Production and Utilization of Hemicelluloses from Renewable Resources for Sustainable Advanced Products

Sárossy, Zsuzsa

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Zsuzsa Sárossy
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PhD Thesis
Zsuzsa Sárossy
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Biosystems Division
Risø DTU National Laboratory for Sustainable Energy
Technical University of Denmark
Abstract

Vast amounts of by-products are generated every year from agricultural crop production and hence great quantities of polysaccharides remain underutilized. The polysaccharides from agricultural by-products can be separated and used in the form of new materials. This thesis is devoted to the possibility of using hemicelluloses for special polysaccharide film applications in the packaging sector, starting from hemicellulose isolations from a side product of agricultural processes, hemicellulose characterization and assessing material properties and the potential use of hemicellulose films in later applications.

First, water-soluble hemicelluloses of rye bran were extracted with a high-temperature treatment combined with enzymatic starch removal. After the hot water extraction, non-soluble fibers and protein fractions were separated and the washed fiber fraction was further treated with alkali (NaOH) solution with different solid to liquid ratios. The ratio of arabinoxylans (~65%) and β-glucans (~25%) was similar in the water-extracted and alkali-extracted materials; however, their arabinose/xylose (Ara/Xyl) ratio differed. The alkali-extracted arabinoxylan was less substituted with an Ara/Xyl ratio of 0.35, while the water-extracted material had an Ara/Xyl ratio of 0.54.

In order to analyse the monosaccharide composition of the isolated hemicelluloses, a method based on gas chromatography-mass spectrometry analysis of acetylated methyl glycosides was developed. The derivatives of the monosaccharides, arabinose, glucose and xylose were studied in detail using $^{13}$C-labeled analogues as internal standards for identification and quantitative analysis. The electron impact-induced fragmentation of all the studied monosaccharides was studied and quantification was made using extracted fragment ion pair (unlabeled and labeled monosaccharide fragment) intensities. Using the intensity ratios obtained from the ion chromatograms, accurate quantification of monosaccharide constituents from selected hemicelluloses was demonstrated.

The effect of co-extracted mixed linkage β-glucans on films cast from the isolated rye hemicelluloses was studied. Water-extractable mixed linkage β-glucans (BG) were isolated from oat bran using a similar process as was used for isolation of rye hemicelluloses. The β-glucan content of the rye hemicellulose isolate was reduced to less than 5% by a selective lichenase treatment. The material properties of films prepared from the rye hemicellulose isolate and lichenase-treated rye hemicellulose (WE-AX) as such, or with varying amounts of added BG (20:80; 50:50; 80:20 ratios) were studied. Prior removal of β-glucan from the isolate decreased the tensile strength of the films significantly as well as the elongation at break. Addition of BG to the purified WE-AX resulted in an increase in the tensile strength and elongation at break of the films. In contrast, the presence of BG had no clear effect on the oxygen permeability of the films. Both pure rye WE-AX and pure BG films showed excellent oxygen barrier properties (0.9-1.0 cm$^3$·μm/m$^2$·d·kPa). However, the water vapour permeability increased with addition of increasing amounts of BG to WE-AX. Considering the mechanical and barrier properties of the films, it was shown that higher β-glucan contents have positive effects when practical applications of cast arabinoxylan-β-glucan films are considered.
In order to improve the mechanical and barrier properties of rye arabinoxylan films, nanocomposite films of arabinoxylan and a fibrous nanoclay (sepiolite) were prepared. The films contained 2.5 – 10% added nanoclay and showed high transparency, especially with lower sepiolite contents (2.5 and 5%). The nanoparticles were well embedded in the arabinoxylan matrix as shown by scanning electron microscopy. FTIR (Fourier transform infrared) spectroscopy provided some evidence for hydrogen bonding between sepiolite and the arabinoxylan matrix. Mechanical testing showed greatly improved tensile strength and Young's modulus in the nanocomposite films. However, unlike layered nanoclays, addition of sepiolite fibers did not reduce the oxygen permeability of reinforced films.
Resumé


Først blev vandopløseligt hemicellulose ekstraheret fra rugklid i en høj-temperatur process kombineret med enzymatisk fjernelse af stivelse. Derefter blev de uopløselige fibre adskilt fra proteinfraktionen, hvorefter de blev vasket og yderligere behandlet med alkali (NaOH) under forskellige tørstofsforhold. Forholdet mellem arabinoxylaner (~ 65%) og β-glucaner (~ 25%) var ens i de med vand udvundne og de med alkali udvundne materialer; dog var deres arabinose/xylose forhold (Ara/Xyl) varierede. De med alkali udvundne produkter var mindre substitueret med et Ara/Xyl forhold på 0.35, mens de med vand udvundne produkter havde et Ara/Xyl forhold på 0.54.


Effekten af co-extraherede ”mixed-linkage” β-glucaner på film støbt af den isolerede rug-hemicellulose blev undersøgt. Vand-ekstraherbare ”mixed-linkage” β-glucaner (BG) blev isoleret fra havreklid ved hjælp af en prosess lignende den, der blev brugt til isolering af rug-hemicellulose. β-glucanindholdet i isolater fra rug-hemicellulose blev reduceret til mindre end 5% ved en selektiv lichenasebehandling. Materialeegenskaberne af film fremstillet fra isolater fra både rug-hemicellulose og lichenase-behandlede isolater (WE-AX) som sådan, rene eller med varierende mængder af tilsat BG (20:80, 50:50, 80:20), blev undersøgt. Fjernelse af β-glucan fra isolaterne formindskedte trækstyrken af filmene væsentligt og ligeledes forlængelsen ved brud. Tilsætning af BG til de oprensede WE-AX resulterede i en stigning i trækstyrke og forlængelse ved brud af filmene. I modsætning hertil havde tilstedevedærelsen af BG ingen klar effekt på ilt-permabiliteten. Både ren rug WE-AX og ren BG film viste fremragende iltbarriere egenskaber (0.9 til 1.0 cm³·μm/m²·d·kPa). Gennemtrængeligheden af vanddamp blev øget ved tilsætning af stigende mængder af BG til WE-AX. Ved betragtning af de mekaniske egenskaber og af
barriereegenskaber af filmene, blev det påvist, at et højere $\beta$-glucan-indhold har en positiv indvirkning, når de praktiske anvendelser af de støbte arabinoxylan-$\beta$-glucan-film er taget i betragtning.

For at forbedre de mekaniske egenskaber og barriereegenskaberne af rug-arabinoxylan-film, blev en nano-komposit-film af arabinoxylan og en fibrøs nanoclay (sepiolit) undersøgt. Filmene indeholdt fra 2.5 til 10% nanoclay og de viste høj transparenthed, især ved lavere sepiolit indhold (2.5 og 5%). Nanopartiklerne var godt forankret i arabinoxylan-matricen som vist ved scanning elektronmikroskopi. FTIR (Fourier transform infrared) spektroskopi har sandsynliggjort hydrogenbindinger mellem sepiolit og arabinoxylan-matricen. Mekanisk afprøvning viste væsentligt forbedret trækstyrke og Youngs moduli ved nano-kompositfilmen. I modsætning til lagdelte nanoclays, reducerede tilægningen af sepiolit fibre ikke ilt-permabiliteten af kompositen.
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Abbreviations

Ara/Xyl – arabinose-to-xylose ratio
Araf – arabinofuranose
AsFFFI - asymmetric flow field –flow fractionation
AEC – anion exchange chromatography
AX - arabinoxylan
BG- oat mixed-linkage β-glucan
CE – capillary electrophoresis
DMSO – dimethyl sulfoxide
ESI-MS - electrospray ionization mass spectrometry
FTIR – Fourier transform infrared
GC-FID – gas chromatography-flame ionization detection
GCMS – gas chromatography-mass spectrometry
GGM - galactoglucomannan
HPAEC-PAD – high performance anion exchange chromatography-pulsed amperometric detection
HPLC – high performance liquid chromatography
HPSEC – high performance size exclusion chromatography
LSD - least significance differences
MALDI-TOF-MS - matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MMT - montmorillonite
mPEG - poly(ethylene glycol) methyl ether
Mw – weight average molecular weight
NMR – nuclear magnetic resonance
OP – oxygen permeability
OTR – oxygen transfer rate
RAX – rye arabinoxylan
RH – relative humidity
SEM - Scanning electron microscopy
TFA - trifluoroacetic acid
WE-AX – water-extracted arabinoxylan
WEH – water-extracted hemicelluloses
WIS – water-insoluble rye fiber
WVP – water vapor permeability
WVTR – water vapour transmission rate
Xylp – xylopyranose
1. Introduction

Hemicelluloses represent a huge amount of the woody and grass biomass cell walls and have the potential to become an enormous resource for new biopolymers and biomaterials. However, these vast amounts of polysaccharides have not been exploited and the potential of hemicelluloses has not been realized and applied on an industrial scale. Xylan-type hemicelluloses are the most abundant hemicelluloses in plants (Ebringerová and Heinze, 2000).

There are plenty of agricultural by-products available with currently low value as excellent sources of xylans, such as corn husk, corncobs, sunflower hulls, brewer's grain, wheat, rye and oat bran or sugarbeet pulp (Izydorczyk and Biliaderis, 2006). As rye (*Secale cereale*) is a cereal traditionally grown in the northern and eastern regions of Europe, including Denmark, a significant amount of bran is produced every year. The rye production of Denmark is above 115 000 tons per year, most of which is used for human consumption (42%) or as animal feed or feedstock for alcohol distilling (Sahlstrom and Knutsen, 2010). Considering that roughly 20-35% of the grains are separated as bran during milling, an estimated 40 000 tons of rye bran is available for use in Denmark each year.

Xylans can be utilized as purified polysaccharides or also as relatively unpurified extracts, containing proteins, phenolic substances, other polysaccharides, which may in some cases enhance certain material properties and lower production costs (Ebringerová, et al., 2005). The isolation processes for such hemicelluloses from the mentioned agricultural by-products will necessarily involve physical fractionation of the different cell wall components. Isolation of one single fraction is not economically feasible, especially if the process would not provide high yields of the desired product. Nevertheless, by-products such as cereal brans are rich sources of not only soluble and insoluble dietary fibers but also proteins, oligosaccharides and antioxidant aromatic components. Thus, placing the isolation into a biorefinery concept could be beneficial in respect of sustainability as well as for economic reasons.

During the last decade there has been continuously increasing interest in exploiting xylan-type hemicelluloses in food packaging and other applications. Indeed, there have been a number of
reports discussing films prepared from xylans extracted from wheat bran, rye grains, barley husks, corn hulls and bran, oat spelt or aspen and beech (Gröndahl and Gatenholm, 2007, Mikkonen, et al., 2009, Sternemalm, et al., 2008, Zhang, et al., 2011, Zhang and Whistler, 2004). Films from agricultural by-products have been shown to provide good oxygen and/or grease barrier films and their application as oxygen barriers in multilayer packaging materials has also been suggested (Gröndahl and Gatenholm, 2007).

The study presented in this thesis was carried out at Risø-DTU (Risø National Laboratory for Sustainable Energy, Technical University of Denmark) and at the University of Helsinki, Finland. The PhD project was funded by the Technical University of Denmark. The aim of the work was to find suitable raw materials among different agricultural by-products for hemicellulose isolations. The processes were planning to obtain relatively pure, high molar mass hemicelluloses and to understand their structural conformation. The goal for the isolated hemicelluloses was to produce materials of later relevance as industrially useful packaging films or coatings in special applications. The film properties of isolated hemicelluloses were to be investigated as such and with additional reinforcement, and an attempt was made to understand the effect of hemicellulose purity on the material properties.

The thesis includes four papers attached to the main study. In Paper I, two different hemicellulose isolation processes from rye bran are investigated and the chemical composition and molar mass of the isolated materials are compared. In Paper II, the monosaccharide composition of the isolated hemicelluloses was analyzed in detail. Structural and film properties of the isolated hemicelluloses were investigated in Paper III, as well as the effects of β-glucans on the arabinoxylan film mechanical and barrier properties. Hemicellulose nanocomposite films were prepared in Paper IV and potentially improved mechanical and barrier properties were studied.
2. Literature review

2.1 Plant cell walls and hemicelluloses

2.1.1 Plant cell walls

The plant cell wall is a particular natural composite which has excellent material properties. These properties are due to the unique conformation of the wall structure, composed of the main cell wall constituents: cellulose, lignin and hemicelluloses (Ebringerová and Heinze, 2000). A "skeleton" structure is created in the plant cell walls to assist withstanding large tensile and compressive forces and also to be sufficiently flexible and highly load-bearing (Burton, et al., 2010). All plant cell walls have a similar basic structure, a fibrillar phase of cellulose microfibrils located in a matrix phase, which contains polysaccharides, structural proteins, glycoproteins, phenolic components and lignin (Harris and Smith, 2006). The complex polysaccharides and structural proteins link the long, crystalline cellulose ribbons in the cell walls which wind around in several layers in each cell (Carpita and Gibeaut, 1993). Cellulose, as the most common component of plant cell walls is built up of unbranched and unsubstituted (1→4)-β-D-glucose units. The molecular chains of these building units form an extended, ribbon-like conformation which allows parallel packing of the chains. These chains form non-covalent microfibrillar complexes through inter- and intramolecular hydrogen bonds and these cellulose microfibrils become reinforcing rods in the cell walls (Burton, et al., 2010, Fincher and Stone, 2004). Lignin is a polyphenolic molecule which assists the wall become resistant to compressive forces and restricts the passage of small molecules (Burton, et al., 2010).

Two major types of cell walls, primary and secondary, can be differentiated on the basis of cell wall development. Primary cell walls are deposited while the cells are actively growing, whereas secondary cell walls are placed onto the primary walls when the cells have attained their final shape and are usually thicker (Harris and Smith, 2006). Two distinct types of primary cell walls can be found in different plant sources. Dicot plants and monocots other than grasses have type I walls, while the Poaceae (grasses) and related monocot families have special, type II cell walls (Figure 1). Secondary
cell walls can be further classified into walls containing lignin and walls without lignin content (Harris and Smith, 2006).

Many cell wall models have been suggested to describe the behaviour of the cell wall components and the chemical and physical bonds and forces creating the wall structure. According to the cell wall model of McCann and Roberts (McCann and Roberts, 1991) there are non-covalent interactions between primary cell wall polysaccharides, cellulose, hemicellulose and pectin. The main principle claims that the cell wall is strengthened as there is a particular non-covalent interaction between glycans, which cross-links cellulose microfibrils (Scheller and Ulvskov, 2010). The cell wall matrix phase of polysaccharides and proteins is responsible for providing flexibility as well as some additional mechanical support for the cell but its porosity also allows the diffusion of water and other smaller molecules through the cell wall (Burton, et al., 2010). Thus hemicelluloses act as "glue" molecules that hold the microfibrils together and through interactions with other cell wall components contribute to the appropriate physical properties of the cell walls (Carpita and Gibeaut, 1993, Scheller and Ulvskov, 2010). Hemicelluloses can also play a role as seed storage polysaccharides, such as mixed-linkage β-glucans in cereal grains. The cell walls in grasses contain very different matrix polysaccharides and protein constituents than the cell walls of other flowering plants (Carpita and Gibeaut, 1993). Figure 1 illustrates a cell wall type II structure where cellulose microfibrils are embedded into a matrix phase of polysaccharides and structural proteins.

2.1.2 Hemicelluloses

The term "hemicellulose" was first suggested by Schulze for polysaccharides which were extracted from plants with dilute alkali and were similar to cellulose, but could be hydrolyzed by dilute mineral acids yielding pentose sugars in addition to hexoses (Schulze, 1891). The phrase hemicellulose is now generally used to refer to non-starch polysaccharides found in plant cell walls in connection with cellulose (Ebringerová, et al., 2005). Hemicelluloses are heteropolysaccharides and they represent up to 50% of the biomass of annual and perennial plants. While the cellulose basic chemical structure is identical in plants, the composition of hemicelluloses can vary within tissues of
the same plant (e.g., roots, stems, leaves and seeds) and between different plant species (Albertsson and Edlund, 2011).

Figure 1. Illustration of the Type II cell wall (Carpita and Gibeaut, 1993).

In addition, there can be variations in the amounts of hemicelluloses relative to other cell wall components, like cellulose (Burton, et al., 2010). In contrast to cellulose, hemicelluloses are amorphous, often branched and have different solution properties (Albertsson and Edlund, 2011). Hemicellulose chain substitution also varies, which is illustrated by the wide range of molar masses for different hemicellulose types.

Hemicelluloses are usually divided into four major groups in view of their structural conformation: D-xyloglycans (xylans), mannans, mixed-linkage β-glucans and xyloglucans (Ebringerová, et al., 2005). The monosaccharide building blocks of hemicelluloses can be pentose and hexose sugars as well as acid sugars (Figure 2). The most common hexoses are D-glucose, D-galactose and D-mannose, while
the pentoses mainly comprise D-xylose and L-arabinose. Rhamnose and fucose can also be found in certain hemicellulose types.

![Pentoses](image1)

![Hexoses](image2)

![Uronic acids](image3)

**Figure 2.** Hemicellulose building units consisting of C5 and C6 monosaccharides and acid sugars.

### 2.2 Importance and abundance of xylans

Xylans are the most widespread hemicelluloses and the second most abundant polysaccharides in plants (Ebringerová and Heinze, 2000). Xylan-type hemicelluloses can be found in several varieties in terrestrial plants and algae and they dominate in monocot plants. Table 1 illustrates the monosaccharide composition and xylan type of hemicellulosic samples from different plants.

Xylans show a great structural variety as they fulfill a variety of roles and functionalities in the cell walls of different plant tissues. Xylans are usually built up from a β-(1→4) linked Xylp
(xylopyranose) backbone chain, substituted with sugar units such as α-L-arabinofuranose and O-acetyl groups or 4-O-methyl-glucuronic acid residues, while the occurrence of homoxylns is very rare.

**Table 1.** Examples of xylan types in different plant materials and monosaccharide compositions.

<table>
<thead>
<tr>
<th>Raw material</th>
<th>Xylan type</th>
<th>Monosaccharide composition (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ryegrass leaves</td>
<td>galactoarabinoxylans, L-arabino (4-O-methyl-D-glucurono)xylans</td>
<td>Ara 19.8  Xyl 26.0  Glc 24.2  Uronic acid 9.1  Man 19.1  Gal 4.4</td>
<td>(Xu, et al., 2007)</td>
</tr>
<tr>
<td>Wheat straw</td>
<td>arabinoglucuronoxylan</td>
<td>Ara 15.1  Xyl 63.2  Glc 16.2  Uronic acid 5.5  Man 0.5  Gal 4.4</td>
<td>(Sun, et al., 2000)</td>
</tr>
<tr>
<td>Aspen wood chips</td>
<td>4-O-methyl-glucuronoxylan</td>
<td>Ara 16.7  Xyl 41.1  Glc 3.9  Uronic acid 0.5  Man 4.3</td>
<td>(Gabrielli, et al., 2000)</td>
</tr>
<tr>
<td>Rye bran</td>
<td>arabinoxylan</td>
<td>Ara 45.1  Xyl 46.1  Glc 4.5  Uronic acid 3.1  Man 4.3</td>
<td>(Ebringerová, et al., 1994)</td>
</tr>
<tr>
<td>Birch wood chips</td>
<td>4-O-methyl glucuronoxylan</td>
<td>Ara 3.2  Xyl 66.9  Glc 1.4  Uronic acid 4.2</td>
<td>(Westbye, et al., 2007)</td>
</tr>
<tr>
<td>Corn fiber</td>
<td>arabinoglucuronoxylan</td>
<td>Ara 13.7  Xyl 17.2  Glc 5.6  Uronic acid 2.2</td>
<td>(Benkő, et al., 2007)</td>
</tr>
<tr>
<td>Rice husk</td>
<td>arabinoxylan</td>
<td>Ara 1.8  Xyl 15.9  Glc 34.0  Uronic acid 2.2</td>
<td>(Vegas, et al., 2008)</td>
</tr>
<tr>
<td><em>Palmaria palmata</em></td>
<td>homoxylan</td>
<td>Ara 37.1  Xyl 3.6  Glc 2.2  Uronic acid 2.2</td>
<td>(Deniaud, et al., 2003)</td>
</tr>
<tr>
<td>(red seaweed)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a:* % (w/w) of total extracted material  
*b:* % (w/w) in original wood sample  
*c:* sugar composition is expressed as polymeric glucan, xylan and arabinan  
*d:* arabinoxylan is acetylated, acetyl groups:1.6%  

Monosaccharides: Ara-arabinose, Xyl-xylose, Glc-glucose, Man-mannose, Gal-galactose

Among the herbaceous plants, cereals like wheat, rye and barley typically have a less complex structure, containing arabinose substituents, when compared to rice, sorghum, finger millet or maize bran which contain galactopyranose or uronic acid side branches on the xylan main chain (Izydorczyk and Biliaderis, 2006). Hardwood (e.g., birch, eucalyptus, aspen) xylans can have a high content of acetylation besides the methyl-glucuronic acid residues while softwood xylans carry no acetyl substituents. Xylan is usually ester-linked to lignin through the glucuronic acid side chains in woody tissues (Ebringerová and Hromádková, 1999). Short oligosaccharide substituents can also be found in heteroxylans and, in grasses, α-arabinofuranosyl substituents can be esterified with hydroxycinnamic acids as illustrated in Figure 3 (Burton, et al., 2010).
The xylose units in arabinoxylans are substituted with arabinofuranose residues at position C3 or at both the C3 and C2 positions (Fincher and Stone, 2004), while in glucuronoxylan the methyl-glucuronic acid residues are linked to the xylose units at the C3 position. The distribution pattern of the xylan substituents was proposed to be non-random and this presumably reflects the functional diversity of xylans in plant tissues, contributing to their different solubility, enzymatic degradability and interactions with other cell wall components (Ebringerová and Heinze, 2000, Fincher and Stone, 2004).

2.3 Hemicelluloses of cereal cell walls and their biological role

Non-starch polysaccharides from cereal grain cell walls include cellulose, (1→3, 1→4)-β-D-glucans, heteroxylans, glucomannans, xyloglucans, pectic polysaccharides, and callose (Fincher and Stone, 2004). These polysaccharides are key components in the cell walls and have different functions during grain development, dormancy and also after germination. After the grain reaches its mature state, the endosperm cells are dead but they are packed with starch and storage proteins and usually have thin cell walls. In contrast, the aleurone cells are still alive at maturity, contain a high amount of
lipid droplets and protein bodies and have thick cell walls (grain components are illustrated in Figure 4). Non-cellulosic polysaccharides, especially xylans and mixed-linkage β-glucans comprise a large part of the walls of the starchy endosperm and the aleurone layer (Fincher and Stone, 2004). Cereal heteroxylans are typically arabinoxylans or glucuronoarabinoxylans. A great portion of the acidic arabinoxylans appears usually in the outer layers of the grains, in the husk and bran parts (Izydorczyk and Biliaderis, 2006). The cellulose content of the pericarp and seed coat layer are higher than that of the endosperm and can reach amounts as high as 30% by weight (Fincher and Stone, 2004).

Figure 4. Microscopic picture of the rye kernel (The Nordic Rye Group) (a) Microstructure of parts of intact rye grain (b) Protein appears red, cell walls rich in β-glucan appear light blue and lignified cell walls of the fruit coat yellowish-brown (Kamal-Eldin, et al., 2009).

The arabinose substitution frequency depends on the plant species, the cell wall type and the different grain layers and is shown by the ratio of arabinose/xylose units (Ara/Xyl). This ratio indicates the degree of branching and can vary in the range 0.1-1.1 (Fincher and Stone, 2004, Izydorczyk and Biliaderis, 2006). Glitsø and Knudsen found that rye arabinoxylans in the cell walls of the pericarp
fraction had a higher Ara/Xyl ratio (0.93) than that of the aleurone-rich fraction (0.59) (Glitsø and Bach Knudsen, 1999). In certain cases, the Ara/Xyl ratios are only slightly different; however, NMR (nuclear magnetic resonance) studies can reveal differing substitution patterns on the xylan chain. Virkki et al. found an Ara/Xyl ratio of 0.56 for high-viscosity wheat arabinoxylan while the Ara/Xyl ratio of rye arabinoxylan was 0.5 (Höije, et al., 2008, Virkki, et al., 2008). Pitkänen et al. investigated the fine structure of these hemicelluloses and found that roughly one-third of the \( \beta-D-Xylp \) (xylopyranose) residues are monosubstituted in wheat and two-thirds are disubstituted with \( \alpha-L-Araf \) (arabinofuranose) units. In rye the substitution pattern is reversed, and two-thirds of the xylose units are monosubstituted (Pitkänen, et al., 2009). Pastell et al. studied the substitution of the arabinoxylan chain in eight different cereal by-products by combining NMR and HPAEC-PAD (high performance anion exchange chromatography-pulsed amperometric detection) techniques (Pastell, et al., 2009). Arabinoxylans extracted from wheat, rye and oat brans showed similar substitution patterns, with both mono- and di- arabinose substitution and it appeared to be characteristic for bran-originated arabinoxylan (AX) to possess significant amounts of Araf doubly substituted Xylp units. Other arabinoxylans, originating from more lignified plant tissues such as rice and barley husk, oat spelt or corn cob have been found to contain mostly mono-substituted xylan chains.

The linear polysaccharides, \((1\rightarrow3), (1\rightarrow4)\) mixed-linkage \( \beta \)-glucans (henceforward referred to as \( \beta \)-glucans) are built up of \( \beta-D-glucopyranosyl \) units. Unlike cellulose, they contain both \((1\rightarrow3)\) and \((1\rightarrow4)\) glycosidic linkages which provide different solution and material properties (Albertsson and Edlund, 2011). \( \beta \)-Glucans play a role in cell expansion in primary cell walls and are probably also energy storage polysaccharides in the cells. \( \beta \)-Glucans have not been found in dicot plants but in the Poales order and their most common occurrence is in Poaceae (grasses) (Carpita and Gibeaut, 1993, Fincher and Stone, 2004, Scheller and Ulvskov, 2010). \( \beta \)-Glucans occur in the sub-aleurone and endosperm parts of the cereals, where the ratios of occurrence depend on the cereal type, and can represent up to 70% by weight of the cell wall composition (Fincher and Stone, 2004). In oats, \( \beta \)-glucans are concentrated in the sub-aleurone layers while a more even distribution can be found in rye and barley grains between the sub-aleurone layers and the endosperm (Cui, et al., 2000). Cereal cell walls hold varying amount of arabinoxylans and \( \beta \)-glucans. While barley and oats are rich in \( \beta \)-
glucans, rye and wheat contain higher amounts of arabinoxylan. Some examples of cereal hemicellulose contents are shown in Table 2.

Table 2. Arabinoxylan and β-glucan content of cereal milling fractions.

<table>
<thead>
<tr>
<th>Cereal type</th>
<th>Cell component</th>
<th>Arabinoxylan content (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>β-glucan content (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>rye</td>
<td>bran</td>
<td>35.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.4</td>
<td>(Nilsson, et al., 1996)</td>
</tr>
<tr>
<td></td>
<td>flour</td>
<td>7.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.3</td>
<td>(Nilsson, et al., 1996)</td>
</tr>
<tr>
<td>wheat</td>
<td>bran</td>
<td>22.6</td>
<td>1.2</td>
<td>(Maes and Delcour, 2002)</td>
</tr>
<tr>
<td>barley</td>
<td>hulled, flour</td>
<td>4.5</td>
<td>4.5</td>
<td>(Izydorczyk and Dexter, 2008)</td>
</tr>
<tr>
<td>oat</td>
<td>hulled, flour</td>
<td>8.0-8.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.7-5.7</td>
<td>(Wood, et al., 1989)</td>
</tr>
</tbody>
</table>

<sup>a</sup>: percent of weight  
<sup>b</sup>: result is given as dietary fiber

The structure of β-glucans from different origins has been reported in several studies and differences are often characterized by the molar ratio of cellotriosyl to cellotetraosyl units as the major products from specific enzymatic hydrolysis (lichenase) (Cui, et al., 2000, Roubroeks, et al., 2000, Wood, et al., 1994). It has been shown that after such enzymatic treatment, over 90% of the β-D-glucopyranose residues are arranged as blocks of two or three consecutive (1→4)-linked units separated by a single (1→3)-linked unit. The ratio of cellotriosyl to cellotetraosyl units was reported to be 4.5, 3.3, 3.0 and 2.2 for wheat, barley, rye and oat β-glucans respectively (Cui, et al., 2000, Cui, et al., 2000, Wood, et al., 1994). The structure of a trisaccharide (cellotriosyl) unit is shown in Figure 5. The arrangement and ratio of cellotriosyl and cellotetraosyl units are important issues controlling the solution and material properties of mixed-linkage β-glucans. The gelation ability of β-glucan solutions can be assigned to the formation of stable junction zones arising from the presence of consecutive cellotriosyl units.
Even though the chemical composition of cereal cell walls and the structure of individual components have been explored, there is little knowledge about the orientation and interaction between the cell wall polymers in their native state in plant cells (Cui, et al., 2000). Cell wall integrity is maintained by extensive non-covalent interactions, especially hydrogen bonds between the cellulose microfibrils and the matrix phase. The cell walls are built up mostly of polysaccharides but also other components are present such as proteins and lignin in the secondary cell walls. Aromatic (hydroxycinnamic) acids, most commonly ferulic acid and p-coumaric acid to a lesser extent are present in grass cell walls and are ester-linked to the arabinofuranosyl residues. Dehydro-diferulate bridges also occur, especially in the pericarp and seed coat cells, which crosslink the arabinoxylan chains. The cross-linking through ester bonds between the arabinoxylan chain and ferulic acids is thought to be a defence mechanism against microorganism or herbivore attack to digest the walls (Carpita and Gibeaut, 1993, Fincher and Stone, 2004, Scheller and Ulvskov, 2010). Arabinoxylans can also be covalently associated with proteins, through ester-linked ferulic acid units which are linked to the tyrosine amino acid of the cell wall proteins.

Figure 5. Trisaccharide unit of a mixed-linkage β-glucan structure adapted from Cui and Wang, 2009.
2.4 Isolation of xylans

Potential xylan sources are woody materials, such as wood meal and shavings or forest chips and annual plant residuals such as straw, stalks, husks, bran, and hulls as by-products of a range of agricultural processes (Ebringerová and Heinze, 2000). The liberation of the xylan components from the cell walls of woody and lignified grass tissues is usually restricted by the presence of lignin as well as a strong network composed of ester and ether-linked lignin-carbohydrate complexes. Hydrogen bonds between polysaccharide components also hinder the release of hemicelluloses (Ebringerová and Heinze, 2000). Extraction of hemicelluloses from plant cell walls is typically carried out using neutral or alkaline solvents, the latter being the most common method (Vuorinen and Alen, 1999). Several processes have been introduced at a laboratory scale for hemicellulose isolations from grain crops and from cereal brans, involving water and alkali extraction as well as other combinations such as alkali and hydrogen peroxide, alkali and chlorite solutions or dimethyl sulfoxide (Ebringerová and Heinze, 2000). In addition, pilot-scale isolation of cereal xylans has been demonstrated, indicating the feasibility of scaling up to an industrial level (Bataillon, et al., 1998, Delcour, et al., 1999, Faurot, et al., 1995, Hollmann and Lindhauer, 2005).

2.4.1 Extractions with water

Water extraction allows the isolation of high molar mass hemicelluloses and helps to reserve the native hemicellulose structure although the resulting yields are relatively low (Ebringerová and Heinze, 2000). These lower yields often result because of difficulties in carrying out extraction of cereal bran xylans. Such difficulties may arise because hemicelluloses are bound to lignin or cellulose through ferulic acid bridges and also because of hydrogen bonding between the non-substituted xylose residues and the cellulose chains (Maes and Delcour, 2001, Nilsson, et al., 1996, Vinkx and Delcour, 1996). The cross-links are non-covalent and not very strong chemical bonds nevertheless, if present in large numbers, they can result in insolubility under mild extraction conditions (Izydorczyk and Biliaderis, 2006).
As indicated in a number of reports, only 20-40% (w/w) of cereal grain hemicelluloses is typically water-extractable (Hansen, et al., 2004, Nilsson, et al., 2000, Vinkx and Delcour, 1996). A general method has been demonstrated for water extraction by Bengtsson and Åman (Bengtsson and Åman, 1990). Water extraction may be applied at different temperatures in the 25-100°C range and also at high pressure in certain cases in order to develop more efficient extraction methods. Schooneveld-Bergmans et al. isolated glucuronoarabinoxylans from wheat bran with cold water and also with steam explosion. Cold water extraction yielded only 1% hemicellulose isolate as a percentage of the starting bran with a purity of 54%. Steam explosion, which was attempted to solubilize polymeric feruloylated glucuronoarabinoxylans from destarched bran resulted in higher yields (20 to 31%); however, the extract purity was lower (approximately 50% sugar content) and the degree of polymerization decreased with increasing temperature (Schooneveld-Bergmans, et al., 1999). Roos et al. used steam pretreatment to isolate hemicelluloses from barley husks and found that polymeric xylan was extracted; however, the M_w rapidly decreased with increasing temperature and treatment time (Roos, et al., 2009). Treatments involving steam (180-200 °C) can be beneficial in reaching higher yields and enabling the process to extract hemicelluloses with acetyl groups still attached to the xylan chains, although without care in choosing proper treatment conditions, autohydrolysis of hemicelluloses can be initiated by the release of acetic acid from acetylated xylans. For such extractions, steaming conditions need to be mild in order to avoid degradation of the desired polysaccharides (Glasser, et al., 2000).

2.4.2 Extractions with alkali

Yields of extracted hemicelluloses can be greatly improved under alkaline conditions. Sodium hydroxide, potassium hydroxide and barium hydroxide solutions are generally used for alkaline extractions. Alkaline extractions can cause deacetylation in certain hemicelluloses, in which case the original carbohydrate structure will not be preserved. In contrast to the use of sodium or potassium hydroxide solutions, selective arabinoxylan extraction, avoiding the co-isolation of β-glucans, can be performed with barium hydroxide solution (Bergmans, et al., 1996, Nilsson, et al., 1996). Presumably due to the presence of Ba^{2+} ions the β-glucans remain insoluble; however, the mechanism of selective
extraction is not clear. Separation of arabinoxylans and β-glucans can also be performed via precipitation with saturated ammonium sulfate or through enzymatic digestion (Gruppen, et al., 1991, Izydorczyk and Biliaderis, 1995, Ragaee, et al., 2008b, Roubroeks, et al., 2000).

2.4.3 Complex extraction processes combined with lignin removal

Alkaline extractions are often associated with lignin removal in lignified tissues in husks or brans using sodium hypochlorite, chlorine or hydrogen peroxide treatments (Hollmann, et al., 2009, Maes and Delcour, 2001). Bataillon et al. used chlorite delignification prior to alkali extraction from wheat bran and found that the method was also applicable on a pilot scale (Bataillon, et al., 1998). Higher yields may be obtained from lignified materials using dimethyl sulfoxide as a delignifying agent but the use of this solvent is not ideal in pilot scale or industrial isolation processes (Ebringerová and Heinze, 2000). As a consequence, a range of multi-step extraction processes have been proposed for such polysaccharide isolations, involving water and alkali extractions as well as the use of other solvents, combined with purification steps (Bergmans, et al., 1996, Izydorczyk and Biliaderis, 1995, Karppinen, et al., 2001, Lazaridou, et al., 2008, Maes and Delcour, 2002, Nilsson, et al., 1996, Schooneveld-Bergmans, et al., 1999). Glasser et al. employed additional steps such as prehydrolysis and delignification before alkaline extraction of barley husks, which resulted in good purity and lower polydispersity at adequate yield (48 and 43% isolated xylan in w/w % of xylan content of biomass respectively for prehydrolyzed and delignified barley husk samples). However, a steam explosion treatment resulted in severe hemicellulose depolymerization on barley husks (Glasser, et al., 2000). Bergmans et al. applied autoclaving (one hour at 121°C), alkaline peroxide and chlorite delignification as pretreatments before alkali extraction of wheat bran. These treatments were not effective in increasing the extract yields from water-insoluble material (Bergmans, et al., 1996).
2.4.4 Enzyme-aided extractions

Many of the isolation processes cannot provide the required yield, purity or simplicity and therefore researchers have turned to alternative ways of extraction such as enzymatic degradation of certain cell wall components using β-glucanases, arabinofuranosidases, endoxylanases, and ferulic acid esterases (Izydorczyk and Biliaderis, 2006). Figuera-Espinoza et al. examined the enzymatic release of high molecular weight arabinoxylans from rye bran using different pure endo-xylanases and studied the effect of using arabinofuranosidase, endo-β-D-glucanase or ferulic acid esterase (Figueroa-Espinoza, et al., 2004). It was shown that complementary physical pretreatments are necessary besides the use of enzymes. Faulds et al. used an enzyme preparation from *Humicola isolens* called Ultraflo which hydrolyzed ferulic and coumaric acids from brewer’s spent grain and wheat bran (Faulds, et al., 2004). The residues after the enzyme treatment showed little or no degradation of α-cellulose and arabinoxylan, which might be useful in supporting isolation processes.

2.4.5 Additional treatments and purification of hemicellulose isolates

Additional treatments like ultrasonication or microwave can be of benefit by providing separation of co-extracted starch and proteins from isolated hemicelluloses. Hollmann et al. showed that ultrasonication reduced the time required for alkali-based extraction of arabinoxylans from wheat bran (Hollmann, et al., 2009). Ebringerová found that besides significantly shorter extraction times, higher yields could be reached using an ultrasound-assisted two-step extraction procedure involving aqueous alkaline treatment of corn bran (Ebringerová and Hromádková, 2002).

Following or during isolation procedures, purification of crude hemicellulose isolates is usually necessary. Purification processes typically involve inactivation of endogenous enzymes in the case of water extractions and the use of hydrolytic enzymes to degrade starch and protein contaminants, followed by separation of the hydrolysis products from the hemicellulose isolate. Amylase enzymes, such as α-amylase and amyloglucosidase are applied for starch degradation and protein removal is generally carried out with protease enzymes (Dervilly, et al., 2000). Proteins have been separated not
only through the use of enzymes but also using montmorillonite clays (Crowe and Rasper, 1988, Hartmann, et al., 2005). Delcour et al. used a clay treatment step at acidic pH and showed that 21.7% protein contamination could be completely removed from an arabinoxylan isolate (Delcour, et al., 1999). Ragaee et al. used a phenol treatment on isolated β-glucans from rye whole meal to reduce protein contamination (Ragaee, et al., 2008a). Protein content decreased from 6.4% to less than 1% as a result of this treatment.

Separation, concentration and precipitation of extracted hemicelluloses can be done using ethanol, other alcohols (e.g., isopropanol) or different salt solutions. Separation can be performed with various filtration and membrane techniques. The most commonly used separation membranes at a laboratory scale are dialysis membranes with different Mw cut-off values. Ultrafiltration is a very efficient separation method which can be scaled up to pilot and industrial scales. Krawczyk et al. used ultrafiltration and following diafiltration in order to concentrate and purify barley husk arabinoxylan (Krawczyk, et al., 2008). Arabinoxylan and β-glucan are known to reduce filtration efficiency in beer production and cause fouling problems during filtration and thus an ultrafiltration membrane made of surface-modified polyvinylidene fluoride has been used in the study of Krawczyk et al. Ultrafiltration (UF) and diafiltration significantly increased the purity of the extracted polysaccharides, as the filtrate after UF constituted 40% of the solids and approximately 70% after the diafiltration steps.

Extraction procedures for xylan production on an industrial scale are still under development. The extraction processes should be suitable for certain raw material types and take into account the intended end use. The isolations as well as the purification processes need to deliver high yields in order to be cost efficient.
2.5 Chemical characterization of xylans

2.5.1 Monosaccharide analyses

Accurate procedures for monitoring polysaccharide isolation processes and the composition of final isolates are essential. Cell wall polysaccharide sugar components are generally analyzed by degradation of the polymers followed by monosaccharide analysis. Polysaccharide degradation is normally carried out by acid hydrolysis, acid methanolysis or enzymatic hydrolysis. The enzymatic hydrolysis results in oligosaccharides hence its use can be limited for analytical investigations. Alternatively, enzyme mixtures or special oligosaccharide analytical techniques may be used (e.g., anion exchange chromatography). In enzymatic treatments, hydrolysis conditions are mild and undesirable secondary reactions can be avoided. In contrast, acid hydrolysis provides significantly more harsh reaction conditions, which is often necessary to successfully liberate sugar monomers from lignin components and crystalline cellulose. Acid hydrolysis as well as acid methanolysis may end with incomplete hydrolysis of the samples. Acid hydrolysis can also lead to sugar decomposition and loss of acid sugar components (De Ruiter, et al., 1992, Puls, 1993, Virkki, et al., 2008, Willfor, et al., 2009). Therefore, polysaccharide degradation conditions are necessarily a compromise between total depolymerization and minimum degradation of the liberated sugar components (Willfor, et al., 2009).

2.5.2 Comparison of acid hydrolysis and acid methanolysis

Acid methanolysis is superior to acid hydrolysis in respect to hemicellulose and pectin analyses (Bertaud, et al., 2002). Cellulose is only slightly degraded during the methanolysis process, so the detected glucose units originate mostly from non-cellulosic polysaccharides. In contrast, acid hydrolysis completely hydrolyses cellulose, resulting in a high amount of glucose released (Sundberg, et al., 1996). Methanolysis provides high yields of monosaccharides which are converted into methyl glycosides during the reaction with hydrochloric acid in anhydrous methanol. In addition to neutral sugar analysis, methanolysis allows the determination of uronic acid residues, acid sugars which are converted into methyl glucuronosides and which do not suffer degradation as in acidic hydrolysis conditions (Bertaud, et al., 2002, Vuorinen and Alen, 1999). As advantages of the methanolysis
technique, only small sample quantities are needed (~10 mg) and the method is highly sensitive and replicable (Sundberg, et al., 1996); however, a long sample preparation time is needed.

The resulting monosaccharides are determined by chromatographic or colorimetric methods. Determination of monosaccharides or oligosaccharides with HPLC (High Performance Liquid Chromatography) and AEC (anion exchange chromatography) does not require sample derivatization, although the selection of the most appropriate detector is critical. Pulsed amperometric detection (PAD) is sensitive and most commonly used with AEC for monosaccharide and oligosaccharide determination. GC (gas chromatography) analysis requires derivatization of the sugars in order to increase their volatility and thermal stability. The most common derivatization method is based on per(trimethylsilylation) (Vuorinen and Alen, 1999). Higher separation and sensitivity can be reached with the use of GCMS systems.

A number of research groups have made an effort to compare different types of hemicellulose analyses and detection methods as well as reaction conditions and modifications. The raw material and the type of polysaccharide of interest have to be taken into consideration when the analysis method is chosen. Sundberg et al. compared acid hydrolysis and acid methanolysis and found that methanolysis was more suitable for analysis of cellulose-containing samples. Crystalline hemicelluloses such as crystalline glucomannans or trapped hemicelluloses in the ordered cellulose structure can hinder the efficiency of acid methanolysis and lower the measured sugar content. Willför et al. made an inter-laboratory study comparing acid hydrolysis, acid methanolysis and enzymatic hydrolysis of five different raw materials and applied different chromatographic and detection techniques such as GC-FID (Gas chromatography-flame ionization detection), GCMS, HPAEC-PAD and HPAEC-borate analysis (Willfor, et al., 2009). It was found that samples containing xylan and uronic acids could generally be more accurately analyzed with acid methanolysis; however, acid hydrolysis conditions are needed to degrade crystalline polysaccharides. On the other hand, strong acidic solutions can degrade labile sugars and hence, for total sugar composition analysis, both methods are necessary. Consideration also needs to be given to the appropriate calibration method as a function of starting plant type. Hilz et al. analyzed xyloglucan samples from blackcurrants with different separation techniques, including HPAEC, RP-HPLC, and CE (capillary electrophoresis), as well
as MALDI-TOF-MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) and ESI-MS (electrospray ionization mass spectrometry) for oligosaccharide analysis (Hilz, et al., 2006). It was found that oligomers obtained after enzymatic degradation could be efficiently analyzed with all the techniques except for RP-HPLC. Combination of these techniques allows even oligosaccharide profiling and further structural studies on oligosaccharides, originating from hemicelluloses. De Ruiter et al. analyzed uronic acid-containing polysaccharides using sulphuric acid, TFA (trifluoroacetic acid) hydrolysis, and acid methanolysis combined with TFA hydrolysis (De Ruiter, et al., 1992). The best results were found using the combined methanolysis-TFA method while the TFA hydrolysis alone was not sufficient for complete hydrolysis. Using the TFA hydrolysis, decomposition of acid sugars was detected while incomplete degradation of pectin was discovered when analyzing galacturonic acid-containing pectic materials. When applying acid methanolysis prior to acid hydrolysis, higher sugar recoveries were reached than with the use of acid hydrolysis and more efficient cleavage of glycosidic linkages were noticed and less sugar decomposition occurred.

2.5.3 Linkage analysis

For detailed structural analysis and the determination of linkage positions of glycosidic linkages between sugar residues, permethylation analysis can be performed. In this technique, polysaccharides are derivatized to form methyl ethers, which is followed by hydrolysis, peracetylation and analysis by GCMS. The analysis starts with methylation of the free hydroxyl groups, followed by the hydrolysis or methanolysis of the polysaccharide. The reaction is typically carried out in a dry solvent such as dimethyl sulfoxide (DMSO) while the methylating agent is usually methyl iodide. Using sodium hydride, reaction occurs with the DMSO solvent and formation of the dimsyl anion (methyl sulfinyl carbanion) (Hakomori, 1964, Price, 2008). Esters, reducing sugars and uronic acid structures can be labile under the basic conditions used during this reaction. Reducing end groups can be protected with sodium borohydride-mediated conversion to alditol end groups. Permethylation was applied by Dervilly et al. (Dervilly, et al., 2002), combining the analysis with $^1$H NMR spectroscopy for determining the substitution pattern on the xylan chain in barley arabinoxylan. Revanappa et al. revealed with the methylation analysis of Hakomori that the xylose residues were present in three
forms in wheat hemicelluloses: un-substituted, monosubstituted and di-substituted. The position of arabinofuranosyl residues on the xylan backbone has also been determined (Hakomori, 1964, Revanappa and Salimath, 2010).

Linkage analysis can also be made by 1D or 2D NMR analysis. In comparison to the chromatographic techniques applied for sugar analysis, NMR spectroscopy has the advantage of providing excellent resolution of individual sugars, including minor components, no need for sample derivatization and is a non-invasive, fast method. The NMR signals can be assigned according to location on the main chain, the side chain or the functional groups of the sample and give structural information on specific sites (Kiemle, et al., 2004). The two- or three-dimensional techniques can be used to determine the primary and secondary structures and conformation of oligosaccharides and polysaccharides (Kajiwara, Kanji, Miyamoto, Takeaki, 2004). Both $^1$H and $^{13}$C NMR have been used to investigate the anomeric protons and carbons of arabinoxylans and β-glucans in rye hemicelluloses (Cyran, 2010, Nilsson, et al., 2000, Roubroeks, et al., 2000, Virkki, et al., 2005). The $^1$H and $^{13}$C chemical shifts may give an indication of the linkage type if the chemical shifts for the specific linkage have been reported previously (Duus, et al., 2000). Using $^1$H and $^{13}$C NMR spectroscopy, information can be obtained regarding the substitution pattern on the xylose chain as well as the positions of arabinose substitution (Cyran, 2010).

2.6 Utilization of xylans—films from cereal or grass hemicelluloses

Cereal hemicelluloses, arabinoxylans and β-glucans in particular, have numerous potential uses, but only a few have been developed to an industrial scale. The dietary health benefits of arabinoxylans and β-glucans have lately been discovered and food and pharmaceutical products with these hemicelluloses have been prepared. Xylitol derived from xylose has been widely used as an artificial sweetener. There is also extensive research interest in C5 sugars such as xylans as feedstocks for ethanol fermentation processes: however, economically feasible processes are still being sought. Cereal hemicelluloses can be used in functional food formulations in several ways, such as
fermentable substrates for probiotic microorganism growth, as dietary fibers, as prebiotics or as encapsulation materials for probiotics (Charalampopoulos, et al., 2002).

Numerous research papers have been produced about the use of hemicelluloses in food-related applications. For example, it has been shown that xylans contribute to the mechanical properties of the dough and the texture of bread and baked products. Arabinoxylans increase the water absorption and gas retention in dough and thus assist loaf volume development and can be used as additives in bread-making (Ebringerová and Hromádková, 1999, Ebringerová, et al., 2005, Izydorczyk and Biliaderis, 1995). Cereal β-glucans have long been known for their beneficial physical and physiological properties and β-glucan products have been commercially accepted as functional and bioactive components (Ebringerová, et al., 2005). β-Glucans are applicable as food hydrocolloids as a result of their viscosity-enhancing effects and gelling ability under certain conditions. Thus, they are used in the food industry as thickening agents or even as fat mimetics. Oatrim™, an enzymatically hydrolyzed oat flour or bran product containing β-glucans has been used as a fat mimetic in oatmeal cookies, processed meats, sauces and beverages (Lazaridou, et al., 2007). Glucagel™ is a β-glucan gel formed from low molecular weight barley β-glucans and is used as a fat mimetic in bakery and dairy products as well as in edible films (Cui and Wang, 2009).

Cereal hemicelluloses are being tested in pharmaceutical products as well. β-Glucans have important therapeutic effects in terms of coronary heart disease and can assist in the reduction of blood cholesterol and the glycemic response (Charalampopoulos, et al., 2002). Xylans have been shown to have valuable biological effects such as a suppressive effect on blood pressure, hypocholesterolemic activity and "antinutritive" effects (xylan-type hemicelluloses and β-glucans are excellent natural agents in avoiding the development of obesity due to their ability to increase the viscosity of the gut contents and slow enzymatic action and digestion) as dietary fibers (Ebringerová, et al., 2005). Arabinogluuronoxylans with immuno-stimulating activity have been isolated from herbal plants. Other pharmaceutical-related effects such as the anti-tumor effects of xylan-type hemicelluloses have been shown and xylan sulfates are proven to be efficient substitutes for heparin (Ebringerová and Hromádková, 1999). Maize bran arabinoxylans were cross-linked by peroxidase and formed a three-dimensional network (Methacanon, et al., 1998). In the presence of water, a hydrogel
was produced and found to be suitable as a wound management aid. This polysaccharide material was commercialized as Sterigel™.


2.7 Materials from arabinoxylans and β-glucans

2.7.1 Impact of molecular structure on material properties

The molecular structure of hemicelluloses ultimately determines the properties of the materials prepared from these polysaccharides. For example, hemicellulose solubility and rheological properties are strongly related to structure. For the preparation of biomaterials, structural properties, such as chemical composition, degree of polymerization, degree of substitution and the substitution pattern should be considered. Substitution of the xylan chain can be a key factor influencing water solubility. Side groups of arabinoxylans can prevent hydrogen bonding and also render the polysaccharide water-soluble. Sternemalm and Höije et al. chemically and enzymatically modified rye flour arabinoxylan in order to observe the effect of arabinose substitution on cast film properties. An increase in arabinose content resulted in a plasticizing effect but negatively affected the oxygen permeability of the films (i.e., the permeability increased). Crystallinity increased and water solubility decreased with decreasing arabinose substitution (Höije et al., 2008, Sternemalm, et al., 2008). Zhang et al. cast arabinoxylan films with Ara/Xyl ratios between 0.2 and 1.3 (Zhang, et al., 2011). In this work it was shown that water-soluble arabinoxylan with a high degree of substitution was completely amorphous, while the other arabinoxylans with reduced substitution showed clear diffractions in the X-ray analysis indicating that the unsubstituted regions of the chains may crystallize, while the higher substituted areas of the xylan chain remain amorphous. Lower arabinose substitution also generally
denotes decreased water solubility and reduced hydrophilicity. Accordingly, the physico-chemical properties of xylan-based materials depend significantly on their substitution pattern. Rheological properties are also often studied in relation to preparation of hemicellulose hydrogels and gelling properties. Cereal flour arabinoxylan and corn bran xylan formed gels only after oxidation (hydrogen-peroxide treatment), which could be explained by cross-links formed between ferulic acid units and xylan and/or proteins (Ebringerová, et al., 2005, Izydorczyk and Biliaderis, 1995). Cereal arabinoxylans could be used as drug carriers by transforming the xylan into a hydrogel. Carvajal-Millan et al. prepared cross-linked wheat arabinoxylan with laccase and examined the release of a test protein, ovalbumin from the formed polysaccharide network (Carvajal-Millan, et al., 2006). The cross-linking method allowed gel formation without modifying the protein. The protein release rate and quantity could be adjusted by varying the amount of the entrapped protein.

2.7.2 Arabinoxylan and β-glucan films and barrier properties

Hemicellulose films show outstanding gas barrier properties and, as shown in Table 3, can show mechanical properties comparable to some conventional plastics used in food packaging; however, without modification, hemicellulose films are both brittle and hygroscopic (i.e, also poor moisture barriers). A number of approaches have lately been investigated as means to enhance such properties by chemical or physical modification techniques.

The oxygen barrier properties of hemicellulose films are comparable to those of commonly used barrier plastics such as ethylene vinyl alcohol. The mechanical properties of arabinoxylan films have been demonstrated and their application as oxygen barriers in multilayer packaging has been suggested (Gröndahl and Gatenholm, 2007). Xylan-type hemicelluloses from agricultural crops or from wood have been used for biodegradable film production. For example, arabinoxylans from corn hulls and bran, rye flour, oat spelt, barley husks and wheat bran have been used in studies on film casting from aqueous solutions (Gröndahl and Gatenholm, 2007, Höije et al., 2008, Mikkonen, et al., 2009, Sternemalm, et al., 2008, Zhang, et al., 2011, Zhang and Whistler, 2004). Commercially available arabinoxylans from wheat and rye have lately been studied for several purposes and applications such as biodegradable films or coatings have been included. Agricultural by-products such as oat spelt
arabinoxylan have been shown to provide good oxygen and/or grease barrier films in applications where moderately high water vapor permeability is required (Mikkonen, et al., 2009). Edible films have been made by blending arabinoxylans with other components such as lipids, agar and cassava and biodegradable films have also been cast from mixtures of galactoglucomannan and konjac glucomannan (Mikkonen, et al., 2008, Peroval, et al., 2002, Phan The, et al., 2009a).

The rheological properties of β-glucan solutions have been studied but their use to provide free-standing films or edible films has scarcely been investigated. Tejinder et al. prepared edible films from β-glucans originating from barley and oats (Tejinder, 2003). The films had good mechanical properties but were less effective moisture barriers than other typical edible films, like casein or gluten-based films and were less effective moisture barriers than arabinoxylan films (Mikkonen, et al., 2009, Peroval, et al., 2002, Tejinder, 2003).

2.7.3 Composite films

Several types of composite materials have been made using xylan and other polysaccharides such as mannans or cellulose, proteins, lipids or even nanoclays (Peroval, et al., 2002, Phan The, et al., 2009a, Umemura and Kawai, 2008, Ünlü, et al., 2009). Wheat gluten and xylan films were prepared and it was shown that xylan could be used without significant changes in the gluten film properties. The advantage of this approach is that the use of xylan could reduce the cost of gluten film production (Kayseriliyoglu, et al., 2003).

Stevanic et al. added bacterial nanocellulose to rye arabinoxylan with decreased arabinose substitution. The cellulose addition resulted in cohesive and optically transparent composite films which were stronger and stiffer than the pure xylan films. The films showed better mechanical properties (68 MPa tensile strength while the pure AX had a tensile strength of 58 MPa) using the arabinoxylan with non-reduced arabinose substitution; however, the water barrier properties were not significantly improved (Stevanic, et al., 2011).
<table>
<thead>
<tr>
<th>Source of hemicellulose</th>
<th>Addition of plasticizer (w/w%)</th>
<th>Test temperature (°C)</th>
<th>Relative humidity (%)</th>
<th>Moisture content (%)</th>
<th>WVTR (10^{-3} \text{ g m}^{-2} \text{ s}^{-1})</th>
<th>WVP (10^{-11} \text{ g m}^{-2} \text{ Pa}^{-1} \text{ s}^{-1})</th>
<th>Tensile strength (MPa)</th>
<th>Youngs’ modulus (MPa)</th>
<th>Strain at break (%)</th>
<th>Oxygen permeability (\text{cm}^3 \mu\text{m}/\mu\text{m}^2 \text{ day kPa})</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barley husks Ara/Xyl:0.22</td>
<td>23</td>
<td>50</td>
<td>35.5</td>
<td></td>
<td>50.3</td>
<td>2930</td>
<td>2.5</td>
<td>0.16</td>
<td>(Gröndahl and Gatenholm, 2007)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corn fiber</td>
<td>21</td>
<td>65</td>
<td></td>
<td></td>
<td>4.2 ± 1.5</td>
<td>21 ± 9</td>
<td>(Mikkonen, et al., 2008)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rye (Ara/Xyl:0.5)</td>
<td>23</td>
<td>50</td>
<td>10.97 ± 0.23</td>
<td></td>
<td>52.4</td>
<td>1750</td>
<td>4.7</td>
<td>2.0</td>
<td>(Höije, et al., 2008)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corn hulls</td>
<td>22</td>
<td>54</td>
<td></td>
<td></td>
<td>4.7</td>
<td>53.8</td>
<td>1316</td>
<td>6.2</td>
<td>(Zhang and Whistler, 2004)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rye (commercial)</td>
<td>30</td>
<td>50</td>
<td>~14</td>
<td></td>
<td>58 ± 11</td>
<td>2500 ± 400</td>
<td>8.1 ± 3.3</td>
<td>(Stevanic, et al., 2011)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wheat bran WS-ES WS-EI WI</td>
<td>25</td>
<td>50</td>
<td>~9.5</td>
<td>~12</td>
<td>~13.5</td>
<td></td>
<td></td>
<td></td>
<td>(Zhang, et al., 2011)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corn bran</td>
<td>15% glycerol</td>
<td>25</td>
<td>22 for the WVP tests 54</td>
<td></td>
<td>3.92</td>
<td>17.7</td>
<td>26.5±4.1</td>
<td>72.4 ± 35.2</td>
<td>7.4 ± 2.9</td>
<td>(Peroval, et al., 2002)</td>
<td></td>
</tr>
<tr>
<td>No information</td>
<td>15% glycerol</td>
<td>25</td>
<td>22 for the WVP tests 57</td>
<td></td>
<td>13.7</td>
<td>22.3</td>
<td>5.46</td>
<td>(Phan The, et al., 2009a)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Oat spelt</td>
<td>10% glycerol 10% sorbitol</td>
<td>22 for the WVP tests 54</td>
<td>11.2 ± 0.5</td>
<td>11.1 ± 0.2</td>
<td>2.08</td>
<td>0.81</td>
<td>3.89</td>
<td>1.27</td>
<td>28</td>
<td>17</td>
<td>1100 700</td>
</tr>
<tr>
<td>Oat</td>
<td>Changing moisture content in films</td>
<td>23</td>
<td></td>
<td></td>
<td>20-80</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oat</td>
<td>30% glycerol</td>
<td>100</td>
<td></td>
<td></td>
<td>68.8-80.8</td>
<td>6.27-7.72</td>
<td>1500-6000</td>
<td>1-22</td>
<td>(Tejinder, 2003)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Examined material</td>
<td>Addition of plasticizer (w/w%)</td>
<td>Test temperature (°C)</td>
<td>Relative humidity (%)</td>
<td>Moisture content (%)</td>
<td>WVTR (10^{-9} \text{ g m}^{-2} \text{ s}^{-1})</td>
<td>WVP (10^{-11} \text{ g m}^{-2} \text{ Pa}^{-1} \text{ s}^{-1})</td>
<td>Tensile strength (MPa)</td>
<td>Youngs' modulus (MPa)</td>
<td>Strain at break (%)</td>
<td>Oxygen permeability ((\text{cm}^3 , \mu\text{m} / \text{m}^2 \text{ day} , \text{kPa}))</td>
<td>Reference</td>
</tr>
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<td>------------------</td>
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<td>----------------------</td>
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<td>---------------------------------</td>
<td>----------</td>
</tr>
<tr>
<td>Cellophane</td>
<td></td>
<td>25</td>
<td>22</td>
<td></td>
<td></td>
<td>4.54 ± 0.134</td>
<td></td>
<td></td>
<td></td>
<td>(Phan The, et al., 2009b)</td>
<td></td>
</tr>
<tr>
<td>EVOH(^a)</td>
<td></td>
<td>23</td>
<td>85 for WVP tests</td>
<td>0</td>
<td></td>
<td>0.0012-0.0035</td>
<td>0.01-0.1</td>
<td></td>
<td></td>
<td>(Lange, et al., 2003)</td>
<td></td>
</tr>
<tr>
<td>PVAL(^b)</td>
<td></td>
<td>23</td>
<td>85 for WVP tests</td>
<td>0</td>
<td></td>
<td>0.000347</td>
<td></td>
<td></td>
<td></td>
<td>(Lange, et al., 2003)</td>
<td></td>
</tr>
<tr>
<td>PP(^c)</td>
<td></td>
<td>23</td>
<td>85 for WVP tests</td>
<td>50</td>
<td></td>
<td>0.023-0.046</td>
<td></td>
<td></td>
<td></td>
<td>(Lange, et al., 2003)</td>
<td></td>
</tr>
</tbody>
</table>

a: arabinoxylan  
b: WS-ES: water and ethanol soluble hemicelluloses; WS-EI: water-soluble, ethanol insoluble; WI: water-insoluble  
c: β-glucan  
d: temperature and relative humidity regarding all the applied tests  
e: tensile strength expressed in N  
f: EVOH=Ethylene vinyl alcohol  
g: PVAL=Poly(vinyl alcohol)  
h: PP=Polypropylene
Recent studies have reflected a growing interest in cellulose addition to hemicellulose for films with increased strength. Sulfonated and hydrochloric acid-treated cellulose whiskers were added to oat spelt xylan and the film containing whiskers, especially sulfonated whiskers, showed significantly higher tensile strength (from ~2 MPa tensile strength increased to ~7 MPa with 10% whisker addition) (Saxena, et al., 2009). However, these increased strength values were considerably lower than such values found previously for oat spelt arabinoxylans (Mikkonen, et al., 2009). Péroval et al. added lipids to arabinoxylan films in order to improve their water barrier efficiencies (Péroval, et al., 2002). The water vapor permeability of the films was slightly decreased; however, the mechanical properties showed some negative changes as the tensile strength and elongation of the films decreased. Hydrogenated palm oil was shown as the best additive for arabinoxylan with composite films presenting stable particle size distribution, low contact angle values and similar elongation values to the pure arabinoxylan films. Mikkonen et al. mixed corn arabinoxylan (cAX) with galactoglucomannan (GGM) to examine the mechanical properties and thermal behavior of the cast films and found that cAX and GGM formed homogeneous blend films, but they were sensitive to high relative humidity and the mechanical properties were not improved relative to pure GGM films (Mikkonen, et al., 2008).

There is an extensive history of research on clay-polymer nanocomposites over the past 20 years and this has included a few studies on xylan-clay film properties (Viota, et al., 2010, Ünlü, et al., 2009). From this research it was shown that layered nanoclay addition can result in better mechanical and barrier properties when the nanoclay is well distributed in the xylan matrix. Ünlü et al. made films using corn cob xylan and an unmodified montmorillonite clay (NaMt) and studied the rheological, thermal and morphological film properties (Ünlü, et al., 2009). Electrokinetic, rheological and crystallinity measurements suggested that interactions took place on the NaMt surface but that the xylans were not oriented into the clay galleries. The authors proposed that such biocomposites could be applicable in cosmetics, as thickeners or in cleaning agents.

Xylans can be considered for various other material applications. For example, Silva et al. coated magnetic microparticles with xylan from corn cobs to prevent gastric dissolution of the magnetite, as magnetic particles may be used as contrast agents or markers for monitoring gastrointestinal functions (Silva, et al., 2007). During the coating preparation, iron salts and xylan were co-precipitated in alkaline medium followed by emulsification/cross-linking reactions. After
120 minutes dissolution time, the xylan coated microparticles were still protected, as xylan did not dissolve at gastric pH (pH 1). Hence, the xylan coating was able to shield the magnetite and can be a promising material for oral administration.

The increasing knowledge and the growing willingness to develop new biopolymer-based materials will lead to an increasing application of xylan and xylan derivatives. Hemicelluloses having such a diverse structure, give plenty of possibilities for enzymatic and chemical modifications directed to particular uses (Ebringerová and Hromádková, 1999). With modification of xylan-type hemicelluloses, new materials with excellent properties can be produced; however, more research is needed in order to find these valuable properties and raise the production to an industrial scale (Ebringerová and Heinze, 2000).

In this context, the research described in this thesis was designed to improve our understanding of the structure and location of hemicelluloses in the cereal cell walls as well as their connection to other wall components, to isolate hemicelluloses from the cereal walls and to evaluate the potential use of such hemicelluloses in film applications.
3. Aims of the study

The overall aim of the study was to find suitable raw materials for hemicellulose isolations which could be applied for biopolymer production with focus on applicability as food packaging materials.

The main objectives of the individual studies were:

- Isolation of high molecular weight hemicelluloses from agricultural side products
- Characterization of the isolated hemicelluloses, particularly sugar composition analysis, with different methods
- Evaluation of film characteristics from the isolated arabinoxylans and β-glucans as well as mixtures of them
- Study on new arabinoxylan/clay films with improved material properties
4. Experimental

Methods and analyses are briefly discussed below and the detailed methodology is outlined in the attached papers. Materials or methods applied during the studies and are not described in the papers are presented in the methods section of the thesis. References are given to the corresponding paper, where the detailed methodology can be found.

4.1 Materials

Rye and oat bran were prepared from whole grains. Rye (*Secale cereale*) cultivar Carotop was grown in Denmark in 2008. Rye grains from this crop were disc milled and the fine flour fraction was discarded. Milling fractions in the range of 0.1 mm to 1.0 mm were obtained and the composition of each fraction was analyzed. Material with particle size in the range 0.25-1.0 mm and with a mean diameter of 0.5 mm was used as rye bran material for hemicellulose extractions. Similarly, grains from the oat (*Avena sativa*) cultivar Revisor grown in Denmark in 2008 were disc milled and after fine flour separation a fraction with particle size 0.25-1.0 mm was obtained and used for isolations. All the fractions between 0.1 mm and 1.0 mm in particle size were analyzed for their mixed-linkage β-glucan content.

Labeled monosaccharides, D-Arabinose-1-13C, D-glucose-13C6, D-glucose-1-13C, D-xylose-13C5, D-xylose-1-13C, 2,3,4,6-tetra-acetyl-α-D-glucopyranosyl bromide and L-arabinose, D-glucose were purchased from Sigma-Aldrich. D-xylose was purchased from Merck (Darmstadt, Germany). These monosaccharides were used for calibration purposes in carbohydrate analysis (Paper II).

Arabinoxylan (Lot 20601) from rye flour was purchased from Megazyme International Ireland Ltd. (Bray, Ireland). The arabinoxylan was a high viscosity polysaccharide with Ara/Xyl ratio = 0.64 and purity ~90% and was used for film casting (Paper IV). Birch wood xylan was purchased from Sigma Aldrich and hemicellulose monosaccharide components were analyzed by acid methanalysis and acetylation (Paper II).

Thermostable α-amylase Termamyl SC was obtained from NovoZymes A/S (Bagsvaerd, Denmark) and Amyloglucosidase (EC 3.2.1.3 from *A. niger*) from Megazyme International Ireland Ltd. (Bray, Ireland). These enzymes were used during the hot water extractions of rye and oat bran.
hemicelluloses. Lichenase (endo-1,3(4)-β-Glucanase) (EC 3.2.1.73 from Bacillus sp.) was purchased from Megazyme International Ireland Ltd. and was applied for enzymatic removal of mixed-linkage β-glucans from the isolated rye bran hemicelluloses (Paper III).

The nanoclay sepiolite was purchased from Sigma-Aldrich and was used to produce reinforced xylan films (Paper IV). The sepiolite powder contains approximately 13% Mg (on dry weight basis) with the unit cell formula of Mg$_2$H$_2$Si$_3$O$_9$·xH$_2$O.

4.2 Isolations

Three types of extraction process were applied to rye bran in order to isolate high molar mass hemicelluloses: hot water extraction, alkaline treatment and wet oxidation. Hot water extraction was used for oat hemicellulose isolations. No additional delignification step was used in any of the isolations because initial raw material analysis tests indicated no significant amounts of lignin content in the starting materials.

4.2.1 Hot water extraction of rye and oat bran hemicelluloses

The isolation processes are shown in Figure 6 and described in detail in Papers I and III. The solubilization of water-extractable hemicelluloses was facilitated by using a combination of heat, α-amylase enzyme treatment and wet milling. Wet milling reduced the size of starch granules and bran fibers and provided an easier path for the enzyme to act on the substrate. The solubilized hemicelluloses and liquefied starch were separated from waxes, fibers and proteins using centrifugation. Subsequent amylglucosidase enzyme treatment allowed further degradation of starch and the resulting syrup was dialyzed in order to separate glucose residues from the isolated hemicelluloses. Excess ethanol used for precipitation of hemicelluloses allowed the precipitation of high molar mass polysaccharides (e.g., starch).
Figure 6. Isolation of water-and alkaline hemicellulose extracts from rye bran.
4.2.2 Alkali extraction of rye bran fibers

The rye bran fiber fraction (WIS) of the separated water-insoluble fraction was extracted with NaOH according to the method of Ragaee et al. (Ragaee, et al., 2008b). The bran fibers were washed with water and dried at 45°C overnight prior to NaOH extraction. Alkaline extraction was performed using 1 M NaOH with continuous magnetic stirring and solid-to-liquid ratios (w/v) of: 1:10; 1:35; 1:70. The supernatant was separated by centrifugation and neutralized with 5 M HCl. The hemicellulose-containing supernatant was then dialyzed (MWCO 12000-14000 Da) against deionized water. Hemicelluloses in the dialyzed liquid were precipitated with ethanol and then freeze dried.

4.2.3 Wet oxidation of rye bran

Wet oxidation was applied to isolate hemicelluloses from the unprocessed rye bran. 60 g dry material (rye bran, particle size range: 0.25-1 mm) was placed in a loop reactor as described by Schmidt and Thomsen (Schmidt and Thomsen, 1998), with addition of 6.5 g Na$_2$CO$_3$ and one liter water. The bran material was treated for 10 minutes at 195°C in the presence of oxygen while the pressure was adjusted to 12 bars. After the treatment, the material was filtered, and the volume of the unwashed filtrate was 1.0 l. The pH of the filtrate was adjusted to 4.5 and then the polysaccharides in the filtrate were precipitated with an equal volume of ethanol. After storage at 4°C overnight the precipitate was collected after centrifugation (approx. 6000 g, 20 minutes). Isolated polysaccharides were washed with a 1:1 (v/v) mixture of ethanol and water, resuspended in water and then freeze dried.

4.2.4 Wet oxidation of rye bran fibers

In addition to wet oxidation of the unprocessed rye bran, the separated fiber fraction after hot water extraction was also used in wet oxidation experiments. After the hot water extraction, rye bran slurry was centrifuged and the fiber fraction (WIS material shown in Figure 6) was separated, washed with water at 40°C and dried in an oven at 40°C. The fibers were treated similarly as the bran material.
4.3 Enzymatic treatment

Hot water extraction of rye hemicelluloses allows the parallel isolation of two types of hemicelluloses, arabinoxylans and β-glucans. β-Glucan co-extraction may be avoided by using special extracting solvents such as Ba(OH)$_2$, or specific enzymes can be used for β-glucan removal. In this study, lichenase enzyme (E.C. 3.2.1.73; endo-1,3(4)-β-glucanase) was used in order to degrade the mixed-linkage β-glucans in rye hemicellulose isolates (Paper III). The enzyme specifically cleaves the (1→4) linkages of the 3-substituted glucose residues, producing oligosaccharides which are mostly tri- and tetrasaccharides (Izydorczyk and MacGregor, 2000, Roubroeks, et al., 2000). The treatment was followed with dialysis for 48 hours against MilliQ water with a cut-off value of 12000-14000 Da. The dialysis was applied to remove the monomeric and oligomeric sugars from the samples. The water-extracted samples enriched in arabinoxylan were freeze dried after the dialysis process.

4.4 Raw material characterization methods

4.4.1 Sugar components, lignin content

Rye oat bran sugar composition and Klason lignin content were determined using a 72% sulphuric acid hydrolysis. During the prehydrolysis step, 1.5 ml sulphuric acid was added to 0.16 g rye and oat bran or rye bran fiber sample at 30°C for one hour. By this procedure, the cell wall structure is loosened and oligosaccharides are produced. The acidic mixture was then diluted with water and heated up to 120°C for one hour, the oligosaccharides further cleaved and monosaccharides produced. The supernatant contained these monosaccharides, which were measured by HPLC. The solid residue after the hydrolysis was separated by filtration and ashed at 550°C. The Klason lignin content was calculated as the difference in weight between the dry solid residue after hydrolysis and the residue after ashing.
4.4.2 Starch and mixed-linkage β-glucan content

The starch content of rye and oat milling fractions as well as that of rye and oat bran was measured and calculated. Starch was hydrolyzed enzymatically after dissolution of the various samples. An α-amylase enzyme was used for specific liquefaction of starch and later amyloglucosidase enzyme was added in order to degrade the resulting glucose oligomers. Glucose content was determined by HPLC.

Mixed-linkage β-glucans were measured quantitatively using a Megazyme mixed-linkage β-glucan assay procedure. β-Glucan-containing samples were suspended and hydrated in a buffer solution at pH 6.5, treated with lichenase enzyme and the solid fraction was separated. The aliquot was then incubated with β-glucosidase enzyme to degrade the glucose oligomers into monomers and the amount of the produced glucose was measured colorimetrically using an oxidase/peroxidase reagent.

4.4.3 Protein content

Protein content in rye and oat bran, rye and oat fibers, separated protein and wax fraction after hot water extraction and isolated rye and oat bran hemicelluloses was calculated from the total amount of measured nitrogen in the samples (5.83 x N). The total nitrogen content was determined with an EA 1110 CHNS-O elemental analyzer (CE Instruments, Wigan, UK) at 1800°C combustion temperature.

4.4.4 Extractives

Soxhlet extraction of rye and oat bran was carried out for 24 hours using 96% (v/v) ethanol in order to determine the lipid content according to the ASTM Standard E1690 (ASTM, 2008).

4.4.5 Ash

Ash content was determined as the mass difference of dry (water-free) material before and after heating to 550°C for three hours. The measured ash content represents the inorganic content of the plant material to be analyzed.
4.4.6 Monosaccharide analyses

The monosaccharide composition of isolated hemicelluloses was measured using several methods. These methods were based on degradation of polysaccharides into oligo- and monosaccharides using either acid hydrolysis (dilute sulphuric acid) or acid methanolysis (HCl in water-free methanol). Monosaccharide content was then analyzed by liquid or gas chromatography. For HPLC analysis, polysaccharide samples were hydrolyzed using a dilute (8 v/v %) sulphuric acid solution at 120°C. Samples were derivatized in order to produce volatile components for GC analysis. The derivatization methods were trimethylsilylation or acetylation of methyl glycosides, produced in the acid methanolysis procedure. The trimethylsilylated sugars were analyzed using GC (Paper III) and acetylated monosaccharides were analyzed with GCMS (Paper II). Ara/Xyl ratio was calculated for all the isolated hemicelluloses, so as to provide information about the arabinose substitution on the xylan chains.

Monosaccharide standards (D-glucose, D-galactose, D-xylose, D-arabinose,) and acid sugars (D-galacturonic acid, D-glucuronic acid) were subjected to acid methanolysis and acetylation so as to be used in calibration and carbohydrate identification. In order to demonstrate the feasibility of the method on polysaccharides, dextran samples (used for quantitative analysis) with different molecular weight (1080 Da, 5000 Da, 9890 Da, 12000 Da) were also analyzed.

4.4.7 Molar mass analysis

The molar masses of isolated hemicelluloses from rye bran, as well as that of the purified rye arabinoxylan, were determined by high performance size exclusion chromatography (HPSEC) and the molar mass of isolated oat β-glucan was determined with asymmetric flow field–flow fractionation AsFiFFF (Paper III). SEC systems separate the molecules according to their sizes in solution (hydrodynamic volumes). This hydrodynamic volume depends on the molecular conformation in solution. Thus, it is important that the studied samples are well dissolved in the eluent and molecular aggregation is avoided. Different solvents were applied in the molar mass analyses presented here. An aqueous alkaline eluent (0.01 M NaOH) was applied for SEC in the analysis of hemicelluloses isolated with hot water extraction or alkaline extraction (Paper I) and wet oxidation treatments, while DMSO was used for analysis of hot water-extracted
hemicelluloses (Paper III). Weight-average molecular weight ($M_w$) determinations were based on conventional calibration using a range of pullulan standards or based on the light scattering/viscometry method using a $dn/dc$ value of 0.064 ml/g (for the samples in DMSO). An aqueous eluent was used for oat β-glucan molar mass characterization and the samples were analyzed in an AsFlFFF system. Molar mass calculations were made according to Gómez, et al., 1997, and a $dn/dc$ value of 0.151 ml/g was used.

### 4.4.8 NMR analyses

Detailed structural analysis of polysaccharides is commonly carried out using proton NMR ($^1$H-NMR) and/or $^{13}$C-NMR. The signals obtained give information about the linkages between the monosaccharide constituents in the polysaccharide chains.

One dimensional $^1$H-NMR spectra were collected for purified arabinoxylan sample from rye bran using a Varian Unity 500 spectrometer (Varian NMR Systems, Palo Alto, CA, USA), at 600 MHz with cryo probe. The sample was exchanged three times with D$_2$O prior to the measurement and finally dissolved in pure D$_2$O. A presaturation pulse sequence was used to minimize the DHO signal. The $^1$H chemical shifts (ppm) were referenced to an internal acetone signal at 2.225 ppm.

### 4.5 Film characterization

#### 4.5.1 Preparation of films

Polysaccharides were mixed into MilliQ water for 24 hours at 40 °C under magnetic stirring at a concentration of 10 g/l for film casting (Paper III). Arabinoxylan was mixed into MilliQ water for four hours, one hour at 90 °C and three hours at 60°C, under magnetic stirring for films in the study on effects of β-glucan content on arabinoxylan films (Paper IV). The prepared suspensions were degassed by ultrasonication under vacuum for five minutes and cast on Teflon plates or Teflon-coated Petri dishes.

In order to prepare nanocomposite films (Paper IV), sepiolite nanoclay was mixed into MilliQ water at a concentration of 2.5 g/l and the suspension was treated with an ultrasonic processor under magnetic stirring for one hour. The prepared arabinoxylan solution and nanoclay
suspensions were mixed, further stirred and ultrasonicated and then cast on coated Petri dishes. Plasticized films were prepared as well using 30% (on dry weight basis) poly(ethylene glycol) methyl ether (mPEG). Films were dried at 23 °C and 50 % relative humidity (RH) and conditioned for at least four days before analyses (Paper IV).

4.5.2 Thickness

The film thicknesses were measured using a micrometer. A micrometer screw gauge (Lorentzen & Wettre, Kista, Sweden) or Mega-Check Pocket Coating Thickness Meter (List-Magnetik, Leinfelden-Echterdingen, Germany) were used. The thickness of the test pieces was measured at 5-10 points with μm precision and an average thickness was calculated.

4.5.4 Light transmission of films

The light transmission of arabinoxylan-sepiolite nanocomposite films (Paper IV) was measured using an Ultrospec 2100pro UV–visible spectrophotometer (Biochrom Ltd, Cambridge, UK) in the 190-890 wavelength range. The light transmission values of the films were normalized to a film thickness of 30 μm using the Lambert-Beer law.

4.5.5 Mechanical properties

Mechanical properties were measured with an Instron 4465 (Paper III) or an Instron 5944 (Paper IV) universal testing machine at 23 °C and 50% RH on films conditioned prior to testing. The crosshead speed was 5 mm/min, the initial distance between the grips was 50 mm in all measurements and a load cell of 100 N or 50 N was used. Tensile strength, elongation at break and Young’s modulus were determined for ten test pieces and the average of the results was calculated. Test samples were prepared with a rectangular shape, 10 mm wide and approximately 100 mm in length with a testing length of 45 mm.
4.5.6 Oxygen permeability

The oxygen transmission rate (OTR) of test films was measured with an OPT-5000 Oxygen Permeability Tester (PBI-Dansensor A/S, Ringsted, Denmark) containing a ceramic solid-state oxygen sensor. Measurements were performed at 23 ± 0.03°C and 50 ± 2% RH. The films were placed in a permeability chamber which consisted of an upper (feeding) and a bottom (receiving) chamber. Dry nitrogen containing less than 0.1 ppm oxygen was used as carrier gas and pure oxygen served as the test gas. On the feeding chamber side, oxygen flows and the film sample is opposed to the pure oxygen. On the bottom chamber side pure nitrogen gas reaches the film sample. The amount of diffused oxygen is measured by the oxygen sensor. The oxygen permeability (OP) was calculated by multiplying the measured OTR with the thickness of the films and dividing with the pressure value of the measuring chamber. The method was used for OP measurements on films from rye hemicelluloses and oat β-glucans (Paper III) and arabinoxylan-sepiolite nanocomposite films (Paper IV).

4.5.7 Water vapour permeability

The water vapour permeability was measured by a gravimetric method, according to the ASTM E 96/E 96M – 05 standard (ASTM, 2005). Films were sealed to aluminium cups, containing dry CaCl₂ as a desiccant and cups were placed into a climate chamber with controlled relative humidity and temperature. This set-up included an air gap of 6 mm between the desiccant and the underside of the film. The cups were weighed 8-10 times over the period of four days. Calculations were performed according to the method of Mikkonen et al. (Mikkonen, et al., 2010). The water vapour transmission rate (WVTR) was calculated from the linear regression of the slope of weight gain vs. time by dividing the slope by the test cell mouth area. The water vapour permeability (WVP) was obtained by multiplying the WVTR by the thickness of the film and dividing it by the water vapour partial pressure difference between the two sides of the film. The method was used for WVP measurements on pure hemicellulose and blended hemicellulose films (Paper III) and pure and nanocomposite films (Paper IV).
4.5.8 Microscopy

Scanning electron microscopy (SEM) was used to image the nanoclay distribution or aggregation of clay particles in arabinoxylan-sepiolite nanocomposite films (Paper IV). Images were taken after tensile testing with a AURIGA® FIB-SEM instrument (Carl Zeiss, Oberkochen, Germany).

4.5.9 X-ray diffraction

X-ray diffraction was used to study crystallinity changes in RAX-sepiolite composite films and comparison was made with pure RAX and sepiolite powder. A Siemens D5000 X-ray diffractometer (Siemens Analytical and X-Ray Instruments Inc., Madison, WI, USA) equipped with a Co (λ = 0.179 nm) tube and a diffracted beam monochromator was used. Diffractograms were collected in the 2θ range of 3–30° using a step size of 0.05° and a counting time of 20 sec.

4.5.11 Water absorption

The water content of xylan films was analyzed gravimetrically. Films pieces were equilibrated at 23°C and three different relative humidities: 50% RH in a climate chamber and 75.5% and 98% RH by using desiccators containing saturated NaCl solution and water respectively (Höije, et al., 2008). The water content of the films was determined after drying the film pieces at 105°C for 24 hours (Paper III) or one hour (Paper IV) and weighing (Veiga-Santos, et al., 2007). The equilibrium moisture content was calculated as the weight of the absorbed water in the film sample at equilibrium divided by the dry weight:

\[
\text{Moisture uptake} = 100 \times \frac{W_{\text{moist}} - W_{\text{dry}}}{W_{\text{dry}}}
\]

where \( w_{\text{moist}} \) is the film sample weight equilibrated at 50% RH and \( w_{\text{dry}} \) is the dry sample weight.

4.5.12 Statistical analyses

The differences between mean results of the mechanical tests and equilibrium moisture contents were analyzed using one-way ANOVA and least significance differences analysis for film samples from rye bran hemicelluloses and oat β-glucans and their blended films (Paper III). The analyses were done at p<0.05 level using the Statistica 8.0 (StatSoft, Inc., Tulsa, USA) software.
5. Results and discussion
In this chapter the main findings of the project are summarized and discussed.

5.1 Raw materials

The raw materials investigated were the bran fractions of two cereal types, rye and oats. Whole grains were processed in order to separate the bran fraction for hemicellulose isolations. The milled grains were separated into six size fractions after sifting. These different fractions of oat and rye grains showed changes in their composition. For example, different $\beta$-glucan contents were found in the milling fractions of oat bran as shown in Figure 7. The $\beta$-glucan content clearly decreased with decreasing particle size. Most probably, different cell wall layers can be found in the larger particles whereas a higher ratio of the endosperm fraction is present in the smaller components. A similar behavior was observed for the location of $\beta$-glucans in oat grain cell walls. In oat grain, $\beta$-glucans mainly occur in the sub-aleurone layer, while in barley and rye grains, a more even distribution between the aleurone and endosperm regions has been shown (Cui and Wang, 2009). Such great differences as in the case of oat milling fractions were not observed in the $\beta$-glucan contents of the milled rye fractions in this study (1.6% measured $\beta$-glucan content for rye fine flour); however, the $\beta$-glucan content decreased with decreasing particle size. Rye bran and rye fine flour has significantly lower $\beta$-glucan contents than oat grains. The measured arabinoxylan content of milled rye fractions showed small differences as well with decreasing particle size (data not shown).

Milled fractions were combined to prepare the bran materials used for hemicellulose extractions in order to have relatively high available hemicellulose content, nevertheless sufficiently high amounts of material were obtained from the grains. Approximately 50% (by weight) of the milled grains constituted fine flour, with a particle size below 0.15 mm, while the fraction with particle sizes in the range 0.25-0.5 mm composed 25-30% of the processed grains. The fractions with particle size between 0.25 and 1.0 mm were combined and used as the bran material for hemicellulose extraction in the case of both rye and oat grains.
The composition of the studied cereal brans is shown in Table 4. The grain composition presented here shows the climate effects of this year, and the impacts of the specific growing area, the cereal varieties, harvesting time and storage conditions, which may all affect the amounts of different components present. The rye bran composition (Table 4) showed a rather low (~13 % w/w) arabinoxylan content, which was likely a result of the process used to mill the grains. The grains were processed using a disc mill as an alternative to industrial roller milling, which could explain the high starch content and thus the low hemicellulose content in the raw material. Cereal bran materials prepared and reported in other studies were processed using industrial roller milling which can provide a good separation of the bran and aleurone layers of the grains from the starchy endosperm parts. These bran materials show a higher arabinoxylan content (20-25% by weight) and lower starch content (17-28% by weight) than found in this study and as indicated in Table 4 (Kamal-Eldin, et al., 2009, Rakha, et al., 2010). The disc milling would provide a slightly different bran structure with a higher amount of starch granules originating from the endosperm part of the grains. The non-starch glucan in rye bran was comprised mainly of β-glucan (2.8% w/w) based on glucose and starch contents (Table 4) and presumably also cellulose.

The oat bran was also obtained using a disc milling process, which provided a bran material containing 47% starch (Table 4), comparable to that reported in previous publications (Butt, et al.,
The measured β-glucan content was 7.5% which is also in the range of values reported earlier (Immerstrand, et al., 2009, Johansson, et al., 2000).

Table 4. Composition of rye and oat bran (Percent of dry weight ± standard deviation).

<table>
<thead>
<tr>
<th>Component</th>
<th>Rye bran</th>
<th>Oat bran</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylose(^a)</td>
<td>8.6 ± 0.2</td>
<td>4.0 ± 0.1</td>
</tr>
<tr>
<td>Arabinose(^a)</td>
<td>4.0 ± 0.1</td>
<td>1.6 ± 0.0</td>
</tr>
<tr>
<td>Glucose(^a)</td>
<td>60.2 ± 0.6</td>
<td>57.0 ± 1.3</td>
</tr>
<tr>
<td>Starch</td>
<td>49.6 ± 2.9</td>
<td>47.0 ± 0.3</td>
</tr>
<tr>
<td>β-glucan</td>
<td>2.8 ± 0.1</td>
<td>7.5 ± 1.3</td>
</tr>
<tr>
<td>Klason lignin</td>
<td>4.6 ± 0.2</td>
<td>4.5 ± 0.8</td>
</tr>
<tr>
<td>Protein</td>
<td>10.8 ± 0.1</td>
<td>16.2</td>
</tr>
<tr>
<td>Extractives</td>
<td>9.9 ± 0.2</td>
<td>12.8 ± 0.1</td>
</tr>
<tr>
<td>Ash</td>
<td>1.4 ± 0.1</td>
<td>2.7</td>
</tr>
<tr>
<td>Total</td>
<td>99.5</td>
<td>98.8</td>
</tr>
</tbody>
</table>

\(^a\): Carbohydrate components are presented as anhydro-sugars

5.2 Hemicellulose isolations

The aim was to isolate hemicelluloses from the raw materials with high molar mass and a relatively intact structure without significant chain degradation. The idea was to apply methods which could be implemented at pilot- or industrial scale or as part of a biorefinery system. Furthermore, the extraction processes were aimed at achieving relatively high yields and the design of processes which could operate in a cost-effective way with moderate purification costs. Thus different isolation processes were tested and the mentioned aspects were considered. The desired hemicelluloses might then be used in special applications such as films or coatings, so the molecular structure and composition, molar mass and appearance were evaluated.

5.2.1 Hot water extraction of hemicelluloses from rye and oat bran

The isolation procedure using a high-temperature treatment allowed the recovery of water-extractable hemicelluloses. Fibers, proteins and waxes were separated after the high-temperature treatment; however, additional protein separation was necessary using an autoclave treatment for protein precipitation. The remaining high protein content (8.5 %) could be a
consequence of existing covalent linkages between arabinoxylan chains and proteins. There are
varying reports in the literature. For example Ragaee et al. found that water-extracted
arabinoxylan contained 3-5% (w/w) proteins even after enzymatic digestion (Ragaee, et al., 2001),
but in contrast other researchers found the fraction after proteinase digestion contained 61-65 %
proteins (Cyran and Saulnier, 2005). The specific presence and composition of aromatic
constituents (e.g., ferulic acid) could have blocked the enzymatic action and this would be
consistent with an association between the polysaccharides and proteins in the cereal cell walls.
Further purification of the isolated hemicelluloses was needed and consisted primarily of the
removal of starch residuals.

The monosaccharide composition of hemicelluloses isolated from rye bran (Table 5)
showed a high content of xylose and arabinose, which was related to high arabinoxylan content
(approx. 65 w/w% of the isolated material) in the samples. Some glucans (24%), which were
mostly β-glucans (17%), were co-extracted with the arabinoxylans. Water-extracted arabinoxylans
represented 25% of the total arabinoxylan content of the bran which showed an efficient
extraction yield considering the low amount of water-extractable hemicelluloses present in rye
bran. However, extraction yield calculated from the starting bran material (3.2% w/w) was rather
low, which is partly due to the low hemicellulose content of the starting rye bran and a high
content of starch (50%) in the used bran. The losses during the fraction separations, dialysis and
precipitation further decreased the yield. Ragaee et al. found that 22 to 33 wt% of the total
arabinoxylan content of different rye meals was water-extractable (Ragaee, et al., 2001). Cyran et
al. achieved slightly higher total hemicellulose yields (~ 4%) than found in this study (Cyran and
Saulnier, 2005).

The composition of the isolated oat β-glucan showed significantly less protein (3.6%)
relative to the rye hemicellulosic materials. A low amount of arabinoxylan (~3%) was co-extracted,
while the remaining carbohydrate content was mainly composed of glucose. The measured β-
glucan content was lower than the measured amount of glucose (91%), which could be due to the
presence of some starch. The purity of the β-glucan extract (81% measured β-glucan content) was
comparable to values previously found for water-extracted materials, varying in the range 74-95% (Johansson, et al., 2000, Skendi, et al., 2003). The extraction yield of β-glucan-rich hemicelluloses
was 4% from the starting oat bran, which is somewhat higher than that of rye bran hemicelluloses

5.2.2 Alkali extraction of hemicelluloses from rye bran fibers

After the hot water extraction process, the water-insoluble material consisted of a fraction rich in proteins and waxes and a fiber fraction, which represented 27% of the starting rye bran. The major building components of the water-insoluble (WIS) fibers were polysaccharides. Almost 30% (w/w) of the fiber fraction was arabinoxylan; however, there was also a large amount of Klason lignin (10.3%). The measured total anhydro-glucose content of WIS was 30.1% (w/w), which probably contained a significant amount of cellulose and starch residuals, whereas the β-glucan content was only approx. 15% (w/w) of the total glucose content (measured β-glucan content: 4.7%). The washing process decreased the amount of glucose residues by 34%, proving that a significant amount originated from starch degradation.

![Figure 8: Monosaccharide composition of NaOH-extracted rye fibers with solid to liquid ratios: 1:15, 1:35 and 1:70.](image)

NaOH solutions were mixed with the WIS fraction to see the effect of the solid to liquid ratio on the isolation efficiency. Figure 8 shows the sugar components of the NaOH-extracted fiber material. The average monosaccharide compositions of the Ax 1:15 and Ax 1:70 samples were
almost identical, while the Ax 1:35 showed slightly lower average results from all the sugars. However, the overall yield was the highest for the isolated arabinoxylan Ax 1:35 material, giving 66% of the total arabinoxylan content of the washed fiber material while the corresponding yields for Ax 1:15 and 1:70 were 41% and 45% respectively (data not shown). These reported percentages were derived from the arabinoxylan content of the washed fibers and the arabinoxylan content of the isolated materials, measured with acid hydrolysis and HPLC analysis. Cyran et al used 1 M NaOH solution for arabinoxylan extraction and found that some arabinoxylan structures were closely associated with cellulose. This meant that the use of stronger alkaline solutions (e.g., 4 M NaOH) was necessary (Cyran and Saulnier, 2007). Ragaee et al. showed that higher concentrations of NaOH could dissolve more β-glucans (Ragaee, et al., 2008a); however, this also induced depolymerization of the polysaccharides. The polysaccharide content of the isolated materials for Ax 1:15, Ax 1:35 and Ax 1:70 was 91%, 76% and 90% respectively as determined by acid hydrolysis and HPLC analysis, suggesting the presence of smaller amounts of other components like proteins and Klason lignin. The presence of Klason lignin was indicated as well by the darker colour of the alkaline-extracted materials. Considering the amount of chemicals needed, the 1:35 treatment would be the most beneficial solution for such extractions, since this experiment showed the highest hemicellulose yield.

5.2.3 Rye bran hemicellulose extractions with wet oxidation

Two types of rye materials, rye bran and WIS material (see Figure 6) were processed using a hydrothermal treatment. This process resulted in a liquid fraction with a brown colour, rich in solubilized starch. The monosaccharide composition of this liquid fraction showed 48.1 g glucose, 6.0 g xylose, 1.5 g galactose and 5.4 g arabinose (in one liter liquid fraction). The high measured glucose content was a reflection of the liquefied starch and the Ara/Xyl ratio (0.90) indicated the presence of a highly branched arabinoxylan. The analysis was made before dialysis. After dialysis and precipitation, the monosaccharide analysis showed the presence of glucose and a low amount of arabinoxylan (Table 5). The high glucose content measured with a dilute sulphuric acid hydrolysis on this precipitated material indicated that the starch content was not degraded to short oligomers or monomers during the wet oxidation process, hence was not separated from arabinoxylan during dialysis and was precipitated as well.
**Wet oxidation treatment of rye fibers**

The WIS material was pretreated using wet oxidation as the rye bran. The obtained material after dialysis and ethanol precipitation showed a significantly lower glucose content and higher arabinoxylan content compared to the rye bran. The brown colour of the sample indicated the presence of lignin. The lower monosaccharide content also pointed to the presence of other components such as proteins or waxes.

### 5.2.4 Comparison of isolated hemicelluloses using different extraction processes

The monosaccharide composition and extraction yield of different isolation processes is shown in Table 5. The results suggest that the hemicellulose material with the highest purity was isolated using hot water extraction. A similar amount of glucose residues was found in the alkali-extracted material, suggesting the presence of β-glucans in the alkali-extracted material and also in the water-extracted isolate. The calculated Ara/Xyl ratios showed some variation, although it can be concluded that the materials originating from the fiber fraction of rye bran have a lower arabinose substitution than the materials from rye bran, containing parts of the endosperm region of the rye grains. The resulting Ara/Xyl ratio (0.54) in hot water-extracted hemicelluloses was in agreement with that found in other water-extracted arabinoxylans from rye (Bengtsson and Åman, 1990, Delcour, et al., 1999, Ragaee, et al., 2001). The alkali-extracted material showed an Ara/Xyl ratio of 0.34. Such decrease in branching was observed in previous studies in the outer layers of the rye grains compared to an intermediate milling fraction or whole flour (Nilsson, et al., 1996). Since the rye bran in this study contained a significant amount of the endosperm region while the used fiber fraction mainly consisted of the aleurone region and the seed coat, our findings are similar to the results found in the work of Nilsson et al.
Table 5. Monosaccharide composition of materials extracted from rye bran or rye water-insoluble fibers (WIS).

<table>
<thead>
<tr>
<th>Monosaccharide (weight %)</th>
<th>Rye bran (Hot water extraction)</th>
<th>WIS (Alkali extraction)</th>
<th>Rye bran (Wet oxidation)</th>
<th>WIS (Wet oxidation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>glucose</td>
<td>23.5</td>
<td>22.5</td>
<td>93.6</td>
<td>25.3</td>
</tr>
<tr>
<td>xylose</td>
<td>42.9</td>
<td>37.9</td>
<td>8.1</td>
<td>28.7</td>
</tr>
<tr>
<td>galactose</td>
<td>-</td>
<td>1.2</td>
<td>0.0</td>
<td>0.9</td>
</tr>
<tr>
<td>arabinose</td>
<td>23.5</td>
<td>12.7</td>
<td>3.7</td>
<td>6.7</td>
</tr>
<tr>
<td>fructose</td>
<td>-</td>
<td>-</td>
<td>3.7</td>
<td>-</td>
</tr>
<tr>
<td>Ara/Xyl</td>
<td>0.54</td>
<td>0.34</td>
<td>0.46</td>
<td>0.23</td>
</tr>
</tbody>
</table>

a: alkali extraction, applied solid-to-liquid ratio 1:35

Molar mass analysis was performed for all isolated hemicellulosic materials, using 0.01 M NaOH solution as eluent and solvent. The calculated $M_w$ values (Table 6) showed that the hemicellulose isolates had a high molecular weight and were isolated as polymeric materials. A relatively high dispersity was observed for all the samples and especially for the alkaline- and wet oxidation-extracted hemicelluloses. Hemicellulose isolates prepared with wet oxidation showed a clear decrease in molar mass, compared to the materials obtained by the two other extraction processes. Material obtained using wet oxidation showed a low arabinose substitution, low yield and a partly degraded hemicellulosic structure. Thus, this method was not used further for hemicellulose extractions.

Alkali extraction resulted in a material with high $M_w$ and a relatively high yield (66% w/w of the total arabinoxylan content). The xylan chain was less substituted than arabinoxylan produced with hot water extraction as indicated by the ratio of measured arabinose and xylose residues. However the alkaline-extracted material showed limited water solubility and possessed a brownish colour, hence the water-extracted material was favoured for later production of hemicellulose-based films.
Table 6. Molar mass averages ($M_w$, $M_n$) and dispersity index ($M_w/M_n$) of rye hemicellulose isolates.

<table>
<thead>
<tr>
<th></th>
<th>Hot water extraction</th>
<th>Alkali extraction</th>
<th>Wet oxidation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rye bran</td>
<td>WIS</td>
<td>WIS</td>
</tr>
<tr>
<td>$M_w$ (g/mol)</td>
<td>365000</td>
<td>273400</td>
<td>71100</td>
</tr>
<tr>
<td>$M_n$ (g/mol)</td>
<td>193200</td>
<td>105600</td>
<td>29500</td>
</tr>
<tr>
<td>$M_w/M_n$</td>
<td>1.9</td>
<td>2.6</td>
<td>2.4</td>
</tr>
</tbody>
</table>

5.3 Monosaccharide compositional analysis of water-extracted hemicelluloses

5.3.1 Acid methanolysis and acetylation of selected monosaccharides and acid sugars for identification

In order to establish a more accurate and selective monosaccharide compositional analysis for the isolated hemicelluloses, acid methanolysis was tested and the obtained methyl glycosides were analyzed with GCMS after derivatization. The applied derivatization procedure was acetylation. The general method used as derivatization after acid methanolysis is trimethylsilylation as opposed to acetylation (Bertaud, et al., 2002, Doco, et al., 2001, Willfor, et al., 2009). The acetylated derivatives showed excellent chromatographic properties enabling the separation of $\alpha$- and $\beta$-anomers as well as the furanose and pyranose forms of the monosaccharides. These derivatives could provide advantages in comparison to silylated monosaccharides by, for example, higher stability towards hydrolysis, simpler isotopic patterns, and relatively low molecular weights.

In order to obtain some experimental support for the assignment of the $\alpha$- and $\beta$-anomers observed in the chromatograms, methyl-2,3,4,6 tetra-O-acetyl-$\beta$-D-glucopyranoside was prepared. The analysis revealed only a single component, enabling an unambiguous assignment of the $\beta$-anomer and hence indirectly for the $\alpha$-anomer as well. All other $\alpha$- and $\beta$-anomer pairs are assumed to show an identical order of separation. The separation of the anomers is shown in Figure 9 where the pure $\beta$-anomer and a derivatized sample of a glucose standard are illustrated with mixed $\alpha$- and $\beta$-anomers.
5.3.2 Cleaving efficiency and recovery after methanolysis of dextran polymers

The cleaving efficiency of the methanolysis treatment was tested using dextran polymers with known molar masses. The tested dextran samples had molecular masses as follows: 1080, 5000, 9890, 12000 g/mol. The samples were treated the same way as the monosaccharide and hemicellulose samples and sorbitol was added as internal standard in the same amount as the dextran samples (approximately 10 mg). The results showed that the obtained amount of glucose residues correlated well with the amount of glucose in the dextran polymers, indicating the very effective cleaving found when using the acid methanolysis procedure. The ratio of the obtained peak areas of dextran standards and sorbitol resulted in a consistent value of each applied dextran standard, also suggesting sufficient cleaving efficiency and no evidence for undesired side reactions (data not shown).

![Graph showing separation of α- and β-anomers of methyl-2,3,4,6 tetra-O-acetyl-D-glucopyranoside.]

**Figure 9.** Separation of α- and β-anomers of methyl-2,3,4,6 tetra-O-acetyl-D-glucopyranoside.

5.3.3 Chromatographic pattern of selected monosaccharides and $^{13}$C labeled monosaccharides

The retention times of the monosaccharide (arabinose, glucose and xylose) derivatives were determined as well as their mass spectra in order to identify other carbohydrates in mixtures arising from the hemicelluloses. The monosaccharide standards were expected to account for the major part of the extracted hemicelluloses. Isotope dilution was applied, $^{13}$C labeled monosaccharides were analyzed and derivatized using the same procedure as for the monosaccharide standards. The applied labeled compounds were regarded as internal standards.
and used for quantification of the analyzed monosaccharides in the hemicellulose samples. The use of isotope dilution may be advantageous since the analytical equipment (normally mass spectrometry) reveals high sensitivity, precision and the use of the isotope-labeled compound as internal standard may improve chromatography due to the carrier effect.

The number of peaks on the obtained chromatograms for each monosaccharide was two or four according to the formed α- and β-anomers and furanose and pyranose ring forms. Figure 10 shows the chromatographic patterns of the pure monosaccharides D-glucose, D-xylose and L-arabinose compared with their labeled counterparts. Very similar patterns including peak ratios and retention times are apparent when comparing the ion chromatograms of the labeled and unlabeled monosaccharides.

5.3.4 Quantification of monosaccharides using 13C-labeled compounds

The electron impact (EI)-induced fragmentations of the studied monosaccharide derivatives were followed using the obtained mass spectra. The fragmentation pattern of these compounds has been studied since 1963 (Biemann, et al., 1963, Kulkarni, et al., 1986, Vouros, et al., 1999, Denekamp and Sandlers, 2005). The mass spectra of the monosaccharide derivatives were generally very comparable to those found in earlier reports and databases.

For quantification of the studied monosaccharides, extracted ion chromatograms were used. Such data may be used if the peaks have a reasonable intensity, the m/z value ensures a good selectivity, and a logical fragmentation pathway is apparent. The glucose derivative (methyl-2,3,4,6 tetra-O-acetyl-D-glucopyranoside) exhibited a relatively strong signal at m/z 243 (mass spectra not shown). This ion has previously been assigned to the consecutive loss of methyl formate and an acetoxyl radical, as supported by deuterium labeling (Biemann, et al., 1963, DeJongh and Biemann, 1963, Guvermont, et al., 1990). The mass shifts observed here in the spectra of methyl-2,3,4,6 tetra-O-acetyl-D-glucopyranoside-13C6 (m/z 243 to m/z 248) and methyl-2,3,4,6 tetra-O-acetyl-D-glucopyranoside-13C1 (m/z 243 unchanged) were in complete agreement with this previous assignment. The m/z 243/248 was chosen for EIC (extracted ion chromatogram) analysis of glucose.
Figure 10. Total ion chromatograms of acetylated methyl glycoside derivatives of: (a) D-glucose-$^{13}\text{C}_6$; (b) D-glucose; (c) D-xylose-$^{13}\text{C}_5$; (d) D-xylose; (e) D-arabinose-$^{13}\text{C}-1$; (f) L-arabinose.
The xylose derivative (methyl-2,3,4 tetra-O-acetyl-D-xylopyranoside) exhibited a strong signal at m/z 170 and initiated a series of eliminations of ketene, (i.e., m/z 170, 128 to 86). This ion has likewise been discussed in terms of a consecutive loss of methyl formate and acetic acid (Biemann, et al., 1963, DeJongh and Biemann, 1963). The m/z 128/132 pair was chosen for EIC analysis of xylose.

The arabinose derivative (methyl-2,3,5 tri-O-acetyl-D-arabinofuranoside) exhibited a strong signal at m/z 217 (mass spectra not shown). A clear shift in mass is observed in the spectra of methyl-2,3,5 tri-O-acetyl-D-arabinofuranoside-1-13C (m/z 217 to 218). This is in agreement with a single cleavage stabilizing the product ion as an oxocarbenium ion. The m/z 217/218 pair was chosen for EIC analysis of arabinose.

The isotope equilibrium was checked by plotting the ion intensity ratio of the added standard/13C labeled compound against the mixed molar ratio of added standard/13C labeled compound (Figure 11). Linear regression analysis was performed for glucose (calibration equation: \( y = 0.9112 x; R^2 = 0.9962 \)) due to no contribution from the labeled compound to m/z 243 and no contribution from the unlabeled compound to m/z 248. Similarly, linear regression was applied for xylose (calibration equation: \( y = 1.0267 x; R^2 = 0.9999 \)). In the case of arabinose, the unlabeled

Figure 11. Monosaccharide standard/13C labeled compound standard plots.
derivative has an abundance of 89 % at $m/z$ 217 and a natural abundance of 9 % at $m/z$ 218. This has the consequence that a linear regression analysis cannot be formed. Instead, a second order polynomial analysis was used (calibration equation: $y = -0.035 x^2 + 0.8952 x; R^2 = 0.9983$). A good correlation between the peak areas and added natural monosaccharide was apparent. There are minor deviations with respect to recovery, which is assigned to a slight non-equilibrium between the $\alpha/\beta$ anomers and the furanose/pyranose forms. This is taken into account using the equations obtained by regression analyses.

5.3.5 Monosaccharide analysis of selected hemicelluloses

The developed carbohydrate analysis method was applied for the water-extracted hemicelluloses from rye bran as well as for a commercial arabinoxylan, isolated from rye grains. The total ion chromatograms of the examined hemicellulose samples showed the presence of the expected monosaccharides glucose, xylose and arabinose. Glucose provided two peaks corresponding to the $\alpha$- and $\beta$-configurations of the monosaccharide pyranose ring form, while xylose showed four peaks, relating to the furanose and pyranose forms and the $\alpha$- and $\beta$-anomers. The presence of arabinose was also apparent in the chromatogram and, in addition to the two pyranose peaks, only one furanose peak was detectable in both cases. This is supposedly because the $\beta$-furanose peak eluted in a similar time-window as the $\alpha$-xylopyranose. Identification of the monosaccharides was based on their retention times and mass spectra. The labeled monosaccharides were present as internal standards, added in known amounts, and the typical ion fragments were used for their identification. The monosaccharides glucose, xylose and arabinose were quantified by the ratio of the measured peak area of the unlabeled and labeled component for the chosen fragment ions. Figure 12 shows the GCMS chromatogram obtained for the water-extracted hemicellulose sample from rye bran (WEH). A minor peak appeared at 21.081 minutes which was identified as D-galactose based on previous investigation of monosaccharides. Another small peak appeared at 20.99 minutes, which corresponded to an $\alpha$-glucofuranose ring. The identification was apparent from the mass spectra since the labeled compound was present showing a signal at $m/z$ 221 while the unlabeled compound showed a signal at 217.
The amount of recovered carbohydrates varied between 72 and 92% for the hemicellulose samples. High carbohydrate contents were expected, especially in the commercially available RAX (reported purity over 90% in both cases); however in some cases the carbohydrate content was measured below 80%. The measured monosaccharide content of the hemicellulose sample WEH contained arabininoxylan and a significant amount of glucose (Table 7), which as shown earlier may be attributable to mixed-linkage β-glucans. The carbohydrate composition of RAX was measured in other studies and similar results were for example reported by Pitkänen et al. for the contribution of the various monosaccharides to the total carbohydrate content (33% arabinose, 66% xylose and 1% glucose) (Pitkänen, et al., 2009).
Table 7. Monosaccharide composition (%) of rye hemicelluloses and birch wood xylan and their arabinose-to-xylose ratios.

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Monosaccharide composition (%)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Xyl</td>
<td>Ara</td>
<td>Glc</td>
<td>Ara/Xyl</td>
</tr>
<tr>
<td>WEH</td>
<td>47.9 ± 1.8</td>
<td>26.8 ± 1.4</td>
<td>25.3 ± 0.5</td>
<td>0.56 ± 0.05</td>
</tr>
<tr>
<td>RAX</td>
<td>64.2 ± 1.6</td>
<td>35.0 ± 1.4</td>
<td>0.8 ± 0.2</td>
<td>0.55 ± 0.03</td>
</tr>
</tbody>
</table>

Ara=arabinose, Xyl=xylose, Glc=glucose
n=2

In summary, the hemicellulose analysis method outlined here can be used to identify hemicellulose components and provide reliable quantitative data.

5.4 Enzymatic treatment of isolated rye bran hemicelluloses and investigation of the resulting arabinoxylan structure

Lichenase enzyme treatment was applied to the hot water-extracted hemicelluloses (WEH) in order to remove β-glucan contamination and enrich the WEH in arabinoxylan. The monosaccharide composition and Ara/Xyl ratio of the WEH and the lichenase enzyme-treated hemicelluloses (WE-AX) are summarized in Table 8. The analysis results showed that the enzymatic treatment was efficient as the WE-AX contained about 1% of β-glucan. There was also a minor amount of other residual glucose-containing impurity. The measured Ara/Xyl ratio of WE-AX did not change significantly when compared to that of WEH, providing evidence that the lichenase treatment did not affect the arabinoxylan structure.
Table 8. Composition of rye hemicelluloses WEH and lichenase-treated hemicelluloses (WE-AX).

<table>
<thead>
<tr>
<th>Sample type</th>
<th>WEH</th>
<th>WE-AX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrate content (%)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>89.5</td>
<td>83.1</td>
</tr>
<tr>
<td>Monosaccharide composition (%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xyl</td>
<td>42.9 ± 2.6</td>
<td>49.6 ± 2.0</td>
</tr>
<tr>
<td>Ara</td>
<td>23.1 ± 1.1</td>
<td>28.4 ± 1.0</td>
</tr>
<tr>
<td>Glc</td>
<td>23.5 ± 2.1</td>
<td>5.2 ± 0.2</td>
</tr>
<tr>
<td>Ara/Xyl ratio</td>
<td>0.54</td>
<td>0.57</td>
</tr>
<tr>
<td>Measured β-glucan content&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17.1 ± 0.5</td>
<td>1.3 ± 0.6</td>
</tr>
</tbody>
</table>

<sup>a</sup>: corresponding to the sum of Xyl, Ara, Glc
<sup>b</sup>: percent of weight ± standard deviation; n=3
Ara=arabinose, Xyl=xylose, Glc=glucose

Structural changes in the polysaccharides were further investigated and molar mass distributions in WEH and WE-AX were determined using HPSEC in a DMSO-based eluent. Results are shown in Table 9.

Table 9. Molar mass averages ($M_w$, $M_n$), dispersity index ($M_w/M_n$), and sample recovery of isolated rye hemicellulose WEH and WE-AX.

<table>
<thead>
<tr>
<th>Sample type</th>
<th>WEH&lt;sup&gt;a&lt;/sup&gt;</th>
<th>WE-AX&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>$M_w$ (g/mol)</td>
<td>270000</td>
<td>232000</td>
</tr>
<tr>
<td>$M_n$ (g/mol)</td>
<td>153000</td>
<td>190000</td>
</tr>
<tr>
<td>$M_w/M_n$</td>
<td>1.8</td>
<td>1.2</td>
</tr>
<tr>
<td>Sample recovery %</td>
<td>75</td>
<td>66</td>
</tr>
</tbody>
</table>

<sup>a</sup>: samples measured by HPSEC in a DMSO-based eluent

The WEH material showed no UV-absorbing peaks, such as phenolic acids eluting with the arabinoxylan (chromatogram not shown). The broader refractive index peaks found for the WEH sample, reflect the presence of β-glucans. Average molar masses ($M_w$, $M_n$) were calculated from the light scattering signals. The calculated molar mass values (Table 9) for the WE-AX sample were in accordance with previously analyzed water-extracted rye arabinoxylan reported by others (Pitkänen, et al., 2009). The average molar mass value of the WEH sample was slightly higher than
that of the WE-AX sample, which is consistent with the higher molar mass of β-glucans in comparison with arabinoxylans.

Proton NMR analysis was performed on the WE-AX sample in order to examine the substitution pattern on the xylan chain. The α-anomeric region of the 1H-NMR spectrum is presented in Figure 13. The spectrum showed three major peaks, a first at 5.39 ppm, indicating the α-L-Araf (1→3) substituent linked to the β-D-Xylp residue on the xylan chain, a second peak at 5.28 ppm, referring to the (1→3) linked arabinofuranose residues, and a third at 5.22 ppm, referring to the (1→2) linked α-L-Araf residues attached to the xylan chain (Pitkänen, et al., 2011). It can be concluded from the proton NMR spectrum and peak intensities that the substituted xylose units were mainly monosubstituted with arabinose residues at the C3 position, whereas the disubstituted xylose residues were less abundant and the arabinose substitution occurred at the C3 and C2 positions. Small overlapping signals at 5.22 and 5.28 ppm were observed, which are typical for the complex arabinoxylan structure and originate from α-L-Araf residues attached to two consecutive substituted xylose units. The small signals at 4.76 and 4.75 ppm might originate from the minor amount of β-glucan residues in the hemicellulose sample.

Pitkänen et al. reported 1H-NMR studies carried out on rye arabinoxylan extracted from whole grains and found that approximately one-third of β-D-Xylp residues are disubstituted and two-thirds are monosubstituted with α-L-Araf residues (Pitkänen, et al., 2009). These results as well as the overall appearance of the reported spectra correlate well with the results found in this study, and suggest that both the molar mass distribution and the substitution pattern of our WE-AX were similar to that found by Pitkänen et al.
5.5 Film casting and film properties from isolated hemicelluloses

5.5.1 Film forming and appearance

Films were cast from the isolated hemicellulose material WEH, the lichenase enzyme-treated material WE-AX, and from oat β-glucans (BG). Further, blended films were made from WE-AX and BG with weight ratios of 20:80, 50:50 and 80:20. The cast films were evaluated by their appearance, mechanical and barrier properties and the effect of the β-glucan present in the isolated rye hemicellulose was investigated.

All of the cast films were cohesive but brittle without addition of plasticizer. Film cracking occurred especially when preparing test samples of films containing mixtures of WE-AX and BG. Films from WEH were transparent but the WE-AX films had a tawny colour. This colour presumably arose from particle precipitation after the lichenase enzyme treatment. The BG samples formed transparent films with some opaque areas. A blended film, WE-AX:BG 80:20, was prepared in order to model the composition of the WEH and to study the effect of a similar amount of added β-glucan on the arabinoxylan film. Illustrations of the blended films WE-AX:BG 80:20 and 20:80 can be seen in Figure 14. Some particle aggregation can be seen in the high WE-AX-containing sample, which would also explain the finding that the amount of added WE-AX determined the colour of the blended films. The thickness of the films varied between 40 and 60 μm.
Tensile strength and elongation at break was measured and Young’s modulus was calculated from the tensile test results of all the cast film types. First, the results of the one-component films were evaluated. A significant (p<0.05) decrease in mechanical properties was observed due to removal of β-glucan from WEH (Figure 15). Average tensile strength dropped from 32.8 MPa to 15.7 MPa, although it should be noted that the standard deviation was high for the test samples. A significant decrease in elongation at break from 10.6% to 6.1% was also observed. A lower decrease was seen for the average Young’s modulus values. BG films showed tensile strength (average: 33.0 MPa) similar to that of the WEH films, but lower elongation at break (7.8%) (Figure 15).

Skendi et al. measured tensile strength values for oat β-glucan films in the range 20-40 MPa and somewhat lower elongation at break values than those of the BG films in our study. In that case, the tested oat β-glucans had lower measured molar mass, varying between 180 000 and 300 000 g/mol (Skendi, et al., 2003). Previous tensile strength results reported for arabinoxylan films without addition of external plasticizer have been higher than the WEH film test results described here. Höije et al. and Stevanic et al. measured tensile strength of 52.4 MPa and 58 MPa respectively for rye arabinoxylan films without plasticizer addition (Höije, et al., 2008, Stevanic, et al., 2011). While the measured tensile strength was higher, the films measured by Höije et al.

Figure 14. Optical microscopic images of blend films of rye arabinoxylan and oat β-glucans. (a): WE-AX:BG 80:20; (b):WE-AX:BG 20:80. The scale shown on the images is 500 μm.

5.5.2 Mechanical properties of cast films
showed a lower elongation at break (4.7%) than the WEH and WE-AX films in this study, whereas Stevanic et al. reported a higher elongation at break (8.1%). These differences are surprising since the structure of the rye arabinoxylan used in this study and applied by Höije and Stevanic et al. was shown to be very similar by HPSEC, NMR and monosaccharide analyses. A slight difference in such strength and elongation values in the various studies and the other WEH properties can be caused by variations in the water content of the test films as a consequence of differing relative humidities and other test conditions. Water has a plasticizing effect on biopolymer-based films and thus decreases the tensile strength and increases the elongation at break values (Mikkonen, et al., 2008).

Aggregation and precipitation of oligosaccharides originating from β-glucans has been previously observed as a consequence of lichenase treatment (Lazaridou, et al., 2008). It was shown that arabinoxylan is also present in this precipitate and is generated by the hydrolysis of β-glucans (Izydorczyk and MacGregor, 2000). In our work, some particle aggregation was visible in solution and in the cast WE-AX films after the lichenase treatment. Partly insoluble material may also have originated from denaturated proteins in the WE-AX material due to boiling after enzymatic treatment. These aggregated particles could act as defects in the WE-AX films and contribute to the early breaking of the samples during tensile testing. This effect could contribute to the drastic decrease in tensile properties of WE-AX compared with WEH.

Höije studied the properties of two alkali-extracted arabinoxylan fractions from barley fiber with varying β-glucan content (Höije, 2008). Treatment with NaOH resulted in extraction of both arabinoxylans and β-glucans, whereas Ba(OH)$_2$-extraction was selective for arabinoxylans. In the research carried out by Höije, the presence of β-glucan had a positive effect on film flexibility. Films containing both arabinoxylans and β-glucans (β-glucan contents = 19.2 and 28.3% of dry matter) showed twice as high elongation at break values (~6 and 7%) as the pure arabinoxylan films. These findings are in good agreement with those from the present study; however, Höije did not report differences in the tensile strength of the pure arabinoxylan films and the arabinoxylan films enriched in β-glucans.
Figure 15. Mechanical properties: tensile strength, elongation at break and Young’s modulus of pure and blended films.

Tensile properties of blended films were also investigated. The addition of BG to WE-AX showed a positive effect on the tensile strength and made the films significantly stronger. The tensile strength of the WE-AX sample after 20% addition of BG increased from 15.7 MPa to 31.2 MPa (Figure 15a), which was somewhat lower than the value obtained for the WEH sample originally containing about 20% (w/w) β-glucans. No significant difference was detected in the tensile strengths of the WE-AX:BG 20:80, WE-AX: BG 80:20 blended films, and the pure BG film. The elongation at break values of the blended films (Figure 15b) were all higher than those of the pure WE-AX or BG films. The elongation at break increased with increasing content of added BG. WE-AX films containing 20% added BG gave slightly lower elongation at break results than the WEH films (Figure 15), although BG addition had a positive effect on the elongation of the films. Films from the WE-AX:BG 80:20 blend showed similar Young’s modulus values to the WEH samples.
There was no clear trend in Young’s moduli as a function of BG loading in the mixed films (Figure 15c).

Addition of BG significantly improved the tensile strength and the elongation at break of the WE-AX films. This may be a result of intermolecular or physical interactions between the blended polysaccharides. Izydorczyk et al. reported the possibility of hydrogen bonds between certain unsubstituted fragments of the arabinoxylan chain and cellulose-like sections of the β-glucan chain, which are more or less longer β-(1→4) connected glucose residues. However, these interactions are assumed for the polysaccharides located in the plant cell walls. They are less apparent in solution where interactions are probably blocked, since attachment of the mentioned polysaccharide segments are probably hindered due to the stiff conformation of the polymers (Izydorczyk and MacGregor, 2000).

5.5.3 Barrier properties of cast films

Barrier properties, as reflected by the oxygen and water vapour permeabilities of cast films, were studied. In this respect, the properties of pure arabinoxylan and β-glucan-containing films were investigated as well as the effect of β-glucan addition.

Water vapour permeability was studied at a RH gradient 0/52% and in such circumstances the WEH films showed a significantly lower WVP than all the other studied films (Figure 16). The films from BG samples provided a significantly higher permeability value and the calculated WVP values increased with increasing BG content. The WVP value of the WE-AX:BG 80:20 film was similar to that of the WE-AX film. Thus, similar WVP values to those found for the WEH film could not be obtained by mixing WE-AX and BG in the appropriate ratio. The measured water vapour transmission rate (WVTR) and WVP values of the WEH samples were lower than or within the same range as those previously reported for corn hull and corn bran arabinoxylan samples (Peroval, et al., 2002, Zhang and Whistler, 2004). The WVP of BG was higher in this study than that of the other samples; however, it was still lower than the value of 59 g·mm/kPa·m²·d, previously found for isolated barley β-glucan films (Tejinder, 2003). Higher values for β-glucan films were expected since the polysaccharide has a higher water affinity and a higher possible number of binding sites to form hydrogen bonds in the presence of water than arabinoxylan
This suggests that the presence of β-glucan should not reduce the water vapour permeability of the arabinoxylan films, although the β-glucan-containing WEH film samples showed lower values than the WE-AX films. This might be due to more dense packing of the polysaccharide components or molecular interactions between the polymers, giving better moisture barrier properties to the WEH films.

![Water Vapour Permeability of Pure and Blended Films](image)

**Figure 16.** Water vapour permeability of pure and blended films.

All the studied films showed low oxygen permeability values which were generally similar. The OP of the WEH and WE-AX films were below 1.0 cm$^3\cdot$μm/m$^2\cdot$d·kPa, suggesting that the presence of β-glucans had no significant effect on oxygen barrier properties. The presence of incompletely dissolved and thus aggregated particles, zones of the films with changing thickness, and pinholes might have contributed to the noted differences (values between 1.30 and 1.97 cm$^3$·μm/m$^2$·D·kPa for the blended films) in permeability values between the one- and two-component films. In previous studies, the oxygen permeability of arabinoxylan films was found to be very low, in the same range as poly(vinyl alcohol) and slightly higher than that of ethylene vinyl alcohol (EVOH), both of which are commonly used as barrier plastics (Lange, et al., 2003). Rye arabinoxylan films were studied by Höije et al. and provided an oxygen permeability of
2.0 cm$^3$ μm/m$^2$·d·kPa (Höije et al., 2008) at 50% RH, which is in good agreement with the results reported here.

5.5.4 Film moisture contents

The equilibrium moisture contents (EMC) of cast films were studied at three different relative humidities and data are shown in Table 10. At 50% RH the BG films showed the highest water absorption as expected based on previous studies (Tejinder, 2003). It was also the case that BG films showed significantly higher EMC values than WEH and WE-AX films at 75% RH. Much higher EMC values were found at 98% RH for all film samples, confirming the previous finding that arabinoxylans are very hygroscopic and highly water absorbent at high RH (Gröndahl, et al., 2004). As shown in Table 10 the WEH films presented the highest water absorption (55.6%), which was surprisingly high relative to the values obtained for the WE-AX, BG and blended films.

The water sorption behavior of arabinoxylan and β-glucan films was observed by Ying et al. and found to be similar to that reported here (Ying, et al., 2011b). Water content and the glass transition temperature of arabinoxylan and β-glucan films were examined after conditioning at RH values between 11 and 91%. For RH values between 11 and 75%, the β-glucan films had higher moisture contents; however, the tendency was reversed at 91% RH, which is in good agreement with our findings. Such differences are thought to occur due to different motions of the carbohydrate chains. The average distances between the chains are shorter in the linear β-glucan than in the xylan chains with arabinose substitution, possibly causing stronger dipolar interactions in the β-glucan films (Ying, et al., 2011a). Thus β-glucan chains might form a more compact structure than arabinoxylans, resulting in fewer available OH groups for water binding and this could result in lower water absorption in films containing β-glucans at high humidities.
Table 10. EMC of pure WEH, WE-AX and BG films, and three blend films of WE-AX and BG at different relative humidities.

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Water content (%)a</th>
<th>50% RH</th>
<th>75% RH</th>
<th>98% RH</th>
</tr>
</thead>
<tbody>
<tr>
<td>WEH</td>
<td>12.3 ± 1.3b</td>
<td>13.4 ± 1.4b</td>
<td>55.6 ± 3.4b</td>
<td></td>
</tr>
<tr>
<td>WE-AX</td>
<td>11.9 ± 0.3c</td>
<td>13.7 ± 1.3c</td>
<td>41.5 ± 0.1b,c,e</td>
<td></td>
</tr>
<tr>
<td>WE-AX + BG 80:20</td>
<td>10.1 ± 0.5b,c,d,f</td>
<td>14.4 ± 1.0</td>
<td>38.1 ± 3.7b,d</td>
<td></td>
</tr>
<tr>
<td>WE-AX + BG 50:50</td>
<td>11.9 ± 0.6d,e</td>
<td>15.0 ± 1.5</td>
<td>36.5 ± 3.8b,c,e</td>
<td></td>
</tr>
<tr>
<td>WE-AX + BG 20:80</td>
<td>10.9 ± 0.1e</td>
<td>14.8 ± 1.0</td>
<td>42.4 ± 2.1b,e</td>
<td></td>
</tr>
<tr>
<td>BG</td>
<td>13.4 ± 1.5c,f</td>
<td>15.9 ± 0.3b,c</td>
<td>43.9 ± 0.6b,d,e</td>
<td></td>
</tr>
</tbody>
</table>

a: percent of weight ± standard deviation; n=3
b,c,d,e: values with the same superscript letters in the same column are not statistically different (p<0.05)

In summary, the presence of β-glucans was found to have an effect on the material properties of arabinoxylan films. In particular, the WEH material containing co-extracted arabinoxylans and β-glucans formed stronger films with higher elongation than films with reduced β-glucan content. A positive effect on mechanical properties was also observed when β-glucans were artificially added to arabinoxylans prior to film casting. The increase in tensile strength might be predicted based on the more linear structure of β-glucans as compared to the branched nature of the arabinoxylans. The higher water affinity of β-glucans might at the same time introduce a plasticizing effect as a result of higher EMCs in mixed films, which would explain the increase in elongation at break under tensile load. The greater water affinity of the β-glucans could also partly explain variations in the water vapour barrier properties of the various films. Considering the mechanical and barrier properties of the films, this research has shown that higher β-glucan contents might have generally beneficial effects when practical applications of cast xylan/β-glucan films are considered.
5.6 Nanocomposites from hemicelluloses and a fibrous nanoclay

Nanocomposite films were cast using rye endosperm arabinoxylan and sepiolite nanoclay. The carbohydrate content of the applied arabinoxylan was measured and found to be approximately 82% and had Ara/Xyl ratio of 0.57. The structure of this arabinoxylan material was recently studied by $^1$H NMR and it was found that the xylan main chain is mainly monosubstituted by arabinose residues and that double substitution is rather rare (Pitkänen, et al., 2011). The applied nanoclay, sepiolite, has a hydrated magnesium silicate structure, which is illustrated in Figure 17. Alternating longitudinal blocks and channels (tunnels) are found in the sepiolite structure, producing long needles. The blocks are built up of two layers of tetrahedral silica, sandwiching an octahedral magnesium oxide-hydroxide sheet in the centre (Chivrac, et al., 2010, Esteban-Cubillo, et al., 2008). The octahedral sheet is discontinuous, hence allowing the formation of tunnel-like micropores (channels), running parallel to the fiber-axis. The channels in the sepiolite structure are filled with coordinated water bound to the magnesium ions at the edges of the octahedral sheets and zeolitic water associated with the clay structure by hydrogen bonding inside the tunnel-like pores. Silanol (Si-OH) groups appear at the external surface of the sepiolite structure (Chivrac, et al., 2010, Kuang, et al., 2003, Sakizci, et al., 2011). The numerous silanol groups naturally provide sites for hydrogen bonding and Van der Waals interactions with the hydroxyl groups of the arabinoxylan structure in the nanocomposite, contributing to the reinforcing effect of sepiolite. Arabinoxylan molecules however most likely will not fit inside the inner tunnels of the sepiolite structure. A homogeneous distribution of the sepiolite particles in the arabinoxylan film matrix and interactions, such as hydrogen bonds, were expected to result in enhanced mechanical properties, and potentially improved barrier properties. Such structural changes and film properties were investigated.
5.6.1 Visual film properties and light transmittance

All cast films were cohesive and the thickness of the films varied between 25 and 45 μm. Light transmittance showed a general decrease with increasing clay content. The pure RAX films were not entirely transparent due to some impurities in the material, which may have been because of minor quantities of proteins precipitated during sample dissolution or lignin contamination. A significant amount of lignin was not expected in the applied RAX, since the material was water-extracted from rye grains and only minor amounts of aromatic components, such as ferulic acids were present. The light transmittance of the films is shown in Figure 18. The pure RAX and films containing lower (2.5 and 5%) amounts of added sepiolite showed a minor inflection in light transmissions at 260 nm. This could be attributable to some aromatic compounds such as proteins or lignin absorbing in the ultraviolet region, since the typical absorption of aromatic compounds such as aromatic amino acids or ferulic acid occurs in the range 200-300 nm (Li, et al., 2011). The observed differences suggest that 5% or higher amounts of
added clay were not well dispersed and therefore introduced some opacity (Rhim, 2011). This effect was more noticeable at 10 and 20% sepiolite loadings.

![Graph](image)

**Figure 18.** Light transmittance of arabinoxylan and arabinoxylan-sepiolite nanocomposite films.

### 5.6.2 Film morphology

The sepiolite distribution and presence of clay aggregates were studied with FIB-SEM (focused ion beam scanning electron microscopy) after tensile tests on the film samples. Figure 19a shows the distribution of added 2.5 wt% sepiolite to the RAX matrix while Figure 19b illustrates the nanocomposite film sample containing 5 wt% added sepiolite. The images show well-embedded clay nanoparticles with no pull-outs, illustrating strong binding between the sepiolite and the arabinoxylan chains. The lower level of clay addition is apparent in the image of the RAX-sepiolite 97.5:2.5 film which contains half of the amount of added sepiolite as the film RAX-sepiolite 95:5. The RAX matrix shows an arabinoxylan chain structure with nodules in the background. Stevanic et al. prepared arabinoxylan-bacterial nanocellulose composite films with the same RAX material used in our study and found a similar nodular morphology (Stevanic, et al., 2011). The diameter of nanoclay particles is approximately 100 nm based on the images, which size shows an appropriate scale to values reported in earlier studies (Fernandes, et al., 2011).
Figure 19. SEM images of arabinoxylan-sepiolite nanocomposites, RAX-sepiolite 97.5:2.5 (a) and RAX-sepiolite 95:5 (b).

Crystallinity changes were investigated in the nanocomposites compared to the pure arabinoxylan and sepiolite structures. A crystalline structure was not expected to be found for the arabinoxylan applied in this study as it was shown in previous reports that highly substituted cereal arabinoxylans are amorphous. With addition of a nanoparticle having a crystal structure, the formed new crystal structure of the nanocomposites was investigated.

The XRD spectra of the films and sepiolite powder are shown in Figure 20. The characteristic (110) reflection of sepiolite appeared at 8.6° (corresponding to 1.19 nm) in the low angle range (Perraki and Orfanoudaki, 2008). This peak at 8.6° corresponds to the internal channel reflections of the
fibrous structure (Chivrac, et al., 2010). The RAX film and RAX films containing sepiolite showed a broad band at ~ 22° due to the amorphous nature of the arabinoxylan. Displacement of the peak at 8.6° of the sepiolite diffractogram was observed in the XRD patterns of the nanocomposite films. Such displacement could be related to the presence of oriented molecules inside the sepiolite channels; however, in the case of a polysaccharide matrix such as arabinoxylan, as well as the minor changes observed, the peak shifting is probably due to sample positioning and reflection geometry changes from sample to sample. However, the occurrence of the diffraction peak at 8.6° as well as the broad RAX band may reflect the presence of a well separated network of sepiolite fibers in the RAX matrix.

5.6.3 Mechanical properties of films

The reinforcing effect of sepiolite nanoclay addition was examined by tensile testing. The efficiency of plasticization using mPEG addition was also studied. Unfilled arabinoxylan films without plasticizer addition showed a stress at break of 42.5 MPa (Figure 21), which is similar to but slightly lower than values reported previously for this material and higher than found for WEH and WE-AX rye hemicelluloses in our previous study (32.8 MPa for WEH, see chapter 5.5). For example, Höije et al. and Stevanic et al. measured tensile strengths of 52.4 MPa and 58 MPa respectively for rye arabinoxylan under the same RH, while the temperature was slightly higher (30°C) in the tensile tests of Stevanic et al. (Höije, et al., 2008, Stevanic, et al., 2011).
Addition of sepiolite clay resulted in a very significant increase in film strength and stiffness (Figure 21). As an example, addition of 2.5% sepiolite to RAX gave an increase in Young's modulus from 2.3 to 3.9 GPa and an increase in tensile strength from 42.5 to 73.6 MPa. Further addition of clay showed only a minor additional increase in the Young's modulus values and no significant increase in the tensile strength values. No statistically significant differences were detectable in the strain at break values of the pure RAX and RAX-sepiolite composites without plasticizer addition. The addition of mPEG plasticizer had the expected effect of decreasing film stiffness and strength while increasing the elongation values. In the case of the RAX-sepiolite films with added mPEG, a slight increase in the Young's modulus and a decrease in the tensile strength values were observed with increasing sepiolite content. The elongation at break values showed values approximately four to five times higher than those of the non-plasticized films. A decreased elongation could be seen with 5 and 10% sepiolite content in the films.

The reinforcing effects of sepiolite addition have been investigated with other biopolymers such as chitosan and starch. Chivrac et al. incorporated 3 and 6% (wt) sepiolite into starch and a cationic starch matrix and found an increase (up to ~2.5 times higher values compared to pure
wheat starch) in the Young’s moduli with clay addition (Chivrac, et al., 2010). Mechanical properties of starch-based nanocomposites were compared with addition of a layered-type of nanoclay, montmorillonite. It was found that the fibrous sepiolite increased the Young’s modulus and the tensile strength to a greater extent than montmorillonite addition, presumably due to the morphology of the clay, the increased crystallinity of the nanocomposites and more favoured interactions between the starch matrix and the sepiolite. Similar behaviour was found in gelatin films when sepiolite was compared with layered silicates (Fernandes, et al., 2011). A reinforcing effect was observed similar to previous results reported in the literature. Due to the presence of numerous silanol groups on the surface of the sepiolite fibers, strong interaction can be developed with the film matrix when the sepiolite is well dispersed. Darder et al. (2006) showed that sepiolite addition could double the Young’s modulus; however, this finding was obtained in films containing very high sepiolite loadings (Darder, et al., 2006). The high sepiolite loadings (3 to 91 g chitosan per 100 g of sepiolite) resulted in highly fragile films, hence the strain at break values of such films could not be measured and tensile strength values were not reported.

As concluded from the XRD and FTIR results (results not shown), interaction, presumably through hydrogen bonding between the sepiolite fibers and the xylan chains took place and these interactions contributed to a great increase in the stiffness of the material. It is apparent from the results that above a clay content of 5 wt%, fiber aggregation is more probable, which will contribute to the breakage of the films and tensile strength and Young’s modulus values will not increase further. We may however assume that the sepiolite fibers are themselves able to form a network, hence providing a reinforced nanocomposite material with superior mechanical properties compared to the pure arabinoxylan matrix. Considering the high aspect ratio of sepiolite particles and the fiber length and diameter, a percolation threshold can be calculated. Since the dimensions of sepiolite fibers show a great variation in different reports (Bilotti, et al., 2008, Fernandes, et al., 2011, Tunc, et al., 2011), a range of calculated values can be obtained for the percolation threshold between 0.6% and 3.5% if a cylindrical sepiolite shape is assumed ($P_c=0.7/r$, where $P_c$ is the percolation threshold for cylindrical shaped particles and $r$ is the aspect ratio, calculated from the ratio of length and diameter of sepiolite fibers) (Yudin, et al., 2008).
The calculation suggests that an added amount of sepiolite, which is above the critical percolation threshold of approximately 2% (v/v) would result in highly increased strength and stiffness in the nanocomposite materials. As supported by the tensile data presented here, films containing sepiolite at loadings much above the estimated $P_c$ do not show significant further increases in tensile strength and stiffness.

5.6.4 Barrier properties of films

Water vapour barrier properties of cast xylan/sepiolite films were studied by measuring the water vapor permeability of the films. The measured values were $2.6 \pm 0.3$, $2.5 \pm 0.0$, $3.3 \pm 0.4$ and $3.1 \pm 0.3$ (g·mm/kPa·m²·day) for the RAX, RAX-sepiolite 97.5:2.5, 95:5 and 90:10 films respectively. These data show that sepiolite addition up to 10% had no significant influence on
water vapour permeability. Even though sepiolite could theoretically create a longer and more difficult path for water molecules to diffuse through the films, sepiolite fibers are hydrophilic, embedded into a hydrophilic and poor water barrier matrix and hence a positive effect in terms of reduced water vapour permeability is not seen. Further, the barrier effect commonly noted when plate-like clays are well dispersed in biopolymer films is not observed here, which is consistent with the different fibrous morphology of sepiolite and less likelihood that water vapour permeability will be reduced through a tortuous path effect (Grunlan, et al., 2004).

The effect of sepiolite nanoclay addition on the arabinoxylan film oxygen permeability was studied and the results were 0.18 ± 0.05, 0.38 ± 0.23, 0.17 ± 0.13 and 0.19 g·mm/kPa·m²·day for RAX, RAX-sepiolite 97.5:2.5, 95:5 and 90:10 films respectively. Low results were expected since hemicellulose films have been proven to be excellent oxygen barriers, showing values in the range 0.16-3.2 cm³·μm/m²·day·kPa for similar hemicellulose film types (Gröndahl and Gatenholm, 2007, Höije, et al., 2008, Mikkonen, et al., 2009). In this case, as with water vapour, sepiolite addition had no significant effect on oxygen permeability and, as above, the lack of a tortuous path effect when using a fibrous clay as an additive may provide an explanation.

In summary, transparent and highly reinforced arabinoxylan-sepiolite nanocomposites were prepared. Arabinoxylan films containing 2.5-10% sepiolite were cast from aqueous suspensions and characterized by various physical methods. As expected, sepiolite films were well embedded in the xylan matrix as illustrated by scanning electron microscopy. X-ray diffraction showed the characteristic basal peak for sepiolite which showed no significant shift at the different loadings in the xylan film, which is consistent with the results of other results on biopolymer-sepiolite nanocomposite films (Chivrac, et al., 2010, Darder, et al., 2006). Mechanical testing revealed very significant increases in Young's moduli and tensile strength, which exceeded previously reported values for xylan/nanocellulose films. Unlike layered nanoclays, addition of sepiolite fibers did not reduce the water vapour or oxygen permeability of test films.
6. Summary in relation to the aims of the study

In the study presented in this thesis, hemicelluloses were isolated from cereal brans using different processes. The fine structure and composition of these hemicelluloses were determined with various analysis methods. Polysaccharide films were cast and strength, barrier and morphological properties were studied in order to evaluate the possibilities of using cereal hemicelluloses in special material applications.

Several methods were proven to be efficient for high molar mass hemicellulose isolations, without initiating significant degradation of the arabinoxylan structure. However, hot water extraction provided a material with higher molar mass, less arabinose substitution and higher water-solubility than the material isolated with alkali. Hemicelluloses obtained by wet oxidation had a significantly decreased molar mass, since the applied high temperature and pressure resulted in a partly degraded structure. Hot water extraction was shown to be applicable for hemicellulose extractions from cereal brans, as this method is mild and does not require high amounts of applied chemicals; however the resulting yields are rather low. Therefore, a non-purified material (isolated with hot water extraction) might be used for further processing or complementary methods would be needed to isolate other types of hemicelluloses such as alkali-extractable xylans or β-glucans or other components from cereal brans, like proteins or fibers, in order to make extraction processes economically feasible on a larger scale. The water-extracted hemicelluloses were then used for further analysis and also for film casting.

The composition and structure of the hot water-extracted hemicelluloses were analyzed in detail. In order to follow the efficiency of the isolation processes and differentiate between the compositions of the different hemicellulose types, accurate, relatively fast and easily implemented analysis methods are required. For complete structural analysis, the application of several techniques is also necessary. A new carbohydrate analysis method showed similar results to those found earlier, but it was also proven that this method, based on acid methanolysis, is very sensitive towards hemicelluloses and very small sample amounts could therefore be analyzed. It was found that rye bran hemicelluloses mainly constituted of arabinoxylan in which the xylan main chain is to a large extent mono-substituted with arabinose units and that double substitution is
less significant. The other hemicellulose type isolated from rye bran was mixed-linkage β-glucans, which were co-extracted with arabinoxylans.

The isolate obtained with hot water extraction contained biopolymer contaminants, a minor amount (8 wt%) of proteins and a significant amount (22 wt%) of mixed-linkage β-glucans besides the extracted arabinoxylans. The isolation process would be more cost effective if such contaminants had no negative effects on the properties of the target materials and there was no need for their specific removal. Thus, the effects of β-glucan content present in the rye isolate and artificially added β-glucan from oat bran were studied in terms of rye arabinoxylan film properties. It was found that β-glucan contamination improved the mechanical properties and lowered the water-vapour permeability of the films, but had no significant effect on oxygen permeability. These differences might occur as a result of more dense packing of the two polysaccharides (arabinoxylan and β-glucans) in the cell walls, physical or chemical interactions between them or their different configurations since β-glucans have a more linear structure compared to the branched arabinoxylans. However, the details of the interactions between the two polysaccharides were beyond the scope of this study and need further investigation. This fundamental study on the effect of the presence of β-glucans on arabinoxylan behaviour suggested that additional purification steps involving β-glucan removal might be undesirable for some film applications. It could be advantageous to study the effects of other contaminants like proteins, glucose oligomers from starch, or even the structure and chain length of these oligomers/polymers. The film studies on the water-extracted hemicelluloses also confirmed that the chosen isolation processes were suitable for providing films with very low oxygen permeability values.

As indicated in the Introduction to the thesis, the eventual application of hemicellulose films could be in materials for the packaging sector, in which particular mechanical and barrier properties are often required (e.g., for food packaging). For that reason, the study on arabinoxylan/sepiolite films was included as a potential new direction for development of films with enhanced properties. The results of this study have provided new knowledge and shown that very significant increases in film strength and stiffness are possible, albeit at the cost of reduced extension under load. Furthermore, unlike the case of layered MMT nanoclays, the addition of sepiolite fibers was not an effective way of enhancing gas barrier properties. In the case of
hemicellulose films, the oxygen barrier properties are already quite impressive; however, for many practical applications, significant reduction in water vapour barrier properties is also needed and MMT addition or a combination of MMT and sepiolite may be needed for this reason. Easier handling of hemicellulose films will be possible through addition of plasticizers such as mPEG, although with trade-offs in terms of other mechanical and barrier effects. The lack of thermoplasticity will necessarily limit use of xylan films (e.g., in multi-layer laminates), reinforced or not, until such time as new thermoplastic derivatives can be developed and implemented. Finally, it should be noted that the use of nanoclays in polymer films, especially in respect to food packaging, is coming under increased scrutiny in terms of any migration effects and toxicological implications and any future such use of sepiolite nanoclay will need to be considered in this light.

It was shown in this study that a by-product from the milling industry can be successfully used for heteropolysaccharide isolations and for further utilization in bio-based materials, for instance for food packaging. Knowledge was gained about the structural conformation of the polysaccharides and connection was made between hemicellulose types and film properties. A novel method of nanoclay addition was successfully applied in hemicellulose film casting, and significantly enhanced mechanical properties were seen. Even though the isolation process requires further development and the prepared and reinforced hemicellulose films need to gain higher resistance in highly humid environments in order to be industrially useful materials; it is my hope that this work shows a possible direction of utilizing components of agricultural by-products as new polymeric materials and contributes to the knowledge of researchers bringing hemicelluloses to the centre of attention as potential raw materials for industrially useful biopolymers.
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References


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Paper I

ISOLATION AND CHEMICAL CHARACTERIZATION OF HEMICELLULOSES FROM RYE BRAN

Sárossy Zs., Egsgaard H., Plackett D.*

Biosystems Division, Solar Energy Programme, Risø National Laboratory for Sustainable Energy, Technical University of Denmark, DK-4000 Roskilde, Denmark, Tel: +45 2132 6261, zssa@risoe.dtu.dk

ABSTRACT: Several methods have been suggested for hemicellulose isolation from cereals and cereal brans and extraction processes for water soluble hemicelluloses have been developed for soluble dietary fibers in the food industry. Using these methods, water soluble hemicelluloses can be readily extracted as high molecular weight polymers and the relevance of these materials can be evaluated in non-food applications. Water-soluble hemicelluloses of rye bran were extracted with a high-temperature treatment combined with enzymatic starch removal. After the hot water extraction, non-soluble fibers and protein fractions were separated and the washed fiber fraction was further treated with alkali (NaOH) solution with different solid to liquid ratios. The ratio of arabinoxylans (~65%) and β-glucans (~25%) were similar in the water-extracted and alkali-extracted materials, however their ara/xyl ratio differed. The alkali-extracted arabinoxylan was less substituted with an ara/xyl ratio of 0.35, while the water-extracted material had an ara/xyl ratio of 0.54. High molar mass hemicellulose materials were isolated with an average molecular weight of 700,000 g/mol with both isolation processes.

Keywords: hemicelluloses, agricultural residue, isolation

1 INTRODUCTION

Cereal brans such as those extracted from wheat and rye are by-products of the conventional milling process. They are mainly used as animal feed [1] although many industrial applications can be found such as in viscosity modifiers, gelling agents or tablet binders [2]. Furthermore, there is an increasing interest in using hemicellulose-rich dietary fibers from cereal brans for human consumption. Arabinoxylans and mixed linkage β-(1→3), 1→4)-D-glucans (henceforward denoted as β-glucans) are constituents of cereal dietary fibers and have been studied because of their medicinal effects. They can reduce the occurrence of diabetes, cardiovascular diseases or colon cancer and also reduce blood cholesterol and glucose levels [2-4]. Xylans show excellent emulsifying properties and can be applied as protein foam stabilizers [5]. Considering the molecular and physicochemical properties of these molecules, there is considerable potential for formation of value-added materials. The hemicellulose-rich cereal brans are potential sources for biopolymers due to their arabinoxylan content.

The bran forms the outer parts of the grains including several layers of the grain coat (e.g. the pericarp, cuticle, the testa (or the seed coat) and the aleurone layer). Commercial bran preparations also contain variable amounts of the starchy endosperm and germ depending on the milling process [6, 7]. The total arabinoxylan content of the bran is usually higher than that in the endosperm and therefore milling leads to different fractions with different hemicellulose contents. Although more arabinoxylan can be found in the bran, the amount of water-extractable arabinoxylan is higher in the endosperm-rich fractions [7].

Rye bran hemicelluloses are mostly arabinoxylans and β-glucans to a minor extent and both are embedded and bound with other components in the secondary cell walls. Rye water-extractable arabinoxylans typically have a chain of (1→4)-linked β-D-xylpyranose units containing L-arabinofuranose residues connected principally to the C3 or C2 and C3 positions [7]. The arabinoose units can be ester-linked to ferulic acid residues which may form diferulic acid bridges neighboring with arabinoxylan chains [8]. Arabinoxylans can probably also be covalently associated with proteins through ester-linked ferulic acids and tyrosine residues [8, 9]. In cereal grain cell walls, the arabinoxylan is closely associated with mixed linkage β-glucans as well, especially in the endosperm part [10].

Extraction of hemicelluloses from the cell walls is based on their solubility and can be carried out in neutral or alkaline solutions [11]. Hence, hemicelluloses are divided into two fractions: water-soluble and water-insoluble [12]. Difficulties in carrying out water extraction of cereal bran xylans may arise because hemicelluloses is bound to lignin or cellulose through ferulic acid bridges and also because of hydrogen bonding between the non-substituted xylose residues and the cellulose chains [7, 13, 14].

Several processes have been introduced for hemicellulose isolation from grain crops and from cereal brans, involving water and alkali extraction as well as other combinations such as alkali and hydrogen peroxide, alkali and chlorite solutions or dimethyl sulfoxide [15]. In addition, pilot-scale isolation of cereal xylans has been demonstrated, indicating the feasibility of scaling up to an industrial level [16-19].

Only 20-40% (w/w) of cereal grain hemicelluloses is typically water-extractable [7, 20, 21]. Water extraction allows the isolation of high molar mass hemicelluloses and helps preserve the hemicellulose structure although the resulting yields are relatively low [15]. A general method has been demonstrated for water extraction by Bengtsson and Åman [22]. Yields can be highly improved by extraction with other solvents, most commonly applied under alkaline conditions. Such treatments can cause deacetylation in the case of certain hemicelluloses so the original structure will not then be preserved. Selective arabinoxylan extraction, avoiding the co-isolation of β-glucan, can be performed with barium hydroxide solution contrary to sodium or potassium hydroxide solutions [14, 23]. Separation of arabinoxylans and β-glucans can also be performed via precipitation with saturated (NH₄)₂SO₄ or through enzymatic digestion [24, 25].

Alkaline extractions are often associated with lignin removal using sodium hypochlorite, chlorine or hydrogen peroxide treatments [13, 26]. Higher yields can be obtained from lignified materials using dimethyl sulfoxide as a delignifying agent but the use of this solvent is not applicable in pilot scale or industrial
isolation processes [15]. As a consequence, a range of multi-step extraction processes have been proposed for such polysaccharide isolations [14, 23, 25, 27-30]. Additional enzyme treatments are usually necessary to obtain a high-purity hemicellulose extract but the presence of components such as starch and proteins can in addition hinder the isolation of xylans [31, 32]. Amylase enzymes, such as α-amylase and amyloglucosidase are applied for starch degradation and protein removal is generally carried out with protease enzymes [33]. Additional treatments like ultrasonication can be of benefit by providing separation of co-extracted starch and protein from the isolated hemicelluloses. Hollmann et al. showed that ultrasonication reduced the extraction time of alkali-treated arabinoxylans from wheat bran [26].

The present study was aimed at isolation of high molar mass hemicelluloses from rye bran. These hemicelluloses might then be used to produce industrially useful biodegradable materials. The extractability, chemical composition and structure of the water-extractable hemicelluloses was examined based on a hot water isolation process, while the residual water-insoluble material was subjected to an alkaline treatment. The molar mass distribution of the isolated hemicelluloses and the effect of isolation method on hemicellulose structure were studied.

2 EXPERIMENTAL

2.1 Materials
Rye (Secale cereale) cultivar Carotop was grown in Denmark in 2008. Rye grains were disc milled and the fine flour fraction was separated. Material with particle size in the range 0.25-1.0 mm and with a mean diameter of 0.5 mm was used for hemicellulose extraction. The bran fraction was analyzed in accordance with the procedures described below.

Thermostable α-amylase Termamyl SC was obtained from Novozymes A/S (Bagsvaerd, Denmark). Amyloglucosidase (EC 3.2.1.3 from A. niger) was purchased from Megazyme International Ireland Ltd. (Bray, Ireland).

2.2 Composition of rye bran
Total sugar composition was determined by HPLC analysis after sulphuric acid hydrolysis. In this procedure, 1.5 ml of 72% H2SO4 was added to 0.16 g sample and analysis after sulphuric acid hydrolysis. In this procedure, Total sugar composition was determined by HPLC. Rye (Secale cereale) cultivar Carotop was grown in Denmark during the extraction procedure. Water-insoluble material (WIS), bran fibers, proteins and waxes were separated from the supernatant syrup by centrifugation (approx. 6000 g for 15 minutes). The syrup (water soluble – WS) fraction was treated again with Termamyl SC for 45 minutes at 95°C. Treated sugar syrup was autoclaved for 5 minutes at 120°C and α-amylase was deactivated. Further separation of precipitated proteins was performed by centrifugation (approx. 6000 g, 10 minutes). The pH of the samples was reduced to 4.5 with 5 M HCl. Enzymatic digestion of glucose oligomers was performed with amyloglucosidase (dosage: 0.2 w/v% of residual starch mass), starch was gelatinized for 45 minutes at 95°C. Fragmentation of particles was carried out using a wet mill (Mannesmann, Remscheid, Germany) during the extraction procedure. Water-insoluble material (WIS), bran fibers, proteins and waxes were separated from the supernatant syrup by centrifugation (approx. 6000 g for 15 minutes). The syrup (water soluble – WS) fraction was treated again with Termamyl SC for 45 minutes at 95°C. Treated sugar syrup was autoclaved for 5 minutes at 120°C and α-amylase was deactivated. Further separation of precipitated proteins was performed by centrifugation (approx. 6000 g, 10 minutes). The pH of the samples was reduced to 4.5 with 5 M HCl. Enzymatic digestion of glucose oligomers was performed with amyloglucosidase (dosage: 0.2 w/v% of residual starch mass) at 60°C for 45 minutes. Sugar syrup was diaлизed (MWCO 12000-14000 Da) at room temperature for 24 hours against water to remove the glucose and oligomer units originating from starch. The aqueous extract was collected and precipitated with an equal volume of ethanol (96% v/v) and left overnight at 4°C. The precipitate was collected after centrifugation (approx. 6000 g, 20 minutes). Hemicellulose gum was washed with a 1:1 (v/v) mixture of ethanol and water. The gum was then resuspended in distilled water and freeze dried. The fiber fraction of the separated WIS material was extracted with NaOH according to Ragaee et al [24]. The bran fibers were washed with 500 ml, centrifuged for 15 minutes at approx. 6000 g and dried at 45°C overnight. NaOH extraction was performed with 1 M NaOH at 25°C for 2 hours with continuous magnetic stirring at fiber to liquid ratios of: 1:10; 1:35; 1:70. The mixture was
neutralized with 5 M HCl after the treatment and centrifuged for 20 minutes at approx. 4000 g. The hemicellulose-containing supernatant was dialyzed (MWCO 12000-14000 Da) for 24 hours at room temperature against deionized water and hemicelluloses were precipitated with ethanol then freeze dried as described above.

![Figure 1: Hemicellulose isolation procedure starting from rye bran](image)

### 2.4 Sugar composition of the isolated material

Monosaccharide composition was determined by HPLC analysis. Before analysis, samples were treated with 4% (w/v) sulphuric acid and autoclaved for 10 minutes at 121 °C. Samples were neutralized with CaCO₃ and filtered (45 μm) for HPLC analysis. Samples were washed as follows: 25 ml Millipore water, 25 ml 2M HCl, 25 ml Millipore water, 25 ml 5% NaHCO₃, 25 ml Millipore water, while the organic phase was isolated and then dried with anhydrous Na₂SO₄.

Monosaccharide standards (D-glucose, D-xylose, D-arabinose) and acids (D-galacturonic acid, D-glucuronic acid) were treated with the above described method as well. The derivatized sugars were analyzed by GC-MS using a Hewlett Packard HP 6890 gas chromatograph interfaced to a HP5973 Mass Selective Detector (Agilent, Denmark). A sample of 1 μl was injected using an HP 7683 auto sampler (Agilent, Denmark) and introduced in a split mode (1:20). The source and rod temperature were 230 °C and 150 °C respectively. The products were separated using a 0.32 mm i.d. x 30 m WCOT fused silica column coated with VF-23ms at a thickness of 0.25 μm (Analytical, Denmark). The carrier gas was He at a flow rate of 1.2 ml/min. Separation of a wide range of products was achieved using a temperature program from 70 °C to 250 °C. Full mass spectra were recorded every 0.3 s (mass range m/z 40 – m/z 450). Products were identified using NIST search engine, version 2.0 f. (Agilent, Denmark).

### 2.6 Size exclusion chromatography analysis

Molar mass determinations were carried out using size exclusion chromatography (SEC). Samples were dissolved in 1M NaOH (4 mg/ml) by stirring overnight, diluted four times in the eluent (0.01 M NaOH, 50 mM NaCl, pH 12) and filtered using 0.45 μm syringe filters (PTFE) before analysis. Samples (200 μl) were injected on a TSK-Gel G4000PW column (7.5 x 600 mm, ToSoHaas, King of Prussia, USA) with a TSK-Gel G2500PW guard column (7.5 x 600 mm). The eluent flow rate was 0.5 ml/min. Three detectors were used to monitor the resulting peaks: a light scattering detector (Model 270 dual detector, Viscotek Corp.), a differential refractometric detector (Shimadzu) and a UV-VIS photodiode array detector (Shimadzu). Conventional calculations were made using TriSEC 3.0 software (Viscotek Corp.). Data were referred to pullulan standards in the molar mass range of 5600-1.6 mill g/mol.

## 3 RESULTS AND DISCUSSION

### 3.1 Composition of rye bran

The composition of rye bran is shown in Table 1. The high starch content of ~50% w/w should be noted. This amount is in contrast with the starch content of cereal brans provided by industrial mills, which typically vary in the range of 13-28% w/w [6, 14, 30]. The grains were processed using a disc mill instead of industrial roller milling which may explain this difference starch content and probably reduced the hemicellulose content in the raw material [4, 6]. The disc milling supposedly provided a slightly different bran structure with a higher amount of starch granules originating from the endosperm. Starch molecules are attached to the aleurone layer of the grains and were separated by the milling. The starchy endosperm particles likely originated mainly from the outer parts of the endosperm, the subaleurone and prismatic cells, since the inner endosperm parts were separated and recovered as fine rye flour [31]. As a result, the pentosan content of the bran material was lower (~13% w/w) when disc milling was used. The non-
Starch glucan was comprised mainly of β-glucan (2.8% w/w) and also cellulose. Nilsson et al. reported a higher amount of β-glucans (3.4%) which was nevertheless in a very similar range. Rakha et al. found 4.4% β-glucan, while Kamal-Eldin et al. measured 5.3% in a rye bran from Finland [4, 6, 14]. The cellulose and Klason lignin contents were in accordance with the results given for rye bran in previous reports [6]. The ash content of the material was lower than previously measured values (2.8-6.5% w/w) as was the measured protein content [6, 31]. Minerals are in general concentrated in the bran fraction with the lowest mineral content in the endosperm part of the grains. Compositional differences therefore occurred due to the different milling procedures, which will influence the amount of endosperm particles in the material.

### Table 1: Composition of rye bran

<table>
<thead>
<tr>
<th>Component</th>
<th>g/100 g dry material</th>
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<tbody>
<tr>
<td>Xylan</td>
<td>8.6 ± 0.2</td>
</tr>
<tr>
<td>Arabinan</td>
<td>4.0 ± 0.1</td>
</tr>
<tr>
<td>Glucan (starch)</td>
<td>49.6 ± 2.9</td>
</tr>
<tr>
<td>Glucan (non-starch)</td>
<td>10.6</td>
</tr>
<tr>
<td>β-glucan</td>
<td>2.8 ± 0.1</td>
</tr>
<tr>
<td>Klason lignin</td>
<td>4.6 ± 0.2</td>
</tr>
<tr>
<td>Protein</td>
<td>10.8 ± 0.1</td>
</tr>
<tr>
<td>Extractives</td>
<td>9.9 ± 0.2</td>
</tr>
<tr>
<td>Ash</td>
<td>1.4 ± 0.1</td>
</tr>
</tbody>
</table>

#### 3.2 Water-extractable hemicelluloses

The isolation procedure shown in Figure 1 using a high temperature treatment allowed the recovery of water-extractable hemicelluloses. The wet milling had the effect on making the starch granules more available for the amylase enzyme when compared with previous extractions, starch granules were more available to the α-amylase and the final glucose content decreased by approx. 20% (data not shown). Fibers, proteins and waxes were separated after the high temperature treatment; however, additional protein separation was necessary. Remaining proteins were precipitated during the autoclave treatment and separated by centrifugation. Further purification of the isolated hemicelluloses was needed and consisted primarily of the removal of starch residuals. After amyloglucosidase enzyme treatment, dialysis was performed. Dialysis was applied in order to remove mono- and oligosaccharides as well as buffer ions, peptides and proteins which were smaller than the membrane cut-off value (12000-14000 Da). Fractional precipitation of the isolated hemicelluloses was not applied to avoid further loss of the material and decreasing yields. Delcour et al. applied a clay treatment in order to reach further purified material with protein separation which left no residual proteins in the arabinoxylan material [19]. With the applied method, a 93.4% pure arabinoxylan was isolated which is a higher purity than described earlier by Bengtsson and Åman (72%) [22]. Faurot et al. applied a heat treatment to precipitate water-soluble proteins from the supernatant phase after water extraction from wheat flour, which treatment led to relatively high protein content in the pentosan extracts, varying from 30% to 50% w/w [18]. The high protein contents could be further decreased with proteinase treatment since the precipitation and separation by centrifugation resulted in significant residual protein amounts.

Water-extracted arabinoxylans represented 25% of the total arabinoxylan content of the bran which showed an efficient extraction yield considering the low amount of water-extractable hemicelluloses. Ragaee et al found that 22 to 33% of the total arabinoxylan content of different rye meals was water extractable [39]. However extraction yield calculated from the starting bran material was rather low, since the isolated material was only 2.7% w/w of the starting bran. The losses during the fraction separations, dialysis and precipitation further decreased the yield. Cyran et al. reached slightly higher yields, approximately 4% [31]. The resulting sugar composition (Table 2) showed a major amount of xylose and arabinose and a significant amount of glucose monomers. Presumably, the dialysis process removed most of the degraded starch molecules and the extraction method allowed the co-isolation of β-glucans. The resulting 0.54 ara/xyl ratio was in agreement with other water-extracted arabinoxylans isolated from rye [19, 22, 39]. The high protein content might be a consequence of existing covalent linkages between arabinoxylan chains and proteins. Ragaee et al. found that water-extracted arabinoxylan contained 3-5% (w/w) proteins even after enzymatic digestion [39], while Cyran et al found the fraction after proteinase digestion to be enriched with 61-65% proteins [31]. The specific presence and composition of aromatic constituents could have blocked the enzymatic action and this would be consistent with an association between the polysaccharides and proteins in the cereal cell walls.

### Table 2: Composition of water-extracted hemicelluloses

<table>
<thead>
<tr>
<th>Component</th>
<th>g/100 g dry material</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total monosaccharides</td>
<td>92.1</td>
</tr>
<tr>
<td>Arabinose</td>
<td>22.5</td>
</tr>
<tr>
<td>Xylose</td>
<td>41.7</td>
</tr>
<tr>
<td>Glucose</td>
<td>23.6</td>
</tr>
<tr>
<td>Galactose</td>
<td>1.6</td>
</tr>
<tr>
<td>Fructose</td>
<td>2.8</td>
</tr>
<tr>
<td>ara/xyl</td>
<td>0.54</td>
</tr>
<tr>
<td>Protein</td>
<td>11.4</td>
</tr>
<tr>
<td>Yielda</td>
<td>3.0</td>
</tr>
</tbody>
</table>

a: Expressed as weight percentage of rye bran

#### 3.3 Alkaline-extractable hemicelluloses

After the water extraction process, the amount of water-insoluble material was approx. 40% (w/w), which consisted of a fraction rich in proteins and waxes and a fiber fraction. Nilsson et al. isolated polysaccharides from 3 milling fractions of rye, a bran, an intermediate and a flour fraction [14]. After water extraction, the remaining WIS material constituted 50% of the starting bran and 25% for the intermediate fraction and found a starch content of less than 2% in these fractions. The WIS fiber fraction was separated (Figure 1) and the composition was analyzed and is shown in Table 3. The obtained fiber fraction made up 27% of the starting rye bran. This separated amount of fibers was in good agreement with previously found yields after extraction.
with α-amylase and proteinase enzyme treatment on rye grain outer layers [40]. The major building components of the WIS fibers were polysaccharides. Almost 30% w/w of the fiber fraction was arabinoxylan; however there was also a large amount of Klason lignin and a high proportion of proteins present in the fraction. A fairly high percentage of the measured glucose residues originated from cellulose and starch residues since the β-glucan content was only approx. 15% (w/w) of the total glucose content. The washing process decreased the amount of glucose residues by 34%, proving that a significant amount originated from starch degradation. The protein content was ~13% and higher than that previously measured in the study by Cyran et al [40].

Table 3: Composition of the WIS material (before alkaline treatment)

<table>
<thead>
<tr>
<th>Component</th>
<th>g/100 g dry material</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>18.4</td>
</tr>
<tr>
<td>β-glucan</td>
<td>4.7</td>
</tr>
<tr>
<td>Total monosaccharide</td>
<td>60.9</td>
</tr>
<tr>
<td>Arabinose</td>
<td>9.1</td>
</tr>
<tr>
<td>Xylose</td>
<td>18.4</td>
</tr>
<tr>
<td>Glucose</td>
<td>33.4</td>
</tr>
<tr>
<td>Klason lignin</td>
<td>10.3</td>
</tr>
</tbody>
</table>

Different ratios of the washed rye fiber and 1 M NaOH solutions were mixed to see the effect of the solid to liquid ratio on the isolation efficiency. In order to separate the resulted small molecules, dialysis was used. Figure 2 shows the sugar components of the NaOH extracted fiber material. The ara/xyl ratios (data not shown) were in the same range in all cases (0.35, 0.34 and 0.36 respectively), which suggested that the xylan chain was less substituted than in case of the water-extracted arabinoxylan. The decrease in branching was observed in previous studies in the outer layers of the rye grains compared to an intermediate milling fraction or a whole flour [14]. The monosaccharide composition of the Ax 1:15 and Ax 1:70 were almost identical while the Ax 1:35 showed slightly lower results from all the sugars. However the overall yield was the highest for the isolated arabinoxylan Ax 1:35 material, giving 66% of the total arabinoxylan content of the washed fiber material while only 41% and 45% were the results with the materials Ax 1:15 and Ax 1:70 respectively (data not shown). Cyran et al used 1 M NaOH and even stronger 4 M NaOH solution for arabinoxylan extraction and found that some arabinoxylan structures were closely associated with cellulose and therefore the use of stronger alkaline solutions than 1 M NaOH was necessary [40]. Ragae et al. showed that higher concentrations of NaOH could dissolve more β-glucans [41]; however, this also induced depolymerization of the polysaccharides. The polysaccharide content of the isolated materials for Ax 1:15, Ax 1:35 and Ax 1:70 was 91%, 76% and 90% respectively, suggesting the presence of smaller amounts of other components like proteins and Klason lignin [41]. The presence of Klason lignin was indicated as well by the material’s darker color. Cyran et al found that rye arabinoxylans were associated with proteins and lignin components and these were present in 1 M NaOH extracts even after water extraction with α-amylase and proteinase [40]. Considering the amount of chemicals needed, the 1:35 treatment would be the most beneficial solution for such extractions, since this experiment showed the highest hemicellulose yield.

Figure 2: Monosaccharide composition of NaOH extracted rye fibers with solid to liquid ratios: 1:15, 1:35, 1:70

3.4 Monosaccharide analysis of isolated hemicelluloses

A more thorough investigation of the monomer composition of the water-extracted material was performed by acid methanolysis, sugar derivatization and GC-MS detection. Derivatization involved peracetylation using acetic anhydride. Acetylation was applied as derivatization for the methyl glycosides although the most common method is per(trimethylsilyl)ation. Contrary to silylation, the prepared acetates were very stable so they could provide more information for a longer period than sylilated products and might be used for further branching studies as well.

Figure 3: GC-MS chromatogram of isolated hemicellulosic material after acid methanolysis and acetylation. Peaks 1-5 represent xylose and arabinose, peaks 6-8 represent glucose residues.

The analysis method has the potential to be more suitable for hemicellulose analysis than acid hydrolysis since the sugar acids are protected and detectable after chemical modification. The obtained chromatogram can be seen in Figure 3. Peaks 1-5 show arabinose and xylose while peaks 7-9 are hexose sugars, including glucose. Glucose suffered degradation during the analysis; however, the ratio of the degradation products remained...
the same in several repeat analyses. Sugar identification and quantification was done by analysis based on the peak retentions and peak areas compared with previously analyzed mono-and polysaccharide standards. The drawback of this analysis is that during sample preparation, a mixture of α- and β-anomers as well as pyranose and furanose forms were obtained, hence no information on such structures could be obtained since up to four isomers of one single sugar unit could be identified [42].

Figure 4: Monomer composition, expressed as % of the total sugar amount with acid methanolysis and acid hydrolysis

![Graph showing monomer composition](image)

The wet chemical analysis involving use of dilute sulphuric acid and HPLC analysis showed the presence of glucose, xylose, and arabinose as major compounds as well as the presence of fructose (Table 2). The relative sugar composition was calculated and the comparison of results based on acid methanolysis and hydrolysis is shown in Figure 4. Comparing the two sugar analysis procedures a higher xylose and glucose content could be found with acid methanolysis. The arabinose level was lower in the case of methanolysis and the xylose amount was very similar which could result from incomplete degradation of the arabinoxylan structure in the case of acid methanolysis. Sundberg et al. compared acid methanolysis and acid hydrolysis results for wood hemicelluloses and found a higher xylose, mannose and glucose content when using hydrolysis, assuming that cellulose glucose units were also cleaved [38]. It was shown that most of the glucose units formed by methanolysis originate from non-cellulosic components as the method does not degrade crystalline cellulose. This is in contrast with results from acid hydrolysis. Willför et al. compared different carbohydrate analysis methods performed in different laboratories and found that methanolysis was a more suitable method for xylan and uronic-acid-containing sample analyses, in which labile sugars were not degraded as they are during acid hydrolysis [43]. However, acid methanolysis enables analysis of both neutral and acidic carbohydrates in one run and provides excellent separation of the obtained sugars. Although methanolysis data are more reproducible, a longer sample preparation time is needed. Further, the separation capability and sensitivity of the GC-MS system is higher than that of the HPLC system. Acid methanolysis has been shown to be a suitable method for isolated hemicellulose analysis and has been applied to different raw materials such as wood, pulp fibres, or wheat straw samples. (Sundberg, 1996, Bertaud, 2002, Willför, 2009, Virkki, 2008, Pitkanen, 2008).

3.5 SEC - molar mass distribution

Molar mass analysis of the isolated hemicelluloses was analyzed by SEC. Conventional calibration was used for molar mass calculations based on the response of a range of pullulan standards. Hemicellulosic materials tend to form aggregates in solution. Since this behavior likely occurs during the analysis, the light scattering signal may lead to false molar mass calculations.

The obtained chromatogram indicated that high molar mass materials were isolated in both cases, using the water extraction and the alkaline treatment. Figure 5 shows the refractive index (RI) signals of the water-extracted and the Ax 1:35 alkali-extracted materials. The chromatograms showed a slightly higher hydrodynamic volume for the water-extracted material. The calculated average molar masses of the isolated hemicelluloses were in a similar range (Mw= 729 900 g/mol for the water-extracted and Mw= 744 600 g/mol for the Ax 1:35 material). A smaller amount of low molecular weight components could be seen, although those signals might be partly covered by the unbalanced signal of the eluent. Rather wide peaks are observable especially in case of the water-extracted material, indicating a mixture of molecules having a wide range of different molar masses. Such behavior was observed previously by Cyran et al. for water-extracted cereal arabinoxylans [31]. Additionally the high polydispersity of the two studied hemicellulosic materials refers to a wide range of molar masses (Pd=6.84 and 4.83 for water-extracted material).

Molar mass distribution of isolated cereal hemicelluloses has been thoroughly investigated. The calculated molar mass often depends on the SEC system, the eluent and the calculation method, so the measured average molar mass can vary between 2 x 10^5 and 9 x 10^5 g/mol [31, 44]. Pitkänen et al. found a lower weight average molecular weight (246 400 g/mol) of water-extracted rye arabinoxylan which was dissolved in DMSO [44], while Cyran et al. found fractions of water-extracted rye hemicelluloses with 9.34 x 10^4 and 5.49 x 10^5 g/mol dissolved in a NaNO₃ solution [31].

Figure 5: SEC profiles of water-extracted hemicelluloses and alkaline-extracted Ax 1:35 material

![Graph showing SEC profiles](image)

4 CONCLUSIONS

Hemicellulose extractions from rye bran were performed using hot water and alkaline treatments. The original rye bran material was rich in starch, containing a
fairly high amount of the endosperm part of the grains. The hot water-extracted material was treated with starch degrading enzymes followed by dialysis and the resulting material contained mainly arabinoxylan (~65%) and co-extracted β-glucans (~20%). The remaining water-unextractable material was alkali-extracted and this resulted in a material with a similar content of arabinoxylan and β-glucan as the water-extracted material. The alkali-extracted material had a lower arabinose substitution with a lower ara/xyl ratio (0.35) than the water-extracted material (0.54). Acid methanlysis was proven to be a suitable method for monosaccharide analysis. Acid methanlysis resulted in a slightly different monosaccharide composition than acid hydrolysis. It showed a higher xylose and glucose content and a lower arabinose level. Methanlysis data could be more reproducible and the sensitivity of the GC-MS system is higher than that of the HPLC system to detect the chemically modified monomers. High molecular weight materials (~700 000 g/mol) were isolated; the extraction procedure did not have a significant effect on the molecular weight. The polydispersity of the two types of hemicellulosic however showed some differences (Pd=6.84 for alkali-extracted and 4.83 for water-extracted material). High molar mass hemicelluloses can be isolated with a similar structure using a hot water or an alkaline treatment however the extraction yields are higher with alkali extractions. The choice of extraction processes should be dependent on the application purposes for the hemicellulosic materials.

5 REFERENCES

6 ACKNOWLEDGEMENTS

Anne Belinda Thomsen and Mark Lawther are acknowledged for supervising the extraction processes. We are grateful to Ingelis Larsen and Lotte Nielsen for technical assistance.
Paper II

Carbohydrate analysis of hemicelluloses by gas chromatography-mass spectrometry of acetylated methyl glycosides

Zsuzsa Sárossya, David Plackettb, Helge Egsgaard*

aDepartment of Chemical and Biochemical Engineering, Technical University of Denmark, P.O. Box 49, 4000 Roskilde, Denmark

bDanish Polymer Centre, Department of Chemical and Biochemical Engineering, Technical University of Denmark, Søltofts Plads, 2800 Kgs. Lyngby, Denmark

*Corresponding author. Tel.: +45 46775440
E-mail address: heeg@risoe.dtu.dk
Abstract

A method based on gas chromatography-mass spectrometry analysis of acetylated methyl glycosides was developed in order to analyse monosaccharides obtained from various hemicelluloses. The derivatives of monosaccharide standards, arabinose, glucose and xylose were studied in detail and $^{13}$C-labeled analogues were used for identification and quantitative analysis. Excellent chromatographic separation of the monosaccharide derivatives was found and identification of the anomeric configuration was feasible through a prepared and identified pure methyl-2,3,4,6 tetra-O-acetyl-$\beta$-D-glucopyranoside. The electron impact mass spectrum and fragmentation path was studied for each monosaccharide derivative. Fragment ion pairs of labeled and unlabeled monosaccharides were used for quantification; $m/z$ 243/248 for glucose, 128/132 for xylose and 217/218 for arabinose. Using the intensity ratios obtained from the extracted ion chromatograms, accurate quantification of monosaccharide constituents of selected hemicelluloses was demonstrated.

Keywords: acid methanolysis, hemicellulose, arabinoxylan, stable isotope, methyl glycoside, gas chromatography-mass spectrometry

Introduction

Hemicelluloses, cellulose and lignin are the main components in plant cell walls with cellulose providing the structural strength, lignin supporting this strengthening function and hemicelluloses binding with pectin to cellulose to form a network of cross-linked fibers. Hemicelluloses are heteropolysaccharides and can represent up to 50% of the biomass in annual and perennial plants. While cellulose has the same elementary chemical structure in all higher plants, hemicellulose structure and composition can be highly diverse, varying within tissues of the same plant, within individual plant species and in content relative to other cell wall components, like cellulose [1]. Common hemicellulose building blocks are pentoses and hexoses as well as sugar acids. Xylan-type hemicelluloses are the most widespread and can be found in several varieties in terrestrial plants and algae. Xylans are the dominant form of hemicelluloses found in monocot plants [2]. These hemicelluloses are usually built up from $\beta$-(1→4) linked xylopyranose (Xylp) backbone chains substituted with sugar units such as arabinofuranose and O-acetyl groups or 4-O-methyl-glucuronic acid residues. The
occurrence of homoxylans is quite rare. Among the herbaceous plants, cereals like wheat, rye and barley have a less complex hemicellulose structure, containing only arabinose substituents on the xylan main chain; however, the α-arabinofuranosyl substituents can be esterified with ferulic acid [1, 3]. Hardwood (e.g., birch, eucalyptus, aspen) xylans can have a high content of acetylation in addition to methyl-glucuronic acid residues, while softwood xylans carry no acetyl substituents. Cereal water-extractable arabinoxylans typically have a chain of (1→4)-linked β-D-xylopyranose units containing arabinofuranose residues connected to the C(OH)-2 or C(OH)-3 position [4]. In cereal grain cell walls, arabinoxylan is closely associated with mixed linkage (1→3, 1→4) β-D-glucans.

The compositional analysis of hemicelluloses is generally undertaken by degradation of the polymers followed by monosaccharide analysis. Polysaccharide degradation is commonly carried out by acid hydrolysis, acid methanolysis or enzymatic hydrolysis. The enzymatic hydrolysis route results in incomplete hydrolysis so its use is limited for analytical investigations. However, enzymatic hydrolysis conditions are mild and undesirable secondary reactions can therefore be avoided. Acid hydrolysis as well as acid methanolysis may end with incomplete hydrolysis of the samples if the reaction conditions are inadequate or acid hydrolysis may lead to decomposition of the sugars [5-8]. Acid methanolysis is superior to acid hydrolysis in respect to hemicellulose and pectin analyses. While acid hydrolysis degrades cellulose, this does not occur to any large extent when methanolysis is used. Thus, most of the glucose formed by methanolysis, originates from non-cellulosic components [9]. Methanolysis cleaves the glycosidic linkages which results in high yields of sugar monomers. In addition, acid methanolysis allows the determination of uronic acid residues, which are esterified by this process and do not suffer degradation as under acid hydrolysis.
conditions [10, 11]. The resulting monosaccharides are generally determined by chromatographic methods. Determination of monosaccharides or oligosaccharides with HPLC (high-performance liquid chromatography) and AEC (anion exchange chromatography) does not require sample derivatization, although the selection of the appropriate detector system is critical. Pulsed amperometric detection (PAD) is sensitive and is most commonly used with AEC for monosaccharide and oligosaccharide determination [11]. In comparison, the separation capability and sensitivity of GC-MS systems are generally higher than those obtained using LC methods. GC analysis requires derivatization of the sugars, which is usually based on monosaccharide/methyl-glycoside per(trimethylsilyl)ation [11]. Besides the silylation reaction, other types of derivatization can be applied, such as trifluoracetylation [12] or peracetylation [13-15]. Utilization of mass spectrometry together with GC has great advantages in carbohydrate analysis, generating well resolved chromatograms that allow the identification and quantification of the studied compounds, even if they are present in very small amounts [16].

The acid methanolysis reaction raises a problem with the anomeric center in sugars since it has an equilibrating effect on the \( \alpha \) and \( \beta \)-anomers, originating from the oligo-and polysaccharides. After the methanolysis procedure, both anomers are present which is accompanied by the isomerisation of the pyranoside and furanoside forms, hence the determination of the original structural information becomes impossible. As a result, up to four isomers of one sugar unit can be found. Laine et al. realized that the occurrence of these four isomers can become an advantage, since the ratio of the isomers after acid methanolysis is almost constant. Therefore, identification can be based on more than a single peak [17].
In the research described here, acid-catalyzed methanolysis was applied to reference monosaccharides and various hemicellulosic products. The resulting methyl glycosides were acetylated and then analysed by GC-MS. $^{13}$C-labeled monosaccharides were used as chemical internal standards, thereby facilitating quantitative analysis. The aspects of stable isotope dilution analysis as well as isotope equilibrium are discussed. The quantitative analyses were based on extracted ion chromatograms using ions which maximized selectivity and sensitivity. The performance of the method was demonstrated by analysis of commercial hemicelluloses as well as hemicelluloses isolated from rye bran.

**Experimental**

**Materials**
D-Arabinose-1-$^{13}$C, D-glucose-$^{13}$C$_6$, D-glucose-1-$^{13}$C, D-xylose-$^{13}$C$_5$, D-xylose-1-$^{13}$C, 2,3,4,6-tetra-acetyl-$\alpha$-D-glucopyranosyl bromide, L-arabinose, D-glucose, hydrogen chloride – methanol solution (1.25 M), cross-linked-poly(4-vinylpyridine) and birch wood xylan (BWX) were all purchased from Sigma-Aldrich (Steinheim, Germany). D-xylose was obtained from Merck (Darmstadt, Germany). Arabinoxylan (Lot 20601) from rye flour (RAX 2) was purchased from Megazyme International Ireland Ltd. Hemicelluloses from rye bran (RAX 1) were isolated as previously described [18]. Rye bran slurry (bran to water ratio = 1:7 w/v) was treated with Termamyl SC (dosage: 0.6 U/g residual starch) at pH 6.0 with continuous stirring for 45 minutes at 95°C. Fragmentation of particles was carried out using a wet mill (Mannesmann, Remscheid, Germany) during the extraction procedure. The supernatant syrup was separated by centrifugation (approx. 6000 g for 15 minutes) and treated again with Termamyl SC for 45
minutes at 95°C. The sugar syrup was autoclaved for five minutes at 120°C. The pH of the samples were reduced to 4.5 with 5 M HCl. Enzymatic digestion of the resulting glucose oligomers was performed with amyloglucosidase (dosage: 0.006 U/g residual starch) at 60°C for 45 minutes. The glucose-rich syrup fraction was dialyzed (MWCO = 12000-14000 Da) at room temperature for 24 hours against water. The aqueous extract was collected and precipitated with an equal volume of ethanol (96% v/v) and left overnight at 4°C. The precipitate was collected after centrifugation (approx. 6000 g, 20 minutes). Isolated hemicelluloses were washed with a 1:1 (v/v) mixture of ethanol and water. These water-extracted hemicelluloses were then resuspended in water and freeze dried.

Sample preparation

13C-labeled monosaccharide samples and anhydrous D-glucose, D-xylose and L-arabinose were dissolved in MilliQ water. Stock solutions were made at concentrations of 2 mg/ml. Series of five samples of D-glucose, D-xylose and L-arabinose respectively were prepared with the addition of a constant amount of the corresponding 13C compound. Thus, a constant volume (0.5 ml) was taken from the 13C stock solutions, whereas volumes between 0 and 1 ml were taken from the solutions of the unlabeled monosaccharides. The prepared solutions were frozen and freeze dried prior to acid methanolysis.

Preparation of methyl-2,3,4,6 tetra-O-acetyl-β-D-glucopyranoside

100 mg of 2,3,4,6 tetra-O-acetyl-α-D-glucopyranosyl bromide was dissolved in 10 ml methanol. The solution was left at ambient temperature overnight and was then neutralized with 50 mg NaHCO₃. 25 ml chloroform was added and the solution was washed with 100 ml MilliQ water. The organic phase was isolated
and anhydrous Na$_2$SO$_4$ was added to absorb traces of water. The prepared glucopyranoside solution was used without further purification.

**Acid methanolysis and acetylation**

Carbohydrate composition was determined by GC-MS after acid methanolysis and acetylation of the sugar samples. Hemicellulosic samples (approx. 10 mg) were degraded to monosaccharides using 5 ml 1.25 M HCl in methanol [9]. The samples were kept at 100°C overnight in test tubes sealed with Teflon-lined metal screw caps. The test tubes were placed in protective metal tubes for safety reasons. 4-vinylpyridine (approx. 0.85 g) was added to each reaction mixture to trap HCl and the samples were filtered and washed with 5 ml methanol. After evaporation of methanol, samples were dissolved in 4 ml of an acetic anhydride-pyridine mixture (1:4) and kept at 100°C for 30 minutes. After cooling to ambient temperature, 20 ml chloroform was added to the mixture. The samples were then washed sequentially as follows: 25 ml Millipore water, 25 ml 2M HCl, 25 ml Millipore water, 25 ml 5 % NaHCO$_3$, 25 ml Millipore water, and the organic phase was isolated. The organic phase was dried with anhydrous Na$_2$SO$_4$.

**GC-MS analysis of derivatized monosaccharides**

The derivatized sugars were analysed by GC-MS using a Hewlett Packard HP 6890 gas chromatograph interfaced to a HP5973 Mass Selective Detector (Agilent, Denmark). 1 μl sample volumes were injected by a HP 7683 auto sampler (Agilent, Denmark) and introduced in a split mode (1:20). The source and rod temperature was 230 °C and 150 °C, respectively. The products were separated using a 0.32 mm i.d. x 30 m WCOT fused silica column coated with VF-23ms at a thickness of 0.25 μm (Analytical, Denmark). The carrier gas was He at a flow rate of 1.2 ml/min. Separation of a wide range of products was
achieved using a temperature program from 70 °C to 250 °C. Full mass spectra were recorded every 0.3 s (mass range $m/z$ 40 – $m/z$ 450). Products were identified using NIST search engine version 2.0 f. (Agilent, Denmark).

Results and discussion

Derivatization and chromatography

The derivatization of monosaccharides (i.e., transformation to methyl glycosides followed by acetylation) is to a great extent a standard procedure. However, the use of poly(4-vinylpyridine) to trap the acid catalyst leads to enhanced appearance of the pyranose forms, as well as suppressing the formation of by-products (e.g. peracetates). The use of pyridine however resulted in a high yield of furanose-based saccharides in which the ratio of furanose and pyranose forms depended on the time of reaction.

The derivatives exhibited excellent chromatographic properties enabling the separation of the $\alpha$- and $\beta$-anomers as well as the furanose and pyranose forms. The derivatives present some advantages in comparison to silylated monosaccharides by, for example, higher stability towards hydrolysis, simpler isotopic patterns, and relatively low molecular weights. However, silylation provides a proven and rapid preparation method [19].

In order to obtain some experimental support to assign the $\alpha$- and $\beta$-anomers observed in the chromatograms, methyl-2,3,4,6 tetra-O-acetyl-$\beta$-D-glucopyranoside was prepared. The analysis revealed only a single component, enabling an unambiguous assignment of the $\beta$-anomer and hence indirectly for the
α-anomer as well. All other α- and β-anomer pairs are assumed to show an identical order of separation.

The retention times for conceivable structures of the monosaccharide derivatives were determined as well as their mass spectra in order to identify other carbohydrates in mixtures arising from the hemicelluloses. The retention times obtained for the individual monosaccharides and the assigned structures are summarized in Table 1. These monosaccharides are expected to account for the major part of the investigated hemicelluloses. However, it is assumed that the method can be extended in a straightforward way to other monosaccharides and sugar acids.

The number of peaks for each monosaccharide was two or four according to the formed α- and β-anomers and furanose and pyranose ring forms. Figure 1 shows chromatographic patterns for the pure monosaccharides D-glucose, D-xylose and L-arabinose compared with their labeled counterparts. Very similar patterns including peak ratios and retention times are apparent when comparing the ion chromatograms of the labeled and unlabeled monosaccharides in Fig. 1.

**Mass spectrometry of acetylated monosaccharides**

The electron impact-induced fragmentation of similarly derivatized monosaccharide compounds was studied as early as 1963 by Biemann et al. and has later been studied in detail by collision-induced fragmentation and theoretical methods by several authors [13, 15, 20-24]. The interpretation of the spectra is rather difficult due to the fact that the signals for the molecular ion and the first fragments are typically of very low intensity. This aspect is illustrated by the
spectrum of xylose, cf. Figure 2, where the first ion at highest mass appears at 170 m/z and hence the upper third of the spectrum escape detection. However, an important aspect is the unambiguous determination of carbon content in the ions using the 13C labeling cf. Figure 2 and some details become apparent concerning fragmentation of the derivatized xylose, e.g. to which extent the monosaccharide carbons participate in the fragmentation. The obtained mass spectra of the derivatives of monosaccharides are generally very comparable to those found in earlier reports and databases and the obtained and found fragment ions follow these described routes.

**Extracted ion chromatograms of monosaccharides**

The ions to be used for analytical purposes in the extracted ion chromatograms should have a reasonable intensity, the m/z value should ensure a good selectivity, and a logical fragmentation pathway should be apparent. The derivatives all have the acetyl cation (m/z 43) as a dominant base peak. Thus, as shown in Figure 2, due to the normalization procedure, peaks with a relative intensity of 5-10 % may provide acceptable sensitivity and dynamic range. In the present study, 13C labeling has been used exclusively to avoid significant isotope effects and hydrogen exchange reactions.

The mass losses of the different monosaccharide derivatives are represented in Figure 3. The glucose derivative (methyl-2,3,4,6 tetra-O-acetyl-D-glucopyranoside) exhibits a relatively strong signal at m/z 243 (mass spectra not shown). This ion has previously been assigned to the consecutive loss of methyl formate and an acetoxy radical as supported by deuterium labeling [13, 20, 24]. The mass shifts observed here in the spectra of methyl-2,3,4,6 tetra-O-acetyl-D-
glucopyranoside-\textsuperscript{13}C\textsubscript{6} (m/z 243 to m/z 248) and methyl-2,3,4,6 tetra-O-acetyl-D-glucopyranoside-\textsuperscript{13}C\textsubscript{1} (m/z 243 unchanged) were in complete agreement with this previous assignment. The m/z 243/248 was chosen for EIC (extracted ion chromatogram) analysis of glucose.

The xylose derivative (methyl-2,3,4 tetra-O-acetyl-D-xylopyranoside) exhibits a strong signal at m/z 170 and initiates a series of eliminations of ketene, (i.e., m/z 170, 128 to 86). This ion has likewise been discussed in terms of a consecutive loss of methyl formate and acetic acid [13, 20]. The shifts in mass observed, cf. Figure 2, in the spectra of methyl-2,3,4 tri-O-acetyl-D-xylopyranoside-\textsuperscript{13}C\textsubscript{5} (m/z 128 to m/z 132) and methyl-2,3,4 tri-O-acetyl-D-xylopyranoside-1-\textsuperscript{13}C (m/z 128 and170 are unchanged) (not shown) were in complete agreement with this assignment. The m/z 128/132 pair was chosen for EIC analysis of xylose.

The arabinose derivative (methyl-2,3,5 tri-O-acetyl-D-arabinofuranoside) reveals a strong signal at m/z 217 (mass spectra not shown). A clear shift in mass is observed in the spectra of methyl-2,3,5 tri-O-acetyl-D-arabinofuranoside-1-\textsuperscript{13}C (m/z 217 to 218). This is in agreement with a single cleavage stabilizing the product ion as an oxonium ion. The m/z 217/218 pair was chosen for EIC analysis of arabinose.

In general, the pyranose forms generate the most intense signals and were chosen for glucose and xylose. On the other hand the simple fragmentation of furanoses may have benefits in terms of a more straightforward assignment but with lower selectivity.
**Stable isotope dilution and isotope equilibrium**

The isotope dilution method may advantageously be applied to problematic analyses due to the inherent benefits (e.g., the isotope-labeled compound has to be regarded as the perfect internal standard, the analytical equipment (normally mass spectrometry) reveals high sensitivity, precision and dynamic range). In addition, the use of the isotope-labeled compound as an internal standard may improve chromatography due to the carrier effect. It should be emphasized that when isotope equilibrium has been obtained, the ratio between internal standard and analyte cannot be changed. This means that work-up of samples does not require determination of recovery. On the other hand, isotope equilibrium is an essential aspect, in particular when the analyte may undergo chemical isomerization. This is obviously the case with monosaccharides.

The isotope equilibrium was checked by plotting the ion intensity ratio of the added standard/$^{13}$C labeled compound against the mixed molar ratio of the added standard/$^{13}$C labeled compound (Fig. 4). Linear regression analyses were performed for glucose due to no contribution from the labeled compound to $m/z$ 243 and no contribution from the unlabeled compound to $m/z$ 248. Similarly, linear regression was applied for xylose. In the case of arabinose, the unlabeled derivative has an abundance of 89% at $m/z$ 217 and a natural abundance of 9% at $m/z$ 218. This has the consequence that a linear regression analysis cannot be formed. Instead, a second order polynomial analysis was used. The results from the regression analyses are given in Table 2. A good correlation between the peak areas and added natural monosaccharide is apparent. There are minor deviations with respect to recovery, which is assigned to a slight non-equilibrium between the $\alpha/\beta$-anomers and the furanose/pyranose forms. This is taken into account using the equations obtained by regression analyses.
**Hemicellulose component analysis**

The GC chromatograms of the examined hemicellulose samples showed the presence of the expected monosaccharides glucose, xylose and arabinose. Glucose provided two peaks corresponding to the $\alpha$- and $\beta$-configurations of the monosaccharide pyranose ring form, while xylose showed four peaks, relating to the furanose and pyranose forms and the $\alpha$- and $\beta$-anomers. Arabinose was present to a lesser extent than xylose, which was apparent in the chromatogram and, in addition to the two pyranose peaks, only one furanose peak was detectable in all cases. This is supposedly because a furanose peak elutes in a similar time as the earlier xylopyranose, thus these two peaks overlap and these arabinose/xylose peaks are not considered for quantitative work. Identification of the monosaccharides was based on their retention times. The labeled monosaccharides were present as internal standards, added in known amounts, and the typical ion fragments were used for their identification. In the hemicellulose samples, the monosaccharides glucose, xylose and arabinose were quantified by the ratio of the measured peak area of the unlabeled and labeled component for the chosen fragment ions.

Figure 5 shows the GC-MS chromatogram obtained for a water-extracted hemicellulose sample from rye bran (RAX 1). A minor peak appears at 21.081 minutes. Analysing the mass spectrum of this compound, a hexose fragmentation could be traced and no labeled compound was found. Based on previous investigation of monosaccharides, including D-galactose, the retention time corresponded to that found earlier (data not shown). Another small peak appeared at 20.99 minutes, which corresponded to an $\alpha$-glucofuranose anomer.
The identification was apparent from the mass spectra since the labeled compound was present showing a signal at $m/z$ 221 while the unlabeled compound showed a signal at 217.

The amount of recovered carbohydrates varied between 72 and 92% for the hemicellulose samples. Two hemicelluloses (RAX 2 and BWX) are commercial products available and high carbohydrate contents were expected (reported purity over 90% in both cases); however, in some cases the carbohydrate content was measured to be below 80%. The measured monosaccharide content of the hemicellulose sample RAX 1 contained arabinoxylan and a significant amount of glucose attributable to mixed-linkage $\beta$-glucans. The monosaccharide content of the RAX 1 material was analysed by acid methanolysis and trimethylsilylation followed by GC-FID (unpublished data) and a xylose content of 48.0% (percentage of measured monosaccharides), arabinose content of 25.8% and glucose content of 26.2% were found. Comparing the two analyses, no significant differences could be found in the obtained values for monosaccharide composition. The carbohydrate composition of RAX 2 was measured in other studies and similar results were for example reported by Pitkänen et al. for the contribution of the various monosaccharides to the total carbohydrate content (33% arabinose, 66% xylose and 1% glucose) [25]. In summary, the hemicellulose analysis method outlined here can be used to identify hemicellulose components and provide reliable quantitative data.
Conclusions

Acid catalyzed methanolysis and acetylation of intermediary methyl-glycosides originating from hemicellulose samples was carried out and the reaction products analysed with GC-MS. The analytical method was proven to be highly sensitive and applicable for monosaccharide compositional analysis of hemicellulose samples. The derivatives showed excellent chromatographic separation, allowing distinction of the anomeric configuration of the furanose and pyranose forms. Differentiation of α- and β-anomers was confirmed by preparation of a pure β-anomer (methyl-2,3,4,6 tetra-O-acetyl-β-D-glucopyranoside). Retention times were collected for the various labeled and unlabeled monosaccharide standards and peaks were assigned for monosaccharide identification. $^{13}$C-labeled compounds as internal standards were proven to be applicable in quantitative analyses. The electron impact-induced fragmentation of all the studied monosaccharides was followed and fragment ions of $m/z$ 243 and 248 were used for glucose quantification, whereas the ion pair 128/132 was chosen for xylose and the ion pair 217/218 for arabinose. A linear regression was found between the intensity ratio and the mixed molar ratio of the monosaccharide standards and the $^{13}$C-labelled compounds in the case of xylose and glucose. However, such linearity cannot be found for arabinose, since a 9% natural abundance occurs at $m/z$ 218. Using the intensity ratios obtained from the extracted ion chromatograms, accurate quantification of monosaccharide constituents of selected hemicelluloses was demonstrated.
Acknowledgements

The Technical University of Denmark is thanked for financial support. Hanne Wojtaszewski is acknowledged for assistance with sample preparation and GC-MS analyses.

References


Figure captions

**Fig. 1** Total ion chromatograms of acetylated methyl glycoside derivatives of: (a) D-glucose-\(^{13}\)C; (b) D-glucose; (c) D-xylose-\(^{13}\)C; (d) D-xylose; (e) D-arabinose-\(^{13}\)C-\(^{1}\); (f) L-arabinose

**Fig. 2** The EI-MS fragmentation pathway of acetylated methyl glycosides of D-xylose (a) and D-xylose-\(^{13}\)C (b)

**Fig. 3** Diagnostic ions of monosaccharide derivatives, glucose (a), xylose (b) and arabinose (c)

**Fig. 4** Monosaccharide standard/\(^{13}\)C labeled compound standard plots

**Fig. 5** Total ion chromatogram obtained from a rye hemicellulose sample. The assigned peaks refer to: Xyl(1)=\(\alpha\)-xylofuranose; Xyl(2)=\(\alpha\)-xylopyranose; Xyl(3)=\(\beta\)-xylofuranose; Xyl(4)=\(\beta\)-xylopyranose; Ara(1)=\(\alpha\)-arabinofuranose; Ara(2)=\(\beta\)-arabinofuranose; Ara(3)=\(\alpha\)-arabinopyranose; Ara(4)=\(\beta\)-arabinopyranose; Glc(1)=\(\alpha\)-glucopyranose; Glc(2)=\(\beta\)-glucopyranose
Table 1 Chromatographic retention times, ring and assigned anomeric configurations and area percentages for authentic monosaccharides

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention time (min)</th>
<th>Ring configuration and assigned anomeric configuration</th>
<th>%a</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-glucose</td>
<td>21.776</td>
<td>pyranose, α</td>
<td>68.9</td>
</tr>
<tr>
<td></td>
<td>22.105</td>
<td>pyranose, β</td>
<td>31.1</td>
</tr>
<tr>
<td>D-xylose</td>
<td>18.164</td>
<td>furanose, α</td>
<td>7.5</td>
</tr>
<tr>
<td></td>
<td>18.532</td>
<td>pyranose, α</td>
<td>33.8</td>
</tr>
<tr>
<td></td>
<td>18.897</td>
<td>furanose, β</td>
<td>10.5</td>
</tr>
<tr>
<td></td>
<td>18.981</td>
<td>pyranose, β</td>
<td>48.2</td>
</tr>
<tr>
<td>L-arabinose</td>
<td>18.280</td>
<td>furanose, α</td>
<td>12.3</td>
</tr>
<tr>
<td></td>
<td>18.538</td>
<td>furanose, β</td>
<td>34.7</td>
</tr>
<tr>
<td></td>
<td>18.630</td>
<td>pyranose, α</td>
<td>34.6</td>
</tr>
<tr>
<td></td>
<td>19.244</td>
<td>pyranose, β</td>
<td>18.4</td>
</tr>
</tbody>
</table>

a: area % based on total ion chromatograms
Table 2 Regression analyses of arabinose, glucose and xylose calibrations

<table>
<thead>
<tr>
<th>Monosaccharide</th>
<th>EIC ion pairs (m/z)</th>
<th>Calibration equation</th>
<th>Coefficient of determination (r²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabinofuranose</td>
<td>217/218</td>
<td>$y = -0.035 x^2 + 0.8952 x$</td>
<td>0.9983</td>
</tr>
<tr>
<td>Glucopyranose</td>
<td>243/248</td>
<td>$y = 0.9112 x$</td>
<td>0.9962</td>
</tr>
<tr>
<td>Xylopyranose</td>
<td>128/132</td>
<td>$y = 1.0267 x$</td>
<td>0.9999</td>
</tr>
</tbody>
</table>
Table 3 Monosaccharide composition (%) of rye hemicelluloses and birch wood xylan and their arabinose-to-xylose ratios

<table>
<thead>
<tr>
<th>Sample type</th>
<th>RAX 1</th>
<th>RAX 2</th>
<th>BWX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xyl</td>
<td>47.9 ± 1.8</td>
<td>64.2 ± 1.6</td>
<td>98.3 ± 0.2</td>
</tr>
<tr>
<td>Ara</td>
<td>26.8 ± 1.4</td>
<td>35.0 ± 1.4</td>
<td>-</td>
</tr>
<tr>
<td>Glc</td>
<td>25.3 ± 0.5</td>
<td>0.8 ± 0.2</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>Ara/Xyl ratio</td>
<td>0.56 ± 0.05</td>
<td>0.55 ± 0.03</td>
<td>-</td>
</tr>
</tbody>
</table>

Ara=arabinose, Xyl=xylose, Glc=glucose
n=2
Figure 1

Retention time (min)

Intensity (a. u.)

A

B

C

D

E

F
Figure 3

A

\[
\begin{align*}
&\text{AcO} \quad \text{O} \quad \text{OCH}_3 \\
&\text{AcO} \quad \text{OAc} \\
&\text{M}=362
\end{align*}
\]

-60 m/z 302 → -59 m/z 243

B

\[
\begin{align*}
&\text{AcO} \quad \text{O} \quad \text{OCH}_3 \\
&\text{AcO} \quad \text{OAc} \\
&\text{M}=290
\end{align*}
\]

-60 m/z 230 → -60 m/z 170 → -42 m/z 128

C

\[
\begin{align*}
&\text{AcO} \quad \text{O} \quad \text{OAc} \\
&\text{OAc} \quad \text{OCH}_3 \\
&\text{M}=290
\end{align*}
\]

-73 m/z 217
Figure 4

![Graph showing molar ratio of unlabeled/\(^{13}\)C labeled monosaccharides against unlabeled/\(^{13}\)C labeled monosaccharide peak areas. Different symbols represent different monosaccharides: Arabinose (diamonds), Xylose (upward-pointing triangles), and Glucose (downward-pointing triangles).]
Paper III

Sárossy, Zs., Tenkanen, M., Pitkänen, L., Bjerre, A.B., Plackett, D.: Extraction and chemical characterization of rye arabinoxylan and the effect of β-glucan on the mechanical and barrier properties of cast arabinoxylan films. Submitted to *Food Hydrocolloids*. 
Extraction and chemical characterization of rye arabinofuranose and the effect of β-glucan on the mechanical and barrier properties of cast arabinofuranose films

Zsuzsa Sárossy, Maija Tenkanen, Leena Pitkänen, Anne Belinda Bjerre, David Plackett *

a Biosystems Division, Bioenergy and Biorefinery Programme, National Laboratory for Sustainable Energy, Technical University of Denmark (Risø DTU), P.O. Box 49, 4000 Roskilde, Denmark

b Department of Food and Environmental Sciences, University of Helsinki, P.O. Box 27, FIN-00014, Helsinki, Finland

c Danish Technological Institute, Gregersensvej, 2630 Tåstrup, Denmark.

*Corresponding author. Tel.: +45 46775487
E-mail address: dapl@risoe.dtu.dk

Abstract

Water-extractable hemicellulose fractions (WEH), containing approximately 65% arabinofuranoses (WE-AX) and 20% mixed-linkage β-glucans were isolated from rye bran. In addition, water-extractable mixed linkage β-glucans (BG) were isolated from oat bran as a reference material. The β-glucan content of the rye hemicellulose isolate was reduced to less than 5% by a selective lichenase treatment.
Rye hemicelluloses, WEH and WE-AX had arabinose-to-xylose ratios of 0.54 and 0.57 and weight-average molecular weight \( (M_w) \) of 270 000 and 232 000 g/mol respectively. The \( M_w \) of BG was higher at 386 000 g/mol. The material properties of films prepared from the rye hemicellulose isolate and WE-AX as such, or with varying amounts of added BG (20:80; 50:50; 80:20 ratios) were studied. Prior removal of \( \beta \)-glucan from the isolate decreased the tensile strength of the films significantly as well as the elongation at break. Addition of BG to the purified WE-AX resulted in an increase in the tensile strength and elongation at break of the films. In contrast, the presence of BG had no clear effect on the oxygen permeability of the films. Both pure rye WE-AX and pure BG films showed excellent oxygen barrier properties (between 0.9-1.0 cm\(^3\)-\(\mu\)m/m\(^2\)-d-kPa). However, the water vapor permeability increased with addition of increasing amounts of BG to WE-AX. To our knowledge, this is the first study on the effect of \( \beta \)-glucans on the material and permeability properties of arabinoxylan-based films.

Keywords: rye bran, oat bran, arabinoxylan, \( \beta \)-glucan, films, material properties

1. Introduction

Agricultural crop by-products contain vast quantities of different polysaccharides, which are often underutilized and from which value can be obtained in the form of new materials. As an example, cereal bran represents 15-30% of the grain and contains various other polysaccharides besides starch, which are easily accessible and could be utilized in smart new applications. Bran is an important part of the human diet due to its high dietary fiber content and is also used in animal feed.
Rye (*Secale cereale*) is a cereal traditionally grown in the northern and eastern regions of Europe, adding diversity to the cereal market. In the past decade, rye growth has fluctuated in the Nordic countries and in the European Union; however, in the last few years an increase in rye production has occurred in the EU, and this now represents approximately half of the world production (9.87 million tonnes in the EU and 18.2 million tonnes in the world in 2009). Production in the Nordic countries (970 000 tonnes in 2009) provides approximately one-tenth of the production in the EU, where most of the output comes from Eastern European countries (FaoStat Crops Production., 2011). Considering that roughly 20-35% of the grains are separated as bran during milling, an estimated 300 000 tonnes of rye bran is available for use in the Nordic countries each year.

Non-starch polysaccharides from cereal grain cell walls include cellulose, (1→3, 1→4)–β-D-glucans (henceforward referred to as β-glucans), heteroxylans, glucomannans, xyloglucans, pectic polysaccharides, and callose (Fincher & Stone, 2004). Although these polysaccharides usually constitute less than 10% of the grain, they influence the grain quality. Polysaccharides, together with proteins and phenolics, compose the plant cell wall matrix phase in which cellulose microfibrils are embedded. Extensive non-covalent interactions, especially hydrogen bonds, between the cellulose microfibrils and the matrix phase maintain the integrity of the cell wall (Fincher & Stone, 2004; Roubroeks, Andersson, & Åman, 2000). The main polysaccharide constituents of rye cell walls are arabinoxylans, β-glucans and cellulose (Ragaee et al., 2001). While the whole grains contain 7-12% arabinoxylan and around 1-2% β-glucans, the bran fraction can hold up to 37% arabinoxylan and 3.4% β-glucans (Roubroeks, Andersson, & Åman, 2000; Nilsson, Saulnier, Andersson, & Åman, 1996).

Extraction of hemicelluloses from plant cell walls is typically carried out using neutral or alkaline solvents (Vuorinen & Alen, 1999). A portion of the cell wall material can be extracted by
water while the non-water-extractable material can be obtained by using various other processes and solvents (Cyran, 2010). The most commonly used isolation method is based on alkaline solvents. The arabinoxylans are mostly extracted with NaOH and β-glucans are co-extracted using this method. The co-extracted β-glucans can be removed by fractional precipitation or enzymatic degradation; however, the precipitation techniques do not always provide complete separation of the polysaccharides. Another approach is to use more selective extracting solvents, such as Ba(OH)₂ solutions, which preferentially extract arabinoxylans while most of the β-glucans remain insoluble (Roubroeks, Andersson, & Åman, 2000; Gruppen, Hamer, & Voragen, 1991). In the case of water-extraction processes, both water-soluble arabinoxylans and β-glucans are isolated and are believed to be loosely bound at the cell wall surface. The difficulties associated with water extraction of xylans are probably a result of covalent and non-covalent interactions among arabinoxylans and between the xylans and other cell wall components, such as phenolic acids, proteins or even β-glucans (Aspinall & Sturgeon, 1957; Moore et al., 1996).

The structure of water-extractable arabinoxylans from rye has been studied extensively. It has been found that approximately half of the xylopyranosyl residues in the xylan main chain are substituted at O-3 and about 2% of the residues are substituted by arabinofuranosyl units at both the O-2 and O-3 positions. Further structural studies showed that the branch points were predominantly isolated residues (36%) or small blocks of two residues (62%), so the distribution pattern was not random in the chains and consisted of a range of structures (Aspinall & Sturgeon, 1957; Vinkx & Delcour, 1996; Åman & Bengtsson, 1991; Bengtsson & Åman, 1990; Nilsson et al., 2000). The flexibility of less-substituted arabinoxylans might permit intermolecular alignment over short sequences of unsubstituted xylose residues by hydrogen bonds (Ragae et al., 2001; Izydorczyk &
Biliaderis, 1995). Izydorczyk et al. studied possible molecular interactions between arabinoxylans and β-glucans and found evidence of spontaneous and strong intermolecular association between unsubstituted regions of xylan chains and cellulose-like β-(1 → 4)-linked fragments from the β-glucan chains (Izydorczyk & MacGregor, 2000). These associations are thought to be based on hydrogen bonds. Such non-covalent associations, as well as the extent of cross-interactions between β-glucans and arabinoxylans in the plant cell walls, might contribute to the poor water-extractability, solubility and enzymatic indigestibility of these polysaccharides and, in addition, to the overall cell wall mechanical properties (Izydorczyk & MacGregor, 2000; Lazaridou, Chornick, Biliaderis, & Izydorczyk, 2008).

Xylan-type hemicelluloses from agricultural crops or from wood have been studied for biodegradable film production. For example, arabinoxylans from corn hulls and bran, rye flour, oat spelt, barley husks and wheat bran have been used in studies on film casting from aqueous solutions (Zhang & Whistler, 2004; Sternemalm, Höije, & Gatenholm, 2008; Hoije et al., 2008; Mikkonen et al., 2009; Gröndahl & Gatenholm, 2007; Zhang et al., 2011). The excellent oxygen barrier properties and good mechanical properties of arabinoxylan films have been demonstrated and their application as oxygen barriers in multilayer packaging has been suggested (Gröndahl & Gatenholm, 2007). Rye flour arabinoxylan has been chemically and enzymatically modified in order to observe the effect of arabinose substitution on film properties. Decreasing arabinose content positively affects the oxygen permeability of the films, giving lower values. Crystallinity has been shown to increase with decreasing arabinose substitution (Sternemalm, Höije, & Gatenholm, 2008; Hoije et al., 2008). Agricultural by-products such as oat spelt arabinoxylan can provide excellent oxygen and/or grease barrier films in applications where moderately high water vapor permeability is required (Mikkonen et al., 2009).
Edible films have been made by blending arabinoxylans with other components such as lipids, agar and cassava and biodegradable films have also been cast from mixtures of galactoglucomannan and konjac glucomannan (Peroval, Debeaufort, Despre, & Voilley, 2002; Phan The, Debeaufort, Voilley, & Luu, 2009; Mikkonen et al., 2008).

The rheological properties of β-glucan solutions have been extensively studied, but their use to provide free-standing films or edible films has scarcely been investigated. Tejinder et al. prepared edible films from β-glucans originating from barley and oats (Tejinder, 2003). The films had good mechanical properties but were less effective moisture barriers than casein-, gluten- or arabinoxylan-based films (Mikkonen et al., 2009; Peroval, Debeaufort, Despre, & Voilley, 2002).

In this study, hemicelluloses were isolated from cereal bran fractions using hot water extraction. The idea was to avoid harsh or environmentally degrading processes and to apply a cost-effective method. The isolated hemicelluloses might then be used to produce industrially useful packaging films or coatings in special applications. If no fractional precipitation is applied, hot water extraction of cereal bran results in a mixture of arabinoxylans and β-glucans. Given that the cost of purifying the crude isolated hemicelluloses could be relatively high and might restrict further practical development, the aim of the study described here was to examine the effect of β-glucan content on the material properties of arabinoxylan-based cast films.
2. **Materials and methods**

2.1. **Materials**

Rye (Secale cereale) cultivar Carotop was grown in Denmark in 2008. Rye grains from this crop were disc milled and the fine flour fraction was separated. Material with particle size in the range 0.25–1.0 mm and with a mean diameter of 0.5 mm was used for hemicellulose extraction. Similarly, grains from the oat (Avena sativa) cultivar Revisor grown in Denmark in 2008 were disc milled and a fraction with particle size in the range 0.25–1.0 mm was separated and used for isolations. Thermostable \( \alpha \)-amylase Termamyl SC was obtained from Novozymes A/S ( Bagsvaerd, Denmark). Amyloglucosidase (EC 3.2.1.3 from A. niger) and lichenase (endo-1,3(4)-\( \beta \)-Glucanase) (EC 3.2.1.73 from Bacillus sp.) were purchased from Megazyme International Ireland Ltd. (Bray, Ireland). All other chemicals and reagents used were of analytical grade.

2.2. **Composition of rye and oat bran**

Total sugar composition of rye and oat bran was determined by HPLC analysis after sulphuric acid hydrolysis. First, 1.5 ml of 72% \( \text{H}_2\text{SO}_4 \) was added to a 0.16 g sample and the sample was pre-hydrolyzed for 60 minutes at 30 °C. Second, after dilution of the hydrolyzate with MilliQ water (42 ml), the liquid samples were autoclaved at 120°C for 60 minutes. Filtered liquids were analyzed on an HPLC column (Aminex HPX-87H, Bio-Rad, Hercules, CA, USA), while the solid residue was heated to 550°C to determine the lignin ash content (Kaar & Brink, 1991; Thygesen et al., 2005). Glucose, xylose and arabinose contents were determined from the liquid phase. For HPLC analysis, 40 \( \mu \)l samples were injected at a temperature of 63°C and flow rate of 0.6 ml/min (eluent 4 mM...
H$_2$SO$_4$). All HPLC analytes were detected by a refractive index (RI) detector (Shimadzu, Japan).

Samples were analyzed in duplicate.

Starch content was determined using a Laboratory Analytical Procedure (LAP) of NREL (Issue Date: 07/17/2005) (Sluiter & Sluiter, 2008). In this case, 100 mg of milled sample and starch standard was weighed and 0.2 ml ethanol was added to aid sample dispersion. Subsequently, 2 ml of dimethyl sulfoxide (DMSO) was added and the tubes were placed in a briskly boiling water bath for five minutes. Then, 2.9 ml 3-morpholinopropane-1-sulfonic acid (MOPS) buffer and 0.1 ml thermostable $\alpha$-amylase were added, mixed and the mixture was incubated in a boiling water bath for six minutes, with stirring every two minutes. Tubes were placed in a 50°C water bath and 4 ml sodium acetate buffer (pH 4.5), followed by 0.1 ml amyloglucosidase enzyme, was added and mixed well. After incubating for 30 minutes at 50°C, samples were removed and centrifuged for 10 minutes at 2000 g. Samples for glucose determination were analyzed in triplicate by HPLC.

Mixed linkage $\beta$-glucan content was analyzed by the method of McCleary and Codd (AOAC Method 995.16) using a Megazyme assay kit (Megazyme International) (McCleary & Codd, 1991). Experiments were performed in triplicate.

Soxhlet extraction of rye and oat bran was carried out for 24 hours using 96% (v/v) ethanol in order to determine the lipid content according to the ASTM Standard E1690, 2008 (ASTM, 2008). The ash content was measured gravimetrically after heating at 550°C.

The protein content of rye bran was calculated from the total amount of measured nitrogen in the samples (5.83 x N). The total nitrogen content was determined with an EA 1110 CHNS-O
elemental analyzer (CE Instruments, Wigan, UK) at 1800°C combustion temperature. Rye and oat bran chemical compositions are summarized in Table 1.

2.3. Isolation of rye bran hemicelluloses

Rye bran slurry (bran to water ratio = 1:7 w/v) was treated with Termamyl SC at pH 6.0 with continuous stirring. After addition of α-amylase (dosage: 0.6 U/g residual starch), starch was gelatinized for 45 minutes at 95°C. Fragmentation of particles was carried out using a wet mill (Mannesmann, Remscheid, Germany) during the extraction procedure. Rye bran fibers, proteins and waxes were separated from the supernatant syrup by centrifugation (approx. 6000 g for 15 minutes). The syrup fraction was treated again with Termamyl SC for 45 minutes at 95°C. Treated sugar syrup was autoclaved for five minutes at 120°C and α-amylase was deactivated. Further separation of precipitated proteins was performed by centrifugation (approx. 6000 g, 10 minutes). The pH of the samples were reduced to 4.5 with 5 M HCl. Enzymatic digestion of the resulting glucose oligomers was performed with amyloglucosidase (dosage: 0.006 U/g residual starch) at 60°C for 45 minutes. The glucose-rich syrup fraction was dialyzed (MWCO = 12000-14000 Da) at room temperature for 24 hours against water to remove the glucose and oligomer units originating from starch. The aqueous extract was collected and precipitated with an equal volume of ethanol (96% v/v) and left overnight at 4°C. The precipitate was collected after centrifugation (approx. 6000 g, 20 minutes). Isolated hemicelluloses were washed with a 1:1 (v/v) mixture of ethanol and water. These water-extracted hemicelluloses (WEH) were then resuspended in water and freeze dried.
2.4. Isolation of oat bran β-glucans

Oat bran was Soxhlet-extracted with 96% (v/v) ethanol for six hours in order to remove lipids. The procedure for isolation of β-glucan (BG) followed the steps for hemicellulose isolation from rye bran described above.

2.5. Lichenase treatment of water-extracted rye bran hemicelluloses

Enzymatic treatment was performed in order to remove mixed-linkage β-glucans from the WEH material and enrich it in arabinoxylan. Enzymatic hydrolysis was performed with 0.2 U lichenase/g hemicelluloses. The enzymatic hydrolysis was carried out in 0.1 M sodium phosphate buffer, pH 6.9 for 24 hours at 50°C. The enzyme was deactivated after the treatment by boiling the solution for 10 minutes. The treatment was followed with dialysis for 48 hours against MilliQ water with a cut-off value of 12000-14000 Da. The dialysis was applied to remove the monomeric and oligomeric sugar units from the samples. The water-extracted samples enriched in arabinoxylan (WE-AX) were freeze dried after the dialysis process.

2.6. Monosaccharide analysis

Monosaccharide composition analysis of isolated WEH and WE-AX was performed using gas chromatography after acid methanolysis of the samples according to Sundberg et al. (Sundberg, Sundberg, Lillandt, & Holmbom, 1996). Hemicellulosic samples (approx. 10 mg) were degraded to monosaccharides using 2 ml 2M hydrochloric acid in anhydrous methanol (prepared by addition of 16 ml of acetyl chloride to anhydrous methanol in a 100 ml volumetric flask). The samples were kept at 100°C in pear-shaped flasks for three hours and then neutralized with pyridine and diluted with
methanol. The samples (600 μl) were trimethylsilylated prior to GC analysis using 200 μl
TMSCl/BSTFA/pyridine (1:100:100) at 60°C for 30 minutes. After evaporation, heptane (1 ml) was
added. The monosaccharide standards (D-xylose, D-arabinose and D-glucose) were treated the same
way as the samples and were diluted to make a calibration curve. The samples and the standards were
analyzed in triplicate. The GC instrument was an Agilent 6890N GC system with a flame ionization
detector (FID). The column was an Agilent J&W 122-1032G DB-1 (30 m × 0.25 mm × 0.25 μm;
Agilent Technologies, Foster City, CA). The temperature program was as follows: oven temperature
150°C, ramped at 2°C/min to 186°C and at 1°C/min to 200°C and at 20°C/min to 300°C. The injection
volume was 1 μL and the split ratio was 20:1.

Monosaccharide composition analysis of isolated oat bran β-glucan was determined by HPLC
analysis. Before analysis, samples were treated with 4% (w/v) sulphuric acid and autoclaved for 10
minutes at 121 °C. Samples were neutralized with CaCO3 and filtered (45 μm) for HPLC analysis.
Samples (40 μl) were injected at a temperature of 85°C and flow rate of 0.6 ml/min (eluent: Millipore
water) on an Aminex HPX-87P (Bio-Rad, Hercules, CA, USA) column.

2.7. Molar mass analyses

Molar mass analysis of isolated WEH and WE-AX were carried out by HPSEC (high
performance size exclusion chromatography) analysis using DMSO-based eluent (Pitkänen, Virkki,
Tenkanen, & Tuomainen, 2009). Samples for HPSEC analysis were dissolved in DMSO containing
0.01 M LiBr at room temperature for four days and filtered using 0.45 μm syringe filters before
analysis (GHP Acrodisc 13, Pall Corp., Ann Arbor, MI). Sample concentration was 3 mg/ml and the
injection volume was 100 μl. The HPSEC equipment consisted of a pump module (GPCmax, Viscotek
Corp., Houston, TX), a UV-detector (Waters 486 Tunable Absorbance Detector, Milford, MA, USA), a combined light scattering and viscometric detector (270 Dual Detector, Viscotek Corp.) and a refractive index (RI) detector (VE 3580, Viscotek Corp.). Two Shodex columns were used: LF-804 columns (8 × 300 mm, exclusion limit 2 × 106, Showa Denko, Tokyo, Japan) with a guard column LF-G (4.6 × 10 mm). The light scattering detector included two scattering angles: 7° (low angle light scattering, LALS) and 90° (right angle light scattering, RALS). The flow rate was 1 ml/min and the molar mass averages were calculated based on the light scattering/viscometry method using the dn/dc value of 0.064 ml/g. All the calculations were carried out using OmniSEC 4.5 software (Viscotek Corp.).

Oat BG was characterized with AsFlFFF (asymmetric flow field–flow fractionation) in aqueous eluent. Oat BG was dissolved in water containing 0.1 M NaNO₃ at a concentration of 2 mg/ml and filtered before analysis. Molar mass analysis was performed by using an AF2000 MT instrument (including Postnova Analytics software, Landsberg/Lech, Germany) equipped with multi-angle light scattering (MALS, Brookhaven Instruments Corporation, Holtsville, NY, USA) and refractive index (PN 3150, Postnova Analytics) detectors. The MALS detector contains a 30 mW laser as the light source operating at λ₀ = 660 nm with seven scattering angles (35°, 50°, 75°, 90°, 105°, 130°, 145°). In a separation channel a regenerated cellulose membrane with cut-off value of 10 000 g/mol was used. The cross-flow gradient employed for separation is described elsewhere (Pitkänen, Tenkanen, & Tuomainen, 2011). The sample injection volume was 50 µl. The Berry equation and a dn/dc value of 0.151 ml/g (Gómez et al., 1997) were used for the molar mass calculations.
2.8. Film casting

Freeze-dried polysaccharides (WEH, WE-AX, BG and the mixtures of WE-AX and BG of ratios 80:20, 50:50 and 20:80) were mixed into MilliQ water for 24 hours at 40 °C under magnetic stirring at a concentration of 10 g/l. The suspensions were degassed by ultrasonication under vacuum for five minutes and cast on Teflon plates or Teflon-coated Petri dishes. Films were dried at 23°C and 50% relative humidity (RH) and conditioned for at least four days before analysis. Films were identified in a similar manner as the extracted carbohydrates. The thickness of the test pieces was measured at 5-10 points using a micrometer screw gauge (Lorentzen & Wettre, Kista, Sweden) or Mega-Check Pocket Coating Thickness Meter (List-Magnetik, Leinfelden-Echterdingen, Germany) with µm precision and an average thickness was calculated.

2.9. FT-IR spectroscopy

FT-IR (Fourier transform infrared) spectra were collected using a Perkin Elmer Spectrum One spectrometer. The film samples were dried and milled with liquid nitrogen and pressed into KBr pellets (2 mg sample/ 200 mg KBr). 16 scans were collected in the range 400-4000 cm⁻¹ at a resolution of 2 cm⁻¹.

2.10. Tensile tests

Films were removed from the Teflon-coated Petri dishes and cut into 10 mm wide and approximately 100 mm long pieces with a testing length of 45 mm. Two films were cut from each of the samples into 10 pieces in total for tensile testing. Mechanical properties were measured with an Instron 4465 universal testing machine (Instron Corp., High Wycombe, England) at 23 °C and
50% RH. The crosshead speed was 5 mm/min, the initial distance between the grips was 50 mm and a
100 N load cell was used. Tensile strength, elongation at break and Young’s modulus were determined
for the ten test pieces and the average of the results was calculated.

The differences between mean results of the mechanical tests were analyzed using one-way
ANOVA and least significance differences (LSD) analysis. The analyses were done at p<0.05 level
using the Statistica 8.0 (StatSoft, Inc., Tulsa, USA) software.

2.11. Water vapour permeability tests

The water vapour permeability was measured for three films per sample type according to the
ASTM E 96/E 96M – 05 standard (ASTM, 2005). Films were sealed to aluminium cups, containing
43 g dry CaCl₂ as a desiccant and cups were placed into a 52% RH atmosphere. This set-up included an
air gap of 6 mm between the desiccant and the underside of the film. The aluminium cups were placed
into a desiccator cabinet containing a fan which circulated the air above the samples at a speed of
0.15 m/s. The temperature was kept at 22 °C while the RH was maintained at 52% using saturated
Mg(NO₃)₂ solution. The cups were weighed 8-10 times over a period of four days and the temperature
and RH were measured in the cabinet prior to each weighing using a Rotronic HygroPalm RH meter
(Rotronic AG, Bassersdorf, Switzerland). Calculations were performed according to the method of
Mikkonen et al. (Mikkonen et al., 2010). The water vapour transmission rate (WVTR) was calculated
from the linear regression of the slope of weight gain vs. time by dividing the slope by the test cell
mouth area. The water vapour permeability (WVP) was obtained by multiplying the WVTR by the
thickness of the film and dividing it by the water vapour partial pressure difference between the two
sides of the film. The correction method of Gennadios, Weller and Gooding was used to calculate the water vapour partial pressure at the underside of the film (Gennadios, Weller, & Gooding, 1994).

2.12. Water content and water absorption

Films pieces were equilibrated at 23°C and three different relative humidities: 50% RH in a climate chamber and 75.5% and 98% RH by using desiccators containing saturated NaCl solution and water respectively (Hoije et al., 2008). The water content of the films was determined after drying the film pieces at 105°C for 24 hours and weighing (Veiga-Santos, Oliveira, Cereda, & Scamparini, 2007). All samples were analyzed in triplicate. The differences between mean results were analyzed using one-way ANOVA and LSD analysis at p<0.05 level.

2.13. Oxygen permeability

The oxygen transmission rate (OTR) of films was measured with an OPT-5000 Oxygen Permeability Tester (PBI-Dansensor A/S, Ringsted, Denmark) containing a ceramic solid-state oxygen sensor. Measurements were performed at 23 ± 0.03°C and 50 ± 2% RH. The test samples were placed in a permeability chamber which consisted of an upper (feeding) and a bottom (receiving) chamber. Dry nitrogen containing less than 0.1 ppm oxygen (Alphagaz 2, Air-Liquid Danmark) was used as carrier gas and pure oxygen (N45, Air-Liquid Danmark) served as the test gas. Inlet pressure was set to four bars at the regulator. The oxygen permeability was calculated by multiplying the OTR with the thickness of the films and dividing with the pressure value of the measuring chamber. The oxygen permeability was determined for two replicates of each film type.
3. Results and Discussion

3.1. Isolation and monosaccharide composition of the rye and oat hemicellulose fractions

The rye bran composition (Table 1) showed a rather low (~13 % w/w) arabinoxylan content, which likely occurred due to the milling of the grains. The grains were processed using a disc mill as an alternative to industrial roller milling, which may explain the high starch content and thus the low hemicellulose content in the raw material (Kamal-Eldin et al., 2009; Rakha, Åman, & Andersson, 2010). The disc milling would provide a slightly different bran structure with a higher amount of starch granules originating from the endosperm part of the grains. The non-starch glucan was comprised mainly of β-glucan (2.8% w/w) and presumably also cellulose.

The oat bran was obtained using a disc milling process similar to that applied to rye bran, which provided a bran material containing 47% starch (Table 1), comparable to that reported in previous publications (Immerstrand et al., 2009; Westerlund, Andersson, & Åman, 1993; Butt et al., 2008). The measured β-glucan content was 7.5% which is also in the range of values reported earlier (Immerstrand et al., 2009; Johansson et al., 2000).

The isolation procedure using a high-temperature treatment allowed the recovery of water-extractable hemicelluloses. Fibers, proteins and waxes were separated after the high-temperature treatment; however, additional protein separation was necessary. This was done by precipitation of the proteins during the autoclave treatment.

The monosaccharide composition of WEH (Table 2) showed a high content of xylose and arabinose, which was related to a high arabinoxylan content (approx. 65 w/w% of the isolated material) in the samples. The resulting Ara/Xyl ratio of 0.54 was in agreement with that found in other water-extracted arabinoxylans from rye (Ragaee et al., 2001; Bengtsson & Åman, 1990; Delcour, Rouseu, &
Vanhaesendonck, 1999). Some glucans (24%), which were mostly β-glucans (17%), were co-extracted with the arabinoxylans (Table 2). Water-extracted arabinoxylans represented 25% of the total arabinoxylan content of the bran which showed an efficient extraction yield considering the low amount of water-extractable hemicelluloses present in rye bran. However, extraction yield calculated from the starting bran material (3.2% w/w) was rather low, which is partly due to a high content of starch (50%) in the used bran. Ragaee et al. found that 22 to 33% of the total arabinoxylan content of different rye meals was water-extractable (Ragaee et al., 2001). Cyran et al. achieved slightly higher yields (~ 4%) than found in this study (Cyran & Saulnier, 2005).

In order to remove β-glucan contamination from the arabinoxylan material, lichenase enzyme treatment was applied to WEH followed by dialysis. Lichenase (E.C. 3.2.1.73; endo-1,3(4)-β-glucanase) enzyme can be used for the selective enzymatic degradation of cereal β-glucans. The enzyme specifically cleaves the (1→4) linkages of the 3-substituted glucose residues, producing oligosaccharides which are mostly tri- and tetrasaccharides (Roubroeks, Andersson, & Åman, 2000; Izydorczyk & MacGregor, 2000). The results show that the enzymatic treatment was efficient as the WE-AX sample contains about 1% of β-glucan. There was also a minor amount of other residual glucose-containing impurity. The measured Ara/Xyl ratio of WE-AX did not change significantly when compared to that of WEH, providing evidence that the lichenase treatment did not affect the arabinoxylan structure. Both rye hemicellulosic samples (WEH and WE-AX) showed notable nitrogen content, indicating the presence of proteins (~ 9%).

The composition of the isolated BG is summarized in Table 2. Significantly less protein was found in the oat extract relative to the rye hemicellulosic materials. A low amount of arabinoxylan was co-extracted, while the remaining carbohydrate content was mainly composed of glucose. The
measured β-glucan content was lower than the measured amount of glucose, which could be due to the presence of some starch. The purity of the BG was comparable to previously found values for water-extracted materials, varying between 74-95% (Johansson et al., 2000; Skendi, Biliaderis, Lazaridou, & Izydorczyk, 2003). The extraction yield of oat BG was somewhat higher than that of rye bran hemicelluloses and in good agreement with previously reported yields for water-extracted β-glucans (Westerlund, Andersson, & Åman, 1993; Wood, Siddiqui, & Paton, 1978).

3.2. Molar mass analysis

Molar mass distributions in WEH and WE-AX were determined using high performance size exclusion chromatography (HPSEC) in a DMSO-based eluent and the chromatograms are shown in Figure 1. Molar mass analysis of BG from oats was performed by asymmetric flow field-flow (AsFIFFF) fractionation using a water-based eluent. Molar mass analysis of β-glucans is frequently carried out in aqueous eluents (Roubroeks, Andersson, & Åman, 2000; Tosh, Wood, Wang, & Weisz, 2004; Liu & White, 2011), while xylan molar mass analysis is performed using water or other solvents, such as DMSO. DMSO was recently proven to be a better solvent for cereal arabinoxylans than water, preventing xylan aggregation and chain entanglement (Pitkänen, Virkki, Tenkanen, & Tuomainen, 2009).

The refractive index, viscosity and light scattering peaks (Figure 1a) of the WE-AX sample appeared at the same retention volume. However, in the WEH sample (Figure 1b) the peaks are broader which can especially be seen in the RI signal. UV peaks appeared at lower elution volumes than the other detector signals, thus no significant amount of UV-absorbing components, such as phenolic acids
eluted together with the arabinoxylan. The intensity of the UV signal for the WE-AX material was higher and showed two major peaks, while only one peak appeared in the analysis of the WEH. Such UV-signals may appear because of the presence of lignin or protein impurities. The presence of the lichenase enzyme could also have contributed to the appearance of the second peak. Average molar masses ($M_w, M_n$) were calculated from the light scattering signals. The calculated molar mass (Table 3) for the WE-AX sample was in accordance with previously analyzed water-extracted rye arabinoxylan by Pitkänen et al. (Pitkänen, Virkki, Tenkanen, & Tuomainen, 2009). The average molar mass value of the WEH sample was slightly higher than that of the WE-AX sample, which indicates the higher molar mass of $\beta$-glucans in comparison with arabinoxylans.

The $M_w$ of the BG sample (386 000 g/mol) was higher than that of the WEH and WE-AX (Table 3). The RI recovery value of the analyzed BG sample is lower than those of the arabinoxylan samples. Some of the lower molar mass compounds may go through the AsFIFFF membrane, which has a cut-off value of 10 kDa, and are therefore not detected. Hence, recovery is decreased and a fraction of the low molar mass material is lost during the analysis and excluded from the calculations. A wide range of water-soluble $\beta$-glucan molar masses can be found in previous reports, varying between $2.57\cdot10^3$ – $1.2\cdot10^6$ g/mol (Roubroeks, Andersson, & Åman, 2000; Tosh, Wood, Wang, & Weisz, 2004). Besides the molar mass results, a higher dispersity was found for the BG samples than for the arabinoxylan samples. These differences are in good agreement with previously reported values for oat $\beta$-glucans (Skendi, Biliaderis, Lazaridou, & Izydorczyk, 2003; Roubroeks et al., 2000).
3.3. Film forming and material properties

All of the cast films were cohesive but brittle without addition of plasticizer and cracking occurred especially during test sample preparation of the mixed films of WE-AX and BG. Films from WEH were transparent but the WE-AX films had a tawny colour. The BG samples formed transparent films with some opaque areas. A blended film, WE-AX:BG 80:20, was prepared in order to model the composition of the WEH and study the effect of a similar amount of added β-glucan on the arabinoxylan film. Blended films, WE-AX:BG 50:50 and 20:80, were then cast to examine the effect of increasing amounts of β-glucan on arabinoxylan film properties. The amount of added WE-AX determined the colour of the blend films. The thickness of the films varied between 40 and 60 μm.

3.4. FT-IR spectroscopy

FT-IR spectra were collected to confirm the composition of each of the extracted carbohydrates or mixtures. Pattern differences were investigated between the blend films and the pure WEH, WE-AX and BG samples (Figure 2). In the region 1000-1200 cm\(^{-1}\) one intense ring and side group band could be found in the WEH and WE-AX samples which is typical for arabinoxylans (Figure 2B), while for the BG samples (Figure 2F) two broad bands were found at 1023 and 1071 cm\(^{-1}\) (Ahmad et al., 2010). The maximum absorption band could be assigned to C-OH bending at ~1044 cm\(^{-1}\) for WEH and WE-AX samples. The phenolics and proteins had specific absorption bands in the 1700-1500 cm\(^{-1}\) in all samples. The protein content of the samples could be seen from the amide I (1654 cm\(^{-1}\)) and amide II (1539 cm\(^{-1}\)) bands (Kacuráková et al., 1999). These bands appeared clearly in the WEH and WE-AX spectra and were less visible in the BG spectra, corresponding to the chemical analysis data (Table 2). Comparing the spectra for WEH and WE-AX, a shoulder can be seen in the spectrum for the WEH.
sample at around 1020 cm$^{-1}$, which can be related to two bands assigned to the $\beta$-glucans in this region, arising from (1\(\rightarrow\)4) glycosidic linkages.

The spectra of the blend films show that the intensity and the extent of any changes compared to the spectra of the one-component films were related to the mixing ratio. The typical band in the carbohydrate region at 1044 cm$^{-1}$ broadened in the case of the mixture WE-AX:BG 20:80 showing similar peaks to the BG sample. The high protein content was visible as a result of the addition of WE-AX to the blend film in the mixture WE-AX:BG 80:20. A sharp peak was visible in the spectra of the mixed films at 1384 cm$^{-1}$ which is probably a result of C-H and O-H bending in xylans.

Robert et al. have compared the FT-IR spectra of arabinoxylan and mixed-linkage $\beta$-glucans. $\beta$-Glucans showed an absorption band at 895 cm$^{-1}$ assignable to the $\beta$-(1\(\rightarrow\)4) linkage, while two broad and intense bands and a shoulder appeared at about 1065, 1020, and 990 cm$^{-1}$ (Robert et al., 2005) which is in good agreement with our findings. The FT-IR spectra of a mixture from arabinoxylan, $\beta$-glucan and arabinogalactan structures were also investigated by Robert et al. who found that the absorption bands of the polysaccharides were very close and showed partial overlapping, which would hinder investigations on possible interactions between arabinoxylan and $\beta$-glucans using FT-IR analysis.

3.5. Tensile properties of films

3.5.1. Tensile properties of pure arabinoxylan and $\beta$-glucan films

Tensile strength, Young’s modulus and elongation at break were calculated from the tensile test results on the one-component WEH, WE-AX and BG films. A significant (p<0.05) decrease in mechanical properties was observed due to removal of $\beta$-glucan from WEH (Figure 3). Average tensile
strength dropped from 32.8 MPa to 15.7 MPa, although it should be noted that the standard deviation was high for the test samples. A significant decrease in elongation at break from 10.6% to 6.1% was also observed. A lower decrease was seen for the average Young’s modulus values. BG films showed tensile strength (average: 33.0 MPa) similar to that of the WEH films, but lower elongation at break (7.8%) (Figure 3).

Skendi et al. measured tensile strength values for oat β-glucan films in the range of 20-40 MPa and somewhat lower elongation at break values than the BG films in our study. In that case, the tested oat β-glucans had a lower measured molar mass, varying between 180 000 and 300 000 g/mol (Skendi, Biliaderis, Lazaridou, & Izydorczyk, 2003).

Previous tensile strength results for arabinoxylan films without addition of external plasticizer have been higher than the WEH film test results reported here. Höije et al. and Stevanic et al. measured a tensile strength of 52.4 MPa and 58 MPa respectively for rye arabinoxylan films without plasticizer addition (Höije et al., 2008; Stevanic et al., 2011). However, in these studies, the arabinoxylan originated from rye endosperm, thus the “fine” structure might have been different, even though the average Ara/Xyl ratio and the molecular weight were similar to those reported here. While the measured tensile strength was higher, the films measured by Höije et al. showed a lower elongation at break of 4.7% than the WEH and WE-AX films in this study, whereas Stevanic et al. reported elongation at break of 8.1%. Arabinoxylan films originating from corn hulls showed similar mechanical properties with a tensile strength of 53.8 MPa and an elongation at break of 6.2% (Zhang & Whistler, 2004). A slight difference in such strength and elongation values in the mentioned studies and the WEH properties can be caused by variations in the water content of the tested films as a consequence of differing relative humidities and other test conditions. Water has a plasticizing effect.
on biopolymer-based films and thus decreases the tensile strength and increases the elongation at break values (Mikkonen et al., 2008).

Aggregation and precipitation of oligosaccharides originating from β-glucans has been previously observed as a consequence of lichenase treatment (Lazaridou, Chornick, Biliaderis, & Izydorczyk, 2008). It was shown that arabinoxylan is also present in this precipitate and is generated by the hydrolysis of β-glucans (Izydorczyk & MacGregor, 2000). In our work, moderate particle aggregation was visible in solution and in the cast WE-AX films after the lichenase treatment. Partly insoluble material may also have originated from denatured proteins in the WE-AX material due to boiling after enzymatic treatment. These aggregated particles could act as defects in the WE-AX films and contribute to the early breaking of the samples during tensile testing. This effect could contribute to the drastic decrease in tensile properties of the two arabinoxylan-containing samples, WEH and WE-AX.

Höije has studied the properties of two alkali-extracted arabinoxylan fractions from barley fiber with varying β-glucan content (Höije, 2008). Treatment with NaOH results in extraction of both arabinoxylans and β-glucans, whereas Ba(OH)₂-extraction is selective for arabinoxylans. In the research carried out by Höije, the presence of β-glucan had a positive effect on film flexibility. Films containing both arabinoxylans and β-glucans (β-glucan contents = 19.2 and 28.3% of dry matter) showed twice as high elongation at break values (~6 and 7%) as the pure arabinoxylan films. These findings are in good agreement with those from the present study; however, Höije did not report differences in the tensile strength of the pure arabinoxylan films and the arabinoxylan films enriched in β-glucans.
3.5.2. Tensile properties of blend films

Different amounts of oat BG were added to the WE-AX material to examine in more detail the effect of β-glucan on the mechanical properties of films (Figure 3). The addition of BG significantly increased the tensile strength of the WE-AX films (p<0.05). The tensile strength of the WE-AX sample after 20% addition of BG increased from 15.7 MPa to 31.2 MPa (Figure 3a), which was somewhat lower than the value obtained for the WEH sample originally containing about 20% (w/w) β-glucans. No significant difference was detected in the tensile strengths of the WE-AX:BG 20:80, WE-AX: BG 80:20 blended films, and the pure BG film. However, the 50:50 mixtures of AX and BG showed slightly higher tensile strength values than the other films.

The elongation at break values of the blended films (Figure 3b) were all higher than those of the pure WE-AX or BG films. The elongation at break increased with increasing content of added BG. Surprisingly, the highest value 12.6% was found in the WE-AX:BG 20:80 films which was higher than that recorded for the pure BG films. WE-AX films containing 20% added BG gave slightly lower elongation at break results than the WEH films (Fig. 3), although BG addition had a positive effect on the elongation of the films.

Films from the WE-AX:BG 80:20 blend showed similar Young’s modulus values to the WEH samples (605 MPa for the mixture and 662 MPa for the WEH films with no significant difference at p<0.05). There was no clear tendency observed in the Young’s modulus values with increasing loading of BG in the mixtures (Figure 3c).

Addition of BG significantly improved the tensile strength and the elongation at break of the WE-AX films. This may be a result of intermolecular or physical interactions between the blended polysaccharides. Izydorczyk et al. reported the possibility of hydrogen bonds between certain
unsubstituted fragments of the arabinoxylan chain and cellulose-like sections of the β-glucan chain, which are more or less longer β-(1 → 4) connected glucose residues. However, these interactions are assumed for the polysaccharides located in the plant cell walls. They are less apparent in solution where interactions are probably blocked, since attachment of the mentioned polysaccharide segments are hindered due to the stiff conformation of the polymers (Izydorczyk & MacGregor, 2000). The structure of the externally added BG originating from oat was probably different to that of the rye β-glucan present in the WEH. The structure of β-glucans from different cereals has been reported in several studies and the differences are often characterized by the molar ratio of cellotriosyl to cellotetraosyl segments (Roubroeks, Andersson, & Åman, 2000; Cui, Wood, Blackwell, & Nikiforuk, 2000; Wood, Weisz, & Blackwell, 1994). Rye β-glucans have been reported to possess a higher ratio of cellotriosyl to cellotetraosyl units (~2.8–3.3) than β-glucans from oats (~2.0–2.4). However, this calculated molar ratio depends on the isolation process and cereal varieties as well as the part of the grain used for β-glucan extractions. These structural differences could have an effect on the material properties as well as interactions with arabinoxylans.

3.6. Water content

Water absorption of the one- and two-component films was examined at three different relative humidities and the results are summarized in Table 4. At 50% RH the pure BG films had the highest water content as expected from the previously reported higher water affinity (Tejinder, 2003). Water contents of rye hemicellulosic materials (WEH and WE-AX) were similar while the blended films of WE-AX and BG showed a decreasing tendency in water content compared to the one-component films. At 75% RH the BG films showed higher water content than the WEH and WE-AX films. The water
absorption difference increased the most in the case of the 80:20 and 20:80 WE-AX and BG blend films when compared to the measured water content differences after conditioning at 50% RH. Considerably higher values were found at 98% relative humidity, confirming the previous finding that arabinoxylans are very hygroscopic and absorb a great amount of water in high humidity environments (Gröndahl, Eriksson, & Gatenholm, 2004). The WEH films showed the highest water absorption (55.6%), which was surprisingly high relative to the values obtained for the WE-AX, BG and blended films.

The WEH films in our study gave water absorption values similar to those reported by Höije et al. (54.6%) for a rye arabinoxylan film with a similar Ara/Xyl ratio (Höije et al., 2008). Ying et al. recently reported a study on the mobility of arabinoxylan and β-glucan polymer chains and their interactions with water in thin films (Ying, Saulnier, & Rondeau-Mouro, 2011). Water content and the glass transition temperature of arabinoxylan and β-glucan films were examined after conditioning at relative humidities between 11 and 91%. For RH values between 11 and 75%, the β-glucan films had higher water contents; however, the tendency was reversed at 91% RH, which is in good agreement with our findings. Such differences are thought to occur due to different motions of the polymer chains. The average distances between the chains are shorter in the linear β-glucan than in the xylan chains with arabinose substitution, possibly causing stronger dipolar interactions in the β-glucan films. In effect, β-glucan chains form a more compact structure than arabinoxylans and this could result in lower water absorption of films containing β-glucans at high humidities.
3.7. Water vapor permeability

WEH films showed significantly lower WVP at 0/52 % RH gradient than the WE-AX and the blended films (Table 5). The films from BG samples provided a significantly higher permeability value. The WVP value of the WE-AX:BG 80:20 film was similar to that of the WE-AX film (Table 5). Thus, similar values to those from the WEH film could not be obtained by mixing WE-AX and BG in the appropriate ratio. The WVP values increased with increasing content of added BG and the WE-AX:BG 20:80 film showed a similar value to the pure BG film. The measured WVTR and WVP values of the WEH samples were lower than or within the same range as previously found for corn hull and corn bran arabinoxylan samples (Zhang & Whistler, 2004; Peroval, Debeaufort, Despre, & Voilley, 2002). The WVP of BG was higher in this study than that of the other samples; however, was still lower than the value of 59 g·mm/(kPa·m²·d) previously found for isolated barley β-glucan films (Tejinder, 2003). Higher values for β-glucan films were expected since the polysaccharide has a higher water affinity and a higher possible number of binding sites to form hydrogen bonds in the presence of water (Ying, Saulnier, & Rondeau-Muoro, 2011). This suggests that the presence of β-glucan should not reduce the water vapour permeability of the arabinoxylan films, although the β-glucan-containing WEH film samples showed lower values than the WE-AX films. This might be due to a more dense packing of the polysaccharide components or molecular interactions between the polymers, giving better moisture barrier properties to the WEH films.
3.8. Oxygen permeability

The oxygen permeability (OP) of the WEH and WE-AX films showed similar and low values, below 1.0 cm$^3$·μm/(m$^2$·d·kPa) (Table 6). These values suggest that the presence of β-glucans had no significant effect on the oxygen barrier properties. The measured permeability of pure BG films was indeed also low at 1.2 cm$^3$·μm/(m$^2$·d·kPa). Oxygen permeability values for the blended films were in the same range as the one-component WEH, WE-AX and BG films. The presence of incompletely dissolved and thus aggregated particles, zones of the films with changing thickness, as well as pinholes might have contributed to the noted differences in permeability values between the one- and two-component films. In previous studies the oxygen permeability of arabinoxylan films were found to be very low; in the same range as poly(vinyl alcohol) and slightly higher than that of ethylene vinyl alcohol (EVOH), which are commonly used barrier plastics (Lange, Wyser, & Lange, 2003). Gröndahl measured 0.16 cm$^3$·μm/(m$^2$·d·kPa) for barley husk arabinoxylan films without plasticizer addition, while glycerol-plasticized oat spelt arabinoxylan films were reported to have OP values of 3.0-3.2 cm$^3$·μm/(m$^2$·d·kPa) (Mikkonen et al., 2009; Gröndahl & Gatenholm, 2007). Rye arabinoxylan films were studied by Höije et al. at 50% RH and provided a permeability of 2.0 cm$^3$·μm/(m$^2$·d·kPa) (Höije et al., 2008) which is in good agreement with the results reported here.

4. Conclusions

The presence of β-glucans was found to have an effect on the mechanical properties of arabinoxylan films. In particular, arabinoxylans and β-glucans co-extracted from plant material formed stronger films with higher elongation than films with reduced β-glucan content. A corresponding positive effect was noted when β-glucans were artificially added to arabinoxylans ahead of film
casting. These findings were obtained from a study in which hemicelluloses were isolated from rye bran using hot water extraction, which allowed the separation of a material (WEH) containing 65% arabinoxylan, 20% β-glucans and 9% proteins. When the same procedure was applied to oat bran, a material (BG) consisting of greater than 90% β-glucans was isolated. In order to better understand such effects in cast films, β-glucan was selectively removed from WEH by applying a lichenase enzyme treatment, resulting in samples identified as WE-AX. HPSEC analysis indicated that WEH and WE-AX had similar molecular weights and no UV-absorbing compounds, such as phenolic acids, were present. In comparison, BG had higher Mw and dispersity, as expected for these polysaccharides, and there was some evidence for aggregation during the analysis. FT-IR analysis provided additional proof of the presence of arabinoxylan and β-glucan structures in the co-extracted hemicellulose film samples, while in the blend films the observed intensity and band changes in the FT-IR spectra were a function of the mixing ratio of BG and WE-AX. Although it is hard to fully explain the effect of β-glucan content on arabinoxylan film properties, the increase in tensile strength might be predictable based on the more linear structure of β-glucans as compared to the branched nature of the arabinoxylans. The higher water affinity of the β-glucans might at the same time introduce a plasticizing effect as a result of higher equilibrium moisture contents in mixed films, which would explain the increase in elongation at break under tensile load. In a similar manner, even though the differences are not large, the greater water affinity of the β-glucans could at least partly explain variations in the water vapour barrier properties of the various films. In line with reported observations on other carbohydrates, all the films prepared in this study showed low oxygen permeability. Considering the mechanical and barrier properties of the films, this research has shown that higher β-glucan contents have a generally beneficial effect when practical applications of cast xylan/β-glucan films are considered.
Acknowledgements

Mark Lawther (Danish Technological Institute) is acknowledged for his advice and for supervising the hemicellulose extraction processes. We thank Susanna Heikkinen (University of Helsinki) and Ingelis Larsen (Risø-DTU) for assistance with the monosaccharide compositional analysis and Mari I. Heikkilä (University of Helsinki) for her help in film casting and tensile testing. Financial support from EU COST Action FP0602, which allowed Zs. Sárossy to visit the University of Helsinki, Department of Food and Environmental Sciences for a period of three months during the course of the work reported here, is also gratefully acknowledged. The Technical University of Denmark is thanked for financial support.

References


Figure captions

**Figure 1.** HPSEC chromatograms of rye hemicelluloses WE-AX (A) and WEH (B) samples. Signals: RI- refractive index, VISC – viscometer, RALS - right angle light scattering, UV – ultraviolet

**Figure 2.** FT-IR spectra of pure and blended arabinoxylan and β-glucan films: WEH (A), WE-AX (B), WE-AX:BG 80:20 (C), WE-AX:BG 50:50 (D), WE-AX:BG 20:80 (E), BG (F)

**Figure 3.** Tensile strength (A), elongation at break (B) and Young's modulus (C) of pure WEH, WE-AX, BG and blended WE-AX and BG films
Table 1.

Composition of rye and oat bran (Percent of dry weight ± standard deviation)

<table>
<thead>
<tr>
<th>Component</th>
<th>Rye bran</th>
<th>Oat bran</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylose(^a)</td>
<td>8.6 ± 0.2</td>
<td>4.0 ± 0.1</td>
</tr>
<tr>
<td>Arabinose(^a)</td>
<td>4.0 ± 0.1</td>
<td>1.6 ± 0.0</td>
</tr>
<tr>
<td>Glucose(^a)</td>
<td>60.2 ± 0.6</td>
<td>57.0 ± 1.3</td>
</tr>
<tr>
<td>Starch</td>
<td>49.6 ± 2.9</td>
<td>47.0 ± 0.3</td>
</tr>
<tr>
<td>β-glucan</td>
<td>2.8 ± 0.1</td>
<td>7.5 ± 1.3</td>
</tr>
<tr>
<td>Klason lignin</td>
<td>4.6 ± 0.2</td>
<td>4.5 ± 0.8</td>
</tr>
<tr>
<td>Protein</td>
<td>10.8 ± 0.1</td>
<td>16.2</td>
</tr>
<tr>
<td>Extractives</td>
<td>9.9 ± 0.2</td>
<td>12.8 ± 0.1</td>
</tr>
<tr>
<td>Ash</td>
<td>1.4 ± 0.1</td>
<td>2.7</td>
</tr>
<tr>
<td>Total</td>
<td>99.5</td>
<td>98.8</td>
</tr>
</tbody>
</table>

\(^a\): Carbohydrate components are presented as anhydro-sugars
Table 2.

Isolation yield, monosaccharide composition, Ara/Xyl ratio, protein content and measured β-glucan content of rye hemicelluloses WEH, WE-AX and oat BG samples

<table>
<thead>
<tr>
<th>Sample type</th>
<th>WEH</th>
<th>WE-AX</th>
<th>BG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yield (%)(^a)</td>
<td>3.2</td>
<td>-</td>
<td>4.0</td>
</tr>
<tr>
<td>Carbohydrate content (%)(^b)</td>
<td>89.5</td>
<td>83.1</td>
<td>94.7</td>
</tr>
<tr>
<td>Monosaccharide composition (%)(^c)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xyl</td>
<td>42.9 ± 2.6</td>
<td>49.6 ± 2.0</td>
<td>1.95 ± 0.3</td>
</tr>
<tr>
<td>Ara</td>
<td>23.1 ± 1.1</td>
<td>28.4 ± 1.0</td>
<td>1.46 ± 0.0</td>
</tr>
<tr>
<td>Glc</td>
<td>23.5 ± 2.1</td>
<td>5.2 ± 0.2</td>
<td>91.1 ± 1.5</td>
</tr>
<tr>
<td>Ara/Xyl ratio</td>
<td>0.54</td>
<td>0.57</td>
<td>0.75</td>
</tr>
<tr>
<td>Measured β-glucan content(^c)</td>
<td>17.1 ± 0.5</td>
<td>1.3 ± 0.6</td>
<td>81 ± 1.2</td>
</tr>
<tr>
<td>Protein content (%)(^c,d)</td>
<td>8.5 ± 0.5</td>
<td>9.1 ± 0.1</td>
<td>3.6 ± 0.3</td>
</tr>
</tbody>
</table>

---

\(^a\): weight percent from rye or oat bran  
\(^b\): corresponding to the sum of Xyl, Ara, Glc  
\(^c\): percent of weight ± standard deviation; n=3  
\(^d\): Protein content=total nitrogen x 5.83
Table 3.

Molar mass averages ($M_w$, $M_n$), dispersity index ($M_w/M_n$), sample recovery of isolated rye hemicellulose WEH, WE-AX and oat BG samples.

<table>
<thead>
<tr>
<th>Sample type</th>
<th>WEHa</th>
<th>WE-AXa</th>
<th>BGb</th>
</tr>
</thead>
<tbody>
<tr>
<td>$M_w$ (g/mol)</td>
<td>270000</td>
<td>232000</td>
<td>386000</td>
</tr>
<tr>
<td>$M_n$ (g/mol)</td>
<td>153000</td>
<td>190000</td>
<td>180000</td>
</tr>
<tr>
<td>$M_w/M_n$</td>
<td>1.8</td>
<td>1.2</td>
<td>2.1</td>
</tr>
<tr>
<td>Sample recovery %</td>
<td>75</td>
<td>66</td>
<td>46</td>
</tr>
</tbody>
</table>

a: samples measured by HPSEC in a DMSO-based eluent
b: samples measured by AsFIFFF in an aqueous eluent
Table 4.

Water content of pure WEH, WE-AX and BG films, and three blend films of WE-AX and BG at different relative humidities

<table>
<thead>
<tr>
<th>Sample type</th>
<th>50% RH</th>
<th>75.5% RH</th>
<th>98% RH</th>
</tr>
</thead>
<tbody>
<tr>
<td>WEH</td>
<td>12.3 ± 1.3b</td>
<td>13.4 ± 1.4b</td>
<td>55.6 ± 3.4b</td>
</tr>
<tr>
<td>WE-AX</td>
<td>11.9 ± 0.3c</td>
<td>13.7 ± 1.3c</td>
<td>41.5 ± 0.1bc,e</td>
</tr>
<tr>
<td>WE-AX + BG 80:20</td>
<td>10.1 ± 0.5bc,d,f</td>
<td>14.4 ± 1.0</td>
<td>38.1 ± 3.7bd</td>
</tr>
<tr>
<td>WE-AX + BG 50:50</td>
<td>11.9 ± 0.6dc</td>
<td>15.0 ± 1.5</td>
<td>36.5 ± 3.8bc,e</td>
</tr>
<tr>
<td>WE-AX + BG 20:80</td>
<td>10.9 ± 0.1c</td>
<td>14.8 ± 1.0</td>
<td>42.4 ± 2.1bc,e</td>
</tr>
<tr>
<td>BG</td>
<td>13.4 ± 1.5ef</td>
<td>15.9 ± 0.3bc,e</td>
<td>43.9 ± 0.6bd,e</td>
</tr>
</tbody>
</table>

a: percent of weight ±standard deviation; n=3
b,c,d,e: values with the same superscript letters in the same column are statistically different (p<0.05)
Table 5.

Water vapor transmission rate (WVTR), water vapor permeability (WVP) and corrected water vapor permeability of pure WEH, WE-AX and BG films, and three blend films of WE-AX and BG at approx. 50% relative humidity

<table>
<thead>
<tr>
<th>Type of film</th>
<th>WVTR (g/m²·d)</th>
<th>WVP (g·mm/kPa·m²·d)</th>
<th>Corrected WVP (g·mm/kPa·m²·d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WEH</td>
<td>89 ± 5</td>
<td>2.8 ± 0.4</td>
<td>2.8 ± 0.4</td>
</tr>
<tr>
<td>WE-AX</td>
<td>204 ± 1</td>
<td>7.3 ± 0.5</td>
<td>7.7 ± 0.5</td>
</tr>
<tr>
<td>WE-AX + BG 80:20</td>
<td>224 ± 8</td>
<td>7.4 ± 0.1</td>
<td>7.9 ± 0.1</td>
</tr>
<tr>
<td>WE-AX + BG 50:50</td>
<td>293 ± 8</td>
<td>9.1 ± 0.4</td>
<td>9.9 ± 0.4</td>
</tr>
<tr>
<td>WE-AX + BG 20:80</td>
<td>283 ± 5</td>
<td>12.0 ± 0.0</td>
<td>13.0 ± 0.0</td>
</tr>
<tr>
<td>BG</td>
<td>363 ± 8</td>
<td>11.1 ± 1.1</td>
<td>12.3 ± 1.2</td>
</tr>
</tbody>
</table>
Table 6

Oxygen transmission rate (OTR) and oxygen permeability (OP) of pure WEH, WE-AX and BG films, and three blend films of WE-AX and BG at 50% relative humidity and 23°C

<table>
<thead>
<tr>
<th>Type of film</th>
<th>OTR (ml/m² day)</th>
<th>OP (cm³ μm/m² d kPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WEH</td>
<td>4.25 ± 2.95</td>
<td>0.98 ± 0.32</td>
</tr>
<tr>
<td>WE-AX</td>
<td>2.32 ± 0.87</td>
<td>0.87 ± 0.24</td>
</tr>
<tr>
<td>WE-AX + BG 80:20⁵ᵃ</td>
<td>2.74</td>
<td>1.30</td>
</tr>
<tr>
<td>WE-AX + BG 50:50⁵ᵃ</td>
<td>3.75</td>
<td>1.60</td>
</tr>
<tr>
<td>WE-AX + BG 20:80</td>
<td>7.19 ± 1.79</td>
<td>1.97 ± 0.79</td>
</tr>
<tr>
<td>BG</td>
<td>3.91 ± 1.42</td>
<td>1.23 ± 0.72</td>
</tr>
</tbody>
</table>

⁵ᵃ: only one successful measurement could be made from the samples due to leaking, as a result of defects on the film surface
Figure 1
Figure 2
Figure 3

A: Tensile strength (MPa) vs. Amount of added β-glucan (%)

B: Elongation at break (%) vs. Amount of added β-glucan (%)

C: Young's modulus (MPa) vs. Amount of added β-glucan (%)
Composite films of arabinoxylan and fibrous sepiolite: morphological, mechanical and barrier properties

Zsuzsa Sározya, Thomas O. J. Blomfeldtb, Mikael S. Hedenqvistb, Christian Bender Kochc, Suprakas Sinha Rayd, David Placketta*

a Department of Chemical and Biochemical Engineering, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark

b Department of Fibre and Polymer Technology, KTH Royal Institute of Technology, SE-100 44 Stockholm, Sweden

c Department of Chemistry, University of Copenhagen, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark

d DST/CSIR Nanotechnology Innovation Centre, National Centre for Nano-Structured Materials, Council for Scientific and Industrial Research, P.O. Box 395, Pretoria 0001, Republic of South Africa

*Corresponding author. E-mail: dapl@kt.dtu.dk

Tel.:+45 4525-6817

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Abstract

Hemicelluloses represent a largely unutilized resource for future bio-derived films in packaging and other applications. However, improvement of film properties is needed in order to transfer this potential into reality. In this context, sepiolite, a fibrous clay, was investigated in terms of its use as an additive to enhance the properties of rye flour arabinoxylan. Composite films cast from arabinoxylan solutions and sepiolite suspensions in water were transparent at additive loadings in the 2.5 - 10 wt% range. Scanning electron microscopy showed that the sepiolite was well dispersed in the xylan films and sepiolite fiber aggregation was not found. These observations are consistent with good compatibility between the hydrophilic clay and the hydrophilic matrix and were supported by FTIR spectroscopy which provided some evidence for hydrogen bonding between sepiolite and arabinoxylan. Also consistent with these findings, mechanical testing showed significant increases in film stiffness and strength with sepiolite addition and the tuning of properties that could be realized through poly(ethylene glycol) methyl ether (mPEG) plasticizer addition. Unlike layered nanoclay, sepiolite addition did not significantly influence the thermal degradation or the gas barrier properties of xylan films, which is likely a consequence of sepiolite fiber morphology. Calculations suggested that sepiolite fibers might form a physical network at loadings of 2 volume % or higher and, consistent with this suggestion, the characteristic strong basal peak for sepiolite was detected in X-ray diffraction patterns for all of the arabinoxylan/sepiolite film compositions. In summary, sepiolite was shown to have potential as an additive to obtain stronger hemicellulose films although other approaches, possibly in combination with the use of sepiolite, would be needed if enhanced film barrier properties are required for specific applications.

Keywords: arabinoxylan, sepiolite, composite film, tensile properties, barrier properties
Introduction

Plant polysaccharides derived from by-products of agricultural processes have lately received much attention as substrates for potential future packaging materials.\textsuperscript{1-4} Xylan-type hemicelluloses are the most abundant heteropolysaccharides in agricultural residues and there has been increasing interest in their use in food packaging and other applications.\textsuperscript{5} Indeed, over the past few years there have been a number of reports discussing films prepared from xylans extracted from wheat bran, rye grains, barley husks, corn hulls and bran, oat spelt or aspen and beech.\textsuperscript{1-3, 6, 7} Agricultural by-products such as oat spelt or barley husk arabinoxylans have been shown to provide good oxygen and/or grease barrier films in applications where moderately high water vapor permeability is required.\textsuperscript{1, 2} Hemicelluloses are hydrophilic polysaccharides and films tend to show poor properties in highly humid environments. Depending on the application, mechanical properties may also need improvement.

A potential route to improving mechanical and barrier properties is the addition of nanofillers to form hemicellulose-based nanocomposites. In comparison to unmodified polymers, the characteristic features of nanocomposites generally include higher modulus, increased strength, decreased gas permeability and increased thermal stability. These effects are in large part due to the high surface area of the well-distributed nanoparticles and the resulting increased interfacial area.\textsuperscript{8-10} Clay-biopolymer nanocomposites are of particular interest because of the significant potential to improve material properties; however, in the case of hemicelluloses, the number of publications discussing the effects of clay nanofiller addition remains quite limited.\textsuperscript{10, 11} Xylan-clay films have been made focusing on the use of layered silicate clays such as montmorillonite (MMT). For example, Ünlü et al.\textsuperscript{11} prepared films using corn cob xylan and MMT. Their research using electrokinetic, rheological and crystallinity measurements suggested that interactions took place on the MMT surface and that the xylans were not oriented into the MMT interlayer spaces.

In addition to the use of MMT, the interesting potential of other clays having tubular or fibrous morphologies has been recognized. In the case of sepiolite, a fibrous clay, researchers have investigated
its use as a filler in natural rubber and plastics such as poly(vinyl chloride).\textsuperscript{12} Combinations of sepiolite with biopolymers such as starch, chitosan, gelatin and polylactide have also been investigated.\textsuperscript{9, 13-15} As one example, biocompatible biomaterials were prepared with collagen and sepiolite and used as gel-like complexes.\textsuperscript{16} Improved mechanical properties were found in these sepiolite/biopolymer nanocomposites, which is in general comparable with the improvements obtained with other fiber-like nanofillers (e.g., cellulose whiskers). Chivrac et al.\textsuperscript{9} studied the effects of sepiolite addition on the mechanical properties of starch nanocomposites. In their study a comparison was made between sepiolite and other fibrous fillers, such as cellulose whiskers or cellulose nanofibers, as well as hectorite, a layered nanoclay. Evidence for strong hydrogen bonding was noted at cellulose whisker loadings above the percolation threshold. Reinforcing effects were observed when either cellulose whiskers or cellulose nanofibers were used and in both cases mechanical property improvements were similar to those achieved when using a layered or fibrous clay additive. In these composites, the Young's modulus increased and there was no effect on the strain at break.

Sepiolite has a hydrated magnesium silicate composition with a theoretical unit-cell formula equivalent to Si\textsubscript{12}O\textsubscript{30}Mg\textsubscript{6}(OH)\textsubscript{4}·(H\textsubscript{2}O)\textsubscript{4}·8H\textsubscript{2}O. Alternating longitudinal blocks and channels (tunnels) are found in the sepiolite structure, producing long needles with a high surface area (approximately 374 m\textsuperscript{2}/g).\textsuperscript{8} The blocks are built up of two layers of tetrahedral silica, sandwiching an octahedral magnesium oxide-hydroxide layer in the centre (Figure 1). The composite layers are off-set, hence allowing the formation of tunnel-like micropores (channels), running parallel to the fiber axis. The channels in the sepiolite structure are occupied by water coordinated to the magnesium ions at the edges of the octahedral layers and zeolitic water associated with the clay structure by hydrogen bonding inside the tunnels. Silanol (Si-OH) groups appear at the external surface edges of the sepiolite structure.\textsuperscript{9, 17-19} The numerous silanol groups provide sites for hydrogen bonding and Van der Waals interactions in composites, contributing to the reinforcing effect of sepiolite. From this perspective, the availability of silanol groups for hydrogen bonding with other moieties mostly occurs at the edges.
We focused our work on composite films using arabinoxylan from rye grains and sepiolite at loadings between 2.5 and 10 wt% clay. The objective was to observe potential improvements in the mechanical and barrier properties of arabinoxylan films. Morphology, crystallinity and structural changes in the composite films were studied as well as tensile and barrier properties and these characteristics were compared with those of unreinforced arabinoxylan films. To our knowledge, this is the first report on the production and mechanical/barrier properties of sepiolite-hemicellulose composite films.

**Figure 1.** Transection of the sepiolite structure (adapted from Karakashev et al.²⁰).

**Materials and methods**

**Materials**

Arabinoxylan (Lot 20601) from rye flour (RAX) was purchased from Megazyme International Ireland Ltd. (Bray, Ireland). The arabinoxylan was a high-viscosity sample with reported Ara/Xyl ratio of 0.64, purity ~90% and ash content of 4.5 wt%. Poly(ethylene glycol) methyl ether (mPEG) and sepiolite were purchased from Sigma-Aldrich (Steinheim, Germany). The sepiolite powder from Aldrich has a unit cell...
formula of Mg$_2$H$_2$Si$_3$O$_9$·xH$_2$O and a reported Mg content of approximately 13%.

**Film casting**

Neat RAX and RAX-sepiolite composite films containing the two components in ratios 97.5:2.5, 95:5, 90:10 and 80:20 were prepared, although the last of these, containing 20 wt% sepiolite, was only prepared for tests on light transmission. RAX was mixed into MilliQ water for four hours at 70 °C under magnetic stirring at a concentration of 10 g/l. Sepiolite suspensions were prepared at a concentration of 2.5 g/l in MilliQ water and were sonicated for one hour under magnetic stirring using a UP 400 S ultrasonic processor with a 22 mm diameter tip and a maximum amplitude of 100 μm at 25% output (Hielscher Ultrasonics GmbH, Teltow, Germany). The sepiolite suspension and RAX solution were then mixed in appropriate ratios using magnetic stirring. The mixtures were degassed by ultrasonication for 15 minutes and suitable volumes poured into Teflon-coated Petri dishes for the purposes of film casting.

In selected cases, mPEG plasticizer was added to the mixed RAX-sepiolite formulations prior to ultrasonication at 30 wt% loading on the basis of total RAX-sepiolite weight. mPEG was chosen as the plasticizer as it is non-toxic and may be applied in foods, cosmetics or pharmaceuticals. mPEG has not to our knowledge been used as a plasticizer in previous studies on hemicellulose films. Films were prepared by drying the contents of the Petri dishes at 23 °C and 50 % relative humidity (RH) and held under these conditions for at least four days before analysis. The thickness of the films was measured at 10 points using a Mega-Check Pocket Coating Thickness Meter with μm precision (List-Magnetik, Leinfelden-Echterdingen, Germany) and average film thicknesses were calculated.

**Light transmittance**

The light transmittance of the films was measured for two replicates of each film type over the 190–890 nm wavelength range with an Ultrospec 2100pro UV–visible spectrophotometer (Biochrom Ltd, Cambridge, UK). The light transmittance was normalized to a thickness of 30 μm for all tested films.
Film opacity, normalized to a film thickness of 30 μm and expressed as absorbance × nanometers, was calculated with an integration procedure as similarly reported by Siró et al.22

**Fourier transform infrared (FTIR) spectroscopy**

FTIR spectra were collected using a Perkin Elmer Spectrum One spectrometer (PerkinElmer Life and Analytical Sciences, Shelton, WA, USA). For this purpose, the film samples were tested using an STI Thunderdome attenuated total reflectance (ATR) attachment. Sepiolite powder samples were pressed into KBr pellets (2 mg sample/ 200 mg KBr). Spectra were obtained based on 16 scans collected in the range 400-4000 cm⁻¹ at a resolution of 2 cm⁻¹.

**X-ray diffraction**

X-ray diffraction was used to study structural changes in RAX-sepiolite composite films in comparison to neat RAX film and sepiolite powder. A Siemens D5000 X-ray diffractometer (Siemens Analytical and X-Ray Instruments Inc., Madison, WI, USA) equipped with a Co (λ = 0.179 nm) tube and a diffracted beam monochromator was used. Diffractograms were collected in the 2Θ range of 3–30° using a step size of 0.05° and a counting time of 20 sec. One film sample was tested from each cast film type.

**Microscopy**

The fracture surface morphology of the composites after tensile testing was studied using focused ion beam scanning electron microscopy (FIB-SEM) using an AURIGA® CrossBeam® Workstation (Carl Zeiss, Oberkochen, Germany). The samples were sputtercoated with carbon in a vacuum chamber before examination. The chemical composition of the composites was analyzed with energy dispersive X-ray spectroscopy (EDX, NORAN System Six, Thermo Scientific, Waltham, MA) in the JEOL 7500F SEM (JEOL Ltd., Tokyo, Japan).
**Thermogravimetric analysis (TGA)**

The thermal degradation of films and sepiolite powder was studied under N$_2$ atmosphere at a flow rate of 30 ml/min using a Netzsch TG 209 F3 Tarsus thermogravimetric analyzer (NETZSCH-Gerätebau GmbH, Selb, Germany). The weight of the analyzed film pieces varied between 1-2 mg and alumina pans were used for the measurements. The samples were heated from 36°C to 900°C at a rate of 20°C/min, including an isothermal ramp at 120°C for three minutes.

**Tensile testing**

Tensile testing was performed on rectangular film samples (10 mm x 80 mm) using an Instron 5944 universal testing machine (Instron Corp., High Wycombe, England) with Instron grips series nr. 2712-019, according to the ASTM standard test method D882-09. Film test samples were cut using a Synrad 48-5 laser cutter (Synrad, Mukilteo, WA, USA). Sample testing was performed in a conditioned room at 23 ± 1°C and 50 ± 2 % RH. A load cell of 50 N was used with an extension rate of 5 mm/min and an initial grip distance of 50 mm. A pre-load of 0.1 N (extension rate = 5 mm/min) was applied to ensure measurement of straight samples. Ten specimens were tested from each sample, which were conditioned at 23°C and 50% RH for 120 hours before testing.

Statistical analyses were performed on the tensile test results using Tukey-Kramer HSD (Honestly Significant Difference) tests on data from ten specimens using the statistical software JMP version 5.0.1 (SAS Institute Inc., Cary, NC, USA) at a significance level of 0.05.

**Water vapor permeability**

The water vapor permeability was measured for three films per sample type according to the ASTM 96/E 96M – 05 standard. Films were sealed to aluminum cups, containing 21 g dry CaCl$_2$ as a desiccant. This set-up included an air gap of 6 mm between the desiccant and the underside of the film.
The aluminum cups were placed into a climate chamber with controlled relative humidity and temperature (50% RH and 23°C). The cups were weighed 8-10 times over the period of three days. Calculations were performed according to the method of Mikkonen et al. The water vapor transmission rate (WVTR) was calculated from the linear regression of the slope of weight gain vs. time by dividing the slope by the test cell mouth area. The water vapor permeability (WVP) was obtained by multiplying the WVTR by the thickness of the film and dividing it by the water vapor partial pressure difference between the two sides of the film. The correction method of Gennadios et al. in which the air gap is taken into account, was used as a means of calculating the water vapor partial pressure at the underside of the film. However, data calculated using this correction method gave WVP values which were not significantly different from those calculated without correction.

**Oxygen permeability**

The oxygen transmission rate (OTR) of films was measured with an OPT-5000 Oxygen Permeability Tester (PBI-Dansensor A/S, Ringsted, Denmark) containing a ceramic solid-state oxygen sensor. Measurements were performed at 23 ± 0.03°C and 50 ± 2% RH. The test samples were placed in a permeability chamber which consisted of an upper (feeding) and a bottom (receiving) chamber. Dry nitrogen containing less than 0.1 ppm oxygen (Alphagaz 2, Air-Liquide Danmark) was used as carrier gas and pure oxygen (N45, Air-Liquide Danmark) served as the test gas. Inlet pressure was set to four bar at the regulator. The oxygen permeability was calculated by multiplying the OTR with the thickness of the films and dividing with the pressure value of the measuring chamber. The oxygen permeability was determined for two replicates of each film type.
Results and discussion

Visual properties and light transmittance

All cast films were cohesive and the thickness of the films varied between 25 and 45 μm. Light transmittance showed a general decrease with increasing clay content. All cast films were at least semi-transparent; however, minor color changes were visible as the clay loading was increased to 10 and 20 wt%, as can be seen in Figure 2. The neat RAX films showed some slightly opaque spots, which may have been due to minor quantities of proteins precipitated during sample dissolution or lignin contamination. Significant contamination by lignin was not expected in the RAX since the material was alkali-extracted from rye grains and only minor amounts of aromatic components, such as ferulic acids, which would be characteristic of lignin, were found. Robertson et al. measured the ferulic acid content of the RAX material used in this study at a level of 1.55 mg/g. The light transmittance of the films as a function of wavelength is shown in Figure 3. The neat RAX film and films containing lower amounts of added sepiolite (2.5 and 5 wt%) showed a minor inflection in light transmission at 260 nm. This finding could be attributable to some aromatic compounds such as lignin or proteins absorbing in the ultraviolet region, since the typical absorption of aromatic compounds such as ferulic acid or aromatic amino acids occurs in the range 200-300 nm. The film opacities were calculated and a good correlation was found with the visual appearance. The calculated values were 276.2, 217.3, 328.0, 349.2, 398.5 (AU × nm) for the RAX, RAX-sepiolite 97.5:2.5, 95:5, 90:10 and 80:20 films respectively. These differences suggest that 5 wt% or higher amounts of added clay were not well dispersed and therefore introduced some opacity. This effect was particularly noticeable at 10 and 20 wt% sepiolite loadings.
Figure 2. Images of neat rye arabinxyolan (RAX) and rye arabinxyolan (RAX)-sepiolite composite films, from left to right RAX, RAX-sepiolite 95:5, 90:10 and 80:20. Films were placed on a white paper sheet with printed text.

Figure 3. UV-VIS transmittance of rye arabinxyolan (RAX) and RAX-sepiolite composite films.

Microscopy

The sepiolite distribution and the presence of clay aggregates were studied with FIB-SEM. Figure 4 a shows the distribution of 2.5 wt% sepiolite in the RAX matrix in the cross-section of tensile-tested films and Figure 4 b illustrates the composite film cross-section containing 5 wt% added sepiolite after tensile testing. The images show well-dispersed sepiolite fibers with minimal pull-outs, indicating
strong binding between the sepiolite and the RAX matrix. The lower level of clay addition is apparent from the fewer fibers visible in the image of the RAX-sepiolite 97.5:2.5 film (Figure 4a) which contains half the amount of sepiolite as the film RAX-sepiolite 95:5. The RAX matrix shows a nodular appearance and a similar morphology to that reported by Stevanic et al. in a study on arabinoxylan-bacterial cellulose composite films based on the same RAX. From the FIB-SEM images, the diameter of the sepiolite fibers is estimated at ~100 nm, which is comparable with values reported in an earlier study.

The composition of the composite films was studied by energy-dispersive X-ray spectroscopy (EDX) (Figure 5). As expected, EDX analysis revealed the occurrence of silicon and magnesium in an approximate ratio of 1.5 to 1 (atomic ratio) due to the presence of sepiolite. Phosphorus and calcium, present in the pure RAX and dominating in the ash, were also detected in the films.
Figure 4. FIB-SEM images of rye arabinoxylan (RAX)-sepiolite composites, RAX-sepiolite 97.5:2.5 (a) and RAX-sepiolite 95:5 (b). Scale bar = 200 nm.

Figure 5. Low magnification SEM image and corresponding EDX spectrum for the cross-section of a 95:5 rye arabinoxylan (RAX)-sepiolite composite film.
FTIR spectroscopy

FTIR spectra of sepiolite powder, RAX and RAX-sepiolite films were collected in order to examine the presence of clay in the RAX matrix as well as to study the formation of changes in molecular structure or new chemical bonds. The spectrum of neat RAX (Figure 6) shows a strong absorption band at 1042 cm⁻¹, which is due to C-OH bending.31, 32 A weak absorption band at 900 cm⁻¹ is characteristic of β-(1→4) glycosidic linkages between the xylose units in the RAX structure. A low intensity amide I absorption band appears at approximately 1651 cm⁻¹, which is consistent with low content of protein contamination.33 The absence of an absorption band at 1517 cm⁻¹ indicates that very little, if any, ferulic acid is present.

The FTIR spectrum of sepiolite (Figure 6) shows peaks in the 3400-3650 cm⁻¹ region attributed to OH stretching vibrations of zeolitic and Mg-coordinated water molecules occurring inside the tunnels and bound to the magnesium ions in the mineral structure. Bending vibrations of the zeolitic or channel water molecules contributed to a signal at 1666 cm⁻¹. Peaks associated with Si-O bonds in the tetrahedral sheets were seen between 1300 and 800 cm⁻¹. Si-O-Si bonds contributed to bands at 1215 cm⁻¹ and 1079 cm⁻¹ and in-plane Si-O-Si vibrations were observed at ~1025 and 980 cm⁻¹.13, 34

The spectra of RAX-sepiolite films show some differences when compared with the spectra of the pure components. The shoulder in the spectrum of RAX at 995 cm⁻¹ grew with sepiolite addition and a peak appeared at 984 cm⁻¹ in the spectrum of the RAX-sepiolite 90:10 film, which can be associated with the higher clay content of this film. A slight increase in the intensity of the peak at 1666 cm⁻¹ was also noticed; however, there is overlap here with the RAX amide I band, which hinders interpretation. A peak appearing at 3380 cm⁻¹ in the spectrum of the RAX film shifted towards higher wavenumbers with addition of sepiolite, as was observed for example, at 3435 cm⁻¹ in the spectrum of the RAX-sepiolite 90:10 film. This shift of the OH stretching band is likely indicative of hydrogen bonding interactions between RAX and sepiolite.
Arabinoxylans with a high degree of substitution (Ara/Xyl ratio of 0.5 or greater) are amorphous and show no distinct peaks in the X-ray diffraction pattern. Decreasing the arabinose substitution (Ara/Xyl ratios between 0.37 and 0.2) on the xylan main chain can lead to increasing crystallinity, showing distinct crystalline peaks between 4.9 and 3.3 Å. It is believed that the unsubstituted regions of the chains crystallize while the more highly substituted regions of the xylan chains remain amorphous. The arabinoxylan (RAX) applied in this study was highly substituted (reported Ara/Xyl ratio of 0.64) and therefore the presence of a crystalline structure in the polymer was neither expected nor found. The neat RAX film exhibited a broad peak with a maximum at 4.5 Å (Figure 7 A).

The characteristic (110) diffraction of sepiolite dominates the pattern of the pure sepiolite sample (Figure 7 E) and in addition an unidentified impurity can be detected with a d-spacing of 22.5 Å. A minor but distinct shift of the (110) diffraction can be observed in the mixed films. This shift is caused by the off-set of the effective diffraction plane between the films and the shift scales with the effective density of the films. The maximum of the broad peak in the XRD pattern of the films is also shifted slightly towards higher angles (Figs. 7 C, D, E). This may signify a change in the polymer, but it is also influenced by overlapping with the minor diffraction peaks of the sepiolite.
Figure 7. X-ray diffraction patterns of neat rye arabinoxylan (RAX) (A), sepiolite (E) and composite films of RAX-sepiolite, RAX-sepiolite 97.5:2.5 (B), 95:5 (C) and 90:10 (D). (110) indicates the position of the characteristic peak from sepiolite and U an unidentified mineral in the sepiolite sample.

TGA analysis

The thermal properties of neat RAX and RAX-sepiolite films were studied using TGA under nitrogen atmosphere. Figures 8 A and 8 B show the typical TGA and DTGA curves for RAX, RAX-sepiolite films and sepiolite powder. Sepiolite showed a weight loss of 11.1% in the temperature range 36-900 °C (the temperature range 36-550 °C is shown in Figure 8). Sepiolite loses zeolitic and structural water when it is heated and this is followed by loss of octahedrally coordinated hydroxyl groups (internal Mg-OH) in a four-step process with eventual collapse of the crystal structure. Kuang et al. found that maxima for the rate of weight loss occurred at 60 °C, 260 °C, 510 °C and 800-830 °C and, except for the last two, these changes are shown in Figure 8 A, representing loss of zeolitic water, structural water in two stages and Mg-OH dehydroxylation respectively. In Figure 8 A, initial weight losses of approximately 5% can be seen, which are attributable to release of water. As shown, the arabinoxylan in the RAX and composite film samples started to decompose at ~260 °C. Slight differences in maximum weight loss temperatures (T_max) were observed in the DTGA. For example,
RAX showed a $T_{\text{max}}$ at 291 °C, while $T_{\text{max}}$ for the composite films containing 2.5%, 5% and 10% sepiolite were found at 295 °C, 297 °C and 298 °C respectively. A shift in $T_{\text{max}}$ is commonly observed during thermal analysis of clay-biopolymer films. In one case, a similar temperature increase was found for starch-sepiolite composites in which the $T_{\text{max}}$ temperature increased 3 °C at 3 wt% sepiolite addition and 8 °C at 6 wt% sepiolite addition. Addition of layered nanoclays, such as MMT has also been shown to increase degradation temperatures and in these cases the clay is thought to form an inorganic network, which can acts as a gas transport barrier and thereby hinder the diffusion of pyrolysis gases. As a case in point, Ünlü et al. cast xylan films from corn cob with MMT addition and studied the thermal behavior of such films. A $T_{\text{max}}$ of 284 °C was found for neat xylan but this increased to 303 °C with the addition of 2 g/ml MMT to 7.8·$10^{-5}$ g/ml xylan. It seems reasonable to assume that the fibrous sepiolite would not so easily form a tortuous path to hinder the path of pyrolysis gases and, as a result, the increases in $T_{\text{max}}$ are less significant (Figure 8 B).

![Figure 8](image.png)

Figure 8. TGA (A) and DTGA (B) results for sepiolite, arabinoyxylan (RAX), sepiolite and composites of RAX and sepiolite in the temperature ranges 36-550 °C and 110-550 °C respectively.
Tensile testing

The effect of sepiolite addition on RAX film mechanical properties was examined by tensile testing. The efficiency of plasticization using mPEG addition was also studied. Neat arabinoxylan films without plasticizer addition showed a tensile strength (stress at break) of 42.5 MPa, which is similar to, but slightly lower than, values reported previously for this material. For example, Höije et al.\textsuperscript{35} and Stevanic et al.\textsuperscript{30} measured tensile strengths of 52.4 MPa and 58 MPa respectively for rye arabinoxylan at the same RH, but at a slightly higher temperature in the work of Stevanic et al. Addition of sepiolite resulted in a very significant increase in film strength and stiffness (Table 1). As an example, addition of 2.5 wt\% sepiolite to RAX gave an increase in Young’s modulus from 2.3 to 3.9 GPa and an increase in tensile strength from 42.5 to 73.6 MPa. Further addition of sepiolite showed only a minor additional increase in the Young's modulus values and no significant increase in the tensile strength values. No statistically significant differences were detectable in the strain at break values of the neat RAX and RAX-sepiolite composites without plasticizer addition.

### Table 1. Young's modulus, strain at break and tensile strength of rye arabinoxylan (RAX) and RAX-sepiolite composite films

<table>
<thead>
<tr>
<th>Sample</th>
<th>Young's Modulus (GPa)</th>
<th>Strain at break (%)</th>
<th>Tensile strength (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAX-sepiolite 90:10</td>
<td>4.3 ± 0.1 A</td>
<td>5.0 ± 0.8 C</td>
<td>73.7 ± 5.3 A</td>
</tr>
<tr>
<td>RAX-sepiolite 95:5</td>
<td>4.2 ± 0.2 A</td>
<td>8.1 ± 2.3 C</td>
<td>66.7 ± 2.3 B</td>
</tr>
<tr>
<td>RAX-sepiolite 97.5:2.5</td>
<td>3.9 ± 0.3 B</td>
<td>10.4 ± 3.1 C</td>
<td>73.6 ± 3.2 A</td>
</tr>
<tr>
<td>RAX</td>
<td>2.3 ± 0.2 C</td>
<td>11.9 ± 4.3 C</td>
<td>42.5 ± 6.5 C</td>
</tr>
<tr>
<td>RAX-sepiolite 90:10 + 30% mPEG</td>
<td>1.4 ± 0.1 D</td>
<td>26.7 ± 11.1 B</td>
<td>24.9 ± 5.1 F</td>
</tr>
<tr>
<td>RAX-sepiolite 95:5 + 30% mPEG</td>
<td>1.0 ± 0.2 E</td>
<td>33.3 ± 6.5 B</td>
<td>26.6 ± 2.3 EF</td>
</tr>
<tr>
<td>RAX-sepiolite 97.5:2.5 + 30% mPEG</td>
<td>0.8 ± 0.1 EF</td>
<td>44.4 ± 7.8 A</td>
<td>30.2 ± 6.6 DE</td>
</tr>
<tr>
<td>RAX + 30% mPEG</td>
<td>0.7 ± 0.1 F</td>
<td>42.0 ± 9.7 A</td>
<td>32.8 ± 7.3 D</td>
</tr>
</tbody>
</table>

A,B,C,D,E,F: values with different superscript letters in the same column are statistically different
The addition of mPEG plasticizer had the expected effect of decreasing film stiffness and strength while increasing the elongation values. In the case of the RAX-sepiolite films with added mPEG, a slight increase in the Young's modulus and a decrease in the tensile strength values were observed with increasing sepiolite content. The strain at break showed values approximately four to five times higher than those of the non-plasticized films. A decrease in extensibility could be seen in films with 5 and 10 wt% sepiolite content and 30 wt% mPEG relative to unfilled RAX or RAX films with 2.5 wt% sepiolite containing the same percentage of mPEG. The reinforcing effects of sepiolite addition have been investigated with other biopolymers such as chitosan and starch. Chivrac et al.\textsuperscript{9} incorporated 3 and 6 wt% organomodified sepiolite into plasticized starch and found that the Young's modulus increased by up to a factor of 2.5 relative to unfilled wheat starch. The mechanical properties of various starch-based nanocomposites were compared and it was found that fibrous sepiolite increased the Young's modulus and the tensile strength to a greater extent than MMT addition, presumably due to the morphology of the clay, the increased crystallinity of the nanocomposites and interactions between the starch matrix and the sepiolite. As reported, a slight decrease was found in the strain at break values with sepiolite addition with similar clay loading as applied in our study. Similar behavior when comparing layered silicates with sepiolite was observed in gelatin films.\textsuperscript{14} A reinforcing effect comparable to that reported here was observed and assigned to the presence of numerous silanol groups on the surface of the sepiolite fibers strongly interacting with the carbohydrate matrix. Darder et al.,\textsuperscript{13} in their study on chitosan as matrix, showed that sepiolite addition doubled the Young’s modulus; however, this finding was obtained in films containing very high sepiolite loadings. Such high sepiolite loadings (3 to 91 g chitosan per 100 g of sepiolite) resulted in highly fragile films, hence strain at break was not measured and tensile strength values were not reported. With the addition of high sepiolite loadings, clay aggregation was observed in the cast films through the use of low-temperature SEM.
Soler added MMT clay to arabinoxylan from barley husks and studied the reinforcing effects of MMT; however, significant effects in terms of tensile strength and Young's modulus were not seen. For example, the Young's modulus and tensile strength of arabinoxylan films were 1.3 GPa and 30.8 MPa respectively while the corresponding values for films reinforced with 11 wt% MMT were 2.0 GPa and 31.6 MPa.

Given the results presented here for the reinforcing effects of sepiolite fibers, it is interesting to make comparison with the effects introduced by other fibrous nanofillers. A significant increase in the tensile strength of hemicellulose films was previously seen when using cellulose nanofibers. Since the size of nanofibrillated cellulose is in a similar range to that of sepiolite fibers and both fillers have a high aspect ratio, a similar behaviour may be expected. Hemicellulose-cellulose nanocomposite mechanical properties are summarized in Table 2. Peng et al. recently reported ~300% increase in tensile strength and Young's modulus with 15 wt% nanocellulose addition to bamboo arabinoxylan. In this case, nanocellulose was obtained by multi-homogenization of bleached sisal pulp and had a reported aspect ratio of 50-100, based on a nanofibril diameter of 20 ± 10 nm and length > 1000 nm. Such significant increases in strength and stiffness were, however, not observed when rye arabinoxylan was reinforced using bacterial nanocellulose with similar dimensions as the sepiolite applied in this study. For example, the Young's modulus of arabinoxylan films showed ~ 30% increase in the nanocomposite with 15% bacterial nanocellulose addition.
Table 2. Mechanical properties of xylan-cellulose nanocomposite films

<table>
<thead>
<tr>
<th>Sample</th>
<th>Filler loading (wt %)</th>
<th>Reference</th>
<th>Young's Modulus</th>
<th>Strain at break</th>
<th>Tensile strength</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAX</td>
<td>0</td>
<td>30</td>
<td>2.5 ± 0.4</td>
<td>8.1 ± 3.3</td>
<td>58 ± 11</td>
</tr>
<tr>
<td>RAX + BC</td>
<td>15</td>
<td></td>
<td>3.2 ± 0.5</td>
<td>3.3 ± 0.6</td>
<td>53 ± 7</td>
</tr>
<tr>
<td>AX&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0</td>
<td>38</td>
<td>0.7 ± 0.1</td>
<td>3.4 ± 0.2</td>
<td>11.9 ± 0.9</td>
</tr>
<tr>
<td>AX + NFC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20</td>
<td></td>
<td>3.4 ± 0.1</td>
<td>1.4 ± 0.1</td>
<td>39.5 ± 2.2</td>
</tr>
<tr>
<td>X&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0</td>
<td>39</td>
<td>~22</td>
<td>~22</td>
<td>~5.9</td>
</tr>
<tr>
<td>X + SW&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10</td>
<td></td>
<td>~23</td>
<td>~23</td>
<td></td>
</tr>
</tbody>
</table>

RAX = rye arabinoxylan, BC = bacterial cellulose, AX = arabinoxylan, NFC = nanofibrillated cellulose, X = xylan from oat spelts, SW = sulfonated cellulose whisker

<sup>a</sup>: added sorbitol plasticizer, 25% (percentage based on xylan content)

<sup>b</sup>: added sorbitol plasticizer, 50%

As concluded from the FTIR results, interaction through hydrogen bonding between the sepiolite fibers and the RAX chains can be inferred and these interactions contribute to a great increase in the stiffness of the material. It is also apparent from the results that, above a clay content of 5 wt%, fiber aggregation is more probable, which will contribute to the breakage of the films under tensile load and mean that further increases in tensile strength and Young's modulus are not likely to be detected. We may assume that the sepiolite fibers are themselves able to form a network, hence providing a reinforced composite material with superior mechanical properties compared to the neat RAX matrix. Considering the high aspect ratio of sepiolite fibers, a percolation threshold can be calculated. Since the dimensions of sepiolite fibers show a great variation in different reports<sup>14,40,41</sup>, a range of calculated values can be obtained for the percolation threshold between 0.6% and 3.5% (v/v) if a cylindrical sepiolite shape is assumed (P<sub>c</sub>=0.7/r, where P<sub>c</sub> is the percolation threshold for cylindrical shaped particles and r is the aspect ratio calculated from the ratio of length and diameter of sepiolite fibers).<sup>42</sup> This calculation
suggests that an added amount of sepiolite above the critical percolation threshold of approximately 2% (v/v) would result in highly increased strength and stiffness in the materials. As supported by the tensile data presented here, films containing sepiolite at loadings much above the estimated P_c do not show significant further increases in tensile strength and stiffness.

**Barrier properties**

Water vapor barrier properties of cast RAX-sepiolite films were studied by measuring the water vapor permeability of the films using an ASTM method. The measured values were 2.6 ± 0.3, 2.5 ± 0.0, 3.3 ± 0.4 and 3.1 ± 0.3 (g·mm/kPa·m²·day) for the RAX, RAX + sepiolite 97.5:2.5, 95:5 and 90:10 films respectively. These data show that sepiolite addition up to 10 wt% had no significant influence on water vapor permeability. Even though sepiolite might theoretically create a slightly longer and more difficult path for water molecules to diffuse through the films, sepiolite fibers are hydrophilic, and embedded into a hydrophilic matrix with poor water vapor barrier properties. Hence, a positive effect in terms of reduced water vapor permeability is not seen. Further, the barrier effect commonly noted when plate-like clays are well dispersed in biopolymer films is not observed here, which is consistent with the different fibrous morphology of sepiolite and a lower probability that water vapor permeability will be reduced through a tortuous path effect. WVP values measured for the unfilled RAX reference film were lower or in the same range as values reported previously for unplasticized arabinoxylan films extracted from corn hulls and bran.

The effect of sepiolite addition on RAX film oxygen permeability was studied and the results are summarized in Table 3. RAX films showed low permeability values, between 0 and 1 cm³·μm/m²·d·kPa. Low OP values were expected since hemicellulose films have been proven to be excellent oxygen barriers, with values in the range 0.16-3.2 cm³·μm/m²·day·kPa reported for similar hemicellulose film types. In this case, as with water vapor, sepiolite addition had no significant effect on oxygen permeability and, as above, the lack of a tortuous path effect when using a fibrous clay as an additive
may provide an explanation for these findings.

**Table 3.** Oxygen transmission rate (OTR) and oxygen permeability (OP) of neat rye arabinoxylan (RAX) and RAX-sepiolite composite films at 50% relative humidity and 23°C. Values obtained from two measurements.

<table>
<thead>
<tr>
<th>Type of film</th>
<th>OTR (ml/m² day)</th>
<th>OP (cm³ μm/m² d kPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAX</td>
<td>0.58-0.70</td>
<td>0.15-0.22</td>
</tr>
<tr>
<td>RAX-sepiolite 97.5:2.5</td>
<td>0.80-1.41</td>
<td>0.22-0.54</td>
</tr>
<tr>
<td>RAX-sepiolite 95:5</td>
<td>0.20-0.74</td>
<td>0.10-0.26</td>
</tr>
<tr>
<td>RAX-sepiolite 90:10a</td>
<td>0.69</td>
<td>0.19</td>
</tr>
</tbody>
</table>

*a: only one successful measurement could be made from the samples due to leaking, as a result of defects on the film surface

**Conclusions**

Transparent rye arabinoxylan (RAX) films containing 2.5-10 wt% sepiolite were cast from aqueous suspensions and characterized by various physical methods. FTIR spectroscopy provided some evidence for hydrogen bonding between sepiolite and the RAX matrix. Strong interfacial bonding between sepiolite and the RAX matrix was illustrated by scanning electron microscopy and the distribution of the clay was also shown using EDX imaging. Mechanical testing revealed very significant increases in the Young's modulus and tensile strength as a result of sepiolite addition, which exceeded previously reported values for xylan/nanocellulose or xylan/MMT films. However, unlike layered nanoclays, addition of sepiolite fibers did not reduce the water vapor or oxygen permeability of RAX films and therefore other steps would need to be introduced in order to obtain higher gas barrier properties for specific applications.
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