile it increased as the degree of pearling increased gradually from 10 to 80%. However, beta-glucan content decreased in the outer grain layers fraction as pearling increased from 10 to 32%, but it increased with the increasing degree of pearling from 32% to 80%. All outer grain layer fractions had more protein, lipid and ash contents than the whole barley and pearled grain in both varieties. The highest protein amount was reported for pearling flour removed after 23-25% pearling with 23.9±0.5 and 22.7±0.6% db for Phoenix

and Candle, respectively. Such results indicate that ash, protein and lipid are concentrated in the outer layers, while starch and beta-glucan are mainly present in the inner tissues of barley grain. Similar composition pattern was reported by van Donkelaar et al. (2015), who removed 5, 10, 15 and 25 wt% of the whole barley kernel by pearling and prepared fractions 1-4, respectively, 75% of the original kernel weight remained and made the 5th fraction. Microscopic images were used to identify starch and fibrous fragments in all these fractions. Table 2.1 shows the chemical components present in the whole barley and different fractions obtained by the pearling process. They reported fewer starch granules and more fibrous material in the outer layers. Fractions 3 and 4 had higher amounts of protein, indicating high protein content in the aleurone layer of barley grain. Also, beta-glucan was mostly present in fraction 4 due to the existence of thick cell walls composed of beta-glucan and arabinoxylans (Jadhav et al., 1998). Wang et al. (1997) reported similar results for starch (58.7% db) and protein (11.7% db) contents of whole barley grain, and removal of 32.6 wt% of the kernel after pearling process led to the higher concentrations of starch (71.5% db) in the pearled grain and protein (16.2% db) in the pearling flour, compared to the initial whole grain. van Donkelaar et al. (2015) also reported the highest concentration of fat (7.2±0.3 %db) in fraction 3, due to the existence of germ in this part. Marconi et al. (2000) reported similar data about fat concentration. Fractions 1 and 2 had more ash and insoluble fiber (cellulose, lignin and pentosane) compared to the other fractions and whole kernel due to the presence of the hull

layer. These authors found that fractions 2 and 3 had the highest amount of total arabinoxylan with 21.3±25 and 17±9 (% db), respectively.

Table 2.1. Composition of minor components of whole barley and barley fractions (Adapted from van Donkelaar et al., 2015).

Fraction	Starch (% db)	Beta-glucan (% db)	Protein (% db)	Fat (% db)	Ash (% db)	IF (% db)	A+X (% db)
Fraction 1	2.5	0.3	5.7	1.2	7	82.9	14.8
Fraction 2	7.8	1.3	10.8	3.7	6.59	68.8	21.3
Fraction 3	24.3	3.1	18.3	7.2	5.78	39.5	17
Fraction 4	44.6	4.9	17	4.2	3.79	23.9	9.1
Fraction 5	77.2	4.5	8.2	0.9	0.85	6.3	2.3
Whole barley	62.7	3.6	9.8	1.3	2.2	18.2	6.4

db: Dry basis; IF: Insoluble fiber; A: Arabinose; X: Xylose

Zheng et al. (2011) studied arabinoxylan and beta-glucan concentration patterns in three different fractions of six varieties of hull-less barley. These authors separated the outer layers as bran, aleurone and subaleurone layers known as short, and the remaining part of the kernel made the flour part. Beta-glucan content was 8.12–13.01%, 6.15–7.58%, 2.48–2.95% and 4.96% to 7.62% db for short, bran, flour and whole barley, respectively. While arabinoxylan was mostly present in the bran fraction (7.99–9.59%) compared to the short (2.29–3.86%), flour (1.2–2.29%) and whole barley (2.97–4.73% db). According to Bhatty (1997), beta-glucan concentration depends on the variety of the barley kernel. They reported that beta-glucan was mostly present in the subaleurone layer for low beta-glucan content hull-less barley, while in barley that contains high amounts of beta-glucan, it is present more uniformly in the endosperm. Knuckles and Chiu (1995) and

Rolando et al. (2005) reported the existence of most beta-glucan in the aleurone layer. Izydorczyk et al. (2003) investigated the composition and structural properties of three fractions of barley kernel. These authors used Falcon (normal), CDC Candle (waxy), and CDC 92-55-06-48 (high-amylose) hull-less barley cultivars from western Canada. Barley samples were pearled and 10% of the outer layers of the kernel were removed. Then, pearled grain was ground after passing four corrugated rolls, followed by sieving to obtain flour and coarse fractions (>600 µm and >183 µm). The coarse fraction (>600 µm) was passed through a shorts duster followed by sieving on a 183 µm sieve to obtain flour and a coarser fraction, which made up the bran. Another coarse fraction (>183µm) passed through a shorts duster and sieved on a 183µm, followed by passing a sizing passage, then a coarse fraction was obtained as fiber rich fraction. Fig 2.2 presents the process to obtain different fractions from barley.

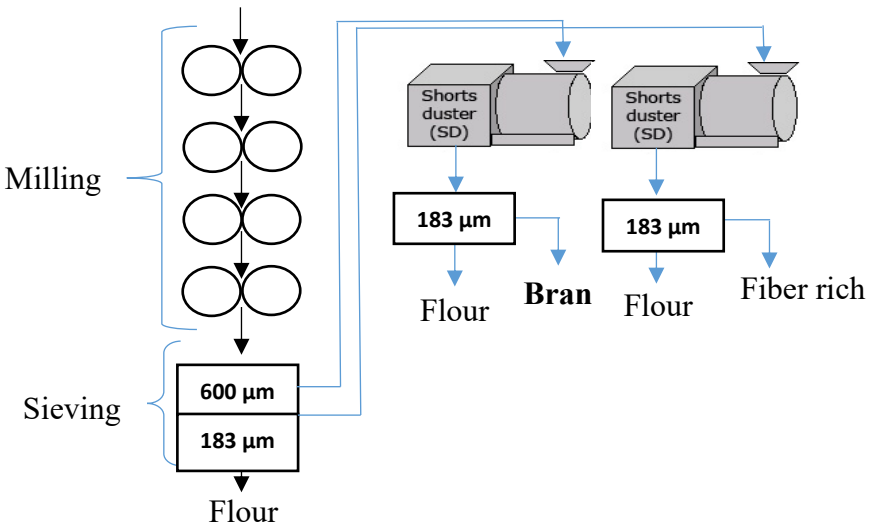


Fig. 2.2. Barley milling flow; SD: shorts duster; bran and fiber-rich fraction.

The highest amounts of total arabinoxylan, protein and ash contents were in the bran compared to the flour, fiber fraction and the whole grain for all three barley varieties. Total arabinoxylan was determined for high amylose, normal and waxy barley brans as 14.27±0.72, 17.21±0.18 and 18.47 ±0.19%, respectively. Also, whole normal and waxy barley and their brans had higher amounts of arabinoxylan than high-amylose barley and its bran fraction, while fiber fraction had the highest amount of total beta-glucan compared to the flour and whole kernel for all three barley varieties.

2.3. Fractionation of barley bran

Barley bran is a by-product and complex material composed of protein, lipid, carbohydrates (non-starch polysaccharides and starch) and ash. The removal of proteins, oil and starch can be achieved through fractionation of the bran, leading to obtain pure non-starch polysaccharides rich in arabinoxylan that can be used for the production of valuable compounds and nutraceuticals.

2.3.1. Extraction of lipids

Bran lipid is a valuable ingredient for food, nutraceutical, pharmaceutical and cosmetic applications (Alonso, 2018). Conventionally, lipid extraction is performed using chemical solvents such as hexane and petroleum ether. These techniques usually need long extraction times and produce large amounts of toxic solvent wastes (Brooks et al., 1998). An alternative technique is supercritical carbon dioxide (SC-CO_2) extraction in which CO_2 is used as a solvent at pressure and temperature above its critical point (31°C and 74 bar). Some properties of CO_2 including density,

viscosity, diffusivity, heat capacity and thermal conductivity change at supercritical conditions. SC-CO_2 has a strong ability to solubilize compounds due to its high density and great penetration power into the solid matrix by low viscosity (Dunford et al., 2003; Saldaña et al., 2002). Some advantages of SC-CO_2 extraction are as follows:

- CO_2 is nontoxic, nonflammable, and low cost.
- Due to the low critical temperature of CO_2, extractions can be performed at mild temperatures, leading to less degradation of heat-sensitive components to obtain extracts with natural properties (Dron et al., 1997).
- CO_2 is released as a gas after extraction through pressure reduction, therefore there is no solvent left in the final product (Henning et al., 1994).
- SC-CO_2 has high mass transfer, leading to fast extraction and high recovery (Wheeler and McNally, 1989; Henning et al., 1994).
- The crude oil obtained by SC-CO_2 has less impurities compared to the conventionally solvent extracted oil (Devittori et al., 2000).

Jung et al. (2012) extracted oil from wheat bran using SC-CO_2 and compared its quality with the oil obtained by hexane extraction. They observed lower acid value (AV), peroxide value (POV) and higher radical scavenging activity in SC-CO_2 extracted oil, which indicates its better quality. Kwon et al. (2010) also investigated SC-CO_2 extraction of oil from wheat bran at temperatures

ranging from 40 to 60ºC and pressures ranging from 100 to 300 bar, with a CO_2 flow rate of 26.81 g/min. At a constant temperature from 40 to 60ºC, they reported an increase in extracted oil from bran with increasing pressure from 100 to 300 bar. Similarly, at constant pressure from 100 to 300 bar, the oil yield increased with temperature rise from 40 to 60ºC due to the increase of oil components vapour pressure and its predominance over the drop in solvent density. They obtained the highest amount of oil at 60ºC and 300 bar with a yield of 2.69 g/12 g of wheat bran. Soares et al. (2016) studied the effect of pressure (150-250 bar) and temperature (40-80°C) on the extraction yield of rice bran oil using SC-CO_2. In that study, total oil content of the raw material was 15.44±0.11 wt%, which was determined by Soxhlet extraction using hexane. They showed an increase in extraction yield with pressure rise from 150 to 250 bar at 40 and 80°C due to the increase in density and solvating power of CO_2, resulting in solubility enhancement of rice bran oil. However, when the temperature increased from 40 to 80°C at 150 and 250 bar, the extraction yield of oil decreased due to the reduction of CO_2 density and solvation power. They reported the highest yield (12.68 wt%) and oil recovery (82.12 wt%) obtained at 40°C and 250 bar. The decrease in extract amount with increasing temperature at low pressures indicates a crossover behavior of the solubility isotherms, which was reported by Dunford and Temelli (1997), who reported a decrease in the amount of extract from canola flakes at 207 bar and increase at 414 and 620 bar with increasing temperature.

2.3.2. Starch and protein removal

Starch is one of the most abundant carbohydrates in barley grain and it ranges from 62 to 77% of the grain dry weight (Bhatty and Rossnagel, 1998). However, starch is mostly concentrated in the barley endosperm. Bran, which is composed of outer layers, contains less amount of this polysaccharide. This polysaccharide is composed of glucose monomers connected with α-(1-4) and α-(1-6) linkages. Starch is made up of a mixture of amylose (15-20%), a linear chain polymer, and amylopectin (80-85%), which is a branched molecule. Removal of starch was performed using an enzymatic approach, including alpha-amylase hydrolysis (Yu et al., 2018), and a combination of alpha-amylase and amyloglucosidase hydrolysis of barley bran (Karimi et al., 2018). Enzymatic starch removal using alpha-amylase was based on the hydrolysis of α-(1-4) linkages and produced maltose, maltotriose and branched dextrins. Further, they are converted to glucose by amyloglucosidase and non-starch polysaccharides are precipitated with ethanol, followed by its separation from the liquid and drying to obtain a destarched product.

Removal of protein was also investigated using enzymatic hydrolysis with protease in wheat bran (Swennen et al., 2005) and rye bran (Flack et al., 2014). Another approach is alkaline treatment using NaOH reported for protein removal from barley (Wang et al., 2010; Song et al., 2012). In lignocellulosic materials, protein is attached to the hemicellulose via covalent bonds. Protein can be solubilized with alkali and further precipitated at isoelectric pH (Zhu et al., 2010). Song et al.

(2012) reported the removal of protein from barley bran using alkaline treatment (3 N NaOH) at pH of 9.5 for 1 h. They centrifuged the solution and adjusted the pH of supernatant to 4.5 with (2 N HCl) to precipitate the protein. They recovered 33 g of protein from 100 g of barley bran. Solubilization and removal of protein from barley outer layers (mainly bran and germ) was also reported by salt and alkaline solutions. Wang et al. (2010) reported the maximum recovery of protein (64% of initial protein content) using alkaline solution (0.5M NaOH) at pH of 11.5. The protein recovery using salt solution (1M NaCl) was 25-30%, which was lower compared to the protein recovery using alkaline solution (64%).

2.4. Oligosaccharides

According to the international carbohydrate community, oligosaccharides are carbohydrate polymers composed of 2-10 monosaccharide units linked by O-glycosidic bonds (Roberfroid and Slavin, 2000), although carbohydrates with 2-20 monosaccharide units are also considered as oligosaccharides according to Rohrer (2002).

2.4.1. Classification

Oligosacharides are classified into two groups. Primary oligosaccharides are synthesised from a mono or oligosaccharide and a glycosyl donor by the action of a glycosyl transferase (Kandler and Hopf, 1980). Secondary oligosaccharides are those formed by hydrolysis of higher oligosaccharides, polysaccharides, glycoproteins, and glycolipids.

2.4.2. Physicochemical properties of oligosaccharides

Oligosaccharides are water soluble and their sweetness is 0.3–0.6 times as sucrose. The sweetness of the oligosaccharide product relies on the chemical structure, the degree of polymerization and the presence of mono and disaccharides in the product mixture (Playne and Crittenden, 1996); Voragen, 1998). Oligosaccharides with longer chain length have less sweetness than the molecules with shorter chain. Therefore, due to the low sweetness of oligosaccharides, they can be used in the food products with the restriction of sucrose usage (Roberfroid and Slavin, 2000). Compared with mono- and disaccharides, oligosaccharides can provide more viscosity, improved body and mouthfeel of food products due to their higher molecular weight, compared to mono- and disaccharides (Playne and Crittenden, 1996).

2.4.3. Health benefits of oligosaccharides

Oligosaccharides are non- digestible carbohydrates and cannot be hydrolyzed by human intestinal digestive enzymes due to the arrangement of anomeric carbon atoms (C1 or C2) of the monosaccharide units and the nature of their glycosidic bonds (Roberfroid and Slavin, 2000). Therefore, they have potential use as prebiotics that are fermented in the colon by anaerobic microorganisms to release energy, metabolic substrates, lactic and short-chain carboxylic acids (Quigley, 2010). Besides, they promote the growth of probiotics that are live microorganisms associated with health benefits on the host such as *Bifidobacteria* and *Lactobacilli* (Qiang et al.,

2009). They improve the intestinal absorption of minerals such as calcium, magnesium and iron. They have beneficial effect on the carbohydrate and lipid metabolism, leading to a decrease in cholesterol, triglyceride and phospholipid concentrations in the blood, reducing the risk of diabetes and obesity (Mussatto and Mancilha, 2007). Oligosaccharides have been studied widely both as food ingredients and pharmacological supplements. Many studies showed that arabinoxylan oligosaccharides (AXOS) have antidiabetic effect due to the decrease of blood glucose level and insulin response (Charalampopoulos et al., 2002; Grootaert et al., 2007; Muzzarelli, 2009). Wang et al. (2011) showed that supplementation of 5% wheat bran xylo-oligosaccharides in the high fat diets can be effective in controlling body weight, improving blood glucose and lipid levels of blood in rats.

2.4.4. Production of oligosaccharides

Bioactive oligosaccharides are produced by either synthesis (with enzymatic or chemical approach) or polysaccharide depolymerization (with physical, chemical or enzymatic methods).

2.4.4.1. Chemical and biochemical synthesis of oligosaccharides

The chemical or biochemical synthesis of oligosaccharides is difficult to control compared to the synthesis of other biopolymers such as peptides and nucleic acids due to the existence of monomeric unit combinations (Barreteau et al., 2006). The chemical glycosylation reaction occurs

between a glycosyl donor and a glycosyl acceptor with only one free hydroxyl group (Barreteau et al., 2006). Fig. 2.3. illustrates the steps of chemical synthesis of oligosaccharides.

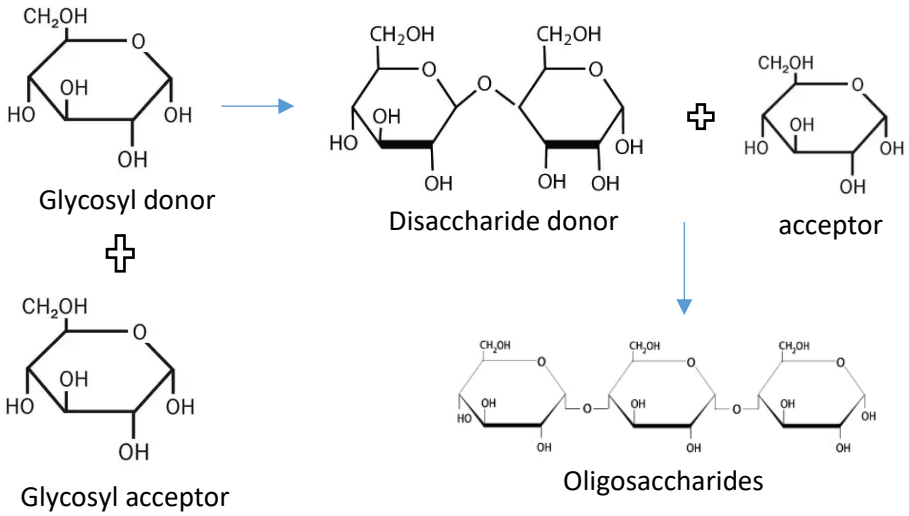


Fig. 2.3. Chemical synthesis of oligosaccharides (Adapted from Barreteau et al., 2006).

2.4.4.2. Enzymatic glycosylation

Large scale oligosaccharides production is performed using enzymes, including glycosidases and glycosyltransferases. Glycosyltransferases catalyse the transfer of sugar units from activated donor molecules to acceptor molecules, forming glycosidic bonds. Many bioactive oligosaccharides are formed by the enzymatic approach such as fructo-oligosaccharides (FOS) from sucrose using fructosyltransferase (Sangeetha et al., 2005), galactosyl-oligosaccharides from lactose using beta-galactosidase (Czermak et al., 2004) and gentio-oligosaccharides from glucose by transglycosylation (Playne and Crittenden, 1996).

2.4.4.3. Polysaccharide depolymerization

Oligosaccharides are produced using chemical treatments like acid hydrolysis. Enzymatic hydrolysis of polymers is also employed as the main method to produce oligomers (Barreteau et al., 2006). Xylo-oligosaccharides (XOS) are the most common oligomers produced at industrial scale by the hydrolysis of polysaccharide xylan, which is obtained from lignocellulosic materials such as hardwoods, corn cobs, straws, bagasse, hulls, malt cakes and bran. Different methods are used to produce this type of oligosaccharides, including: i) isolation of xylan from lignocellulosic material by chemical treatments, and further enzymatic hydrolysis of xylan to xylo-oligosaccharides; and ii) hydrolysis of xylan to xylo-oligosaccharide by water or dilute mineral acid solutions (Vázquez et al., 2000).

2.4.4.4. Hydrothermal production of oligosaccharides from hemicellulosic biomass

Hemicelluloses are considered the second abundant polysaccharide in lignocellulosic materials. They are composed of various polymers, including xylans, mannans, xyloglucans, arabinogalactans, galactomannans, or mixed β-glucans (Gullon et al., 2014). These compounds have amorphous structure and connect the cellulose and lignin in the biomass (Bajpai, 2016). They have potential for use as feedstocks to produce novel valuable oligosaccharides (Moniz et al., 2016). There is a growing interest for this component due to unexplored properties and

applications. Moreover, few hemicellulosic oligosaccharides are produced in the industry, for instance commercial xylooligosaccharides are manufactured by Suntory Ltd. in Japan using a combined alkaline extraction of hemicelluloses from biomass and enzymatic hydrolysis, which leads to a poor substitution pattern such as glucose or arabinose chains in the obtained oligosaccharides (Moniz et al., 2016). Hydrothermal technologies are suitable to obtain valuable compounds from biomass in which water is used at high temperatures and pressures without the addition of catalysts. Hemicellulose is selectively solubilized by hydrothermal processing (160-200°C, 30-50 bar, 5-60 min,) and oligosaccharides with a wide distribution of degree of polymerization (DP) and high variety of substituents are obtained (Moniz et al., 2016). In addition, hydrothermal treatment is an environmentally friendly process because only water and feedstock are reagents with no other chemicals added (Moure et al., 2006). However, besides hemicellulose thermal solubilization, various side reactions occur, including saponification of acetyl groups, removal of extractives such as waxes, phenols, aromatics and other chemical components that are not chemically bound to components of biomass, solubilization and removal of ash, removal of some acid-soluble lignin fractions, Maillard reaction between proteins and sugars and formation of new products. Therefore, the final product is composed of different complex materials with undesired and non-saccharide compounds and further purification is needed to refine hydrothermal liquors and improve the purity of oligosaccharides before their application (Vegas et al., 2004; Garrote et al., 2007). Autohydrolysis, subcritical hydrolysis and supercritical hydrolysis are the

most studied hydrothermal techniques for the production of value-added compounds from cereal by-products. Using autohydrolysis, biomass was treated with hot water at high temperature (150–220°C), pressure (5–20 bar), and solvent/feed ratio (5–15 mL/g) (Ruiz et al., 2017). Earlier, steam explosion has been used for the pretreatment of biomass before xylan extraction and enzymatic hydrolysis.

2.5. Subcritical water technology

In the subcritical water (SCW) process, water is used as a solvent at temperatures and pressures above its boiling point (100°C, 1 bar) and below its critical point (374°C, 221 bar). Applied pressure helps to maintain water in the liquid state. Fig. 2.4 illustrates the phase diagram of water.

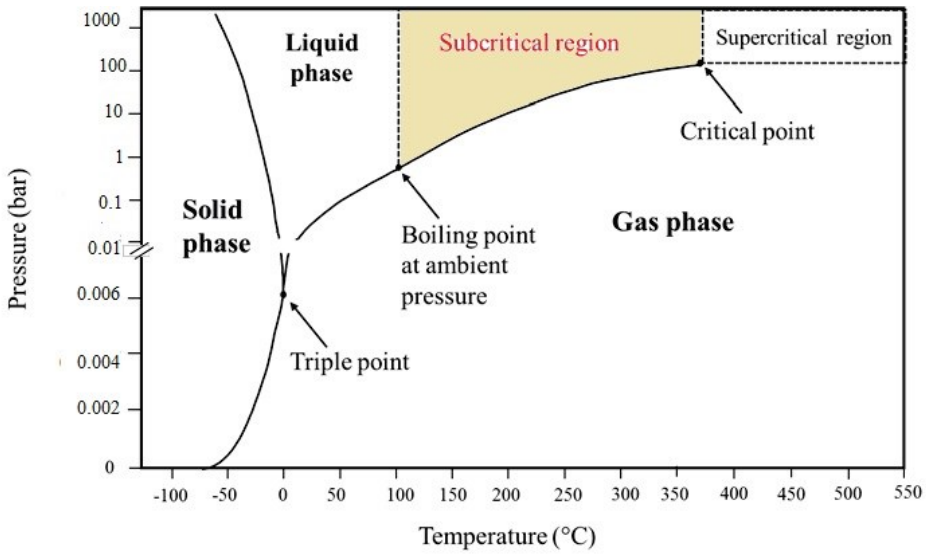


Fig. 2.4. Phase diagram of water (Adapted from Morphy and Koop, 2005; Brunner, 2014).

Table 2.2 shows physical and chemical properties of subcritical water compared with water at ambient condition. The physicochemical properties of water, such as the density, viscosity,

dielectric constant and ionic product, changes considerably under subcritical conditions due to the increase of temperature and pressure compared with the properties under normal conditions. Due to the disruption of hydrogen bonds and reduction of electrostatic interactions between the water molecules, and between water and surrounding molecules, its dielectric constant (ε) decreases from 78.5 to 27.1 and 18.2 by increasing the temperature from 25°C (1 bar) to 250°C (50 bar) and 330°C (300 bar) (Moller et al., 2011). Therefore, this dielectric constant becomes similar to less or non-polar solvents at room temperature, such as methanol (ε=32.6) and 1-butanol (ε=17.8) at 25°C/1 bar (Curren and King, 2009). Also, mass transfer improves at elevated temperatures due to the decrease of water viscosity (Moller et al., 2011). In addition, permittivity and surface tension of water declines, while there is an increase in its diffusivity.

Table 2.2. Physical and chemical properties of water at ambient and subcritical conditions.

Properties	Ambient water	Subcritical water
Temperature (°C)	0-100	100-374
Density (g cm^{-3})	0.997 (25°C)	0.958 (101°C, 1.1 bar) 0.692 (330°C, 300 bar)
Viscosity (μPas)	884 (25°C)	277 (101°C) 50.4 (371°C)
Heat capacity ($J\ g^{-1}\ K^{-1}$)	4.2 (25°C)	4.2, (101°C) 69, (371°C)
Dielectric constant	78.5 (25°C, 1 bar)	27.1 (250 °C, 50 bar) 18.2 (330°C, 300 bar)
Compressibility	No	Slightly increased, but still a liquid at 370°C
Ion product ($mol^2\ L^{-2}$)	10^{-14} increased to 10^{-12} at 100 °C	Increases from 10^{-12} (100°C) to 10^{-11} (300°C)

2.5.1. Subcritical water production of oligosaccharides

Subcritical water treatment is a promising technology for conversion of polysaccharides of different agricultural by-products into high value oligosaccharides. Table 2.3 presents maximum oligosaccharides production using different hydrothermal treatments.

Khuwijitjaru et al. (2014) used successfully SCW at temperatures of 100–300°C, solvent/solid of 10 (mL/g), for 15 min to hydrolyze the mannan polysaccharides of coconut meal into mono and oligosaccharides and obtained manno-oligosaccharides (72% of total carbohydrates) in the liquid extracts collected at 250°C. Wang et al. (2014) also treated apple pomace and citrus peel using subcritical water to obtain pectin with low molecular weight. They reported the highest pectin yield from citrus peel and apple pomace (21.95% and 16.68%, respectively) in the liquid extracts. Klinchongkon et al. (2015) investigated the production of oligosaccharides from passion fruit peel by subcritical water with temperatures in the range of 100–245°C, and reported the highest total oligosaccharide yield (20.5 and 21.2%) at 150 and 175°C. Martinez et al. (2010) obtained the highest pectic oligosaccharides yield of 25.1% by hydrothermal treatment of orange peel waste at 160°C, with a liquid to solid ratio of 12 kg water/kg of dry feedstock.

Table 2.3. Production of oligosaccharides from agricultural by-products using different hydrothermal treatments.

Raw material	T (°C)	t (min)	P (bar)	Solvent /solid (mL/g)	System	OS (DP)	OS Recovery/yield (%)	Reference
Corn cob	200	10	30	7	Continuous SCW	XOS, 2-10	57.8	Makishima et al. (2009)
Coconut meal	250	15	NR	10	Batch SCW	MOS, 2-6	20.4 (% raw material)	Khuwijitjaru et al. (2014)
Orange peel	160	NR	NR	12	Autohydrolysis	POS	25.1 (% raw material)	Martínez et al. (2010)
Sugarcane bagasse	200	10	NR	10	Autohydrolysis	XOS, 2-5	50.4	Zhang et al. (2018)
Wheat bran	200	NR	NR	10	Autohydrolysis	XOS, >2	70	Rose and Inglett (2010)
Wheat straw	180	30	NR	10	Autohydrolysis	XOS, >2	44	Ruiz et al. (2011)
Wheat straw	180	NR	10	-	Autohydrolysis	XOS, >2	50	Rodriguez et al. (2019)
Corn stover							70	
Sugarcane bagasse							80	

T: Temperature, t: Time, P: Pressure, OS: Oligosaccharides, DP: Degree of polymerization.

MOS: Manno-oligosaccharides, POS: Pectic-oligosaccharides, XOS: Xylo-oligosaccharides, SCW: Subcritical water.

NR: Not reported. XOS Recovery (%): (mass of XOS/mass of initial xylan) x 100.

In the SCW media, hydronium ions generated by dissociation of water and acetyl groups are released from the hemicellulose to catalyze the hydrolysis and produce shorter oligomers and consequently decrease the molecular weight of the polymer (Krogell et al., 2013). Also, hemicellulose has amorphous structure and its molar mass is lower than cellulose, therefore it is more accessible for hydrolysis under SCW media. Usually, SCW treatments are performed with batch reactors (Krogell et al., 2013), semi-continuous reactors (Gallina et al., 2016) and slurry continuous reactors (Makishima et al. 2009). Biomass can be hydrolyzed in batch and semi-continuous systems without additional milling, but slurry continuous reactors require very small particles of 200-300 μm to make a pumpable slurry. In a semi-continuous system, the residence time of the liquid phase can be changed by different flow rates; however, the residence time of the solid is much larger, as solid is kept in the reactor until the complete extraction of soluble materials (Gallina et al., 2017).

There are two different types of hemicellulose: one is easy to remove and the other one is strongly associated with cellulose and difficult to remove, only after removal of cellulose at temperatures above 240°C (Gallina et al., 2016). Moreover, hemicellulose hydrothermal fractionation in SCW is a complex phenomenon with different stages. In the first stage, hemicellulose is cleaved inside the biomass particle due to the random breaking down of bonds, which forms progressively shorter oligomers. Then, oligomers which are short enough to extract are solubilized and transferred to the liquid phase. Meanwhile, acetic acid is produced by deacetylation of hemicellulose, leading to

pH reduction of water. These two phenomena occur for hemicellulose dissolution and further mass transfer between the biomass and the liquid (Cabeza et al., 2016). In the next stage, monomeric sugars can be produced and released from the polymer or obtained from further hydrolysis of oligomers. As the process continues, sugars can be converted to degradation products such as furfural and hydroxyl methyl furfural (HMF) or other substances. Also, sugars degradation can release acetic acid in the liquid phase. The possible pathways of hemicelluloses fractionation in a hydrothermal process is illustrated in Fig. 2.5.

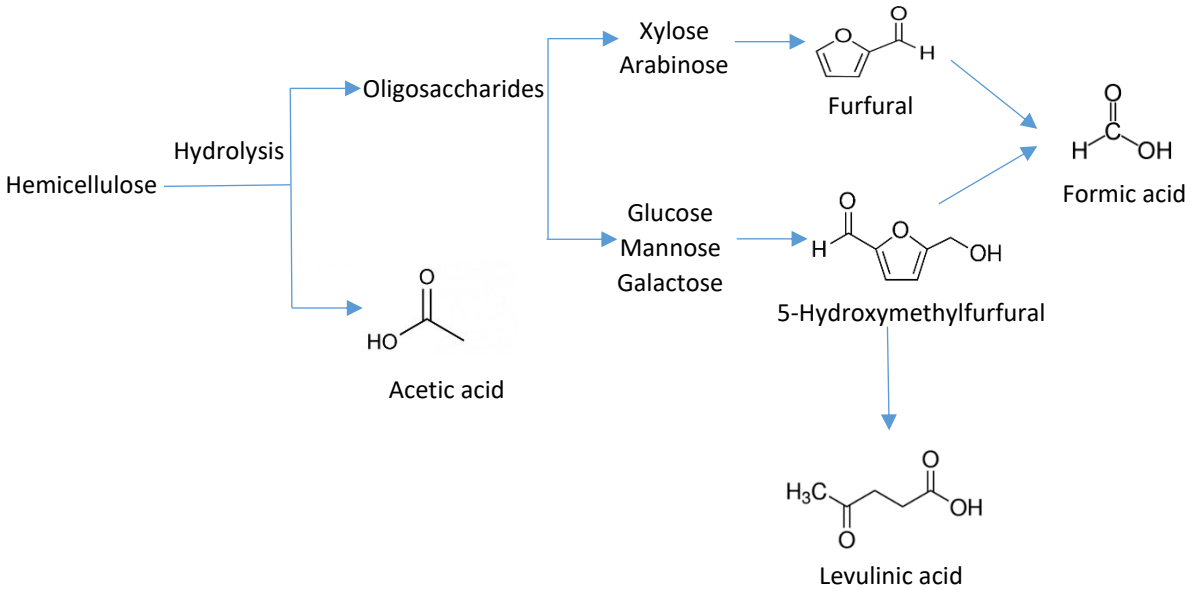


Fig. 2.5. Reaction pathways for hemicellulose hydrolysis and formation of degradation products in SCW (Adapted from Ruiz et al., 2017).

Makishima et al. (2009) investigated the recovery of corn cob hemicellulose using a continuous flow type reactor with 29.9 and 3.4%db of xylan and arabinan, respectively, for 9-12 min at 200, 205 and 210°C with a pressure of 30 bar and 13.5 wt% slurry as a feed in the reactor. In all

treatments, xylo-oligosaccharides with DP2-DP10 (XO_L) and DP>11(XO_H), xylose, glucose, arabinose and furfural were found in the liquid extracts. They concluded that the yield of XO_H decreased with increasing temperature, while the yield of XO_L, xylose and furfural increased.

Yedro et al. (2017) used a semi-continuous SCW system to extract hemicelluloses from Holm oak *Quercusilex* with 25 g feed, flow rate of 150 $L.h^{-1}$ and temperatures between 130 and 170°C. The molar mass decreased by increasing temperature and the largest hemicelluloses were extracted at the shortest reaction times. They showed reduction in the molar mass of hemicelluloses as a function of time in all the experiments. The molar mass was significantly reduced at 160 and 170°C compared to the initial value at the beginning of the extraction process. They reported that a mixture of lower molar mass hemicelluloses was obtained in the liquid extracts. In their study, the lowest molar mass of 1.8 kDa was obtained at 170°C after 60 min, where 60% yield of total hemicellulose was obtained.

2.6. Production of oligosaccharides by enzymatic depolymerization

Enzymatic depolymerization of polysaccharides for oligosaccharide production has been widely reported in the literature. Different prebiotic oligosaccharides were produced with enzymes, including malto-oligosaccharides (MOS) using α-amylase on starch; isomalto-oligosaccharides by action of α,β-amylase and α-glycosidase on starch (Mussatto and Mancilha, 2007); fructo-oligosaccharides (FOS) using inulinase on inulin and fructans (Cazetta et al., 2005), oligo-galacturonides by the action of pectinase, pectolyases, and other polygalacturonases on the pectic substrate (Holck et al., 2011).

Hemicellulose, mainly composed of xylan and arabinoxylan has been converted into xylo-oligosaccharides (XOS) using different cereal by-products such as corn cob (Chapla et al., 2011), wheat bran (Zhao and Dong, 2016), wheat straw (Akpinar et al., 2009) and sugarcane bagasse (Jayapal et al., 2013) and β,1-4 xylanases. There is a growing interest for XOS production with DP of 2-5, especially xylobiose due to their faster fermentation by probiotic microorganisms to improve the gastrointestinal environment (Gullon et al., 2011). Moniz et al. (2016) reported that the fermentation of high DP 9–21 oligosaccharides was slower than the one of low DP (4–6) oligosaccharides. XOS are usually produced from xylan rich materials using chemical fractionation of the raw material to isolate xylan with further enzymatic hydrolysis (Teng et al., 2010; Akpinar et al., 2007). Hardwood (e.g., birchwood, beechwood), corn cob, straw, bagasse,

rice hull, malt cake, and bran are the typical raw materials rich in xylan that are used for the production of XOS (Vazquez et al., 2000).

Hemicellulose**,** which is rich in xylan, can be solubilized using strong alkaline **s**olutions such as NaOH, KOH, $Ca(OH)_2$, ammonia or a mixture of these compounds, and the solubilized fraction is recovered from the suspension by further processing. Alkaline treatment hydrolyzes ester linkages between the hemicelluloses and other structural components, leading to dissolve hemicelluloses and lignin and disrupts the cell wall of lignocellulosic materials by swelling the cellulose and decreasing cellulose crystallinity (Ebringerova and Heinze, 2000; Sun and Tomkinson, 2002). Acetyl groups and uronic acids are separated from the hemicellulose by saponification during alkaline extraction, which leads to the structural change in the final extracted hemicellulose (Zheng et al., 2009). In some cases, the raw material has been pre-treated with oxidizing agents, salts or alcohols to remove lignin or pectic substances. Organic compounds such as acids and alcohols can be used to precipitate and recover the solubilized hemicelluloses and hemicellulose-degradation products (Vazquez et al., 2000).

Consequently, this complex hemicellulosic material is hydrolyzed using the synergistic action of different enzymes and compounds such as XOS with reduced DP are produced. Generally, endo-β-1-4-xylanases degrade xylan by attacking the β-1,4-bonds between xylose units to produce XOS, and β-xylosidase converts lower-DP XOs into monomeric xylose. To avoid the production of

xylose, enzyme complexes with low exo-xylanase and/or β-xylosidase activity are desired (Uffen, 1997). Table 2.4 presents performed treatments on various raw materials and maximum XOS production using enzymatic hydrolysis (xylanase). Wang et al. (2009) examined 10 family endo-1,4-beta-xylanase from *Bacillus subtilis* for the production of XOS from wheat bran. In this study, insoluble dietary fiber was isolated from deproteinized and destarched wheat bran using alpha amylase and protease to hydrolyze starch and protein, respectively. The yield of XOS (mixture of xylose (X), xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5) and xylohexaose (X6) was 13.36% of raw material and 31.32% of isolated insoluble dietary fiber. Flack et al. (2014) reported the production of arabinoxylan-oligosaccharides mixture (AXOS) from rye bran using thermostable xylanase GH10 (RmXyn10A) and GH11 (pentopan). Rye bran was autoclaved at 121°C for 15 h for the solubilisation of AX. The yield of X2, X3, X4, A3X, A2XX mixture was 10% and 15% of arabinoxylan content in rye bran, for RmXyn10A and pentopan, respectively. Chapla et al. (2011) extracted xylan from corncobs by four different treatments, including dilute acid (H_2SO_4), sodium hypochlorite, NaOH and autoclaving, then the extracted xylan was hydrolyzed by *A. feotidus* xylanase. The highest yield of XOS was for xylobiose (X2) 1.2±0.9 mg/mL and xylotriose (X3) 1.5±0.1 mg/mL obtained by NaOH treatment of raw material. Akpinar et al. (2009) examined cotton stalk, tobacco stalk, sunflower stalk and wheat straw to produce XOS by extracting xylan with KOH solution followed by hydrolysis with endoxylanase from *Aspergillus niger* and *Trichoderma longibrachiatum*. The yield of total XOS

from *A. niger* was higher than *T. longibrachiatum*. Yields of xylobiose and xylotriose obtained from tobacco stalk, cotton stalk, sunflower stalk and wheat straw were in the following order: 1.56±0.03, 1.18±0.01; 1.03±0.29, 0.80±0.026; 1.49±0.01, 1.20±0.01; 0.78± 0.07 and 0.71±0.07 mg/mL. Zhao and Dong (2016) reported the production of XOS (DP=2-4) with cellulase enzyme for hydrolyzing the cell walls followed by extraction of xylan with NaOH solution. Commercial xylanase (pentopan) was used and 51 (mg/g raw material) XOS mixture was obtained. Jayapal et al. (2012) obtained yields of X2 and X3 of 0.56 mg /mL and 0.79 mg/mL, respectively, from sugarcane bagasse. The xylan was extracted with NaOH solution and steam explosion, and then hydrolyzed by xylanase enzyme from *Trichoderma viridae.*

Table 2.4. Production of XOS from agricultural by-products using enzymatic hydrolysis.

Raw material	Treatments	XOS	Yield/Recovery	Reference
Corn cob	Steam explosion (165°C for 35 min)	DP 2-5	75 (%w/w xylan in corn cob)	Liu et al. (2018)
Corn cob	Alkali extraction (NaOH 1.25 M)	DP 2-5	X2: 1.2 mg/mL X3: 1.5 mg/mL X4: 1 mg/mL X5: 1.4 mg/mL Total XOS: 107 mg/g raw material	Chapla et al. (2011)
Eucalyptus	Lignin removal: sodium chlorite (0.93%, w/v) + acetic acid (0.31% v/v) (70°C for 4h) Alkali extraction of xylan: H_2O_2 6% (w/v) + NaOH 2-5 M	DP 2 and 4	3.39 mg/mL 30.8 % (%w/w xylan in eucalyptus)	Mafei et al. (2019)
Poplar	Lignin removal: hydrogen peroxide (30%, w/w + acetic acid (99%, w/w) (60°C for 2 h)	DP 2-4	19.8 (%w/w xylan in poplar)	Hao et al. (2019)
Rye bran	Heat treatment (Autoclave at 121°C for 15h)	DP 2-4	Xylanase GH10 enzyme: 15% Pentopan enzyme: 10% w/w of AX content in rye bran	Flack et al. (2014)
Sugarcane bagasse	Alkali extraction (NaOH 12%+steam)	DP 2-3	X2: 1.15 mg /mL X3: 0.56 mg/mL	Jayapal et al. (2013)
Wheat straw	Alkali extraction of xylan (KOH 24%)	DP 2-6	X2: 0.78 mg/mL X3: 0.71 mg/mL	Akpinar et al. (2009)
Wheat bran	Screening-cellulase-colloid milling-alkali extraction	DP 2-4	57 mg/g raw material	Zhao and Dong (2016)

DP: Degree of polymerization, XOS: Xylo-oligosaccharides, XOS Recovery (%): (mass of XOS/mass of initial xylan) x 100.

2.7. Xylo-oligosaccharides (XOS) separation and purification

Xylo-oligosaccharides produced by hydrothermal or enzymatic treatment are a mixture composed of a wide DP range of oligomers, monomers or non-saccharide compounds. Therefore, purification and separation are required to remove undesired compounds such as high molecular weight compounds, enzymes, monomers and acids to obtain a pure XOS that can be used as a prebiotic or pharmaceutical compound with anti-allergy, anti-infection and anti-inflammatory properties (Vazquez et al., 2005). Moreover, studies showed that oligomers with low DP (preferentially 2-4) have better prebiotic potential, because they can be utilized in higher amounts by beneficial bacteria (Chen et al., 2016). Therefore, it is required to purify the obtained XOS mixture to obtain oligomers with a desired DP range.

Various techniques have been studied to refine the XOS mixture, including solvent extraction with ethyl acetate, vacuum evaporation (Vazquez et al., 2005; Alonso et al., 2003), adsorption with activated carbon, acid clay, bentonite, diatomaceous earth, aluminum hydroxide or oxide, titanium and silica (Zhu et al., 2006; Pellerin et al., 1991) and membrane separation (Swennen et al., 2005; Vegas et al., 2006). Among all these separation technologies, membrane separation including ultra and nanofiltration has been reported as a promising method to purify XOS mixture.

For membrane purification, the basis for separation is molecular size, although solute–membrane interactions, chemical and electrostatic properties of the sample influence the permeability of the

membrane. Materials with molecular weight in the range of 1 to 1000 kDa are retained in the retentate by specific ultrafiltration membranes, while salts and water pass through to recover compounds of interest in the permeate. Nanofiltration is effective to retain the compounds with molecular weight in the range of 200-1000 Da (Singh et al., 2019). Ultrafiltration was used to remove compounds with high molecular weight and nanofiltration was performed to separate monomers and acids from the XOS mixture.

Membrane separation can be performed with dead-end or cross-flow filtration systems. The dead-end filtration is a batch process, in which feed is forced vertically to the membrane and the retained materials are accumulated on the surface of the membrane. In cross-flow filtration, feed flows with a constant turbulent flow parallel to the membrane, avoiding the accumulation of materials on the membrane surface (Calabro and Basile, 2011).

Singh et al. (2019) proposed a sequential purification of XOS mixture obtained from almond shell by autohydrolysis at 200°C for 5 min, followed by endo-xylanase treatment (10 U, 36 h). First, they used a dead-end ultrafiltration system with a membrane of 1 kDa molecular weight cut-off to separate compounds with high molecular weight like enzymes in the retentate fraction. The obtained permeate had 90.18 and 95.69% of initial xylobiose and xylotriose, respectively. Monomers were also recovered in the permeate with 92.42, 96.56 and 95.47% for xylose, glucose and arabinose, respectively. Then, they used a further nanofiltration system through a 250 Da

membrane to remove monomers from xylobiose and xylotriose mixture obtained in the resulting permeate from 1 kDa membrane. After passing the permeate through 250 Da membrane, 80% of xylobiose and 41% of xylotriose with respect to their amounts in the feed were recovered in the retentate, besides monomers including xylose, glucose and arabinose were obtained with 54, 38.7 and 40.8% recovery, respectively. As a result, they recovered 80% of xylobiose and 41% of xylotriose in the final retentate, along with the removal of 46% xylose, 61.3% glucose and 59.2% arabinose.

Rico et al. (2018) produced XOS from peanut shells using a stainless-steel reactor and a liquid-to solid ratio of 8 kg water/kg dry shell under non-isothermal conditions at 190-220°C. Then, a nanofiltration system with 3.5 bar and a 300 Da membrane was used to remove low molecular weight compounds such as monosaccharides, acetic acid, and some non-saccharide compounds in the hydrolysates obtained under the best autohydrolysis conditions. Peanut shells were composed of 20.87, 12.38, 0.89 and 42.68 wt% of oven dry mass glucan, xylan, arabinan and lignin, respectively. They obtained the highest amount of XOS with a concentration of 7.60 g/L at 210°C and the liquors obtained were further processed by nanofiltration. After nanofiltration, the concentration of XOS increased from 40.71 g/100g non-volatile compounds of the starting autohydrolysis solution to 52.88 g/100g non-volatile compounds of the retentate obtained. They observed a significant drop in the acetic acid concentration from 12.0 to 4.79 g/100 g nonvolatile

compounds and no significant differences were reported in the concentrations of monosaccharides and soluble lignin.

Table 2.5 shows different methods used for the purification of XOS produced by enzymatic and hydrothermal treatments. It also summarizes the recovery of obtained XOS with specific DP range in the final purified fraction.

Activated carbon adsorption is another method that has been used to remove monosaccharides and acids like acetic acid from the XOS mixture. Activated carbon has a large internal surface area and highly porous structure, which allows adsorb materials from the liquid and gas phase (Jankowska et al., 1991). Its surface area ranges typically from 800 to 1500 m^2/g (Bansal and Goyal, 2005). Monosaccharides and acids are not adsorbed on activated carbon and hence are removed easily from the hydrolysate. Further, adsorbed XOS with DP $\geq$ 2 can be eluted with different ethanol concentrations to obtain the desired purified oligomers. At the same time, activated carbon treatment contributes to decoloration of the XOS solution, especially when it was produced by hydrothermal treatment from plant materials. Because of final transparent color, XOS can be used as additives in the processed foods, drinks and pet foods.

Chen et al. (2014) obtained XOS (DP2-DP9) from *Miscanthus x giganteus* by autohydrolysis at 180°C/20 min (65% w/w xylan) and 200°C/5 min (69% w/w xylan). They added activated carbon with 1%, 5%, 10% and 20% (w/v) of the liquid volume and placed the mixture in a shaker at 100

rpm for 60 min. Further, they separated the activated carbon and washed it using distilled water to remove monomers. Then, ethanol elution was carried out using solutions with concentrations of 5%, 30%, 50%, 70% and 95% (v/v) to obtain the adsorbed XOS. They evaluated separate fractions obtained from ethanol washing and reported that XOS with DP 2, 3, 4, 5, 6, 7, 8 and 9 were recovered at 70.5, 86.9, 66.3, 56.2, 48.9, 39, 33.3 and 33.3%, respectively. The maximum recovery of oligomers was achieved in the first 5, 30 and 50% ethanol eluted fractions. For example, xylobiose (DP 2) was recovered at 31.6, 27 and 8.1% by 5, 30 and 50% ethanol elution, respectively. Xylotriose was obtained at 11.7, 38.5 and 22.6% by 5, 30 and 50% ethanol elution, respectively. Xylobiose and xylotriose were recovered by ethanol elution in high amounts compared to the rest of the other XOS. Chapla et al. (2012) also purified the XOS mixture (DP 2-5) obtained from corn cob using xylanase hydrolysis. They used 10% (w/v) activated carbon and the bound fractions of XOS were eluted with a gradient of 30–100% (v/v) ethanol. They concluded that around 80% of XOS were recovered in the ethanol elution fractions, and xylobiose and xylotriose had the highest recovery among the other types of XOS.

Table 2.5. Purification of XOS produced from agricultural by-products using enzymatic and hydrothermal hydrolysis.

Raw material	XOS production	Purification	Recovery (%)	Reference
Almond shell	Autohydrolysis + endoxylanase	1 kDa, ultrafiltration, 250 Da nanofiltration	X2: 80 X3: 41	Singh et al. (2019)
Birch wood	Autohydrolysis	1 kDa, ultrafiltration	X2-X6: 37	Rivas et al. (2016)
Corn cob	Endoxylanase	Activated carbon	X2-X5: 80	Chapla et al. (2012)
Corn stover	Endoxylanase	Activated carbon	X2-X4: 54.8	Zhu et al. (2006)
Cotton stalk	Endoxylanase	1, 3 kDa, ultrafiltration	X2: 89 (1, 3 kDa) X3: 93 (1 kDa) X3: 96 (3 kDa)	Akpinar et al. (2007)
Miscanthus x giganteus	Autohydrolysis	Activated carbon	X2: 70.5 X3:86.9 X4:66.3 X5:56.2	Chen et al. (2014)
Peanut shell	Autohydrolysis	300 Da, nanofiltration	X2-X6:75.7	Rico et al. (2018)
Wheat bran	Endoxylanase	1 kDa, ultrafiltration	X2+X3: 57.55	Zhao and Dong. (2016)

X2: Xylobiose, X3: Xylotriose, X4: Xylotetraose, X5: Xylopentaose, X6: Xylohexaose.

Recovery was calculated with respect to the initial individual XOS in the hydrolysates.

Chapter 3. Xylo-oligosaccharides production from barley bran using subcritical water technology and enzymatic hydrolysis

3.1. Introduction

Bran composed of outer tissues of barley (pericarp, testa, aleurone, subaleurone layers and germ) is rich in ash, protein and lipids, while starch and beta-glucan were concentrated in the inner tissues of barley grain.

After cellulose, hemicelluloses comprise the highest amount of plant cell wall polysaccharides in lignocellulosic materials (Peng et al., 2012), and are tightly linked to cellulose with non-covalent linkages (Anwar et al., 2014). Hemicelluloses are amorphous polysaccharides composed of xylose, arabinose, glucose, galactose and mannose, with different structures and the average degree of polymerization (DP) is in the range of 80–200 DP (Saha, 2003; Ebringerova, 2005). Organic acids, such as D-glucuronic acid, 4-O-methyl-D-glucuronic acid, and D-galacturonic acid are also present in the structure of these polysaccharides (Sjostrom, 1993). Arabinoxylan (AX), the most abundant hemicellulose in cereal by-products, was high in barley bran (14-30%). Gong et al. (2012) reported 14.21-21.06% of AX in the bran of three Tibetan hull-less barley varieties. In another study, hull-less barley bran was composed of 31.65±0.21 (% w/w) AX and 0.76±0.07 (% w/w) beta-glucan and other polysaccharides, for which values were not reported (Guo et al., 2019).

Fractionation of cereal by-products to remove lipid, starch and protein was reported in the literature. Lipid extraction was carried out traditionally using chemical solvents such as hexane and petroleum ether. Instead of the conventional method, supercritical CO_2, a green technology is widely used in recent years. Starch removal was performed using different methods, including enzymatic hydrolysis by alpha-amylase for barley (Izydorczyk et al., 1998; Yu et al., 2018), wheat bran (Ruthes et al., 2017), Termamyl in wheat bran (Maes and Delcour, 2002) and combination of alpha-amylase and amyloglucosidase for barley bran (Karimi et al., 2018), screening with mesh sieves to obtain fractions with different particle sizes of wheat bran (Zhao and Dong, 2016) and hot water extraction of wheat bran (Gullon et al., 2014). Removal of protein was also studied by enzymatic hydrolysis using Neutrase for wheat bran (Swennen et al., 2005) and protease for rye bran (Flack et al., 2014), alkaline solutions with NaOH for barley (Wang et al., 2010; Song et al., 2012) and salt and alcohol solutions for barley (Wang et al., 2010). Yan et al. (2019) removed lipids from wheat bran using refluxing petroleum ether twice for 6 h, then starch and protein were sequentially removed using hydrolysis with alpha-amylase and papain, respectively, followed by air drying in an oven at 50°C for 12 h. Alonso (2018) proposed a sequential process for wheat bran fractionation, including lipid extraction by supercritical CO_2 at 70°C and 300 bar, starch removal by alpha-amylase using ultrasound-assisted extraction at 55°C and 7 min, and protein removal by NaOH treatment at 80°C and pH of 9.3.

Hemicellulose was solubilized selectively using mild hydrothermal treatments at 160-200°C, 15-45 min with different systems, including batch, semi-continuous and continuous, where a mixture of oligomers with a wide range of molecular weight distribution was obtained (Cocero et al., 2018; Yedro et al., 2017). In general, hemicellulose was hydrolyzed into high molecular weight oligomers (DP>11), low molecular weight oligomer (DP 2-11), monomers, furfural and degradation products (Makishima et al., 2009).

Different hydrothermal approaches have been used for the production of oligosaccharides from different matrices, such as autohydrolysis of corncobs, almond shells, olive stones, rice husks, wheat straw, and barley straw at 179°C for 23 min (Nabarlatz et al., 2007); wheat straw at 120-200°C for 30 min (Chen et al., 2017); barley hull at 207°C (Vegas et al., 2005), hazelnut shell at 150-200°C for 20-46 min (Surek and Buyukkileci, 2017).

Subcritical water technology (SCW) is considered a green method for hydrolysis of lignocellulosic biomass, such as cereal by-products. Subcritical water is used as a solvent at temperatures above 100°C under pressure to keep water in liquid state and below its critical point 374°C/221 bar (Smith, 2002). Using SCW, hydronium ions produced by autoionization of water at high temperatures are responsible for the hydrolysis reactions and breaking of hemicellulosic chains (Gullon et al., 2009). The release of O-acetyl groups from hemicellulose produces organic acids, such as acetic and uronic acids, which promote the hydrolysis of hemicelluloses and formation of

oligosaccharides from hemicellulosic materials (Alvarez et al., 2014). Oligosaccharides production with SCW has been studied for passion fruit peel (Klinchongkon et al., 2015); wheat bran (Ruthes et al., 2017) and coconut meal (Khuwijitjaru et al., 2014). Pronky and Mazza (2010) investigated the fractionation behavior of barley straw using SCW treatment with a flow type reactor at 165°C, flow rate of 115 mL/min, and a solvent-to-solid ratio of 60 mL/g. Among oligomers characterized, xylo-oligosaccharides (32.28% db) and gluco-oligosaccharides (6.32% db) had the highest amounts in the liquid extract. Makishima et al. (2009) hydrolyzed corn cob (29.9% db xylan and 3.4% db arabinan) using a continuous SCW process for 9-12 min at a pressure of 30 bar. They obtained the highest yields of xylo-oligosaccharides with a degree of polymerization (DP) > 11 at 200°C/9 min (23.7%) and DP of 2-11 at 205°C/9 min (57.8%). But, to the best of my knowledge, there is no study on barley bran fractionation and hydrolysis in SCW media. Therefore, the main objective of this study was to investigate the process conditions for the fractionation of barley bran aiming to remove lipids and starch, enriching the residual meal in protein and carbohydrates, from which protein is removed later from destarched bran. Then, this barley bran fraction was hydrolyzed with SCW and enzyme to obtain oligosaccharides. Hydrolysates were characterized for the structural carbohydrates and xylo-oligosaccharides (XOS) contents.

3.2. Materials and methods

3.2.1. Raw material and chemicals

Whole barley grains were separated using 1.0 mm sieve from barley bran sample (Falcon variety) provided by the company (Progressive Foods Inc., Edmonton, AB, Canada). The bran fraction with a maximum particle size of 1.0 mm was ground, vacuum packed and stored at -20°C for the further fractionation process.

Thermostable alpha-amylase (E-BLAAM), amyloglucosidase (E-AMGDF) and endo-1,4-β-D-xylanase (E-XYAN4) from *Aspergillus niger* were purchased from Megazyme (Wicklow, Ireland). Xylooligosaccharide standards, xylobiose (X2), xylotriose (X3), xylotetraose (X4) and xylohexaose (X6) were obtained from Megazyme (Wicklow, Ireland). All sugar standards, D(+) glucose, D(+) xylose, D(+) galactose, L(+) arabinose, and D(+)mannose with a purity of ≥ 96% and Alpha tocopherol standard were purchased from Sigma Aldrich (St. Louis, MO, USA).

3,5-Dinitrosalicylic acid (98%) and DL-lactic acid (90%) were purchased from Sigma Aldrich (St. Louis, MO, USA). HPLC grade water was purchased from Fisher Scientific (Ottawa, ON, Canada). Analytical grade sulfuric acid (72%) and petroleum ether were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Liquid CO_2 was purchased from Praxair (Edmonton, AB, Canada).

3.2.2. Proximate compositional analysis of barley bran, and defatted, destarched and deproteinized fractions

All proximate compositional analysis was performed at least in duplicate.

3.2.2.1. Moisture content

Moisture content was measured using a gravimetric method 935.29 (AOAC, 2000). Briefly, barley bran (2 g) was weighed with an analytical balance (Mettler Toledo, Mississauga, ON, Canada) in a pre-weighed aluminum container and dried in an oven (Model 655G, Fisher Scientific Iso Temp, Toronto, ON, Canada) at 105°C for 3 h. Then, the container with dried bran was weighed after cooling in a desiccator. The moisture content was calculated using equation (3.1).

$$\text{Moisture content } (\%) = 100 \times \left[\frac{X-Y}{X}\right] \quad (3.1)$$

where, X= weight (g) of the sample before drying, and Y= weight (g) of the sample after drying.

3.2.2.2. Ash content

Ash content of the barley bran was measured according to the official method 936.07 (AOAC, 2000). Approximately, 1 g sample was weighed in the clean and pre-weighed porcelain crucibles. Then, crucibles containing samples were transferred to the muffle furnace (Model F-A1730, Thermolyne Corporation, Dubuque, IA, USA) and kept at 550°C overnight. The crucibles were then removed from the muffle furnace and cooled in a desiccator. The crucibles were weighed after cooling and the ash content was calculated according to equation (3.2).

$$Ash\ content\ (\%\ w/w) = \left[\frac{\mathrm{X-Y}}{\mathrm{Z-Y}}\right] \times 100 \quad (3.2)$$

where, X = weight of the crucible and ash, Z = weight of crucible and sample, and Y = weight of crucible.

3.2.2.3. Protein content

Protein content was determined using a Leco TruSpec nitrogen analyzer (Model FP- 428, Leco Instruments Ltd., Mississauga, ON, Canada). Barley bran (0.1 g) was weighed into an aluminum foil cone and then pressed to form a pellet. The sample was placed into the loading head followed by sealing the system and purging the sample to avoid the entry of air during the loading phase. The sample was combusted inside a furnace at 950°C by pure oxygen. Orchard leaves and ethylene diamine tetra acetic acid (EDTA) were used as standards to calibrate the system. The nitrogen content of the sample was measured and multiplied by a conversion factor of 6.25 previously reported for barley (Temelli, 1997). The final protein content was calculated using equation (3.3).

$$Protein\ content\ (\%) = Nitrogen\ content\ \times 6.25 \quad (3.3)$$

3.2.2.4. Lipid content

The lipid content of barley bran was determined according to the AACC method 30-25 (AACC, 1982) using the Goldfisch extraction unit (Labconco, Kansas City, MO, USA). Approximately 2 g of barley bran was weighed into the extraction thimbles (25 mm I.D × 80 mm length, Whatman International Ltd., Maidstone, England). Petroleum ether (40 mL) used as a solvent was added to the sample inside the thimble. A similar thimble containing only 40 mL of petroleum ether was used as a blank at 60°C for 6 h. The extraction thimbles were placed into the Goldfisch extraction unit and preweighed clean extraction beakers were used for collecting the solvent and dissolved

lipid. The system ran at a temperature of 60°C for 6 h. The weight of the beakers containing the lipid was recorded after cooling and used to calculate the lipid content according to equation (3.4).

$$Lipid\ content\ (\%) = \left[\frac{X-Y}{Z}\right] \times 100 \quad (3.4)$$

where, X= weight of the beaker containing the lipid, Y= weight of the empty beaker, and Z= weight of the sample.

3.2.2.5. Carbohydrates

The total carbohydrate content of barley bran was calculated by deduction of the sum of the percentage of moisture, ash, protein and lipid contents from 100% using equation (3.5).

$$Total\ carbohydrates\ (\%) = 100\% - (moisture\% + ash\% + protein\% + lipid\ \%) \quad (3.5)$$

3.2.2.6. Starch content

The starch content of barley bran was determined according to the AOAC method 996.11 using the "Total starch assay kit" (Megazyme, Wicklow, Ireland). First, 95% of powder barley bran (1 mm) passed through the 0.5 mm sieve to obtain the appropriate particle size for this starch determination. Then, 100 mg of sample was weighed in duplicate into the glass test tubes (16 × 120 mm). Ethanol 80% (0.2 mL) was added to wet the samples and then vortexed. Then, 3 mL of diluted thermostable alpha-amylase in 100 mM sodium acetate buffer (300 U) was added to each tube followed by incubation in a boiling water bath for 6 min. The tubes were stirred using a vortex after 2, 4 and 6 min. Then, 0.1 mL amyloglucosidase (330 U) was added and the tubes containing the enzyme were placed in a water bath at 50°C for 30 min. After incubation, the entire content of

the tube was transferred to the 100 mL volumetric flask and the volume was adjusted to 100 mL by addition of 96.7 mL distilled water. The solution was mixed, and 1 mL of the obtained solution was transferred into 2 mL micro-centrifuge tubes and centrifuged at 3000*g* for 10 min. Duplicate aliquots (0.1 mL) of the clear supernatant from each tube was transferred to the glass culture tubes. D-Glucose standard solutions (0.1 mL) and water were used as the control sample and reagent blank solution, respectively. The GOPOD reagent (3 mL) was added to each tube (including the D-glucose control and reagent blank) and incubated at 50°C for 20 min. Then, the absorbances of each sample and control were measured at 510 nm against the reagent blank. The content of total starch was calculated using equation (3.6).

$$Starch\ content\ (\%) = A \times F \times \left[\frac{FV}{0.1}\right] \times \left[\frac{1}{1000}\right] \times \left[\frac{100}{W}\right] \times \left[\frac{162}{180}\right] \quad (3.6)$$

where, A = absorbance of the sample against the reagent blank, FV= final volume (100 mL), 0.1 = volume of sample analyzed, (1/1000) = conversion from µg to mg, (100/W) = starch content as a percentage of sample, W = sample weight (mg), (162/180) = a factor to convert from free D-glucose, determined in the experiment, to anhydroglucose, that is present in starch.

$$F = \left[\frac{100\ \mu g\ of\ D-glucose}{Absorbance\ for\ 100\ \mu g\ of\ D-glucose\ standard}\right] \quad (3.7)$$

3.2.2.7. Beta-glucan content

Beta-glucan content was measured according to the AOAC method 995.16 using the "Mixed linkage beta-glucan assay kit" (Megazyme, Wicklow, Ireland). First, 95% of barley bran passed through the 0.5 mm sieve to obtain the appropriate particle size for the experiment. Then, 80-120 mg of barley bran was weighed in duplicate into the glass test tubes (16 × 120 mm) and 0.2 mL ethanol (50%) was added to aid dispersion. Sodium phosphate buffer 20 mM (4 mL) was added and the content was mixed using a vortex for 3 min. Test tubes were placed in a boiling water bath and incubated for 1 min, vortexed again for 3 min and incubated at 100°C for an additional 2 min. Then, the tubes were incubated at 50°C for 5 min and lichenase (0.2 mL) was added followed by incubation at 50°C for 1 h with regular vigorous vortexing (3-4 times). Sodium acetate buffer (200 mM, 5 mL) was added to the tubes and mixed using a vortex in order to adjust the pH to 4. Then, the tubes with the samples were placed at room temperature (22°C) for 5 min and centrifuged at 1000*g* for 10 min. An aliquot (0.1 mL) of the content of the tube was transferred into three culture tubes and beta-glucosidase (0.1 mL) was added to two of these tubes. Sodium acetate buffer (0.1 mL) was added to the third tube to prepare the blank. The GOPOD reagent (3 mL) was added to each tube and incubated at 50°C for 20 min. Finally, all tubes were removed from the water bath and the absorbances were measured at 510 nm against the blank. The beta-glucan content was calculated using equation (3.8).

$$Beta - glucan\ (\%) = A \times F \times \left[\frac{FV}{0.1}\right] \times \left[\frac{100}{W}\right] \times \left[\frac{162}{180}\right] \times \left[\frac{1}{1000}\right] \quad (3.8)$$

where, A = absorbance of the sample against the blank, FV= final volume (9.4 mL), 0.1 = volume of sample analyzed, (1/1000) = conversion from µg to mg, (100/W) = beta-glucan content as a percentage of sample, W = sample weight (mg), (162/180) = a factor to convert from free D-glucose determined in the experiment, to anhydroglucose, that occurs in β-glucan.

$$F = \left[\frac{100\ \mu g\ of\ D{-}glucose}{Absorbance\ for\ 100\ \mu g\ of\ D{-}glucose\ standard}\right] \quad (3.9)$$

3.2.2.8. Determination of structural carbohydrates and lignin

The NREL standard analytical procedure was used for the determination of structural carbohydrates and lignin (Sluiter et al., 2008). First, 92% of barley bran was passed through the 0.25 mm sieve to obtain the appropriate particle size for the experiment. Then, 300 mg of the sample was weighed and transferred into the Pyrex pressure tubes, sulfuric acid 72% (3 mL) was added and mixed with the sample using vortex for 5 min. The tubes were placed in a water bath at 30°C and incubated for 1 h with continuous stirring using magnetic stirrers. After completion of hydrolysis, deionized water (84 mL) was added to dilute the acid to a 4% concentration. Then, the tubes were sealed and placed in an autoclave at 120°C for 1 h. After the autoclave cycle, the tubes containing hydrolysates were cooled at room temperature (22°C). The empty porcelain porous bottom crucibles (Coors #60531) were placed in the muffle furnace at 575°C for ashing, followed by cooling in the desiccator and the weights were recorded. The hydrolysates were vacuum filtered using crucibles and filtrates were captured. The remaining solids were transferred out the pressure tubes using 50 mL hot deionized water. Soluble lignin in the filtrate was measured using the

spectrophotometer at 320 nm. Then, the filtrates were neutralized using calcium carbonate in order to adjust the pH between 5 and 6 followed by passing through 0.22 µm filters and the content of sugars was determined by HPLC. Structural carbohydrates, including xylose, galactose, arabinose, and glucose in the filtrate were determined by HPLC using a Shimadzu LC-10AD pump (Shimadzu Co., Kyoto, Japan) equipped with an autosampler (SIL- 10A), a refractive index detector (RID-10A) (Shimadzu Co., Kyoto, Japan) and Aminex HPX 42-P column (dimension: 300 mm X 7.8 mm; average particle size: 25 µm; Bio-Rad laboratories, CA, USA) at 85°C with HPLC grade water as the mobile phase at a flow rate of 0.6 mL/min. The sugar concentrations were determined by comparison against a set of known sugar standards. The concentration of the polymeric sugars was calculated from the concentration of the corresponding monomeric sugars, using an anhydro correction of 0.88 for xylose and arabinose and 0.90 for glucose and galactose. The crucibles containing acid insoluble residue were dried in an oven at 105°C overnight and weighed after cooling in the desiccator. Then, one of the crucibles was placed in the muffle furnace at 575°C overnight and cooled in the desiccator and the weight was recorded. The insoluble residue from the other crucible was used for the protein quantification using the Leco nitrogen analyzer. Polymeric sugars, acid soluble and insoluble lignin contents were determined using equations (3.10), (3.11) and (3.12).

$$Polymeric\ sugars\ (\%) = \left[\frac{Monomeric\ sugars\ concentration \times anhydro\ correction \times 86.73 \times 1/1000}{ODW}\right] \times 100 \qquad (3.10)$$

$$Acid\ soluble\ lignin\ (\%) = \left[\frac{A \times 86.73 \times D}{W \times 30}\right] \times 100 \qquad (3.11)$$

$$Acid\ insoluble\ lignin\ (\%) = \left[\frac{(X-Y)-(Z-Y)-P}{ODW}\right] \times 100 \quad (3.12)$$

where, X = weight of crucible plus dry residue (g), Y= weight of crucible (g), Z= weight of crucible plus ash (g), P = weight of protein (g), ODW = [(weight of sample (g) × total solids (%)) / 100], A = Absorbance of the sample at 320 nm, 86.73 = volume of the filtrate (mL), D = dilution factor, W = weight of sample (mg) and 30 = absorptivity of biomass at 320 nm.

3.2.3. Lipid extraction from barley bran

3.2.3.1. Lipid extraction from barley bran using supercritical CO_2 (SC-CO_2) within a 10 mL extraction vessel

Barley bran (3.0 g) was used to study the process conditions for maximum removal of lipid using the supercritical CO_2 extraction system (ISCO SFX 220, Lincoln, NE, USA) (Fig. 3.1). First, the sample was weighed and loaded inside the extraction cell, which had filters at the bottom and top. Then, the cell was inserted into the extraction chamber. Cooler was turned on to reach 0°C so that CO_2 is in the liquid to be pumped. The one-way valve was opened between CO_2 tank and the extraction chamber. The working temperature was set on the extractor. Then, the desired pressure and time were set. After reaching the desired temperature and pressure, the dynamic extraction started. The extraction was performed at different pressures of 200 and 300 bar and temperatures of 40 and 70°C with 2 mL/min of CO_2 flow rate (at pump condition). All extractions were performed in duplicate and total lipid extract was collected every 30 min in pre-weighed glass

tubes placed in an ice bath. The tubes with the lipid extracted were kept at room temperature (22°C) for 20 min prior to the gravimetric quantification.

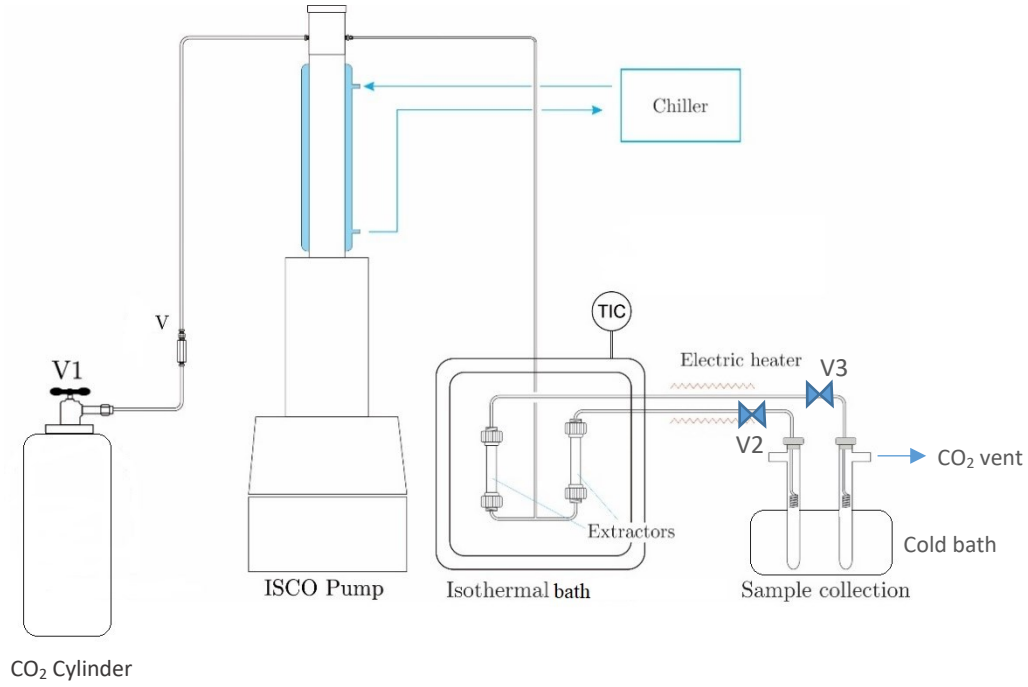


Fig. 3.1. Supercritical CO_2 extraction equipment with 10 mL extraction vessel: V= check valve, V1= cylinder valve 1, V2 and V3 = micro metering valves, TIC= temperature indicator controller.

The yield and recovery of extracted lipids were calculated using equations (3.13) and (3.14), respectively.

$$Yield\ (\%) = \left[\frac{Weight\ of\ extracted\ lipid}{Initial\ mass\ of\ bran}\right] \times 100 \quad (3.13)$$

$$Recovery\ (\%) = \left[\frac{Weight\ of\ extracted\ lipid}{Initial\ mass\ of\ lipid\ in\ bran}\right] \times 100 \quad (3.14)$$

3.2.3.2. Tocopherol analysis

The concentration of α-tocopherol was measured in the lipid samples obtained at different pressures (200 and 300 bar) and temperatures (40 and 70°C) for 30 min extraction. Briefly, 36 mg of lipid was dissolved in 1 mL hexane and passed through the 0.22 µm filter. Then, 20 uL of sample was injected into a HPLC system equipped with a fluorescence detector RF-535 (Shimadzu Co., Kyoto, Japan), a Supelcosil-LC-Diol column (25 cm × 4.6 mm, 5 µm, Supelco, Oakville, ON, Canada). The mobile phase was a mixture of *n*-hexane/isopropanol (99.4:0.6, vol/vol) with a flow rate of 1 mL/min. The concentration of α-tocopherol was quantified using the standard and reported as µg α-tocopherol per mL of sample.

3.2.3.3. Lipid extraction from barley bran using SC-CO_2 within a 300 mL extraction vessel

SC-CO_2 extraction of lipid from barley bran was carried out using a laboratory scale equipment (Newport Scientific Inc., Jessup, Md., USA) described by Bozan and Temelli (2002). Barley bran (40 g) was loaded inside the basket and mixed with glass beads (3 mm in diameter) to improve the extraction process. Glass wool was used at both ends to hold the sample and the basket was placed inside the 300 mL extraction chamber. Extraction temperature was maintained within ± 2°C using a thermocouple placed inside the extractor, a temperature controller and a heating jacket placed around the extractor. A back-pressure regulator was used to maintain the pressure constant during the extraction process. Lipid extraction was performed at the optimum temperature and pressure

(70°C and 300 bar) obtained previously using the 10 mL SC-CO_2 system at a flow rate of 1.5 L/min (measured at ambient conditions) for 8 h. The cumulative amounts of extracts were reported as a function of time.

3.2.4. Starch removal from defatted barley bran

Defatted barley bran (100 g) was used for enzymatic starch removal with heat stable alpha-amylase and amyloglucosidase according to the AOAC Method 991.43 with some modifications. The bran was mixed with potassium phosphate buffer (0.05 M, pH of 6.5) in a ratio of 1:10 w/v and incubated with alpha-amylase (150 U g^{-1} of defatted bran) for 40 min at 98-100°C. Then, HCl (0.561 N) was added for pH adjustment to 4.1- 4.8, followed by incubation with amyloglucosidase (660 U g^{-1} of defatted bran) at 60°C for 30 min. The polysaccharides were precipitated using the addition of 4 times volume of 95% ethanol with a total of 4 L for each batch and separated by centrifugation at 5000*g* for 15 min followed by drying in an oven at 40°C overnight. The dried sample was ground in a centrifugal mill (Retsch, Haan, Germany) to obtain a powder with a maximum particle size of 1.0 mm, then vacuum packed and stored at -20°C. This defatted and destarched sample was used as a feed material for the SCW hydrolysis.

3.2.4.1. Subcritical water hydrolysis

The defatted and destarched sample (2 g) was hydrolyzed using a SCW system. Hydrolysis was carried out in a semi-continuous flow type equipment similar to the one described by Ciftci and Saldaña (2015). The main components of the SCW system are high performance liquid chromatograph (HPLC) pump a pre-heater, a stainless steel high pressure reactor, a digital pressure gauge, a cooling system an oven and a back pressure regulator (Fig. 3.2). Defatted and destarched barley bran (2 g) was mixed with 20 g of glass beads (2.3 mm diameter) and then loaded into the reactor, which had inlet and outlet filters of 20 µm. The system was heated by the oven and its temperature was monitored by a digital thermometer during the process. Distilled water was first degassed in an ultrasound bath for 20 min and delivered with the HPLC pump at a 5 mL/min flow rate to the preheating section and then passed through the sample inside the reactor vessel. Pressure of the system was maintained constant using the back-pressure regulator. The extracts were collected in vials after passing through the cooling system placed after the reactor and stored at -18°C for further analysis. The experiments were carried out at temperatures of 120–200°C, a constant pressure of 50 bar for 15, 30 and 60 min. Pressure was selected above the maximum pressure studied in the literature (30 bar) for the production of oligosaccharides using SCW. In general, the main role of pressure in SCW is to maintain water in the liquid state. The changes in pH values after hydrolysis were recorded in the liquid extracts. All experiments were performed

in duplicates. The solid residue left in the reactor vessel after each experiment was dried in an oven at 40°C overnight.

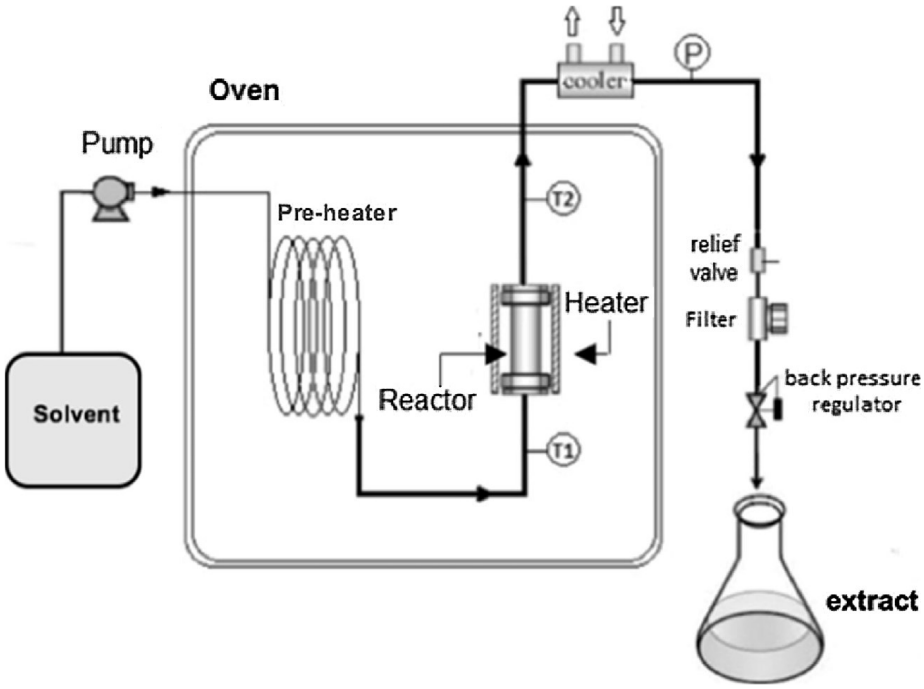


Fig. 3.2. Subcritical fluid system: P= pressure gauge, and T1 and T2= thermocouples.

3.2.5. Protein removal from defatted and destarched bran

Defatted and destarched bran (20 g) was used for protein removal and polysaccharides extraction with alkaline treatment (Song et al., 2012). Briefly, the bran was mixed with sodium hydroxide (3 N) at a liquid to solid ratio of 20:1 v/w, for almost 6 h at 60°C under constant stirring. After extraction, the solid residue was separated from the liquid extract by centrifugation at 5000*g* for 15 min. Then, the soluble protein was precipitated by adjusting the pH to 4.5 in the liquid extract by adding HCl (0.5 N) and the precipitated protein was removed from the supernatant by

centrifugation at 5000*g* for 10 min. Ethanol (90%, 800 mL) was added to the second obtained supernatant and polysaccharides were precipitated overnight at 4°C. Then, the precipitated fraction was dried in an oven at 40°C overnight. The dried sample was ground in a centrifugal mill (Retsch, Haan, Germany) to obtain a powder with a maximum particle size of 1.0 mm, then vacuum packed and stored at -20°C for further analysis. This bran fraction was used for enzymatic and SCW hydrolysis.

3.2.5.1. Enzymatic hydrolysis of defatted – destarched and deproteinized bran

3.2.5.1.1. Substrate and enzyme preparation

Sodium acetate buffer (100 Mm) with pH of 4.5 containing bovine serum albumin (0.5 mg/mL) was used for enzymatic hydrolysis. The buffer was prepared by the addition of 5.71 mL acetic acid (1.049 g/mL) to 900 mL distilled water and the pH was adjusted to 4.5 using NaOH (1 N). Then, the bovine serum albumin (500 mg) was added and the total volume was adjusted to 1L using distilled water. Defatted – destarched and deproteinized bran (2.2 g) was added to 90 mL of buffer and dispersed at 50°C by stirring. The solution volume was adjusted to 100 mL with the same acetate buffer. Two endo beta-xylanase concentrations (50 and 200 U) were prepared by dilution of 50 and 200 µL of the initial enzyme in 3950 and 3800 µL of sodium acetate buffer and stored in an ice bath for further use.

3.2.5.1.2. Enzymatic hydrolysis with endo beta-xylanase

Defatted – destarched and deproteinized bran solution (10 mL) in two series of 50 mL Falcon tubes (10 tubes in each series) were incubated at 40°C for about 5 min. Then, diluted endo-xylanase (0.2 mL) from 50 U solution was added to the contents of first tube series and the same amount of 200 U enzyme solution was added to the second series of tubes. Two tubes without enzyme were used as control samples. All tubes were incubated in a water bath at 40°C with continuous agitation. Two tubes from both series were taken out from the water bath at different time intervals (1, 4, 8 and 16 h) and incubated at 100°C for 5 min to inactivate the enzyme. Then, the aliquots and control samples were transferred to the 1 mL micro centrifugal tubes and centrifuged at 5000*g* for 5 min. Then, supernatants from centrifuged solutions were taken and kept at 4°C for further analysis.

3.2.6. Characterization of hydrolysates

3.2.6.1. pH determination

The pH of the liquid extracts was measured using a pH meter (Denver Instrument, Model 220, USA) at all temperature investigated and 50 bar for 15, 30 and 60 min.

3.2.6.2. Reducing sugar determination

Liquid extracts obtained at 50 bar and 120, 140, 160, 180 and 200°C with residence times of 15, 30 and 60 min were used for reducing sugar determination according to the method described by Miller (1959). First, the DNS reagent was prepared by dissolving 3,5-dinitrosalicylic acid (10 g) and sodium sulfite (0.5 g) in 250 mL NaOH (1.0 N) and adjusting the volume to 1 L by miliQ

water. Then, this solution was filtered using Whatman paper and kept in a dark bottle at 4°C. The DNS reagent (1 mL) was chilled in an ice bath for 5 min inside the test tube and mixed with 0.5 mL of hydrolysate and the total volume was adjusted to 2 mL by adding miliQ water. Then, the solution was mixed using a vortex and chilled for 10 min followed by incubation in a boiling water bath (95-98°C) for 5 min. After chilling (15 min), the tube was placed in a water bath at room temperature (22°C) for 15 min and the absorbance was measured using a spectrophotometer at 575 nm against the blank. The calibration curve was prepared using xylose standard solutions containing 0-5 mg xylose/mL.

3.2.6.3. Total sugars determination

The concentration of total sugars was determined according to the NREL standard analytical procedures (Sluiter et al., 2006). The SCW extracts were post hydrolyzed to break all the oligomers into monomers followed by HPLC analysis. Briefly, 20 mL of each liquid extract was hydrolyzed with 697 µL of sulfuric acid (72%wt). Then, the hydrolysates were incubated in an autoclave at 121°C for 1 h in sealed glass pressure tubes. The solution obtained was neutralized using calcium carbonate in order to adjust the pH between 6 and 7 followed by passing through 0.22 µm filters and the contents of sugars (glucose, xylose, arabinose and galactose) were determined by HPLC. Determination of mass of sugars (structural carbohydrates) in destarched bran was described in Chapter 3 (Section 3.2.2.8) and recovery of sugars in the extracts was calculated using equation (3.15).

$$Recovery\ (\%) = \left[\frac{Mass\ of\ sugars\ in\ the\ extract}{Initial\ mass\ of\ sugars\ in\ destarched\ bran}\right] \times 100 \quad (3.15)$$

3.2.6.4. Monosaccharides and organic acids determination

The monosaccharides of SCW extracts were determined using a HPLC system equipped with a refractive index detector (RID-10A) (Shimadzu Co., Kyoto, Japan) and a BioRad Aminex HPX-87H column (300 × 7.8 mm, Bio-Rad laboratories, CA, USA) with 0.005 M sulfuric acid as the mobile phase at a flow rate of 0.6 mL/min at temperature of 65°C for 50 min. The contents of monosaccharides (glucose, xylose and arabinose) and organic acids (lactic acid, acetic acid) were determined by HPLC using calibration curves that were prepared with standard solutions (See Appendix A).

3.2.6.5. Xylo-oligosaccharides determination by HPLC

Xylo-oligosaccharides were measured using a HPLC system equipped with a refractive index detector (RID-10A) (Shimadzu Co., Kyoto, Japan) and a Shimadzu LC-10AD pump (Shimadzu Co., Kyoto, Japan). Xylo-oligosaccharides were eluted with HPLC grade water as the mobile phase in the Aminex HPX 42-A column (300 mm × 7.8 mm, 25 µm, Bio-Rad laboratories, CA, USA) with de-ashing and micro-guard carbo-p cartridges. Elution was performed at 85°C at a flow rate of 0.4 mL/min for 45 min. The concentration of each oligosaccharide was quantified using peak areas compared with standard oligosaccharides including xylobiose (X2), xylotriose (X3), xylotetraose (X4) and xylohexaose (X6) and reported as mg/mL oligosaccharides. The XOS recovery or XOS production by xylan conversion to XOS was determined by equation (3.16).

$$Xylo-oligosaccharides\ recovery\ (\%) = \left[\frac{Mass\ (g)\ in\ the\ liquid\ extract\ (enzymatic\ and\ SCW)}{Mass\ of\ initial\ xylan\ (g)}\right] \times 100 \quad (3.16)$$

3.2.7. Statistical analysis

Data are shown as mean ± standard deviation according to the results obtained from at least duplicate experiments and analysis. Two- and one-way analysis of variance (ANOVA) and the multiple comparisons of the means with Tukey's test at $p < 0.05$ were performed using Minitab version 18.0 (Minitab Inc., State College, PA, USA) at 95% confidence interval.

3.3. Results and discussion

3.3.1. Lipid extraction with supercritical CO_2 using 10 and 300 mL extraction vessels

The SC-CO_2 extraction of lipids from barley bran at three extraction conditions are shown in Fig. 3.3 (a,b) in terms of the extract weight (g) and extract yield (%) using 10 mL vessel. Fig. 3.3 c presents the extract weight (g) using 300 mL vessel. Different fractions were collected in tubes every 30 min for a total of 120 min. The lipid content of barley bran was 9.46 ± 0.27% using petroleum ether for 6 h based on Goldfisch determination. Using SC-CO_2, the majority of lipid was removed in the first 30 min of extraction (solubility effect), but no significant increase ($p > 0.05$) was observed after 60, 90 and 120 min (mass transfer predominance) at 300 bar/40°C and 300 bar/70°C. At 200 bar/40°C, the amount of lipid extracted increased significantly ($p < 0.05$) from 30 to 60 min with the increasing of CO_2 mass, leading to the higher solubilization of lipid and no change was observed from 60 to 120 min. The total amount of lipid obtained was

significantly ($p < 0.05$) influenced by temperature and pressure. The total lipid extracted after 30 min increased using high pressures from 200 bar (0.19 g) to 300 bar (0.24 g) at a constant temperature of 40°C due to the increase of CO_2 density, which leads to the higher solubility of lipid (0.24 g).

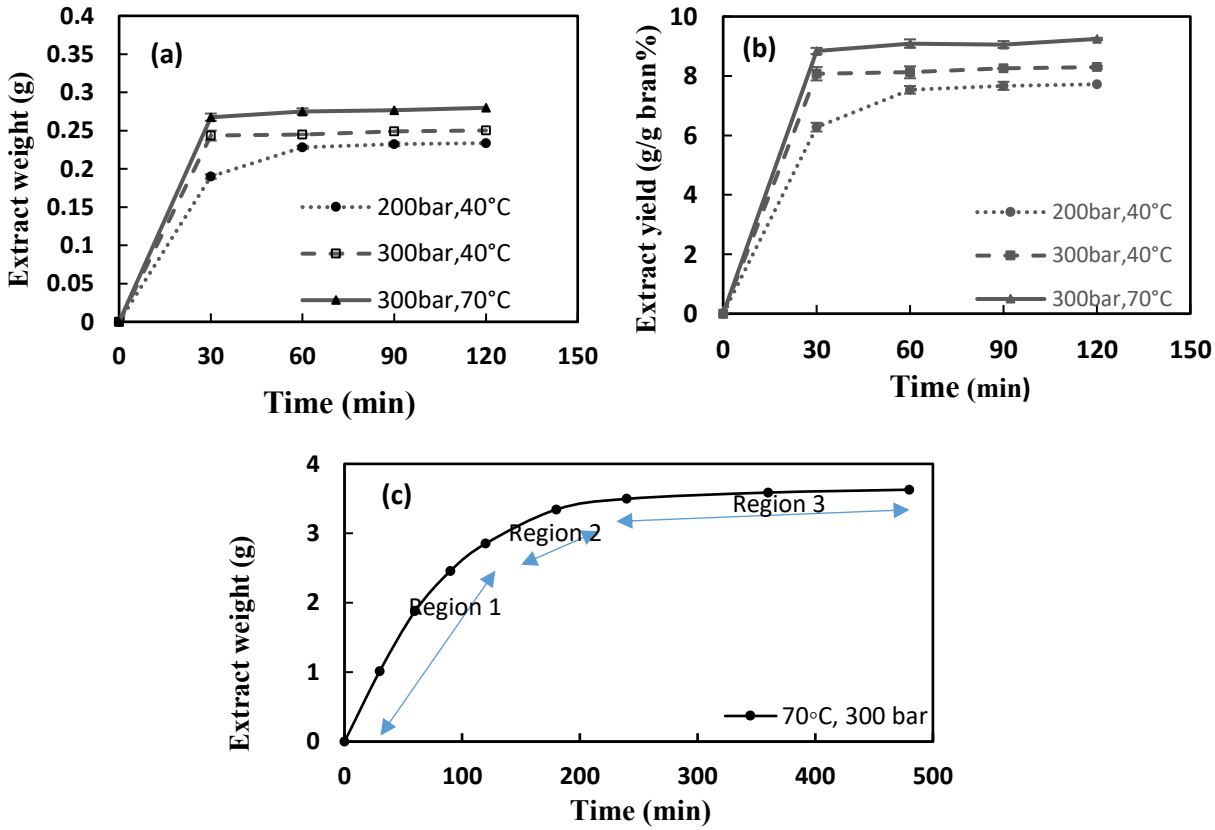


Fig. 3.3. Lipid extraction from barley bran using supercritical CO_2: (a) extract weight (g) using 10 mL vessel, (b) extract yield within 10 mL vessel and (c) extract weight (g) using a 300 mL vessel.

Saldaña et al. (2002) investigated the extraction of cocoa butter from Brazilian cocoa beans using SC-CO_2 at a temperature of 50°C, pressures of 152 and 248 bar and a flow rate of 1.3 g/min.

Pressure had a significant effect on the extraction yield of cocoa butter, where it increased three times from 4.5 to 13% by pressure rise with the same CO_2 mass (2.6 kg).

Prado and Saldaña (2014) studied the effect of pressure and temperature for flax hulls (26% lipid) oil recovery at three pressures (100, 200 and 300 bar) and two temperatures (40 and 60°C) at a flow rate of 3.15×10^5 kg/s for 120 min. They reported the increase of lipid extraction yield by pressure rise from 100 to 300 bar (almost 9 to 14% db) at 40°C and (almost 5 to 17% db) at 60°C. With an increase in temperature, the lipid yield increased at a high pressure of 300 bar but decreased at low pressures of 100 and 200 bar, which reflects the crossover behavior that was also reported by Fattori et al. (1988).

An increase from 0.24 to 0.26 g of extracted lipid was achieved when extraction was carried out at 40 and 70°C, respectively at a constant pressure of 300 bar and 30 min. These results agree with previous data reported by Temelli et al. (2013), who studied the effect of pressure and temperature on lipid recovery from 35% barley pearling flour. The authors reported an increase in the amount of total extract from 0.19 to 0.23 g after 180 min with a pressure rise from 240 to 450 bar. The density of CO_2 decreased at high temperatures (0.9 g/mL at 40°C/300 bar versus 0.7 g/mL at 70°C/300 bar). However, the vapor pressure and diffusivity of the lipid increased with temperature. In this study, after 120 min, yields of 7.7%, 8.3% and 9.3% were obtained for 200 bar/40°C, 300 bar/40°C and 300 bar/70°C, respectively (Fig. 3.3 b). The lowest lipid recovery was about 66% at 200 bar and 40°C for 30 min while the highest lipid recovery was 98% at 300 bar and 70°C for

120 min. Therefore, predominance of solute vapour pressure over solvent density was observed similarly to the reported by Azevedo et al. (2008) and Kwon et al. (2010) for SC-CO_2 extraction of green coffee oil and wheat bran oil, respectively.

As 300 bar and 70°C was the best condition to obtain the highest lipid recovery from barley bran, this condition was used for the large scale extraction vessel (300 mL), and the extraction curve is shown in Fig. 3.3 c. The SC-CO_2 extraction curve as a function of time has three main regions. In the first region, the free lipid on the surface of the barley bran is solubilized in SC-CO_2 and transferred by convection at a constant extraction rate region (solubility predominant). In general, 50 to 90% of total lipid is extracted during this period. The second region shows the falling extraction rate, in which free lipid available on the surface is reduced and diffusion of CO_2 starts to solubilize the rest of lipid inside the particles. The last region is the diffusion-controlled region, and mass transfer mechanism is mainly related to the diffusion of CO_2 and lipids inside the barley bran matrix (Fig. 3.3). The highest weight of lipid (3.62 g) corresponding to 95.4% from 40.2 g of initial sample was obtained after 480 min extraction. However, there was no significant change in the weight of the extracted lipid after 240 min.

From Fig 3.4, the ratio between the mass of total CO_2 to the mass of feed was 15.7 for a 30 min extraction using a 10 mL extraction vessel with a lipid recovery of 94% at 300 bar/70°C. Using the 300 mL extraction vessel, after 4 h extraction at the same solvent/feed ratio of 15.7, the recovery was 92%. Using 10 mL vessel and 3 g sample, with 2 mL/min of flow rate at pump

conditions (CO_2 density at 300 bar/70°C is 0.7879 g/mL, NIST Chemistry WebBook), the mass of CO_2 used was 47.27 g after 30 min extraction. Using 300 mL vessel and 40.2 g sample, with a flow rate of 1.4-1.5 L/min at ambient condition, the mass of CO_2 used was 47.22 g (CO_2 density at ambient condition is 0.00178 g/mL, NIST Chemistry WebBook) after 4 h extraction 640.8 g.

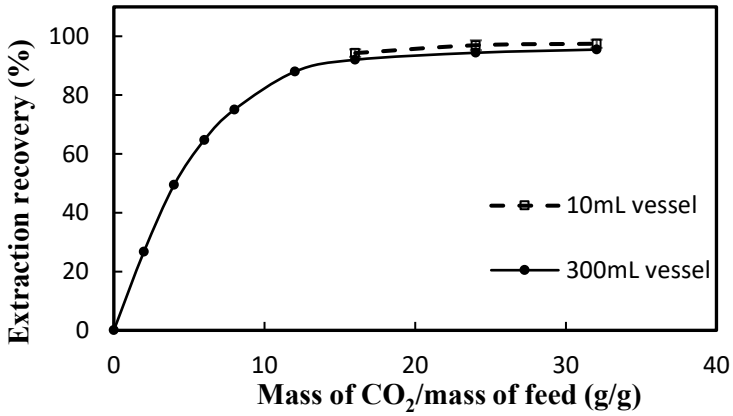


Fig. 3.4. Lipid recovery as a function of CO_2/feed (g/g) ratio at 300 bar and 70°C.

Water extraction or moisture removal is another phenomenon which takes place during SC-CO_2 lipid extraction and final defatted product has lower moisture content compared to the initial material. Dunford and Temelli (1997) reported the reduction of moisture in defatted canola flakes after SC-CO_2 lipid extraction. In this study, moisture content was reduced in the final defatted bran compared to the initial barley bran and related results are discussed in Chapter 3 (Section 3.3.2) The effect of temperature and pressure on the α-tocopherol content in the extracted lipids is shown in Fig. 3.5. The amount of α-tocopherol was 420.86 µg/g lipid at 200 bar and 40°C, then it increased to 613.91 µg/g lipid at 300 bar and 40°C. The increase of α-tocopherol content with pressure increase was due to the increase in CO_2 density and its solvating power. The amount of

α-tocopherol did not change at 300 bar and 70°C (605.66 µg/g lipid). Therefore, the best condition to obtain α-tocopherol was 300 bar and 40°C.

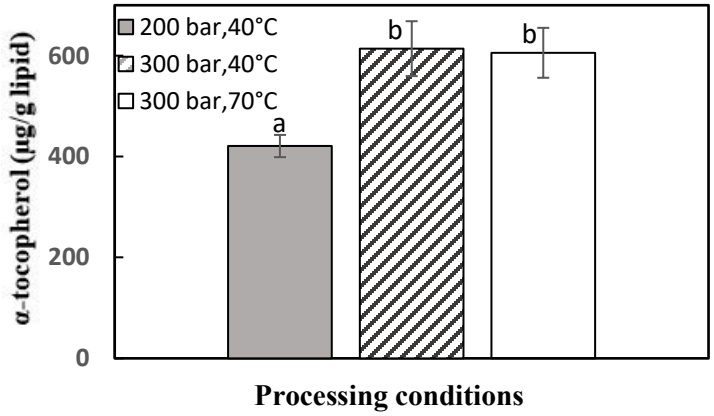
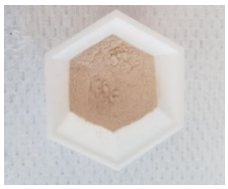


Fig. 3.5. Content of α-tocopherol in lipids extracted at different temperatures and pressures for 30 min within 10 mL vessel. Means with different letters are significantly different at $p<0.05$.

3.3.2. Proximate composition of raw, defatted and defatted-destarched bran

Moisture, fat, ash, protein, starch, and beta-glucan contents were reported in Table 3.1 for bran**,** defatted and defatted-destarched bran (DF-DS bran). Supercritical CO_2 lipid extraction was successful in reducing the lipid content of barley bran from 9.4 to 0.35%, while the protein content increased significantly from 25.7 to 28.6%. Moreover, moisture content dropped to 3.02% in defatted bran. Dunford and Temelli (1997) observed the reduction of moisture content of cooked canola from 12.7 to 9% and preheated canola from 15.9 to 12.2% at 75°C and 414 bar while extracting lipid from canola flakes. In another study, lipid was extracted from wheat germ by SC-CO_2 at 55°C and 380 bar where the moisture content reduced from 8 to 5% and the protein content increased from 31 to 36% in defatted cake (Panfilli et al., 2003).

Table 3.1. Composition of barley bran.

Constituent	bran (% w/w)	Defatted bran (% w/w)	Defatted-destarched bran (% w/w)
Moisture	5.3 ± 0.1^{a}	3.0 ± 0.2^{b}	2.7 ± 0.0^{b}
Protein	25.7 ± 0.3^{c}	28.6 ± 0.1^{b}	31.2 ± 0.6^{a}
Lipid	9.4 ± 0.2^{a}	0.3 ± 0.0^{b}	ND
Ash	4.1 ± 0.2^{b}	5.9 ± 0.0^{a}	6.9 ± 0.2^{a}
Starch	10.4 ± 0.6^{a}	12.0 ± 0.4^{a}	1.0 ± 0.1^{b}
Beta-glucan	1.8 ± 0.1^{a}	ND	2.6 ± 0.4^{b}
Image			

Data are expressed as mean ± standard deviation of two replicates.
[a-b] Different letters in the same row indicate significant differences ($p < 0.05$), ND: Not determined.

Structural carbohydrates were quantified in defatted and defatted-destarched bran (Table 3.2). The difference between the composition of defatted and defatted-destarched bran is illustrated in Fig. 3.6.

Table 3.2. Structural carbohydrates in defatted and defatted-destarched bran.

Constituent	Defatted bran (db %)	Defatted-destarched bran (db %)
Glucan (with beta glucan and starch)	29.40 ± 0.61^{a}	19.17 ± 0.93^{b}
Xylan	8.36 ± 0.17^{b}	12.83 ± 0.35^{a}
Galactan	3.06 ± 0.21^{a}	3.65 ± 0.15^{a}
Arabinan	9.55 ± 0.48^{b}	13.47 ± 0.61^{a}
Lignin	8.13 ± 0.32^{a}	7.17 ± 0.53^{a}

Data are expressed as mean ± standard deviation of two replicates.
[a-b] Different letters in the same row indicate significant differences ($p < 0.05$).

Enzymatic destarching was performed using alpha-amylase to hydrolyze α-(1→4) linkages and produce maltose, maltotriose and branched dextrins, followed by their conversion to glucose by ±amyloglucosidase and further precipitation of non-starch polysaccharides with ethanol. This enzymatic method was effective in reducing starch content to almost 1%. Total glucose content (glucan), determined after acid hydrolysis of mainly beta-glucan, starch and cellulose reduced from 29.40 to 19.17% compared to the defatted bran due to the removal of starch. However, beta-glucan, xylan and arabinan increased to 2.66, 12.83 and 13.47%, respectively. The reason why beta-glucan increased could be due to the method used for its determination (enzymatic hydrolysis) where glucose coming from only beta-glucan was accounted in the measurement (Fig. 3.6). Also, by removing starch, the other components increased.

Enzymatic removal of starch and further precipitation of non-starch polysaccharides was reported in the literature. Aguedo et al. (2013) removed starch from wheat bran using alpha-amylase treatment at 90°C for 90 min, where the glucan content was reduced from 10.23 to 3.08% db in the final destarched bran. Wang et al. (2009) treated wheat bran using a combination of alpha-amylase and amyloglucosidase to obtain destarched bran. In that study, starch content reduced from 14.21 to 0.23, while the amounts of xylan and arabinan increased from 15.66 to 42.78 and from 8.72 to 19.45% (db), respectively. Ruthes et al. (2017) removed starch from wheat bran before subcritical water hydrolysis to obtain beta-gluco-oligosaccharides and arabinoxylo-oligosaccharides. The authors reported an increase from 57 to 60.8 mg/g (db) for beta-glucan and

from 289.6 to 405.3 mg/g (db) for arabinoxylan content after removal of starch, however reduction of glucan from 45.5 to 35.7 was observed (% of total carbohydrates).

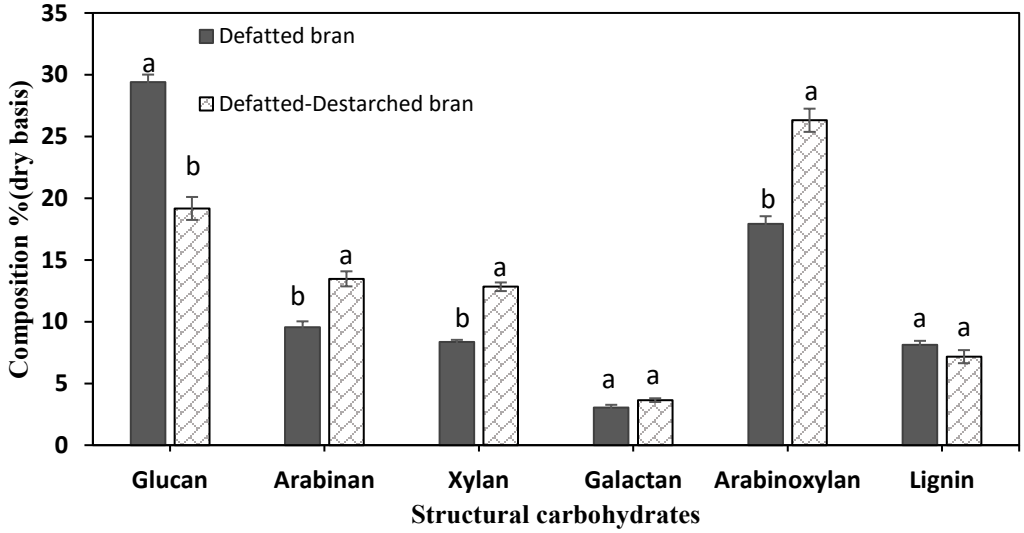


Fig. 3.6. Structural carbohydrates of defatted bran and defatted-destarched bran. Means within the same group of structural carbohydrates with different letters are significantly different at $p<0.05$.

3.3.3. Characterization of SCW hydrolysates

3.3.3.1. Change in pH

The pH values of the SCW extracts as a function of time are shown in Fig. 3.7. Initially, a pH value of 6.24 for barley bran was measured in the first 10 min using the SCW equipment with distilled water at ambient condition with a flow rate of 5 mL/min as the control. For all the other SCW extracts, after 15 min, the pH reduced sharply, showing only minor changes until the end of the process. Released acetyl groups in the hemicellulose are decreased with time and the continuous incoming water (Gallina et al. 2016). The trend is similar at all conditions investigated and the

temperature had the strongest influence in the pH change. The highest pH of 5.76-5.85 was obtained at 120°C, while the lowest pH of 3.89-4.37 was obtained at 200°C due to the higher extent of depolymerization of hemicellulose and greater deacetylation with further formation of acetic acid. The pH change was in agreement with Sarkar (2013), who studied the extraction of total carbohydrates and phenolic compounds from barley hull using subcritical water treatment. They reported a pH of 7.12 in the extract collected at 120°C, and acidic pH of 3.86 in the extract collected at 180°C. Release of acetyl groups from hemicellulosic sugars and formation of acetic acid increase the concentration of hydronium ions and reduce the pH in the subcritical water media (Fig. 3.7). Gallina et al. (2018) reported a pH change in hot pressurized water pre-treatment of 10 different types of wood at 160ºC, a flow rate of 150 L h^{-1}, pressure of 9 bar and solvent to feed ratio of 150:1 (mL/g). They observed that the highest increase in the concentration of H_3O^+ ions was within the first 40 min, with minor change until the end of the process. Acetic acid promotes the breaking down of extracted hemicellulose and increases the yield of reducing sugars. Also, acetic acid aids hydrolysis of the oligomeric fractions and reduces the molecular weight of extracted hemicellulose (Garrote et al., 2001). In this study, acetic acid was not detected in the liquid extracts obtained at 120 and 160°C. However, acetic acid was detected at conditions of 180°C (5.96±0.86 mg) and 200°C (10.83±0.93 mg) in the extracts collected after 15 min that led to the pH reduction from 5.76 to 4.06 and 3.89 at 120, 180 and 200°C, respectively. Also, lactic acid was detected in the liquid extracts obtained at high temperatures and could be responsible for

the increase of acidity. Lactic acid was quantified as 46±7.14 mg and 70.32±2.29 mg in the SCW extracts obtained at 180 and 200°C, respectively, after 15 min extraction time (Fig. 3.8). Lactic acid has important applications in the food industry such as mild acid flavor agent, pH regulator or preservative in olives and pickled vegetables (Komesu et al., 2017).

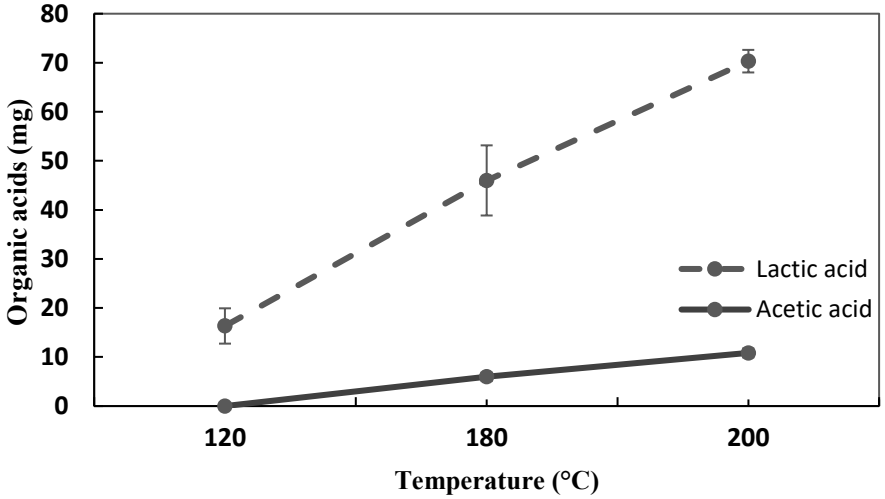


Fig. 3.7. pH values of the SCW extracts at different temperatures and time.

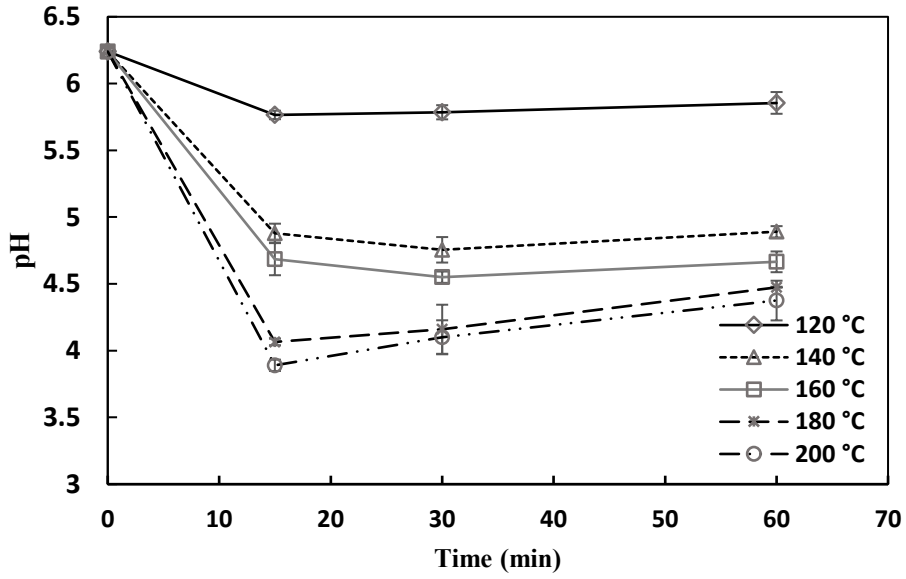


Fig. 3.8. Organic acids in the SCW extracts obtained at different temperatures after 15 min.

3.3.3.2. Reducing sugar content of SCW hydrolysates

The effect of temperature (120-200°C) on reducing sugar yield at a constant flow rate (5 mL/min) and pressure (50 bar) is shown in Fig. 3.9. The DF-DS bran is mainly composed of biopolymers, including glucan (19.17% db) and arabinoxylan (26.31% db) (Fig. 3.6), which were hydrolyzed in the SCW media and converted to smaller compounds with reducing ends. An increase in these reducing sugars shows a higher rate of depolymerization and formation of low molecular weight compounds. Zhao and Saldaña (2019) studied the hydrolysis of cassava starch and chitosan by SCW treatment at temperatures of 75, 100, 125 and 150°C and pressures of 50, 85, 120 and 155 bar. They reported an increase of reducing end yield around five times in extracts collected at 150 °C compared with the extracts at 75°C, which indicated the hydrolysis of these polymers and formation of smaller compounds with reducing ends.

Hydronium ions produced from water auto-ionization led to hemicellulose depolymerization by cleavage of glycosidic linkages and release of *O*-acetyl groups, which form acetic and uronic acids. These acids accelerate the hydrolysis of hemicellulose, further producing reducing sugars (Alvarez et al., 2014). The temperature had a significant effect on the hydrolysis rate and promoted the breaking down of polymers, leading to more reducing sugar production. The formation of reducing sugar was less significant at 120 and 140°C. Increasing the temperature from 160 to 180°C led to higher reducing sugar yield from 2.63 to 7.28%, 4.43 to 10.09% and 6.99 to 12.17% for 15, 30 and 60 min extraction time, respectively.

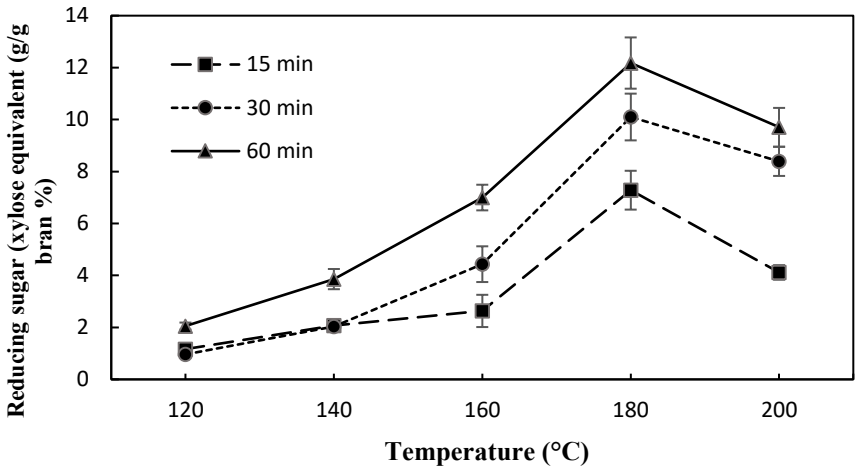


Fig. 3.9. Yield of reducing sugar after SCW hydrolysis at different temperatures and times at 5 mL/min water flow rate and 50 bar.

The effect of temperature on the reducing sugar yield is due to the thermal effects and increase in the concentration of H^+ and OH^-, which promotes the acid-catalyzed hydrolysis of cellulose and hemicellulose and formation of more reducing sugars. However, at elevated temperature of 200°C, the yield declined to 4.11, 8.39 and 9.7% for 15, 30 and 60 min extraction time, respectively. Pourali et al. (2010) reported similar results in the hydrolysis of rice bran using a batch SCW system over a temperature range of 180–360°C for 10 min, where the total soluble sugars yield increased with temperature and reached the maximum amount of almost 200 mg/g db at 190°C, and then decreased sharply at temperatures above 200°C. This reduction could be an evidence of conversion of sugars into other secondary compounds, such as hydroxymethyl furfural (HMF), furfural, acetic acid, levulinic acid, formic acid, lactic acid, etc. (Abaide et al. 2019; Pinkowska et al., 2011). In this study, increased levels of acetic acid and lactic acid were detected in the

hydrolysates collected at 180 and 200°C, leading to a decrease of reducing sugars and further reduction in pH, which was explained in Chapter 3 (Section 3.3.3.1). According to Fig. 3.7, pH values decreased in the liquid extracts obtained at 200°C from an initial pH of 6.2 to almost pH of 3-4. However, this further increase in acidity did not improve the yield of reducing sugars, as organic acids like lactic acid was produced as a result of sugars degradation. Phaiboonsilpa and Saka (2011) investigated the effect of acetic acid addition on the hydrolysis of Japanese beech in the SCW media and reported no improvement of hemicellulose hydrolysis by additional acetic acid (0.1–3%) compared to the control sample (0%), which showed the auto formation of acetic acid during the hydrolysis of hemicellulose. Similar findings were obtained by Ciftci and Saldaña (2015), who hydrolyzed sweet blue lupin hull in SCW and reported that the yield of hemicellulosic sugars in the extracts increased from 160°C (64.2%) to 180°C (85.5%), and then decreased at 200 and 220°C. Abaide et al. (2019) obtained fermentable sugars from rice husk by SCW hydrolysis using a semi-continuous system with at a flow rate of 15 mL/min, 30 g feed, pressure of 250 bar and different temperatures (180, 220, and 260°C) for a total reaction time of 15 min. They reported an increase in reducing sugar yield from 0.8 to 18.3 g/100 g rice husk at 180 and 220°C and a reduction to 5.5 g/100 g rice husk at 260°C, where the lower sugar content at 260°C was associated with the increase of acetic acid concentration at 220 and 260°C from 4.5 to 21.9 g/L due to the degradation of sugars.

The yield of reducing sugar increased significantly ($p < 0.05$) with increasing solid residence time at 180°C from 15 min (7.28%) to 30 min (10.09%) and no statistical change was observed at 60 min (12.17%). Improvement of reducing sugar yield after 30 min extraction was due to the complete soaking and more interaction of the feed and dissolution of biopolymers from surface of the matrix, enhancing their extraction and depolymerization, leading to a higher yield of reducing sugars. Overall, the highest reducing sugar yield was obtained at 180°C and 30 min (10.09±0.90%) and 180°C and 60 min (12.17±0.98%).

Mayanga et al. (2018) investigated the hydrolysis of brewer's spent grains in a semi-continuous SCW system at 140, 160, 180 and 210°C with flow rates of 10 and 20 mL min^{-1} and 150 bar. These authors reported a significant increase in reducing sugar yield with temperature rise from 140 to 210°C. They obtained the maximum reducing sugar yields (6.4 g reducing sugar/100 g) at 210°C for 56 min (10 mL min^{-1}) and 28 min (20 mL min^{-1}), which were less than the yield in this study. Also, they did not observe reduction of reducing sugars at temperatures above 180°C, probably because of the higher flow rate, which led to the short residence and contact time of brewer's spent grains with water to produce acids. Therefore, the hydrolysis rate of hemicellulose was reduced (Abdelmoez et al., 2014; Zhu et al., 2011).

3.3.3.3. Total sugars composition

The production of reducing sugars indicates the trend of hydrolysis based on temperature and time. Further quantifications are needed to determine the individual sugars as monomeric units after post hydrolysis with sulfuric acid. Table 3.3 summarizes sugars composition of SCW hydrolysates obtained in this study, including arabinose, xylose, galactose, glucose and total sugars and the resulting pH of the hydrolysates. Figs. 3.10 and 3.11 show the recovery (%) of individual and total sugars obtained after SCW treatment at 15, 30 and 60 min as a function of temperature from 120 to 180°C. The recovery was calculated with respect to the initial weight of structural carbohydrates in the destarched bran.

The main sugars obtained from the DF-DS bran were xylose, glucose and arabinose. Glucose from hydrolysis of hemicellulose, cellulose and starch was obtained in the first 15 min with 10.5, 10.6, 13.8 and 16.5% recovery at 120, 140, 160 and 180°C, respectively. There was an increase in the glucose recovery only when the temperature increased from 120 to 140°C, but there was no significant difference at 160 and 180°C after 30 and 60 min extraction times. The maximum recovery of glucose was 29.86% at the best condition of 140°C/ 50 bar/ 5 mL/min/ 60 min, without any noticeable change up to 160 and 180°C (Fig. 3.10 a).

Table 3.3.a. Sugar composition after SCW treatment.

Processing conditions		SCW extracts						
T (°C)	t (min)	pH	Glucose (mg)	Galactose (mg)	Xylose (mg)	Arabinose (mg)	Ratio (p/g)	Total sugars (mg)
120	15	5.76	40.2±2.1^{e}	12.3±0.5^{de}	14.5±0.4^{h}	21.4±3.6^{g}	0.89	88.6±2.4^{e}
120	30	5.78	59.3±5.8^{d}	15.3±1.62^{de}	21.4±1.4^{gh}	32.7±3.2^{fg}	0.91	128.9±12.2^{e}
120	60	5.85	101.2±6.6^{ab}	41.9±1.4^{b}	46.6±2.8^{ef}	73.9±3.4^{e}	1.19	263.7±11.4^{c}
140	15	4.88	40.6±2.8^{e}	13.6±0.1^{de}	16.3±2.78^{gh}	27.1±3.9^{fg}	1.06	97.7±9.5^{e}
140	30	4.75	89.13±3.1^{bc}	28.8±1.5^{c}	53.2±4.3^{ef}	72.7±4.8^{e}	1.41	243.9±13.9^{c}
140	60	4.89	114.5±3.4^{a}	44.9±1.3^{ab}	84.5±4.2^{d}	126.4±2.2^{c}	1.84	370.3±1.8^{b}
160	15	4.68	53.1±4.5^{de}	12.17±0.3^{e}	35.1±5.5^{fg}	37.4±4.4^{fg}	1.36	137.8±7.0^{de}
160	30	4.55	83.0±1.9^{c}	24.9±1.1^{c}	77.8±4.3^{d}	90.5±4.1^{d}	2.02	276.4±11.7^{c}
160	60	4.66	103.6±3.2^{ab}	44.8±0.7^{ab}	112.6±5.0^{c}	147.6±5.2^{b}	2.51	408.7±14.2^{b}
180	15	4.06	63.6±3.7^{d}	17.5±0.23^{d}	58.9±2.1^{e}	43.3±2.8^{f}	1.60	183.49±4.2^{d}
180	30	4.16	80.3±0.9^{c}	39.9±2.74^{b}	126.8±6.7^{b}	99.3±5.2^{d}	2.81	376.2±25.3^{b}
180	60	4.33	102.8±5.0^{ab}	47.8±0.07^{a}	169.8±9.4^{a}	174.±4.5^{a}	3.34	487.8±10.6^{a}

T: temperature; t: time, Data are expressed as mean ± standard deviation of two replicate

$^{a-h}$ Different letters in the same column indicate significant differences ($p < 0.05$), p/g: pentose/glucose.

Total sugars: Glucose + galactose + arabinose + xylose, Recovery is expressed as (%) based on each structural carbohydrate present in the feed material.

Table 3.3.b. Sugar recovery after SCW treatment.

Processing conditions		SCW extracts				
T (°C)	t (min)	Glucose (%)	Galactose (%)	Xylose (%)	Arabinose (%)	Total sugars (%)
120	15	10.5±0.5^{e}	16.9±0.7^{de}	5.6±0.1^{h}	7.9±1.3^{g}	9.0±0.2^{e}
120	30	15.4±1.5^{d}	21.0±2.2^{de}	8.3±0.5^{gh}	12.1±1.2^{fg}	13.1±1.2^{e}
120	60	26.3±1.7^{ab}	57.5±1.9^{b}	18.1±1.0^{ef}	27.4±1.2^{e}	26.8±1.1^{c}
140	15	10.6±0.7^{e}	18.6±0.2^{de}	6.3±1.0^{gh}	10.4±1.4^{fg}	9.9±0.9^{e}
140	30	22.6±0.8^{bc}	39.4±2.1^{c}	20.7±1.6^{ef}	26.9±1.7^{e}	24.8±1.4^{c}
140	60	29.8±0.8^{a}	61.5±1.8^{ab}	32.9±1.6^{d}	46.9±0.8^{c}	37.6±0.1^{b}
160	15	13.8±1.1^{de}	16.6±0.5^{e}	13.6±2.1^{fg}	13.8±1.6^{fg}	14.0±1.4^{de}
160	30	21.6±0.5^{c}	34.2±1.6^{c}	30.3±1.6^{d}	33.6±1.5^{d}	28.1±1.1^{c}
160	60	27.0±0.8^{ab}	61.4±0.9^{ab}	43.8±1.9^{c}	54.7±1.9^{b}	41.6±1.4^{b}
180	15	16.5±0.9^{d}	24±1.7^{d}	22.9±0.8^{e}	16.0±1.0^{f}	18.6±0.43^{d}
180	30	20.9±0.2^{c}	54.7±3.7^{b}	51.2±2.6^{b}	36.8±1.0^{d}	38.2±2.5^{b}
180	60	26.8±1.3^{ab}	65.4±0.9^{a}	63.5±3.6^{a}	64.6±1.6^{a}	49.6±1.0^{a}

T: temperature; t: time, Data are expressed as mean ± standard deviation of two replicates.

$^{a-h}$ Different letters in the same column indicate significant differences ($p < 0.05$), p/g: pentose/glucose.

Total sugars: Glucose + galactose + arabinose + xylose, Recovery is expressed as (%) based structural carbohydrate present in the feed material.

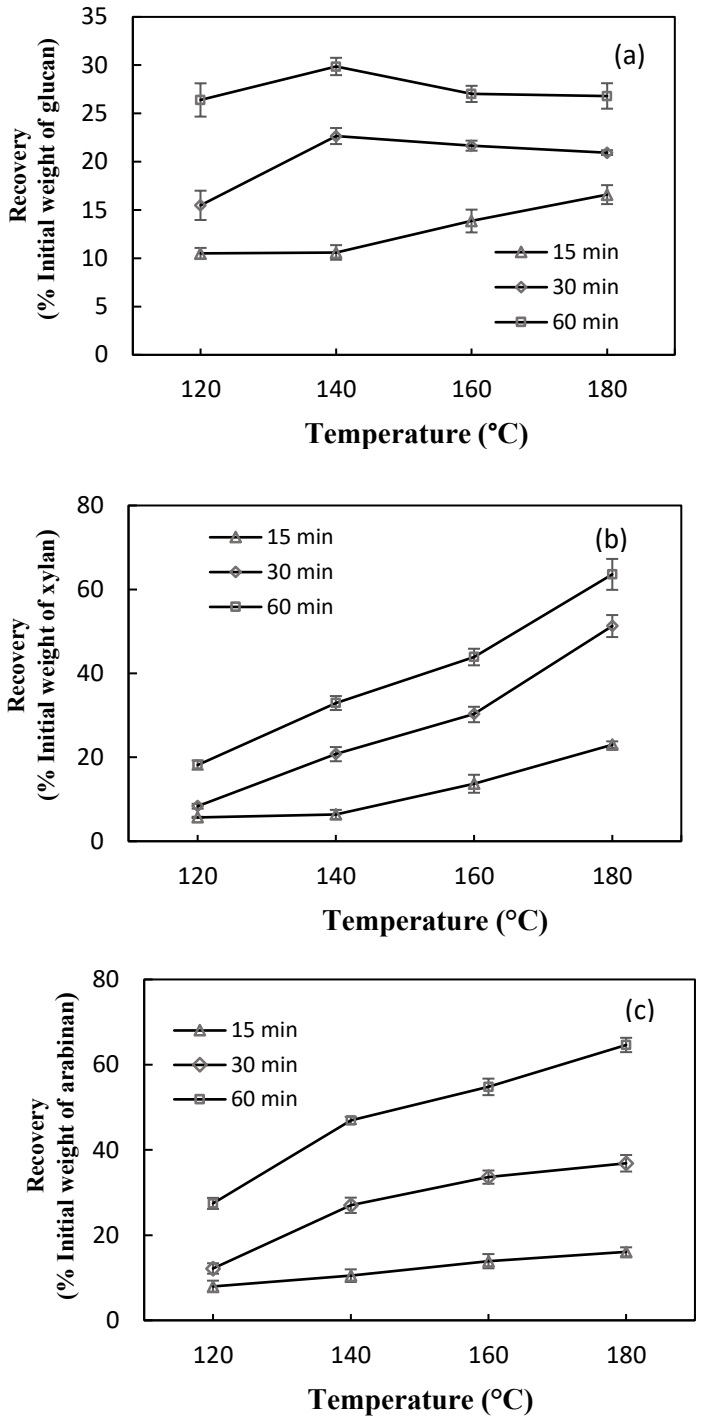


Fig. 3.10. Total recovery of: (a) glucose, (b) xylose, and (c) arabinose in the SCW extracts at 50 bar and 5 mL/min.

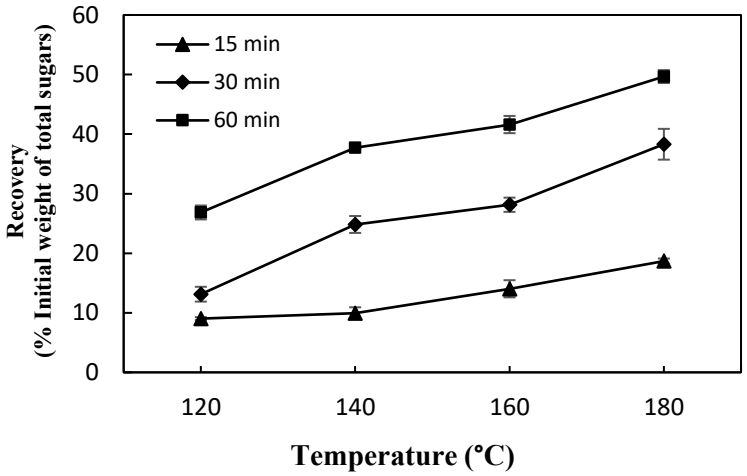


Fig. 3.11. Total recovery of sugars (glucose, xylose, arabinose and galactose) in the SCW extracts at 50 bar and 5 mL/min.

In the SCW condition, low temperatures of 100-135°C are sufficient to obtain the maximum amount of glucose as a free monosaccharide or coming from starch hydrolysis or from the degradation of hemicelluloses, mainly beta-glucan (Cara et al., 2012; Benito-Roman et al., 2013). Therefore after 30 and 60 min, no increase in the recovery of glucose was observed.

Recovery of pentose sugars, including xylose and arabinose, which are the main constituents of hemicellulose increased with an increase of temperature. The amount of xylose was determined as 14.5, 16.3, 35.1 and 58.9 mg in the hydrolysates obtained after 15 min reaction time at 120, 140, 160 and 180°C, respectively. Also, there is a continuous increase with temperature rise from 120 to 180°C after 30 and 60 min (Fig. 3.10 b). In terms of arabinose recovery, a similar trend was observed after 30 and 60 min extraction (Fig. 3.10 c). For the liquid extracts obtained at 180°C and 60 min, xylose and arabinose contents were 169.8±9.4 and 174.1±4.5 mg (70.31% of total

sugars), corresponding to a maximum recovery of 63.58 and 64.62%, respectively. Gallina et al. (2016) hydrolyzed eucalyptus globulus wood in a semi-continuous reactor with 5 g feed, flow rate of 5 mL/min and pressure of 100 bar for 90 min and reported a similar trend with an increase in the recovery of pentose sugars (arabinose and xylose) from 0 to 67.40% at 135 and 180°C, respectively. They obtained pentose sugars with similar recovery at 180°C and 90 min compared to that in this study at 180°C and 60 min. This might be due to the lower solvent-to-feed ratio used (90 mL/g) compared to this study (150 mL/g). Yu et al. (2013) used SCW treatment to obtain sugars from sugarcane bagasse with 45.2% glucan, 23.6% xylan and 2.1% arabinan, using 35 g of the raw material (5% w/v in water) inside a batch reactor for 20 min. They recovered the highest level of xylose (74.3%) at 160°C, and then a reduction was observed by increasing the temperature to 180 and 200°C with 60 and 15% recovery, respectively. They reported an increase in the concentration of degradation products such as furfural and acetic acid from approximately 1 to 2.5 g/L and 1 to 1.7 g/L in the liquid extracts obtained at 180 and 200°C, respectively. In this study, xylose recovery reduction was not observed at 180°C, because in a semi-continuous SCW system, the liquid flows rapidly out of the reactor, minimizing degradation of sugars compared to the batch system.

Ruthes et al. (2017) also extracted sugars from destarched wheat bran using 1 g of raw material at 100, 120, 140 and 160°C for 15 min and pH of 5, 7 and 9 by addition of formic acid or NaOH.

They obtained the highest extraction yields at 160°C and pH 7 with the total recovery of arabinose and xylose (55.1%).

Low recovery of glucose compared to xylose and arabinose might be due to the fact that cellulose is one of the sources of glucose, which is difficult to hydrolyze at temperatures below 240°C. According to Table 3.3, the presence of glucose was higher than the pentose sugars (xylose and arabinose) in the fractions obtained at low temperature (120°C). However, the ratio between the content of pentoses and glucose increased at elevated temperatures (>140°C) and it reached to 3.34 at 180°C/60 min. Ruthes et al. (2017) reported a similar behavior for the extraction of sugars from destarched wheat bran. In their study, glucose had higher concentrations at lower temperatures of 100°C with 530 (mg/g DW of the extracts) compared to the pentose sugars (arabinose and xylose) with 200 mg/g (mg/g DW of the extracts). Moreover, they observed a similar increase in the ratio between pentose sugars and glucose from 0.37 to 3.6 when temperature increased from 100 to 160°C. Therefore, polysaccharides composed of glucose, including beta-glucan and starch were easier to hydrolyze than arabinan and xylan in the SCW media. This phenomenon was also reported by Benito-Roman et al. (2013), who hydrolyzed wheat bran using a semi-continuous rector with 10-12 g feed, pressure of 20 bar, flow rate of 4 g/min, temperatures of 110-175°C for 105 min. In their study, free glucose or glucose from starch hydrolysis was extracted easily at 110 and 135°C with 150 and 80 mg obtained in the total extract, while trace amounts of xylose and

arabinose were observed at these temperatures. However, the concentration of pentose sugars increased dramatically at 160 and 180°C with approximately 100 and 320 mg, respectively.

The effect of temperature on the recovery of total sugars after SCW treatment (Fig. 3.11) was similar to the trend observed for the reducing sugars (Fig. 3.9). Total sugars recovered after SCW treatment was in the range of 88.6 and 487.8 mg corresponding to 9.02 and 49.66% recovery in the liquid extracts at 120°C/15 min and 180°C/60 min, respectively. Extraction time of 15-60 min had a significant effect on the total amount of sugars at all temperatures investigated. For example, 88.6 and 263.7 mg of total sugars were obtained in the liquid extracts after 15 and 60 min at 120°C. By increasing the time, the solvent-to-feed ratio increased leading to hydrolysis of polysaccharides, resulting in a significant influence on the recovery of sugars. The effect of temperature was significant between 120°C (9.02, 13.12, 26.84% recovery) and 180°C (18.67, 38.29, 49.66% recovery) for 15, 30 and 60 min, respectively, and there was no difference between the total amount of sugars obtained at 140 and 160°C.

Sunphorka et al. (2012) reported sugar extraction from rice bran and de-oiled rice bran using SCW in a semi-continuous reactor with a flow rate of 0.5 mL/min, pressures of 40-100 bar and temperatures of 150 and 250°C for 5 and 60 min. The authors reported an increase in total sugars yield by an increase of time from 5 min (18.3 mg/g rice bran) to 60 min (165.1 mg/g rice bran) in the extracts for treatment at 150°C and 0.5 mL/min. Also, they reported an increase from 18.3 to

94.3 mg/g of total sugars in the extracts obtained at 150 and 250°C for 5 min and 165.1 to 119 mg/g for 60 min, which showed the positive effect of time on the total sugars yield.

3.3.3.4. Xylo-oligosaccharides (XOS) and monosaccharides by HPLC

Xylo-oligosaccharides with different degrees of polymerization (DP) were determined by HPLC for all the hydrolysates obtained at 120, 140, 160, 180 and 200°C. Standards of xylose (X1), xylobiose (X2), xylotriose (X3), and xylohexaose (X6) were used to determine the molecular weight (MW) and degree of polymerization (DP) calibration curve (see Appendix A, Fig. A2 a,b). Chromatograms obtained from DF-DS bran hydrolysis by SCW at temperatures in a range of 120-200°C are shown in Fig. 3.12 a-e. Different peaks with retention times in the range of 7.4 to 26.2 min corresponding to DP 1-12, and MW of 150 – 1698 Da were identified in the liquid extracts. For the liquid extracts obtained at 120 and 140°C, only three peaks with retention times of 7.24 (compounds with DP $\geq$ 12), 23.2 and 26.5 min were found and any peaks related to the XOS with DP<12 were not detected (Fig. 3.12 a,b). Only SCW hydrolysates obtained at 160, 180 and 200°C showed peaks related to XOS.

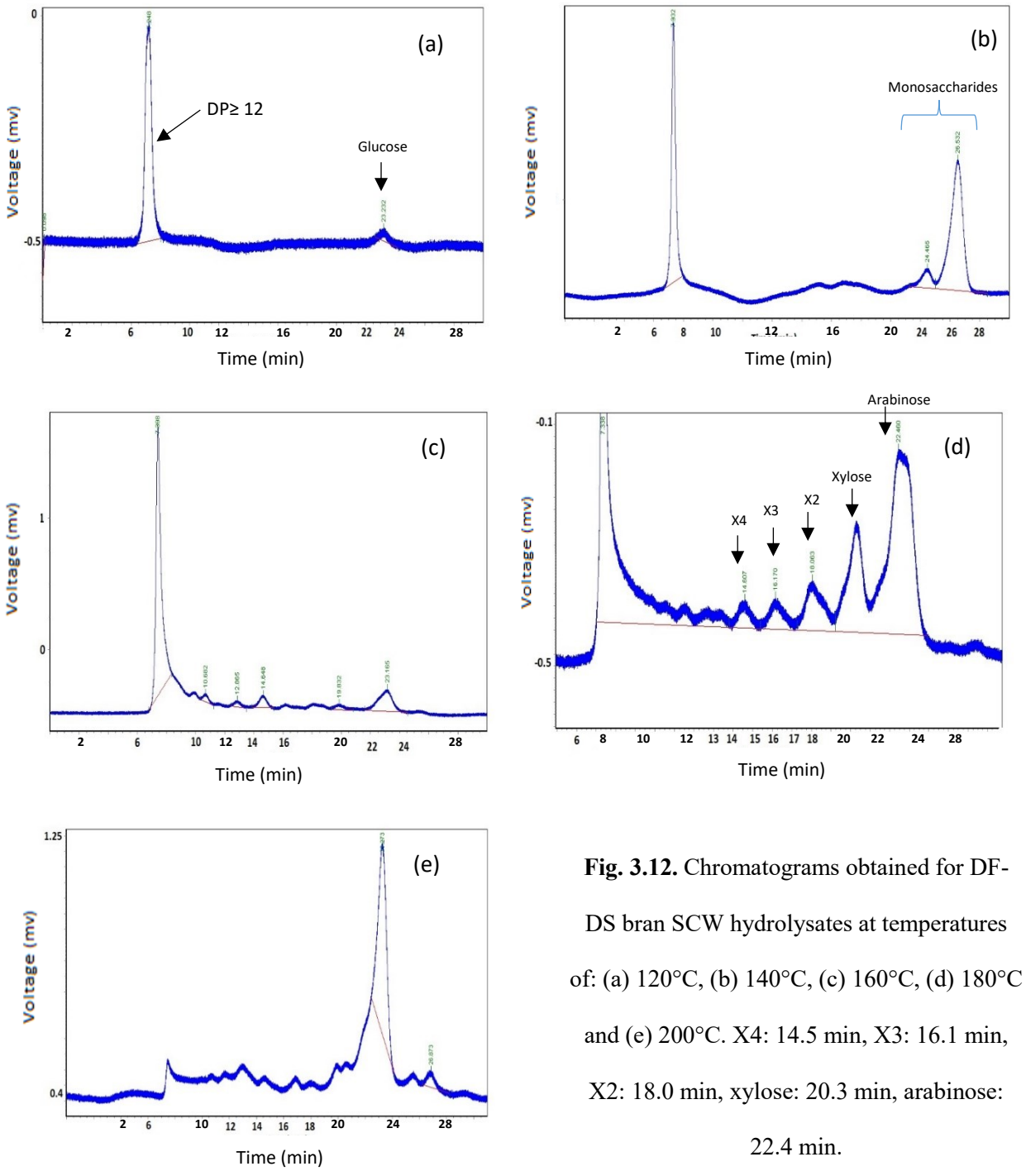


Fig. 3.12. Chromatograms obtained for DF-DS bran SCW hydrolysates at temperatures of: (a) 120°C, (b) 140°C, (c) 160°C, (d) 180°C and (e) 200°C. X4: 14.5 min, X3: 16.1 min, X2: 18.0 min, xylose: 20.3 min, arabinose: 22.4 min.

Table 3.4 summarizes the contents and recovery of XOS such as xylobiose, xylotriose and xylotetraose and liberated monosaccharides, including glucose, xylose and arabinose, which were determined in the hydrolysates collected at 160, 180 and 200°C. Total recovery of XOS in the liquid extracts at 180°C obtained at different times is shown in Fig. 3.13.

For all the extracts collected at 120°C, a large peak of the high-molecular weight fraction with DP≥12 and glucose as monosaccharide were present (Fig. 3.12 a). Xylose, arabinose and XOS were not detected at 120°C/50 bar. However, glucose was detected with contents of 2.83±0.19, 4.63±0.56 and 7.80±0.47 mg after 15, 30 and 60 min, respectively. Studies showed that glucose as a free monosaccharide or coming from starch hydrolysis was obtained at relatively low temperatures of 100-135°C (Benito-Roman et al., 2013). Also, at 160°C, free glucose was quantified with contents of 8.94±1.4 and 11.04±1.15 mg in the extracts obtained at 15 and 30 min, respectively. Generally, hydrolysis of cellulose is difficult at mild temperatures (<250°C) (Prado et al., 2014). Therefore, glucose in the hydrolysates obtained at mild extraction conditions was derived probably from the degradation of hemicelluloses, mainly beta-glucan (Cara et al., 2012). A decrease in the amount of glucose was observed at 180 and 200°C, where trace amounts were detected.

Table 3.4. Recoveries of XOS and monosaccharides in the SCW hydrolysates at 50 bar/5 mL/min.

Processing conditions		Xylo-oligosaccharides Content (mg)				Xylo-oligosaccharides recovery (%)				Monosaccharides Content (mg)		
T (°C)	t (min)	X2	X3	X4	Total XOS	X2	X3	X4	Total XOS	Glucose	Xylose	Arabinose
160	**30**	ND	Trace	12.5±1.0^{c}	12.5±1.9^{c}	ND	Trace	4.8±0.3^{d}	7.3±0.7^{c}	11±1.1	ND	13.4±2.8^{b}
180	**15**	8.0±1.0^{b}	14.9±1.8^{c}	7.7±0.7^{d}	30.7±3.6^{b}	3.1±0.4^{b}	5.8±0.7^{c}	3±0.2^{c}	11.9±1.4^{b}	ND	6.3±0.7^{b}	24.2±1.1^{a}
180	**30**	40.0±1.3^{a}	46.1±0.4^{b}	26.2±1.0^{b}	112.5±2.8^{a}	15.6±0.5^{a}	17.9 ±0.1^{b}	10.2±0.4^{b}	43.8±1.1^{a}	ND	14.9±0.1^{a}	26.3±2.9^{a}
180	**60**	11.2±1.5^{b}	55.4±0.1^{a}	46.7±1.4^{a}	113.4±1.0^{a}	4.3±0.5^{b}	21.5±0^{a}	18.2± 0.5^{a}	44.2±0.0^{a}	ND	13.3±1.7^{a}	23.3±2.0^{a}
200	**15**	Trace	Trace	Trace	Trace	ND	ND	ND	ND	ND	11.5±0.5^{ab}	ND
200	**30**	Trace	Trace	Trace	Trace	ND	ND	ND	ND	ND	12.6±0.1^{a}	ND

Temperature, t: time, ND: Not detected; X2: xylobiose, X3: xylotriose, X4: xylotetraose, Total XOS: X2+X3+X4. XOS Recovery (%): (mass of XOS/mass of initial xylan) x 100.

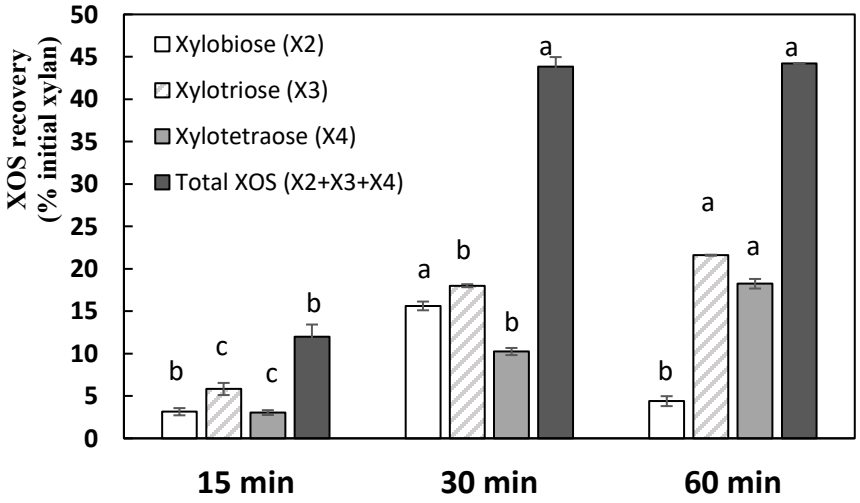


Fig 3.13. Total recovery of xylo-oligosaccharides (XOS) in the SCW extracts at 180°C, 50 bar and 5 mL/min. Means within the same group of XOS with different letters are significantly different at $p<0.05$.

The liberation of arabinose started at 160°C with 8.83 and 16.2 mg in 15 and 30 min times, respectively, while xylose was not detected as free monomer at these conditions. This behavior indicated that arabinose groups are liberated easier than xylose at lower temperatures from the arabinoxylan backbone, confirming that arabinose groups are located at the side chains of hemicellulose and therefore are more accessible for the hydronium ion attack and are separated from the xylan backbone before xylan starts depolymerizing (Kalnins, 2017).

A similar trend was observed for arabinose, which was degraded at 200°C. However, xylose was still stable at high temperatures of 180 and 200°C, indicating higher thermal sensitivity of arabinose compared to xylose (Carvalheiro et al., 2004). This is in agreement with the results

obtained by Mayanga et al. (2018), who hydrolyzed brewer's spent grains in a semi-continuous SCW reactor. They reported an increase in the yield of xylose from 0.3 to 1.1 g/100g feed and arabinose from 1.2 to 2.4 g/100g feed in the liquid extracts obtained at 140 and 180°C, respectively. However, they observed the degradation of arabinose liberated by temperature rise from 180 to 210°C with the yields of 3.1 to 2 g /100 g feed, which was more heat-sensitive compared to xylose with the yields of 0.8 to 1.6 g /100 g feed. Carbohydrate-derived organic acids such as lactic, formic, acetic, succinic and levulinic acids were detected as degradation products at high temperatures of 210°C (Gallina et al., 2016; Pinkowska et al., 2011). Fig. 3.14 shows the reaction pathways of glucose and fructose in subcritical water and conversion to lactic acid. In this study, 46 and 70.32 mg of lactic acid with 5.96 and 10.83 mg of acetic acid were determined at 180°C/15 min and 200°C/15 min, respectively (Fig. 3.8).

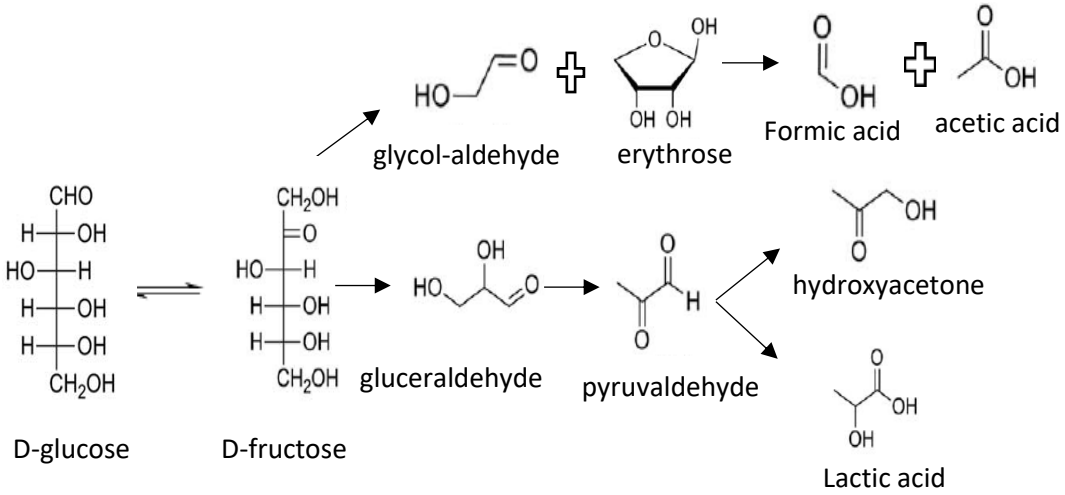


Fig. 3.14. Reaction pathways for conversion of glucose and fructose to organic acids in subcritical water media.

In the liquid extracts collected at 120 and 140°C xylan started to solubilize in the liquid fraction due to the presence of xylose after post hydrolysis with sulfuric acid (Fig. 3.10 b), while xylose and XOS were not detected, indicating that solubilized xylan had high molecular weight and temperature was still not sufficient to hydrolyze the dissolved xylan to smaller oligomers. When the temperature increased to 160°C, the area of a large peak related to the compounds with DP≥12 raised and formation of XOS started (Fig. 3.12 c). For the liquid extracts collected at 160°C/15min, XOS was not detected as probably the residence time was not enough to hydrolyze and convert the feed to smaller oligomers. However, the presence of reducing sugars at 2.63% (Fig. 3.9) at this condition (160°C/15min) could be because of the liberation of monosaccharides including glucose with 8.94 mg and arabinose with 8.83 mg. Trace amounts of xylotriose (X3) and 12.5 mg of xylotetraose (X4) were detected with an increase in the residence time in the extracts obtained at 160°C/30 min.

According to Table. 3.4, a large content of XOS was found in the hydrolysates obtained at 180°C, where the area of the large peak (DP≥12) reduced, indicating the cleavage of large fraction or arabinoxylan hydrolysis and production of xylobiose, xylotriose, xylotetraose and xylose. For the liquid extracts collected at 180°C/15 min, 8.05±1.09, 14.93±1.83, 7.79±0.72 and 30.78±3.65 mg of X2, X3, X4 and total XOS were obtained, corresponding to 3.14, 5.81, 3.03 and 11.99% initial xylan recovery.

By increasing the residence time to 30 min, the recovery of X2, X3, X4 and total XOS improved significantly to 15.61, 17.99, 10.23 and 43.85% initial xylan, respectively, which indicated the increase in the rate of hydrolysis and formation of high contents of XOS due to an increase in the solvent-to-feed ratio, leading to hydrolysis of polysaccharides. Moreover, the molecular weight of hemicellulose obtained reduced as a function of time in the SCW media due to more deacetylation (Yedro et al., 2017).

A similar trend was observed for reducing sugars (Fig. 3.9). However, a further increase in residence time to 60 min did not have an effect to improve the content of total XOS, but increased the contents of X4 and X3 with a reduction in the content of X2. Studies showed that using a semi-continuous SCW hydrolysis of walnut and cherry, fractions with very low molecular mass were produced at the beginning of the process (first 20 min) due to the liberation of acetic acid from acetylated hemicellulose, which helped the hydrolysis and formation of shorter oligomers. Later within 20-60 min, temperature effect led to the breaking of non-acetylated hemicelluloses and the production of longer oligomers (Gallina et al., 2018).

After increasing the temperature up to 200°C, the peak for high molecular weight compounds disappeared and XOS was not detected (Fig 3.12 e). However, large amounts of organic acids such as lactic acid and acetic acid were determined, which indicates the degradation of sugars at this condition (Fig 3.8).

Chen et al. (2017) reported the production of oligosaccharides in SCW media from wheat straw with 41.23% cellulose, 27.70% hemicellulose and 17.99% lignin, using a 100 mL batch reactor with 5.0 g of dewaxed straw and 50 mL of water (solvent-to-feed ratio: 10 mL/g) at 120, 140, 160, 180 and 200°C for 0.5 h. They reported a similar composition of the liquid extracts with xylobiose, xylotriose, xylotetraose and xylopentaose as the main XOS produced and liberated monomers, including arabinose, xylose and glucose. In that study, XOS production started at 120°C with 0.25 g/kg raw material for X2-X6 and further increased up to 180°C, where a maximum total XOS of 61.69 g/kg was obtained, composed of xylobiose (5.37 g/kg), xylotriose (4.25 g/kg), xylotetraose (2.27 g/kg), xylopentaose (1.55 g/kg), xylohexaose (0.15 g/kg), and XOS with DP > 6 (48.10 g/kg). Then, similar to this study, they reported the degradation of XOS with DP>6 at 200°C. They also observed a similar upward trend for the liberation of arabinose up to 180°C, and then a significant reduction at 200°C. Similar to this study, they detected acetic, lactic and formic acids as degradation products with the highest total amount at 200°C with 39.41, 12.20 and 14.76 g/kg, respectively. XOS formation from 2 g destarched barley bran at 180°C/30 min in this study with the yields of 20, 23 and 13 mg/kg for X2, X3 and X4, respectively, were higher than those obtained by Chen et al. (2017), probably due to the higher solvent-to-feed ratio (75 mL/g), or lower feed lignin content (7.17%) of the barley bran used in this study.

The XOS recovery values from different raw materials using hydrothermal treatments are summarized in Table 3.5. XOS recovery obtained in this study was higher or lower compared to

the different studies in the literature; however, comparisons should be performed with care considering the differences in the hydrolysis system and oligosaccharides definitions. For example, in this study XOS recovery was calculated based on the mass of oligosaccharides with DP 2-5, which have been shown to have the highest prebiotic effect (Okazaki et al., 1990; Moura et al., 2008). These results indicate that temperatures above 160°C are needed to hydrolyze the xylan polymer to form lower molecular weight XOS.

Table 3.5: Xylo-oligosaccharides recovery from selected literature raw materials and corresponding extraction conditions.

Biomass	T (°C)	t (min)	P (bar)	Solvent /solid (mL/g)	System	XOS (DP)	XOS (recovery) (%)	Reference
Corn cob X:29.5% A:3.3% L:18.7% G:38.5%	190	15	NR	8	Autohydrolysis	ND	58	Nabarlatz et al. (2004)
Corn cob G:29.7% X:29.9% A:3.4% L: 12.7%	200	10.7	30	7	Continuous SCW	2-10 >11 Total	57.8 23.7 82.2	Makishima et al. (2009)
Wheat straw G:37.4% X:29.4% A:1.9% L: 23.6%	180	30	NR	10	Autohydrolysis	DF	44	Ruiz et al. (2011)
Wheat straw G:31.5% X:19.8% A:2.8% L: 5.9%	179	23	NR	16	Autohydrolysis	ND	43	Nabarlatz et al. (2007)
Barley straw G:30.8% X:19.8% A:3% L: 16.7%				16			43	
Corn stover G:37.8% X:21.3% A:1.6% L: 17.8%	200	10	NR	20	Batch SCW	1-30 >30	28.1 10	Yang and Wyman (2008)
				10	Semi continuous SCW	1-30 >30	20.3 27.9	
				125	Semi continuous SCW	1-30 >30	9.1 64.2	
Oat spelt xylan X:78.9%				20	Batch SCW	1-30 >30	30.1 43	
				10	Semi continuous SCW	1-30 >30	0.3 91.8	
				125	Semi continuous SCW	1-30 >30	0.4 90.8	

Table 3.5. Continued.

Biomass	T (°C)	T (min)	P (bar)	Solvent /solid (mL/g)	System	XOS (DP)	XOS (recovery) (%)	Reference
Wheat bran G:31.7% X+ A: 18.4%	200	NR	NR	10	Autohydrolysis	DF	70	Rose and Inglett (2010)
Sugarcane bagasse	200	10	NR	10	Autohydrolysis	2-5	50.4	Zhang et al. (2018)
Wheat straw	180	NR	10	NR	Autohydrolysis	DF	50	Rodriguez et al. (2019)
Corn stover							70	
DF-DS bran	180	30	50	75	Semi continuous SCW	2-4	43.8	This study
Deproteinized bran	180	30	50	75	Semi continuous SCW	2-4	78.4	This study

T: Temperature; t: Time; P: Pressure; G: glucan; X: xylan; A: arabinan; L: lignin.

ND: Not detected, NR: Not reported.

DF: Calculated based on the difference between monomers concentration after acid hydrolysis refers to DP two or higher, XOS Recovery (%): (mass of XOS/mass of initial xylan) x 100.

3.3.4. Removal of protein from defatted–destarched bran

Alkaline treatment of lignocellulosic materials and agricultural by-products is an efficient method for the removal of protein and hemicellulose (Sun et al., 1995; Wang et al., 2010). Uronic and ester groups are hydrolyzed under alkali conditions, leading to solubilization of hemicellulose in the liquid medium. Also, cellulose swells with a decrease in its crystallinity, which helps better extraction of hemicellulose (Sun and Cheng, 2002). Alkali treatment also leads to the separation of structural linkages between lignin and carbohydrates with disruption of the lignin structure (Carvalheiro et al., 2008). Protein, which is also associated closely with the hemicellulose via covalent bonds, can be solubilized with alkali and further precipitated at isoelectric pH and removed from the mixture (Zhu et al., 2010). The alkaline extraction (NaOH, 3 N) resulted in a deproteinized and arabinoxylan-rich fraction with a yellow-white color (Fig. 3.15).

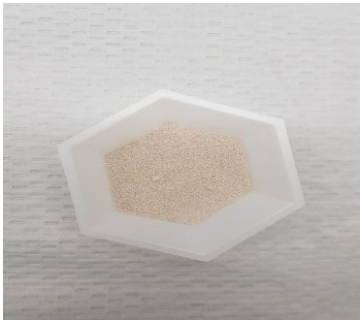


Fig. 3.15. Dried deproteinized fraction obtained after protein removal.

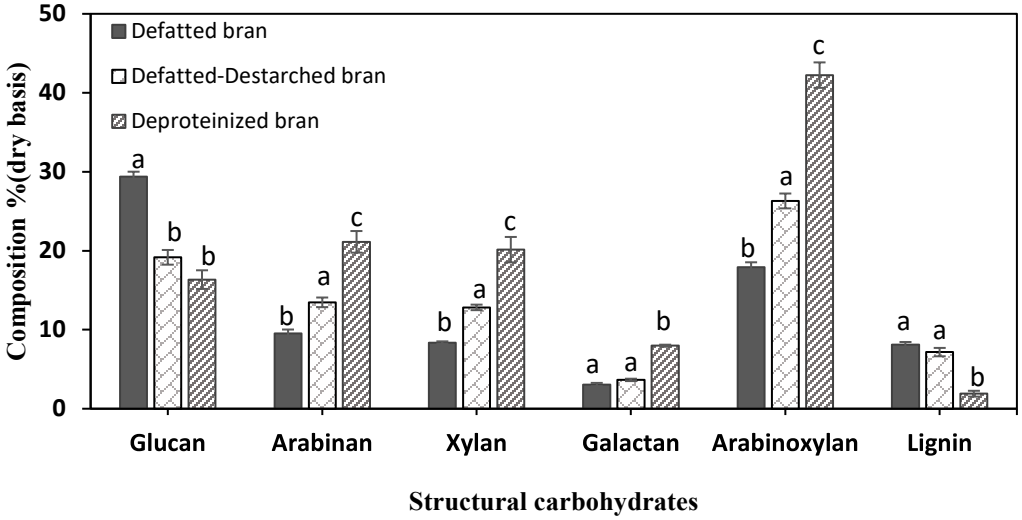


Fig. 3.16. Structural carbohydrates of defatted, defatted-destarched and deproteinized bran. Means within the same group of structural carbohydrates with different letters are significantly different at $p<0.05$.

The concentration of structural carbohydrates of DF, DF-DS and DP bran is provided in Fig. 3.16. Arabinan, xylan and arabinoxylan concentrations increased in the isolated deproteinized fraction in comparison with DF and DF-DS bran. Arabinoxylan was recovered (86.42% of the initial amount in DF-DS bran) in the final dried deproteinized bran with 42.24±1.61% (db). However, lignin and protein reduced to 1.9±0.37 and 4.62±0.59% (db), respectively. The deproteinized fraction had a high percentage of glucose (16.35%, db), which might be due to the linkage of glucose to arabinoxylan already reported in the literature (Verbruggen et al., 1995).

Qiu et al. (2017) extracted hemicellulose from defatted and destarched sorghum bran using alkaline solution (NaOH 50% w/v) at 85°C and precipitated the major arabinoxylan fraction using two volumes of ethanol with further separation using vacuum filtration. They reported the presence

of xylose, arabinose and glucose as the main monomeric sugars with concentrations of 41.52, 35.08 and 7.07 relative mole%, respectively. Kiran et al. (2013) also investigated the alkali extraction of the xylan rich fraction from cotton stalk, sunflower stalk, corn cob, wheat straw and rice hull. They reported a highly branched structure in the composition of rice hull hemicellulose with different types of sugar moieties, including 67, 14 and 8.7% xylose, arabinose and glucose in the xylan fraction obtained.

According to Ebringerova et al. (2005), hemicellulose in rice hull has a complex glucuronoarabinoxylan structure. The recovery of xylan varies in different lignocellulosic materials and depends on the presence and amount of lignin, hydrogen bonds, cellulose content, alkali type and its concentration (Donor and Hicks, 1997). Japayal et al. (2013) recovered around 85% of initial xylan from sugarcane bagasse using an alkali treatment with 12% sodium hydroxide. They mentioned that high xylan recovery could be related to the low content of lignin (6.04 ± 0.06%) in their raw material.

In this study, lignin content was reduced significantly (76%) in the deproteinized fraction due to the alkaline treatment. Decrease of lignin content and the increase of surface area were reported by Kim et al. (2003) from corn stover, Sun et al. (1995) from wheat straw and Kang et al. (2012) from rapeseed straw due to the cleavage of alpha-ether linkages between hemicellulose and lignin (Spencer and Akin, 1980).

Studies showed that lignin removal can improve the efficiency of the xylanase enzyme for the hydrolysis of xylan. Selig et al. (2009) reported a dramatic increase in the xylan conversion to xylose from 5% to almost 50% using xylanase treatment for 72 h in corn stover, when lignin content was reduced from 15.8 to 5.4% (db) by alkaline treatment.

The effect of lignin content on hemicellulose recovery using hydrothermal treatment was also investigated in the literature. Gallina et al. (2018) observed that hemicellulose extraction from different raw materials by a semi-continuous SCW at 160°C with a pressure of 9 bar and a flow rate of 150 L/h, depends on the structural composition and lignin content of the feed material. They reported that 40% hemicellulose was recovered with from eucalyptus that had 25% lignin. On the other hand, maple, cedar and plane wood with over 38% lignin showed low hemicellulose recoveries of 9.7, 10.9 and 18.8%, respectively. This hindering effect of lignin on hydrothermal hemicellulose extraction was also reported by Yedro et al. (2015). Lignin contains phenylpropane units strongly attached to hemicellulose and cellulose that holds them together (Maurya et al., 2015). It is hydrophobic, resistant to water penetration and enzymatic degradation, thus protecting the cellulose and hemicellulose (Mokhothu and John, 2015). Therefore, lignin diminishes the accessibility to hemicellulose for hydrolysis by xylanase. Also, its content negatively affects the hemicellulose extraction, leading to low recovery of xylan during hydrothermal treatment.

Mass balance

Two batches of defatted-destarched bran were produced from DF bran (Fig. 3.17) and two batches of deproteinized bran were produced from DF-DS bran (Fig. 3.18). The following formulas were used to calculate the extraction yield, arabinoxylan and total sugar recovery of destarched bran and deproteinized bran and the results are provided in Tables 3.6 and 3.7.

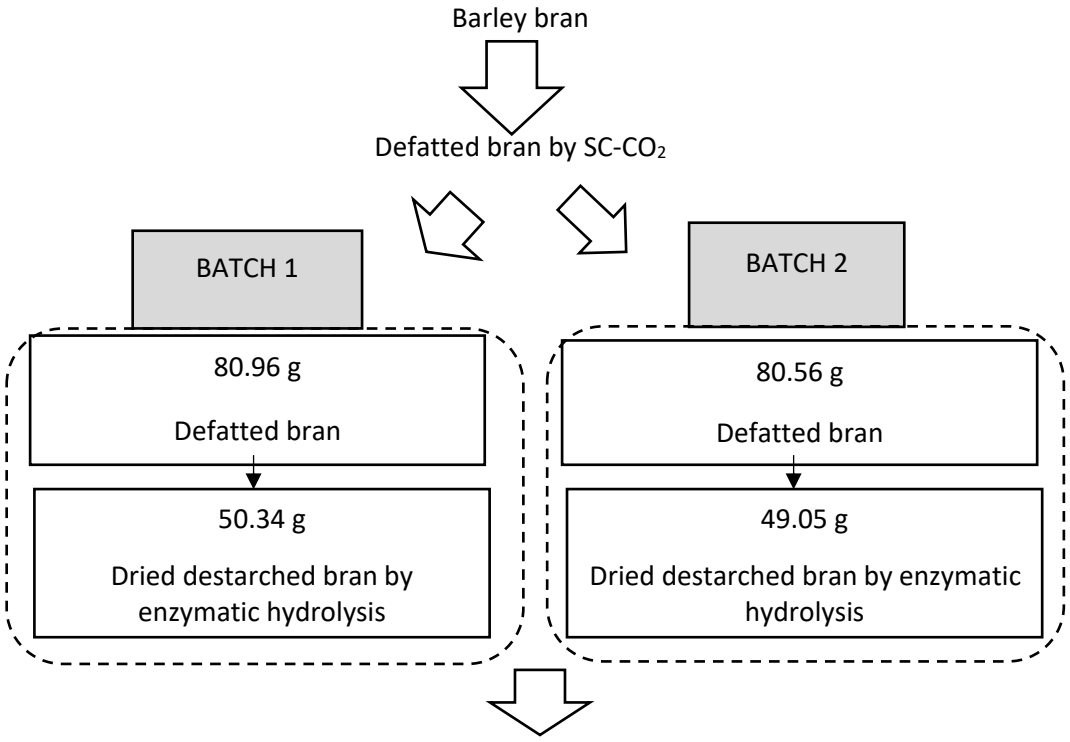
$$Extraction\ yield\ (\%) = \left[\frac{Dry\ weight\ of\ destarched\ bran\ (g)}{Dry\ weight\ of\ defatted\ bran\ (g)}\right] \quad (3.15)$$

$$Arabinoxylan\ recovery\ (\%) = \left[\frac{Weight\ of\ arabinose\ and\ xylose\ (db)\ \ in\ destarched\ bran\ (g)}{weight\ of\ arabinose\ and\ xylose\ (db)\ in\ defatted\ bran\ (g)}\right] \quad (3.16)$$

$$Total\ sugar\ recovery\ (\%) = \left[\frac{Weight\ of\ total\ monosaccharides\ (arabinose,\ \ xylose,\ \ glucose\ and\ galactose)\ in\ destarched\ bran(db)\ (g)}{Weight\ of\ total\ monosaccharides\ (db)\ in\ defatted\ bran(g)}\right] \quad (3.17)$$

Dried destarched brans with masses of 50.34 and 49.05 g were obtained from 80.96 and 80.56g of defatted bran in batches 1 and 2, corresponding to 62.38 and 61.08% yield, respectively. An average of total sugar recovery was around 60.22%. However, more arabinoxylan was recovered (90.69%) after starch enzymatic digestion by alpha-amylase during 40 min at 98-100°C followed by amyloglucosidase treatment for 30 min at 60°C. Lower extraction recovery of total sugar compared with arabinoxylan was due to the starch hydrolysis and removal of glucose (Table 3.6).

Dried deproteinized bran with masses of 11.33 and 10.87 g were obtained from 20.12 and 20.07g of DF-DS bran using sodium hydroxide (3 N) for 6 h at 60°C in batches 1 and 2 corresponding to 56.27 and 54.12% yield, respectively. High amounts of arabinoxylan (86.42%) and total sugars (72.72%) were recovered after protein removal (Table 3.7).

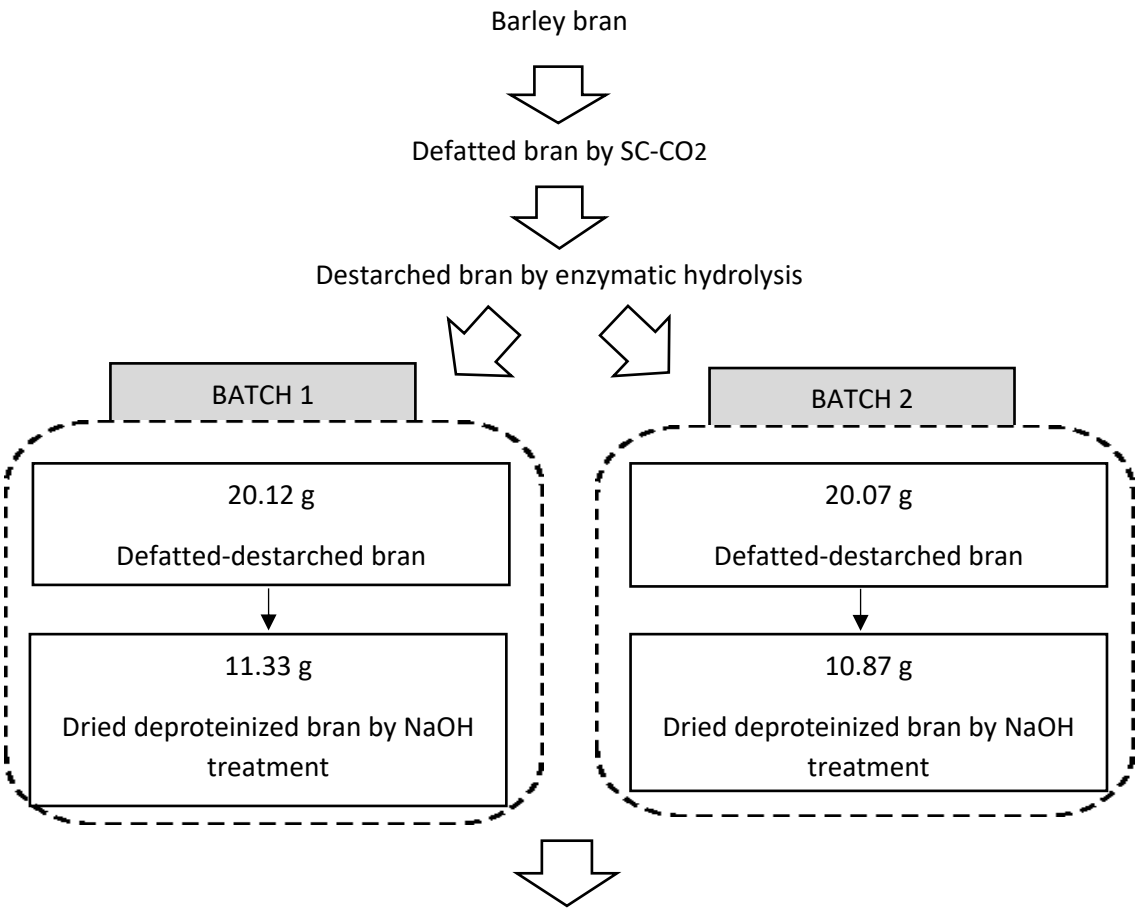


Batch number	Structural carbohydrates composition of dried destarched bran (%db)			
	Xylose	Arabinose	Glucose	Galactose
1	13.02 ± 0.44	13.96 ± 0.38	19.81± 0.93	3.76 ± 0.08
2	12.64 ± 0.20	12.97 ± 0.35	18.53 ± 0.27	3.54 ± 0.13

Fig. 3.17. Overview of the structural carbohydrates of defatted-destarched bran.

Table 3.6. Extraction yield and sugars recovery of defatted-destarched bran.

Batch number	Extraction yield (%)	Arabinoxylan recovery (%)	Total sugars recovery (%)
1	62.38	94.23 ± 2.89	62.61± 0.22
2	61.08	87.37 ± 0.94	57.83 ± 0.83



Batch number	Structural carbohydrates composition of dried deproteinized bran (%db)			
	Xylose	Arabinose	Glucose	Galactose
1	21.28 ± 0.5	22.10 ± 0.45	17.19 ± 0.52	8.08 ± 0.19
2	19.00 ± 1.13	20.15 ± 0.12	15.52 ± 1.19	7.89 ± 0.04

Fig. 3.18. Overview of the structural carbohydrates of deproteinized bran

Table 3.7. Extraction yield and sugars recovery of deproteinized bran.

Batch number	Extraction yield (%)	Arabinoxylan recovery (%)	Total sugars recovery (%)
1	56.27	90.44 ± 1.71	76.40 ± 1.82
2	54.12	82.41 ± 2.20	69.04 ± 0.78

3.3.5. Characterization of enzymatic hydrolysates

3.3.5.1. Reducing sugar

Fig. 3.19 shows reducing sugars production from deproteinized bran with xylanase using two enzyme concentrations of 2.5 and 10 U and four reaction times (1, 4, 8 and 16 h). The yield of reducing sugars in the control sample, which is defined as 22 mg/mL deproteinized bran dispersion (10 mg/mL or 1% w/v arabinoxylan solution) without enzyme was determined as 0.24±0.04%. As observed from the hydrolysis with xylanase (2.5 U), the hydrolysis rate of xylan was fast up to 1 h period, where reducing sugar yield increased significantly to 4.78±0.23%, indicating the production of smaller compounds, which had more reducing ends. Then, the yield of reducing sugars increased to 5.82±0.19% after 4 h incubation and stopped increasing after this period, reaching a plateau. This reduction in hydrolysis rate was probably due to the diminishing of accessible hydrolysis sites in the xylan chain or decrease of endo xylanase activity because of the end product inhibitory effect (Akpinar et al., 2009).

Using 10 U of xylanase, reducing sugars yield was determined as 4.99±0.07, 6.61±0.03, 6.58±0.05 and 6.66±0.00% after 1, 4, 8 and 16 h incubation, respectively. Increasing the concentration of enzyme to 10 U improved the yield of reducing sugars after 4, 8 and 16 h. The same behavior was observed after 4 h incubation and no significant change was detected up to 16 h (Fig. 3.19).

A similar pattern was reported by Akpinar et al. (2009), who studied the hydrolysis of xylan from wheat straw using xylanase from *A. niger*. They incubated 10 mL of 2% xylan solution in citrate buffer pH 4.6 at 40°C for 48 h with 1 U of enzyme and observed a sharp increase in the concentration of reducing sugars up to 8 h (23 mM) and no significant change was reported after this period.

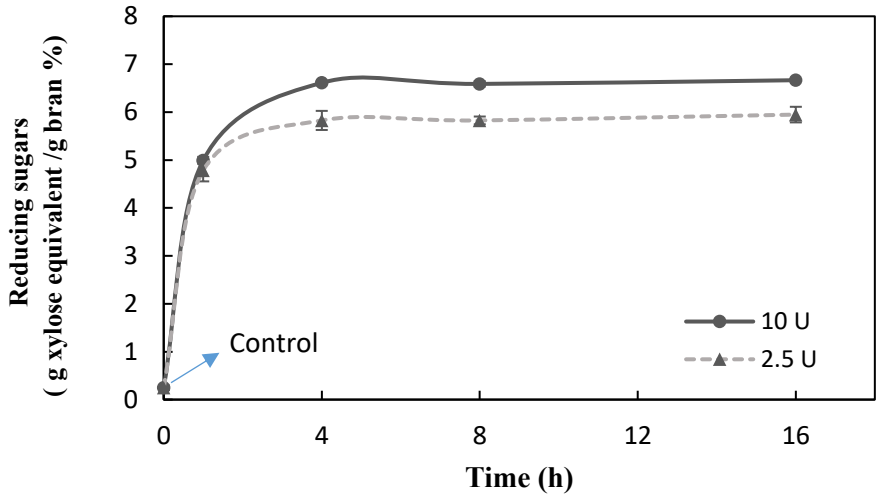


Fig. 3.19. Yield of reducing sugars for enzymatic hydrolysates.

3.3.5.2. Xylo-oligosaccharides (XOS) content by HPLC

Xylo-oligosaccharides with different degrees of polymerization (DP) were determined using HPLC for all the enzymatic hydrolysates obtained with 2.5 and 10 U concentrations of xylanase. Xylobiose (X2) and xylotriose (X3) were the most abundant XOS with a trace amount of xylohexaose (X6) produced by enzymatic incubation and their contents plus recoveries are shown in Table 3.8.

Table 3.8. Recoveries of XOS obtained after enzymatic hydrolysis.

Hydrolysis conditions		XOS content				XOS recovery			
Enzyme concentration (U)	Time (h)	Xylobiose (X2)	Xylotriose (X3)	Xylohexaose (X6)	Total XOS (X2+X3+X6)	Xylobiose (X2)	Xylotriose (X3)	Xylohexaose (X6)	Total XOS (X2+X3+X6)
2.5	1	0.14 ± 0.05^{C}	0.43 ± 0.07^{B}	0.23 ± 0.05^{B}	0.81 ± 0.06^{B}	0.35 ± 0.1^{C}	0.93 ± 0.15^{B}	0.5 ± 0.12^{B}	1.73 ± 0.13^{B}
	4	0.57 ± 0.10^{B}	1.08 ± 0.12^{A}	0.37 ± 0.03^{A}	2.03 ± 0.19^{A}	1.22 ± 0.21^{B}	2.31 ± 0.27^{A}	0.8 ± 0.07^{A}	4.35 ± 0.41^{A}
	8	1 ± 0.06^{A}	0.80 ± 0.16^{AB}	0.26 ± 0.00^{AB}	2.07 ± 0.23^{A}	2.15 ± 0.13^{A}	1.71 ± 0.34^{AB}	0.55 ± 0.01^{AB}	4.42 ± 0.5^{A}
	16	1.10 ± 0.04^{A}	1.13 ± 0.12^{A}	0.21 ± 0.01^{B}	2.45 ± 0.15^{A}	2.35 ± 0.09^{A}	2.42 ± 0.26^{A}	0.46 ± 0.02^{B}	5.25 ± 0.33^{A}
10	1	8.16 ± 0.28^{c}	5.91 ± 0.36^{ab}	0.67 ± 0.03^{a}	14.74 ± 0.11^{c}	17.43 ± 0.6^{c}	12.63 ± 0.77^{ab}	1.44 ± 0.07^{a}	31.51 ± 0.24^{c}
	4	13.14 ± 0.7^{b}	7.4 ± 0.68^{a}	0.56 ± 0.06^{ab}	21.11 ± 0.08^{a}	28.09 ± 1.51^{b}	15.81 ± 1.46^{a}	1.20 ± 0.13^{ab}	45.11 ± 0.18^{a}
	8	13.44 ± 0.2^{b}	5.75 ± 0.25^{b}	0.45 ± 0.08^{bc}	19.65 ± 0.45^{b}	28.72 ± 0.43^{b}	12.3 ± 1.59^{b}	0.96 ± 0.18^{bc}	41.99 ± 0.97^{b}
	16	15.13 ± 0.35^{a}	3.12 ± 0.25^{c}	0.63 ± 0.00^{ab}	18.88 ± 0.62^{b}	32.33 ± 0.76^{a}	6.66 ± 0.55^{c}	1.34 ± 0.01^{ab}	40.36 ± 1.33^{b}

XOS Recovery (%): (mass of XOS/mass of initial xylan) x 100.

Figs. 3.20 a,b also illustrate the XOS recovery from initial xylan using 10 and 2.5 U of xylanase, respectively. Using 2.5 and 10 U of xylanase, the content of total XOS increased significantly from 1 h to 4 h, from 0.81 to 2.03 and from 14.74 to 21.11 mg, respectively. There was no change after this period, similar to the behavior with reducing sugars yield (Fig. 3.19). Xylobiose (X2) and xylotriose (X3) had higher concentrations compared with xylohexaose (X6) (Faryar et al., 2015; Flack et al., 2014; Akpinar et al., 2009).

Xylobiose content showed a significant increase by incubation time at 1, 4 and 8 h with 0.14, 0.57 and 1 mg, corresponding to 0.3, 1.2 and 2.15% recovery with no significant change after 16 h (2.5 U). Xylotriose had a similar concentration behavior with an increase from 0.43 to 1.08 mg at 1 and 4 h incubation and no statistical difference after this period. However, using a higher concentration of enzyme (10 U), xylobiose content increased progressively from 8.16 to 13.14, 13.44 and 15.13 mg after 1, 4, 8 and 16 h, which corresponded to 17.43, 28.09, 28.72 and 32.34% recovery, respectively. Xylotriose was determined as 5.91 mg after 1 h incubation and did not show any significant change after 4 h, while it reduced to 5.75 and 3.12 mg after 8 and 16 h incubation, respectively. The reduction of xylotriose after 16 h could be related to the further conversion of this compound to xylobiose by longer incubation time (8-16 h). Therefore, by increasing the enzyme concentration (10 U), after 16 h incubation, more xylobiose was obtained as a result of further xylotriose hydrolysis.

The DP of the XOS produced depends on the enzyme variety, since some endo-xylanases produced xylose and xylobiose from xylan, while some other types liberated mostly XOS with higher DP. For example, the xylanase from *Streptomyces,* with an optimal temperature of 60°C and pH of 6 attacked on xylan backbone and produced X4 and X3, followed by conversion to X2 and xylose. However, the *Trichoderma* xylanase provided a mixture of X2-X5 but no xylose was produced (Reilly, 1981).

Structure of the extracted xylan plays an important role in the production of XOS with different DP and might influence the level of enzymatic hydrolysis, the yield of reducing sugars and XOS produced (Kiran et al., 2013). Faryar et al. (2015) produced XOS from alkali extracted wheat straw using K80R variant of endo-xylanase with 1% (w/v) xylan solution and 0.48 U of enzyme. They reported the production of xylobiose and xylotriose as the most abundant XOS liberated as a result of incubation at 50, 55 and 60°C. They observed a progressive increase of xylobiose yield up to 15 h (25%), while xylotriose increased until 3 h with further lowering up to the end of the process. In that study, 60°C was the optimal temperature to produce the highest amount of total XOS with a yield of 35% (initial xylan), which was lower than the highest XOS recovery in this study (45.11±0.18%) (Fig. 3.20). This difference might be due to the usage of a lower concentration of enzyme (0.48 U) and different type of xylanase. Akpinar et al. (2009) investigated the XOS production from extracted xylan obtained from tobacco stalk, sunflower stalk, cotton stalk and wheat straw using two different types of endo-xylanase from *Aspergillus niger* and *Trichoderma*

longibrachiatum. They performed the hydrolysis of alkali extracted xylan by mixing 1 U of each enzyme with 10 mL of 2% xylan at 40°C with *A. niger* and at 50°C with *T. longibrachiatum* (1 U, 40°C). They found that xylobiose and xylotriose were the most predominant XOS produced by these two types of enzymes. However, the enzyme from *A. niger* produced more X2 and X3 compared to the enzyme from *T. longibrachiatum*, which produced more xylose. The hydrolysate obtained from enzymatic hydrolysis of wheat straw xylan, with 79.9% xylose and 10.8% arabinose, had 0.786 and 0.719 mg/mL X2 and X3, respectively. In this study, 0.74 and 1.51 mg/mL were the maximum amounts for X3 and X2, respectively. The higher content of xylobiose might be due to the usage of enzyme at high concentration (10 U). Akpinar et al. (2009) obtained more X2 and X3 from tobacco stalk, sunflower stalk, cotton stalk xylan than wheat straw xylan. Because wheat straw xylan had a more branched arabinoxylan structure with a higher amount of arabinose chain. Branched arabinoxylan was not accessible for hydrolysis by xylanase from *A. niger* and less X2 and X3 were produced.

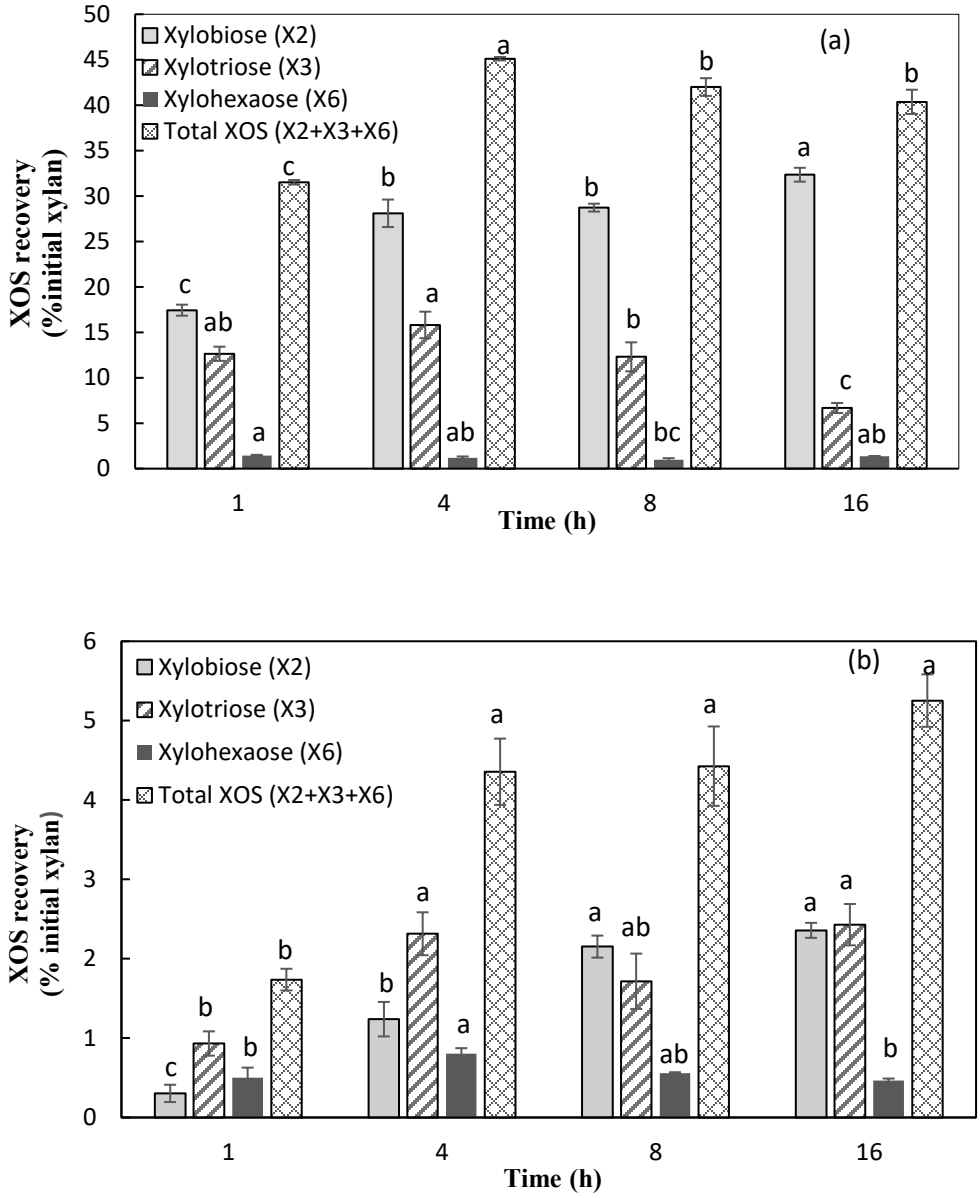


Fig. 3.20. Recovery of xylo-oligosaccharides (XOS) after enzymatic hydrolysis using: (a) 10 U xylanase and (b) 2.5 U xylanase. Means within the same group of XOS with different letters are significantly different at $p<0.05$.

3.3.6. Characterization of subcritical water hydrolysates from deproteinized bran

3.3.6.1. Reducing sugar

Reducing sugar was determined in the SCW hydrolysates collected at 180°C/30 min/50 bar/5 mL/min. This hydrolysis was performed to compare the processes of subcritical water versus enzymatic hydrolysis for the same deproteinized samples for the production of XOS. Deproteinized bran from batch 1 with 21.28 and 22.10% (db) xylose and arabinose, respectively, were selected for hydrolysis with SCW. This fraction was also used for the enzymatic hydrolysis.

The yield of reducing sugars was determined as 22.19±1.66% deproteinized bran, which was significantly higher than the yield of enzymatic hydrolysis (6.61±0.03% deproteinized bran) showing more depolymerization of deproteinized bran with SCW.

3.3.6.2. Xylo-oligosaccharides (XOS) content by HPLC

Fig. 3.21 a,b show the chromatograms obtained from deproteinized bran hydrolyzed by SCW (180°C, 50 bar and 30 min) and xylanase enzyme (10 U, 40°C and 4 h). The X4, X3 and X2 contents were determined in SCW hydrolysate of deproteinized bran, together with other oligosaccharides in the hydrolysate. The SCW produced XOS with a wide range of DP composed of DP > 6; DP = 2-6 and DP < 2. A peak with a retention time of 13.4 min might correspond to X5 which was not identified. Enzymatic hydrolysis led to the production of X2, X3, X5 and X6, while X4 was not detected in any of the samples. Maximum xylotriose obtained by enzymatic hydrolysis

was determined as 7.4±0.68 mg (15.81% recovery), while SCW hydrolysate at 180/50 bar/30 min had 16 times xylotriose (120.6±9.9 mg with 28.3% recovery). Also, total XOS recovery was higher in the SCW hydrolysates with 78.45% compared to the enzymatic hydrolysis, which was only 45.11% (Fig. 3.22). Moreover, the enzymatic process was more time-consuming (4 h) than the SCW process (30 min) to obtain the maximum amount of total XOS from the same initial deproteinized bran. Xylanase activity depends on the nature of the substrate including side chain molecules and length, the degree of branching, and the presence of substituents (Reilly, 1981; Li et al., 2000). Arabinose and acetyl side groups can interfere with the endo-xylanase function and eliminates its cleaving ability leading to less production of oligomers (Akpinar et al., 2009; Polizeli et al., 2005).

Obtaining lower amounts of XOS and resulting reducing sugars after longer time enzymatic hydrolysis (4 h) compared to the fast SCW hydrolysis (30 min) is probably due to the complex and heterogeneous structure of the xylan extracted from DF-DS bran, which had arabinose groups (22.10% db) located as side chains attached to the xylan backbone. However, SCW hydrolysis was able to separate arabinose groups in the form of monomers from deproteinized bran (102.8±6.55 mg) in the liquid hydrolysate at 180°C/50 bar/30 min. Reduction of accessible hydrolysis sites in the xylan chain or decrease of endo-xylanase activity because of the end product inhibitory effect after

specific time (4 h in this study) could be another factor that had a negative influence on the production of XOS by xylanase treatment (Akpinar et al., 2009).

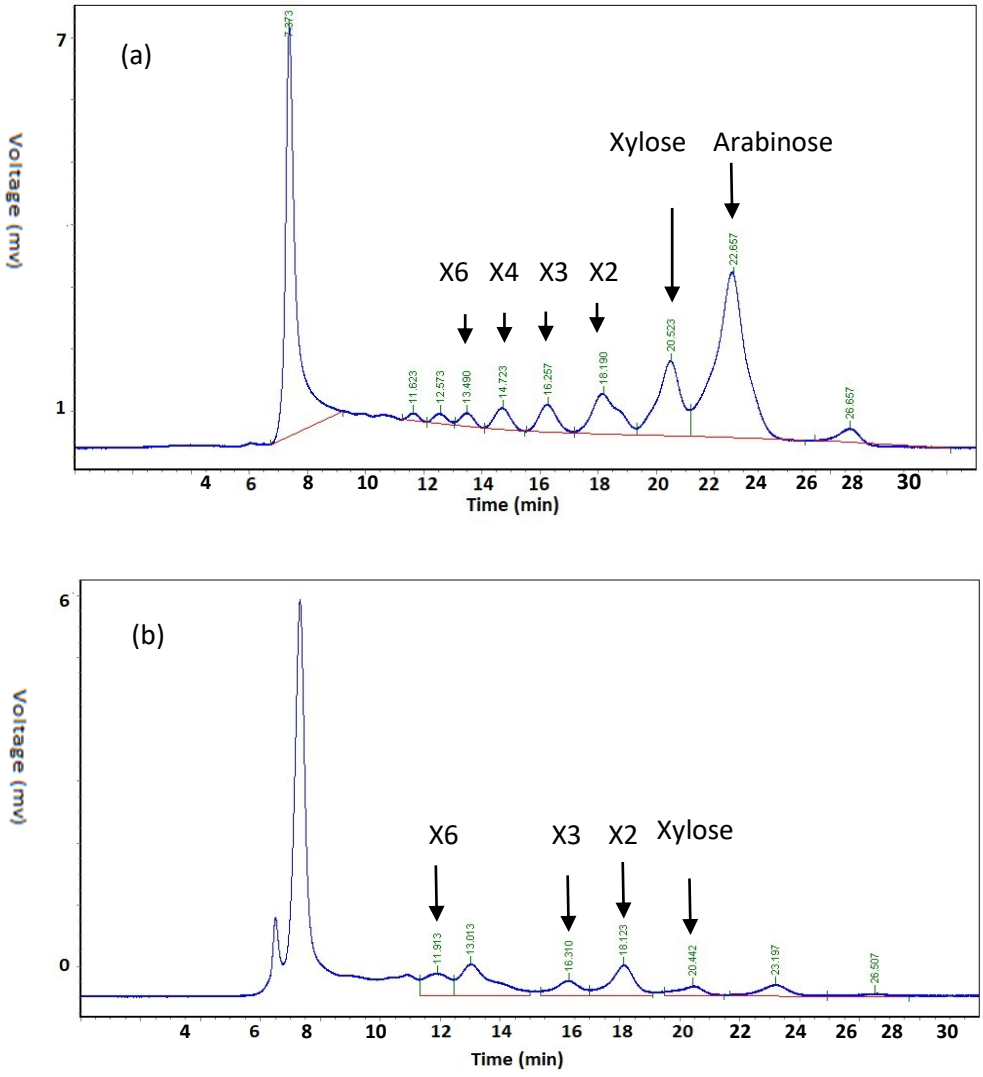


Fig. 3.21. Chromatograms obtained from deproteinized bran hydrolysis by: (a) SCW (180°C/50 bar/30 min) and (b) xylanase enzyme (10 U/4 h).

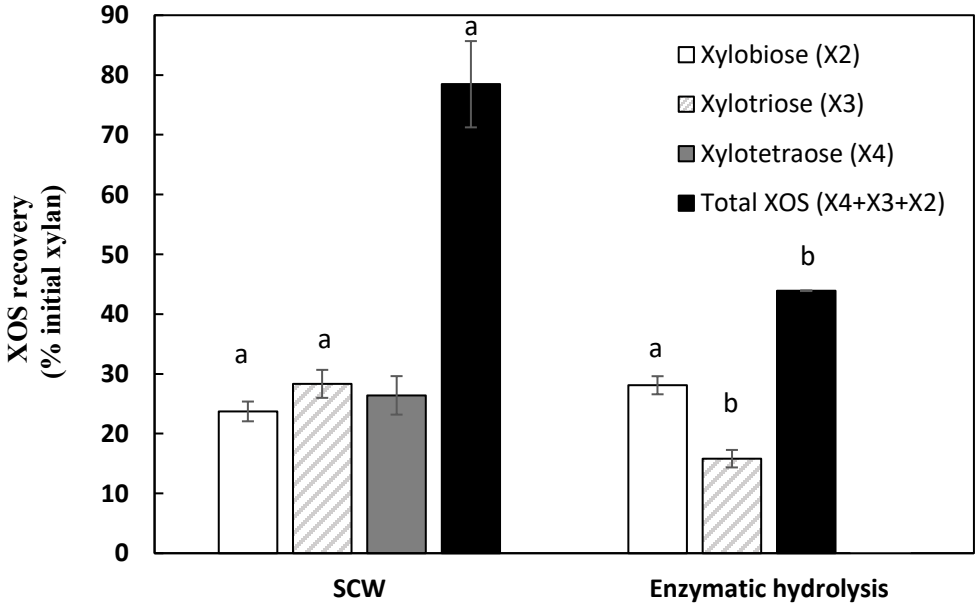


Fig. 3.22. Recovery of xylo-oligosaccharides (XOS) in the SCW and enzymatic hydrolysates from deproteinized bran. Means within the same group of XOS with different letters are significantly different at $p<0.05$.

Rose et al. (2010) investigated the hydrothermal treatment of wheat bran using autohydrolysis in a batch reactor at a temperature range of 170 to 220°C. They obtained arabino-xylooligosaccharides (AXOS) with the highest recovery around 70% (initial xylan%), composed of different compounds with DP < 3 (31.6%), DP = 3-10 (26%) and DP > 10 (42.4%). The recovery of wheat bran oligosaccharides using autohydrolysis was also higher than the recovery of AXOS released after enzymatic treatment with xylanase.

Mathew et al. (2018) used wheat bran xylan extracted after removal of starch and protein with alpha-amylase and protease treatment, respectively, for the production of XOS with xylanase (pentopan) with a ratio of 0.18 U/g bran at 50°C and pH 6. They reported the production of X2

and X3 as major XOS with a maximum amount of total XOS obtained after 28 h (22.8% w/w recovery of the total initial xylan).

Jacobsen and Wyman (2002) studied hydrothermal hydrolysis of sugarcane bagasse using batch reactors with a mixture of water and bagasse with different final solid contents of 0.5, 1.0, 3.0, 7.0, and 10.0 wt % at 200°C for 20 min. They recovered the maximum XOS yield between 70 and 75% within 10 min hydrolysis. Brienzo et al. (2010) used sugarcane bagasse xylan extracted from dewaxed sugarcane bagasse by alkaline hydrogen peroxide and further precipitated with ethanol. They used 2% xylan solution with 60 U/g of xylanase from *Thermoascus aurantiacus* for 3, 6, 12, 24, 48, 72, and 96 h of hydrolysis. Xylobiose was the major XOS produced with the maximum recovery of 38.48±0.99% after 96 h, while xylotriose and xylopentaose were found in trace amounts. These studies also confirmed that enzymatic hydrolysis led to a lower yield of XOS obtained for a longer time compared to the SCW treatment.

In terms of the other types of oligosaccharides produced using SCW and enzymatic hydrolysis, manno-oligosaccharides (MOS) were obtained from coconut meal. Khuwijitjaru et al. (2014) produced MOS from coconut meal by SCW treatment using a batch-type vessel at temperatures in the range of 100–300°C, solvent-to-feed ratio of 10 mL/g, for 15 min. The maximum oligosaccharides were obtained at 250°C after 14 min treatment (MOS with DP of 2–6, 72% of total carbohydrates) and a small amount of oligosaccharides with DP>6 (8% of total

carbohydrates). However, in another study by Kanatani et al. (2012), who used beta-1,4-mannanase enzyme for the production of MOS from coconut flour after defatting with hexane and washing with hot water, they obtained hydrolysates with mainly mannobiose (64.9%) and other monosaccharides.

The results obtained in this thesis and the literature studies discussed confirmed that SCW acts efficiently, compared to the enzymatic method for the hydrolysis and breaking the polysaccharides with a complex structure with several side chains. SCW has the ability to cleave the substituents attached to the backbone, which makes it more accessible for hydrolysis leading to higher amounts of oligomers with wider range of DP.

Table. 3.9. Amounts of xylo-oligosaccharides (XOS) obtained by SCW and enzymatic hydrolysis.

	Xylo-oligosaccharides content (mg)					Xylo-oligosaccharides recovery (%)				
Type of hydrolysis	**Xylobiose**	**Xylotriose**	**Xylotetraose**	**Xylohexaose**	**Total XOS**	**Xylobiose**	**Xylotriose**	**Xylotetraose**	**Xylohexaose**	**Total XOS**
SCW	100.9±7.0[a]	120.6±9.9[a]	112.4±13.7	ND	334± 30.7[a]	23.7±1.6[a]	28.3± 2.3[a]	26.4 ± 3.2	ND	78.4 ± 5.5 [a]
Enzymatic (xylanase)	13.1±0.7[b]	7.4±0.6[b]	ND	0.5±0.06	21.1±0.08[b]	28.09±1.5[a]	15.8 ±1.4[b]	ND	1.2 ± 0.1	45.1 ±0.18[b]

ND: Not detected, Data are expressed as mean ± standard deviation of two replicates.

[a-d] Different letters in the same column indicate significant differences ($p < 0.05$). XOS Recovery (%): (mass of XOS/mass of initial xylan) x 100.

3.4. Conclusions

This study provided a sequential process for the production of xylooligosaccharides (XOS) from barley bran, including lipid removal, starch removal and protein removal with further hydrolysis of the obtained fraction (defatted- destarched and deproteinized bran) using SCW and enzymatic approach.

Lipid removal was successful using a SC-CO_2 system (10 mL vessel) with the highest recovery of 94% at 300 bar/70°C using a ratio of 15.7 (mass of CO_2/mass of feed) after 30 min. Scale-up of this process was also performed using a 300 mL vessel at the same condition (300 bar/70°C) with the maximum lipid recovery of 92% after 4 h extraction at the same solvent-to-feed ratio.

Starch removal from defatted bran was performed using enzymatic hydrolysis with a combination of alpha-amylase (150 U/g DF bran/98-100°C/40 min) and amyloglucosidase (660 U/g DF bran/60◦C/30 min) with further precipitation of non-starch polysaccharides using ethanol. Defatted-destarched bran resulted in only 1.03% starch compared to the initial bran (10.4%), along with an increase of xylose from 8.3 to 12.83% db.

SCW was used for the hydrolysis of DF-DS bran at 120-200°C/50 bar/5 mL/min for 15-60 min. Reducing sugars yield increased with the elevation of temperature and time, and the maximum yield was obtained at 180°C/30 min (10.09±0.90%). XOS was obtained only at 160 and 180°C, with the maximum amounts at 180°C/30 min (40 mg of X2, 46.1 mg of X3, 26.2 mg of X4 and

112.5 mg of total XOS) and 180°C/60 min (11.2 mg of X2, 55.4 mg of X3, 46.7 mg of X4 and 113.4 mg of total XOS).

Deproteinized bran was also obtained after alkaline treatment to remove protein, resulting in a fraction rich in xylan (20.14% db) and arabinan (21.13% db) with a final protein content of 4.62%. This fraction was hydrolyzed by enzymatic treatment using endo-xylanase and SCW treatment. The maximum total XOS obtained by xylanase hydrolysis was 21.11 mg after 4 h (45.11% recovery from initial xylan). SCW hydrolysis produced 334.02 mg XOS (78.4% recovery), which was higher than that for the enzymatic hydrolysis. Therefore, the results obtained suggest SCW as a green technology to produce xylo-oligosaccharides with a range of DP 2-6 with prebiotic effect in a short time from hemicellulosic fraction derived from biomass. It is possible to improve the hydrolysis and obtain more XOS content by manipulating the reaction temperature and time. Moreover, SCW was more effective to break down and form XOS compared to the enzymatic hydrolysis at the investigated processing conditions.

Chapter 4. Xylo-oligosaccharides purification of SCW-treated barley bran using membrane separation followed by activated carbon adsorption

4.1. Introduction

Xylo-oligosaccharides (XOS) have different applications in food, medical, and pharmaceutical industries (Vazquez et al., 2000; 2005). As food ingredients, XOS are stable under acidic conditions, and have resistance to heat (Moure et al., 2006). Since they behave as non-digestible oligosaccharides (NDO), XOS might be used as soluble dietary fiber that has low calorie, helping to maintain a healthy weight and reducing the risk of diabetes and heart disease (Crittenden and Playne, 1996). Moreover, XOS with the desired DP range of 2-6 have been reported to have prebiotic effect (Okazaki et al., 1990; Fooks and Gibson, 2002). A prebiotic is a food component that: i) resists gastric acidity hydrolysis by digestive enzymes, and gastrointestinal absorption; ii) is fermented by intestinal microbiota; and iii) enables a selective stimulation of the growth of intestinal bacteria that has health effects, such as bifidobacteria and lactic acid bacteria. Therefore, XOS are considered as a healthy food ingredient in functional foods. Okazaki et al. (1990) showed the effect of a mixture of xylose (22%), xylobiose (58%), xylotriose (13%), and other saccharides (7%) for the growth and *in vitro* fermentation by *B. adolescentis, B. longum,* and *B. infantis*. In that study, *B. adolescentis*, which is available in most adults' intestines showed a strong ability to use both xylobiose and xylotriose as energy sources and its percentage of total bacterial counts

increased from 10 to 32% after addition of XOS mixture to the culture medium. In another study, rice husk was autohydrolyzed at 205°C with a ratio of 8 kg water/kg of oven-dried rice husk. XOS was produced with a DP range of 2-6 (39.93 wt% non-volatile compounds) in the final hydrolysate. Further, the obtained XOS mixture was used to stimulate the growth of bifidobacteria. The XOS was consumed at the end of the fermentation and the highest utilization corresponded to xylotriose (90%), followed by xylobiose (84%), xylotetraose (83%), and xylopentaose (71%). These results confirmed that XOS with DP 2 and 3 were desirable substrates for bacteria compared to the XOS with higher DP (≥4) (Gullon et al., 2008).

When XOS were produced using hydrothermal treatments, including SCW, autohydrolysis and steam explosion, a variety of compounds such as monosaccharides, organic acids, furfural and hydroxymethylfurfural from dehydration of pentose and hexose sugars, protein and lignin-derived products were found in the final hydrolysates (Aachary and Prapulla, 2011). Also, after enzymatic hydrolysis, the obtained hydrolysates had impurities of high molecular weight components, such as xylanase enzyme, starch, pectin, protein and tannin (Zhao and Dong, 2016).

To obtain XOS with high purity (75-95%) for food applications, with effective prebiotic properties, multistage treatments are needed. Several techniques such as vacuum evaporation, solvent extraction like ethyl acetate, solvent precipitation, physical adsorption and membrane separation have been used to purify hydrolysates containing a mixture of produced XOS plus other impurities

(Moure et al., 2006). Solvent extraction has been employed for the refining of liquors obtained from barley hull autohydrolysis containing 19.56 kg XOS, using ethyl acetate with a liquor to solvent mass ratio of 1:1 kg/kg to remove non-saccharide components such as phenolics, waxes, alcohols and extractive derived materials. XOS was recovered (16.80 kg) in the aqueous phase after evaporation of the solvent and dissolved volatile compounds. Then, solvent precipitation with ethanol, acetone and 2-propanol at a solvent to liquor mass ratio of 5:1 kg/kg, has been used. Further, the solid fraction that precipitated was collected by filtration and 15.94 kg (94% recovery) was the maximum amount of XOS recovered in the dried precipitate obtained with the addition of acetone (Vegas et al., 2005).

The use of activated carbon adsorption is another approach for the purification of XOS due to the stronger adsorption of higher DP oligosaccharides and less adsorption of xylose (DP1) to the activated carbon, which could be effective to remove monomers from oligomers in the mixture. Zhu et al. (2006) used activated carbon adsorption followed by ethanol elution of XOS in the xylanase enzymatic hydrolysates obtained from corn cob and corn stover. They added activated carbon to the hydrolysates in the range of 1-10% of the liquid weight, while shaking at room temperature at 200 rpm for 30 min. Then, activated carbon was separated from the solution and eluted twice with 100 mL of ethanol 15% and 30% v/v. The maximum recovery of XOS was 34.5% with 10% activated carbon and elution with ethanol 15% (100 mL).

Membrane separation, mainly ultrafiltration and nanofiltration, has been used as a promising technology for the refining and concentrating of different oligosaccharides, such as fructo-oligosaccharides (Li et al., 2004), soybean oligosaccharides (Kim et al., 2003), pectic oligosaccharides (Iwasaki and Matsubara, 2000), and chito-oligosaccharides (Jeon and Kim, 1998). Membrane separation driven by a pressure gradient works based on the size of compounds, which leads to the concentration of molecules with different molecular weights and degree of polymerization. This technique has advantages, including low energy usage (Goulas et al., 2003), no need for chemical solvents, easy modification of the processing parameters, such as pressure, temperature, feed flow rate, agitation and ease of scale-up (Drioli, 2004; Cano and Palet, 2007; Czemark et al., 2004). Some operational parameters, which affect the efficiency of the process are: i) the type of organic membranes, mainly derivatives of propylene, ethylene, cellulose and polyamide, ii) inorganic membranes such as ceramic membranes, and iii) operating parameters such as temperature, pressure, pH and the concentration of the feed solution (Pinelo et al., 2009).

Polysulfone is one of the main materials in polymeric membranes due to its mechanical, thermal and chemical stability. It is used as flat sheets or hollow fibers, for microfiltration and ultrafiltration processes. However, these membranes are hydrophobic and have strong protein adsorption capacity, which lead to the fouling of the membrane and pore plugging, resulting in a reduction of water flux and separation efficiency (Wavhal and Fisher, 2005). Different methods have been applied for polysulfone membrane surface modification, which increase their hydrophilic

properties such as bonding the monomers like acrylic or gallic acid (Wavhal and Fisher, 2005), and blending with a hydrophilic polymer like poly (vinylpyrrolidone) (Lu et al., 2007) or cellulose acetate (Mahendran et al., 2004).

Membrane separation was carried out successfully for XOS purification produced by enzymatic and hydrothermal hydrolysis with the recovery in a range of 50-90% (Gullon et al., 2010; Akpinar et al., 2007; Vegas et al., 2006). Swennen et al. (2005) compared ultrafiltration and ethanol precipitation for the purification of arabinoxylo-oligosaccharides produced by endoxylanase hydrolysis of destarched and deproteinized wheat bran. Ethanol precipitation was carried out by the addition of pure ethanol to arabinoxylo-oligosaccharides hydrolysates in three steps. First, ethanol was added to the hydrolysate to a concentration of 60% (v/v) and materials were precipitated overnight. Obtained precipitate was separated from the supernatant, dried and made up the first fraction. Then, ethanol was added to the supernatant to obtain the concentration of 60-90%, and the final dried precipitate from this step made up the second fraction. In the last step, ethanol was added to the second supernatant to reach the concentration of 90%. The precipitate was recovered as described above and the supernatant made up the third fraction. They also tested membranes with a molecular weight cut-off of 5, 10 and 30 kDa at a constant pressure of 4 bar, using a stainless-steel dead-end ultrafiltration cell in a batch mode, in which the feed flows to the membrane perpendicularly. The cell, equipped with a magnetic stir bar to minimize the deposition of the particles on the membrane surface, was filled with 300 mL of enzymatically hydrolyzed

wheat bran and left for 5–8 h at room temperature, where 200 mL of permeate was collected. They reported the presence of arabinoxylo-oligosaccharides with DP 3, 5 and 6 in the permeate and DP 11, 12 and 15 in the retentate of membranes with 5, 10 and 30 kDa. Also, they concluded that more substituted components such as arabinose chains, which were less accessible for enzymatic hydrolysis and thus have higher molecular mass were concentrated in the retentate fractions, while less substituted components were found in the permeate fractions. The ultrafiltered fractions containing XOS with DP 3, 5 and 6 were more heterogeneous, polydisperse and less strictly compared to the fractions obtained with ethanol precipitation. Moreover, they reported higher recovery of arabinoxylo-oligosaccharides in the fractions obtained from ultrafiltration, where 86% was recovered in the retentate of 5 kDa membrane, while the highest recovery by ethanol precipitation was achieved in the third fraction with 47.2%.

The objective of this study was to evaluate ultrafiltration using membranes with two different molecular weight cut off of 3 kDa and 1 kDa to fractionate XOS obtained from barley bran because xylobiose, xylotriose and xylotetraose have molecular weight lower than 1 kDa. Then, they can pass through these two ultrafiltration membranes. For this purpose, defatted-destarched barley bran SCW hydrolysate was passed through both membranes to remove high molecular weight compounds, and recover maximum XOS, mainly xylobiose, xylotriose and xylotetraose in the permeate. Then, the membrane with the higher XOS mass recovery (1 kDa) was used for the purification of the SCW hydrolysate obtained from the deproteinized bran. Mass of oligomers in

the obtained permeates and retentates were quantified to calculate the recovery of xylobiose, xylotriose and xylotetraose.

Further, a model system using a solution (PreticX^{TM} XOS + xylose) was used to evaluate the effect of nanofiltration and activated carbon adsorption to remove xylose as a monomer from the XOS mixture, including xylobiose, xylotriose and xylotetraose. Nanofiltration was performed using two different membranes with 150 and 150-300 Da molecular weight cut off because xylose with the molecular weight of 150 Da can pass through the membrane and be collected in the permeate. Mass of oligomers and xylose in the obtained permeate and retentate fractions were quantified.

The permeate obtained from 1 kDa ultrafiltration membrane after passing SCW treated deproteinized bran, had monomers, including xylose and arabinose, plus XOS. Activated carbon adsorption was studied to remove monomers from this permeate.

4.2. Materials and methods

The Minimate™ tangential flow filtration (TFF) system was kindly provided by Pall Corporation (Mississauga, ON, Canada). The setup, shown in Fig 4.1 included a peristaltic pump, tubings, valves or clamps, pressure gauge, sample reservoir and a capsule membrane. Minimate TFF capsule membranes with a molecular weight cut off (MWCO) of 3 kDa and 1 kDa with modified polyethersulfone material, dimensions of 20 cm x 3.8 cm x 1.8 cm, pH range of 1-14 and the maximum operating pressure of 4 bar were purchased from Pall Corporation (Mississauga, ON,

Canada). Nanofiltration flat sheet membranes Trisep TS80 with a pore size of 150 Da, thin film polyamide material, size of 1016×305 mm, thickness of 130-170 µm, pH range of 1-12, maximum operating pressure of 41 bar and Cynder NFX with a pore size of 150-300 Da, pH range of 3-10.5, size of 1000×1000 mm were purchased from Sterlitech (Kent, WA, USA). PreticX^{TM} XOS obtained from corn was provided by AIDP Inc. (Los Angeles, CA, USA). Sodium hydroxide (NaOH) was purchased from Sigma Aldrich (St. Louis, MO, USA). Xylo-oligosaccharide standards, xylobiose (X2), xylotriose (X3), xylotetraose (X4) and xylohexaose (X6) were purchased from Megazyme (Wicklow, Ireland). D (+) Xylose, D (+) and L (+) arabinose, with a purity of ≥ 96% were purchased from Sigma Aldrich (St. Louis, MO, USA). HPLC grade water was purchased from Fisher Scientific (Ottawa, ON, Canada). Granular activated carbon with the mesh size of 20-60 was purchased from Sigma Aldrich (St. Louis, MO, USA).

4.2.1. Purification by ultrafiltration

The cross-flow ultrafiltration was carried out using the Minimate™ TFF System at a constant pressure of 1 bar and room temperature (22°C) for 2 h. The images of the ultrafiltration system, membranes and overall diagram of the system is shown in Fig. 4.1.

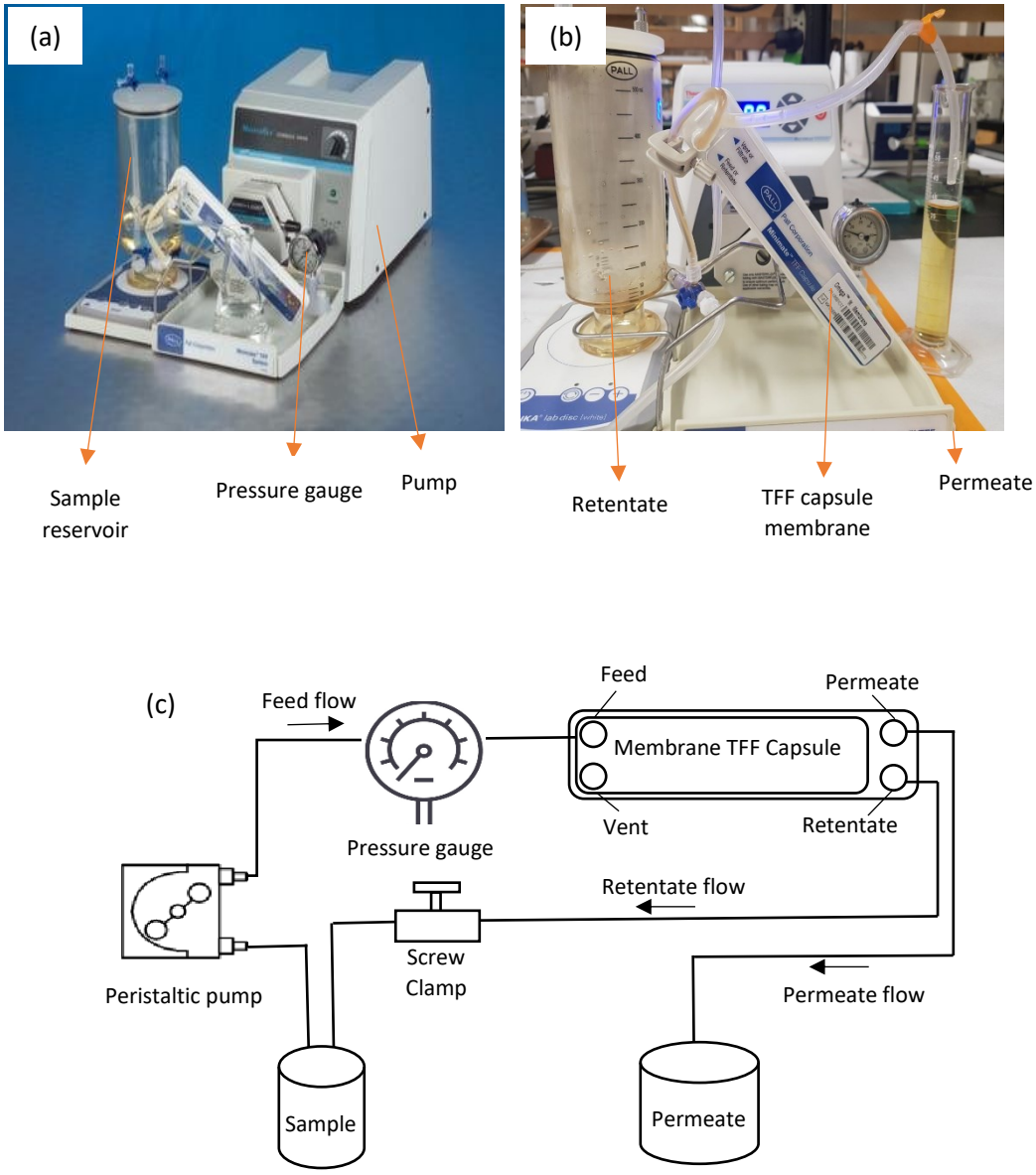


Fig. 4.1. Minimate TFF Capsule System: (a) ultrafiltration system, (b) membrane, and (c) overall diagram.

First, the TFF reservoir was connected with plastic tubings to the pump and a pressure gauge. Then, the capsule membrane was assembled in the system connecting to the pressure gauge and the sample reservoir. Tubing was connected to the permeate port of the membrane to collect the permeate fraction and another plastic tubing was used for passing the retentate stream to the reservoir. A screw clamp was used for controlling the pressure up to 4 bar.

After setting up the TFF system, flushing was performed with distilled water to remove the storage solution of the TFF capsule membrane system before use and prevent contamination of the sample with the storage solution (0.5 N NaOH). For this purpose, the retentate tubing was directed into the waste collection vessel and the screw clamp on the retentate line was fully opened. The sample reservoir was filled with 500 mL of distilled water, the pump was turned on and the speed increased until the feed gauge pressure showed approximately 1.38 bar. The retentate side of the membrane was flushed with water until a total of 250 mL was collected inside the waste collection vessel. Then, the pump speed was reduced to 0 and the pump was turned off.

For water flushing of the permeate side, screw clamp on the retentate line was closed and the pump was turned on until the feed gauge pressure read approximately 1.38 bar to flush the permeate and the rest of water (250 mL) has been removed from the permeate port and collected.

4.2.1.1. Ultrafiltration of the defatted-destarched bran SCW hydrolysate

The hydrolysate of defatted-destarched bran obtained from the SCW treatment at 180°C, 50 bar and 30 min was selected for use as feed for the ultrafiltration with 1 and 3 kDa membranes. This hydrolysate sample was obtained at the best condition of SCW processing as described in Chapter 3 and had the highest amount of total XOS (112 mg) compared to the other hydrolysates collected from SCW treatment of defatted-destarched bran.

The hydrolysate of defatted-destarched bran obtained from the SCW treatment at 180°C, 50 bar and 30 min was passed through the 0.22 µm filter to remove particulate materials. The TFF system was rinsed with water before using it to remove trapped air and clean it. Then, 52 mL of the filtered hydrolysate was passed through the 1 kDa membrane for 2 h at pressure of 1 bar. After 2 h, 40 mL of the permeate was collected and 10 mL of the retentate was kept in the reservoir. Another 52 mL of the same filtered hydrolysate was passed through the 3 kDa membrane at the same condition described above, 40 mL of the permeate and 10 mL of the retentate were obtained. The fractions obtained from 1 kDa membrane were recorded as permeate and retentate A, and fractions obtained from 3 kDa membrane were recorded as permeate and retentate B.

Distilled water (42 mL) was added into the retentate liquid obtained from 1 kDa membrane and passed through the same 1 kDa membrane again to obtain an additional permeate (40 mL) recorded as permeate C. Retentate C was diluted by distilled water and ultrafiltrated with the same procedure as described for permeate A. Finally, permeate and retentate D were collected. All the retentates

and permeates obtained were analyzed for XOS content by HPLC using the Aminex 42-A column described in Chapter 3 (Section 3.2.6.5)**.** First, the mass (mg) of obtained XOS was determined in all fractions. Then, the recoveries of individual and total XOS were calculated according to equation (4.1).

$$Xylooligosaccharides\ recovery\ (\%) = \left[\frac{Mass\ (mg)\ of\ XOS\ in\ the\ permeate\ or\ retentate\ fraction}{Mass\ (mg)\ of\ initial\ total\ XOS\ in\ the\ feed}\right] \times 100 \qquad (4.1)$$

4.2.1.2. Ultrafiltration of the deproteinized bran SCW hydrolysate

The SCW hydrolysate obtained from deproteinized bran at 180°C, 50 bar and 30 min as described in Chapter 3, that had the highest amount of total XOS (334 mg) was ultrafiltrated using 1 kDa membrane at the same condition used for the hydrolysate from defatted-destarched bran as described above. The first permeate (40 mL) and retentate (10 mL) obtained were recorded as permeate and retentate E. The fractions collected after the second ultrafiltration step were recorded as permeate and retentate F. The third fractions obtained were recoded as permeate and retentate G. The recovery of XOS was quantified using equation (4.1) based on HPLC analysis results.

4.2.1.3. Cleaning of the ultrafiltration system

Cleaning of the Minimate filtration system was performed to remove the fouling and residual materials from the membrane after each experiment. The reservoir was filled with 500 mL NaOH (0.3 N). Then, the retentate valve was closed and the permeate tubing was placed into the waste collection vessel. The pump was turned on, the cleaning solution was pumped to reach the pressure of 1.38 bar until collecting 200 mL in the waste collection vessel. Then, the pump was turned off.

The retentate valve was opened and the screw clamp was closed. The pump was turned on and the cleaning solution was recirculated for 30 min. After draining the retentate, the reservoir was filled with distilled water and it was recirculated for 1 h to remove the remaining NaOH solution and completely clean the system.

4.2.2. Purification of the model system of PreticX^{TM} XOS + xylose solution by nanofiltration

A model system composed of XOS without impurities was used to study the effect of nanofiltration for xylose removal. A semi-continuous flow type nanofiltration system was used as shown in Fig. 4.2 a, employing a high performance liquid chromatograph (HPLC) pump (Model REAXUS 6010R, Teledyne Isco, NE, USA), a stainless steel reactor of 2 cm diameter × 11.6 cm length and a controlling valve.

Two nanofiltration membranes with the MWCO of 150 Da (TS-80) and 150-300 Da (Cynder) were used in the outlet of the reactor to remove monosaccharides and increase the XOS proportion compared to the total content of monomers in the solution.

A solution was prepared from PreticX^{TM} XOS (1.36 g) and xylose (0.54 g) in 500 mL distilled water. An aliquot of the solution (43 mL) was used, which was composed of 77.1±0.3, 69.6±0.84, 33.8±0.06, 80.9±0.3 and 180.6±1.07 mg of xylobiose, xylotriose, xylotetraose, xylose and total XOS, respectively.

Pure water flux and conditioning were performed for each membrane. Membranes were cut from flat sheet rolls according to the internal size of the reactor and allowed to soak in water at room temperature for at least 30 min, then loaded into the stirred cell reactor (Fig. 4.2b). Deionized water was pumped through the reactor and membrane for 45 min with a starting pressure of 10 bar, at a flow rate of 0.5 mL/min, at room temperature until flux was relatively constant. Then, the reactor was opened and deionized water was discarded. The feed reservoir was filled with 43 mL of XOS + xylose solution and pumped at the same flow rate, temperature and pressure. After running for 45 min, 10 mL of permeate was collected and 30 mL remained inside the reactor as the retentate. Both permeate and retentate fractions were analyzed by HPLC to determine the amounts of xylobiose, xylotriose, xylotetraose and xylose. Recoveries of total XOS were calculated according to equation (4.1).

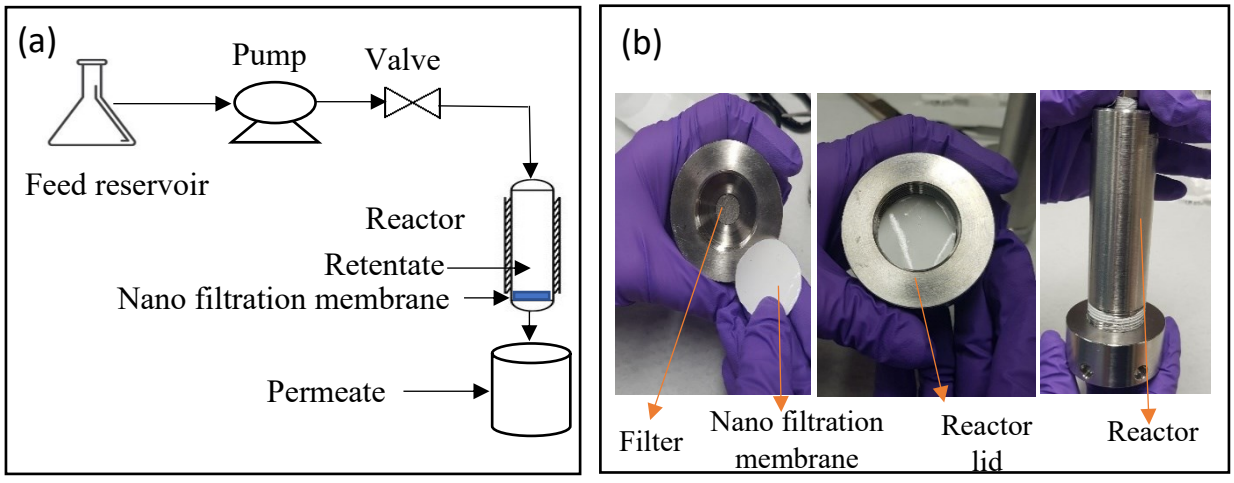


Fig. 4.2. (a) Semi-continuous nanofiltration system and (b) membrane loading inside the reactor.

4.2.3. Purification of PreticX^{TM} XOS + xylose solution by activated carbon adsorption method

This adsorption method was performed according to Zhu et al. (2006) to remove monosaccharides from the solution by adsorption of XOS on to the activated carbon. Activated carbon powder was mixed with the PreticX^{TM} XOS + xylose solution (43 mL) at 10 and 15% of the liquid weight corresponding to 4.11 and 6.16 g, respectively, inside 50 mL Falcon tubes. Then, the mixture was shaken at room temperature at 200 rpm for 30 min to stabilize the carbohydrate adsorption on activated carbon. The activated carbon was separated from the solution by vacuum filtration using a 50-mL Pyrex crucible filter and washed with distilled water 4 times (4×21 mL). The obtained washed liquid was taken and recorded as water fraction A (15% activated carbon) and water fraction B (10% activated carbon). Then, the carbon cake was washed twice with ethanol 15% (2×20 mL) followed by ethanol 30% (2×20 mL). The obtained washed liquid was taken and ethanol was evaporated using liquid nitrogen and the rest of the sample was recorded as ethanol fraction A (15% activated carbon) and ethanol fraction B (10% activated carbon). The water and ethanol fractions were analyzed by HPLC to determine the amounts of xylobiose, xylotriose, xylotetraose and xylose.

4.2.4. Purification and decolorization of permeates E and F by activated carbon adsorption method

Permeates E and F obtained after ultrafiltration using 1 kDa membrane of deproteinized bran SCW hydrolysate were treated by activated carbon adsorption with 10 mL starting feed solution mixed with 1.2 g of activated carbon powder (10% of the liquid weight) as described above. The activated carbon was washed with distilled water (24 mL) after separation, and collected fraction was recorded as water fraction. Then, ethanol washing was performed using ethanol 15% (2×5 mL) followed by ethanol 30% (2×5 mL). After ethanol evaporation, the obtained fraction was recorded as ethanol fraction. Fractions obtained from permeate E were recorded as ethanol and water fraction C and fractions obtained from permeate F were recorded as water and ethanol fraction D.

Xylose, arabinose and total XOS recoveries were calculated in the water and ethanol fractions according to equations (4.2). (4.3) and (4.1), respectively.

$$Xylose\ recovery\ (\%) = \left[\frac{Mass\ (mg)\ of\ xylose\ in\ the\ ethanol\ or\ water\ fraction}{Mass\ (mg)\ of\ initial\ xylose\ in\ the\ feed}\right] \times 100 \qquad (4.2)$$

$$Arabinose\ recovery\ (\%) = \left[\frac{Mass\ (mg)\ of\ arabinose\ in\ the\ ethanol\ or\ water\ fraction}{Mass\ (mg) of\ initial\ arabinose\ in\ the\ feed}\right] \times 100 \qquad (4.3)$$

4.2.5. Xylooligosaccharides and monosaccharides determination by HPLC

Xylo–oligosaccharides and monosaccharides were quantified using a HPLC system described in Chapter 3 Section (3.2.6.5) with an Aminex HPX 42-A column at 85°C and a flow rate of 0.3 mL/min for 45 min.

4.2.6. Statistical analysis

Data are shown as mean ± standard deviation according to the results obtained from at least duplicate experiments and analysis. One-way analysis of ANOVA and the significance difference of data at $p < 0.05$ was performed using Minitab version 18.0 (Minitab Inc., State College, PA, USA) at 95% confidence interval.

4.3. Results and discussion

4.3.1. Xylooligosaccharides content after ultrafiltration of defatted-destarched bran SCW hydrolysate

The SCW hydrolysate (52 mL) obtained from defatted-destarched bran at 180°C, 50 bar and 30 min was ultrafiltrated using membranes of 3 kDa and 1 kDa MWCO. This sample had 8.96, 14.52 and 13.01 and 36.51 mg of X4, X3, X2 and total XOS, respectively. Also, it had a peak area of 243156 for the compounds with high molecular weight with DP > 12.

After the first filtration process, the large peak of high molecular weight compounds (DP>12) almost disappeared in the permeates obtained. Its area reduced to 8254 and 5584 in the permeates B and A after passing through the 3 and 1 kDa membranes, respectively. This peak indicated the presence of high molecular weight compounds with molecular weight more than 1 and 3 kDa that might be soluble lignin, or unhydrolyzed polysaccharides.

In other studies, ultrafiltration was used for the removal of high molecular weight compounds from hydrothermal hydrolysate obtained from corn cob at 200-205°C, 30 bar and 9-12 min using a continuous SCW process (Makishima et al., 2009) and enzymatic hydrolysate obtained from wheat bran extracted xylan using endoxylanase with 0.8, 1.6 and 5 U on 720 mg xylan suspended in 232 mL sodium acetate buffer at 30°C (Swennen et al., 2005).

Fig. 4.3 shows the mass balance after ultrafiltration of the SCW defatted-destarched bran hydrolysate with 1 and 3 kDa MWCO membranes. Xylobiose, xylotriose and xylotetraose were recovered in the permeate B obtained from 3 kDa with amounts of 5.44, 6.70, 4.51 mg, respectively, with 16.66 mg for total XOS. After passing the SCW hydrolysate by 1 kDa membrane, 6.87, 8.37, 4.02 and 19.27 mg X2, X3, X4, and total XOS were obtained in the permeate A.

Fig. 4.4 a,b shows the total recovery of xylobiose, xylotriose, xylotetraose and total XOS obtained after ultrafiltration of SCW defatted-destarched bran hydrolysate using 1 and 3 kDa membranes.

According to the One-way ANOVA analysis, total XOS recovered was significantly different between membranes with 1 kDa (19.27 mg/52.79% recovery) and 3 kDa (16.66 mg/45.63% recovery) cutoff, which was higher in the permeate A obtained by 1 kDa. This phenomenon can be related to the accumulation of high molecular weight compounds layer on the surface of the membrane with high molecular weight cutoff of 3 kDa due to the high flux of permeate, leading to the flow reduction and increasing the retention of low molar mass XOS in the retentate fraction (Nabarlatz et al., 2007). Total XOS recovered (52.79%) in the permeate A obtained after passing 1 kDa membrane and 6.90 mg remained in the retentate A, which corresponded to 18.92% recovery. Therefore, retentate A (10 mL) was diluted by the addition of deionized water (42 mL) and passed through the same membrane again to recover the rest of XOS in the second liquor permeate noted as permeate C (Fig. 4.3).

In permeate C, 1.15, 1.83, 0.94 and 3.93 mg of X4, X3, X2 and total XOS were quantified that corresponded to the recovery of 3.15, 5.01, 2.60 and 10.77%, respectively. Retentate C was ultrafiltrated one more time by the addition of deionized water (42 mL). However, trace amounts of XOS was recovered in the permeate D.

Zhao and Dong (2016) also purified XOS solution containing X2 and X3 obtained from endo-xylanase enzymatic hydrolysis of wheat bran xylan using 1 kDa membrane. They recovered 57.55% of XOS (X2 and X3) in the permeate after 3 times 1 kDa membrane filtration. In another

study, Akpinar et al. (2007) purified XOS obtained by endo-xylanase enzymatic hydrolysis of xylan from cotton stalks, which was composed of X6, X5, X2 and X3. They used 10 kDa disc membrane for the separation of enzymes and other high molecular weight polysaccharides. Then, the obtained permeate was passed through the 3 and 1 kDa disc membranes separately to remove long chain XOS>X5 from the rest and collect permeates with purified XOS with DP < 5. In the final permeate, the recovery of X2 was 89% with both membranes and the recovery of X3 was 93 and 96% by 1 and 3 kDa, respectively. Therefore, they concluded that the use of both membranes could be an appropriate choice to refine XOS, because there was no significant difference in the recovery of X2 and X3. However, the retention of X5 and >X5 were higher with 3 kDa membrane and recoveries of >X5 oligomers in permeate of 3 and 1 kDa were 62 and 57%, respectively. They did not observe any reduction in the recovery of X2 and X3 in the permeate with the use of higher molecular weight cut off membrane of 3 kDa. This might be because of the separation of endo-xylanase enzyme and high molecular weight molecules with 10 kDa membrane before passing by the low molecular weight cutoff membranes (3 and 1 kDa). But, in this study, there was a significant difference in the recovery of total XOS (X4+X3+X2) in the permeate obtained by 1 kDa membrane. Probably, because the hydrolysate was passed through the 1 and 3 kDa membranes directly and no previous filtration was performed to remove high molecular weight compounds.

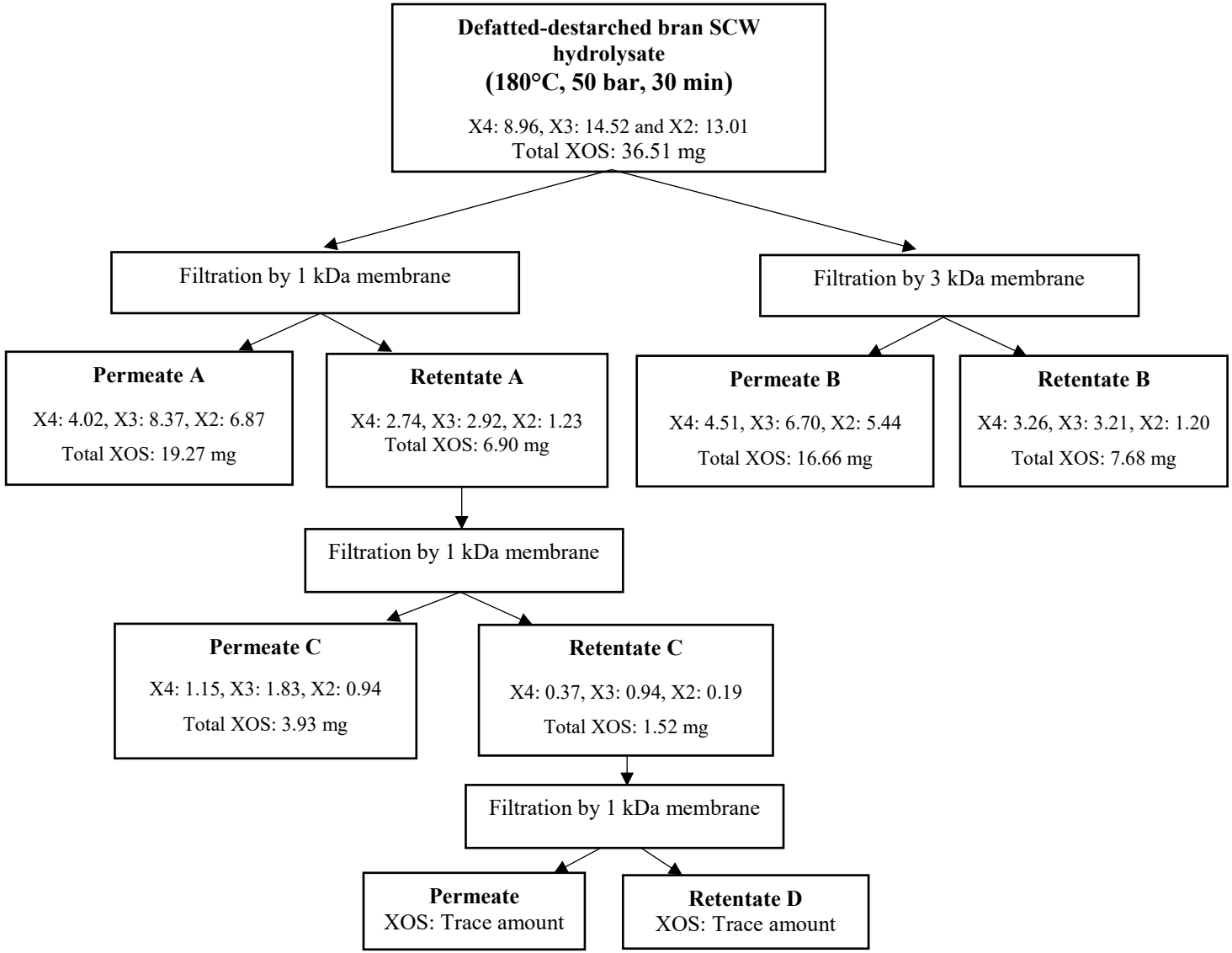


Fig. 4.3. Mass balance after ultrafiltration of defatted-destarched bran SCW hydrolysate with 1 and 3 kDa membranes.

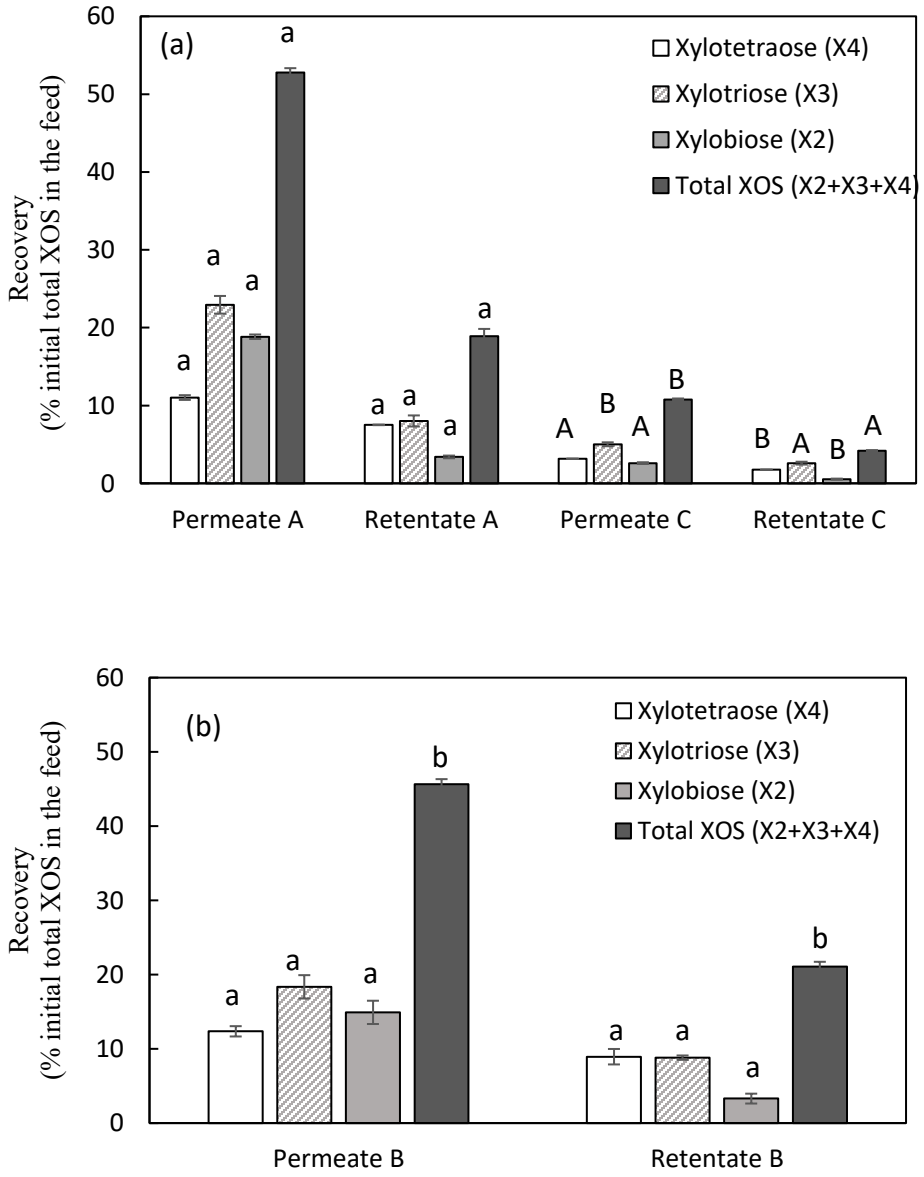


Fig. 4.4. Ultrafiltration of defatted-destarched bran SCW hydrolysate using: (a) 1 kDa and (b) 3 kDa membranes. Means within the same group of XOS with different letters are significantly different at $p<0.05$ (A-B) for Fig 4.4a between permeate C and retentate C, (a-b) for Fig. 4.4a-b between permeate A and permeate B, retentate A and retentate B.

4.3.2. Xylooligosaccharides content after ultrafiltration of deproteinized bran SCW hydrolysate

Membrane with the molecular weight cut off 1 kDa was used for the filtration of deproteinized bran SCW hydrolysate to recover XOS in the obtained permeate, because 1 kDa membrane was tested for the filtration of defatted-destarched bran SCW hydrolysate and total XOS recovery was higher in the permeate A compared to the permeate B obtained after ultrafiltration with 3 kDa membrane (Fig. 4.4).

The starting deproteinized bran SCW hydrolysate had 47.13, 49.14 and 34.74 mg of X4, X3 and X2, respectively. Also, the big peak related to the molecules with DP > 12 had an area of 655080.

Fig. 4.5a shows the filtration process of deproteinized bran SCW hydrolysate. Retentate remained in the feed reservoir and permeate was collected in the cylinder. Two permeates E and F were collected after the two-step ultrafiltration process and they were more clear and lighter in color compared to the feed (Fig. 4.5b).

Fig. 4.6 illustrates the chromatograms (with 0.3 mL/min HPLC flow rate) related to the deproteinized bran SCW hydrolysate and obtained fractions after the ultrafiltration process including permeate and retentate E, permeate and retentate F. Ultrafiltration by 1 kDa membrane was effective to separate high molecular weight compounds (DP>12) by reduction of the peak area from 327540.8 to 35952.8 in the permeate E. The contents of XOS in the permeate E were reported

as 23.94, 30.17, 19.16 and 73.28 mg corresponding to 18.25, 22.99, 14.60 and 55.85% recovery for X4, X3, X2 and total XOS, respectively. However, some XOS were still present in the retentate E with 27.20, 25.62, 21.67 and 25.14% recovery of X4, X3, X2 and total XOS, respectively. Permeate F had 5.61, 5.58, 4.95 and 16.15 mg corresponding to 4.28, 4.25, 3.77 and 12.32% recovery for X4, X3, X2 and total XOS, respectively, and the retentate F had only traces of XOS, which was not possible to quantify. Retentate and permeate G had also trace amounts of XOS. Therefore, the major amount of XOS was recovered in the permeate E (73.28 mg) and the rest was obtained in the permeate F (16.15 mg) (Fig. 4.7).

Fig. 4.8 shows the recovery of XOS obtained from deproteinized bran SCW hydrolysate of the filtration by 1 kDa membrane. In total, 81.07% of total XOS was recovered in the two collected permeates (E and F) obtained by 1 kDa ultrafiltration with trace amounts left in the retentate F.

Singh et al. (2019) autohydrolyzed almond shell at (200°C, 5 min), followed by endo-xylanase treatment (10 U, 36 h). They employed a dead-end (batch) ultrafiltration system using a stirred cell with membrane with 1 kDa molecular weight cut-off at 6.8 bar to separate compounds with high molecular weight such as enzymes by their rejection in the retentate fraction. They separated 96-97% of the initial feed as permeate. Time was not reported in that study. The obtained permeate had 90.18 and 95.69% of xylobiose and xylotriose. Monomers were also recovered in the permeate with 92.42, 96.56 and 95.47% for xylose, glucose and arabinose, respectively. Higher recovery of

oligomers including xylobiose and xylotriose in the permeate in their study compared to this study (55.85% recovery of total XOS in first permeate E) might have some reasons: i) they used higher pressure (6.8 bar) compared to this study (1 bar), and ii) they separated 96-97% of the initial feed as a permeate, in this study only 76% was removed in the first ultrafiltration process. Therefore, higher pressure and higher permeate volume with respect to the initial feed volume, could be effective and help to recover higher amount of XOS in the permeate obtained by ultrafiltration.

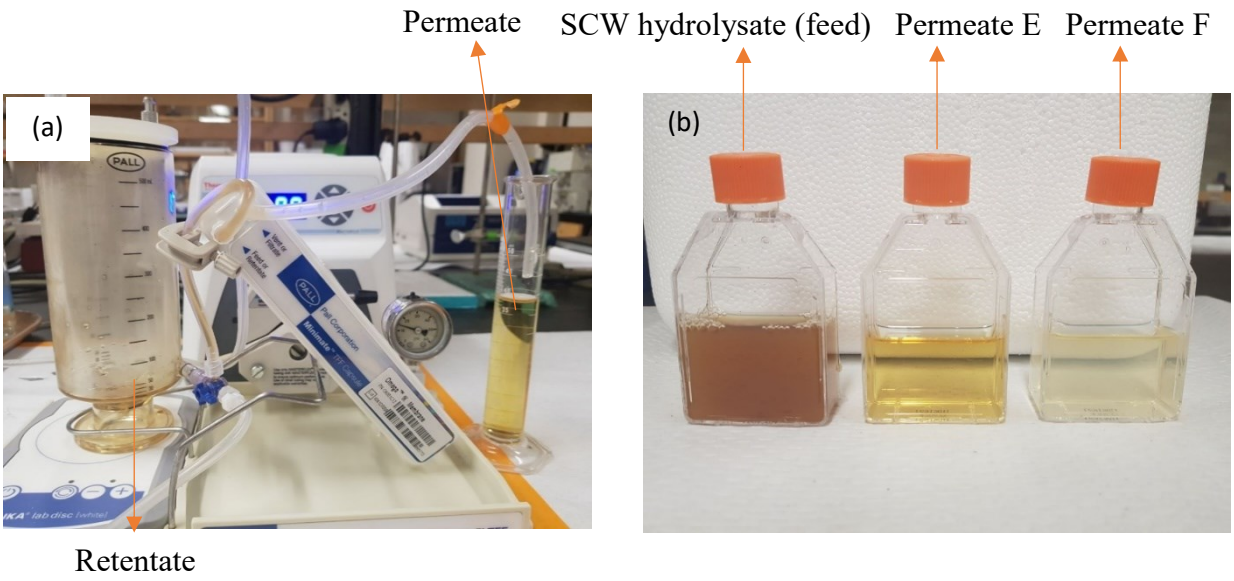
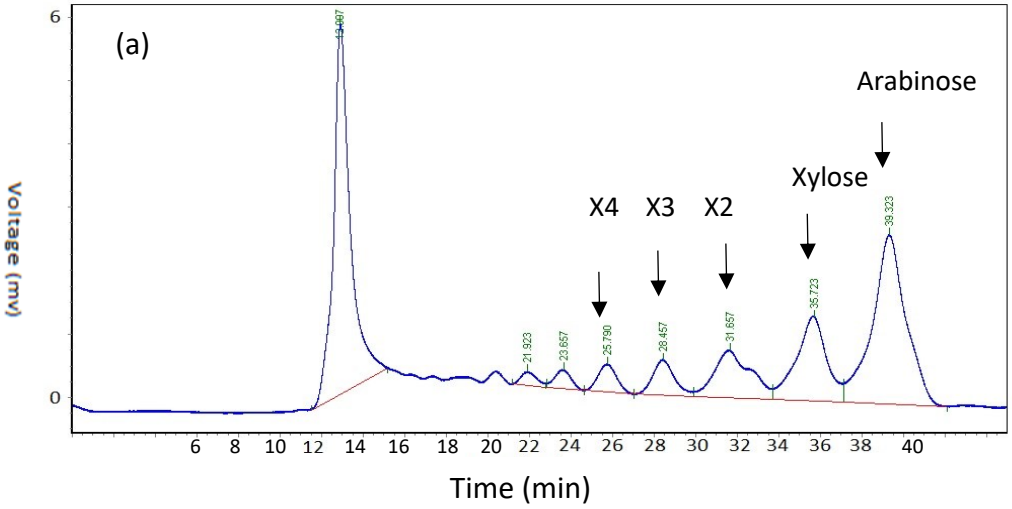
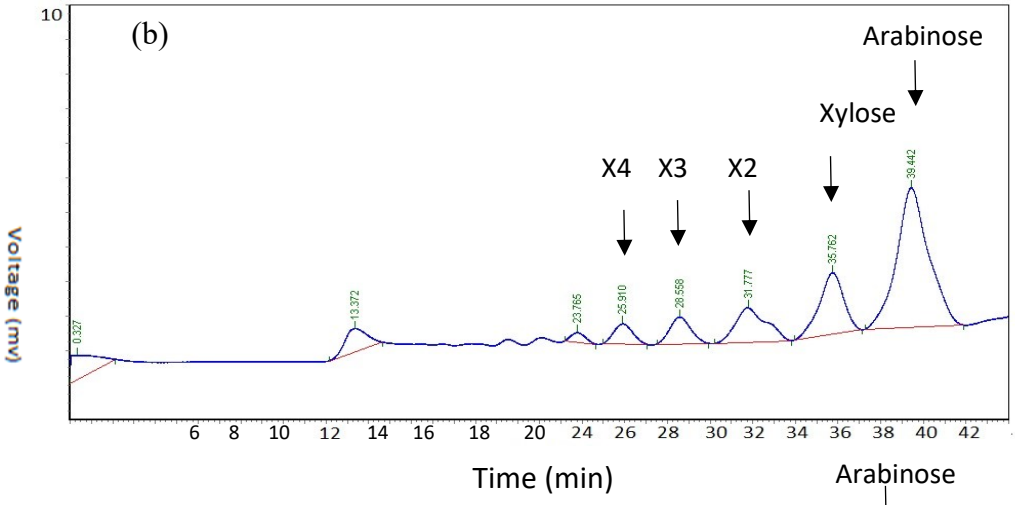
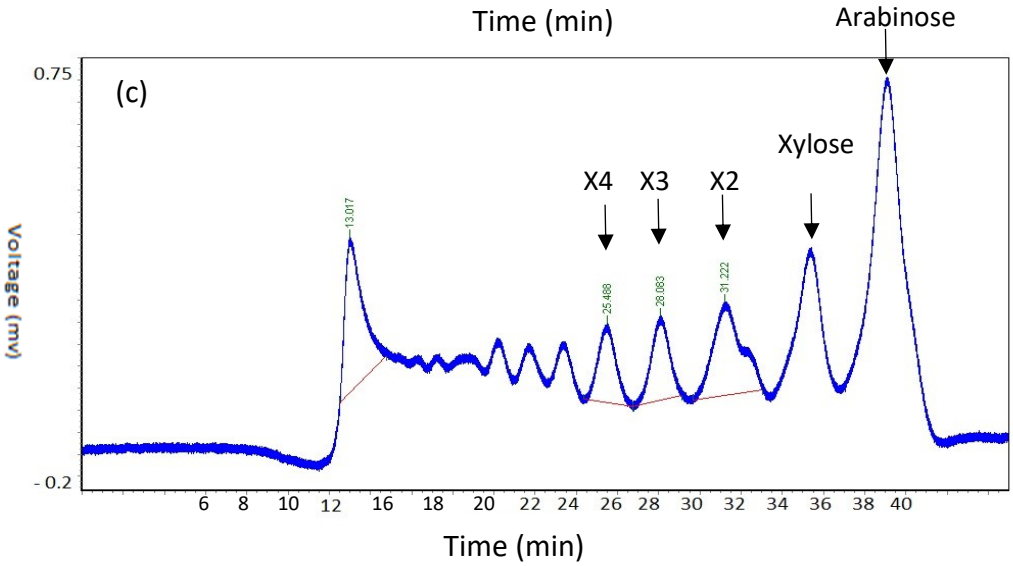


Fig. 4.5. (a) Ultrafiltration of XOS from deproteinized bran SCW hydrolysate and obtained retentate and (b) permeates (E and F) obtained by 1 kDa membrane.


(a)
Arabinose
Xylose
X4
X3
X2
Voltage (mv)
Time (min)


(b)
Arabinose
Xylose
X4
X3
X2
Voltage (mv)
Time (min)


(c)
Arabinose
Xylose
X4
X3
X2
Voltage (mv)
Time (min)

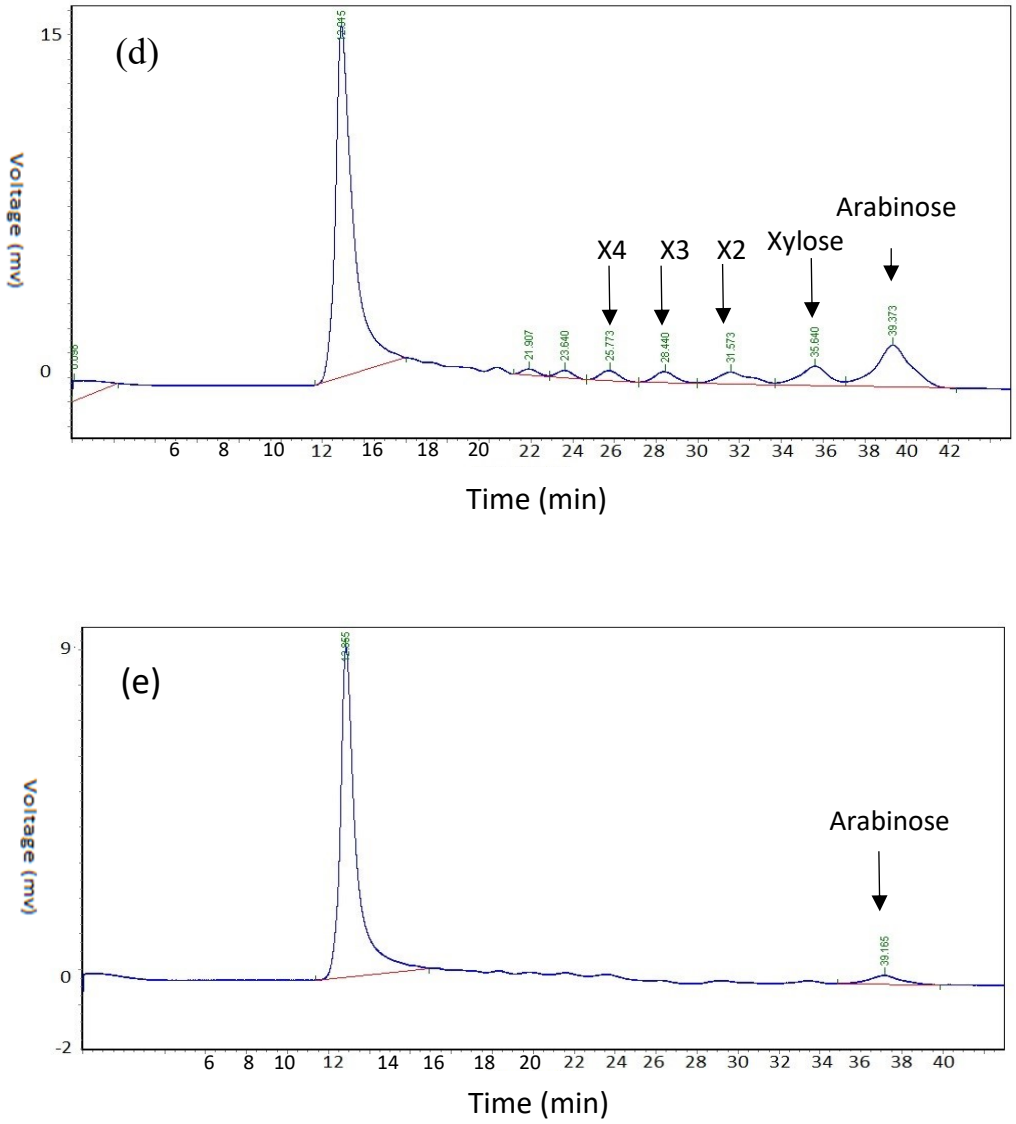


Fig. 4.6. HPLC chromatograms showing xylobiose (X2), xylotriose (X3), xylotetraose (X4) and arabinose obtained after the ultrafiltration process through 1 kDa membrane: (a) deproteinized bran SCW hydrolysate; (b) permeate E; (c) retentate E; (d) permeate F; and (e) retentate F.

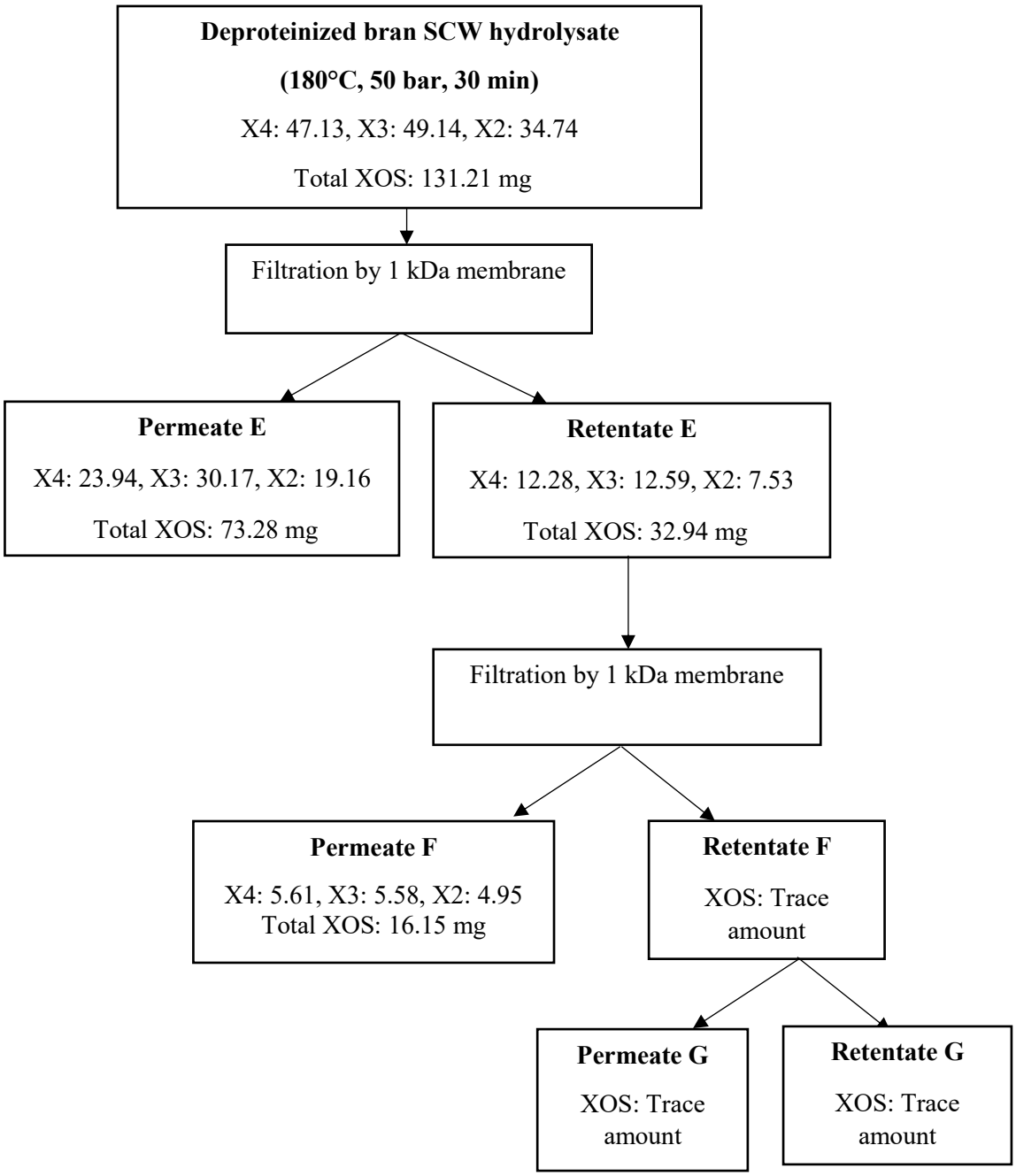


Fig. 4.7. Mass balance after ultrafiltration of deproteinized bran SCW hydrolysate with 1 kDa membrane.

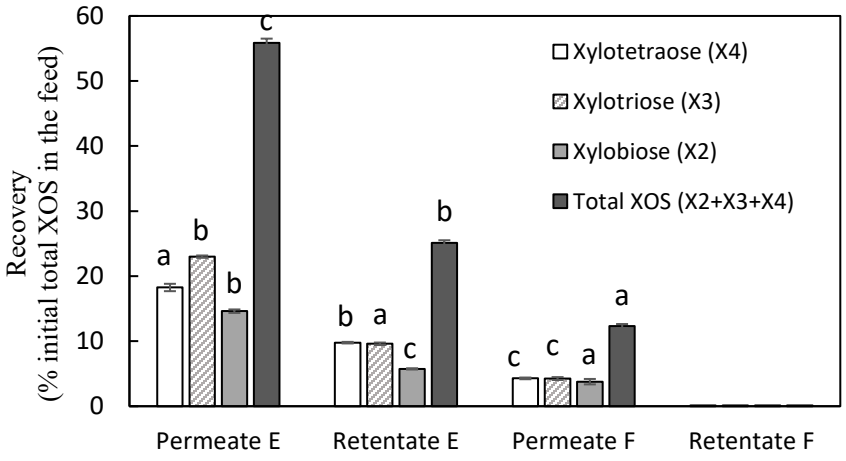


Fig. 4.8. Recovery of XOS from deproteinized bran SCW hydrolysate using 1 kDa membrane. Means within the same group of XOS with different letters are significantly different at $p<0.05$.

4.3.3. Xylooligosaccharides content after nanofiltration and activated carbon treatment of a model system PreticX^{TM} XOS + xylose solution

Nanofiltration and activated carbon adsorption were compared to remove xylose from the XOS in the PreticX^{TM} + xylose solution. Table 4.1 summarizes the XOS, and xylose contents, and the XOS/xylose ratio. It presents the data for the starting 43 mL PreticX^{TM} + xylose solution, permeate and retentate HC, HT obtained after nanofiltration and the water and ethanol fractions A, B obtained after activated carbon adsorption. After nanofiltration of the starting solution using TS-80 (150 Da) and Cynder (150-300 Da) membranes, 10 mL permeate and 30 mL retentate were obtained.

Fig. 4.9 shows the mass balance after nanofiltration and activated carbon adsorption of PreticX^{TM} XOS + xylose solution. After nanofiltration using TS-80 membrane total XOS and xylose contents were obtained in the permeate HT (14.58 and 17.77 mg, respectively) and retentate HT (138.85 and 47.09 mg, respectively). The XOS/xylose ratio, which was determined as 2.23 for the feed solution increased 1.31 times in the retentate to 2.94 and decreased to 0.82 in the permeate, showing partial separation of xylose from XOS after filtration through the TS-80 membrane.

After nanofiltration using the Cynder membrane, 27.20 mg of total XOS and 18.20 mg xylose were recovered in the permeate HC while 114.21 mg of total XOS and 37.68 mg xylose were obtained in the retentate HC. Less recovery of XOS and xylose in the retentate fraction of Cynder membrane was probably due to its larger pore size (150-300 Da) compared to the TS-80 membrane (150 Da), which led to the higher flux of the solution passing through the Cynder membrane and higher recovery of XOS and xylose in the permeate. The XOS/xylose ratio was determined as 3.03 in the retentate of Cynder membrane, which was not significantly different from the ratio in the retentate of the TS80 membrane.

As a result of membrane processing with two different types of nanofilters, 63.21 and 76.85% of total XOS were recovered in the retentate of Cynder and TS80 filters, respectively, with a similar ratio of XOS/xylose.

Using the semi-continuous nanofiltration system with two different membranes was not effective to separate xylose selectively from the total XOS in the retentate fraction. The ratio of XOS/xylose increased from 2.23 only to 2.94 and 3.03 in the retentate of both membranes.

XOS was recovered with higher amounts in the retentate HT (138.85 mg) obtained after nanofiltration through the TS-80 membrane, which had a smaller pore size (150 Da).

Another portion (43 mL) of the PreticX^{TM} + xylose solution was treated with activated carbon adsorption by the addition of 10 and 15 wt% activated carbon to the liquid. After ethanol (15% and 30%) washing and distilled water washing (80 and 85 mL), ethanol and water fractions were obtained, respectively.

As shown in Table 4.1, using 10% activated carbon, higher mass of total XOS (95.93 mg) was eluted with 80 mL ethanol compared to 85 mL water elution that had only 19.87 mg of total XOS. Xylose was more concentrated in the water fraction B (42.24 mg) compared to only 11.63 mg in the ethanol fraction B. Because xylose had little interaction with activated carbon, showing less adsorption on it, which was removed from the XOS-activated carbon complex by water washing. However, XOS with DP 2-4 had stronger adsorption to activated carbon that were recovered by ethanol elution (Pellerin et al., 1991; Zhu et al., 2006). Total XOS content with respect to the xylose content in the ethanol fraction was 8.24, which was higher than this ratio in the two retentates obtained by nanofiltration (2.94 for TS-80 membrane and 3.03 for Cynder membrane).

After addition of 15% activated carbon, the ethanol fraction had 76.72 and 11.81 mg total XOS and xylose, respectively. However, 6.79 and 25.9 mg were determined in the water fraction A. The ratio of XOS/xylose in the ethanol fraction increased to 6.49. Lower recovery of XOS in the ethanol fraction A after treatment with 15% activated carbon was related to the loading of excess carbon that provided more affinity for XOS, and therefore delayed their elution. A similar behaviour was also reported in the literature by Chen et al. (2014), who obtained less amounts of XOS using 20% activated carbon (28.2% recovery of initial XOS) compared to the 10% activated carbon addition, where 40.2% was obtained by ethanol washing in three steps. They used 5, 30 and 50% ethanol to recover the adsorbed XOS on the activated carbon and collected separate fractions.

In the retentates collected after the nanofiltration process, 63.21 and 76.85% of total XOS were recovered by Cynder and TS-80 membranes, respectively, which were higher compared to the recovery in the ethanol fraction B after 10% activated carbon adsorption (53.1%). This data showed that higher amounts of XOS was lost after activated carbon adsorption treatment, because 19.87 mg XOS was separated during water washing. Moreover, the rest of XOS might be captured inside the activated carbon powder and ethanol concentrations (15 and 30%) were not enough for washing and liberate all the XOS adsorbed on the activated carbon.

These results showed that, the use of activated carbon adsorption method with 10% carbon loading was better than the semi-continuous nanofiltration system, due to the higher ratio of XOS/xylose in the obtained ethanol fraction B (8.24). Therefore, activated carbon adsorption with 10% activated carbon loading was used in further experiments for the purification of the obtained permeates (E and F) from ultrafiltration with 1 kDa membrane of deproteinized bran SCW hydrolysate.

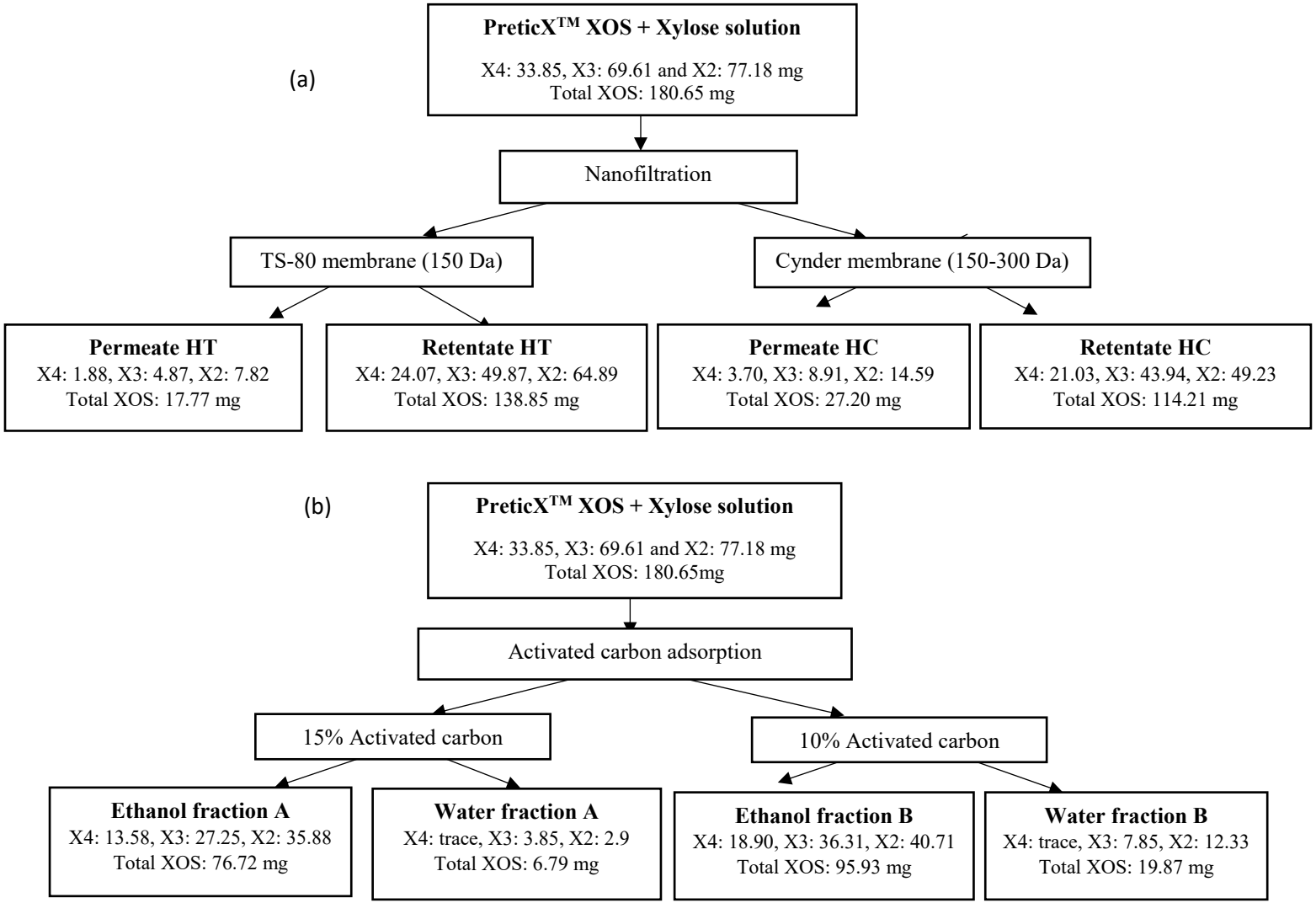


Fig. 4.9. Mass balance after: (a) nanofiltration, and (b) activated carbon adsorption of PreticX™ XOS + xylose solution

Table 4.1. XOS and xylose contents of PreticX™ + xylose solution, the retentate and permeate obtained after membrane filtration, water and ethanol fractions obtained after activated carbon adsorption.

Fraction	Xylobiose (mg)	Xylotriose (mg)	Xylotetraose (mg)	Xylose (mg)	Total XOS (mg)	Ratio (Total XOS/Xylose)
Feed solution (Model system)	77.18±0.30	69.61±0.84	33.85±0.06	80.99±0.30	180.65±1.07	2.23
Method 1: activated carbon adsorption						
Water B	12.33±0.27	7.85±0.28	Trace	42.24±0.8	19.87±0.48	0.47
Ethanol B	40.71±2.17	36.31±1.24	18.90±0.91	11.63±0.94	95.93±1.84	8.24
Water A	2.93± 0.20	3.85± 0.28	Trace	25.94±0.97	6.79± 0.08	0.26
Ethanol A	35.88±0.32	27.25± 0.76	13.58± 0.48	11.81±0.16	76.72± 1.57	6.49
Method 2: Nanofiltration						
Permeate HT	7.82± 0.42	4.87±0.40	1.88±0.18	17.77±0.69	14.58±1.0	0.82
Retentate HT	64.89±0.98	49.87±0.64	24.07±1.27	47.09±1.13	138.85±2.9	2.94
Permeate HC	14.59±0.57	8.91±0.24	3.70±0.27	18.20±0.40	27.20±1.08	1.49
Retentate HC	49.23±2.73	43.94±0.53	21.03±0.77	37.68±0.57	114.21±4.05	3.03

Model system: PreticX™ XOS + xylose solution, permeate and retentate HT obtained from TS-80 membrane, permeate and retentate HC obtained from Cynder membrane, ethanol and water fraction B obtained from 10% activated carbon, ethanol and water fraction A obtained from 15% activated carbon.

4.3.4. Xylooligosaccharide and monomer contents after activated carbon treatment of ultrafiltration permeates E and F obtained from deproteinized bran SCW hydrolysate

Table 4.2 shows the contents of xylobiose (X2), xylotriose (X3), xylotetraose (X4), total XOS, arabinose and xylose of the starting permeate E and F (10 mL out of the total of 40 mL) (Fig. 4.7) used for the activated carbon adsorption. After this process, XOS content, xylose and arabinose were quantified in the water (20 mL) and ethanol fractions (12 mL) obtained and results are presented on Table 4.2.

Fig. 4.11 illustrates the chromatograms related to the permeate E from ultrafiltration by 1 kDa membrane obtained from deproteinized bran SCW hydrolysate, ethanol and water fractions C from activated carbon adsorption (10%). After activated carbon treatment X2, X3, X4, xylose and arabinose were detected in the ethanol fraction obtained. However, the intensity of the peaks related to the monomers were reduced in this fraction.

Fig. 4.12 shows the mass balance after activated carbon adsorption of the ultrafiltration permeates E and F obtained from deproteinized bran SCW hydrolysate. The starting feed permeate E had 4.14, 6.18, 4.38, 14.71, 8.69 and 4.28 mg of xylobiose, xylotriose, xylotetraose, total XOS, arabinose and xylose, respectively. After separation of activated carbon, water washing was performed to remove monomers (arabinose and xylose) and 20 mL water fraction C was obtained.

In this water fraction, trace amounts of X2 and X3 were found and X4 was not detected. Also, 2.36 and 4.44 mg xylose and arabinose were recovered.

After water washing, activated carbon was washed with ethanol 15 and 30% and ethanol fraction was obtained. This step was used for the separation of XOS captured by activated carbon and collection in the ethanol fraction C. This fraction had 2.15, 3.55, 1.95 and 7.66 mg X2, X3, X4 and total XOS, respectively. Total XOS content with respect to the sum of xylose and arabinose contents were 1.13 in the feed permeate E, which increased to 2.94 in the ethanol fraction C, indicating the removal of monomers such as arabinose and xylose from the XOS solution. In the ethanol fraction C, total XOS recovered was 52.10% (of initial XOS), which was higher than the recovery of monomers (30.42 and 16.46% for xylose and arabinose, respectively). Most monomers were present in the water fraction C with xylose (55.11±1.61%) and arabinose (51.15±1.99%) recovery. Because monomers had less interaction with the activated carbon, they were removed by 20 mL water elution before washing with ethanol. Also, decolorization of the permeate E containing XOS occurred after activated carbon adsorption where the ethanol fraction C was completely clear, without any color or precipitate (Fig 4.10). The permeate F obtained after ultrafiltration of retentate E of deproteinized SCW hydrolysate, had 1.23, 1.51, 1.26, 2.62, 3.22 mg of X2, X3, X4, xylose and arabinose, respectively. This permeate was also treated by activated carbon adsorption using the same methodology described for permeate E. In the ethanol fraction, 0.61, 1.37, 0.88 and 0.57 mg X2, X3, X4 and arabinose were detected. The ratio of total XOS to

the sum of arabinose and xylose also increased from 0.68 in the feed to 5.05 in the ethanol fraction D, which was similar to the ethanol fraction obtained for permeate E.

Fig. 4.13 shows the overall scheme of sequential purification of deproteinized bran SCW hydrolysate using ultrafiltration with 1 kDa membrane followed by 10% activated carbon adsorption.

Chen et al. (2014) investigated the purification of xylooligosaccharides produced from *Miscanthus x giganteus* after autohydrolysis at 180°C/20 min and 200°C/5 min using activated carbon adsorption with 1%, 5%, 10% and 20% (w/v) of the hydrolysate volume, shaking at 100 rpm for 60 min. Ethanol elution was performed with concentrations of solutions of 5%, 30%, 50%, 70% and 95% (v/v). They reported the highest XOS recovery of 47.9% (w/w) of initial XOS using 10% (w/v) activated carbon, followed by 35.7% (w/w) of initial XOS using 20% (w/v) activated carbon, which were close to the total XOS recovery in this study (52.10%) with 10% activated carbon use.

Chen et al. (2014) used ethanol washing in five steps with ethanol 5%, 30%, 50%, 70% and 95% (v/v). In total, fractions obtained from 5 and 30% ethanol washing had most of X2, X3 and X4 with 8.2, 49.2 and 41.7% recovery, respectively. After washing with 50% ethanol, 8.1, 22.6 and 17% of the rest captured X2, X3 and X4 in the activated carbon, were eluted in the 50% ethanol fraction.

Zhu et al. (2006) used activated carbon to recover xylooligosaccharides from corn stover and corncob enzymatic hydrolysates, followed by ethanol elution with different concentrations of 15, 30 and 50%. They obtained the highest XOS recovery using 10% activated carbon loading with 34.5, 15.9 and 4.4% recovery by 15, 30 and 50% ethanol elution, respectively. In total 54.8% of total XOS was recovered in all ethanol fractions obtained.

Activated carbon adsorption was also used for the purification of other oligosaccharides like fructooligosaccharides (FOS) produced from enzymatic synthesis of sucrose 50% (w/v) at 50°C for 41 h. The obtained hydrolysate (100 mL), composed of FOS, glucose, fructose and sucrose, was mixed with 5 g of activated carbon, followed by elution with 15% (v/v) ethanol at 50°C. They recovered 80% of the initial FOS, with separation of both fructose and glucose (95%) that led to obtaining a final FOS solution with 92% purity that had only 5-7% sucrose (Kuhn and Filho, 2010).

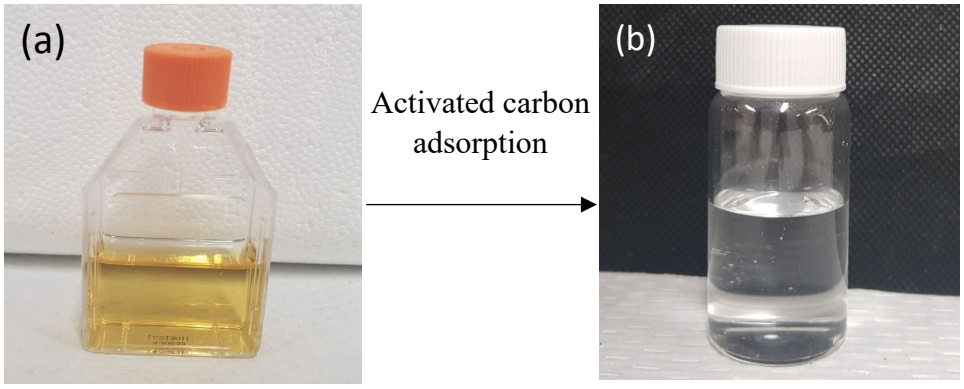


Fig. 4.10. (a) Color of the permeate E from deproteinized bran SCW hydrolysate after ultrafiltration using 1 kDa membrane and (b) ethanol fraction C from permeate E after activated carbon adsorption (10%).

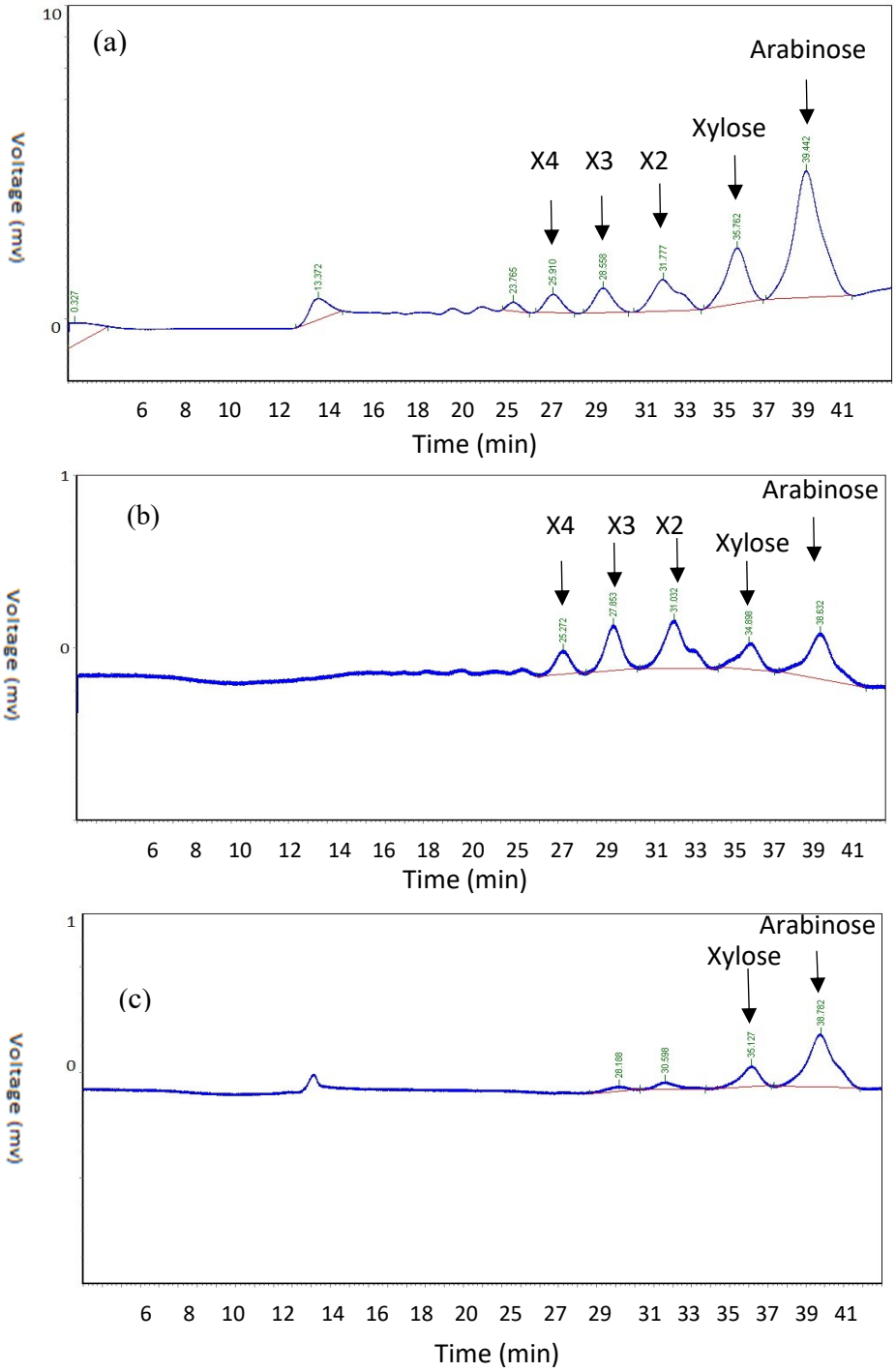


Fig. 4.11. HPLC chromatograms of xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylose and arabinose obtained after activated carbon adsorption treatment. (a) permeate E from ultrafiltration with1 kDa membrane; (b) ethanol fraction C from 10% activated carbon adsorption; and (c) water fraction C from 10% activated carbon adsorption for 30 min.

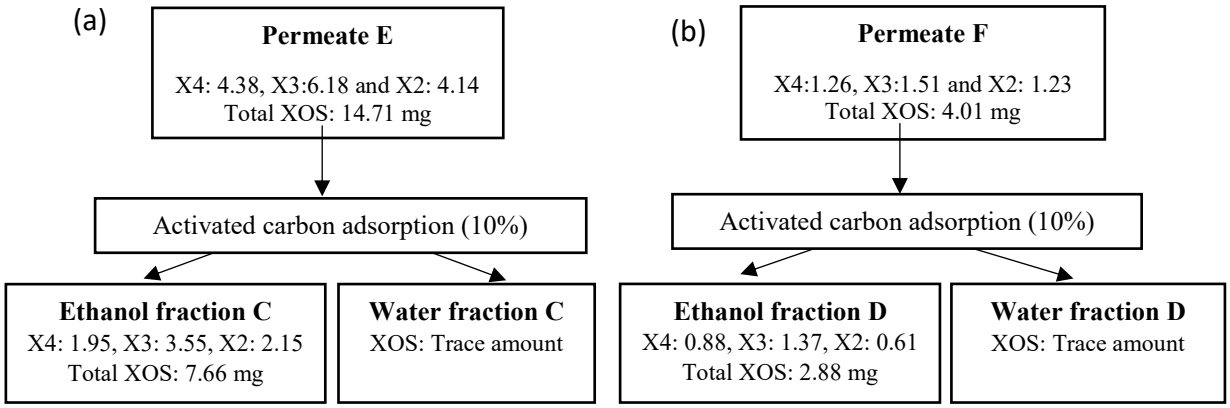


Fig. 4.12. Mass balance after activated carbon adsorption: (a) permeate E and (b) permeate F obtained from ultrafiltration of deproteinized bran SCW hydrolysate.

Table 4.2. XOS, xylose and arabinose contents after activated carbon treatment (10%) of permeates (E and F) obtained from deproteinized bran SCW hydrolysate.

Fraction	Xylobiose (mg)	Xylotriose (mg)	Xylotetraose (mg)	Total XOS (mg)	Arabinose (mg)	Xylose (mg)	Ratio (XOS/ X+A)
Permeate E	4.14±0.37	6.18±0.02	4.38±0.34	14.71±0.74	8.69±0.07	4.28±0.01	1.13
Water C	Trace	Trace	ND	Trace	4.44±0.17	2.36±0.06	-
Ethanol C	2.15±0.24	3.55±0.23	1.95±0.02	7.66± 0.01	1.30±0.15	1.30±0.04	2.94
Permeate F	1.23± 0.03	1.51± 0.01	1.26± 0.04	4.01± 0.02	3.22±0.05	2.62± 0.02	0.68
Water D	Trace	ND	ND	Trace	0.78±0.01	ND	-
Ethanol D	0.61±0.06	1.37±0.1	0.88± 0.00	2.88±0.04	0.57±0.02	ND	5.05

ND: Not detected, A+X: Arabinose + xylose.

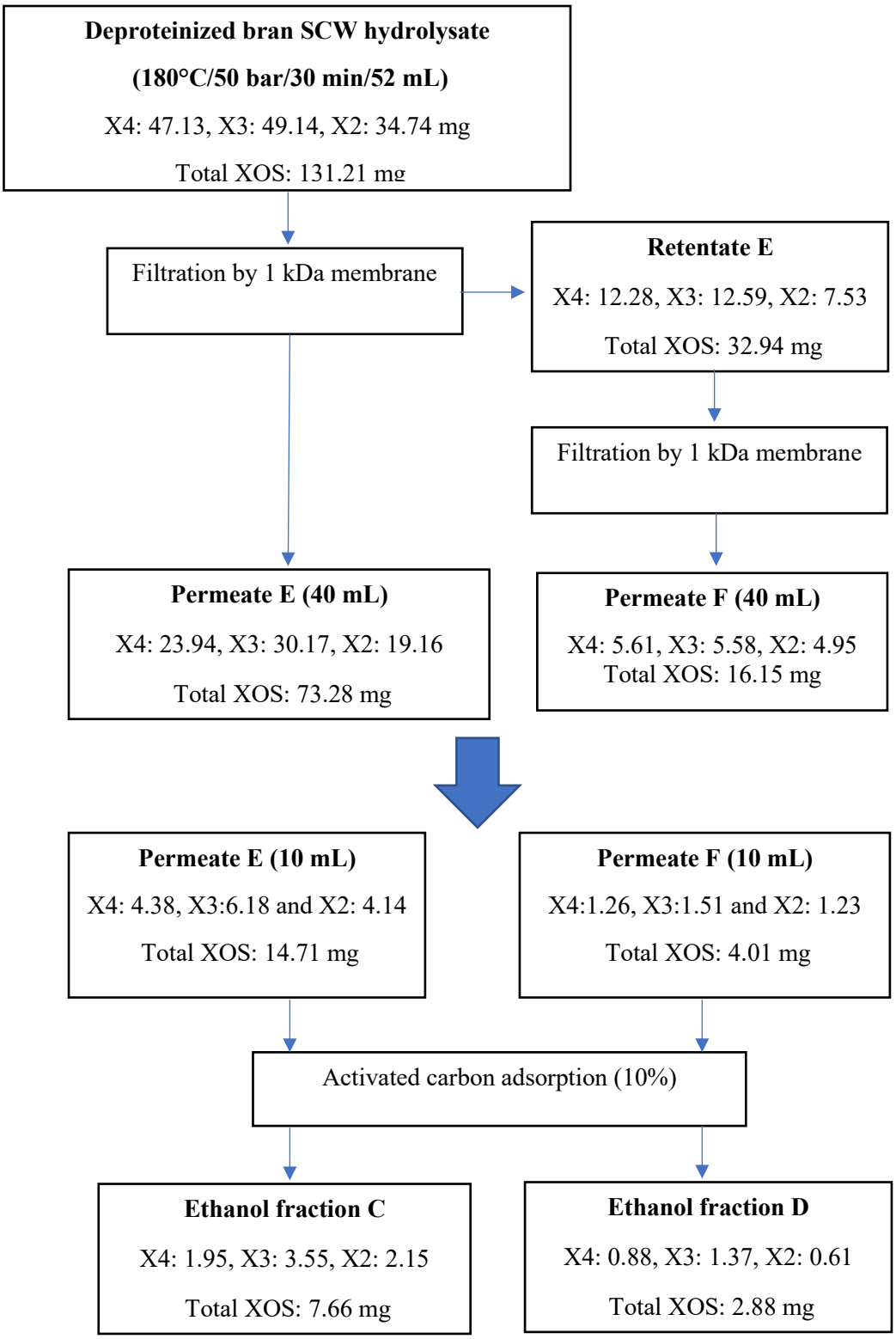


Fig. 4.13. Overall scheme of sequential purification of deproteinized bran SCW hydrolysate using ultrafiltration followed by activated carbon adsorption.

4.4. Conclusions

The SCW hydrolysates obtained at 180°C, 50 bar and 30 min from defatted-destarched bran and deproteinized bran had the highest amount of XOS with a range of DP 2-6, which can have prebiotic activity. However, impurities, such as compounds with high molecular weight derived from other unhydrolyzed polysaccharides such as hemicellulose and lignin were also present in the mixture. Therefore, further processing was needed to purify the XOS mixture and obtain a fraction with the desired molecular weight.

First, ultrafiltration using membranes of 3 and 1 kDa at room temperature and 1 bar were used for the purification of SCW hydrolysate obtained from defatted-destarched bran. Using the 3 kDa membrane, 45.63% of total XOS was recovered in the permeate B, while 52.80% was obtained in permeate A from 1 kDa membrane. Filtration was continued to recover the rest of XOS in retentate A of 1 kDa membrane for two more times where 10.77% of XOS was collected in permeate C. After the third ultrafiltration step, trace amounts of XOS were found in the permeate D and retentate D.

Ultrafiltration using the 1 kDa membrane was performed three times for the SCW hydrolysate of deproteinized bran obtained at 180°C/15 bar/30 min, leading to 131.21 mg of total XOS. Separation of high molecular weight compounds allowed to obtain 73.28 mg of total XOS in the permeate E, corresponding to 55.85% recovery. In the second permeate F, 16.15 mg of total XOS, (12.32% recovery) was recovered. In total, 68% of initial total XOS was recovered from permeates E and F.

PreticXTM XOS + xylose model solution was purified using nanofiltration and activated carbon adsorption. Total XOS was recovered in the retentate of Cynder (63.21%) and TS80 filters (76.85%). In the final ethanol

fraction obtained after 10% activated carbon adsorption (ethanol fraction B), 53.1% of total XOS was obtained. Ratio of XOS/xylose in the final ethanol fraction obtained after activated carbon adsorption (8.24) was higher compared to the retentates after nanofiltration (2.94 for TS-80 and 3.03 for Cynder).

The permeates E and F, obtained after ultrafiltration with 1 kDa membrane of deproteinized bran SCW hydrolysate, were treated by 10% activated carbon adsorption. Total XOS of 52.10%, including X2, X3 and X4 were recovered by 15 and 30% ethanol elution. In this fraction, arabinose and xylose were not completely removed, but the proportion of XOS content with respect to the monomers content increased to 2.94 compared to the 1.13 for the feed.

In conclusion, ultrafiltration using membranes with molecular weight cutoffs of 3 and 1 kDa was evaluated and results showed that it is feasible to design a purification sequence using 1 kDa membrane to purify XOS with desired DP of 2-4 in the permeate fractions with the high molecular weight impurities remaining in the retentate fractions. Further processing of permeate obtained after ultrafiltration using activated carbon adsorption was effective to remove monomers (55% xylose and 51% arabinose) after 20 mL water elution. XOS content with respect to the monomers content increased in the ethanol fraction obtained (2.94), which showed higher recovery of XOS compared to monomers.

Chapter 5: Conclusions and recommendations

5.1. Conclusions

Barley bran is rich in lipid, protein and non-starch polysaccharides, mainly arabinoxylan and beta-glucan. Barley bran is a suitable abundant by-product for the production of high value added xylooligosaccharides (XOS) with prebiotic potential in high demand as a functional food ingredient. Removal of lipid, starch and protein leads to the enrichment of arabinoxylan for further hydrolysis to obtain XOS rich extracts with less impurities. Lipid extraction using supercritical CO_2 as an environmentally friendly process avoids the use of chemical solvents.

The use of enzymatic hydrolysis mainly endo-xylanase is a conventional technique to obtain XOS. But it is time consuming and needs different pre-treatments such as alkaline extraction or steam explosion to obtain a pure xylan, which makes it more accessible for the enzyme activity. As an alternative, subcritical water (SCW) treatment is a quick technique, using water at high temperatures of 100-374°C and pressure to maintain it in the liquid state.

In this thesis research, barley bran with 9.4% lipid content was investigated for the possibility of XOS production using a sequential SC-CO_2, and SCW processing followed by further purification using ultrafiltration and activated carbon adsorption. For this purpose, lipid extraction using SC-CO_2 was performed at different conditions of 200 bar/40°C, 300 bar/40°C and 300 bar/70°C for 30, 60, 90 and 120 min within a 10 mL vessel. Both temperature and pressure had significant effect

on the yield of lipid extracted and the optimum condition was 300 bar/70°C, at which 94.27% of the initial lipid was extracted in 30 min. Using a bigger scale (300 mL) extraction vessel, scale up was studied and after 4 h extraction at the same solvent-to-feed ratio of 15.7, the lipid recovery was 92%. Using higher flowrates, the same lipid recovery can be obtained at shorter time. For example, with a flow rate of 2.4 L/min at ambient condition, (CO_2 density at ambient condition is 0.00178 g/mL, NIST Chemistry WebBook), 640.8 g of CO_2 is used after 2.5 h extraction and the same lipid recovery (92%) could be obtained if lipid has the same solubility.

After lipid extraction, defatted bran with 0.35% lipid, 12% starch and 17.9% arabinoxylan was used for starch removal using an enzymatic approach with a combination of heat stable alpha-amylase and amyloglucosidase, targeting starch hydrolysis and further precipitation of non-starch polysaccharides with ethanol. This process was effective to reduce the starch content in the final destarched bran to 1%, meanwhile enrichment of arabinoxylan to 26.3% was obtained. SCW was then used to study the process parameters such as temperature (120-200ºC) and time (15-60 min) with constant pressure of 50 bar and flow rate of 5 mL/min for the hydrolysis of destarched bran to obtain XOS in the liquid extracts. Reducing sugar yield increased with elevated temperature from 120 (1%) to 180ºC (10%), indicating the hydrolysis of polysaccharides and formation of smaller compounds with reducing ends due to water auto-ionization and releasing of hydronium ions. The maximum reducing sugar yield of 10% g xylose equivalent/g bran was achieved at 180ºC/30 min.

Temperature had a significant effect on the production of XOS. At lower temperatures of 120 and 140ºC XOS (DP 2-4) was not detected and liquid extracts had high molecular weight polymers. Generation of XOS started at 160ºC within 30 min residence time, where 12.5 mg of xylotetraose (DP4) was obtained. At the optimal condition of 180ºC xylobiose (DP2), xylotriose (DP3) and xylotetraose (DP4) were identified. Extraction time was also effective, so that xylobiose content obtained increased from 8 to 40 and 11.2 mg after 15, 30 and 60 min. Xylotriose content increased from 14.9 to 46.1 and 55.4 mg after 15, 30 and 60 min. For xylotetraose, 7.7, 26.2 and 46.7 mg were obtained after 15, 30 and 60 min. It was demonstrated that by increasing time from 15 to 30 min production of all three types of XOS improved. After 60 min extraction, the contents of X4 and X3 increased, while X2 content reduced due to the possibility of shorter oligomers production such as xylobiose at the beginning of the semi-continuous hydrolysis by liberation of acetic acid from acetylated hemicellulose, followed by breaking of non-acetylated hemicelluloses and production of longer oligomers by further hydrolysis in longer reaction time (30-60 min).

For the extracts obtained at 200ºC, reduction of XOS was observed by detecting only trace amounts of X4, X3 and X2, that was confirmed by decreasing the yield of reducing sugars to 4.11, 8.39 and 9.7% for 15, 30 and 60 min, respectively. Within 15 min, generation of degradation products, including acetic acid (10.83 mg) and lactic acid (70.32 mg) probably was responsible for the reduction of reducing sugars and XOS at 200ºC.

Destarched bran with 31.2% protein was treated using NaOH solution, that led to the reduction of protein to 4.62% and increased arabinoxylan concentration to 42.24% db in the final dried deproteinized bran.

Enzymatic hydrolysis of deproteinized bran was performed using endo beta-xylanase with two enzyme concentrations of 2.5 and 10 U and four reaction times (1, 4, 8 and 16 h) at a constant temperature of 40ºC and pH of 4.5. Maximum amount of total XOS (21.11 mg) was produced using 10 U of enzyme after 4 h hydrolysis, and no significant change was observed after 8 and 16 h incubation.

To compare SCW and enzymatic hydrolysis, deproteinized bran was also used for the SCW hydrolysis at 180ºC, 30 min, 5 mL/min and 50 bar, which was the best condition to obtain the highest amount of XOS from destarched bran. As a result, 100.9, 120.6, 112.4 and 334 mg of X2, X3, X4 and total XOS were obtained, respectively, which were higher than the maximum amount of XOS (21.11 mg) produced by the enzymatic hydrolysis. The reason why SCW produced more XOS was related to its ability to break down the side chains of xylan like arabinose with release of 102.8 ± 6.55 mg of this monomer in the liquid extract, while in the enzymatic hydrolysates, arabinose was not detected. Separation of theses side groups makes the xylan backbone more accessible for SCW hydrolysis leading to more XOS.

Purification of the SCW hydrolysates obtained at 180ºC/50 bar/30 min was necessary to remove high molecular weight compounds and monomers, targeting recovery of X2, X3 and X4. For this purpose, ultrafiltration with 3 and 1 kDa molecular weight cutoff membranes were used to remove high molecular weight compounds such as unhydrolyzed polysaccharides from SCW hydrolysate (180/30 min/50 bar/5 mL/min) from destarched bran. The peak related to the high molecular weight compounds disappeared in the final permeates (A and B) obtained using both membranes, but the recovery of total XOS was more in the permeate A obtained after passing through the 1 kDa membrane. SCW hydrolysate obtained from deproteinized bran, which had the highest amount of XOS was filtered using 1 kDa membrane, led to the recovery of 55.85 and 12.32% of total XOS in the permeate E and F, respectively. Permeates E and F were treated with activated carbon for the adsorption of XOS and elution with ethanol. Monomers (arabinose and xylose) that had less adsorption on the activated carbon were removed using elution with water. After activated carbon treatment of permeate E, ethanol fraction C had 52.1% of XOS (of initial XOS), 30.42% of xylose and 16.46% of arabinose. Also, the ratio of XOS with respect to the amount of monomers (arabinose + xylose) increased to 2.94 in the ethanol fraction C compared to the ratio in the initial feed permeate E of 1.13, indicating partial separation of monomers from XOS.

Overall, throughout the research presented in this thesis, the findings are promising for the utilization of barley bran to obtain high value-added products, including lipids, protein and fiber rich fractions. Extracted lipid, rich in tocols and phytosterols, is a suitable ingredient for the

functional foods. Isolated protein is an important ingredient in the food industry with emulsifying and foaming properties. XOS as a low calorie sweetener with prebiotic effect can be obtained from fiber rich fraction by SCW processing, which is an effective green technology. Membrane filtration is a promising technique to purify XOS with a desirable DP range for utilization as a prebiotic in the food industry.

5.2 Recommendations and future work

Some recommendations to advance this research are:

- Identification of XOS substituents attached to the sugar chains, such as acetyl groups, arabinose and glucose can be performed using electrospray tandem mass spectrometry (ESI-MS) or proton nuclear magnetic resonance (1H-NMR).

- Quantification of solubilized lignin in the SCW extracts is recommended to better understand hydrolysate composition.

- Ultrafiltration was performed only at a pressure of 1 bar, which was not sufficient to recover more than 50% of XOS in the first permeate. Therefore, increasing pressure up to 4 bar might be effective to obtain a higher content of XOS in the first permeate, avoiding the use of one more ultrafiltration process to recover more XOS in the second permeate. Because permeate flux increases with elevating pressure, this might help to recover more XOS in the first permeate.

- Nanofiltration using a dead-end system with a continuous stirring could be more effective to purify the XOS mixture from monomers because accumulation of the materials on the surface of the membrane decreases the permeate flux due to clogging. Continuous stirring prevents clogging and might improve the filtration efficiency.

- To use XOS in various applications, the final XOS should be dried by spray and freeze drying.

- The final purified XOS mixture should be investigated in terms of its prebiotic effect *in vitro* and *in vivo.* An *in vitro* study can be performed using gastrointestinal microbiota inoculation with a XOS solution and production of metabolites such as acetic, butyric and propionic acids could indicate its prebiotic efficacy. *In vivo* studies could be first carried out with rodents, and finally based on these results used for human consumption.

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Appendix A

Calibration curves

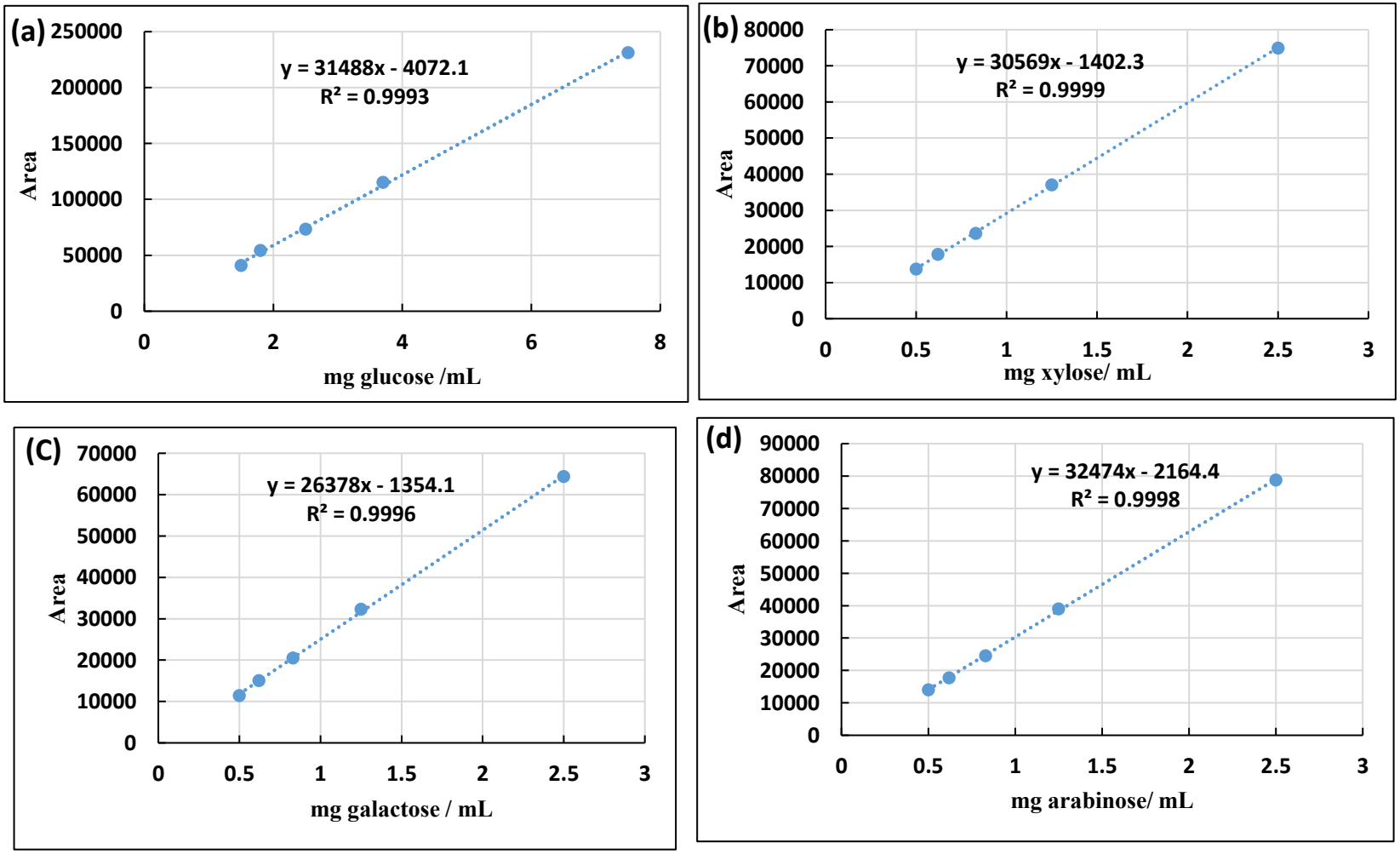


Figure A1. HPLC calibration curves of sugars: (a) glucose, (b) xylose, (c) galactose, (d) arabinose.

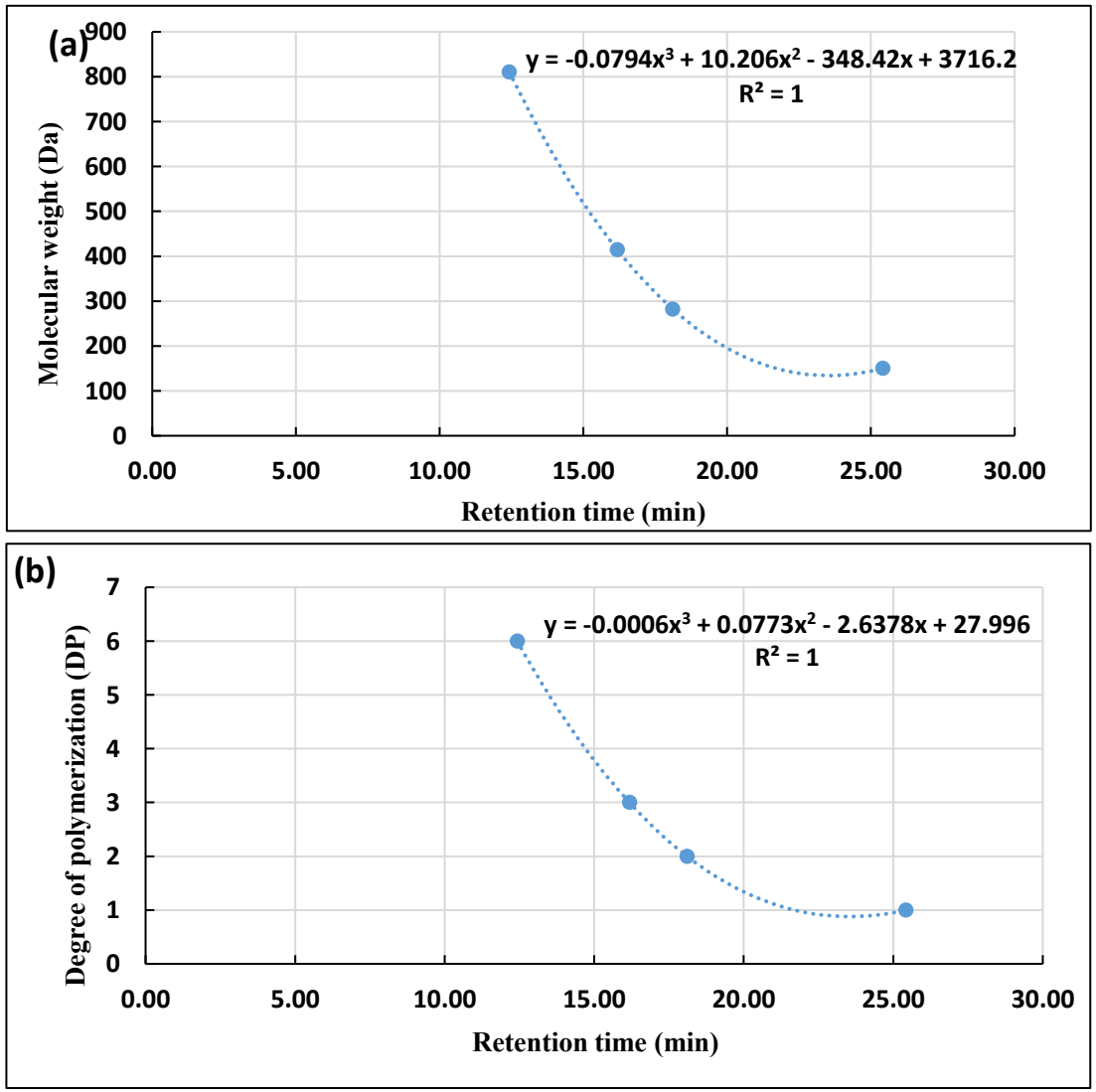


Figure A2. HPLC calibration curves of (a) molecular weight, (b) degree of polymerization of XOS.

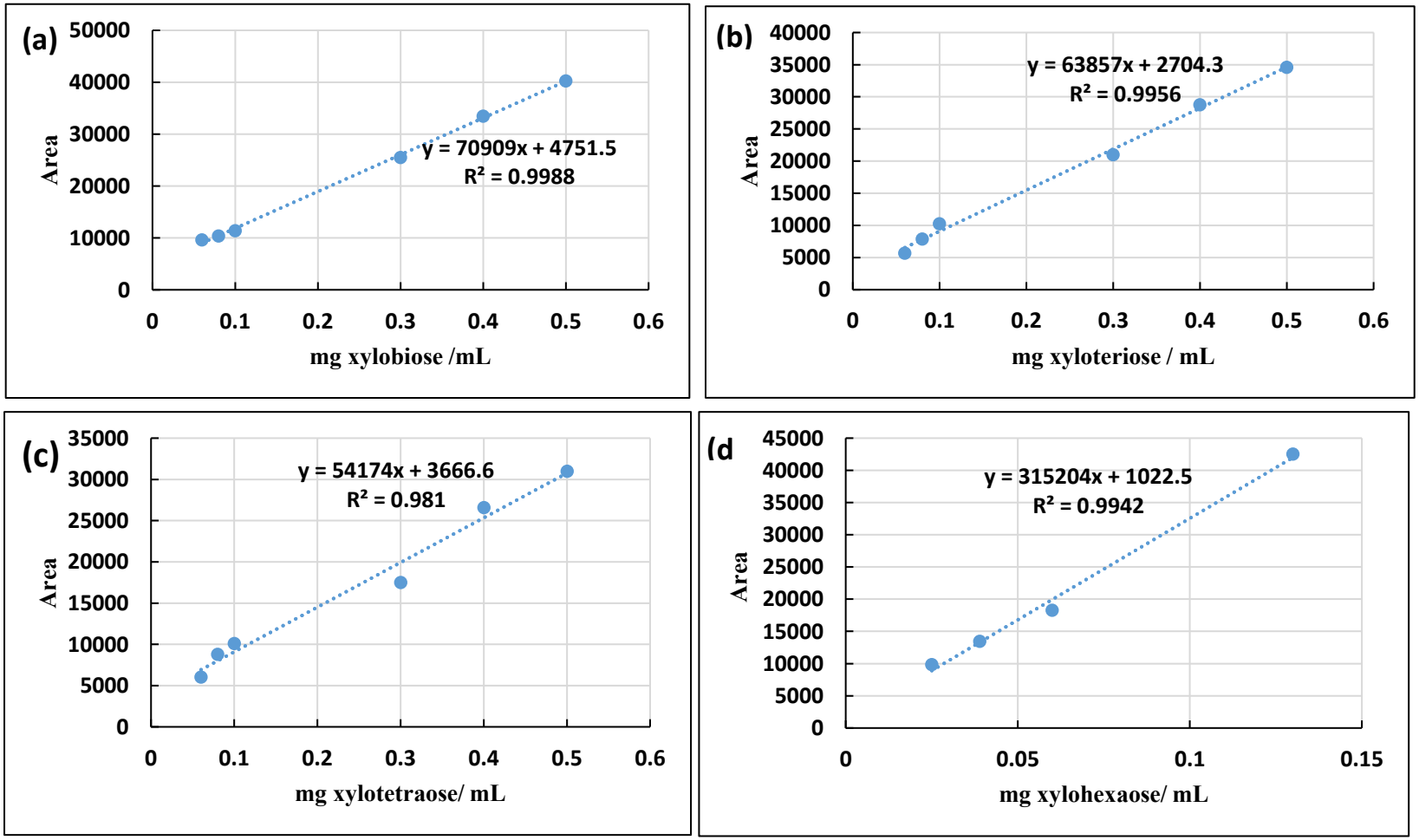


Figure A3. HPLC calibration curves of XOS: (a) xylobiose, (b) xylotriose, (c) xylotetraose, (d) xylohexaose.

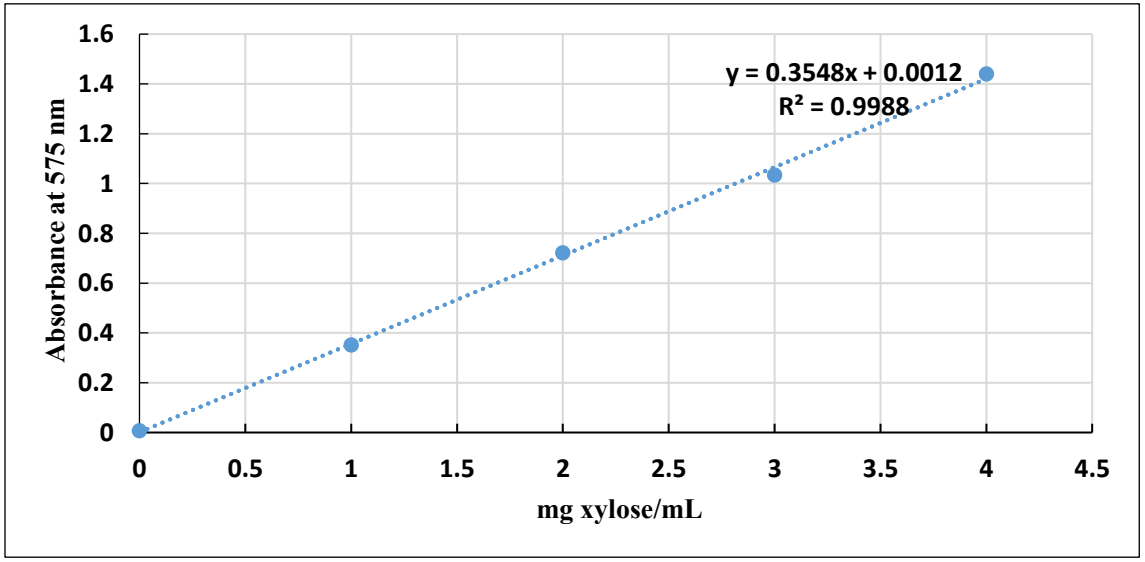
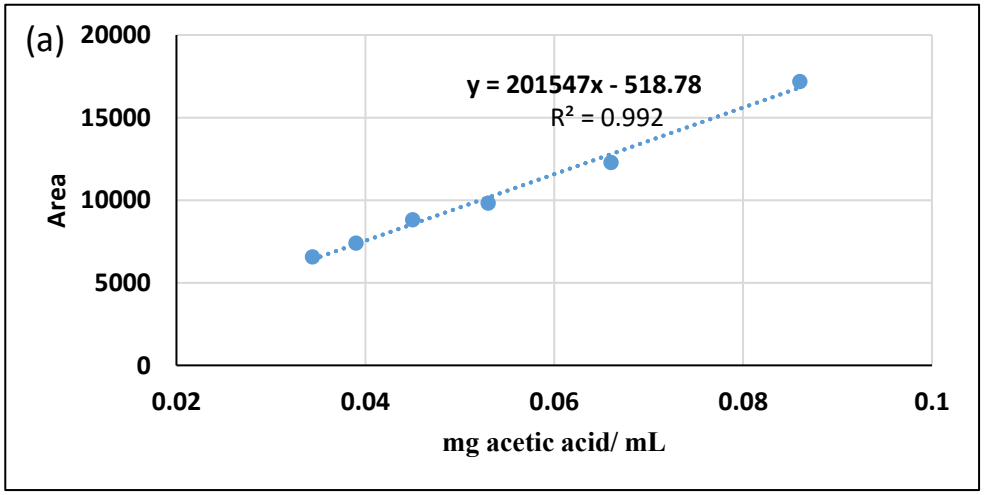


Figure A4. Xylose calibration curve to determine reducing sugar by spectrophotometer.



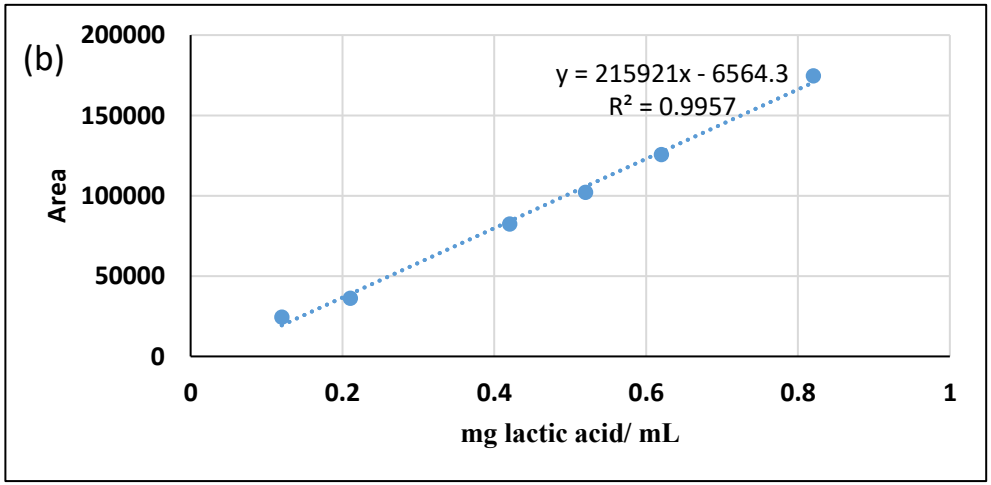


Figure A5. HPLC calibration curves of acids: (a) acetic acid, (b) lactic acid.

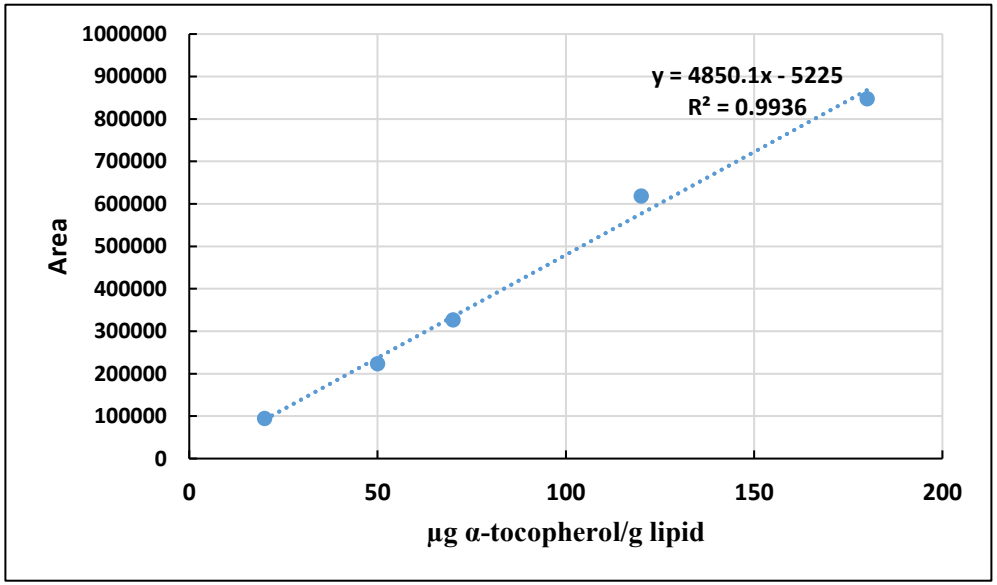


Figure A6. HPLC calibration curve of α-tocopherol.

Appendix B. XOS production using SCW and enzymatic hydrolysis.

Table B1. Moisture content of raw bran and different fractions obtained.

Sample	Replicate	X (g)	Y (g)	Moisture content (%)	Average moisture content (%)
Raw bran	1	2.1725	2.0519	5.55	5.38 ± 0.14[a]
	2	2.0710	1.9608	5.32	
	3	2.0333	1.9256	5.29	
Defatted bran	1	2.0772	2.0195	2.77	3.02 ± 0.22[b]
	2	2.0301	1.9667	3.12	
	3	2.2829	2.2101	3.18	
Defatted-destarched bran	1	2.0054	1.9500	2.76	2.70 ± 0.07[b]
	2	2.1237	2.0674	2.65	
Deproteinized bran	1	2.0564	1.9977	2.85	2.76 ± 0.12[b]
	2	2.0658	2.0106	2.67	

X: weight of sample before drying and Y: weight of sample after drying.

Table B2. Protein content of raw bran and different fractions obtained.

Sample	Replicate	Sample weight (g)	Nitrogen (%)	Protein factor	Protein content (%)	Average protein content (%)
Raw bran	1	0.106	4.0832	6.25	25.52	25.71 ± 0.3^{c}
	2	0.105	4.0726	6.25	25.45	
	3	0.108	4.1824	6.25	26.14	
Defatted bran	1	0.110	4.6032	6.25	28.77	28.60 ± 0.11^{b}
	2	0.103	4.5776	6.25	28.61	
Defatted-destarched bran	1	0.0045	4.9801	6.25	31.12	31.28 ± 0.67^{a}
	2	0.0052	5.0300	6.25	31.43	
Deproteinized bran	1	0.0085	0.7816	6.25	4.88	4.51 ± 0.59^{d}
	2	0.0078	0.7977	6.25	4.98	
	3	0.0058	0.6525	6.25	4.07	
	4	0.0072	0.6580	6.25	4.11	

Table B3. Ash content of raw bran and different fractions obtained.

Sample	Replicate	Sample weight (g)	X	Z	Y	Ash content (%)	Average ash content (%)
Raw bran	1	1.0003	20.3971	21.3533	20.3530	4.40	4.14 ± 0.24^{b}
	2	1.0029	17.5621	18.5256	17.5227	3.92	
	3	1.0084	19.2047	20.1716	19.1632	4.11	
Defatted bran	1	1.0089	9.0121	9.9611	8.9522	5.93	5.96 ± 0.04^{a}
	2	1.0143	9.6306	10.584	9.5697	6.00	
Defatted- destarched bran	1	1.0462	9.0228	9.9978	8.9516	6.81	6.98 ± 0.25^{a}
	2	1.0654	17.5810	18.5701	17.5047	7.16	

X: weight of crucible + ash, Z: weight of crucible + sample, Y: weight of crucible

Table B4. Lipid content of raw bran and different fractions obtained.

Sample	Replicate	Sample weight (g)	Y	X	Lipid content (%)	Average lipid content (%)
Raw bran	1	2.0092	61.3232	61.5132	9.45	9.46 ± 0.27[a]
	2	2.0015	61.0877	61.2829	9.75	
	3	2.0630	57.5708	57.7606	9.20	
Defatted bran	1	2.1310	61.2820	61.2887	0.31	0.35 ± 0.05[b]
	2	2.2100	58.3459	58.3531	0.35	
	3	2.1210	61.2312	61.2401	0.41	

X: weight of beaker + lipid and Y: weight of beaker

Table B5. Starch content of raw bran and different fractions obtained.

Sample	Replicate	Sample weight (g)	ABS 1	ABS 2	ABS (D-glucose standard)	Average ABS (D-glucose standard)	F	Starch content (%)	Average starch content (%)
Raw bran	1	0.115	0.150	0.135	1.065 1.136 1.127	0.01093	90.144	10.0095	10.50 ± 0.68[a]
	2	0.112	0.157	0.147				10.9811	
Defatted bran	1	0.116	0.169	0.169				11.7489	12.07 ± 0.45[a]
	2	0.107	0.164	0.165				12.3917	
Defatted-destarched bran	1	0.100	0.012	0.011				0.9333	1.04 ± 0.14[b]
	2	0.100	0.014	0.014				1.1387	

F: $\frac{100\ (\mu g\ of\ D-glucose)}{Absorbance\ for\ 100\ \mu g\ of\ D-glucose\ standard}$ and ABS: Absorbance at 510 nm.

Table B6. Acid soluble and insoluble lignin contents of different bran fractions obtained.

Sample	Replicate	W (g)	Y	X	Z	Ash (g)	P (g)	ODW	ABS 1	ABS 2	D	AIL (%)	ASL (%)	ASL+AIL (%)	Average ASL+AIL (%)
Defatted bran	1	0.305	26.11	26.13	ND	0.0003	0.005	0.29	0.814	0.906	2	6.72	1.63	8.36	8.13±0.32[a]
	2	0.304	26.15	26.18	26.15	0.0003	0.005	0.29	0.798	0.832	2	6.37	1.52	7.89	
Defatted - destarched bran	1	0.305	28.05	28.08	28.05	0.0006	0.006	0.29	0.769	0.763	2.5	5.74	1.81	7.55	7.17±0.53[a]
	2	0.304	28.71	28.74	ND	0.0006	0.007	0.29	0.798	0.778	2.5	6.23	1.87	8.09	
	3	0.303	25.53	25.55	ND	0.0006	0.006	0.29	ND	ND	-	4.93	-	-	
Deproteinized bran	1	0.302	26.10	26.11	ND	0.0008	ND	0.29	0.760	0.755	1	0.83	0.72	1.56	1.90±0.37[b]
	2	0.300	28.05	28.05	28.05	0.0008	ND	0.29	0.793	0.792	1	0.95	0.76	1.72	
	3	0.305	28.71	28.72	28.71	0.0006	ND	0.29	0.795	0.813	1	1.54	0.76	2.31	
	4	0.306	25.53	25.53	ND	0.0006	ND	0.29	0.797	0.814	1	1.27	0.76	2.03	

W: sample weight, X: weight of crucible + dry residue, Y: weight of crucible, Z: weight of crucible + ash, P: weight of protein, ODW = [(weight of sample (g) × total solids (%))/100], D: dilution, AIL: acid insoluble lignin, ASL: acid soluble lignin, ABS: Absorbance at 320 nm, ND: not detected.

Table B7. Glucan content of different bran fractions obtained.

Sample	Replicate	Sample weight	Glucose (mg/mL)	Glucose (mg/mL)	ODW	Anhydro correction	Average glucan (%)
Defatted bran	1	0.305	1.138	1.098	0.2966	0.9	29.40± 0.61
	2	0.304	1.090	1.130	0.2950	0.9	
Defatted -destarched bran	1	0.305	0.728	0.729	0.2966	0.9	19.17± 0.90
	2	0.304	0.666	0.693	0.2950	0.9	
Deproteinized bran	1	0.302	0.057	0.052	0.2937	0.9	16.35± 1.18
	2	0.300	0.062	0.060	0.2923	0.9	
	3	0.305	0.060	0.066	0.2965	0.9	
	4	0.306	0.069	0.064	0.2975	0.9	

Table B8. Xylan content of different bran fractions obtained.

Sample	Replicate	Sample weight	Xylose (mg/mL)	Xylose (mg/mL)	ODW	Anhydro correction	Average xylan (%)
Defatted bran	1	0.305	0.317	0.323	0.2966	0.8	8.36± 0.17
	2	0.304	0.322	0.332	0.2950	0.8	
Defatted - destarched bran	1	0.305	0.519	0.494	0.2966	0.8	12.83± 0.35
	2	0.304	0.483	0.495	0.2950	0.8	
Deproteinized bran	1	0.302	0.782	0.742	0.2937	0.8	20.14± 1.61
	2	0.300	0.670	0.723	0.2923	0.8	
	3	0.305	0.854	0.827	0.2965	0.8	
	4	0.306	0.804	0.817	0.2975	0.8	

Table B9. Arabinan content of different bran fractions obtained**.**

Sample	Replicate	Sample weight	Arabinose (mg/mL)	Arabinose (mg/mL)	ODW	Anhydro correction	Average arabinan (%)
Defatted bran	1	0.305	0.357	0.353	0.2966	0.8	9.55± 0.48
	2	0.304	0.379	0.390	0.2950	0.8	
Defatted - destarched bran	1	0.305	0.554	0.532	0.2966	0.8	13.47± 0.38
	2	0.304	0.500	0.503	0.2950	0.8	
Deproteinized bran	1	0.302	0.798	0.746	0.2937	0.8	21.12± 1.37
	2	0.300	0.794	0.756	0.2923	0.8	
	3	0.305	0.821	0.870	0.2965	0.8	
	4	0.306	0.853	0.854	0.2975	0.8	

Table B10. Reducing sugar of SCW hydrolysates collected at 50 bar and 0.5 mL/min.

Temperature (°C)	Time (min)	ABS 1	ABS 2	mg xylose/mL	mg xylose/mL	mg xylose	mg xylose	Average yield (g/g bran wt%)	Average yield (g/g bran wt%)
120	15	0.109	0.104	0.303	0.289	22.78	21.73	1.11	1.16 ± 0.07^{g}
	15	0.116	0.117	0.323	0.326	24.26	24.47	1.21	
	30	0.046	0.040	0.126	0.109	18.94	16.40	0.88	0.96 ± 0.11^{g}
	30	0.049	0.052	0.134	0.143	20.20	21.47	1.04	
	60	0.050	0.054	0.137	0.148	41.26	44.64	2.14	2.05 ± 0.13^{efg}
	60	0.052	0.043	0.143	0.117	42.95	35.34	1.95	
140	15	0.201	0.195	0.563	0.546	42.23	40.96	2.08	2.06 ± 0.01^{fg}
	15	0.185	0.207	0.518	0.580	38.85	43.50	2.05	
	30	0.092	0.106	0.255	0.295	38.38	44.30	2.06	2.03 ± 0.05^{efg}
	30	0.088	0.103	0.244	0.286	36.69	43.03	1.99	
	60	0.096	0.102	0.267	0.284	80.15	85.23	4.13	3.85 ± 0.38^{def}
	60	0.088	0.084	0.233	0.244	73.39	70.01	3.58	
160	15	0.206	0.212	0.577	0.594	43.29	44.56	2.19	2.63 ± 0.60^{defg}
	15	0.285	0.299	0.799	0.839	59.99	62.95	3.07	
	30	0.194	0.182	0.543	0.509	81.51	76.43	3.94	4.43 ± 0.68^{d}
	30	0.225	0.243	0.630	0.681	94.61	102.2	4.92	
	60	0.174	0.176	0.487	0.492	146.1	147.8	7.34	6.99 ± 0.49^{c}
	60	0.162	0.155	0.453	0.433	135.9	130.0	6.65	

180	15	0.715	0.765	2.012	2.152	150.8	161.4	7.81	7.28 ± 0.74^{c}
	15	0.605	0.675	1.702	1.899	127.6	141.7	6.75	
	30	0.445	0.530	1.250	1.490	187.7	223.5	10.28	10.09 ± 0.90^{ab}
	30	0.475	0.465	1.335	1.307	200.2	196.0	9.91	
	60	0.266	0.280	0.616	0.785	223.8	235.5	11.47	12.17 ± 0.98^{a}
	60	0.295	0.318	0.828	0.757	248.4	268.0	12.87	
200	15	0.413	0.406	1.160	1.140	87.04	85.56	4.31	4.11 ± 0.28^{de}
	15	0.391	0.353	1.098	0.991	82.39	74.36	3.91	
	30	0.408	0.426	1.146	1.197	171.9	179.5	8.78	8.39 ± 0.56^{bc}
	30	0.388	0.370	1.090	1.039	163.5	155.9	7.99	
	60	0.253	0.233	0.709	0.653	212.9	195.99	10.23	9.70 ± 0.74^{b}
	60	0.225	0.211	0.630	0.591	189.2	177.39	9.17	

[a-g] Different letters indicate significant differences ($p < 0.05$).

Table B11. Reducing sugar of enzymatic hydrolysates using 2.5 U and 10 U xylanase.

Enzyme unit (U)	Time (h)	ABS 1	ABS 2	mg xylose/mL	mg xylose/mL	mg xylose	mg xylose	Average yield (g/g bran wt %)	Average yield (g/g bran wt %)
Control	-	0.018	0.023	0.047	0.061	0.47	0.61	-	0.24 ± 0.04
2.5	1	0.382	0.393	1.07	1.10	10.73	11.04	4.94	4.78 ± 0.23^{c}
	1	0.359	0.365	1.00	1.02	10.08	10.25	4.62	
	4	0.432	0.458	1.21	1.28	12.14	12.87	5.68	5.82 ± 0.19^{b}
	4	0.463	0.471	1.30	1.32	13.01	13.24	5.96	
	8	0.468	0.453	1.31	1.27	13.15	12.73	5.88	5.82 ± 0.08^{b}
	8	0.447	0.456	1.25	1.28	12.56	12.81	5.76	
	16	0.452	0.461	1.27	1.29	12.70	12.95	5.83	5.94 ± 0.16^{b}
	16	0.471	0.478	1.32	1.34	13.24	13.43	6.06	
10	1	0.392	0.398	1.10	1.11	11.01	11.18	5.04	4.99 ± 0.07^{c}
	1	0.385	0.389	1.08	1.09	10.81	10.93	4.94	
	4	0.519	0.519	1.45	1.45	14.59	14.59	6.63	6.61 ± 0.03^{a}
	4	0.506	0.525	1.42	1.47	14.22	14.76	6.58	
	8	0.495	0.530	1.39	1.49	13.91	14.90	6.55	6.58 ± 0.05^{a}
	8	0.517	0.520	1.45	1.46	14.53	14.62	6.62	
	16	0.520	0.522	1.46	1.46	14.62	14.67	6.65	6.66 ± 0.00^{a}
	16	0.521	0.523	1.46	1.47	14.67	14.70	6.67	

ABS:Absorbance at 575 nm, $^{a\text{-}c}$ Different letters indicate significant differences ($p < 0.05$).

Table B12. Lipid extraction using SC-CO_2 within a 10 mL vessel.

Temperature (°C)	Pressure (bar)	Time (min)	Sample weight (g)	X (g)	Y (g)	Z (g)	W (g)	Average cumulative amount of lipid (g)
40	**200**	30	3.0118	34.4624	34.6564	0.194	0.194	0.19 ± 00^{c}
		30	3.0415	34.4567	34.6427	0.186	0.186	
		60	3.0118	35.8721	35.9081	0.036	0.230	0.228 ± 002^{b}
		60	3.0415	34.4648	34.5048	0.04	0.226	
		90	3.0118	34.4264	34.4301	0.003	0.233	0.232 ± 002^{b}
		90	3.0415	35.7737	35.7782	0.004	0.230	
		120	3.0118	34.6228	34.6245	0.001	0.235	0.233 ± 002^{ab}
		120	3.0415	34.4442	34.4456	0.001	0.231	
40	**300**	30	3.0196	35.888	36.1366	0.248	0.248	0.243 ± 007^{ab}
		30	3.0118	34.4116	34.6500	0.238	0.238	
		60	3.0196	34.8711	34.8721	0.001	0.249	0.245 ± 006^{ab}
		60	3.0118	35.888	35.8900	0.002	0.240	
		90	3.0196	34.4301	34.4301	0.000	0.249	0.248 ± 000^{a}
		90	3.0118	36.2221	36.2300	0.007	0.248	
		120	3.0196	35.7775	35.7775	0.000	0.249	0.250 ± 000^{a}
		120	3.0118	34.4575	34.4600	0.002	0.250	

[a-c,] Different letters indicate significant differences ($p < 0.05$).

Table B12. Continued

Temperature (°C)	Pressure (bar)	Time (min)	Sample weight (g)	X (g)	Y (g)	Z (g)	W (g)	Average cumulative amount of lipid (g)
40	**300**	30	3.0196	35.8880	36.1366	0.248	0.248	0.243 ± 007^{B}
		30	3.0118	34.4116	34.6500	0.238	0.238	
		60	3.0196	34.8711	34.8721	0.001	0.249	0.245 ± 006^{B}
		60	3.0118	35.888	35.8900	0.002	0.240	
		90	3.0196	34.4301	34.4301	0.000	0.249	0.248 ± 000^{B}
		90	3.0118	36.2221	36.2300	0.007	0.248	
		120	3.0196	35.7775	35.7775	0.000	0.249	0.250 ± 000^{B}
		120	3.0118	34.4575	34.4600	0.002	0.250	
70	**300**	30	3.0229	34.8700	35.135	0.265	0.265	0.267 ± 003^{A}
		30	3.0300	35.7752	36.0453	0.270	0.270	
		60	3.0229	34.6248	34.6316	0.006	0.271	0.275 ± 004^{A}
		60	3.0300	34.8710	34.8794	0.008	0.278	
		90	3.0229	35.8884	35.8904	0.002	0.273	0.276 ± 003^{A}
		90	3.0300	34.4617	34.4626	0.000	0.279	
		120	3.0229	34.3945	34.4010	0.006	0.280	0.279 ± 0.00^{A}
		120	3.0300	35.8897	35.8897	0.000	0.279	

X: weight of empty tube, Y: weight of tube + lipid, Z: weight of lipid extracted, W: weight of cumulative amount of lipid.

$^{A-B}$ Different letters indicate significant differences ($p < 0.05$).

APPENDIX C. XOS purification using filtration and adsorption.

Table C.1. XOS content and recovery after ultrafiltration of SCW defatted-destarched bran hydrolysate by 3 and 1 kDa membranes.

Membrane			**XOS content (mg)**				**XOS recovery (% total initial XOS)**			
Molecular weight cut off (kDa)	**Fraction**	**DP > 12 (area)**	**Xylotetraose**	**Xylotriose**	**Xylobiose**	**Total XOS**	**Xylotetraose**	**Xylotriose**	**Xylobiose**	**Total XOS**
3	**Permeate B**	8254.5	4.51±0.25[a]	6.70±0.57[a]	5.44±0.57[a]	16.66±0.25[a]	12.36±0.69	18.35±1.57	14.9±1.56	45.63±1.33
	Retentate B	190936	3.26±0.38	3.21±0.10	1.20±0.24	7.68±0.24	8.94±1.04	8.81±0.29	3.30±0.66	21.05±0.66
1	**Permeate A**	5584	4.02±0.11[a]	8.37±0.41[a]	6.87±0.10[a]	19.27±0.9[b]	11.02±0.30	22.93±1.13	18.83±0.29	52.79±0.54
	Retentate A	184522	2.74±0.02	2.92±0.51	1.23±0.12	6.90±0.66	7.51±0.03	8.01±0.71	3.93±0.17	18.92±0.91
	Permeate C	6044	1.15±0.00	1.83±0.09	0.94±0.03	3.93±0.04	3.15±0.01	5.01±0.24	2.60±0.10	10.77±0.12
	Retentate C	43086	0.37±0.00	0.94±0.06	0.19±0.02	1.52±0.03	1.74±0.02	2.59±0.18	0.54±0.06	4.17±0.09

DP: Degree of polymerization, and XOS: Xylo-oligosaccharides.

Table C.2. XOS content after ultrafiltration of deproteinized bran SCW hydrolysate by 1 kDa membrane.

Fraction	DP>12	Xylotetraose	Xylotriose	Xylobiose	Total XOS	Xylotetraose	Xylotriose	Xylobiose	Total XOS
Permeate E	35952	23.94±0.74	30.17±0.23	19.16±0.35	73.28±0.86	18.25±0.56	22.99±0.17	14.60±0.26	55.85±0.65
Retentate E	93727	12.82±0.15	12.59±0.25	7.53±0.12	32.94±0.53	9.77±0.12	9.59±0.19	5.73±0.09	25.10±0.40
Permeate F	22409	5.61 0.16	5.58± 0.28	4.95±0.54	16.15±0.42	4.28±0.12	4.25±0.22	3.77±0.41	12.32±0.32
Retentate F	475094	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace

DP: Degree of polymerization, XOS: Xylooligosaccharides.