# Characterisation of complex xylo-oligosaccharides from xylan rich by-products

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# Characterisation of complex xylo-oligosaccharides from xylan rich by-products

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## ABSTRACT

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Key words: brewery's spent grain, barley, *Eucalyptus* wood, xylan, xylo-oligosaccharides, chromatography, mass spectrometry, NMR spectroscopy.

Hydrolysates obtained by hydrothermal treatment of four xylan rich by-products (wheat bran, brewery's spent grain, corn cobs and *Eucalyptus* wood) were characterised. Depending on the feedstock material studied, the xylan originally present differed in substitution with arabinose, 4-*O*-methylglucuronic acid and *O*-acetyl substituents. Due to a partial release of the various substituents and depolymerisation of the xylan by the hydrothermal treatments performed, a wide variety of differently substituted XOS and xylan-fragments were obtained.

High performance anion-exchange chromatography (HPAEC), reversed phase (RP)-high performance liquid chromatography (HPLC), mass spectrometry (MS), NMR spectroscopy, RP-HPLC-MS and RP-HPLC-NMR showed to be very useful for the separation and characterisation of the detailed structures of the substituted XOS.

The differently substituted XOS in the hydrolysates of brewery's spent grain and *Eucalyptus* wood were separated by anion-exchange and size-exclusion chromatography and characterised in more detail. The XOS in the brewery's spent grain hydrolysate included mainly xylan-fragments substituted with arabinoses and XOS containing only few substituents. Furthermore, arabinoxylan-fragments having *O*-acetyl substitution were present, suggesting the presence of *O*-acetyl in cereal arabinoxylans. The *Eucalyptus* wood hydrolysate contained mainly linear XOS, *O*-acetylated XOS and (*O*-acetylated) XOS substituted with one or two 4-*O*-methylglucuronic acid(s). Additionally, a series of XOS containing both 4-*O*-methyl-glucuronic acid and a hexose, most likely galactose, was identified.

The *O*-acetylated XOS from treated *Eucalyptus* wood were subjected to RP-HPLC-MS/ NMR in order to distinguish the exact positions of the *O*-acetyl substituents. The position of the *O*-acetyl substituent in several xylo-tetramers and xylo-trimers was determined. Additionally, intermolecular *O*-acetyl migration was proven to have occurred in XOS.

One of the strategies to apply XOS in the food-industry is the use as non-digestible oligosaccharides in promoting the growth of beneficial bacteria in the human colon. Linear XOS, arabinose substituted XOS, *O*-acetylated XOS and glucurono-XOS were fermented differently by human intestinal flora as present in human faecal inocula *in vitro*. Also, anti-ulcer tests showed that differently substituents in XOS influence the number of ulcers formed in the stomachs of rats. These results put emphasis on the necessity to be able to perform a detailed elucidation of the structural features of oligosaccharides in general to understand their mechanisms in bioactivity assays more precisely.

# CONTENTS

# CHAPTER 1

# Introduction

## 1.1 Background

The research described in this thesis is part of a larger project aimed at the production of xylo-oligosaccharides (XOS) and xylitol from (treated) agricultural by-products for use in pharmaceutical and food industries, funded by the European Committee. Alternative routes for the utilisation of agricultural by-products are of interest, because the economic value of these by-products as animal feed compounds is decreasing. In order to obtain xylose and XOS, fractionation of xylan rich by-products by a hydrothermal treatment was studied, with emphasis on minimising the environmental impact (avoiding acid, alkali or organosolv additions). The aim of the European-project is to optimise an integrated process for biomass fractionation that could be used for various xylan rich by-products (Fig. 1.1). The by-products studied were wheat bran, brewery's spent grain, corn cobs and *Eucalyptus* wood.

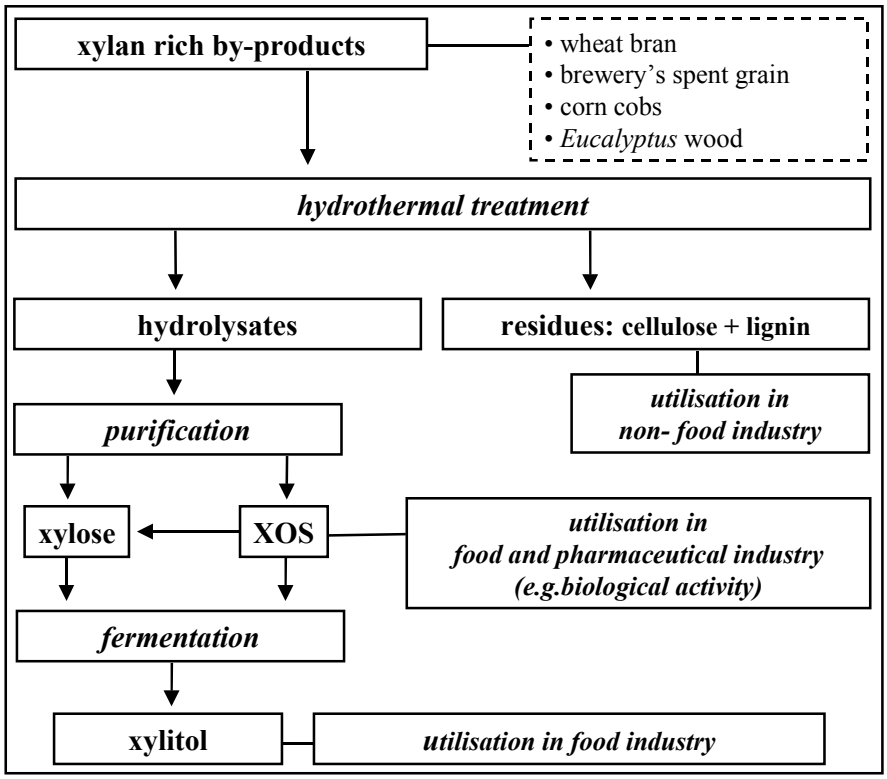


**Figure 1.1** Fractionation of xylan rich by-products by hydrothermal treatment resulting in a soluble (degraded) xylan rich fraction and a ligno-cellulosic residue, and studied within the European-project.

For the resulting residues several possible uses were studied within the European-project, for example the application in paper pulping, in structural materials, sorbents and/ or assembling composite materials (National University of Athens, Greece).

Hydrolysates containing high amounts of xylose were tested to be converted by a fermentation process for xylitol production (Universidade de Vigo-Ourense, Spain). Xylitol

has found applications in chewing gum and in toothpaste, because of its anti-cariogenic properties [*1*]. Furthermore, simultaneous conversion of XOS present in these hydrolysates to monomeric xylose is advantageous for the xylitol production. Therefore, the cloning of β-xylanase, β-xylosidase and α-L-arabinofuranosidase genes into yeast species to be used for fermentation purposes was investigated (INETI, Portugal).

Hydrolysates containing high amounts of XOS were tested for their fermentability by the human intestinal flora, which can be related with a favourable intestinal environment. Other biological properties of XOS, like anti-ulcer properties, were studied as well (INETI, Portugal). However, to test such biological activities it is necessary to have distinct well-characterised series of XOS. In addition, monomeric xylose needs to be separated from the XOS. Monomers can influence the studies of biological properties of XOS and are not regarded as non-digestible oligosaccharides (NDO). Besides monomers undesired (toxic) compounds formed during the hydrothermal treatments need to be excluded.

## 1.2 Occurrence and structural features of xylans

The structural features of the xylans originally present in the by-products used will influence the structures of the XOS formed during hydrothermal treatment. Therefore, in this chapter a summary is given of the structural features of xylans in general and attention is paid to structures present in each of the by-products studied more specifically.

In general, xylans present in the main group of plants, the *Angiospermae* (flowering plants), can be divided in two groups: the (glucurono-)arabinoxylans and glucuronoxylans. Xylan as present in the cell walls of monocotyles (grasses and cereals) consist of linear chains of β-D-(1,4)-linked D-xylopyranosyl residues, which can generally be substituted with α-L-arabinofuranosyl at the 2-*O* and/or 3-*O*-position(s) and α-D-glucopyranosyl uronic acid or its 4-*O*-methyl derivative at the 2-*O*-position [*2,3*]. Few publications describe the presence of *O*-acetyl substituents [*4,5*]. Besides these single unit substituents, a variety of di- and trimeric side chains have been identified as minor constituents of (glucurono-)arabinoxylans. These side-chains can be composed of arabinose only, or can include xylose and galactose as well [*6-11*]. Furthermore, small amounts of ester-linked hydroxycinnamic acid residues, such as coumaric and ferulic acids, are found linked to (some of) the arabinose residues [*6,7,12*].

In spite of these general characteristics, the source from which the xylan is extracted strongly determines the specific features with regard to the type, the amount, position and distribution of substituents over the xylan-backbone (Fig. 1.2). Furthermore, within one plant source different populations of xylans may occur. For example, in arabinoxylan extracted from wheat flour rather high substituted populations as well as less substituted populations have been described (Fig. 1.2) [*13*].

In contrast with the xylan from monocotyles, xylan from dicotyles (hard woods, herbs and woody plants) is an *O*-acetylated 4-*O*-methyl-α-D-glucuronoxylan almost without any

arabinose substitution. On average every tenth xylosyl residue carries an α-4-*O*-methylglucuronic acid residue substituted at the 2-*O*-position [*14-17*]. *O*-acetyl substituents can be located at the 2-*O*- and/ or 3-*O*-positions of the xylosyl residues present. In general, the content of *O*-acetyl is 3-5 % (w/w) of the total wood [*18*].

As an example, the structural models of xylans extracted from various sources are presented in figure 1.2.

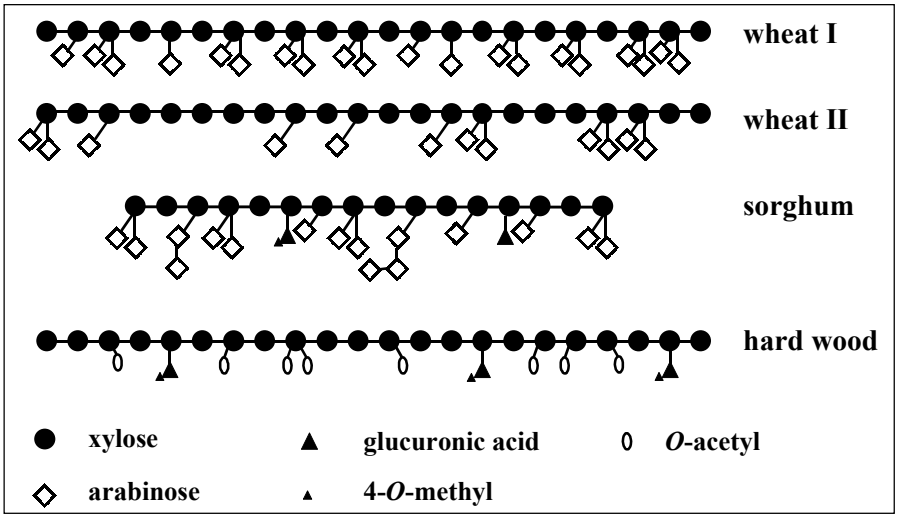


**Figure 1.2** Compilation of structural models for xylans extracted from wheat [*13*], sorghum [*19*] and hardwood [*20*].

## 1.3 Xylan rich by-products: wheat bran, brewery's spent grain, corn cobs and *Eucalyptus* wood

### *1.3.1 Origin and main components of the by-products*

Wheat bran, the non-starchy outer parts of a wheat grain, is produced world-wide as a by-product of the milling of white wheat flour. The bran is used as a feed for cattle or less frequently as a source of dietary fibre [*21,22*]. Wheat bran consists mainly of the cell wall polysaccharides glucuronoarabinoxylans (22-25 % (w/w)), cellulose (7-11 % (w/w)) and β-glucans. Furthermore, in the bran rather high amounts of starch are present (11-30 % (w/w)), besides protein (14-17 % (w/w)) and lignin (3-10 % (w/w)) [*23-26*].

Brewery's spent grain represent an inexpensive and abundant disposable by-products of nearly all breweries. The main use for spent grain is as feed for cattle. However, in literature other possibilities are described, such as ingredient of fibre rich food products, pharmaceutical applications, fuel or compost [*27,28*]. The malt and grain residue obtained

after the liquefaction and saccharification of the starch known as 'spent grain' is essentially a hemicellulosic material. The composition strongly depends on the variety of barley used, malting and brew conditions. Additionally, the use of unmalted grains, like wheat, rice, oat and maize, may influence the composition of the spent grain. However, independent of the precise composition of the spent grain the main components are xylans, ranging from 21-25 % (w/w), cellulose (11-25 % (w/w)), starch (5-26 % (w/w)), lignin (7-19 % (w/w)), fat (7-11 % (w/w)) and protein (21-36 % (w/w)) [*27-30*].

Corn cobs are low grade residues from the agro-industry rich in plant call wall material. Corn cobs are described to be used for the production of ethanol by fermentation [*31*] and for the production of xylitol [*32,33*]. Additionally, corn cobs can be of use in the pulp and paper industries [*34*]. The main components of corn cobs are xylans (30-35 % (w/w)) and cellulose (30-40 % (w/w)) [*34-36*].

*Eucalyptus globulus* wood is a cheap, widespread and largely available resource. Its exploitation for paper pulp production generates large quantities of lignocellulosic residues, which are not currently valorised. Furthermore, forest resources have become increasingly important as renewable sources of raw materials during the last few decades. In general, hardwoods contain 15-35 % of hemicellulose, of which xylan is the main type [*18,37*]. Besides the hemicellulose, cellulose (40 % (w/w)) and lignin (31 % (w/w)) are the main components [*38,39*].

### *1.3.2 Structural features of the xylans present in the by-products*

In general, xylan purified from wheat bran consist of a β-(1→4)-xylan backbone mainly substituted with arabinose residues at 2-*O*- and/or 3-*O*-positions, while some side-chains of (4-*O*-methyl-)α-D-glucuronic acid are present as well [*2*]. However, the ratio of arabinose to xylose, which for arabinoxylans generally represents the degree of substitution, varies for arabinoxylans derived from different parts of the bran. The originally inner part of the wheat bran layer, the aleurone layer, contains arabinoxylan having only few arabinose-substituents (Ara/Xyl-ratio = 0.3). [*40,41*]. Arabinoxylan of the originally outer part of the wheat bran layer, the pericarp, has a much higher degree of substitution (Ara/Xyl-ratio = 1). Besides the arabinose-residues also side-chains of (4-*O*-methyl-)glucuronic acid residues are present, particularly in the bran fraction [*22,41,42*].

Xylan from brewery's spent grain resembles that of wheat bran, although the proportion of arabinose substituents at the 2-*O*-position of the xylosyl residues is much higher compared to wheat bran [*43*]. The ratio of arabinose to xylose ranges from 0.4-0.7 [*28,29*].

Also in corn cobs linear chains of β-D-(1,4)-linked D-xylopyranosyl residues are present, substituted with α-L-arabinofuranosyl at the 2-*O* and/or 3-*O*-position(s). Besides these arabinosyl substituents (4-*O*-methyl-)α-D-glucuronic acid groups are present as substituents as well. Various xylans, with a relatively low Ara/Xyl-ratio (0.03-0.18), are

reported to be present in corn cobs [*34,35*]. The unusual side-chain 2-*O*-β-D-xylopyranosyl-α-L-arabinofuranose is described to be present as well [*34,44*].

Contrary to the arabinoxylans present in the previously described by-products, the xylan present in *Eucalyptus* wood is a typical hard wood xylan: β-(1→4)-xylan backbone substituted with *O*-acetyl- and 4-*O*-methylglucuronic acid groups and practically free of arabinosyl groups [*37,45*].

## 1.4 Processing of xylan rich by-products

An interesting option to utilise xylan rich biomass follows the 'biomass refining' philosophy: raw materials can be fractionated into (degraded) hemicellulose and ligno-cellulosics suitable for different product applications [*46,47*].

Steaming or hydrothermal treatments (150-240 °C) are good methods for the fractionation of the xylans from ligno-cellulosics. Xylan hydrolysis can be improved by a pretreatment of the substrates with diluted acids like sulfuric acid, whereas both cellulose and lignin remain in solid phase [*48-51*]. However, the use of strong acids as pretreatment is not very environmentally friendly and it results in higher processing costs due to the requirement of corrosion-proof equipment. Therefore, a milder hydrothermal treatment may be advantageous.

In the presence of water at elevated temperatures the xylans in the treated products will be hydrolysed and dissolved. Additionally, *O*-acetyl substituents present are (partly) hydrolysed from the xylan, which result in the generation of acetic acid. The acetic acid formed acts as catalyst in the further hydrolysis of the glycosidic linkages in the xylans present. Depending on the operational conditions used, the hydrolysates obtained consist mainly of soluble xylan, XOS and/ or xylose [*52-54*]. To a lesser degree oligomeric fragments of degraded cellulose occur in the hydrolysates. Products of side reactions of pentoses and hexoses, such as furfural and hydroxymethylfurfural (HMF) respectively, can be observed as well [*38,55*].

A number of publications present models or conditions describing the relation between the concentration of (degraded) hemicellulose dissolved and the temperature/ time used in the treatment of various hemicellulose-rich products [*46,51,56-60*]. However, all models have to accept some simplifications due to practical limitations. Little attention is paid to the structural features of the xylans originally present in the treated products, which may influence the parameters used in modelling. Furthermore, detailed structures of the soluble (substituted) xylans and (substituted) XOS obtained during treatment are studied less frequently. The products formed during treatment depend on the xylan-structures originally present in the by-product used for degradation. Therefore, it might be of interest to study by-products containing structurally different xylans during hydrothermal treatment resulting in a variety of XOS with different structural features.

## 1.5 Physiological effects of XOS

Another aim of the European-project was to study possible uses for the different XOS obtained during the treatments described in food and pharmaceutical industries (*vide infra*). One of the options could be the use of XOS as prebiotics through promotion of the growth of beneficial bacteria in the human intestine.

The human gastrointestinal tract constitutes a complex microbial ecosystem comprising several hundred species of bacteria. The colon in particular is densely populated with more than $10^{11}$ bacteria per gram of contents. The majority of bacterial species in the colon belong to the genera *Bacteroides, Ruminococcus, Bifidobacterium, Eubacterium* and *Clostridium* and are able to ferment sugars. Within the intestinal microflora some of these bacteria are believed to be beneficial to the host while others are potentially pathogenic. One of the strategies to selectively increase the number of health promoting bacteria in the colon, mainly *Bifidobacterium* and *Lactobacillus*, is to supply them with oligosaccharides, which are not degraded in the upper gastrointestinal tract and are less assimilated by undesirable flora present [*61*]. The fermentability of a number of oligosaccharides, e.g. XOS, fructo-oligosaccharides and galacto-oligosaccharides, is described in literature [*62-66*].

XOS are non-digestible oligosaccharides (NDO's), which are not degraded by the low-pH gastric fluid, nor by human and animal digestive enzymes and will therefore reach the large bowel intact [*67*]. Furthermore, XOS are considered to specifically increase the number of (beneficial) bifidobacteria in the colon.

In the large bowel the oligosaccharides can be fermented by the intestinal flora into mainly short chain fatty acids (SCFA; acetate, propionate and butyrate), lactate, $CO_2$ and $H_2$ [*63*]. Both the production of SCFA and the increase in bifidobacteria are related with a number of health effects, e.g. bowel function, calcium absorption, lipid metabolism and reduction of the risk of colon cancer [*68,69*].

## 1.6 Fractionation and identification of oligosaccharides

### *1.6.1 Chromatographic techniques*

Various methods concerning the analysis of oligosaccharides have been described and they are mainly based on separation of the oligosaccharides by high-performance liquid chromatography (HPLC) or gas-liquid chromatography after derivatisation [*70*].

A widely used analytical approach for the separation of a variety of oligosaccharides is high-performance anion-exchange chromatography (HPAEC) at high pH (pH  12) combined with pulsed amperometric detection (PAD) [*71*]. In general, structurally related oligosaccharides are well separable by HPAEC, based on differences in their monomeric composition, size and linkages present [*19,71-75*].

HPAEC at pH 5 can also be applied for the separation of oligosaccharides. Under these conditions the separation is mainly based on the number of negative charges per oligomer and charge density within the oligosaccharides [*76*].

In recent literature, the use of reversed phase (RP) chromatography to separate oligosaccharides is described as well [*77-79*]. Separation by reversed phase is mainly based on differences in hydrophobicity of the oligosaccharides, for example resulting from *O*-acetyl substituents present [*80*]. The degree of polymerisation (DP) also influences separation as observed for maltodextrins [*81,82*].

Using the described chromatographic techniques the identification of complex oligosaccharide mixtures remains rather difficult. This is due to the fact that the elution behavior of the various types of oligosaccharides is often rather unpredictable and to the fact that oligomer standards are frequently not available. Consequently, subsequent characterization by using (on-line) mass spectrometry (MS) or NMR is of interest in order to reveal the exact structures of the separated oligosaccharides (§1.6.2). For these purposes it might be of use to separate complex mixtures first by chromatography at a preparative scale. Commonly used techniques are preparative size-exclusion chromatography and preparative anion-exchange chromatography [*83,84*].

### *1.6.2 Spectrometric and spectroscopic techniques*

Innovations in ionisation techniques and mass analysers used in MS together with the development of instruments which are rather easy to control, makes MS a powerful technique for biochemists.

For the analysis of glycoconjugates and oligosaccharides electrospray ionisation (ESI) with an ion trap mass analyser and matrix-assisted laser desorption ionisation (MALDI) combined with a time-of-flight (TOF) mass analyser are very suitable [*76,85-90*]. Optimisation of the ESI process has led to the miniaturisation of the spraying system and has resulted in nanoelectrospray (nanospray). Volumes of analytes less than 1 µl can be introduced into the ion source at very low flow rates of 20-30 nl/min [*91*]. Both ESI and nanospray MS are easily to combine with tandem mass spectrometry. In tandem MS fragmentation of selected oligosaccharides can be performed and therefore is helpful to determine the order of residues within the oligomers. Even underivatised oligosaccharides usually results in good fragmentation spectra giving information on the building blocks of the oligosaccharides analysed [*91,92*]. Structural information by using fragmentation of oligosaccharides can also be obtained by MALDI-TOF MS, using post-source decay (PSD) [*93-95*].

The development of the on-line coupling of HPLC to ESI MS greatly facilitate the identification of unknown oligosaccharides [*96*]. In particular, the on-line coupling of chromatographic methods using MS compatible solvents, e.g. RP-HPLC, with MS are rather easily to perform. HPAEC coupled on-line to MS is more difficult, because of the use of eluent containing high amounts of salts, which can block the MS apparatus or disturb

ionisation of the analytes. Several applications for on-line desalting following HPAEC have been described [*97-101*]. Off-line desalting following (preparative) HPAEC is performed as well in order to obtain pure oligomers, which can be subjected to MS or NMR. However, these off-line desalting methods are rather time-consuming and labour intensive [*76,83*].

Nucleic magnetic resonance (NMR) spectroscopy is a commonly used method in the identification of oligosaccharides as well. Normally, for NMR relatively high amounts of material are needed (~1 mg) and analysis times are rather long compared with chromatographic methods and mass spectrometry. However, NMR provides unquestionable data enabling the complete characterisation of oligosaccharides [*102*]. Already a wide variety of arabino-xylo-oligosaccharides derived from extracted xylans of various sources has been characterised by NMR spectroscopy [*11,83,103-105*].

## 1.7 Aim and outline

The aim of the research described in this thesis was to purify and characterise hydrolysates of four hydrothermally treated xylan rich by-products. Furthermore, the fermentability of the obtained XOS by the microbial flora present in fecal slurries was tested *in vitro*.

The four xylan rich by-products, namely wheat bran, brewery's spent grain, corn cobs and *Eucalyptus* wood, were characterised and subjected to a mild hydrothermal treatment in order to release and degrade the xylan from the starting materials. The hydrolysates were studied in relation to the xylan originally present in the by-product studied (Chapter 2).

Hydrolysates from two hydrothermal treated xylan-rich agrobased materials, *Eucalyptus* wood and brewery's spent grain, were studied in more detail. Hereto, they were fractionated by anion-exchange chromatography and size-exclusion chromatography (Chapter 3). Several pools, representing material with a different type of substitution and/ or DP, were obtained and characterised by their sugar composition. Additionally, the oligosaccharides in the pools described were further identified by high-performance anion-exchange chromatography (HPAEC) and mass spectrometry.

Chapter 4 describes the off-line coupling of analytical HPAEC to MALDI-TOF MS, assisted by on-line desalting and automated sample handling. This method is of help in the identification of oligosaccharides in general and to overcome the rather unpredictable elution behaviour of HPAEC. The system was applied to the analysis of neutral and acidic XOS.

In Chapter 5 the location of *O*-acetyl substituents present in the XOS in hydrolysates of hydrothermally treated *Eucalyptus* wood is determined. To determine the precise location the *O*-acetyl substituents in these XOS a combination of techniques was used.

To compare the physiological effect of the structurally different XOS obtained from the four hydrothermally treated by-products, the XOS were subjected to a fermentation study. XOS (XOS) with various substituents were fermented *in vitro* by faecal inocula (FI) from

four human volunteers to study the influence of substitution on the ability and rate of fermentation and on production of short-chain fatty acids (SCFA) and lactate (Chapter 6).

Finally, in chapter 7 the main findings are summarised and discussed in the context of the research aims and literature.

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# CHAPTER 2

## Hydrothermally treated xylan rich by-products yield different classes of xylo-oligosaccharides

**Abstract**

Four xylan rich by-products, namely wheat bran, brewery's spent grain, corn cobs and *Eucalyptus* wood, were characterised and subjected to a mild hydrothermal treatment in order to release and degrade the xylan from the starting materials. The chemical characterisation of the feedstock materials, with emphasis on the extracted xylan fractions and using enzymatic degradation of these xylans, resulted in rather detailed pictures of the xylans present. Depending on the feedstock material studied, the xylan present was substituted with arabinose, 4-*O*-methylglucuronic acid and *O*-acetyl substituents. During the hydrothermal treatment, arabinose was rather easily removed from the xylan-backbone (wheat bran, brewery's spent grain and corn cobs). The *O*-acetyl substituents were partly released from the feedstocks, becoming available to catalyse the depolymerisation of the xylan. Also, part of the uronic acids were released, mainly during the treatment of *Eucalyptus* wood. Due to the partial release of the substituents and cleavage of the xylan by the treatment performed, a wide variety of xylo-oligosaccharides with different structural features corresponding to the xylan-structure of the original feedstock were obtained. Xylo-oligosaccharides branched with arabinose were identified in the hydrolysate from brewery's spent grain, while in the hydrolysate of corn cobs and *Eucalyptus* wood xylo-oligosaccharides substituted with 4-*O*-methylglucuronic acid were present as well. Additionally, a series of partially *O*-acetylated (acidic) xylo-oligosaccharides was identified in the *Eucalyptus* wood hydrolysate.

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## 2.1 Introduction

Agro-industrial, agricultural and forest by-products, like wheat bran, brewery's spent grain, corn cobs and *Eucalyptus* wood are rich in cellulose and hemicelluloses. Currently, more and more effort is directed towards the re-use of such by-products, considering economic values and environment. Fractionation of these by-products into their main components could be of interest to obtain separate streams useable for different product applications [*1*].

Since the main component of the hemicellulose in the by-products mentioned is xylan, fractionation of these by-products may result in both xylose, an intermediate for the production of xylitol, and a variety of differently substituted xylo-oligosaccharides (XOS). Because of its anti-cariogenic properties xylitol has already been used in food applications, e.g. chewing gum or tooth paste [*2*]. Xylo-oligosaccharides are reported to enhance growth of bifidobacteria and they are frequently defined as prebiotics [*3,4*].

Xylan as present in the cell walls of Gramineae (grasses) consist of a β-D-(1,4)-linked xylopyranosyl backbone, which can be substituted with α-L-arabinofuranosyl on *O*2 and/or *O*3, α-D-glucopyranosyl uronic acid, or its 4-*O*-methyl derivative on *O*2, and *O*-acetyl on (some of) the arabinose or xylose residues. In spite of these general characteristics, the source from which the xylan is extracted strongly determines the specific features with regard to the type, the amount, position and distribution of glycosylic side-chains over the xylan-backbone [*5,6*]. For example, xylan purified from wheat bran is mainly substituted with arabinose residues at 2-*O* and/or 3-*O*, while some side-chains of (4-*O*-methyl-)α-D-glucuronic acid are present as well [*7,8*]. This latter is also the case for corn cobs where even the unusual side-chain 2-*O*-β-D-xylopyranosyl-α-L-arabinofuranose has been described to occur [*9,10*]. Xylan from brewery's spent grain resembles that of wheat bran, although the proportion of arabinose residues at the 2-*O*-position is much higher compared to wheat bran [*11,12*].

In contrast with the xylan from Gramineae, xylan from hardwoods is an *O*-acetylated 4-*O*-methyl-α-D-glucuronoxylan almost without any arabinose substitution. On average every tenth xylose residue carries an α-4-*O*-methylglucuronic acid substituted to *O*-2 [*13*]. A more complex glucuronoxylan purified from *Eucalyptus globulus* Labill has been described by Shatalov et al. [*14*], who reported linkages of both 4-*O*-methyl-α-D-glucuronic acid and 4-*O*-methyl-α-D-glucuronic acid substituted at 2-*O* with α-D-galactose.

An environmentally friendly way to fractionate xylan rich materials is to perform a mild hydrothermal treatment. Such a hydrothermal treatment will result in a selective release and degradation of the xylan of the used resource, leaving the cellulosic residue available for other purposes [*1,15*]. Furthermore, since the xylans in the various by-products are expected to possess rather diverse structures, differences in size and structural features of the oligosaccharides released are expected upon treatment for each material.

In our study wheat bran, brewery's spent grain, corn cobs and *Eucalyptus* wood, were subjected to a mild hydrothermal treatment. The hydrolysates were characterised and the results obtained were related to the composition of the starting materials and the structural

features of the xylans present.

## 2.2 Experimental

### *2.2.1 Feedstock materials*

Wheat bran was obtained from Germen Moagens de Cereais, SA, Vila Franca de Xira (Portugal) and harvested in France (autumn 1997). Brewery's spent grain was supplied from the Brewery Central de Cervejas, Vialonga (Portugal). Corn cobs were supplied by Casa Agrícola Monte Real, Salvaterra de Magos (Portugal) and harvested in Portugal (autumn 1998). Chips of *Eucalyptus* wood were obtained from ENCE Complejo Industrial de Pontevedra Puentenolinos, Lourizan (Galicia, Spain; July 1998).

### *2.2.2 Characterisation and fractionation of feedstock materials*

To remove starch, first the feedstock material (40 g) was suspended in maleic-buffer (360 ml), containing 0.01 M maleic acid, 0.01 M sodium chloride, 0.001 M calcium chloride, 0.05 % (w/v) sodium azide, pH 6.5 and stirred for 1.5h at 100°C. The suspension was cooled till 30 °C and porcine alpha-amylase (Merck; 150 U/ g feedstock), amyloglucosidase of *Aspergillus oryzae* (Sigma; 420 U/ g feedstock) and pullulanase of *Bacillus acidopullulyticus* (Megazyme; 60 U/ g feedstock) were added to degrade most of the starch of the feedstock material (20 hours; 30°C). To remove the glucose formed and other low molecular weight material, ethanol was added till a concentration of 70% was reached. The final alcohol insoluble solids (AIS) were washed with acetone and dried on the air.

AIS was suspended in distilled water (1 : 20 w/v) and extracted for 3 hours at 65 °C under continuous stirring. After centrifugation (10000g; 30 min) the residues were reextracted twice with distilled water and the corresponding supernatants were collected as water soluble solids (WSS). The corresponding residues were recovered as WUS (Water Unextractacle Solids).

WUS was suspended in 200 ml of 4 M potassium hydroxide (KOH)/ 0.26 M $NaBH_4$, for 16 hours at 25 °C under continuous stirring. After centrifugation (10000g; 30 min) the residues were reextracted twice with 4 M KOH (+ 0.26 M $NaBH_4$). The final residues were neutralised with acetic acid, dialysed against distilled water and freeze-dried (KOH res). The corresponding supernatants were collected, neutralised, dialysed and freeze-dried (KOH ss).

### *2.2.3 Enzymatic degradation of the alkali-extractable-fractions (KOHss)*

A solution of alkali-extractable xylan (4 mg KOH ss) or wheat arabinoxylan (4 mg; Megazyme) in 50 mM sodium acetate buffer pH 5 (1 ml) was incubated with endo-(1,4)- β-D-

xylanase I (0.2 µg/ml) for 24h at 30 °C, according to Gruppen et al. [*16,17*]. After inactivation of the enzyme the digests were analysed by HPAEC, HPSEC and MALDI-TOF MS.

### *2.2.4 Hydrolysis of feedstock materials by hydrothermal treatment*

The wheat bran was autoclaved (85 °C; 1h) first, with a ratio of 1 g of feedstock per 10 g of water and starch was substantially removed with the aqueous phase. The autoclave-residue was used to prepare the hydrolysate and corresponding residue of wheat bran by hydrothermal treatment; 155 °C for 60 minutes with a ratio of 1 g of feedstock per 10 g of water (Technical University of Athens, Greece). The brewery's spent grain was autoclaved (100 °C; 1h), with a ratio of 1 g of feedstock per 8 g of water, and starch was substantially removed with the aqueous phase. The autoclave-residue was used to prepare the hydrolysates and residue of brewery's spent grain by hydrothermal treatment; 150 °C for 60 and 120 minutes, both with a ratio of 1 g of feedstock to 8 g of water (INETI, Portugal). To obtain a hydrolysate and residue from corn cobs, the cobs were hydrothermal treated at 160 °C for 75 minutes with a ratio of 1 g of feedstock to 8 g of water (University of Vigo, Spain). The hydrolysate and residue [*1*] of treated *Eucalyptus* wood were prepared at 160 °C for 60 minutes with a ratio of 1g of feedstock to 8 g of water (University of Vigo, Spain).

### *2.2.5 Neutral sugar composition*

The neutral sugar composition was determined by gas chromatography according to Englyst & Cummings [*18*], using inositol as an internal standard. The samples were treated with 72 % (w/w) $H_2SO_4$ (1 h, 30 °C) followed by hydrolysis with 1 M $H_2SO_4$ for 3 h at 100 °C and the constituent sugars released were analysed as their alditol acetates.

### *2.2.6 Uronic acid content*

The uronic acid content was determined as anhydro-uronic acid (AUA) by an automated *m*-hydroxydiphenyl assay [*19,20*] using an autoanalyser (Skalar Analytical BV, Breda, The Netherlands).

### *2.2.7 Acetic acid content*

The degree of acetylation was determined on a SP 8800 system HPLC (Thermo Quest), using an Aminex HPX column [*21*]. The level of acetyl substitution was corrected for the free acetic acid in the sample.

### *2.2.8 Starch content*

Starch was determined enzymatically using the test kit supplied by Boehringer.

### *2.2.9 HPSEC*

High-performance size-exclusion chromatography was performed on three TSKgel columns (7.8 mm ID x 30 cm per column) in series (G4000, G3000, G2500; Tosohaas), in combination with a PWX-guard column (Tosohaas). Elution took place at 30 °C with 0.2 M sodium nitrate at 0.8 ml/min. The eluate was monitored using a refractive index (RI) detector (Shodex RI-71). Calibration was performed using pullulans (Polymerlabs).

### *2.2.10 HPAEC (pH 12)*

High-performance anion-exchange was performed on a Dionex system equipped with a CarboPac PA-1 column (4 mm ID x 250 mm) in combination with a CarboPac PA guard column (3 mm x 25 mm) and PAD-detection [*22*]. Elution (1 ml/min) of the oligomers on the hydrolysates was performed with a combination of linear gradients of 50-90 mM sodium acetate in 100 mM NaOH during 0-5 min, 90-130 mM sodium acetate in 100 mM NaOH during 10 min, followed by a linear gradient to 520 mM sodium acetate in 100 mM NaOH in 15 minutes. For the analysis of arabinose and xylose in the hydrolysates, an isocratic elution (1 ml/min) of 20 minutes was carried out with a solution of 16 mM NaOH. Each elution was followed by a washing and equilibration step. The eluate was monitored using PAD detection.

### *2.2.11 MALDI-TOF mass spectrometry*

For MALDI-TOF MS (Matrix-Assisted Laser Desorption/ Ionisation Time-Of-Flight Mass Spectrometry) a Voyager-DE RP Biospectrometry workstation (PerSeptive Biosystems Inc., Framingham, MA, USA) was used, operated as described by [*23*]. The mass spectrometer was calibrated with a mixture of maltodextrins (mass range 365-2309).

The samples were mixed with a matrix solution (1 µl of sample in 9 µl of matrix), after desalting the samples with anion-exchange material (AG 50W-X8 Resin; Biorad). The matrix solution was prepared by dissolving 9 mg of 2,5-dihydroxybenzoic acid and 3 mg 1-hydroxyisoquinoline in a 1-ml mixture of acetonitrile:water (300 µl:700 µl). Of the prepared (sample + matrix) solutions 1 µl was put on a gold plate and allowed to dry at room temperature.

### *2.2.12 Miscellaneous*

The content of furfural, hydroxymethylfurfural (HMF), formic and levulinic acid was determined by HPLC, with use of an Aminex HPX-87H column and UV-/ RI-detection.

## 2.3 Results and discussion

### *2.3.1 Characterisation of the four by-products and corresponding alkali extracted fractions*

Four xylan rich by-products, namely wheat bran, brewery's spent grain, corn cobs and *Eucalyptus* wood, were characterised. The composition of both the feedstock and their alcohol insoluble solids (AIS) are presented in table 2.1.

**Table 2.1** Yield and composition of xylan rich feedstock materials (FS) and corresponding alcohol insoluble solids (AIS).

| | Wheat bran | | Brewery's spent grain | | Corn cob | | *Eucalyptus* wood | |
|---|---|---|---|---|---|---|---|---|
| | FS | AIS[a] | FS | AIS[a] | FS | AIS[a] | FS | AIS[a] |
| Yield[c] | 100 | 55 | 100 | 84 | 100 | 93 | 100 | 95 |
| Protein[a] | 17 | 9 | 30 | 25 | 3 | 2 | 1 | 1 |
| Sugars (total)[b] | 61 | 36 | 42 | 40 | 71 | 64 | 68 | 67 |
| Ara[a] | 9 | 7 | 8 | 7 | 5 | 3 | 1 | 0 |
| Xyl[a] | 16 | 14 | 15 | 16 | 28 | 26 | 14 | 13 |
| Man[a] | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| Gal[a] | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 |
| Rha[a] | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 |
| Glc[a] (of which starch) | 33 (20) | 12 (2) | 16 (4) | 13 (3) | 33 (0) | 31 (0) | 44 (0) | 44 (0) |
| Uronic acid[a] | 2 | 2 | 2 | 3 | 3 | 3 | 6 | 6 |
| Acetic acid[a] | 0.4 | 0.3 | 0.8 | 0.8 | 3 | 3 | 3 | 3 |

[a] expressed as g of recovered protein, sugar (-residues) or acetic acid from 100 g of feedstock.
[b] neutral sugars + uronic acids expressed as weight percentage (dry matter) of each fraction.
[c] yield as weight percentage per 100 g feedstock material (dry matter).

In wheat bran 41 % of non-starch polysaccharides (neutral sugars + uronic acids + non-starch glucose), 17 % of protein and 20 % of starch was found, corresponding with the 41-60 %, 15-22 % and 10-30 % respectively, reported in literature [*24,25*]. In general, the composition of the brewery's spent grains depends on the brewery's conditions and ingredients used for brewing. In spite of this, the contents found for non-starch polysaccharides (38 %), starch (4 %) and protein (30 %) for the spent grains used in this study fitted within the range as described in literature by Angelino [*26*]. The sugar and protein composition of corn cobs as presented in table 2.1 was similar as described by Pellerin et al. [*27*]. The content of glucose

(44 %) and other sugars (24 %) in the *Eucalyptus globulus* wood resembled that of *Eucalyptus goniocalyx* wood [*28*].

**Table 2.2** Yield and molar composition of xylan rich feedstock materials and corresponding extracts.

| | Yield[a] | Total[b] sugar content | Molar composition | | | | | | | Ara/ xyl[c] | UA/ xyl[c] | Ac/ xyl[c] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Ara | Xyl | Man | Gal | Rha | Glc | UA | | | |
| **Wheat bran** | | 61 | 17 | 30 | 0 | 1 | 0 | 49 | 3 | 0.56 | 0.10 | 0.07 |
| AIS | 100 | 66 | 23 | 44 | 0 | 1 | 0 | 28 | 4 | 0.52 | 0.09 | 0.06 |
| Wss | 10 | 39 | 19 | 30 | 1 | 4 | 2 | 41 | 3 | 0.63 | 0.10 | - |
| KOH ss | 49 | 72 | 22 | 55 | 0 | 2 | 1 | 18 | 2 | 0.40 | 0.04 | - |
| KOH res | 28 | 78 | 25 | 24 | 1 | 2 | 1 | 42 | 5 | 1.04 | 0.21 | - |
| **Brew. SG** | | 43 | 21 | 39 | 0 | 2 | 0 | 33 | 5 | 0.54 | 0.13 | 0.17 |
| AIS | 100 | 49 | 21 | 42 | 0 | 1 | 0 | 31 | 5 | 0.50 | 0.12 | 0.15 |
| Wss | 2 | 28 | 23 | 26 | 2 | 8 | 3 | 30 | 8 | 0.88 | 0.31 | - |
| KOH ss | 34 | 50 | 26 | 54 | 0 | 3 | 2 | 11 | 4 | 0.48 | 0.07 | - |
| KOH res | 24 | 81 | 11 | 14 | 2 | 1 | 2 | 66 | 4 | 0.79 | 0.29 | - |
| **Corn cob** | | 74 | 7 | 43 | 0 | 2 | 1 | 43 | 4 | 0.16 | 0.09 | 0.34 |
| AIS | 100 | 72 | 5 | 46 | 0 | 2 | 1 | 42 | 4 | 0.11 | 0.09 | 0.35 |
| Wss | 1 | 24 | 13 | 18 | 4 | 14 | 4 | 29 | 18 | 0.72 | 1.00 | - |
| KOH ss | 38 | 67 | 9 | 81 | 0 | 2 | 3 | 1 | 4 | 0.11 | 0.05 | - |
| KOH res | 43 | 82 | 4 | 12 | 0 | 0 | 2 | 79 | 3 | 0.33 | 0.25 | - |
| ***Eucalyptus*** | | 71 | 1 | 25 | 1 | 1 | 2 | 63 | 7 | 0.04 | 0.28 | 0.67 |
| AIS | 100 | 73 | 1 | 24 | 1 | 2 | 2 | 63 | 7 | 0.04 | 0.29 | 0.64 |
| Wss | 1 | 13 | 7 | 12 | 14 | 15 | 8 | 21 | 23 | 0.58 | 1.92 | - |
| KOH ss | 7 | 74 | 1 | 81 | 1 | 3 | 2 | 1 | 13 | 0.01 | 0.16 | - |
| KOH res | 80 | 68 | 1 | 15 | 1 | 2 | 2 | 74 | 7 | 0.07 | 0.47 | - |

[a] expressed as weight percentage (dm).

[b] neutral sugars + uronic acids (UA) + *O*-acetyl substituents expressed as weight percentage (dry matter) of each fraction.

[c] ratio mol / mol; Ac = *O*-acetyl substituents

### *2.3.2 Structural characteristics of the alkali-extracts (KOH ss) from the four feedstock materials*

To study the xylan present in the four materials in more detail, alkali-extracts (KOH ss) were prepared. Table 2.2 shows the yield of the extracts and molar sugar composition of the feedstock materials and of their extracts. Furthermore, the molar ratio's of arabinose to xylose, uronic acid to xylose, and *O*-acetyl substituents to xylose are presented, since these ratio's are considered to be a measure for the branching of the xylan.

The yield of glucuronoarabinoxylan (GAX) for the alkali-extraction was calculated as the sum of weights of the recovered arabinose, uronic acid and xylose. For wheat bran, brewery's spent grain and corn cob, most of the GAX was recovered in the KOH-extract (62 %, 45 % and 67 % respectively), while part of the GAX remained in the corresponding residues (25 %, 17 % and 16 % respectively). The alkali extractions of *Eucalyptus* wood resulted in a lower yield of xylans in the alkali-soluble fractions (23 %). The latter extraction was probably hindered by the high lignin content of the wood [*28*], since 56 % of the GAX was recovered in the KOH-residue. Still, we consider our results on the extracted xylan as being representative for most of the (insoluble) xylans in the *Eucalyptus globulus* wood, since our results are rather similar to those obtained by Shatalov et al. [*14*] for the xylan extracted from *Eucalyptus globulus Labill* wood.

Comparing the different by-products several remarks regarding the structural characteristics of the GAX present can be made (table 2.2). In the KOH-extracts of wheat bran, brewery's spent grains and corn cobs, GAX was found having a molar ratio of arabinose to xylose of 0.40, 0.48 and 0.11 respectively, while only small amounts of uronic acids were found. On the other hand, the alkali-extract of *Eucalyptus* wood contained almost no arabinose and much higher levels of uronic acids (UA/Xyl = 0.16). The ratio of *O*-acetyl substituents to xylose was determined for the feedstocks and AIS's. This ratio was found to be much higher for *Eucalyptus* wood (0.64) and corn cobs (0.35) than for wheat bran (0.06) and brewery's spent grain (0.15).

To further study the structural characteristics of the four extracted GAX's, a purified and well characterised endoxylanase [*16*] was used. The molecular weight (Mw) distribution of the alkali-extracts before and after enzymatic degradation by this endoxylanase I is shown in figure 2.1.

Seen from figure 2.1, about one third of the wheat bran and brewery's spent grain xylan was degraded by endoxylanase I to fragments having a Mw < $10^4$. Knowing both the sugar composition of the KOH-extracts and the mode of action of the endoxylanase used, it was suggested that the less degraded xylans present were highly branched with arabinose. Corn cob xylan and *Eucalyptus* xylan were degraded almost completely by endoxylanase I, suggesting a relatively low substitution of most of the xylan. However, the remaining high Mw material of the *Eucalyptus* xylan (Fig. 2.1) probably constitutes a highly substituted acidic xylan. This was substantiated by our finding that a hydrothermal treatment of the *Eucalyptus* wood resulted in a series of xylo-oligosaccharides (DP 4-12) containing two 4-*O*-methylglucuronic acid substituents [*29*].

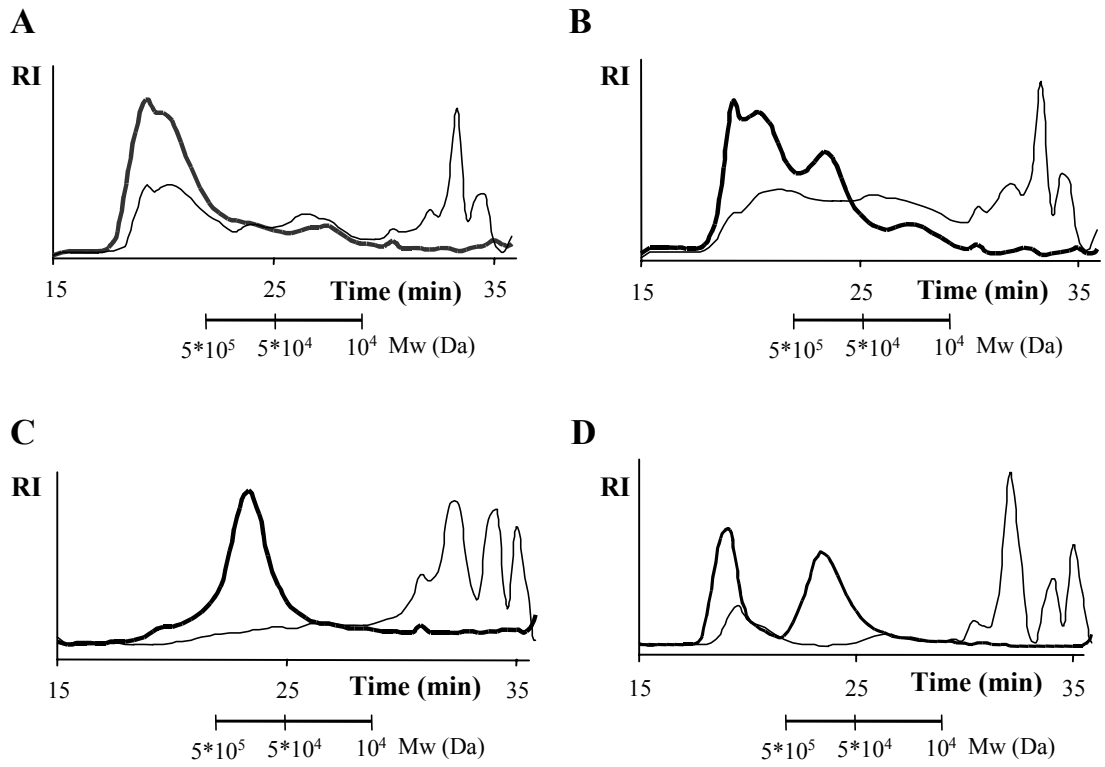


**Figure 2.1** HPSEC elution profiles of the alkali-soluble fractions (KOH ss) of wheat bran (A), brewery's spent grain (B), corn cobs (C) and *Eucalyptus* wood (D); before (bold line) and after (thin line) degradation by endoxylanase I.

The xylo-oligosaccharides formed by endoxylanase I treatment of the KOH-fractions were monitored using HPAEC and MALDI-TOF mass spectrometry. The elution patterns and the main masses from the MALDI-TOF mass spectra (not shown) are presented in figure 2.2.

The HPAEC-elution pattern of the endoxylanase I-digest of wheat flour arabinoxylan, extensively described by Gruppen et al. [*17*] and Kormelink et al. [*16*] was used as a 'standard' for the identification of arabinoxylo-oligosaccharides in our xylan-digests together with the data obtained by MALDI-TOF MS. Endoxylanase I released xylose ($X_1$), xylobiose ($X_2$) and xylotriose ($X_3$) from the xylan-fractions of all materials. Furthermore, in the elution patterns of the degraded wheat bran arabinoxylan, two xylo-oligosaccharides linked with arabinose at *O*-3 were well distinguishable ($X_3A_1$ and $X_2A_1$), of which the masses were confirmed by MALDI-TOF MS as well. Both structures were also present in the endoxylanase-digest of brewery's spent grain xylan and corn cob xylan, in addition to a xylo-oligosaccharide substituted at *O*-2 with arabinose ($X_3A_1$) [*30*].

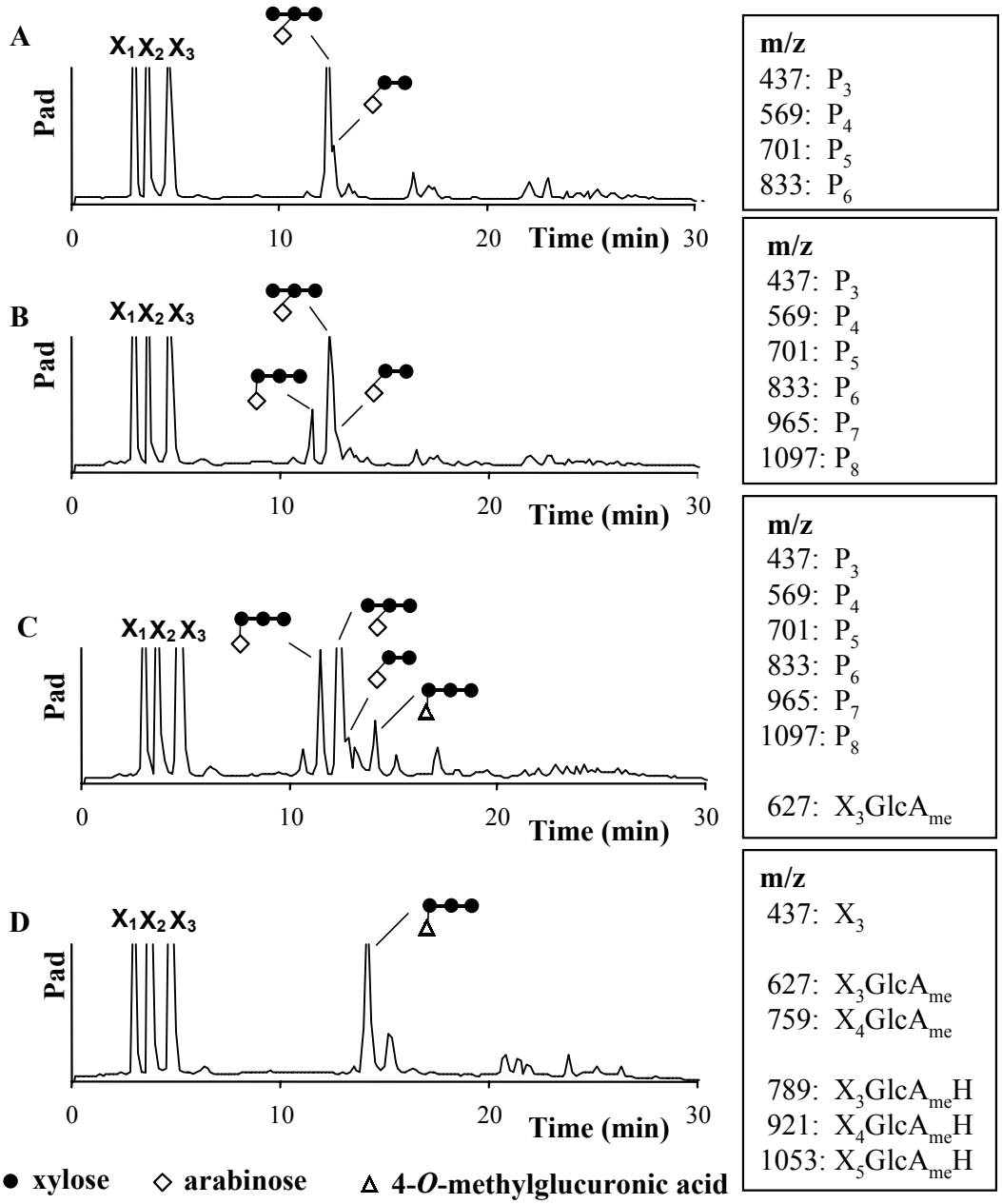


**Figure 2.2** HPAEC elution patterns (on the left) and the main molecular masses (as sodium-adducts) as found by MALDI-TOF mass spectrometry (on the right) of the alkali-soluble fractions (KOH ss) of wheat bran (A), brewery's spent grain (B), corn cobs (C) and *Eucalyptus* wood (D) after degradation by endoxylanase I (P = pentose; X = xylose; $GlcA_{me}$ = 4-*O*-methylglucuronic acid; H = hexose).

The major peak in the elution pattern of the digest of *Eucalyptus* wood corresponded to the acidic oligosaccharide $X_3GlcA_{me}$, confirmed by the MALDI-TOF mass 627 (sodium adduct), present in the digest of corn cob xylan as well [*31*]. Remarkable was the detection of the masses 789, 921 and 1053 (sodium adducts) in the digest of the *Eucalyptus* wood xylan (Fig. 2.2). These masses indicate the presence of a series of xylo-oligosaccharides containing a 4-*O*-methylglucuronic acid *and* an additional hexose as substituent. Shatalov et al. [*14*] have

already described the linkage 2-*O*-α-galactopyronosyl-4-*O*-methyl-α-D-glucuronic acid to be present in xylan from *Eucalyptus globulus labill* wood. In addition, we do not expect the masses 789, 921 and 1053 to be the sodium adducts of $X_3GlcA_2$, $X_4GlcA_2$ and $X_5GlcA_2$ respectively, which is another possibility according to the masses, because we do not have any evidence for the presence of the same series of xylo-oligosaccharides but containing only *one* glucuronic acid.

### *2.3.3 Hydrolysates from the four by-products obtained after hydrothermal treatment; yield and sugar composition*

In order to evaluate the use of hydrothermal treatment to produce xylose and xylo-oligosaccharides from the four by-products, hydrolysates were analysed in detail and the yield of total sugar and of sugar residues was calculated (table 2.3). The figures as presented in table 2.3 include the sugar-residues from both monomeric and oligomeric origin.

**Table 2.3** Yield of sugars and sugar residues in the hydrolysates and corresponding residues after hydrothermal treatment of wheat bran, brewery's spent grain, corn cobs and *Eucalyptus* wood.

| | total[a] | total sugar[b,(c)] | Xyl[b,(c)] | Ara[b,(c)] | GlcA[b,(c)] | Glc[b,(c)] | *O*-acetyl[b,(c)] |
|---|---|---|---|---|---|---|---|
| **Hydrolysates:** | | | | | | | |
| WB II =155 °C; 60 min; 10 g/g | 33 | 13 (22) | 7.2 (27) | 2.3 (16) | 1.0 (33) | 2.6 (11) | 0.1 (25) |
| BSG E1=150 °C; 60 min; 8 g/g | 34 | 17 (35) | 7.9 (42) | 4.1 (45) | 1.0 (33) | 2.8 (16) | 0.3 (38) |
| BSG J =150 °C; 120 min; 8 g/g | 40 | 19 (40) | 9.4 (49) | 4.3 (47) | 1.2 (40) | 3.1 (18) | 0.3 (38) |
| CC. A =160 °C; 75 min; 8 g/g | 37 | 24 (33) | 17 (61) | 2.2 (44) | 1.5 (50) | 1.5 (4) | 1.1 (37) |
| Euc. B2 =160 °C; 60 min; 8 g/g | 20 | 15 (21) | 9.0 (64) | 0.2 (40) | 2.3 (38) | 0.4 (1) | 1.2 (40) |
| **Residues:** | | | | | | | |
| WB II =155 °C; 60 min; 10 g/g | 69 | 31 (47) | 8.3 (31) | 1.4 (10) | 0.7 (23) | 21 (95) | 0.3 (75) |
| BSG J =150 °C; 120 min; 8 g/g | 61 | 23 (48) | 7.3 (38) | 1.2 (13) | 0.6 (20) | 13 (76) | 0.3 (38) |
| CC. A =160 °C; 75 min; 8 g/g | 62 | 44 (59) | 9.9 (35) | 0.6 (12) | 0.6 (20) | 32 (95) | 0.8 (25) |
| Euc. B2 =160 °C; 60 min; 8 g/g | 78 | 50 (70) | 5.5 (39) | 0 (0) | 1.6 (27) | 42 (94) | 0.8 (25) |

[a] yield (dry matter) of 100 g of material subjected to treatment (% (w/w)).
[b] yield of sugar (residue) of 100 g of material subjected to treatment (% (w/w)).
[c] recovery of sugar (residue) as g sugar (residue) of sugar (residue) present in 100 g of material subjected to treatment (% (w/w)).

After hydrothermal treatment, xylose (present as poly- and oligomeric material) was almost completely recovered in the hydrolysates and residues, which indicates that xylose is quite stable during the hydrothermal treatment (table 2.3). However, the recovery of arabinose expressed as the sum of arabinose found in hydrolysates and residues was quite low. This loss of arabinose was reflected in the recovery of GAX (%) from the GAX originally present in

the destarched materials used (AIS), calculated as the sum of arabinose, uronic acid and xylose, which is illustrated by the following data (table 2.3): wheat bran 24 % of (G)AX in the hydrolysate and 24 % in the residue; brewery's spent grain 48 % of (G)AX in the hydrolysate and 30 % in the residue; corn cobs 58 % of (G)AX in the hydrolysate and 31 % in the residue; *Eucalyptus* wood 55 % of (G)AX in the hydrolysate and 34 % in the residue. The total loss of (G)AX during the hydrothermal treatment of wheat bran, brewery's spent grains and corn cobs was mainly due to a loss of arabinose, while of *Eucalyptus* wood it was mainly due to a loss of uronic acids (table 2.3).

The degradation of the sugars, mainly arabinose, in the hydrothermal treated feedstock contributed most likely to the increase in furfural and HMF (5-hydroxymethyl-2-furfural) (results not shown). For brewery's spent grain it was measured that formic acid (0.6 g/l) and levulinic acid (0.05 g/l) were present in the hydrolysates resulting from further degradation of part of the furfural (0.2 g/l) and HMF (0.02 g/l).

**Table 2.4** Sugar composition (mol%) of the hydrolysates and corresponding residues obtained after hydrothermal treatment of wheat bran, brewery's spent grain, corn cobs and *Eucalyptus* wood.

| | Total | Molar composition | | | | | | | Ara/ | UA/ | Ac/ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | sugars[a] | Rha | Ara | Xyl | Man | Gal | Glc | UA | Xyl[b] | Xyl[b] | Xyl[b] |
| **Hydrolysates:** | | | | | | | | | | | |
| WB II | 41 (0) | 0 | 17 | 58 | 0 | 3 | 17 | 5 | 0.29 | 0.09 | 0.04 |
| BSG E1 | 48 (1) | 0 | 27 | 50 | 0 | 3 | 15 | 5 | 0.54 | 0.08 | 0.11 |
| BSG J | 48 (1) | 0 | 24 | 53 | 0 | 3 | 16 | 4 | 0.45 | 0.08 | 0.11 |
| CC A | 63 (3) | 0 | 10 | 74 | 0 | 5 | 6 | 5 | 0.14 | 0.07 | 0.20 |
| Euc. B2 | 71 (6) | 2 | 2 | 70 | 2 | 8 | 2 | 14 | 0.03 | 0.20 | 0.42 |
| **Residues:** | | | | | | | | | | | |
| WB II | 45 (0) | 0 | 5 | 31 | 0 | 0 | 62 | 2 | 0.16 | 0.06 | 0.10 |
| BSG J | 38 (1) | 0 | 7 | 35 | 0 | 0 | 55 | 3 | 0.2 | 0.09 | 0.14 |
| CC A | 69 (1) | 0 | 1 | 27 | 0 | 0 | 70 | 2 | 0.04 | 0.07 | 0.24 |
| Euc. B2 | 63 (1) | 0 | 0 | 13 | 1 | 0 | 82 | 4 | 0 | 0.31 | 0.44 |

[a] neutral sugars + uronic acids (UA) and between parentheses the total content of *O*-acetyl substituents expressed as weight percentage of each fraction (dm).
[b] ratio mol / mol

HPAEC analysis of the hydrolysates (not shown) revealed that quite a proportion of the arabinose and xylose was present as monomer. Therefore, the sugar composition of the hydrolysates was corrected for the presence of monomeric arabinose and xylose (table 2.4 versus 2.5). In the wheat bran hydrolysate the arabinose was *only* present as monomer. Also in the brewery's spent grain hydrolysate, a significant part of the arabinose was present as monomer, but still a ratio of arabinose to xylose of 0.3 was calculated for the oligomers. For the corn cobs and the *Eucalyptus* hydrolysate the ratio of linked arabinose to xylose was

remarkably lower (0.04 and 0 respectively). Furthermore, for the hydrolysate from *Eucalyptus* wood the highest ratio of uronic acids to xylose was found (0.25), together with a noticeable amount of (linked) galactose.

Finally, the ratio's of *O*-acetyl substituents to xylose in the four hydrolysates were calculated. In the hydrolysates from wheat bran and brewery's spent grain this ratio was much lower than the corresponding ratio for the hydrolysates from corn cobs and *Eucalyptus* wood. However, from all four by-products part of the *O*-acetyl substituents were released during the hydrothermal treatment. This release is quite desirable, since the liberated acetic acid catalyses the depolymerisation of the xylan and contributes to an increase in soluble xylan [*1*].

**Table 2.5** Sugar composition (mol%) of the hydrolysates obtained after hydrothermal treatment of wheat bran, brewery's spent grain, corn cobs and *Eucalyptu*s wood; corrected for the presence of monomers.

| | Total | Molar composition | | | | | | | Ara/ | UA/ | Ac/ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | sugars[a] | Rha | Ara | Xyl | Man | Gal | Glc | UA | Xyl[b] | Xyl[b] | Xyl[b] |
| Hydrolysates | | | | | | | | | | | |
| WB II | 33 (0) | 0 | 4 | 64 | 0 | 4 | 21 | 7 | 0.06 | 0.11 | 0.05 |
| BSG E1 | 44 (1) | 0 | 18 | 56 | 0 | 5 | 16 | 6 | 0.32 | 0.11 | 0.11 |
| BSG J | 44 (1) | 1 | 19 | 56 | 0 | 4 | 15 | 5 | 0.34 | 0.09 | 0.11 |
| CC A | 57 (3) | 0 | 3 | 78 | 0 | 5 | 7 | 7 | 0.04 | 0.09 | 0.22 |
| Euc. B2 | 65 (6) | 2 | 0 | 68 | 2 | 9 | 2 | 17 | 0 | 0.25 | 0.48 |

[a] neutral sugars + uronic acids (UA) and between parentheses the total content of *O*-acetyl substituents expressed as weight percentage of each fraction (dm).
[b] ratio mol / mol

### *2.3.4 Molecular weight (Mw) distribution during and after hydrothermal treatment*

The Mw distribution of the soluble material after hydrothermal treatment was monitored using HPSEC (Fig. 2.3).

The treatment performed, designed to obtain a high proportion of oligosaccharides and a low concentration of furfural, resulted in material with masses lower than $10^4$ Da (Rt > 30 minutes; based on pullulans). For the hydrolysate from brewery's spent grain also a remarkable part of the material eluted before 30 minutes. This suggested that, although about 50 % of all GAX became soluble upon treatment, a significant proportion was still having a high Mw.

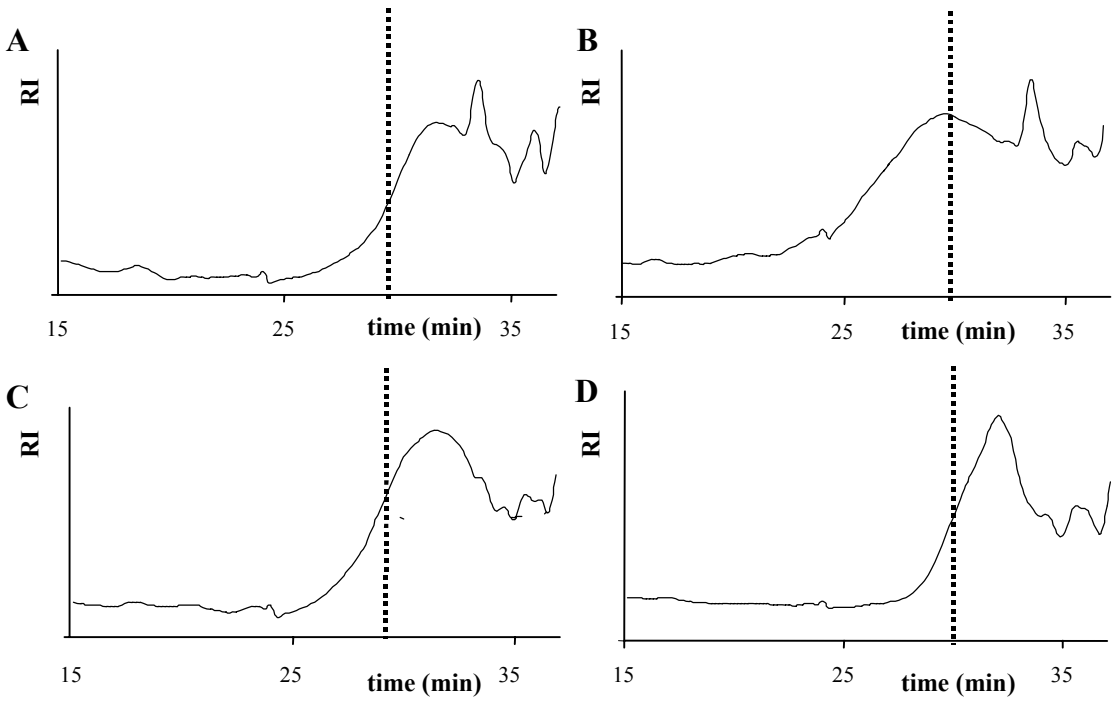


**Figure 2.3** HPSEC elution profiles of the hydrolysates from wheat bran (A), brewery's spent grain (B), corn cobs (C) and *Eucalyptus* wood (D).

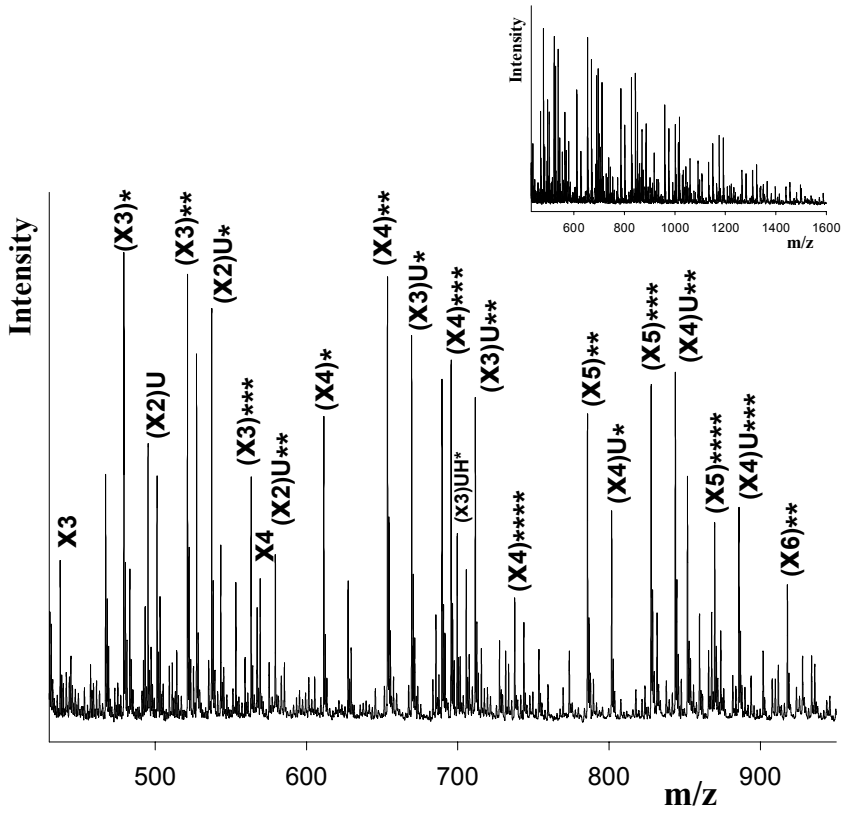


**Figure 2.4** Part of the MALDI-TOF mass spectrum of the *Eucalyptus* wood hydrolysate (X = xylose; U = 4-*O*-methylglucuronic acid; H = hexose; asterix = *O*-acetyl substituent). As illustration the complete spectrum is inserted.

To show the presence of a whole range of oligosaccharides in the hydrolysates, a typical MALDI-TOF mass spectrum is presented in figure 2.4. Several series of xylo-oligosaccharides substituted with *O*-acetyl substituents and 4-*O*-methylglucuronic acid residues were identified (based on the sugar composition of the hydrolysate). In future, research will be carried out to identify the various oligomers present in more detail [*29*].

### *2.3.5 Conclusions*

A rather detailed picture of the structural features of the xylans present in the four feedstock materials was obtained. Our results corresponded well with results previously reported in literature. For wheat bran xylan mainly substituted at O-3 and both O-2 and O-3 with arabinose were detected. The same substitution was present in xylan from brewery's spent grain, but also substitution at O-2 with arabinose was found. In addition to substitution at O-2, O-3 or both O-2 and O-3 with arabinose, also linkages at O-2 with 4-*O*-methylglucuronic acid were found for the xylan from corn cobs. For the *Eucalyptus* wood xylan mainly substitution with 4-*O*-methylglucuronic acid was detected and some indications for the presence of the linkage 2-*O*-α-galactopyronosyl-4-*O*-methyl-α-D-glucuronic acid were obtained.

The four agrobased by-products appeared to be very suitable for studying the effect of hydrothermal treatment on structurally different xylans as well as to recover different series of xylo-oligosaccharides. Arabinose was rather easily split off by hydrothermal treatment from the xylan-backbone of wheat bran, brewery's spent grain and corn cobs. The *O*-acetyl substituents were partly released from the feedstocks, becoming available to catalyse the depolymerisation of the xylan. Also, part of the uronic acids were released during the treatments performed, mainly concerning the treatment of *Eucalyptus* wood.

Due to the partial release of these substituents and cleavage of the xylan by the treatment performed, a wide variety of xylo-oligosaccharides with different structural features depending on the xylan-structure of the original feedstock were obtained. In the hydrolysate from brewery's spent grain xylo-oligosaccharides linked with arabinose were identified, while in the hydrolysate of corn cobs and *Eucalyptus* wood also xylo-oligosaccharides with 4-*O*-methylglucuronic acid residues were present. Additionally, in the *Eucalyptus* wood hydrolysate a series of *O*-acetylated xylo-oligosaccharides was identified. Further structural characterisation of these oligosaccharides will be helpful in studying the mechanism and improving the hydrothermal treatment in the release of xylose and xylo-oligosaccharides. Moreover, these fractions with structurally different xylo-oligosaccharides are very suitable (after purification) to study the effect of different substitution of xylo-oligosaccharides in several biological activity tests and their fermentability by the human intestinal flora. Both the further structural characterisation and the fermentability of the discussed xylo-oligosaccharides will be the subject of further publications.

**Acknowledgement**

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# CHAPTER 3

# Complex xylo-oligosaccharides identified from hydro-thermally treated *Eucalyptus* wood and brewery's spent grain


**Abstract**

Hydrolysates from two hydrothermal treated xylan-rich agrobased materials, *Eucalyptus* wood and brewery's spent grain, were fractionated by anion-exchange chromatography and size-exclusion chromatography. Hereby, several pools were obtained and they were characterised by their sugar composition. Additionally, the oligosaccharides in the pools described were further identified by high-performance anion-exchange chromatography (HPAEC) and mass spectrometry. The hydrothermally treated brewery's spent grain resulted in three pools of which two contained relatively high molecular weight xylan, singly and doubly branched with arabinose [$X_nA_m$], separated from a pool of xylo-oligosaccharides (XOS) less branched with arabinose. The fractionation of the hydrothermally treated *Eucalyptus* wood resulted in a pool, mainly consisting of a series of neutral (*O*-acetylated) XOS [$X_nAc_m$], and three pools containing acidic XOS. Two of these 'acidic' pools contained a series of (*O*-acetylated) xylo-oligosaccharides including *one* 4-*O*-methylglucuronic acid [$X_n(GlcA_{me})_1Ac_m$], while the third 'acidic' pool contained (*O*-acetylated) xylo-oligosaccharides substituted with *two* 4-*O*-methylglucuronic acids [$X_n(GlcA_{me})_2Ac_m$]. Additionally, a series of xylo-oligosaccharides containing both 4-*O*-methylglucuronic acid and a hexose, most likely galactose, was detected in the acidic *Eucalyptus* pools [$X_n(GlcA_{me})_{1or2}Ac_mH$]. Information was obtained about the number of *O*-acetyl substituents linked to the (4-*O*-methylglucurono-) xylo-oligosaccharides. Finally, it was demonstrated with an example that the different substituents to the xylo-oligosaccharides present are of relevance for the fermentability of the xylo-oligosaccharides by human faecal samples.



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## 3.1 Introduction

Agro-industrial and forest by-products, such as brewery's spent grain and *Eucalyptus* wood are rich in cellulose and hemicelluloses. Currently, more and more effort is directed towards the utilisation of such by-products, considering economic values and environment. From this point of view, hydrothermal treatment is of interest because it will result in a selective fractionation of the hemicellulose and cellulose from the by-products, to be used for different product applications [*1,2*].

Recently, we described a first characterisation of the hydrolysates obtained after hydrothermal treatment of brewery's spent grain and *Eucalyptus* wood [*3*]. It was shown that the hydrolysate from brewery's spent grain contains about 48 % (w/w) sugar; 5 % (w/w) as monomeric arabinose, 1 % (w/w) as monomeric xylose and about 42 % (w/w) as polymeric/ oligomeric material mainly composed of arabinose (18 mol%), xylose (56 mol%) and glucose (16 mol%). Glucose and glucose-oligomers most likely originate from the degradation of starch, while xylo-oligosaccharides substituted with arabinose are released from the arabinoxylan, described to be present in barley and brewery's spent grain [*4,5*].

The hydrolysate from *Eucalyptus* wood contains about 71 % (w/w) sugar, of which 2 % (w/w) is monomeric arabinose, 8 % (w/w) is monomeric xylose and 61 % (w/w) are oligosaccharides mainly composed of xylose (68 mol%) and uronic acid (17 mol%). Furthermore, in addition to acetic acid released during hydrothermal treatment many of the *O*-acetyl substituents present in the native *Eucalyptus* wood xylan resist the treatment (6 % (w/w)) resulting in a variety of *O*-acetylated (4-*O*-methyl-glucurono-) xylo-oligosaccharides as identified by matrix assisted laser desorption/ ionisation mass spectrometry (MALDI-TOF MS) [*3,6*]. Such considerable preservation of *O*-acetyl substituents was also observed during steam explosion treatments of birchwood [*7*].

From a nutritional point of view xylo-oligosaccharides usually are considered as non-digestible oligosaccharides (NDO's) and enhance growth of bifidobacteria in the large bowel, which may effect the human gastrointestinal tract beneficially [*8-10*]. However, the influence of several types of substitution of xylo-oligosaccharides on the ability and rate of fermentation by the human intestinal flora and on the production of short chain fatty acids is still unknown. Thus, these questions strengthened the importance of further studies on the structural features of xylo-oligosaccharide in hydrothermal treated substrates, for better understanding the mechanism of xylan-breakdown in addition to studying the fermentability of the xylo-oligosaccharides formed.

In this paper we propose a method to fractionate hydrolysates from brewery's spent grain and *Eucalyptus* wood into several series of differently substituted xylo-oligosaccharides, which are characterised in more structural detail and submitted to fermentation studies.

## 3.2 Experimental

### *3.2.1Hydrothermal treated samples*

Brewery's spent grain was supplied from the Brewery Central de Cervejas, Vialonga (Portugal). The hydrolysates were kindly provided by F. Carvalheiro / F.M. Gírio of INETI (Lisboa, Portugal). Three hydrolysates, prepared from brewery's spent grain at conditions which resulted in a maximum release of oligosaccharides, were equally mixed and further mentioned as the hydrolysate from brewery's spent grain. Chips of *Eucalyptus* wood were obtained from ENCE Complejo Industrial de Pontevedra Puentenolinos s/n Lourizan (Spain, July 1998). The hydrolysates from *Eucalyptus* wood were received gratefully from G. Garrote/ J.C. Parajó of the University of Vigo (Vigo, Spain). Five hydrolysates, all prepared to reach maximal oligosaccharide-concentrations [*1*], were equally mixed and further mentioned as the hydrolysate from *Eucalyptus* wood.

### *3.2.2 Separation of the hydrolysates from brewery's spent grain and Eucalyptus wood by anion-exchange chromatography*

Preparative anion-exchange chromatography was performed using a Biopilot system (APB), equiped with a Source 15 Q (APB) fine line column (1.2 L). The column was activated with a solution of 1 M sodium acetate (pH 5; 5 column volumes (CV)). The excess of (unbounded) ions were removed by elution with water (~ 10 CV). The sample (~ 3.9 g) was applied to the column and the neutral oligosaccharides were separated from the acidic oligomers using the following gradient (25 ml/min): 0-1250 ml → only water; 1250-1750 ml → 0-17 mM sodium acetate buffer (pH 5); 1750-2250 ml → 17-30 mM of buffer (pH 5); 2250-3000 ml → isocratic 30 mM of buffer (pH 5) and 3000-6000 ml → 100 mM of buffer (pH 5). Also in case a gradient was used for elution, the eluent was detected by a Shodex RI-detector (Shodex RI se-72), operable at high flow-rates.

### *3.2.3 Size-exclusion chromatography*

To remove the monomers and dimers from the neutral fractions and to desalt the fractions eluted with a salt gradient from the Source 15 Q-column, a Biopilot system (APB) equipped with a Superdex 30 (APB) column (5 L) was used. Elution was performed with water and from 1000 till 4000 ml fractions were collected every 100 ml. The eluent was detected by a Shodex RI-detector (Shodex RI se-72).

### *3.2.4 Degradation by endoxylanase I*

Samples (4 mg) were dissolved in 50 mM sodium acetate buffer pH 5 (1 ml) and incubated with endo-(1,4)- β-D-xylanase I (0.2 μg/ml) for 24h at 30 °C. The purification and mode of

action of the used endo-(1,4)-β-D-xylanase I from *Aspergillus awamori* is described by Kormelink et al. [*11*]. After inactivation of the enzyme the digests were analysed by HPAEC and HPSEC, calibrated with a well characterised digest of wheat arabinoxylan degraded by the endo-(1,4)-β-D-xylanase I [*11,12*].

### *3.2.5 Fermentation of the pool Euc NI A by human intestinal bacteria*

Fermentation of *Euc* NI A and saponified *Euc* NI A was determined using faecal inocula. Faecal inocula were prepared from fresh faeces in buffered peptone water with cysteine.HCl (0.5 g/l) in approximately 10-fold dilution. A medium consisting of 1 g/l of neutralised bacterial peptone (Oxoid), 8 g/l of sodiumchloride (Merck) and 0.5 g/l of L-cysteine.HCl was adjusted to a pH of 6.7 using a 6N NaOH solution [*13*]. In an anaerobic chamber (atmosphere 80 % $N_2$, 10% $CO_2$, 10% $H_2$) the 10-fold diluted faeces was diluted further (10.000x) with the medium described. A sterile solution of 0.5 % (w/v) *Euc* NI A in thioglycollate broth (sugarfree; Oxoid, CM391) was inoculated with 20 % (v/v) of the 10.000x diluted faecal inocula at 37°C in an anaerobic chamber. Samples were taken at time 0, 1, 4, 10, 24, 48, 72 hours and stored at -80°C. Of each sample enzyme activity was inactivated (5 min, 100°C), centrifugated and the supernatants were diluted 12 times with $H_2O$ before analysis of the reaction products by HPAEC [*10*].

### *3.2.6 Neutral sugar composition*

The neutral sugar composition was determined by gas chromatography according to Englyst and Cummings [*14*], using inositol as an internal standard. The samples were treated with 72 % (w/w) $H_2SO_4$ (1 h, 30 °C) followed by hydrolysis with 1 M $H_2SO_4$ for 3 h at 100 °C and the constituent sugars released were analysed as their alditol acetates.

### *3.2.7 Uronic acid content*

The uronic acid content was determined as anhydro-uronic acid (AUA) by an automated *m*-hydroxydiphenyl assay [*15,16*] using an autoanalyser (Skalar Analytical BV, Breda, The Netherlands).

### *3.2.8 Acetic acid content*

The degree of acetylation was determined on a Spectrophysics apparatus (Thermo Separation Products, U.S.A.), using an Aminex HPX column [*17*]. The content of *O*-acetyl substituents was corrected for the free acetic acid in the samples.

### *3.2.9 HPSEC*

High-performance size-exclusion chromatography was performed on three TSKgel columns (7.8 mm ID x 30 cm per column) in series (G4000 PWXL, G3000 PWXL, G2500 PWXL; Tosohaas), in combination with a PWX-guard column (Tosohaas). Elution took place at 30 °C with 0.2 M sodium nitrate at 0.8 ml/min. The eluate was monitored using a refractive index detector. Calibration was performed using dextrans.

### *3.2.10 HPAEC at pH 12*

High-performance anion-exchange was performed on a Dionex system equipped with a CarboPac PA-1 column (4 mm ID x 250mm) in combination with a CarboPac PA guard column (3 mm ID x 25 mm) and PAD-detection [*18*]. Elution (1 ml/min) of the oligomers in the pools was performed with a combination of linear gradients of 0-150 mM sodium acetate in 100 mM NaOH during 10 min, then 150-450 mM sodium acetate in 100 mM NaOH during 25 min. The oligosaccharides in the samples taken during the fermentation were eluted (1 ml/min) with a combination of linear gradients of 50-90 mM sodium acetate in 100 mM NaOH during 0-5 min, 90-130 mM sodium acetate in 100 mM NaOH during 10 min, followed by a linear gradient to 520 mM sodium acetate in 100 mM NaOH in 15 minutes. Each elution was followed by a washing and equilibration step.

### *3.2.11 MALDI-TOF mass spectrometry*

For MALDI-TOF MS (Matrix-Assisted Laser Desorption/ Ionisation Time-Of-Flight Mass Spectrometry) a Voyager-DE RP Biospectrometry workstation (PerSeptive Biosystems Inc., Framingham, MA, USA) was used, operated as described by [*19*]. The mass spectrometer was calibrated with a mixture of maltodextrines (mass range 365-2309).

The samples were mixed with a matrix solution (1 µl of sample in 9 µl of matrix), after desalting the samples using $H^+$-Dowex AG 50W X8 (Biorad).The matrix solution was prepared by dissolving 9 mg of 2,5-dihydroxybenzoic acid and 3 mg 1-hydroxyisoquinoline in a 1-ml mixture of acetonitrile:water (300 µl:700 µl). Of the prepared (sample + matrix) solutions 1 µl was put on a gold plate and allowed to dry at room temperature.

### *3.2.12 Nanospray mass spectrometry*

Dynamic nanospray was performed on a LCQ Ion-trap (Finnigan MAT 95, San Jose, CA) equipped with a nano-source. Sample was running through a transferring capillary (100 µm ID) and a spraying capillary with an ID of 20 µm at a flow rate of 0.3 µl/min. MS analysis was carried out in the positive mode using a spray voltage of 2 kV and a capillary temperature of 200$^o$C. The capillary voltage was set at 45 kV and the tube lens voltage at 35 kV. $MS^2$ and higher was performed using a window of 1.5-2 m/z and a 30 –35% relative collision energy.

The apparatus and the data were controlled by Xcalibur software. The accuracy of the mass determinations is ±0.3 Da.

Prior to analysis on the LCQ Ion-trap the saponified pools *Euc* AII#, *Euc* AIII# and *Euc* AII# (3 mg/ml) were desalted using $H^+$-Dowex AG 50W X8 (Biorad).

### 3.3 Results and Discussion

Separation of neutral (*O*-acetylated) xylo-oligosaccharides from acidic (*O*-acetylated) xylo-oligosaccharides was performed by preparative anion-exchange chromatography using a Source 15Q-resin. A similar application has been described by Teleman et al. [*20*], who separated an acidic oligosaccharide from neutral ones.

The neutral oligosaccharides present in the hydrolysate of *Eucalyptus* wood were recovered in pool *Euc* NI (neutral I) and were eluted with water only. In this 'neutral' pool, 71 % of the applied sugars was accumulated. Subsequently, the charged oligomers were collected into the four pools *Euc* AI (Acidic I; 17-30 mM sodium acetate-buffer (pH 5)), *Euc* AII (30 mM sodium acetate-buffer), *Euc* AIII (30-50 mM sodium acetate-buffer) and *Euc* AIV (50-100 mM sodium acetate-buffer), with a yield of 13 %, 7 %, 4 % and 3 % of the applied sugar respectively. In pool *Euc* AI, oligosaccharides were combined eluting with a relatively low ratio of uronic acid to xylose (< 0.18). This pool *Euc* AI was not subjected to further purification and analysis, since we were mainly interested in highly substituted xylo-oligosaccharides. The pools *Euc* AII, AIII and AIV, containing oligosaccharides with a rather high ratio of uronic acid to xylose, were desalted by preparative size-exclusion chromatography prior to further characterisation (*Euc* AII#, AIII# and AIV#). Pool *Euc* NI was subjected to preparative size-exclusion chromatography as well, mainly to remove monomers and dimers. All oligosaccharides (> DP2) from pool *Euc* NI were pooled together (*Euc* NI A) representing about 55 % (w/w) of the applied neutral pool.

Similar fractionations were performed with the hydrolysate from brewery's spent grain. By anion-exchange chromatography besides a neutral pool (BSG I) all fractions collected during the gradient with the sodium acetate-buffer were combined into *one* pool (BSG II), since we have already observed before that only few oligomers in this hydrolysate would bind to the column [*3*]. The yield of sugar after this fractionation of the hydrolysate from brewery's spent grain was 90 %, 83 % in BSG I and 7 % in BSG II.

The pools BSG I and II were purified further using preparative size-exclusion chromatography to remove monomers and dimers from BSG I and to desalt BSG II. The SEC elution-pattern of the neutral pool BSG I confirmed our results reported previously that the hydrolysate from brewery's spent grain still possess oligosaccharides with a relatively high molecular weight (Mw) [*3*]. Therefore, pool BSG I was fractionated according to size and the oligosaccharides eluted were combined into three pools (BSG IA, BSG IB and BSG IC). BSG

IA contained the highest Mw material and BSG IC the smaller oligomers. Each of the three pools contained about 11 % (w/w) of the applied neutral pool.

All pools obtained by anion-exchange and size-exclusion chromatography of both hydrolysates were analysed for their sugar composition (table 3.1). The composition of the crude hydrolysates are presented as well.

**Table 3.1** Sugar composition (mol %) of the oligosaccharides-pools obtained from hydrothermal treated *Eucalyptus* wood and brewery's spent grain by anion-exchange and size-exclusion chromatography.

| | Total[a] | Molar composition | | | | | | | Ara/ | UA/ | Ac/ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | sugars | Rha | Ara | Xyl | Man | Gal | Glc | UA | Xyl[b] | Xyl[b] | Xyl[b] |
| *Eucalyptus* wood | | | | | | | | | | | |
| Hydrolysate | 58 (8) | 2 | 0 | 79 | 0 | 8 | 0 | 11 | 0 | 0.14 | 0.59 |
| Pools: | | | | | | | | | | | |
| *Euc* NI A | 78 (11) | 1 | 0 | 86 | 5 | 4 | 4 | 0 | 0 | 0 | 0.51 |
| *Euc* AII# | 68 (9) | 0 | 0 | 85 | 0 | 0 | 0 | 15 | 0 | 0.18 | 0.53 |
| *Euc* AIII# | 59 (7) | 1 | 1 | 66 | 0 | 0 | 0 | 32 | 0.02 | 0.48 | 0.60 |
| *Euc* AIV# | 53 (8) | 0 | 0 | 72 | 0 | 5 | 0 | 23 | 0 | 0.32 | 0.67 |
| Brewery's spent grain | | | | | | | | | | | |
| Hydrolysate | 43 (1) | 1 | 17 | 57 | 0 | 4 | 15 | 6 | 0.30 | 0.11 | 0.12 |
| Pools: | | | | | | | | | | | |
| BSG IA | 88 (1) | 0 | 23 | 52 | 0 | 3 | 22 | 0 | 0.44 | 0 | 0.05 |
| BSG IB | 83 (1) | 0 | 14 | 58 | 0 | 2 | 26 | 0 | 0.24 | 0 | 0.07 |
| BSG IC | 72 (3) | 0 | 12 | 68 | 0 | 1 | 19 | 0 | 0.18 | 0 | 0.12 |
| BSG II# | 44 (1) | 0 | 22 | 63 | 0 | 3 | 4 | 8 | 0.35 | 0.13 | 0.10 |

[a] neutral sugars + uronic acids (UA) and between parentheses the total content of *O*-acetyl substituents expressed as weight percentage of each fraction (dm).
[b] ratio mol/ mol

Pool *Euc* NI A consisted mainly of xylose-residues, while also a rather high level of *O*-acetyl substituents was found (Ac/ Xyl = 0.51). Also in the acidic pools of the hydrolysate from *Eucalyptus* wood (*Euc* AII#, AIII# and AIV#) a rather high amount of *O*-acetyl substituents was determined. Furthermore, as expected, these pools contained quite some uronic acids. The uronic acid-residues were present as 4-*O*-methylglucuronic acid side-chains (*vide infra*), as in most (purified) xylans from hard woods [*21-24*].

The neutral oligosaccharides (DP >2) from the hydrolysate from brewery's spent grain (pool IA, IB and IC) were mainly constituted of xylose-, arabinose- and glucose-residues. The glucose-residues most likely originated from starch degradation products as already mentioned previously. Comparing the arabinose to xylose-ratios of the pools BSG IA, IB and

IC it was remarkable that the material eluting first from the size-exclusion column having a relatively high Mw, also had the highest degree of branching as derived from the ratio of Ara/ Xyl (0.44). From these data it is assumed that in the hydrothermal treatment of brewery's spent grain first arabinose was removed prior to a further hydrolysis of these unsubstituted segments.

To study the structural features of the higher Mw material in the four pools from brewery's spent grain in more detail, a purified and well characterised endoxylanase was used [*11,12*]. The degradation was followed by high-performance size-exclusion chromatography (HPSEC) and the elution-patterns are presented in figure 3.1.

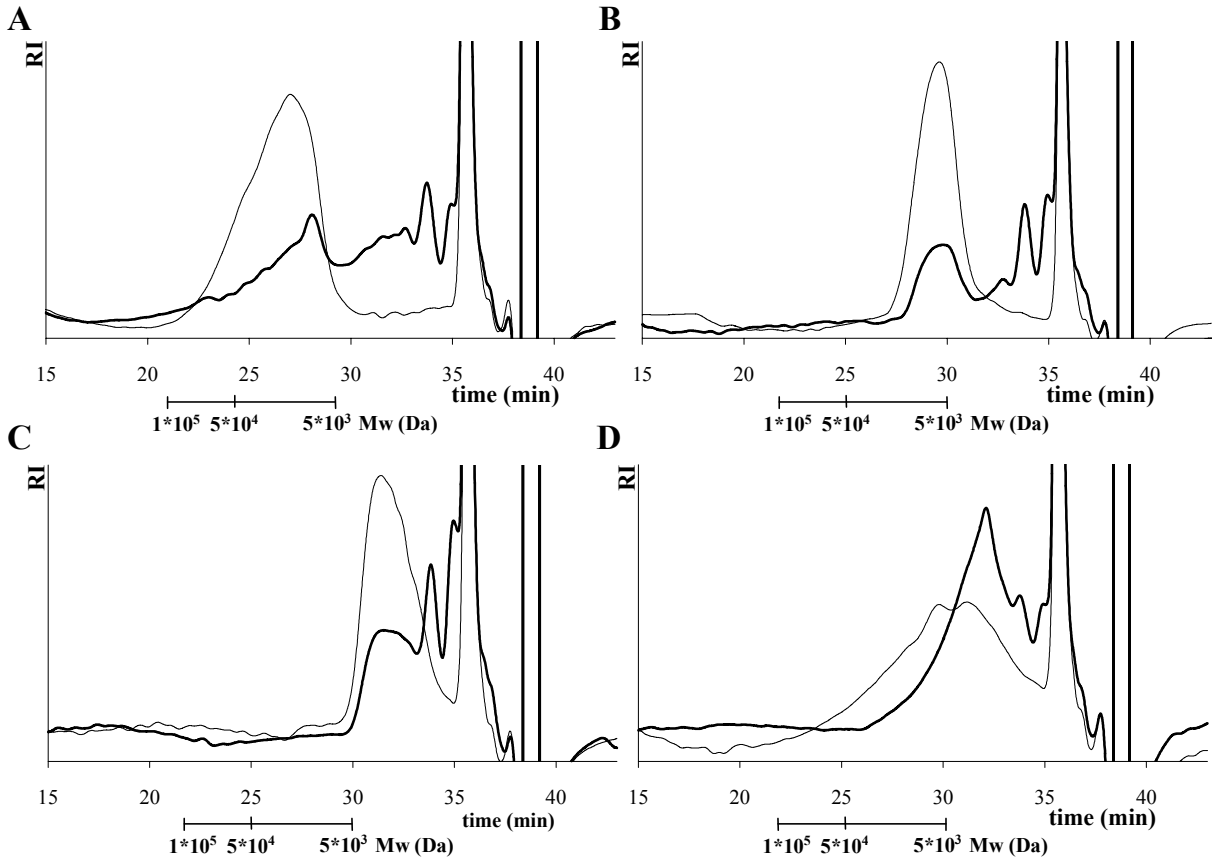


**Figure 3.1** HPSEC elution profiles of the pools BSG IA (A), BSG IB (B), BSG IC (C) and BSG II (D), obtained from the hydrothermal treated brewery's spent grain, before (thin line) and after (bold line) enzymatic degradation by endoxylanase I.

The high Mw material in the pools BSG IA, IB and IC appeared to be degradable quite well by the endoxylanase used. On the other hand, the material present in pool BSG II# was more difficult to degrade, which resulted in only a small decrease of molecular weight. In the latter pool most likely highly branched (glucurono-)(arabino-)xylan-like material was present, being

more difficult to be degraded by the endoxylanase used. The oligosaccharides formed upon degradation of the pools BSG IA, IB and IC by endoxylanase I were further analysed by high-performance anion-exchange chromatography (HPAEC) and compared with the oligosaccharides present before the enzymatic degradation (Fig. 3.2). Apparently, the xylan-like material in pool BSG IA possessed a too high molecular weight to be eluted by HPAEC, while from pool BSG IB and IC some lower Mw material and oligosaccharides were eluted. After endoxylanase-treatment, mainly xylose ($X_1$), xylobiose ($X_2$) and xylotriose ($X_3$) were released in all pools. In pool BSG IC relatively more $X_1$, $X_2$, $X_3$, was released compared to the amount of (branched) oligosaccharides, which were eluted after 10 minutes in the HPAEC elution-pattern. This indicated the presence of less branched material in pool BSG IC compared to pool BSG IB and BSG IA. In the endoxylanase digest of pool BSG IA xylo-oligosaccharides eluted with the same retention time as xylo-oligosaccharides singly or doubly branched with arabinose [*25*]. Therefore, the material originally present in this pool BSG IA was expected to contain more arabinose side-chains than the material in pool BSG IC, as has already been noticed in discussing the sugar composition of these pools.

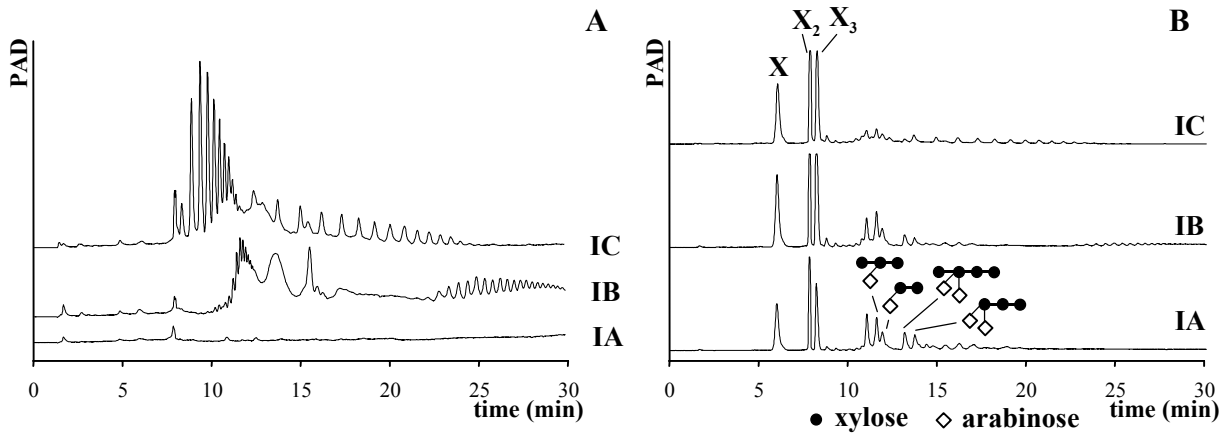


**Figure 3.2** HPAEC elution profiles of the oligosaccharides-pools (IA, IB and IC), obtained from the hydrothermal treated brewery's spent grain, before (A) and after (B) enzymatic degradation (X = xylose).

Further analysis of the oligosaccharides present was performed by MALDI-TOF MS, since this technique has proven to be a powerful method in the analysis of oligosaccharides [*6,26-28*]. The pools BSG IA, BSG IB, BSG IC and BSG II# were all subjected to MALDI-TOF MS. Only for pool BSG IC a clear mass spectrum could be obtained by MALDI-TOF MS (figure not shown), indicating the presence of a homologous series of pentoses (xylose and/ or arabinose (table 3.1)) and hexoses; both ranging from DP 3 - 10.

The pools obtained from the *Eucalyptus* wood hydrolysate were subjected to MALDI-TOF MS as well. The fact that the pools consisted for more than 90 % of xylose, uronic acid

and *O*-acetyl substituents (table 3.1), made the interpretation of the MALDI-TOF mass spectra rather easy.

The mass spectra of the oligosaccharides present in pool *Euc* NI A, before and after removal of alkali-labile esters, are presented in figure 3.3A and 3.3B respectively.

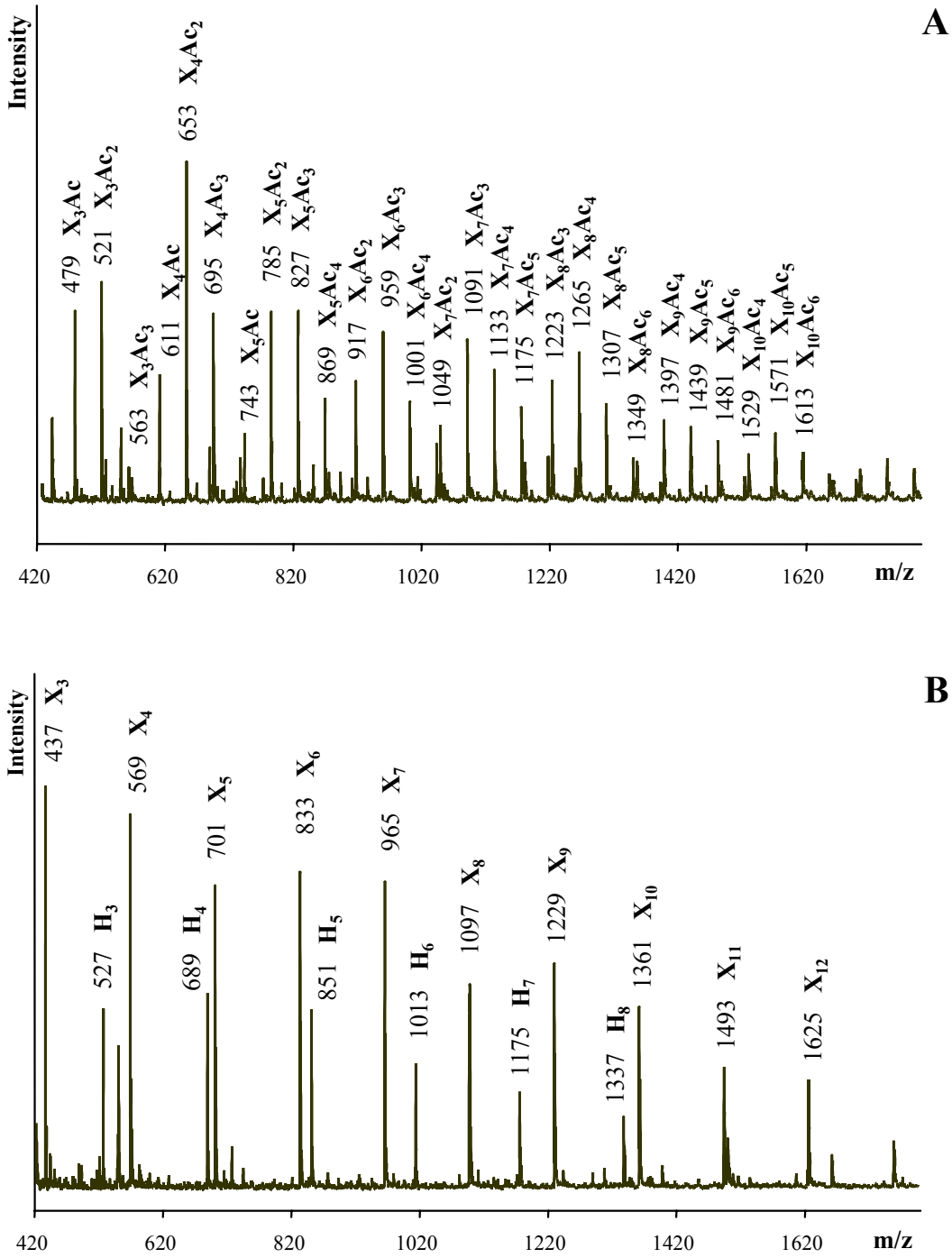


**Figure 3.3** MALDI-TOF mass spectra of the neutral xylo-oligosaccharides (sodium-adducts) obtained from the *Eucalyptus* wood hydrolysate (pool *Euc* NI A), before (A) and after (B) saponification (X = xylose; Ac = *O*-acetyl substituent; H = hexose).

In the *Eucalyptus* pool NI A a large variety of *O*-acetylated xylo-oligosaccharides were present [$X_nAc_m$]. The corresponding series of xylo-oligosaccharides without *O*-acetyl substituents [$X_n$] remained after saponification (Fig. 3.3B). Subsequently, to confirm that the oligomers were built from xylose residues only, endoxylanase I was used to hydrolyse the (saponified) xylo-oligosaccharides. The masses of the series of xylo-oligosaccharides (not *O*-acetylated) disappeared, leaving only the masses of $X_2$, $X_3$ and hexose-oligomers, which confirmed the structures proposed in Fig. 3.3B.

The MALDI-TOF mass spectra of the oligosaccharides present in the 'acidic' pool *Euc* AII#, which were saponified first, are shown in figure 3.4A. In this pool a series of xylo-oligosaccharides branched with one 4-*O*-methylglucuronic acid [$X_n(GlcA_{me})_1$] was detected, in accordance with the sugar composition of this pool (table 3.1). Additionally, in figure 3.4A traces of a series of xylo-oligosaccharides containing both one 4-*O*-methylglucuronic acid and one hexose (H) were observed, most likely corresponding with the 4-*O*-methyl-α-D-glucuronic acid substituted at *O*-2 with α-D-galactose as described by Shatalov et al. [*29*]. This assumption was confirmed by our results obtained in dynamic nanospray MS. In the $MS^2$ spectra of the xylo-oligosaccharides most likely containing both a 4-*O*-methylglucuronic acid and a hexose residue, indeed fragments were observed of these oligosaccharides minus the mass of a hexose residue (162 Da). Subsequently, in the $MS^3$ spectra of the fragments minus the hexose residue fragments were released lacking the mass of a 4-*O*-methylglucuronic acid residue (190 Da). Furthermore, no evidence was found for the presence of two glucuronic acid residues (mass 176 Da each), which would also account for the masses of the series of $X_n(GlcA_{me})_1(H_1)$. The presence of galactoses could not be seen from table 3.1 since the amount of galactoses present is most likely below the detection level of the sugar analysis method used. The same structures as described for the saponified pool were detected in the MALDI-TOF mass spectrum of the non-saponified pool *Euc* AII#, with this difference that each structure was present having one or more *O*-acetyl substituents in addition [$X_n(GlcA_{me})_1Ac_m(H_1)$].

In pool *Euc* AIII#, which was more strongly bound to the anion-exchange column, xylo-oligosaccharides were detected having the same structural features as the oligosaccharides present in pool *Euc* AII#, but of a lower DP (DP 2-4 xyloses). The xylo-oligosaccharides all contained one 4-*O*-methylglucuronic acid or a 4-*O*-methylglucuronic acid plus a hexose, and one or more *O*-acetyl substituent(s) (results not shown). Again, the presence of both a 4-*O*-methylglucuronic acid and a hexose, most likely galactose (*vide infra*), was confirmed by nanospray tandem MS. Thus, in this pool the charge-density per oligomer was rather high, explaining why these oligomers were more strongly bound to the column. The higher charge-density also was reflected in the ratio of uronic acid to xylose (table 3.1).

Pool Euc AIV#, which was bound most strongly to the anion-exchange column, was analysed by MALDI-TOF MS as well (Fig. 3.4B). It was shown that after saponification a

series of xylo-oligosaccharides substituted with *two* 4-*O*-methylglucuronic acid residues [$X_n(GlcA_{me})_2$] was present.

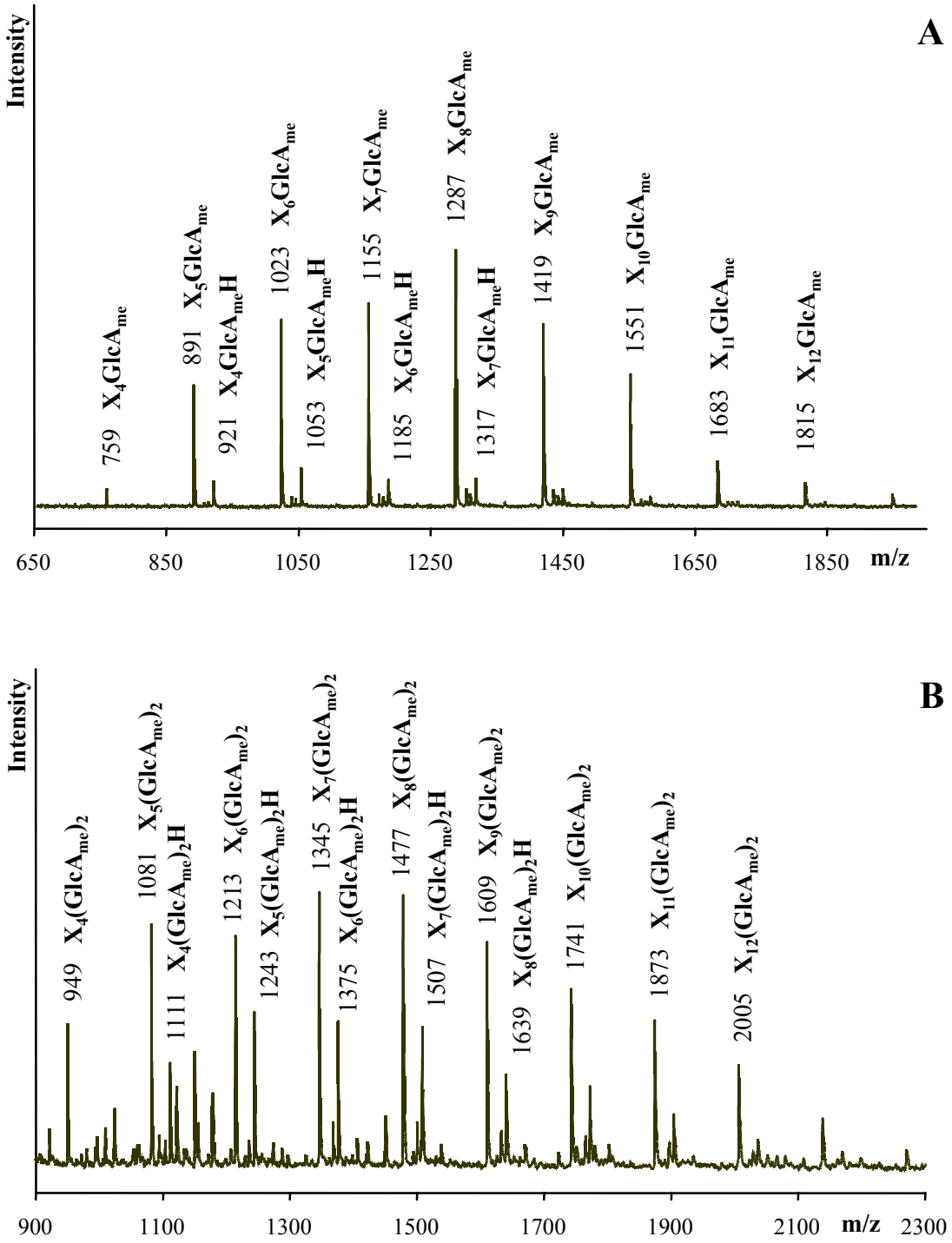


**Figure 3.4** MALDI-TOF mass spectra of the acidic xylo-oligosaccharides (sodium-adducts) obtained from the *Eucalyptus* wood hydrolysate, pool *Euc* AII# (A) and *Euc* AIV# (B), both saponified (X = xylose; Ac = *O*-acetyl substituent; H = hexose; $GlcA_{me}$ = 4-*O*-methylglucuronic acid).

Additionally, a series of similar oligosaccharides having an additional hexose-residue, most likely galactose (*vide infra*), attached was detected in the mass spectrum (Fig. 3.4B). Again, the same structures as described to be present in the saponified pool were detected in the MALDI-TOF mass spectrum of the oligosaccharides present in the non-saponified pool *Euc* AIV#, having one or more *O*-acetyl substituent(s) in addition [$X_n(GlcA_{me})_2Ac_m(H_1)$].

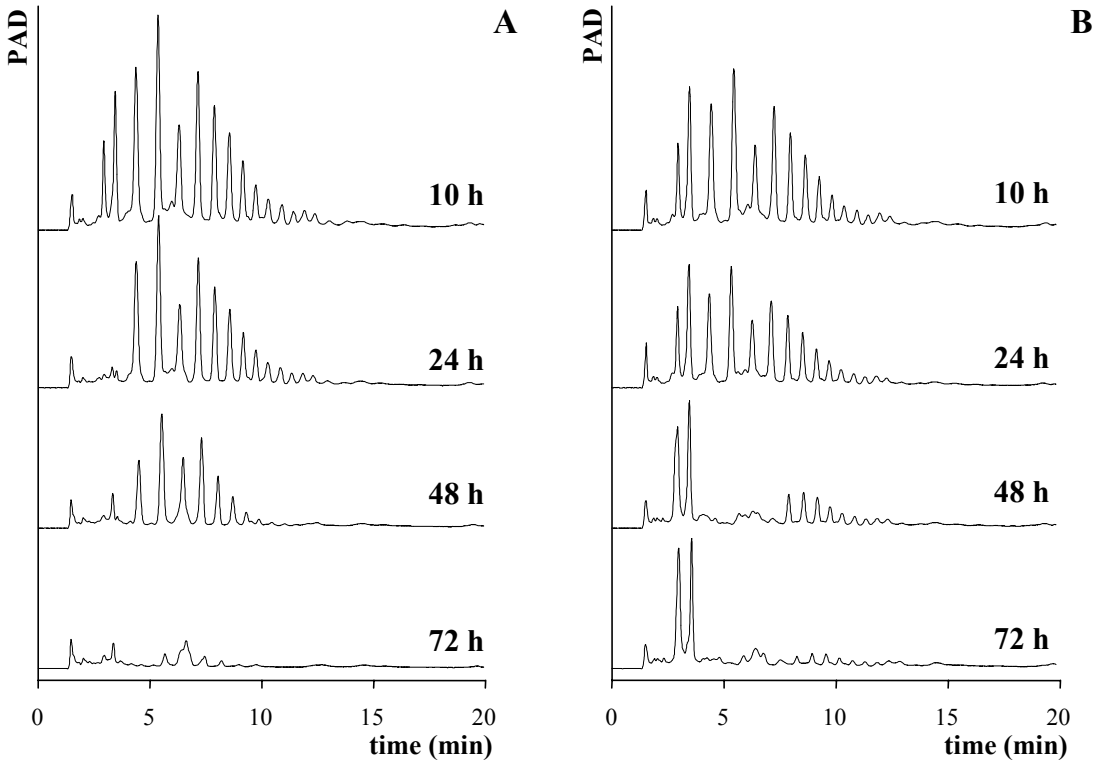


**Figure 3.5** HPAEC elution profiles of *O*-acetylated xylo-oligosaccharides (A) and non-substituted xylo-oligosaccharides (B) during fermentation (72 hours) by a human faecal sample.

In conclusion, separation of the hydrolysates from two hydrothermal treated by-products resulted in several pools of xylo-oligosaccharides differing in the degree and type of branching. Obviously, still a mixture of oligosaccharides was present in each pool to be separated further when complete structural elucidation is needed. Further structural characterisation of these oligosaccharides will be helpful in studying and improving the mechanism of hydrothermal treatment in the release of xylose and xylo-oligosaccharides.

The neutral *O*-acetylated xylo-oligosaccharides from the *Eucalyptus* hydrolysate (*Euc* NI A) have already been subjected to reversed phase chromatography or a TSK-gel amide-column. The *O*-acetylated xylo-oligosaccharides were rather well separated based on the number of *O*-acetyl substituents per oligomer [*30*].

Furthermore, the pools containing xylo-oligosaccharides with various side-chains are very suitable to be used to study the structure-depending effect of xylo-oligosaccharides in

several biological activity tests and during fermentation by the human intestinal flora. An example of the fermentation by a human faecal sample of *O*-acetylated xylo-oligosaccharides (*Euc* NI A) and non-substituted xylo-oligosaccharides (saponified *Euc* NI A) is presented in figure 3.5A and 3.5B respectively. This figure shows the pattern of oligosaccharide digestion analysed by HPAEC and illustrates the potential of the human intestinal flora to ferment (*O*-acetylated) xylo-oligosaccharides *in vitro*. Also, it was demonstrated that non-substituted xylo-oligosaccharides were fermented divergently as compared to the *O*-acetylated ones. The complete results of the fermentation studies will be published in the near future.

**Acknowledgement**

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# CHAPTER 4

# Mass determination of oligosaccharides by matrix assisted laser desorption/ ionization time-of-flight mass spectrometry following HPLC, assisted by on-line desalting and automated sample handling


**Abstract**

The off-line coupling of analytical high-performance anion-exchange chromatography (HPAEC) to matrix assisted laser desorption/ ionization time-of-flight mass spectrometry (MALDI-TOF MS) is described. The system was applied to the analysis of neutral and acidic xylo-oligosaccharides. For MALDI-TOF MS on-line desalting of the HPAEC eluent was performed using an anion self regenerating suppressor (ASRS) in series with a cation self regenerating suppressor (CSRS). The ASRS permitted the exchange of acetate ions with hydroxide ions while the CSRS permitted the exchange of sodium ions with hydronium ions. The continue desalting of the eluent was achieved by the electrolysis of pure water in both suppressors. Following, automated fractionation after HPAEC separation using a 96-well plate fraction collector and computer controlled MALDI-TOF MS sample preparation using a robot are applied as well. The complete process from HPAEC separation at analytical scale to MALDI-TOF MS could be performed most conveniently, giving molecular mass information overcoming the rather unpredictable HPAEC elution behavior of (unknown) oligosaccharides.



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## 4.1 Introduction

A relatively new area of research focuses on the functional properties of oligosaccharides. Some of the qualities attributed to such oligosaccharides are protection against colon cancer bacteria [*1,2*], anti-infective properties [*3*] and stimulation of the growth of beneficial gut bacteria [*1,2*]. Furthermore, the identification of oligosaccharides formed upon enzymatic degradation of polysaccharides has proven to be helpful to elucidate the fine structure of the corresponding polymer [*4*].

HPAEC with pulsed amperometric detection (PAD) is a widely used method for the separation of a variety of oligosaccharides [*5*]. However, since the elution behaviour of the various types of oligosaccharides is rather unpredictable and oligomer standards are frequently not available, the identification of complex oligosaccharide mixtures is difficult [*5*]. Consequently, subsequent characterization by using MS or NMR is required in order to reveal the exact structures of the separated oligosaccharides. Nevertheless, for both techniques usually a labour-intensive sample preparation including a desalting step is needed [*6,7*]. Another disadvantage for NMR analysis is that relatively large amounts (0.5-1 milligrams) of purified material are required [*6*].

From literature it is known that the on-line coupling of HPAEC to MS, using thermospray or electrospray (ES), is successful in the analysis of oligosaccharides [*8,9*]. Nevertheless, the feasibility of a permanent on-line application is low due to the extended experimental set-up. Also, specific knowledge and experience is required to obtain usable mass spectra. Daas et al. [*6*] showed that off-line HPAEC on preparative scale combined with the relatively simply operable MALDI-TOF MS appeared to be quite useful to determine the molecular masses of oligosaccharides in e.g. an enzyme digest. However, due to the high pH of the mobile phase used for HPAEC (pH ≈ 12) oligomers will not be stable for a long time and because of that the pH of the eluent should be neutralised directly after separation. Additionally, large amounts of sodium acetate, which are present in the mobile phase of HPAEC, decrease the signal on the MALDI-TOF mass spectrometer enormously, making desalting of the mobile phase necessary.

On-line membrane suppressors which can remove the sodium from the mobile phase before analysis with thermospray MS [*8,10,11*], or before analysis with MALDI-TOF MS [*12*] have already been described to be successful. But, in all these studies large amounts of regenerating acids (e.g. $H_2SO_4$) were used, which may cause artefacts [*8,11*]. Furthermore, in these applications only the sodium is removed from the mobile phase of HPAEC, leaving the oligosaccharides in acetic acid. This acetic acid disturbs the crystallisation of the samples for MALDI-TOF analysis. In an other study, an on-line microdialysis membrane system was used in combination with ESMS. However, this system was only capable of desalting the eluent within a pH-range between 4 and 11 at a flow of 10-20 µl/min. Also, only oligomers with a mass above 1000 Da remained for further analysis [*13*]. Off-line desalting of preparative HPAEC separated oligosaccharides to perform both ESMS and MALDI-TOF MS was

achieved by acid-catalysed per-*O*-acetylation of the oligosaccharides [*7*]. For sequential analysis a less time-consuming and less laborious sample preparation for MALDI-TOF analysis will be convenient.

In this paper we describe a rapid method for the on-line desalting of HPAEC eluent at analytical scale with the use of a cation membrane suppressor in series connected to an anion membrane suppressor. Subsequent analysis of the separated and on-line desalted oligosaccharides by using MALDI-TOF MS after automated MS sample preparation is described as well.

## 4.2 Experimental

### *4.2.1 Oligosaccharide mixtures*

Xylo-oligosaccharide-mixtures were prepared by hydrothermal treatment of *Eucalyptus* wood (17 minutes; 175 °C) at the Department of Chemical Engineering of the University of Vigo (Spain). The xylo-oligosaccharides (20 mg) were dissolved in 1.2 ml of 0.05 M NaOH and saponified overnight at 4 °C. The mixture was neutralised with 0.6 ml of 0.1 M acetic acid and the final buffer concentration was set to 50 mM NaOAc pH 5 with 0.2 ml of 50 mM NaOAc pH 5. The sugar composition of the mixture was determined as described by Verbruggen et al. [*14*].

### *4.2.2 On-line desalting following HPAEC*

HPAEC was performed on a Dionex system equiped with a CarboPac PA-1 column (250 x 4 mm) in combination with a CarboPac PA guard column (25 x 3 mm) and PAD-detection [*5*]. Elution (1 ml/min) was performed with a combination of linear gradients of 50-90 mM sodium acetate in 100 mM NaOH during 0-5 min, 90-130 mM sodium acetate in 100 mM NaOH during 10 min, followed by 15-min linear gradient to 520 mM sodium acetate in 100 mM NaOH [*15*]. A hundred µl of the saponified sample (15 mg/ml) was injected and separated on the column.

After the detector two desalting units (Dionex) were connected in series (Fig. 4.1). The ultra self regenerating anion suppressor 4 mm-unit (ASRS) was connected first to exchange the sodium ions for hydronium ions ($H_3O^+$). Next, the ultra self regenerating cation suppressor 4 mm-unit (CSRS) was installed to exchange the acetate ions for hydroxide ions ($OH^-$). The continues desalting of the eluent was achieved by the electrolysis of deionized water (8 ml/ min) in both suppressors. Fractions (120 µl) were collected in a 96-well plate, using a Gilson FC-203B fraction collector.

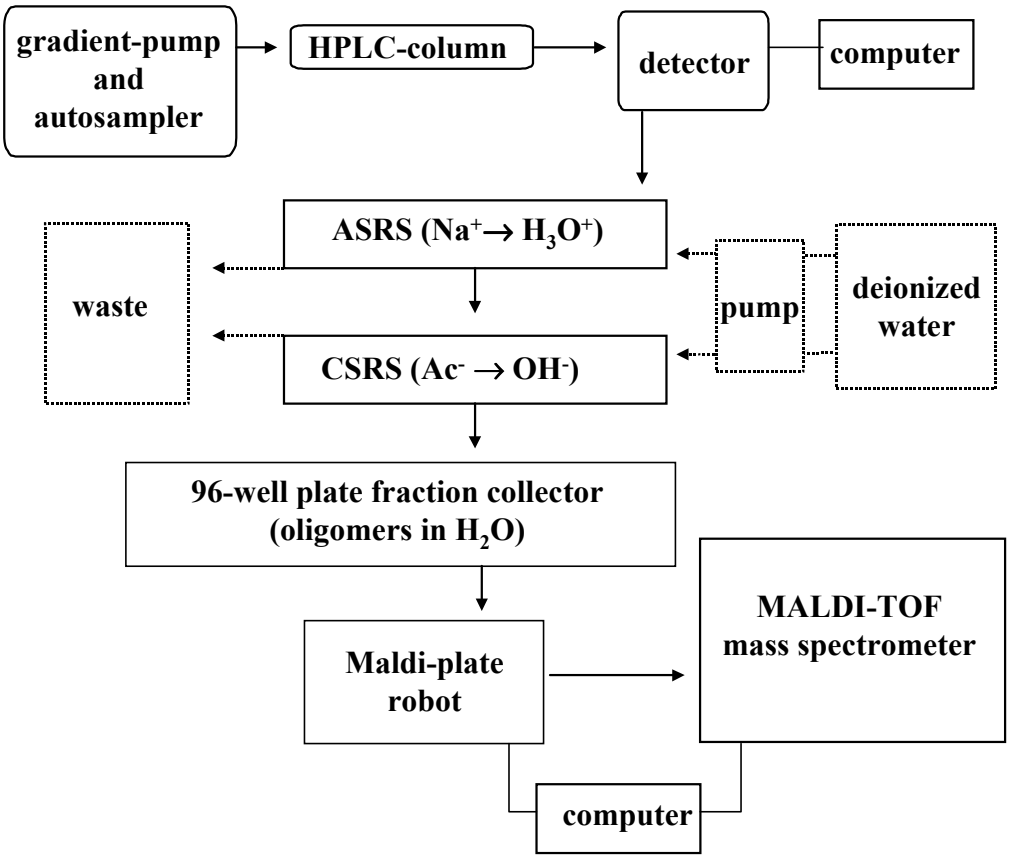


**Figure 4.1** Schematic overview of the experimental setup for automated coupling of HPAEC to MALDI-TOF MS.

### *4.2.3 MALDI-TOF mass spectrometry*

For MALDI-TOF MS analysis, 1 µl of each fraction was automatically transferred from the 96-well-plate to a MALDI-sample-plate and mixed with 1 µl of matrix by using a Symbiot-I robot (PerSeptive Biosystems) and was allowed to dry at room temperature. The matrix solution was prepared by dissolving 9 mg of 2,5-dihydroxybenzoic acid and 3 mg 1-hydroxyisoquinoline in a 1-ml mixture of acetonitrile:water (300 µl:700 µl). MALDI-TOF mass spectra were obtained from all fractions using a Voyager-DE RP workstation (PerSeptive Biosystems), operated as described by Daas et al. [*16*]. The mass spectrometer was calibrated with a mixture of maltodextrines.

## 4.3 Results and discussion

The elution profile of the HPAEC separation of an *Eucalyptus* wood hydrolysate, prepared by hydrothermal treatment, is shown in figure 4.2.

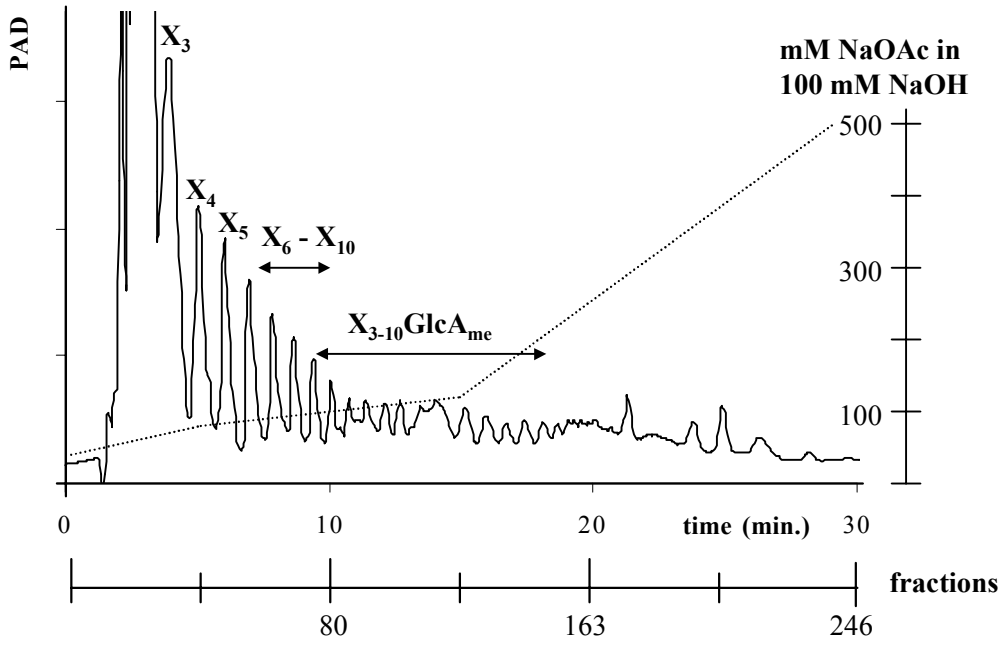


**Figure 4.2** HPAEC elution profile of a xylo-oligosaccharide mixture obtained after hydrothermal treatment of *Eucalyptus* wood, including the used HPAEC gradient and fractionation (X = xylose; $GlcA_{me}$ = 4-*O*-methylglucuronic acid).

The sugar composition of the mixture showed that the oligomers in the mixture mainly consist of xylose and uronic acid (xylose 71 mol%; uronic acid 12 mol%), while hardly any arabinose is present (3 mol%). Van der Hoeven et al. [*10*] described that β-1,4-xylo-oligomers up to DP 10 elute within 10 minutes from the column at the gradient used. Therefore, the first 10 peaks were expected to be xylo-oligomers up to DP 10. Nevertheless, the chromatogram (Fig. 4.2) presents a complex pattern and further characterization was needed to define the separated oligosaccharides. Furthermore, since xylan from *Eucalyptus* wood is reported to be branched with 4-*O*-methylglucuronic acid, xylo-oligosaccharides containing a glucuronic acid are expected to be present in the hydrolysate after hydrothermal treatment [*17*]. While these acidic oligomers all elute after the neutral decamer of β-1,4-xylose residues [*18*], identification of the various components on basis of their elution behaviour was impossible (Fig. 4.2). Subsequent MALDI-TOF MS is performed to confirm the presence and elution time of the expected oligomers.

To perform MALDI-TOF MS, without being hindered by huge amounts of salts, the HPAEC eluent was desalted on-line by using two suppressors in series. The ASRS permitted the exchange of sodium ions for $H_3O^+$ and the CSRS the exchange of acetate ions for $OH^-$. The conductivity of the eluent was measured after collecting 5ml-fractions. Before and after desalting the conductivity was 20 mS and 0.07 mS (0-5 min); 180 mS and 0.06 mS (5-10 min); 190 mS and 0.08 mS (10-15 min); 200 mS and 0.05 mS (15-20 min); 230 mS and 0.52

mS (20-25 min); 260 mS and 6 mS (25-30 min) respectively. These numbers illustrate that the combined use of an ASRS and a CSRS resulted in an almost complete desalting of the eluent, leaving the separated oligosaccharides in almost pure water. However, the concentration of sodium-ions in the desalted eluent was still high enough to form sodium adducts of the oligosaccharides after crystallisation, which is necessary for detection in MALDI-TOF analysis. To allow good performance of MALDI-TOF MS the desalting was sufficient up to fraction 170 (~ 21 min), which corresponds with an eluent concentration of 300 mM NaOAc in 100 mM NaOH. Similar results have been reported by Hoeven et al. [*10*]. They describe that by using two anion membrane suppressors in series the sodium ions could be removed sufficiently to allow thermospray MS detection as long as the sodium concentration in the HPAEC eluent did not exceed 0.4 mol/l [*10*]. The pH of all fractions up to fraction 170 (~ 21 min) had a value between 6 and 7. Only when the ASRS power supply was set at 500 mA and the CSRS power supply at 300 mA the pH of the remaining eluent was kept neutral. The continue desalting of the eluent was achieved by the electrolysis of deionized water in both suppressors. Large amounts of regenerating acids, used in most desalting units described in literature, were avoided in this way [*8,11*].

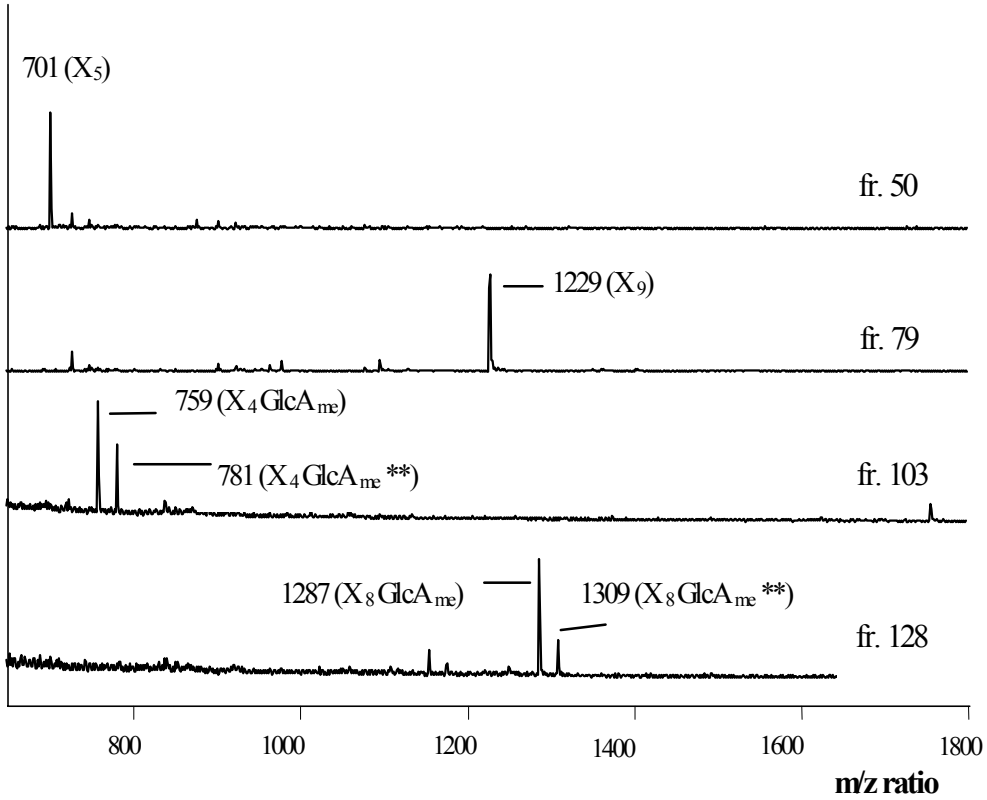


**Figure 4.3** MALDI-TOF mass spectra of four fractions separated by HPAEC of a xylo-oligosaccharide mixture obtained after hydrothermal treatment of *Eucalyptus* wood (see Fig. 4.2).
** indicates the acidic oligomers including two sodium ions.

The separated and desalted fractions were collected on-line in three 96-well plates. Automatical transfer of the fractions from the 96-well plates to MALDI-sample-plates was performed by using a Symbiot-I robot. This process is fully automated and the three plates were prepared for MALDI-TOF analysis within one hour. MALDI-TOF mass spectra were collected for all fractions. Examples of such spectra are shown in figure 4.3. The first ten compounds which were eluted from the column were separated well, giving unambiguous mass spectra. Indeed, the mass spectra confirmed that these compounds were xylose-oligomers with a DP from 3 up to 10. After an elution time of 10 minutes (fraction 80) the mechanism of separation became less distinct, since more homologous series are eluted with decreasing resolution. This resulted in the detection of two or more masses per fraction. However, a series of xylo-oligomers containing one 4-*O*-methyl-glucuronic acid group (DP 2 up to 9) could be clearly distinguished. Spectra of fraction 103 and 128 (Fig. 4.3) show the presence of the acidic xylo-oligosaccharides, represented as single sodium adducts. In addition to the strong signal of the single sodium adduct of the acidic oligomers (e.g. mass 759) also a weaker signal of oligomers including two sodium ions were detected (Fig. 4.3). According to their masses these ions were not doubly charged ions, but single sodium adducts including a second sodium minus a mass of 1. It is anticipated that a hydronium ion from the acid group is exchanged with a sodium and a mass of the sugar plus two sodium ions minus a hydronium occurred.

After separation of the oligomers in the mixture good signals were detected of masses which were less abundantly present and were not recognised in the mass spectrum of the initial mixture (results not shown). This is most likely due to both the higher background in the mass spectrum of the initial mixture and the fact that if the number of components per sample decreases, the laser energy per component increases and therefore also the intensity of the detected mass [*19*].

At this moment the quantification of the oligomers present is still difficult, since complex oligomer standards are hardly available and quantification with MALDI-TOF MS is not possible yet [*19*]. Also, mono- and di-saccharides are not detectable, because MALDI-TOF MS only provides masses above 400 Da, as a result of interference with the matrix.

Because fractions are collected in a 96-well plate the oligosaccharides remain for MALDI-TOF analysis as well as for other MS analysis. Even MS/MS, to characterise the separated oligosaccharides in more detail, could be performed much easier compared to on-line LC/MS analysis because analysis could be performed per fraction. Another possibility to characterise the separated oligomers in more detail is post-source decay fragmentation, observed on a MALDI-TOF apparatus. Molecular masses of fragments, caused by glycosidic cleavages between the monomer residues, can be analysed to obtain information on the original chemical structure of the oligosaccharides [*20*].

Instead of HPAEC at high pH, other HPLC-methods and separation of various (non-sugar) components (e.g. peptides) could be used very easily. Separation of charged

galacturonic acid oligomers already has been carried out in our department using HPAEC at pH 5 [*6,16*]. An advantage of HPAEC at pH 5 instead of at pH 12 is that *O*-acetyl- or methyl-groups will not be removed by the eluent. Coupling to MALDI-TOF MS according to the method described provide data about the number of ester-groups linked to the separated oligomers. The results obtained were promising, although the level of current supply of the CSRS and ASRS to desalt eluent with different compositions may differ for optimal results. Even when elution is performed using only water and/ or organic modifiers (e.g. methanol and acetonitrile), of which no desalting is required, the described, automated method provides a rapid alternative for the characterization of fractionated oligosaccharides.

The HPAEC separation, on-line desalting, automated preparation of the MALDI sample-plates and collecting of the MALDI-TOF mass spectra could all be performed without much effort, allowing a convenient first characterization of all oligomers in a complex mixture. Moreover, only a minimum of sample is required before analysis because the method used is based on separation and identification at an analytical scale.

**Acknowledgement**

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# CHAPTER 5

# Location of *O*-acetyl substituents in xylo-oligosaccharides obtained from hydrothermally treated *Eucalyptus* wood


**Abstract**

A combination of techniques was used to localise the *O*-acetyl substituents in xylo-oligosaccharides, which are present in hydrolysates of hydrothermally treated *Eucalyptus* wood. Reversed phase (RP)-high performance liquid chromatography (HPLC) coupled on-line to both a mass spectrometer and an evaporating light scattering (ELS) detector provided data about the order of elution of the various *O*-acetylated oligomers. The retention of the oligomers on the column depend on the number *and* position of the *O*-acetyl substituents within the xylo-oligosaccharides. One dimensional (1D)- and two dimensional (2D)-$^{1}$H NMR spectroscopy was used to study the structural features of several xylotetramers separated by RP-HPLC, each having one *O*-acetyl substituent. However, for each 'pure' *O*-acetylated xylotetramer a mixture of 2-*O*- and 3-*O*-acetyl substituted xylotetramers was found. *O*-acetyl migration was proven to have occurred in these xylo-oligosaccharides. Mainly *O*-acetyl migration within the same xylosyl residue was observed. An equilibrium was established between the 2-*O*- and 3-*O*-acetyl substituted xylo-oligomers in favour of the 3-*O*-acetylated compound, regardless of the original position (2-*O*- or 3-*O*-) of the *O*-acetyl substituent. RP-HPLC-NMR was performed in order to study the structural features of the acetylated oligomers 'on-line' avoiding *O*-acetyl migration. Finally, the precise location of the 2-*O*- or 3-*O*-acetyl substituent in 6 xylotetramers and 4 xylotrimers separated by RP-HPLC was determined.



*This chapter has been submitted to Carbohydr. Res. by the authors M.A. Kabel, P. de Waard H.A. Schols and A.G.J. Voragen.*

## 5.1 Introduction

The wood of *Eucalyptus* is rich in cellulose and hemicellulose, of which the main hemicellulose present is an acetylated 4-*O*-methylglucuronoxylan [*1*]. Hydrothermal treatment of *Eucalyptus* wood results in a selective fractionation of the hemicellulose and cellulose, which can be used for different product applications [*2,3*].

Recently, we described the characterisation of hydrolysates obtained after hydrothermal treatment of *Eucalyptus* wood. More than 60 % (w/w) of the sugars present in these hydrolysates consists of (*O*-acetyl-)(4-*O*-methylglucurono-) xylo-oligosaccharides [*4,5*]. Up to now the precise location of the *O*-acetyl substituents within the xylo-oligosaccharides (XOS) present remained unassigned.

The positions of the *O*-acetyl substituents can be studied with the help of linkage analysis [*6-8*]. However, more rapid and convenient is the use of NMR spectroscopy. York *et al.* have shown that by using a combination of analytical methods, including mass spectrometry (MS) and NMR spectroscopy, the location of the *O*-acetyl substituents on a nonasaccharide repeating unit of xyloglucan can be determined [*9*]. Several other studies show the presence of *O*-acetyl substituents on xylans or xylan-fragments by directly employing NMR analysis, leaving the exact distribution of the *O*-acetyl substituents over the xylosyl residues out of consideration [*7,10-12*].

To be able to assign the precise location of the *O*-acetyl substituents in each of the XOS present in the *Eucalyptus* hydrolysate by using NMR, the oligomers have to be separated first. In recent literature, the use of reversed phase (RP) chromatography is described: e.g. Pauly *et al.* describe a method to separate acetylated xyloglucan oligosaccharides by RP chromatography [*13*]. Other publications describe the use of RP chromatography in for example the separation of a lichenase digest of β-(1-3,(1-4)-D-glucans [*14*], maltodextrins [*15,16*] or even α-dextrins with the same degree of polymerisation (DP) but differing in the location of the branch point [*17*]. An additional advantage of using RP is that the on-line coupling with MS is easily to perform. This has already been reported to be a good tool in the identification of oligosaccharides [*18-20*].

In this paper we describe the separation and structural features of several acetylated xylo-oligosaccharides obtained from hydrothermally treated *Eucalyptus* wood by using RP-HPLC-$MS^{(n)}$, NMR and RP-HPLC-NMR.

## 5.2 Experimental

### *5.2.1 Acetylated xylo-oligosaccharides*

The neutral acetylated XOS (AcXOS) were obtained as described previously [*4*].

### *5.2.2 Size exclusion chromatography*

To fractionate the AcXOS-mixture (163 mg in 2 ml) a Pharmacia system equipped with a BioGel P-2 column (900 x 26 mm; 200-400 mesh, Bio-Rad Laboratories) thermostated at 60 °C was used. Elution was performed at 0.5 ml/min with water (60 °C) and fractions were collected every 2 ml. The eluent was detected by a Shodex RI-72 detector. Four of these size-exclusion runs were performed to obtain sufficient material of pool $X_4Ac$.

### *5.2.3 MALDI-TOF mass spectrometry*

Matrix assisted laser desorption/ ionisation time-of-flight mass spectrometry (MALDI-TOF MS) was performed as described before [*4*].

### *5.2.4 Reversed phase HPLC - mass spectrometry*

Reversed phase HPLC was performed on a HPLC system (Thermo Separation Products) equipped with a Thermohypersil Aquasil C18 column ((Keystone Scientific); 4.6 mm ID x 150 mm; 3µm) in combination with a Thermohypersil Aquasil C18 guard column (4.6 mm ID x 10mm; 3µm). The RP-column was coupled to a splitter (Dionex) directing 10 % of the eluens to a Sedex 55 evaporating light scattering detector (Sedere), 5 % to a LCQ Ion-trap (Finnigan MAT 95) and the remaining part to a fraction collector or waste. Elution (0.8 ml/min) was performed using a gradient of degassed (Membrane degasser; Thermo Separation Products) methanol in pure water: 0-28 v/v% methanol in 0-70 min. Each elution was followed by a equilibration step (isocratic pure water; 30 min). Pool $X_4Ac$ (20 mg/ml) was subjected to RP chromatography separated in 20 RP-HPLC runs (20 times 100 µl of injection) to obtain enough material of each peak to perform NMR analysis. Fractions (276 µl) were collected by using a fraction collector in the same series of tubes for 5 runs. Finally, all tubes containing similar material were combined.

MS analysis was carried out in the positive mode using a spray voltage of 5.5 kV and a capillary temperature of 200 $^{o}$C. The capillary voltage was set at 42 kV and the tube lens voltage at 20 kV. $MS^n$ was performed using a window of 1.5-2 m/z and a 35–40% relative collision energy. The apparatus and the data were controlled by Xcalibur software. The accuracy of the mass determinations is ±0.3 Da.

### *5.2.5 NMR spectroscopy*

Prior to NMR analysis, the samples were exchanged in $D_2O$ (99.9 atom% D, Cambridge Isotope Laboratories) with intermediate freeze drying. Deuterated samples were dissolved in 190 µl 99.96% $D_2O$ (Cambridge Isotope Laboratories) and inserted in NMR microtubes (Shigemi). NMR spectra were recorded at a probe temperature of 25°C on a Bruker AMX-500 spectrometer located at the Wageningen NMR Centre. $^1$H chemical shifts are expressed in

ppm relative to internal acetone (δ 2.225). 1D $^{1}$H NMR spectra were recorded at 500.13 MHz using 64 scans of 8192 data points and a sweep width of 3000 Hz. The 2D COSY spectra were acquired using the double quantum filtered (DQF) method with a standard pulse sequence delivered by Bruker. The 2D TOCSY spectra were acquired using standard Bruker pulse sequences with a mixing time of 100 ms. For these 2D $^{1}$H spectra 512 experiments of 2048 data points were recorded using 32 scans per increment.

### *5.2.6 Reversed phase HPLC - NMR*

Reversed phase HPLC-NMR was performed on a HPLC system (Bruker) equipped with a Thermohypersil Aquasil C18 column (4.6 mm ID x 150 mm; 3µm) in combination with a Thermohypersil (Keystone Scientific) Aquasil C18 guard column (4.6 mm ID x 10mm; 3µm). Furthermore, the HPLC system was connected to a Bruker peak sampling unit (BPSU; Bruker) following UV detection (205-215 nm; Bruker). Elution (0.8 ml/min) was performed using a gradient of degassed (Membrane degasser; Bruker) methanol in $D_2O$: 0-16 v/v% methanol in 0-40 min. Samples containing mainly $X_4Ac$ (~10 mg/ml) or $X_3Ac$ (~15 mg/ml), obtained by size exclusion chromatography, were applied to RP-HPLC-NMR (100 µl/run). Each elution was followed by a equilibration step (isocratic $D_2O$; 30 min). Samples were transported one by one from the BPSU to the NMR probe, using the same ratio of $D_2O$/ methanol as was necessary for the particular oligomers to elute from the RP-column.
NMR spectra were recorded at a probe temperature of 25°C on a Bruker DPX-400 spectrometer located at the Wageningen NMR Centre. Solvent signals were suppressed with double presaturation using Bruker LC-NMR software. $^{1}$H chemical shifts are expressed in ppm relative to methanol (δ 3.34). 1D $^{1}$H NMR spectra were recorded at 400 MHz using up to 2000 scans of 32768 data points and a sweep width of 8000 Hz. The 2D TOCSY spectrum was acquired using a standard Bruker pulse sequence. 496 experiments of 2048 data points were recorded using 240 scans per increment resulting in a measuring time of 66 hours. The mixing time was 65 ms.

## 5.3 Results and discussion

### *5.3.1 RP-HPLC-MS of acetylated xylo-oligosaccharides*

To be able to study the location of the *O*-acetyl substituents in neutral acetylated xylo-oligosaccharides (AcXOS) obtained from hydrothermally treated *Eucalyptus* wood, the AcXOS-mixture was subjected to RP-high performance liquid chromatography (HPLC). The order in which the oligosaccharides present eluted was rather easily to determine by connecting the RP-column to both an evaporating light scattering (ELS) detector and a mass spectrometer.

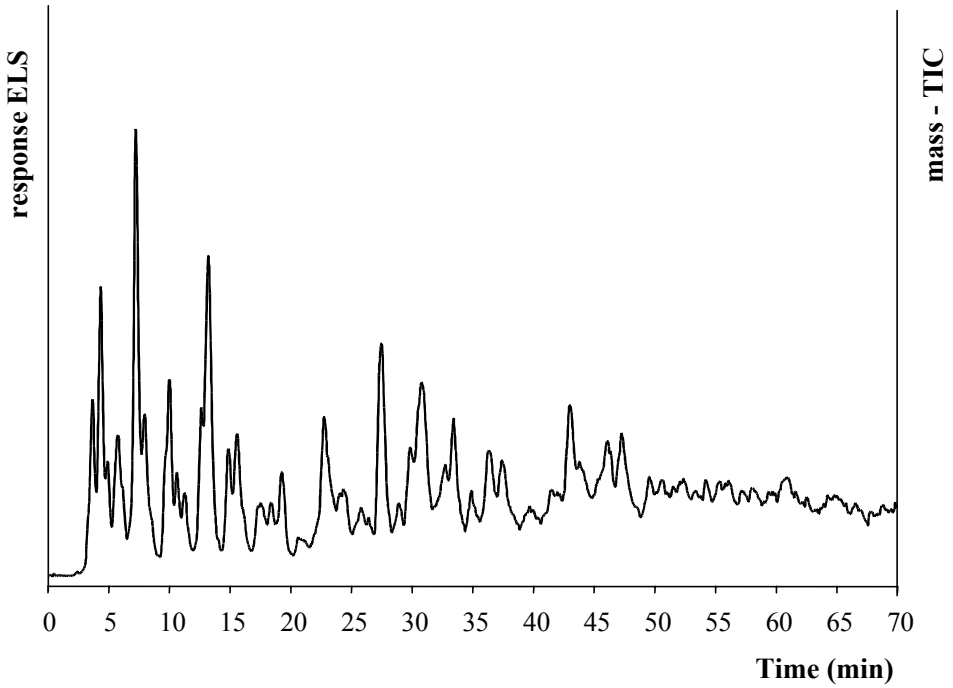


**Figure 5.1** RP-HPLC elution profiles of AcXOS detected by ELSD and MS (total ion current (TIC)).

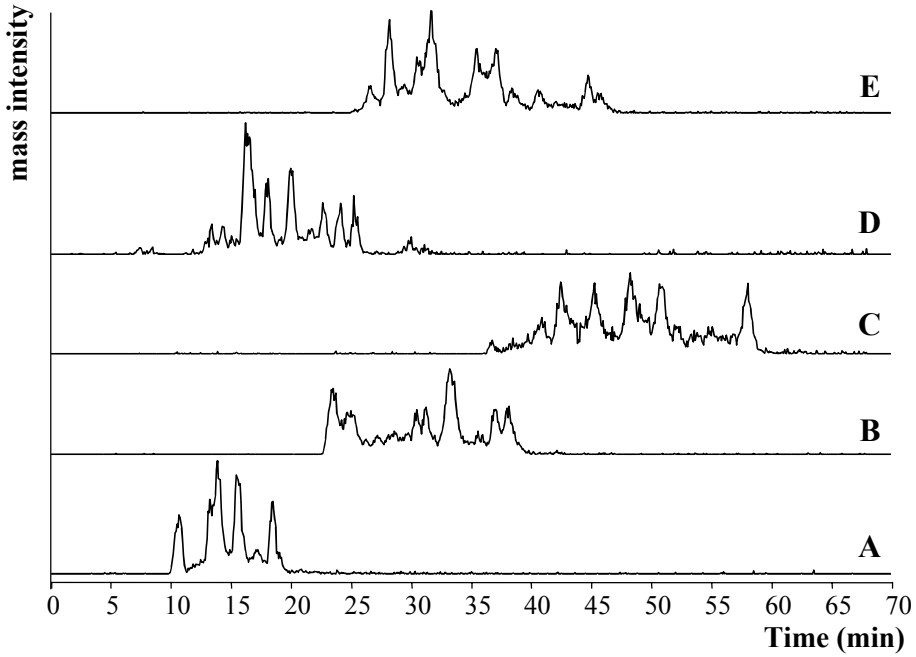


**Figure 5.2** Elution patterns of mass 479 (A; sodium adducted $X_3Ac$), 521 (B; sodium adducted $X_3Ac_2$), 563 (C; sodium adducted $X_3Ac_3$), 611 (D; sodium adducted $X_4Ac$) and 653 (E; sodium adducted $X_4Ac_2$) extracted from the RP-TIC pattern of AcXOS (X = xylose; Ac = *O*-acetyl substituent).

The ELS-elution pattern and the total ion current (TIC)-elution pattern obtained from the separation of AcXOS by RP-HPLC are presented in figure 5.1. Additionally, the MS-software enabled us to extract 'elution patterns per mass' from the original data (TIC) obtained from the mass spectrometer. Five of such 'elution patterns' are shown in figure 5.2.

It was concluded that the number of *O*-acetyl substituents per oligomer influenced their elution by RP-HPLC. The higher the number of *O*-acetyl substituents the higher was the retention of the oligomers on the RP-column used. This resulted in a rather similar retention for oligomers with different DP, but carrying the same number of *O*-acetyl substituents. Similar observations were described by Pauly *et al.* for acetylated xyloglucan oligosaccharides [*13*]. They showed that the presence of an *O*-acetyl substituent on the galactosyl-residue significantly increases the retention time.

Furthermore, various peaks per mass were eluted (Fig. 5.2). This can partly be explained by the fact that in general for RP chromatography pairs of peaks are observed corresponding to the α- and β-anomers of reducing saccharides with a DP ≥ 3 [*21*]. However, separation of α- and β-anomers alone did not explain the number of peaks eluting per mass. We anticipated that AcXOS with differently located *O*-acetyl substituent(s) eluted separately as well.

To determine which of the xylosyl-residues within the various oligomers were acetylated on-line $MS^n$ was performed (not shown). $MS^n$ is reported to be useful in the sequencing of oligosaccharides [*22*]. However, in the AcXOS studied fragmentation was most likely hindered by the *O*-acetyl substituent(s) present. The higher the number of *O*-acetyl substituents per oligomer the less fragments were observed in the $MS^n$-spectra. Too few fragments were present in the $MS^n$-spectra to be able to determine the location of the *O*-acetyl substituent(s) in the xylo-oligomers.

### *5.3.2 Location of the O-acetyl substituent in a xylotetramer by RP-HPLC-MS*

Due to the complexity of the AcXOS-mixture it was decided to study part of the oligomers present in the mixture in more detail. Therefore, the AcXOS-mixture was applied to preparative size-exclusion chromatography (BioGel P2).

The separation by size-exclusion chromatography of the oligomers was rather indistinct, because of the limited resolution on BioGel P2 material of the *O*-acetyl substituted oligomers only slightly differing in Mw; an increase of one *O*-acetyl substituents per oligomer increases the mass with 42 Da.

Fractions containing mainly mass 611 (sodium adduct of $X_4Ac$) as analysed by MALDI-TOF mass spectrometry were combined and freeze-dried. The MALDI-TOF mass spectrum of the combined pool $X_4Ac$ is presented in figure 5.3. Mainly $X_4Ac$ was accumulated in pool $X_4Ac$, although some other AcXOS were still present as well. These AcXOS were not expected to hinder the structural characterisation of $X_4Ac$, because their retention on the RP-column was quite different from $X_4Ac$ (Fig. 5.2).

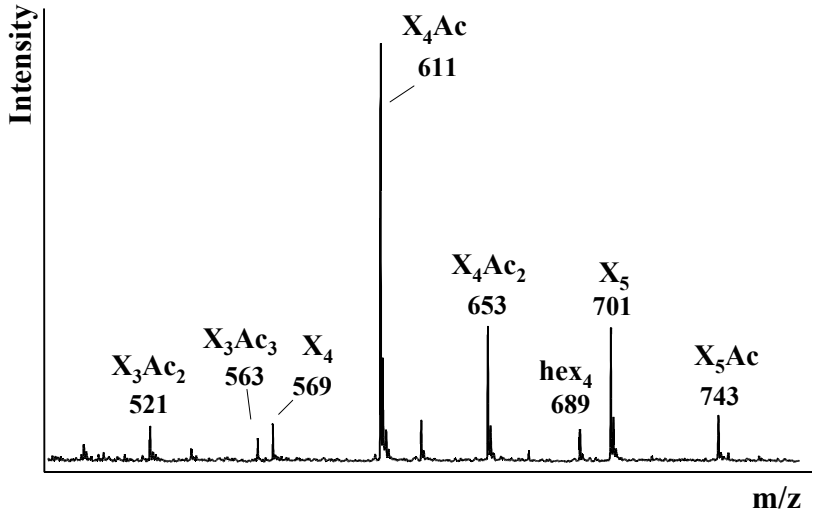


**Figure 5.3** MALDI-TOF mass spectrum of pool $X_4Ac$; masses are presented as sodium adducts (X = xylose; Ac = *O*-acetyl substituent).

Figure 5.4 shows the RP-elution pattern of pool $X_4Ac$ detected by ELS. The 'elution pattern' of peaks having mass 611 extracted from the data obtained by the mass spectrometer is presented as well. Mass spectra of peak A-H (Fig. 5.4) showed that in peak A/B and peak G/H, beside the main $X_4Ac$, only minor signals were detected of mass 479 (sodium adducted $X_3Ac$) and mass 521 ($X_3Ac_2$) respectively. Peaks C, D, E and F solely contained $X_4Ac$.

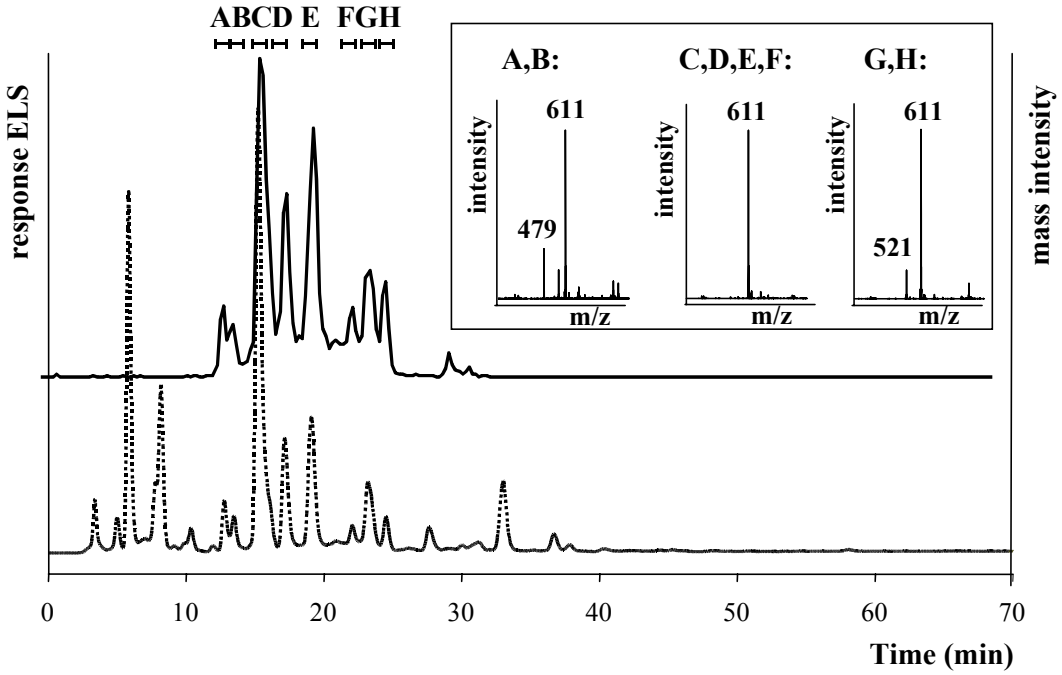


**Figure 5.4** RP-HPLC elution profiles of AcXOS detected by ELSD (----) and the elution patterns of mass 611 extracted from the RP-TIC pattern (—); the (sum of the) mass spectra of the different peaks encoded by A-H are presented in the inserted window.

Again, the data obtained from the fragmentation spectra by $MS^n$ of the peaks A-H (not shown) were not conclusive enough to locate the different positions of the *O*-acetyl substituent in the various xylotetramers. Therefore, the RP-peaks A-H (Fig. 5.4) in correspondingly pool A-H were collected and subjected to NMR spectroscopy. Quite some material of pool $X_4Ac$ (~20 mg) was separated by RP to finally obtain about 1 mg of material for each pool A-H.

### *5.3.3 Location of the O-acetyl substituent in a xylotetramer by NMR*

The pools A, C, D, E, G and H as well as a non-acetylated xylo-oligosaccharide mixture (saponified AcXOS) were subjected to both one-dimensional (1D) and two-dimensional (2D) $^1$H NMR. The $^1$H chemical shifts were assigned based on COSY and TOCSY experiments and are presented in table 5.1.

**Table 5.1** Summary of $^1$H chemical shifts (ppm) of the various xylose and xylosyl residues observed in COSY and TOCSY spectra of pool A, C, D, E, G and H.

| Pool | Xylose residue [a] | $^1$H chemical shifts in ppm | | | | | |
|---|---|---|---|---|---|---|---|
| | | H-1 | H-2 | H-3 | H-4 | H-5ax | H-5eq |
| A; C = E = H; | $\mathbf{X_{red\ \alpha}}$ | 5.18 | 3.55 | 3.83 | 3.76 | 3.98 | 3.76 |
| D = G | $\mathbf{X_{red\ \beta}}$ | 4.58 | 3.24 | 3.55 | 3.78 | 3.38 | 4.05 |
| | $\mathbf{X_I}$ | 4.48 | 3.28 | 3.56 | 3.79 | 3.40 | 4.10 |
| | $\mathbf{X_t}$ | 4.46 | 3.26 | 3.43 | 3.63 | 3.32 | 3.97 |
| C = E = H; | $\mathbf{X_i3}$ | 4.58 | 3.48 | 4.99 | 3.94 | 3.48 | 4.12 |
| D = G | $\mathbf{X_i2}$ | 4.71 | 4.71 | 3.79 | 3.86 | 3.45 | 4.15 |
| C = E = H | ($X_i$2-)$\mathbf{X_{red\ \alpha}}$ | 5.17 | nd[b] | nd[b] | nd[b] | nd[b] | nd[b] |
| | ($X_i$2-)$\mathbf{X_{red\ \beta}}$ | 4.55 | 3.24 | nd[b] | nd[b] | nd[b] | nd[b] |
| | $\mathbf{X_i}$(-$X_i$3) | 4.44 | 3.21 | 3.53 | 3.74 | 3.38 | 4.04 |
| D = G | $\mathbf{X_t}$(-$X_i$3) | 4.42 | 3.17 | 3.42 | 3.6 | 3.32 | 3.94 |
| A | $\mathbf{X_t3}$ | 4.57 | 3.45 | 4.9 | 3.81 | 3.45 | 4.02 |
| | $\mathbf{X_t2}$ | 4.68 | 4.68 | 3.65 | 3.72 | 3.35 | 4.03 |

[a] $\mathbf{X_{red\ \alpha/\beta}}$, reducing end Xyl; $\mathbf{X_i}$, internal Xyl; $\mathbf{X_t}$, non-reducing end Xyl; $\mathbf{X_i3}$, 3-*O*-acetylated internal Xyl; $\mathbf{X_i2}$, 2-*O*-acetylated internal Xyl; ($X_i$2-)$\mathbf{X_{red\ \alpha/\beta}}$, reducing end Xyl substituted (4→1) with $X_i$2; $\mathbf{X_i}$(-$X_i$3), internal Xyl linked (1→4) with $X_i$3; $\mathbf{X_t}$(-$X_i$3), non-reducing end Xyl linked (1→4) with $X_i$3; $\mathbf{X_t3}$, 3-*O*-acetylated non-reducing end Xyl; $\mathbf{X_t2}$, 2-*O*-acetylated non-reducing end Xyl.

[b] not detected.

The proton NMR resonances of non-acetylated xylo-oligosaccharides were used as a reference for reducing end xylose residues ($X_{red\ \alpha}$ and $X_{red\ \beta}$), internal ($X_i$) and non-reducing

end ($X_t$) xylosyl residues. These $^1$H chemical shifts (table 5.1) were in good agreement with values published earlier [*1,23-25*].

Interestingly, the NMR spectra obtained of pool C were similar to the ones of pool E and H. In these spectra particular signals were obtained for a reducing end xylose residue (α and β), of which the H1 was shifted slightly upfield (($X_i2$-)$\mathbf{X_{red\alpha/\beta}}$). An internal xylosyl residue, of which the H1 was shifted upfield was observed as well ($\mathbf{X_i}$(-$X_i3$)). Furthermore, a non-reducing end residue ($X_t$) and both an internal 2-*O*- and 3-*O*-acetylated xylosyl residue ($X_i2$ and $X_i3$) could be distinguished. Therefore, we assumed that the pools C, E and H represented a mixture of xylotetramers (α and β) with the 2-*O*- or 3-*O*-acetyl substituent located on the second xylosyl residue from the reducing end. This assumption was strengthened by the results presented by Van Hazendonk *et al.* [*12*]. They showed that the H1 of a non-acetylated internal xylosyl residue linked to an 3-*O*-acetylated xylose was shifted upfield.

The spectra of pool D were similar to pool G. A particular signal ($\mathbf{X_t}$(-$X_i3$)) was observed in these spectra of a non-reducing end xylosyl of which the H1 was shifted slightly upfield compared to a $X_t$-residue. This upfield-shift was comparable with the upfield-shift observed in the pools C, E and H between the H1 of the $\mathbf{X_i}$(-$X_i3$) and $X_i3$-residue. A $X_{red\ \alpha}$- and $X_{red\ \beta}$-, $X_i3$- and $X_i2$-residue were distinguished as well. Combining these results, it was assumed that the pools D and G contained a mixture of xylotetramers (α and β) with the 2-*O*- or 3-*O*-acetyl substituent located on the third residue (counting from the reducing end residue).

The spectra of pool A contained some particular signals as well. For these particular residues signals were observed corresponding to 2-*O*- and 3-*O*-acetylated xylosyl residues, but of which all $^1$H chemical shifts were shifted upfield compared to a $X_i2$- and $X_i3$-residue. These upfield-shifts corresponded well with the upfield-shifts observed between an $X_i$- and $X_t$-residue. A $X_{red\ \alpha}$- and $X_{red\ \beta}$-residue and a $X_i$-residue were distinguished as well. Therefore, we assumed that pool A represented a mixture of xylotetramers (α and β) with the 2-*O*- or 3-*O*-acetyl substituent located on the non-reducing end xylosyl residue. To our knowledge this is the first time that $^1$H chemical shifts for acetylated non-reducing end xylosyl residues are presented ($X_t2$ and $X_t3$; table 5.1).

The $^1$H chemical shifts observed in the different pools for both the internal 2-*O*-acetylated and internal 3-*O*-acetylated xylosyl residues ($X_i2$ and $X_i3$; table 5.1) corresponded well with values reported in literature [*10,12*]. The $^1$H chemical shifts of xylosyl residues neighbouring $X_i2$- or $X_i3$-residues were partly described in literature [*10,12*]. However, the assignation of the signals to the corresponding xylosyl residues was not performed yet. In the current study we were able to assign the $^1$H chemical shifts to the corresponding xylosyl residue next to a $X_i2$- or $X_i3$ -residue as discussed in the previous part of the text (($X_i2$-)$\mathbf{X_{red\alpha/\beta}}$, $\mathbf{X_i}$(-$X_i3$), $\mathbf{X_t}$(-$X_i3$); table 5.1). The assignation was completed only in combination with the results obtained by RP-HPLC-NMR (§5.3.4).

Quite confusing was the presence of signals corresponding with non-acetylated xylose and xylosyl residues in each position ($X_{red\alpha/\beta}$-, $X_i$- and $X_t$-residue) in all pools analysed. Most likely some deacetylation had occurred (*vide infra*).

It was surprising that each of the pools analysed by NMR contained a mixture of 2-*O*- and 3-*O*-acetylated xylotetramers with the *O*-acetyl substituent at the same xylosyl residue per pool. Therefore, the pools were resubjected to RP-HPLC-MS (Fig. 5.5) and it was shown that more peaks having mass $X_4Ac$ eluted (12-26 min) than the expected two peaks (α and β) per pool [*21*].

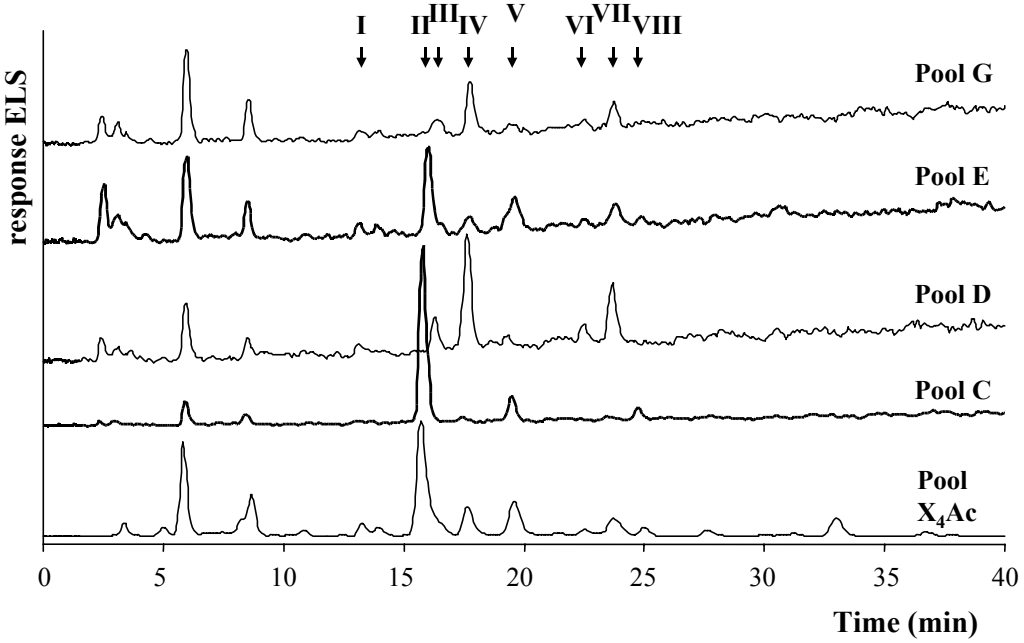


**Figure 5.5** RP-HPLC elution profiles of pool $X_4Ac$, C, D, E, and G detected by ELSD; peaks containing a xylotetramer with one *O*-acetyl substituent are encoded I-VIII.

All oligomers eluting between 12 and 26 min from the RP-column were $X_4Ac$-oligomers, which was confirmed by RP-HPLC-MS (m/z 611 (sodium-adduct); not shown). Furthermore, the same peaks were eluted for pool C and E. Even the intensities of the peaks eluting from pool E were similar to the ones from pool C. The same observations were done for pool D and G. The most reasonable explanation for these results was that *O*-acetyl migration had occurred within the xylotetramer after pooling from RP-HPLC. Unambiguous evidence for this assumption is presented in §5.3.5. Additionally, resubmitting of the pools revealed that besides *O*-acetyl migration indeed deacetylation had occurred as expected from the NMR-results. Both α- and β- xylotetraose were distinguished with a retention time of about 8.5 and 6 minutes respectively in the ELS (Fig. 5.5) and RP-HPLC-MS chromatograms (m/z 569 (sodium-adduct); not shown).

### *5.3.4 Location of the O-acetyl substituent in a xylotetramer by LC-NMR*

We aimed to use RP-HPLC-NMR on-line, since already a mild sample handling starting from collection of fractions till NMR-measurements (evaporation of methanol, freeze-drying and freeze-drying in $D_2O$) most likely promoted *O*-acetyl migration (§5.3.3). Furthermore, for further research it is important to know which RP-peak (Fig. 5.4) belonged to which structure revealed by NMR as presented in §5.3.3.

Both pool $X_4Ac$ and the pools C and D (Fig. 5.4) were applied to RP-HPLC-NMR. Firstly, several separations by RP-HPLC were performed, while automatically the middle part of each peak detected by the UV-detector (210nm) was stored in a separate sample-loop in the Bruker peak sampling unit (BPSU). Subsequently, the material stored in the BPSU was transferred one by one to the NMR-unit and 1D- and/or 2D-$^1$H-NMR spectra were recorded. Five of the 1D-spectra obtained by using RP-HPLC-NMR are shown in figure 5.6. The numbers II-V and VII in figure 5.6 correspond with the peaks II-V and VI in figure 5.5, representing the different xylotetramers with one *O*-acetyl substituent. The peaks I, VI and VIII were too low in concentration in all samples applied to RP-HPLC-NMR to obtain good signals for the NMR-spectra. The peaks coded I-VIII represented similar oligomers as the originally pooled oligomers coded A-H (Fig. 5.4). Therefore, results obtained by the RP-HPLC-NMR performed could be compared well with the NMR-results described in §5.3.3.

The 1D-spectra presented in figure 5.6 showed that the loops corresponding to the oligomers II, IV, V and VII eluted by RP solely contained one $X_4Ac$, also indicated by only one $^1$H chemical shift observed for the $CH_3$ group of the *O*-acetyl substituent present (2.15 or 2.16 ppm) [*10,12*]. The 1D-spectra corresponding to oligomer III showed two *O*-acetylated oligomers (Fig. 5.6). However, together with oligomer III some material of oligomer II was coeluted (Fig. 5.5). The signal detected around 2.05 ppm resulted from an impurity of acetonitrile. The position of the *O*-acetyl substituent (2-*O* or 3-*O*) was rather easy to determine from the 1D-spectra obtained. Namely, the H2 and H3 of the 2-*O*- and 3-*O*-acetylated xylosyl residue respectively gave well distinguishable signals in the anomeric region of the 1D-spectra (see §5.3.3 as well). The anomeric signals of the $X_{red\ \alpha/\beta}$-, $X_i$- and $X_t$-residue shown in figure 5.6 were rather easy to assign as well, since for each pure xylotetramer only four different anomeric protons (residues) could be present per 1D-spectrum. Additionally, part of the signals had already been assigned as described in §5.3.3.

Combining the 1D-results of the purely obtained acetylated xylotetramers (Fig. 5.6) with the TOCSY-results of the pools A-H (§5.3.3), a complete assignation of the $^1$H-shift of the different xylosyl residues was obtained (table 5.1). The structures resulting from this table of the xylotetramers including the location of the *O*-acetyl substituent are presented in figure 5.7.

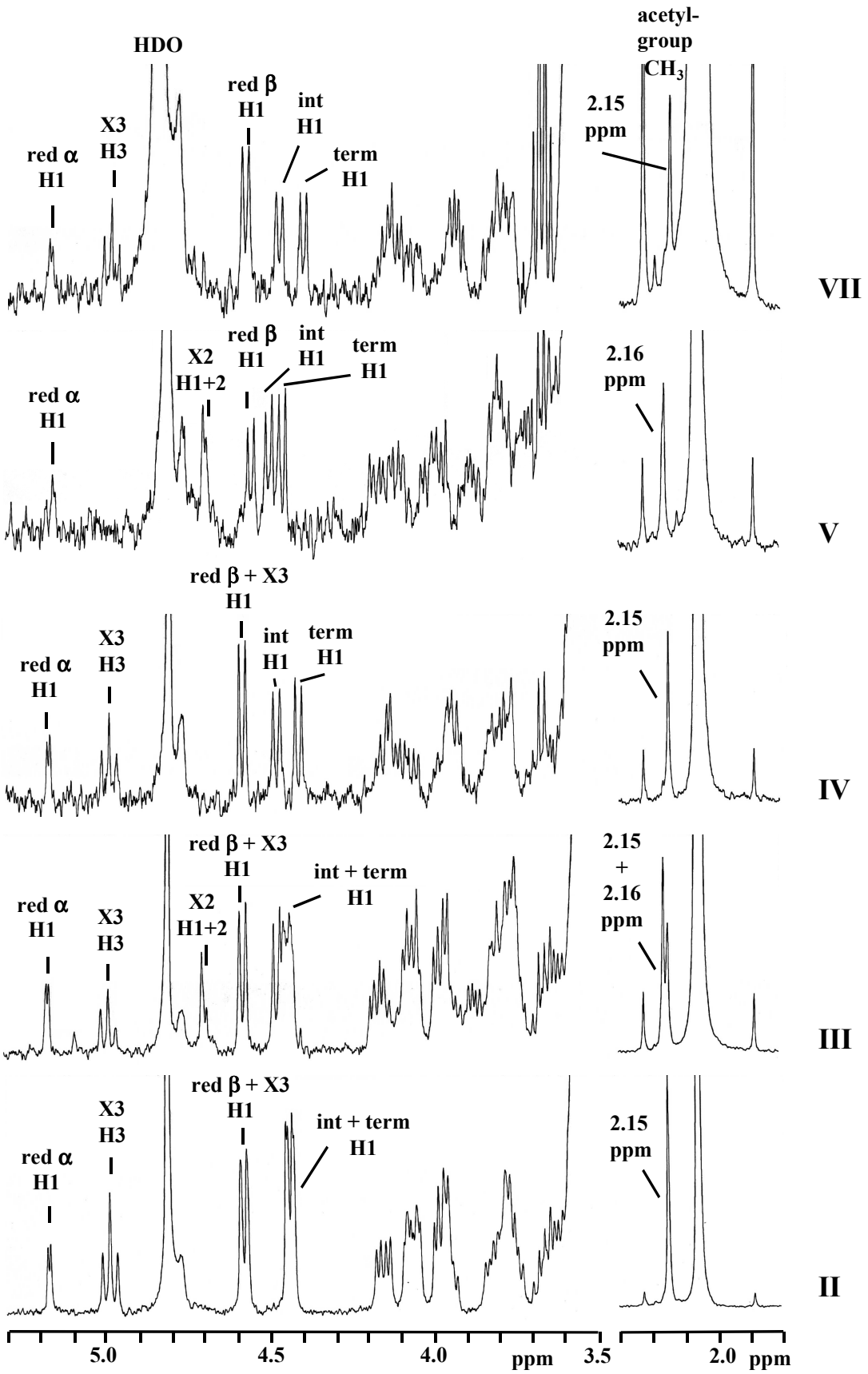


**Figure 5.6** 1D $^{1}$H NMR spectra obtained by RP-HPLC-NMR of peak II-V and VII.

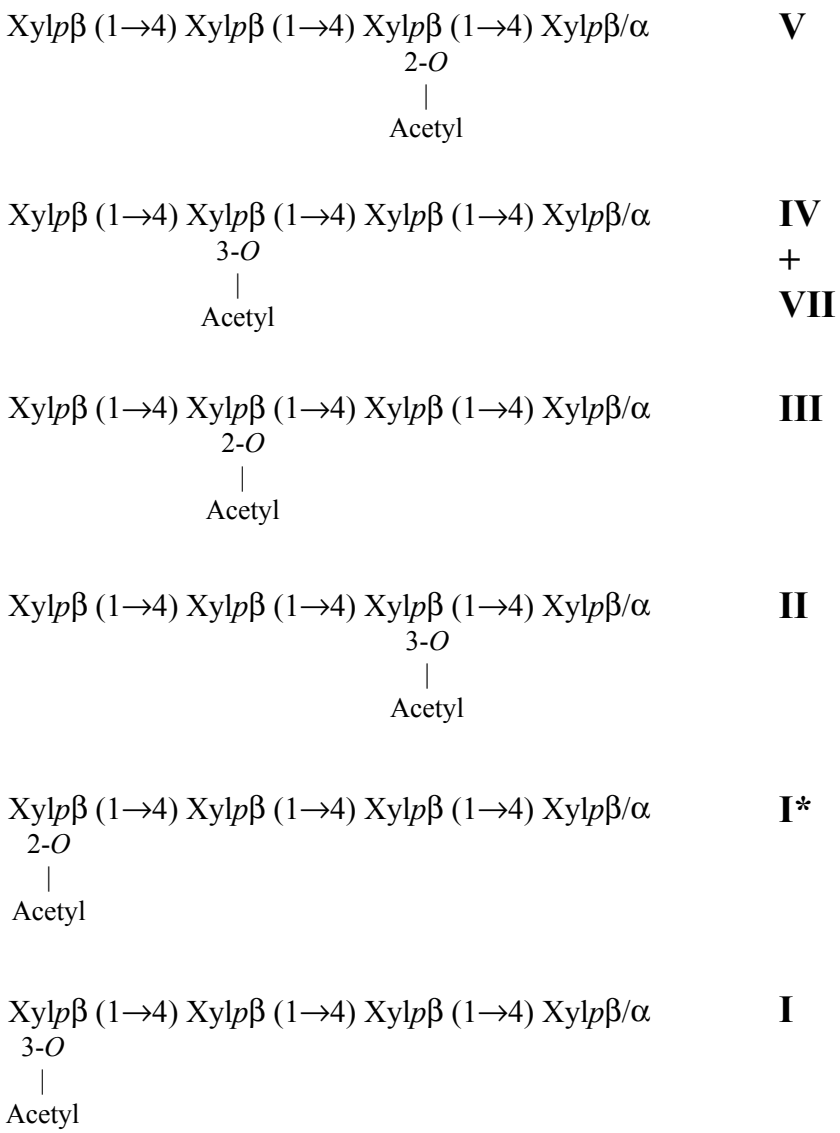


**Figure 5.7** Summary of structures of the various xylotetramers with one *O*-acetyl substituent, eluting in the RP-peaks I-V and VII.

A disadvantage of storing the RP-peaks in the BPSU before NMR analysis is the re-establishment of the α-/ β-equilibrium. Therefore, a distinction between the α- and β-anomeric form separated by RP-HPLC was not obtained by the NMR performed. The amounts of sample were too low for direct RP-HPLC-NMR measurements.

### *5.3.5 Migration of the O-acetyl substituent in a xylotetramer*

The different structures of the acetylated xylotetramers corresponding to the RP-peaks (Fig. 5.7) demonstrated that *O*-acetyl migration had occurred indeed within the pools A-H as had already been suggested in §5.3.3. In pool C and D, which originally contained structure II and IV respectively (Fig. 5.7 & §5.3.4), part of the 3-*O*-acetyl substituents were migrated to the 2-

*O*-position of the same xylosyl residue resulting in structure V and III respectively (Fig. 5.5 & 5.7). Similarly, in pool E, which originally contained structure V (Fig. 5.7 & §5.3.4), part of the 2-*O*-acetyl substituents were migrated to the 3-*O*-position of the same xylosyl residue resulting in structure II (Fig. 5.5 & 5.7). The same equilibrium was reached starting from either the 2-*O*- or the corresponding 3-*O*-acetylated tetramer, in favour of the 3-*O*-acetylated compounds. *O*-acetyl migration was only observed within the same xylosyl residue.

To our knowledge, this is the first time that *O*-acetyl migration was proven in xylo-oligosaccharides in such detail. Reicher *et al.* and Biely *et al.* have already observed that *O*-acetyl migration took place in aqueous solutions of methyl 2-*O*-acetyl-4-*O*-methyl-β-D-xylopyranoside [*26,27*]. Reicher *et al.* did not observe any *O*-acetyl migration in xylan from hardwood (*Mimosa scabrella*) during treatment with aqueous chlorine or hot ethanol [*26*]. However, it is possible that the ratio of 2-*O*- and 3-*O*-acetyl substituents in the xylan is close to that of an equilibrium mixture [*6*], so that any *O*-acetyl migration could not be detected. *O*-acetyl migration is observed as well within the compounds benzyl 2-*O*-acetyl-4-*O*-methyl-β-D-xylopyranoside and benzyl 3-*O*-acetyl-4-*O*-methyl-β-D-xylopyranoside affected by various chemicals [*28*]. Finally, *O*-acetyl migration is reported to occur in acetylated inositol derivatives and xyloglucan oligosaccharides [*13,29*].

Some minor experiments were carried out to study the conditions under which *O*-acetyl migration occurred. Material present in separate loops in the BPSU of the LC-NMR apparatus each containing pure acetylated tetramers in ($D_2O$/ Methanol) were kept for about 1 hour at 70 °C or for about three days at 20 °C. At these conditions no *O*-acetyl migration was observed. So, it is still questionable why the freezedrying and/ or re-dissolving the material in $D_20$ performed already had promoted the *O*-acetyl migration as described in §5.3.3 and §5.3.4.

Taking in account these observations, it was expected that *O*-acetyl migration and deacetylation might have occurred during hydrothermal treatment of the *Eucalyptus* wood as well. Therefore, the position and distribution of the *O*-acetyl substituents in the xylo-oligomers analysed can not be extrapolated directly to the xylan-structures originally present.

### *5.3.6 Location of the O-acetyl substituent in a xylotrimer by LC-NMR*

To present the possibilities of RP-HPLC-NMR confirming the location of *O*-acetyl groups in other xylo-oligosaccharides than xylotetramers, a sample containing mainly *O*-acetylated xylotrimers was analysed.

The *O*-acetyl substituent in xylotrimers present in the AcXOS-mixture (§5.3.1) could be located similarly to the determination of the location of the *O*-acetyl substituent in a xylotetramer. The AcXOS-mixture had already been fractionated by size-exclusion chromatography and also fractions in which mainly a $X_3Ac$ was present were obtained. Four of these fractions were combined to pool $X_3Ac$ and this pool was subjected to RP-HPLC-NMR. The RP-chromatogram resembled the elution pattern as presented in figure 5.2A.

A TOCSY-spectrum was obtained of the material represented by the highest peak in the RP-chromatogram (Fig. 5.8). The signals obtained (Fig. 5.8) were comparable with the $^{1}$H-shifts summarised in table 5.1, which resulted in the characterisation of the two structures presented in figure 5.8. Furthermore, this TOCSY-spectrum was of help in identifying the 1D-NMR spectra obtained of several other acetylated xylotrimers separated by RP-HPLC-NMR. These represented mainly xylotrimers with the 2-*O*- or 3-*O*-acetyl substituent located to the non-reducing end xylosyl residue.

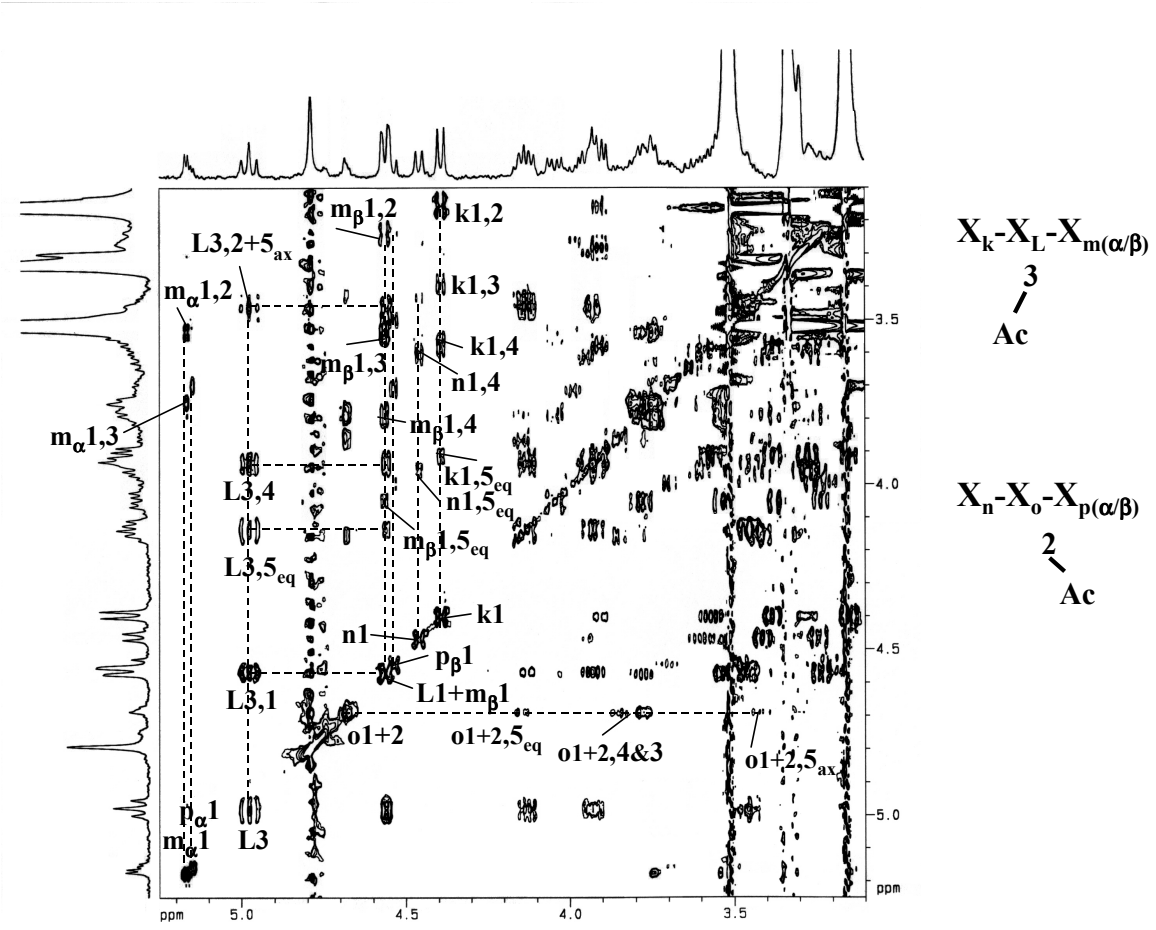


**Figure 5.8** 2D TOCSY spectrum of pool $X_3Ac$. Diagonal peaks and corresponding cross-peaks of the anomeric protons of the various residues (encoded K-P) present are indicated. The two structures deduced from this spectrum are presented as well.

### *5.3.7 Conclusions*

*O*-acetyl substituted xylo-oligosaccharides were separated by using RP-HPLC. An advantage of using RP-HPLC was that only pure water and methanol were used for the separations, facilitating the on-line coupling to a mass spectrometer. High pH high performance anion-

exchange chromatography (HPAEC) using salt gradient up to 1 M, which also is a commonly used technique to separate oligosaccharides [*30,31*], is more difficult to perform on-line with a mass spectrometer. Although, a successful off-line combination of HPAEC with MALDI-TOF MS has been described [*32*]. However, at the high pH used in HPAEC *O*-acetyl substituents will be removed and was no option to use. By using RP-HPLC-MS it became possible to separate and detect xylo-oligomers having the same DP, but in which the *O*-acetyl substituent was located at different positions.

To identify the precise location of the *O*-acetyl substituent in both xylotetramers and xylotrimers RP-HPLC-NMR combined with an intermediate BPSU was used. 1D-NMR spectra were obtained of most of the oligomers by the RP-HPLC-NMR performed. Concentrations used were 50-150 µg per peak for determining 1D-NMR spectra in 2-4 hours and at least 150 µg per peak to obtain a TOCSY-spectrum about 50 hours.

In conclusion, by RP-HPLC-MS, NMR and RP-HPLC-NMR we were able to localise the *O*-acetyl substituent in 6 xylotetramers and 4 xylotrimers. No evidence was found for the presence of an *O*-acetyl substituent located at the reducing end xylosyl residue. To our knowledge for the first time *O*-acetyl migration was proven to have occurred in xylo-oligosaccharides. Mainly *O*-acetyl migration within the same xylosyl residue was observed during NMR-sample pretreatment. An equilibrium was established between the 2-*O*- and 3-*O*-acetyl substituted xylo-oligomers in favour of the 3-O-acetylated compound, regardless the original position (2-*O*- or 3-*O*-) of the *O*-acetyl substituent.

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wheat, that contain the elements →4)[α-L-Ara*f*-(1→3)]-β-D-Xyl*p*-(1→ or →4)[α-L-Ara*f*-(1→2)][α-L-Ara*f*-(1→3)]-β-D-Xyl*p*-(1→. *Carbohydr. Res.* **1991**, *221*, 63-81.

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# CHAPTER 6

# *In vitro* fermentability of differently substituted xylo-oligosaccharides

**Abstract**

Xylo-oligosaccharides (XOS) with various substituents were fermented *in vitro* by faecal inocula (FI) from four human volunteers to study the influence of substitution on the ability and rate of fermentation and on production of short-chain fatty acids (SCFA) and lactate. By all FI used non-substituted XOS (nXOS) and arabino-XOS (AXOS) were fermented faster than the more complex structures of *O*-acetylated XOS (AcXOS) and XOS containing an 4-*O*-methylglucuronic acid group ($GlcA_{me}XOS$). In the first stage (0-40 hours) of the fermentations of nXOS and AXOS mainly acetate and lactate were formed. The fermentations of AcXOS- and $GlcA_{me}XOS$ resulted in a lower lactate production, while the concentration of propionate and butyrate increased. Our results put emphasis on the detailed elucidation of the structural features of NDO's in general to understand the their fermentation-mechanisms more precise.

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## 6.1 Introduction

From a nutritional point of view xylo-oligosaccharides (XOS) usually are considered as non-digestible oligosaccharides (NDO's), which are not degradable by the low-pH gastric fluid, nor by human and animal digestive enzymes and will therefore reach the large bowel intact [*1,2*]. In the large bowel NDO's can be fermented by the intestinal flora into mainly short chain fatty acids (SCFA; acetate, propionate and butyrate), lactate, $CO_2$ and $H_2$ [*3,4*]. Furthermore, NDO's are frequently reported to have an effect on the composition of the colonic flora, which often is the result of an increase in bifidobacteria [*5-7*]. Both the production of SCFA and the increase in bifidobacteria are related with a number of health effects, e.g. bowel function, calcium absorption, lipid metabolism and reduction of the risk of colon cancer [*8-11*].

XOS are reported to be preferentially fermented by bifidobacteria. This was shown *in vitro* experiments as well as *in vivo* [*1,4,12*]. Fermentability studies of XOS were mainly performed using linear and low Mw XOS (degree of polymerisation (DP) < 4). These studies do not answer the question about the influence of substituents to XOS on the mode of fermentation by the human intestinal flora. The importance to be able to distinguish between differently substituted (xylo-) oligosaccharides was recently indicated by Van Laere et al. [*2*], who included linear XOS and arabino-xylo-oligosaccharides (AXOS) in a fermentation study of a range of (complex) plant cell wall derived oligosaccharides. In this study it was found that linear XOS were fermented by more intestinal strains tested as compared to the branched AXOS.

A good source to obtain differently substituted XOS are agro-industrial xylan rich by-products, such as hard woods, brewery's spent grain, corn cobs and wheat bran. Through a mild hydrothermal treatment the xylan present in these by-products is (partly) broken down into a variety of soluble XOS. The structures obtained depend on the structural features of the xylan originally present in the by-products used [*13,14*]. In a former publication we have described the purification and characterisation of several series of XOS obtained from hydrothermal treated brewery's spent grain and *Eucalyptus* wood. These included non-substituted XOS as well as XOS substituted with arabinose or *O*-acetyl and /or 4-*O*-methylglucuronic acid [*15*]. In this paper, we study the fermentation by faecal inocula (FI) of the previously purified series of XOS. The focus of our study is the influence of various substituents of XOS on the ability and rate of fermentation and on the production of SCFA and lactate.

## 6.2 Experimental

### *6.2.1 Xylo-oligosaccharides-mixtures*

The four series of XOS used in this fermentation study were obtained and purified as described previously [*15*]. The italic names between parenthesis refer to the previous publication. Three of the series of XOS (DP 3-15) used were obtained from hydrothermal treated *Eucalyptus* wood: *O*-acetylated XOS (*Euc NI A* → AcXOS), linear XOS (*Euc NI A saponified* → nXOS), and non-acetylated XOS containing a 4-*O*-methylglucuronic acid or 4-*O*-methylglucuronic acid plus a hexose, most likely galactose (*Euc AII# plus AIII# saponified* → $GlcA_{me}XOS$). The fourth series of XOS used was obtained from hydrothermal treated brewery's spent grain. To obtain sufficient amounts of the arabino-xylo-oligosaccharides (AXOS) in the range of DP 3-11, the pool *BSG IB* (containing higher Mw arabinoxylan) was degraded by a purified and well characterised endo-(1,4)-β-D-xylanase III [*16*]. A solution of *BSG IB* in water (2.5 mg/ml) was adjusted to pH 5 with sodium hydroxide and incubated with endo-(1,4)- β-D-xylanase III (0.1 µg/ml) for 30 hours at 40 °C. Finally, the enzyme was inactivated (5 minutes, 100 °C) before using the digest in the fermentation-experiment.

### *6.2.2 Fermentation of XOS*

Fermentation of the nXOS, AcXOS, $GlcA_{me}XOS$ and AXOS was performed using faecal inocula of four human volunteers. Faecal inocula were prepared from fresh faeces in buffered peptone water (Oxoid) with cysteine.HCL (0.5 g/l) in approximately 10-fold dilution. A medium consisting of 1 g/l of neutralised bacterial peptone (Oxoid), 8 g/l of sodium chloride (Merck) and 0.5 g/l of L-cysteine.HCL was adjusted to a pH of 6.7 using a 6N NaOH solution [*17*]. In an anaerobic chamber (atmosphere 80 % $N_2$, 10% $CO_2$, 10% $H_2$) the 10-fold diluted faeces was diluted further (10.000x) with the medium described.

Sterile solutions (S1) were prepared of each of the XOS-mixtures in water (6 % (w/v)) and of pure water as blank, separate from a sterile solution (S2) of bacto yeast nitrogen base in water (Difco; 13.4 % (w/v)). Additionally, a sterile solution (S3) in water was prepared containing a salt-solution (40 % (v/v); $MgSO_4$ (0.2 g/l), $CaCl_2$ (0.2 g/l), $K_2HPO_4$ (1 g/l), $KH_2PO_4$ (1 g/l), $NaHCO_3$ (10 g/l) and NaCl (2 g/l)), casein enzymatic hydrolysate (N-Z-AmineA; bovine milk (Sigma; 5 % (w/v)) and sodiumthioglycolate (Sigma; 0.5 % (w/v)). The three solutions S1, S2, S3 were combined (9.1 ml) in a ratio (v/v) of 17 : 1 : 2 respectively (pH 6-7). The latter solutions (4 substrates and 1 blank) were each inoculated with 20 % (v/v) of the 10.000x diluted faecal inocula (4 inocula) at 37°C in an anaerobic chamber; so, 20 tubes were inoculated. From each tube samples were taken at time 0, 0.5, 7, 19, 27, 43, 55, 67, 75 and 94 hours and stored at -80°C. Of each sample enzyme activity was inactivated (5 min, 100°C), centrifuged and the supernatants were analysed [*2*].

### *6.2.3 Cell material from bacterial growth*

The residue of each sample, obtained after inactivation and centrifugation of the bacterial suspensions (94 hours of fermentation), was mixed with 1 ml of pure water. The turbidity of the mixed solutions was measured by an UV-mini 1240 UV-VIS spectrophotometer (Shimadzu) at 600 nm giving information about the density of cell material from bacterial growth in all samples.

### *6.2.4 Short chain fatty acids and lactate*

The content of the short chain fatty acids (butyrate, propionate and acetate) and lactate were determined on a Spectra Physics 8800 system HPLC, using an Aminex HPX-87H column and detection by refractive index (RI; Spectrasystem RI-150) [*18*].

### *6.2.5 Total sugar content*

The total content of carbohydrates present in the fermentations-experiment was determined by an automated orcinol sulfuric acid assay [*19*] using an autoanalyser (Skalar Analytical BV, Breda, The Netherlands).

### *6.2.6 HPAEC (pH 12)*

To analyse the oligosaccharides present during the fermentations high-performance anion-exchange chromatography (HPAEC) was performed on a Dionex system equipped with a CarboPac PA-1 column (4 mm ID x 250 mm) in combination with a CarboPac PA guard column (3 mm x 25 mm) and PAD-detection [*20*]. Elution (1 ml/min) of the oligomers in the fermentation-samples (diluted 12 times) was performed using the following gradient: 50-90 mM sodium acetate in 100 mM NaOH during 0-5 min, 90-130 mM sodium acetate in 100 mM NaOH during 10 min, 130-520 mM sodium acetate in 100 mM NaOH in 15 minutes. Each elution was followed by a washing (30 min; isocratic 1 M sodium acetate in 100 mM NaOH) and equilibration step (20 min).

### *6.2.7 MALDI-TOF mass spectrometry*

For matrix-assisted laser desorption/ ionisation time-of-flight mass spectrometry (MALDI-TOF MS) of oligosaccharides during fermentation a Voyager-DE RP Biospectrometry workstation (PerSeptive Biosystems Inc., Framingham, MA, USA) was used, operated as described by Daas et al. [*21*]. The mass spectrometer was calibrated with a mixture of maltodextrins (mass range 365-2309 Da as sodium adducts).
The samples were desalted with anion-exchange material (AG 50W-X8 Resin; Biorad) and mixed with a matrix solution (1 µl of sample in 9 µl of matrix). The matrix solution was prepared by dissolving 9 mg of 2,5-dihydroxybenzoic acid and 3 mg 1-hydroxyisoquinoline

in a 1-ml mixture of acetonitrile:water (300 µl:700 µl). Of the prepared (sample + matrix) solutions 1 µl was put on a gold plate and allowed to dry at room temperature.

## 6.3 Results

Four series of differently substituted XOS and a fermentation-blank (without carbohydrate source added) were fermented by faecal inocula (FI) from four human volunteers. All XOS studied are consisted of a β-D-(1,4)-linked xylopyranosyl backbone and vary in DP and type of substituent, as reported previously [*14,15*]. In summary, nXOS are linear (DP 2-11); AcXOS are highly *O*-acetylated (DP 2-11); $GlcA_{me}XOS$ each contain a 4-*O*-methylglucuronic acid group or in addition a hexose, most likely galactose (DP 3-10); AXOS are singly and doubly substituted with arabinose (DP 2-10), while also arabinoxylan-material having a higher molecular weight (Mw) is present (Mw > 1500 Da).

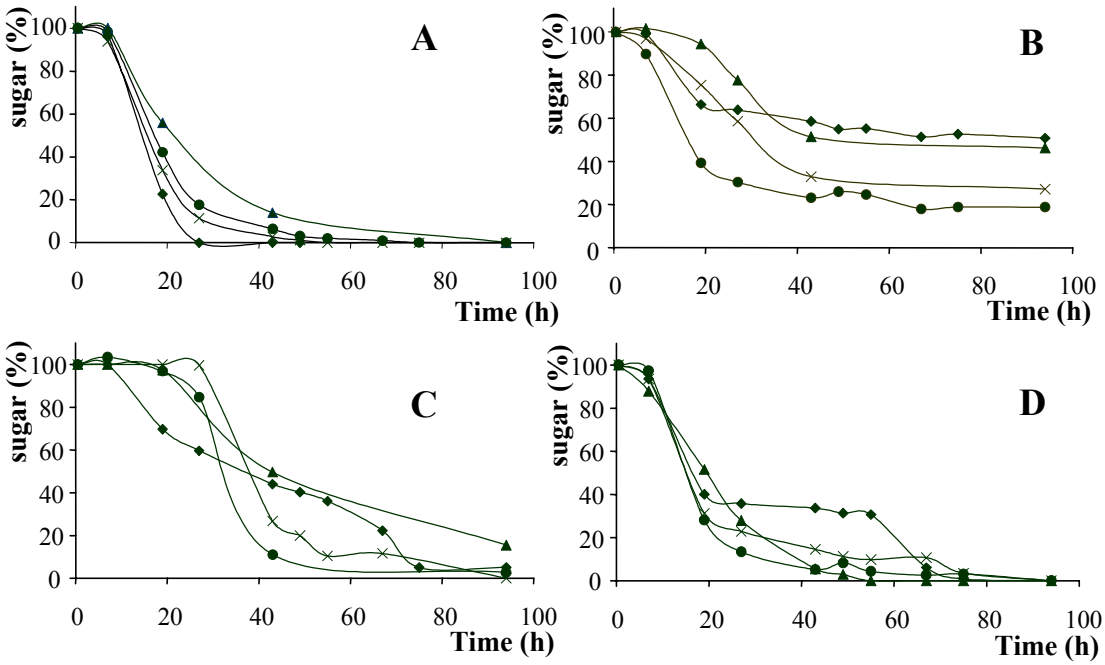


**Figure 6.1** Degradation patterns of nXOS (A), AcXOS (B), $GlcA_{me}XOS$ (C) and AXOS (D) during 94 hours of fermentation by faecal inocula from four persons (● FI 1; ▲ FI 2; × FI 3; ♦ FI 4).

The XOS in the samples taken during the fermentation experiment (till 94 h) were monitored using an automated orcinol sulfuric acid assay, HPAEC and MALDI-TOF mass spectrometry to determine both the total carbohydrates present and individual components. From the results as obtained by HPAEC the amount of nXOS, AcXOS and $GlcA_{me}XOS$ present during fermentation compared to the amount at the start of the fermentation was calculated (total area

%). The relative amount of the AXOS was calculated in a similar way, but from the total carbohydrate content (orcinol sulfuric acid assay). The AXOS contained some higher molecular weight material as well, which could not be separated and quantified by HPAEC. The relative amount of the XOS in the four XOS-mixtures present during the fermentation by four human FI is shown in figure 6.1.

During the first 20 hours of the experiment the pH of all XOS-samples was rather adequate to allow bacteria to grow (pH 5-7). However, during the first 20 hours quite a difference in degradation between the four XOS-mixtures was observed. The susceptibility of the various XOS to fermentation by FI 1, 2 and 3 decreased in the following order AXOS > nXOS > AcXOS > $GlcA_{me}XOS$, whereas by FI 4 the order was nXOS > AXOS > AcXOS > $GlcA_{me}XOS$. However, as can be seen from figure 6.1, the $GlcA_{me}XOS$ were fermented quite well after an adaptation time of about 20 hours (FI 1-3). FI 4 fermented this substrate again divergently; it needed almost no adaptation time, but fermented the $GlcA_{me}XOS$ gradually. Analysis of the fermentation-blanks did not show any carbohydrates to be present or formed during fermentation (0-94 hours).

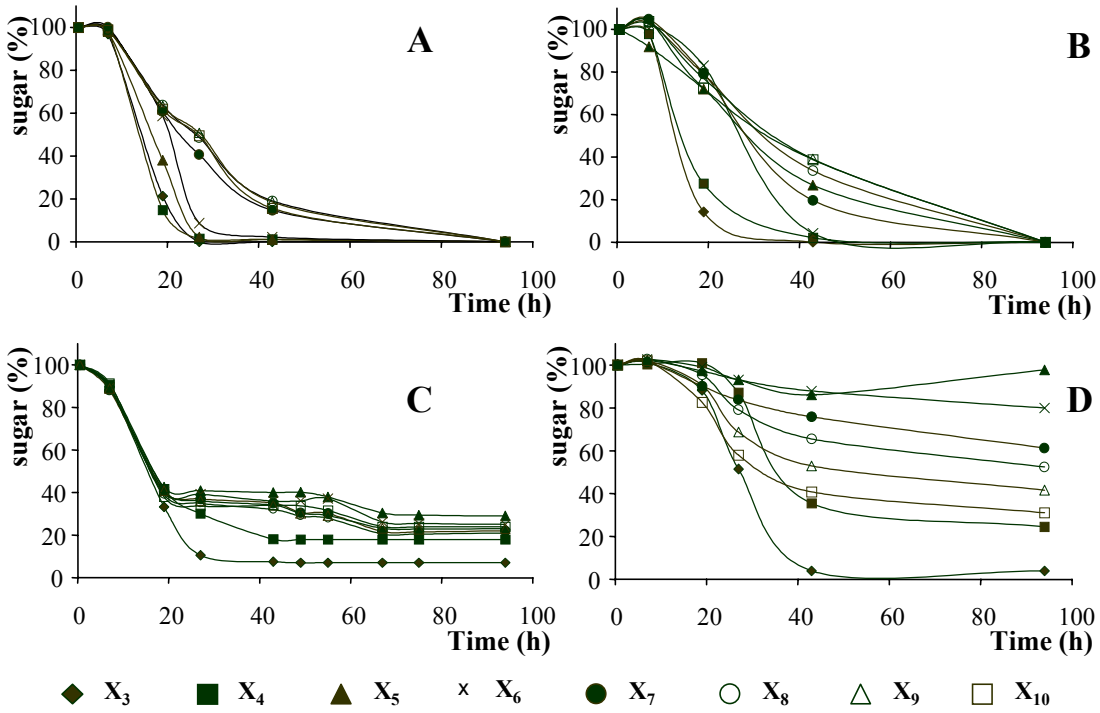


**Figure 6.2** Degradation patterns of different DP's during 94 hours of fermentation of nXOS (FI 1 (A) and FI 2 (B)), and of AcXOS (FI 1 (C) and FI 2 (D).

Figure 6.2 shows the amount of the different XOS present in the nXOS- and AcXOS-mixtures during the fermentation related to the amount present at the start (HPAEC; peak area %) by FI 1 and 2. The results of the fermentations of the different DP's of nXOS and AcXOS by FI 3

and 4 are reported in the text. Analysis of the XOS-composition of the $GlcA_{me}XOS$- and AXOS-mixtures by HPAEC was not accurate enough to estimate the relative amount of these XOS.

Comparing the XOS-profiles of the fermentations of the different DP's of nXOS by FI 1 and 2 during the first 30 hours, it was obvious that DP 3 and 4 were fermented more readily, while DP 8 to 10 were less rapidly fermented. The fermentation pattern of nXOS (DP 3-10) by FI 3 resembled that of FI 1, while FI 4 fermented even the higher DP's of the nXOS in a much shorter time. During the first 30 hours of the fermentation of the AcXOS by FI 1-3 mainly DP 3 was fermented, similarly to the non-substituted DP 3. The AcXOS-fermentation by FI 4 resembled that of FI 2, with the difference that FI 4 was able to ferment DP 4 of the AcXOS in the same rate as DP 3. AcXOS of a higher DP (5-10) were fermented slower (FI 1, 2 and 4). Nevertheless, at the end of these fermentations the amount of DP 5 and 6 was higher than of DP's 9 and 10. This 'accumulation' of DP 5 and 6 was also observed in the fermentation of AcXOS by FI 3. However, in the AcXOS-fermentation by FI 3 the DP's 8-10 were consumed faster than the DP's 3 and 4.

Studying the patterns of degradation of the AcXOS by HPAEC only information was obtained regarding the size of the various oligomers, since at the high pH of the HPAEC-eluents used *O*-acetyl substituents are removed. In contrast, MALDI-TOF MS allows the monitoring of *O*-acetylated XOS, enabling us to follow the fermentation of these oligomers. MALDI-TOF MS also provided data about the degradation of the substituents of the $GlcA_{me}XOS$. As an example, the MALDI-TOF mass spectra of the fermented AcXOS and $GlcA_{me}XOS$ by FI 1 after 0.5, 19 and 43 hours are presented in figure 6.3 and 6.4 respectively. From these figures it can be seen that no masses were observed representing non-substituted XOS. The series of XOS each containing a 4-*O*-methylglucuronic acid plus a hexose (most likely galactose) were accumulated after 43 hours of fermentation. The same observations were noticed for the fermentations of AcXOS and $GlcA_{me}XOS$ by the other three FI (not shown).

The production of SCFA, including acetate, propionate and butyrate, during the fermentation as well as of lactate were measured and for the fermentations by FI 1 and 2 these results are presented in figure 6.5. The results as obtained during the fermentation by FI 3 and 4 are given in the text.

Analysis of all fermentation-blanks showed only after 40 hours some production of SCFA, not exceeding the 20 µmol/ml.

During the first 20 hours of the fermentations of nXOS (FI 1, 2 and 4) and of AXOS (FI 1-3) mainly acetate and lactate were detected in a molar ratio of 1 : 1 respectively. However, during the nXOS-fermentation by FI 3 and AXOS-fermentation by FI 4 some propionate was formed as well, at the expense of lactate (0-19 hours). From 19 till 94 hours of fermentation of nXOS (FI 2-4) and AXOS (FI 1-4) also propionate and/ or butyrate were observed, while the amount of lactate and to a lesser extent of acetate decreased. Only during

fermentation of nXOS by FI 1 (19-94 hours) no propionate or butyrate was detected and the amounts of lactate and acetate remained constant.

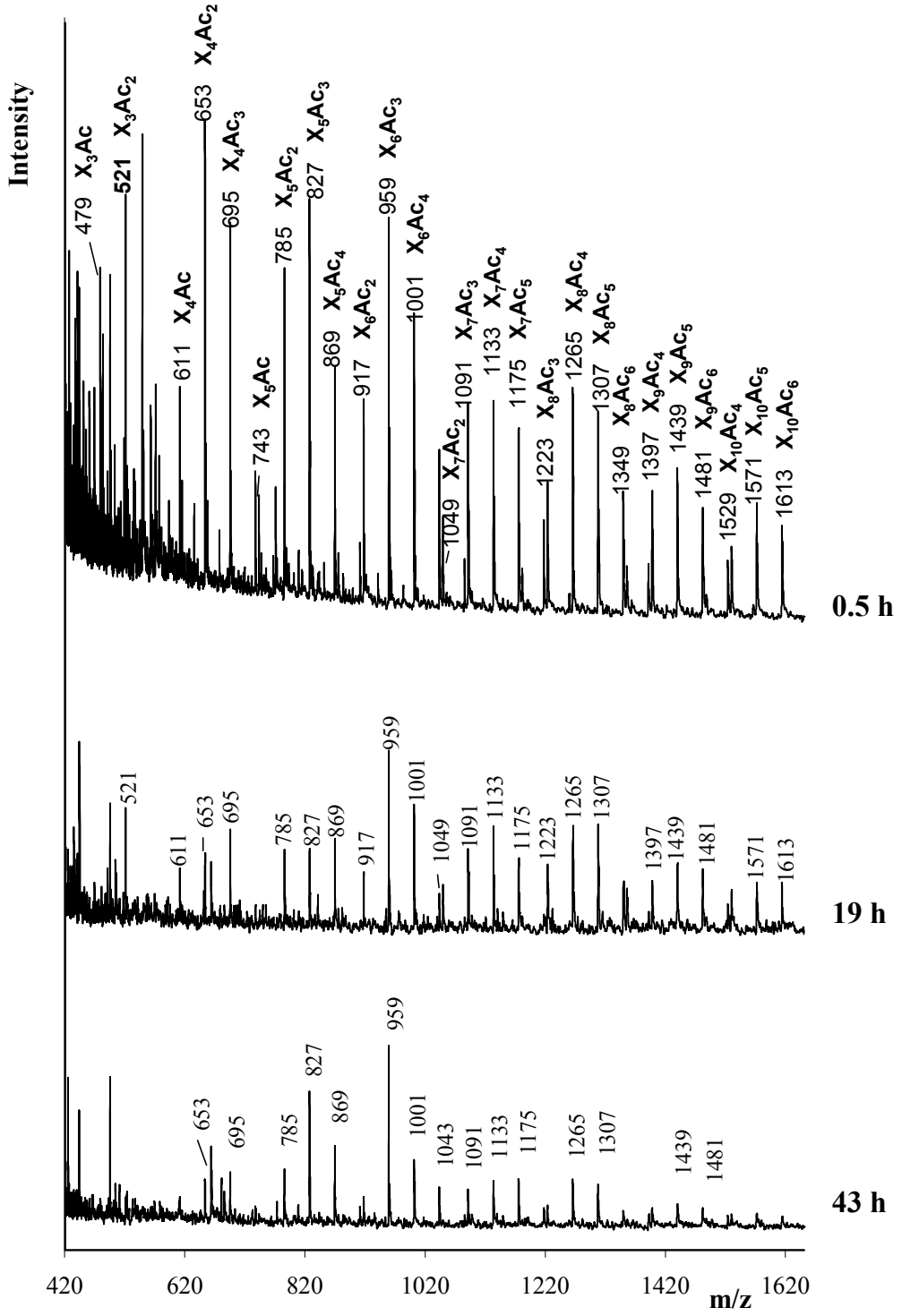


**Figure 6.3** MALDI-TOF mass spectra obtained after 0.5, 19 and 43 hours of fermentation of AcXOS (FI 1); masses of the sodium-adducted AcXOS are inserted (X = xylose; Ac = *O*-acetyl substituent).

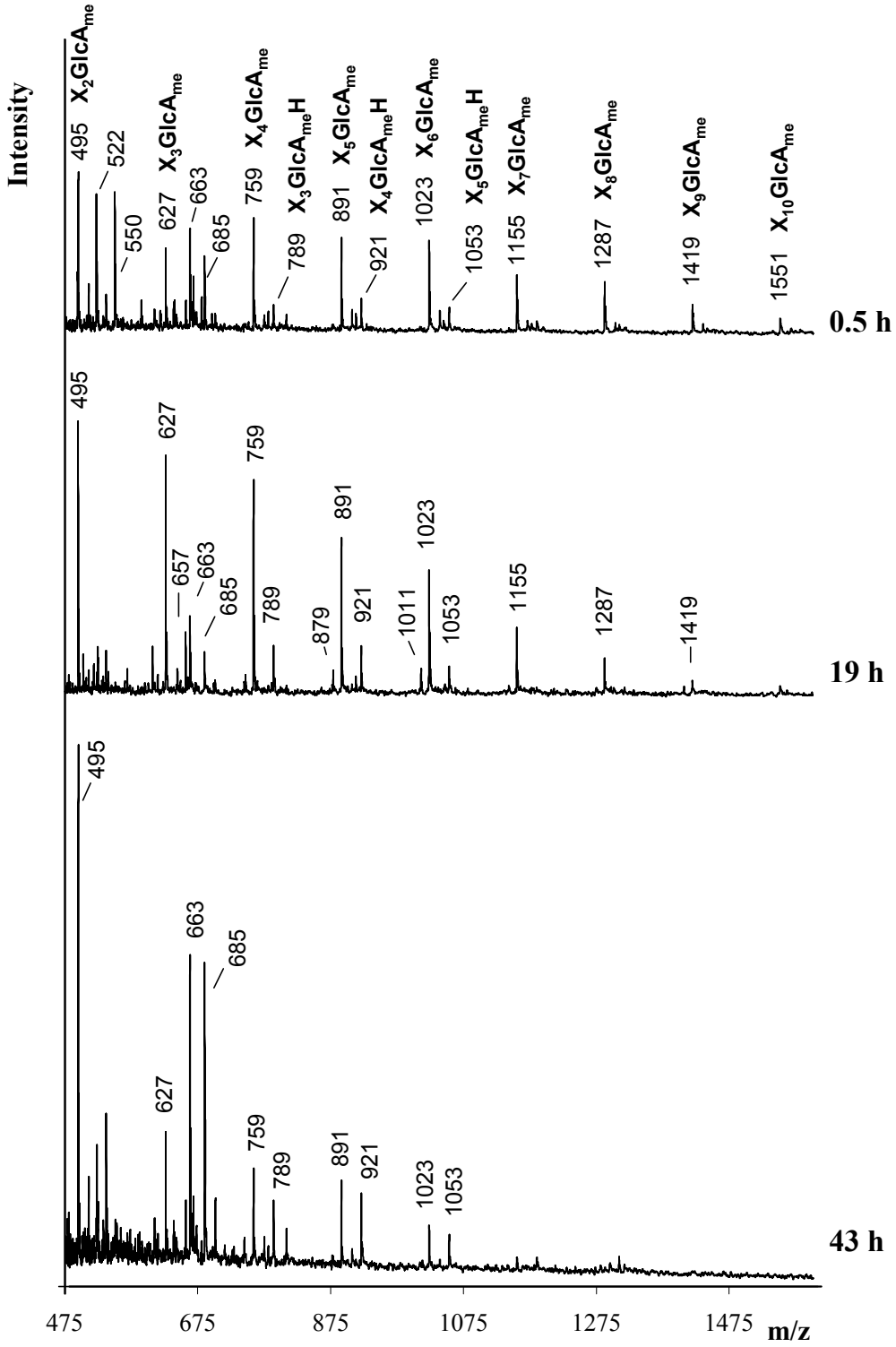


**Figure 6.4** MALDI-TOF mass spectra obtained after 0.5, 19 and 43 hours of fermentation of $GlcA_{me}XOS$ (FI 1); masses of the sodium-adducted $GlcA_{me}XOS$ are inserted (X = xylose; $GlcA_{me}$ = 4-*O*-methylglucuronic acid).

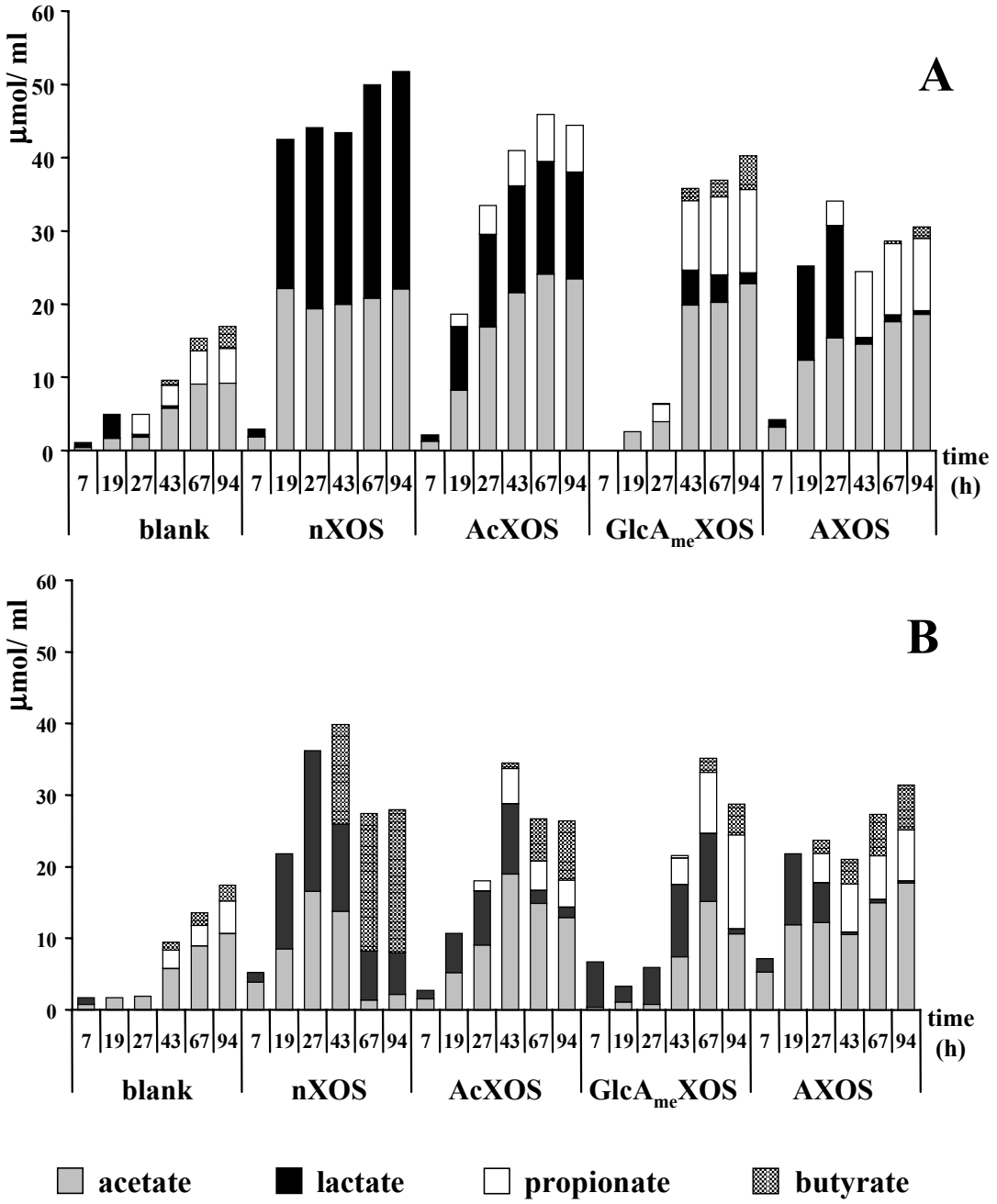


**Figure 6.5** Production of SCFA and lactate during 94 hours of fermentation of a blank, nXOS, AcXOS, GlcAmeXOS and AXOS (FI 1 (A) and FI 2(B)).

During the first 27 hours of the fermentations of AcXOS (pH 5-7) mainly acetate, lactate and propionate were detected in a molar ratio of ~ 1 : 0.8 : 0.2 respectively (FI 1, 2 and 4), but for FI 3 a divergent molar ratio was determined (1 : 0.5 : 0.3). The amount of SCFA and lactate remained quite constant from 27 till 94 hours of the fermentations of AcXOS. However, by FI 2 some butyrate was formed, and a decrease in lactate was detected. During all $GlcA_{me}XOS$-fermentations (pH 5-7), first mainly acetate and lactate were detected, followed by propionate and small amounts of butyrate (0-43 hours). The molar ratio's of acetate, lactate, propionate

and butyrate analysed at 43 hours were 1 : 0.2 : 0.5 : 0.09 (FI 1), 1 : 1.3 : 0.45 : 0.06 (FI 2), 1 : 0.05 : 0.6 : 0.03 (FI 3) and 1 : 0.2 : 0.2 : 0.01 (FI 4). The production of butyrate increased during fermentation of $GlcA_{me}XOS$ from 43 till 94 hours (FI 1-4), while the amount of lactate decreased substantially.

## 6.4 Discussion

Fermentation of the XOS resulted in a decrease of oligomers present and in the production of SCFA and lactate. In all fermentations the increase in cell material from bacterial growth observed corresponded well with the decrease in total amount of XOS present (not shown). The type of bacteria grown were not analysed, since the aim of our study was to compare the fermentation of differently substituted XOS mainly based on degradation patterns and changes in structural features of the XOS tested rather than performing a complete microbiologic study.

During the first 20 hours of the fermentations quite a different degradation pattern for the four XOS-mixtures was observed. The nXOS and AXOS were fermented more rapidly than the $GlcA_{me}XOS$ and AcXOS. However, after an adaptation time the $GlcA_{me}XOS$ were fermented in the same rate as the nXOS and AXOS.

The fact that the fermentation was not pH controlled may have influenced bacterial action. However, the pH after a fermentation-time of 20, 20, 27 and 43 hours of the nXOS, AXOS, AcXOS and $GlcA_{me}XOS$ respectively (pH 5-7) was still rather adequate to allow bacteria to grow. To look at the changes in structure of substituted XOS during fermentation only results obtained of XOS-samples having a pH > 5 were used.

Studying the fermentation of AcXOS and $GlcA_{me}XOS$ by MALDI-TOF MS in more detail, we concluded that the substituents were not easy degradable, because no masses were observed representing non-substituted XOS. Furthermore, it could be concluded that relatively low substituted AcXOS (DP 3 and DP > 7) and $GlcA_{me}XOS$ (DP >7) were fermented preferentially, resulting in an accumulation of relatively high substituted XOS (DP 5-7). This accumulation, caused by degradation of higher DP's into DP 5-7 and a lack of consumption of DP 5 and 6, most likely point at the conclusion that the substituents present delayed or completely hindered fermentation. The 4-*O*-methylglucuronic acid plus hexose containing XOS were hardly fermented at all. During fermentation of all substituted XOS (partial) release of the substituents was immediately followed by rapid fermentation of the remaining xyloses. Similar observations were described by Englyst et al. [*22*] for the fermentation of arabinose side-chains of pectin, xylan and arabinogalactan by mixed populations of human faecal bacteria. These results might point at the suggestion that the kind and amount of substituents per oligomer present influences the rate of fermentation.

SCFA and lactate were formed during the fermentations performed and the total amount of these fermentation products increased with the consumption of total XOS. The

results as presented in figure 6.5 include the amounts of SCFA and lactate produced from the medium components. However, the total concentration of acids produced in the medium without carbohydrate source (fermentation-blank) was less than < 20 μmol/ ml, indicating that most acids were produced from the XOS as substrates. Such a low production of acids in the blanks by a mix of human FI was indicated by Hartemink et al. [*23*] as well.

For all fermentations described a distinction could be made between the first stage of the fermentation (0-40 hours) and a second stage (> 40 hours). In the first stage of the fermentations the pH decreased, while in the second stage the pH remained constant or even increased slightly. In the first stage of the nXOS- and AXOS-fermentations, mainly acetate and lactate were formed. Lactic acid bacteria (e.g. *Lactobacillus* and *Enterococcu*s species) and *Bifidobacterium* sp. may play an important role in this part of the fermentation, as they do not produce butyrate or propionate, but they do produce acetate and lactate [*24*]. A high concentration of acids formed might be desirable since, by a decrease in pH, the growth of potentially pathogenic micro-organisms and growth of putrefactive bacteria will be inhibited [*7,24,25*]. The preference for bifidobacteria to ferment low-substituted XOS, both *in vitro* and *in vivo*, has been described previously [*1,4*]. Contrarily, oat XOS were not selective for bifidobacteria exclusively, since *Bacteroides* sp., *Clostridium* sp., *Lactobacillus acidophilus* and *Klebsiella pneumoniae* also showed moderate growth on these substrates [*2,26*]. Also, the more branched wheat arabinoxylan hydrolysates (singly and double substituted arabinoxylo-oligosaccharides) could only be (partly) fermented by the *Bifidobacterium* sp. and *Bacteroides* sp. tested [*2*]. The latter observations corresponded well with our results that in the first stage of the fermentation of AcXOS and GlcAmeXOS besides acetate and lactate also propionate and some butyrate were formed. This is most likely due to the growth of several intestinal bacteria and not specifically of lactic acid bacteria.

In the second stage of all fermentations, in which less carbohydrate degradation was observed, also propionate and butyrate was produced. Butyrate is reported frequently to be related to several anti-tumour effects *in vitro* and in animal studies of colon cancer [*27-30*]. Butyrate was detected in the fermentations of nXOS, AXOS and $GlcA_{me}XOS$ by almost all FI, but hardly during AcXOS-fermentation. However, butyrate was observed mainly when all XOS were already degraded. Butyrate can be produced directly from carbohydrates by many different intestinal species, especially clostridia. Furthermore, some *Clostridium* species can metabolise lactate to butyrate, carbon dioxide and water [*24*]. Therefore, since in most of the XOS-fermentations lactate decreased in favour of butyrate and all XOS had already been degraded, substantial secondary fermentation through lactate is expected to have occurred.

In conclusion, the nXOS, AXOS, AcXOS and $GlcA_{me}XOS$, obtained from hydrothermally treated xylan rich by-products, were differently fermented by human FI. Both the oligosaccharide degradation patterns of the XOS and the patterns in SCFA and lactate formed depended on the particular structure of the XOS studied. Although this study aimed to reveal differences in structure in relation to fermentation it was also demonstrated that inter-

person variation occurred. Our results suggest that it is useful to correlate detailed structural features of NDO's with their behaviour in fermentation studies, to be able to better understand and control the mechanisms involved.

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# CHAPTER 7

# Concluding remarks

The anti-ulcer properties of XOS will be published by Corvo, M.L., Carvalheiro, F., Lourenço, P., Kabel, M.A., Cruz, M.E., Gírio, F., Schols, H.A.

## 7.1 Introduction

Alternative applications of agricultural by-products are of interest, because their economic value as animal feed compounds is decreasing. One of the possibilities is to perform a hydrothermal treatment of these by-products with emphasis on minimising the environmental impact (avoiding acid, alkali or organosolv additions). Such a treatment will result in a selective fractionation of (partially) degraded hemicelluloses from the ligno-cellulosics present, which can be used for different product applications [*1*].

The aim of the research described in this thesis was to characterise hydrolysates of four hydrothermally treated xylan rich by-products to obtain knowledge about the mechanisms occurring during the treatments. Furthermore, the released and (partly) degraded xylan products were purified and their possible physiological properties studied. The by-products studied are wheat bran, brewery's spent grain, corn cobs and *Eucalyptus* wood.

## 7.2 Hydrothermal treatment of xylan rich by-products

The mild hydrothermal treatment studied is an environmental friendly technology and will result in the fractionation of the different components (ligno-cellulosics and hemicellulose) of the processed by-products. Typical process conditions used for the hydrothermal treatments are temperatures ranging from 150 to 200 °C, 8-10 g of water per g of substrate and process times in a range of 10 to 600 minutes. In these kind of treatments, hemicelluloses are (partly) depolymerised, whereas little alteration is caused in lignin, and cellulose remains almost untouched [*1,2*]. The present work mainly concerned the release and structural features of the hemicelluloses (xylans) in xylan rich by-products by hydrothermal treatment.

### *7.2.1 Effect of hydrothermal treatment on structural features of xylan*

The main products resulting from the depolymerisation of the xylans during hydrothermal treatment are xylose and xylo-oligosaccharides (XOS) [*3,4*]. In chapter 2 the composition of the released XOS was studied in relation to the structural features of the xylans originally present. Therefore, xylans were extracted from the by-products by using alkali, leaving the more difficult extractable xylans in the remaining alkali residues. Subsequently, the composition and main structural features of the alkali extracted xylans were studied. Depending on the source of the by-product various (amounts of) substituents were linked to the xylans present. The molar ratio's of substituents, like arabinose and glucuronic acid, to xylose are generally considered to be a measure for the branching of the xylan [*5*]. However, it would be more appropriate to express the degree of substitution as the ratio of the number of branches attached to xylose residues to the total number of xylosyl residues in the backbone[*6*].

Alkali extracted xylan from wheat bran was mainly substituted at *O*-3 and both *O*-2 and *O*-3 of the xylosyl residues with arabinose. The molar ratio of arabinose to xylose (Ara/Xyl-ratio) found for the alkali extracted xylan was rather low (Ara/Xyl-ratio=0.4) compared to values published in literature (Ara/Xyl-ratio=0.2-1.2) [*7-10*]. Most likely, our extraction performed mainly released rather low substituted xylans present. This was also reflected in a rather high ratio of Ara/Xyl (1.0) found for xylans accumulated in the alkali residues, which represented about 25 % of the xylans originally present.

For xylans from brewery's spent grain and corn cobs arabinosyl substituents were present at *O*-3 or *O*-2 and both *O*-2 and *O*-3 of the xylosyl residues. The alkali extracted xylans from brewery's spent grain and corn cobs had a ratio of arabinose to xylose of 0.5 and 0.1 respectively, while the xylans in the remaining residues had a ratio of Ara/Xyl of 0.8 and 0.3 respectively. Additionally, besides substitution with arabinoses corn cob xylan was substituted with (4-*O*-methyl-)glucuronic acid in a ratio of uronic acid to xylose of 0.1 and 0.3 for alkali extract and residue respectively. These results are in agreement with the structural features of xylans present in brewery's spent grain and corn cobs described previously [*11-16*].

Alkali extracted *Eucalyptus* wood xylan was mainly substituted with 4-*O*-methylglucuronic acid residues (UA/Xyl-ratio=0.16) and some indications for the presence of the linkage 2-*O*-α-galactopyronosyl-4-*O*-methyl-α-D-glucuronic acid were obtained. The presence of the latter substituent has already been described to be present in xylan extracted from *Eucalyptus globulus* Labill [*16*]. However, the extraction with alkali performed was most likely hindered by the high lignin content of the wood [*17*], since 56 % of the xylans originally present was recovered in the remaining residue (UA/Xyl-ratio=0.5). Furthermore, the *O*-acetyl content of the wood was the highest for the four by-products studied (3 % (w/w)) representing a ratio of *O*-acetyl to xylose of 0.65. Usually, for hard woods the content of *O*-acetyl substituents is 3-5 % (w/w) of the total wood [*18*]. These *O*-acetyl substituents are mainly linked to the 2-*O*- and/ or 3-*O*-positions of the xylosyl residues in the backbone of xylan in hard woods [*17,19*].

Taking in account these structural features of the xylans originally present the composition of the hydrolysates obtained after hydrothermal treatment of the four by-products was studied (Chapters 2&3). The results of the composition of hydrolysates of each of the four by-products obtained at similar process conditions are summarised in table 7.1. In this table the estimated molecular weight (Mw) ranges are shown as well. The estimation of the Mw-ranges of the hydrolysates was determined by high-performance size-exclusion chromatography (HPSEC; based on pullulan standards).

The hydrolysate obtained after treatment of wheat bran mainly contained low-substituted XOS and polymeric xylan-fragments. This indicated that during hydrothermal treatment almost all arabinose originally present was removed from the xylans and XOS, and partially converted into degradation products like hydroxymethylfurfural (HMF). In the wheat bran hydrolysate both monomeric xylose and arabinose were present.

**Table 7.1** Sugar content and composition of the hydrolysates obtained after hydrothermal treatment (160 °C; 60 min; 8-10 g/g; Chapter 2) of wheat bran, brewery's spent grain, corn cobs and *Eucalyptus* wood.

| Hydrolysates | Wheat bran | Brewery's spent grain | Corn cobs | *Eucalyptus* wood |
|---|---|---|---|---|
| Mw-range (Da)[a] | $150\text{-}5\cdot10^4$ | $150\text{-}1\cdot10^5$ | $150\text{-}5\cdot10^4$ | $150\text{-}1\cdot10^4$ |
| Monomers (Ara+Xyl)[b] | 8 | 6 | 7 | 9 |
| Oligo- & polymers[b] | 25 | 38 | 50 | 56 |
| Molar composition: | | | | |
| Ara | 4 | 18 | 3 | 0 |
| Xyl | 64 | 56 | 78 | 68 |
| Gal | 4 | 5 | 5 | 9 |
| Glc | 21 | 16 | 7 | 2 |
| UA | 7 | 6 | 7 | 17 |
| Substitution[c]: | | | | |
| Total | 22 | 54 | 35 | 73 |
| Ara | 6 | 32 | 4 | 0 |
| UA | 11 | 11 | 9 | 25 |
| Ac | 5 | 11 | 22 | 48 |

[a] based on HPSEC (pullulan standards).
[b] expressed as weight percentage (dry weight).
[c] amount of substituents per 100 xylosyl residues (Ara = arabinose; UA = uronic acid; Ac = *O*-acetyl).

As was found for wheat bran, during the hydrothermal treatment of brewery's spent grain and corn cob arabinose was released from the xylans present. However, in the hydrolysates of brewery's spent grain still quite some XOS and xylan-fragments containing arabinose were present (Ara/Xyl-ratio=0.3). In the hydrolysates of corn cobs non-substituted and *O*-acetylated xylan-fragments were accumulated. In both the hydrolysates of brewery's spent grain and corn cobs monomeric xylose and arabinose were present as well. During the hydrothermal treatment of *Eucalyptus* wood some non-substituted XOS were released. Furthermore, XOS substituted with 4-*O*-methylglucuronic acid and/ or *O*-acetyl substituents represented a remarkable part of all XOS present.

### *7.2.2 Yield and mechanisms observed during hydrothermal treatment*

The hydrothermal treatments performed resulted in a relatively low release of the monomers arabinose and xylose from the xylans originally present (6-8 %). Furthermore, from the xylans originally present in wheat bran, brewery's spent grain, corn cobs and *Eucalyptus* wood 18, 40, 50 and 45 % were released respectively as XOS or xylan-fragments at the same process conditions. Part of the xylans originally present were recovered in the remaining residues; 24, 30, 31 and 34 % for wheat bran, brewery's spent grain and *Eucalyptus* wood

respectively. The xylan-material not recovered in hydrolysates or residues can be accounted as losses, for example by secondary reactions concerning the conversion of pentoses into furfural and HMF, which occurs at high temperatures [*20*].

Data of hydrothermal treatments of several lignocellulosic products were reviewed by Garrote et al. [*2*]. In most of the cases the recovery of hemicellulose is in the range of 65-82 % of the initial content. However, in general no distinction is made between monomeric/ oligomeric recoveries and detailed characterisation of the recovered hemicellulosic products has not been the aim of many studies yet.

Different degradation patterns were observed depending on the feedstock material subjected to hydrothermal treatment. Arabinose was rather easily split off by hydrothermal treatment from the xylan-backbone of wheat bran, brewery's spent grain and corn cobs. Similar reactivity of arabinose was described during heat treatments of arabinoxylan rich corn stalk [*21*] and arabinogalactan containing coffee beans [*22*]. Furthermore, *O*-acetyl substituents were (partly) released from the xylans present, becoming available to depolymerise the xylan by acid catalysis [*23*]. From the xylans present in brewery's spent grain, corn cobs and *Eucalyptus* wood 26, 38, 35 % of all *O*-acetyl substituents present was released respectively. From xylans present in wheat bran no *O*-acetyl substituents were released at all. However, the original amount of *O*-acetyl substituents present in brewery's spent grain and wheat bran was rather low compared to corn cobs and *Eucalyptus* wood. Therefore, the effect of the release of *O*-acetyl substituents in catalysing the depolymerisation of the xylans by a drop in pH was mainly expected to have occurred during hydrothermal treatments of corn cobs and *Eucalyptus* wood. Part of the uronic acids were released during the treatments performed, this mainly applies to the treatment of *Eucalyptus* wood.

To study the effect of hydrothermal treatment with respect to the products formed of an arabinoxylan and an *O*-acetylated glucuronoxylan rich by-product, the hydrolysates of brewery's spent grain and *Eucalyptus* wood were studied in more detail (Chapter 3). Both hydrolysates were fractionated first by anion exchange chromatography followed by size exclusion chromatography. Hereby, several pools were obtained representing material with different types of substitution and/ or $DP_{(top)}$ (Fig. 7.1). For the $DP_{top}$ of the pools of brewery's spent grain hydrolysate the Mw of the sugar-material having the maximum RI-value during elution by HPSEC was determined ($Mw_{top}$; based on pullulan standards; Chapter 3). It was assumed that this material eluting at the maximum RI-value mainly consisted of arabinoxylan-fragments. Therefore, the $Mw_{top}$ was divided by the residue mass of a pentose (132) resulting in the $DP_{opt}$ (Fig. 7.1). The DP-range of the pools of *Eucalyptus* wood hydrolysates was determined from the MALDI-TOF mass spectra of these pools.

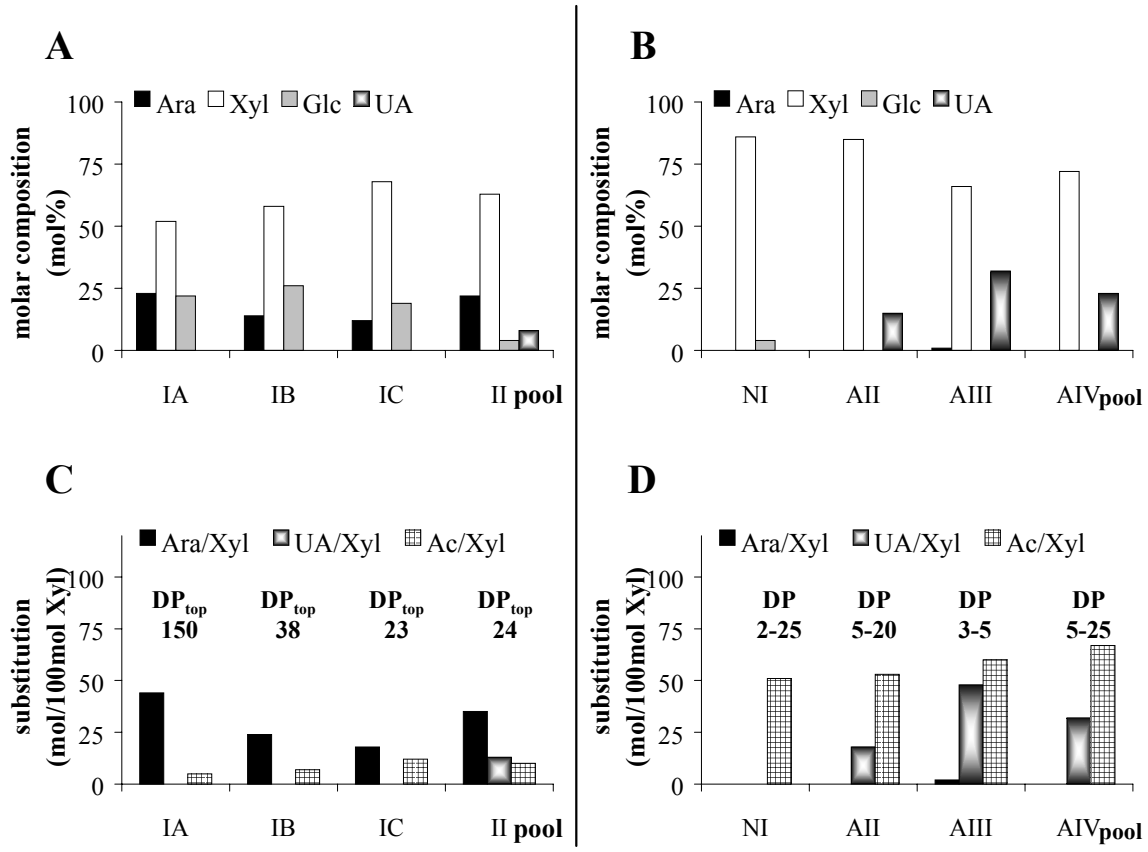


**Figure 7.1** Molar composition (main residues present), substitution (arabinose, uronic acid or *O*-acetyl per 100 xylosyl residues) and DP or $DP_{(top)}$ (degree of polymerisation (at the top of population); calculated as described in text §7.2.2) of pools obtained by anion-exchange and size-exclusion chromatography of hydrolysates of brewery's spent grain (A & C) and *Eucalyptus* wood (B & D) (Chapter 2 and 3).

The hydrolysates of both brewery's spent grain and *Eucalyptus* wood prepared under similar process conditions represented about 50 % of the xylans originally present. However, from *Eucalyptus* wood xylan mainly material was released having a Mw smaller than 5 kDa, while from brewery's spent grain xylan about 35 % of the released material had a higher Mw (Mw 5-500 kDa). Most likely, the partial release of the abundantly present *O*-acetyl substituents in *Eucalyptus* wood indeed had catalysed the depolymerisation of the xylan present (*vide infra*).

Furthermore, for the treatment of the arabinoxylan rich brewery's spent grain it was concluded that the released xylan-fragments having a rather high $DP_{top}$ (150) also represented a rather high Ara/Xyl-ratio (0.44). Released fragments having a lower $DP_{top}$ (38) represented a lower Ara/Xyl-ratio (0.24), while released oligosaccharides ($DP_{top}$ 23) represented an Ara/Xyl-ratio of 0.18. These results may point at the conclusion, that hydrolysis of the xylan backbone mainly occurred after removal of arabinose groups.

In spite of these first observations, the precise mechanisms occurring during hydrothermal treatments influenced by the structural features of the xylans present remained unclear.

## 7.3 Separation and identification of oligosaccharides

### *7.3.1 Chromatographic methods*

To study the material released from the by-products during treatment, the hydrolysates obtained were analysed. Hereto, both high performance anion-exchange chromatography (HPAEC) and high performance size exclusion chromatography (HPSEC) appeared to be useful methods (Chapter 2). HPSEC was performed to study the hydrodynamic volume of the released xylan-fragments, giving information about the depolymerisation of the xylans during treatment. Its use is a fairly well established technique and it has been used to estimate the weight-average molecular weights of (enzymatic degraded) wheat flour arabinoxylans [*5*], the molecular weight distribution of extracted wheat bran arabinoxylans [*7*], and the homogeneity of extracted maize and sorghum (glucurono-)arabinoxylans [*6*].

By using HPAEC, giving much higher resolutions for oligosaccharides compared to HPSEC, (substituted) XOS (DP 2-10) were separated well [*24,25*]. In combination with the HPAEC-elution behaviour of the previously purified and fully identified enzymatically derived XOS from wheat flour xylan [*26-28*], barley xylan [*29*] and sorghum xylan [*30*], (part of) the XOS substituted with arabinose and (4-*O*-methyl-)glucuronic acid present in the hydrolysates studied could be identified (Chapter 2 and 3). Furthermore, to overcome the rather unpredictable elution behavior in HPAEC coupling of HPAEC to mass spectrometry was established to identify the mass of (unknown) oligomers (§7.3.2). The masses of the (unknown) oligomers in the peak fractions in combination with the retention times give valuable information for identifying these oligomers.

A disadvantage of performing HPAEC in the characterisation of oligosaccharides substituted with ester groups (e.g. *O*-acetyl substituents) is that typically eluents are used having a high pH. Hereby, alkali-labile esters (*O*-acetyl) will be removed. To be able to characterise the *O*-acetyl substituted XOS other chromatographic methods were tested.

First, HPAEC at pH 5 was studied. An example of a HPAEC-elution pattern at pH 5 of the complex *Eucalyptus* wood hydrolysate containing (*O*-acetylated) neutral and (*O*-acetylated) negatively charged XOS is shown in figure 7.2. The eluent was collected and combined in six pools, which were subjected to MALDI-TOF MS to reveal the molecular mass of the XOS present (Fig. 7.2). HPAEC at pH 5 in combination with MALDI-TOF MS has recently been used for the separation and identification of oligogalacturonides as well [*31,32*]. From figure 7.2 it can be seen that besides separation of the (*O*-acetylated) neutral and (*O*-acetylated) negatively charged XOS based on the presence of 4-*O*-methylglucuronic acid substituents, also the charge density influenced the separation. XOS containing one 4-*O*-

methylglucuronic acid substituent having a lower Mw were more retained than higher Mw ones having also one 4-*O*-methylglucuronic acid substituent, while XOS containing two 4-*O*-methylglucuronic acid substituents were eluted only at much higher salt concentrations. The conditions used for HPAEC at pH5 were applied to preparative anion-exchange chromatography using Source Q-column material (Chapter 3). Hereby, larger amounts of neutral XOS separated from acidic XOS were obtained.

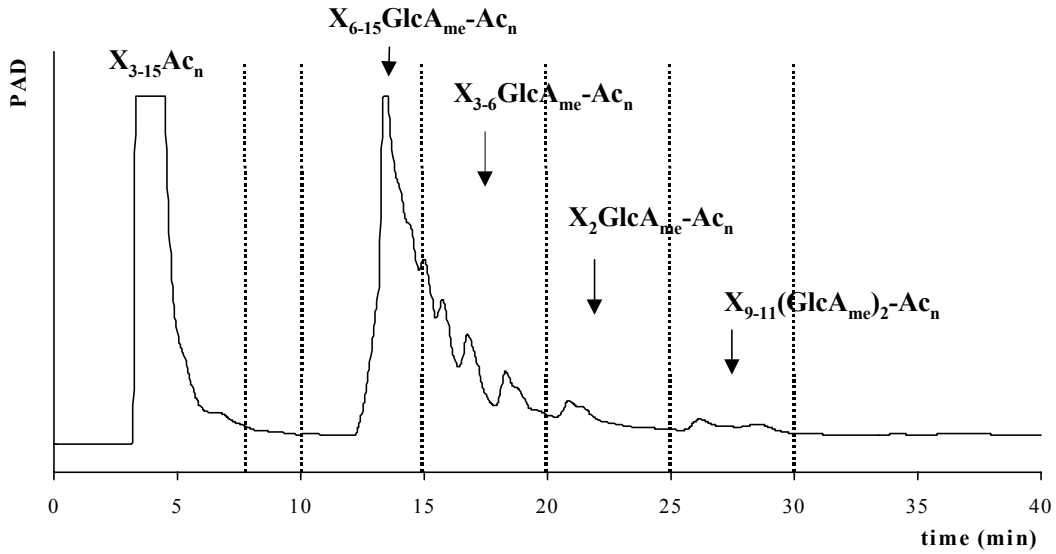


**Figure 7.2** HPAEC (pH 5) elution pattern of an *Eucalyptus* wood hydrolysate; a gradient was used of 0-17 mM of acetic acid during 5 min followed by 17-30 mM of acetic acid during 35 min at 0.5 ml/min (X = xylose; $GlcA_{me}$ = 4-*O*-methylglucuronic acid; Ac = *O*-acetyl).

Further separation of the neutral and *O*-acetylated XOS was performed by using reversed phase (RP) HPLC (Chapter 5). In general, separation by RP-HPLC is based on differences in hydrophobicity [*33*]. The eluens was monitored by using an evaporating light scattering (ELS) detector. ELS detection allows the use of methanol as eluent, while UV detection is not compatible with this eluent. Additionally, by using standards and comparing similar fractions ELS detection also allows quantification of oligosaccharides in different mixtures. The *O*-acetylated XOS present in the hydrolysates of *Eucalyptus* wood were separated based on the number and position of the *O*-acetyl substituents. The higher the number of *O*-acetyl substituents per oligomer the higher was the affinity with the hydrophobic column material. However, XOS having a different DP, but the same number of *O*-acetyl substituents mostly coeluted. Similar observations were described by Pauly et al. for acetylated xyloglucan oligosaccharides [*34*]. They showed that the presence of an *O*-acetyl substituent on the galactosyl-residue significantly increases the retention time.

Another column, a TSKGel amide-80 column (Tosohaas) was tested as well to separate *O*-acetylated XOS. Again, a separation based on the number of *O*-acetyl substituents per oligomer was achieved. In contrary with reversed phase chromatography, on the TSKGel amide-80 column the highly *O*-acetylated oligomers eluted first followed by XOS containing less *O*-acetyl substituents (Fig. 7.3). However, also by using the TSKGel amide-80 column some coelution occurred, which makes the separation of complex oligosaccharide-mixtures more difficult.

RP-HPLC was used in stead of the use of the TSKGel amide-80 column to separate and study the structural features of the more simple and less substituted XOS. Performing RP-HPLC the less toxic methanol could be used as eluent in stead of acetonitril necessary for the elution of the *O*-acetylated XOS from the TSKGel amide-80 column.

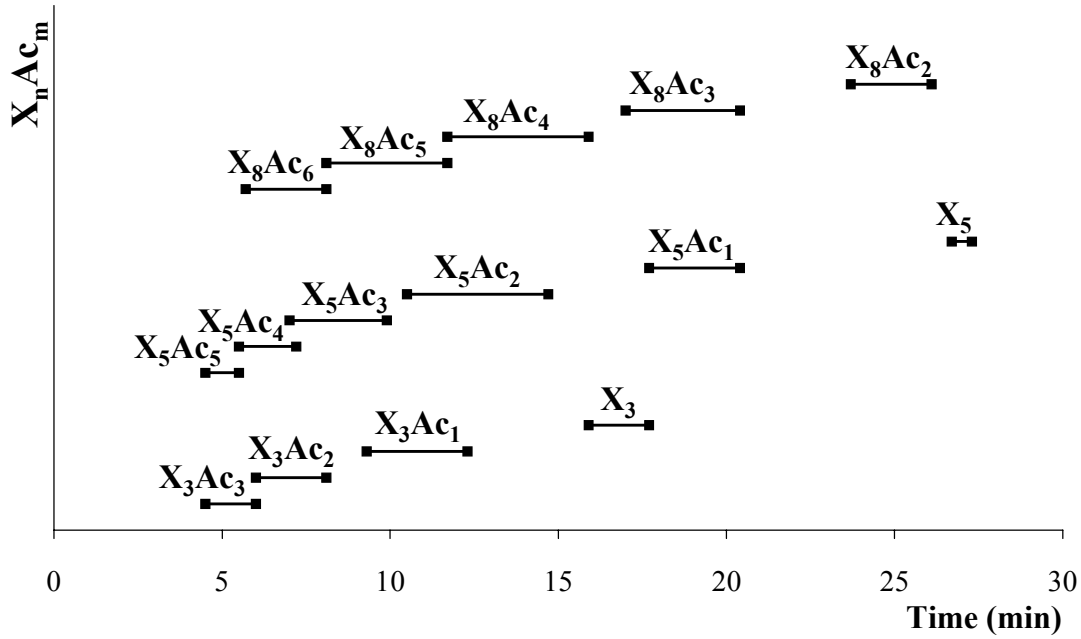


**Figure 7.3** Part of a TSKGel Amide-80 elution pattern of several *O*-acetylated xylo-oligosaccharides monitored by MALDI-TOF MS in fractions collected (0.3 min/fraction); a gradient was used of acetonitril/water from 75/25v/v% to 50/50v/v% in 50 minutes at 1 ml/min (X = xylose; Ac = *O*-acetyl; n = number of xyloses; m = number of *O*-acetyl substituents).

The results obtained by the RP-HPLC as well as the results obtained by using the TSK Gel Amide-80 column reflect the potential of these methods in the separation of oligosaccharides. Additionally, these two methods provided a good alternative for the commonly used HPAEC at high pH, especially for the separation of oligosaccharides substituted with alkali-labile esters. For the separation of complex mixtures a pre-separation by size-exclusion

chromatography might be needed. Also, combining the RP-HPLC and TSKGel Amide-80 separation could be helpful in a complete separation of a variety of *O*-acetylated XOS in complex mixtures.

### *7.3.2 Spectrometric and spectroscopic methods*

In the early nineties nuclear magnetic resonance (NMR) spectroscopy was the preferred technique in order to fully characterise structures of oligosaccharides. Nowadays, both matrix assisted laser desorption/ ionisation time-of-flight (MALDI-TOF) and electrospray MS have become routine and powerful techniques in the identification of structural features of oligosaccharides [*35-37*], even to determine the positions of residues within oligosaccharides [*38,39*].

The arabino-xylo-oligosaccharides mainly present in hydrolysates of brewery's spent grain and corn cobs were difficult to identify in detail by MS, because the mass of an arabinosyl and xylosyl residue is the same (m/z 132). However, the presence of *O*-acetyl and (4-*O*-methyl-)glucuronic residues could be confirmed using MS. Especially, regarding the hydrolysate of *Eucalyptus* wood establishing the number of substituents (*O*-acetyl and 4-*O*-methylglucuronic acid) per oligomer present in a complex mixture was rather straightforward by using MS (Chapter 3).

The off-line coupling of HPAEC to MALDI-TOF MS was established to overcome the rather unpredictable elution behavior of HPAEC (Chapter 4). For MALDI-TOF MS on-line desalting of the HPAEC eluent was performed using an anion self regenerating suppressor (ASRS) in series with a cation self regenerating suppressor (CSRS). The ASRS permitted the exchange of acetate ions with hydroxide ions while the CSRS exchanged sodium ions with hydronium ions. The continuous desalting of the eluent was achieved by the electrolysis of pure water in both suppressors. The desalted eluent was automatically collected by using a 96-well plate fraction collector. Subsequently, the collected fractions were transferred to a MALDI-TOF gold plate using a computer controlled robot. On-line membrane suppressors which can remove the sodium ions from the mobile phase before analysis with thermospray MS [*40-42*], or before analysis with MALDI-TOF MS [*43*] have already been described to be successful. But, in all these studies large amounts of regenerating acids (e.g. $H_2SO_4$) were used, which may cause artefacts [*40,42*]. Furthermore, in these applications only the sodium is removed from the mobile phase of HPAEC, leaving the oligosaccharides in acetic acid. However, high concentrations of acetic acid may disturb the crystallization of the samples for MALDI-TOF analysis. Off-line desalting methods are described in literature as well. These methods concerned mainly rather labor-intensive size exclusion columns [*44*] or ion-exchange columns (e.g. Dowex $H^+$ or $OH^-$ resins (Bio-Rad)) [*26,27*] used to desalt oligosaccharides separated by preparative HPAEC prior to NMR and MS characterisation.

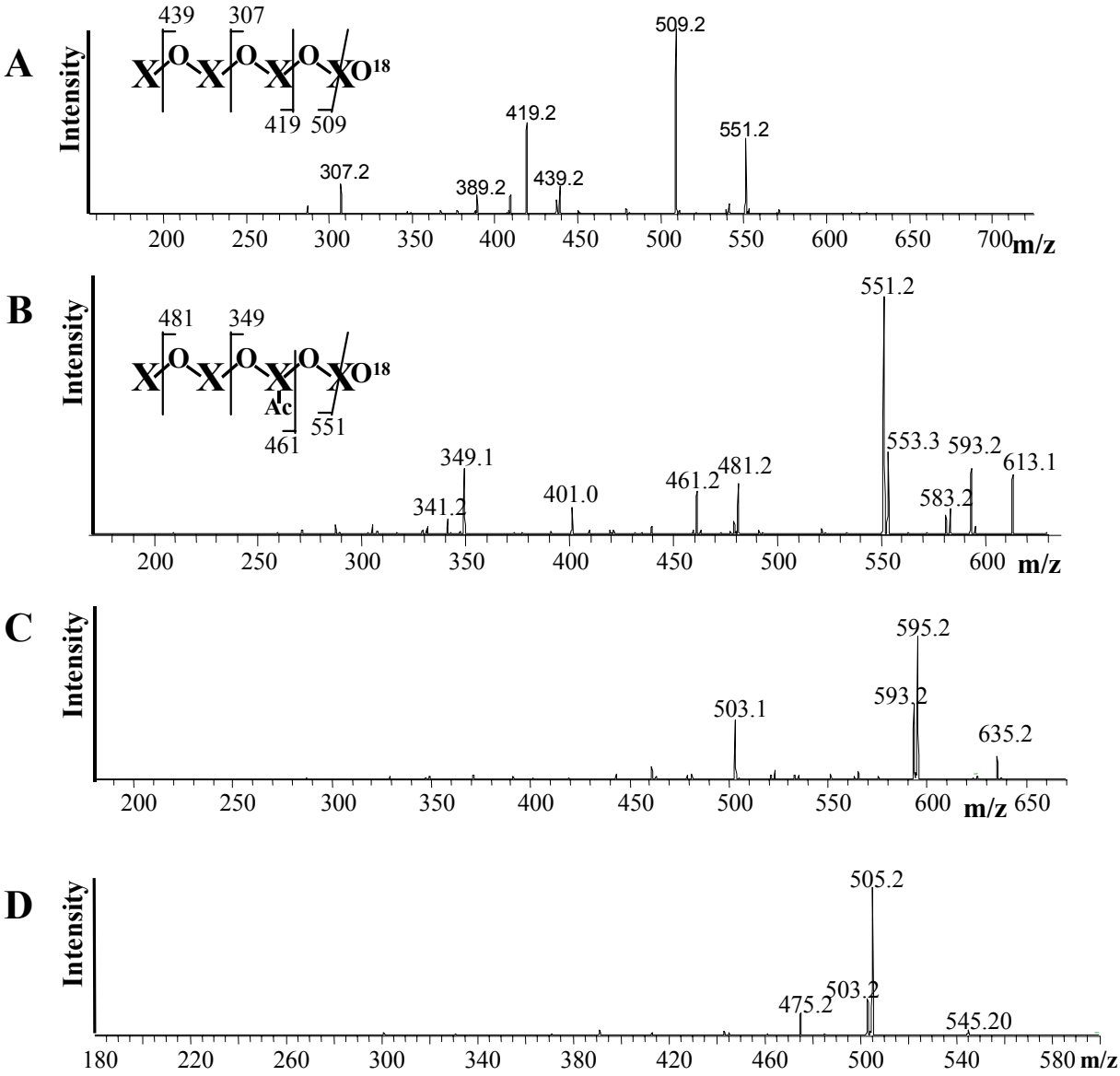


**Figure 7.4** $MS^2$ spectra of an $O^{18}$-labeled (sodium-adducted) xylo-tetramer having zero (A), one (B) or two (C) *O*-acetyl substituents and of an $O^{18}$-labeled (sodium-adducted) xylo-trimer having three *O*-acetyl substituents (D); LCQ Ion-trap (Finnigan MAT 95); 509 (in A) and 551 (in B) = internal (ring)-fragmentation.

An advantage of the use of RP-HPLC in the identification of oligomers is that in general solutions without salts, like pure water and methanol, enable the on-line coupling to a mass spectrometer. RP-HPLC coupled on-line to both an electrospray mass spectrometer and an ELS detector provided information about the order of elution of the various *O*-acetylated xylo-oligomers in the *Eucalyptus* wood hydrolysate (§7.3.1).

Furthermore, tandem mass spectrometry ($MS^n$) is reported to be useful in the identification of the order of sugar residues within oligosaccharides [*38,39*]. On $MS^n$ -analysis

of oligosaccharides a range of fragment ions is observed that are the result of the breaking of one or more bonds in the oligosaccharide ion that was selected as precursor. In general, the glycosidic bond between sugar residues is rather weak and therefore masses of fragment ions that are the result of breaking these bonds are predominantly observed in $MS^n$-spectra. An example of a $MS^2$-fragmentation spectrum of a xylo-tetramer is presented in figure 7.4A, which shows a complete range of fragmentation ions confirming the structure of this xylo-tetramer. However, fragmentation of the *O*-acetylated XOS in the *Eucalyptus* wood hydrolysate studied was most likely hindered by the *O*-acetyl substituent(s) present. The higher the number of *O*-acetyl substituents per oligomer the less fragment ions as a result of breaking glycosidic bonds between the monomers were observed in the $MS^n$-spectra (Fig. 7.4). Therefore, the position of the *O*-acetyl substituted xylose or xylosyl residues within the xylo-oligomers was difficult to distinguish. Contradictory, the $MS^2$-spectrum of the $X_4Ac$ presented in figure 7.4B shows sufficient fragment ions to determine the *O*-acetylated xylosyl residue, which is presented in this figure as well. However, other xylo-tetramers having the *O*-acetyl substituents in a different position (§7.4.2) could not be characterised by $MS^n$, which indicated that the position of the *O*-acetyl seemed to be important as well to obtain enough fragment ions enabling the elucidation of the structures.

The use of the fragmentation of oligosaccharides by using post-source decay (PSD) MALDI-TOF MS was shown by Van Alebeek et al. [*45*]. This technique was reported to permit the determination of the positions of methyl esters or other substituents in the sequencing of methyl-esterified oligogalacturonides [*45*]. Nevertheless, also by using PSD MALDI-TOF MS no fragmentation of *O*-acetylated XOS was obtained.

Since the use of RP-HPLC-MS and $MS^n$ was not successful in the identification of the location of the *O*-acetyl substituents within XOS in the hydrolysate of *Eucalyptus* wood, NMR spectroscopy was used. NMR spectroscopy is a commonly used and powerful method in the identification of oligosaccharides [*27,46-49*]. First, a pool of several xylo-tetramers, each containing one *O*-acetyl substituent, which were located at different positions, was obtained from the *Eucalyptus* wood hydrolysate by size exclusion chromatography. Subsequently, these *O*-acetylated xylo-tetramers were separated by RP-HPLC based on the position of the *O*-acetyl substituent and were analysed by NMR analysis (Chapter 5). In stead of one signal indicating the position of the *O*-acetyl substituent, for each *O*-acetylated xylo-tetramer $^1$H-NMR chemical shifts were obtained corresponding to both 2-*O*- and 3-*O*-acetylated xylosyl residues. In these *O*-acetylated tetramers *O*-acetyl migration was proven to have occurred (§7.4.2). The migration was expected to have occurred during sample pre-treatment for NMR spectroscopy (evaporating methanol at room temperature, freeze-drying and freeze-drying in $D_2O$). To avoid such *O*-acetyl migration RP-HPLC coupled to a NMR unit without further sample treatment was used. Hereby, several *O*-acetylated xylo-tetramers were purified and structurally characterised. The $^1$H-NMR chemical shifts obtained per xylo-tetramer pointed at the presence of 2-*O*- or 3-*O*-acetylated terminal or internal xylosyl residues. The $^1$H-NMR

chemical shifts obtained for 2-*O*- or 3-*O*-acetylated terminal xylosyl residues and for non-acetylated xylose and xylosyl residues influenced by neighbouring *O*-acetylated-xyloses have not been determined before to our knowledge. Teleman et al. [*19*] and Van Hazendonk et al. [*50*] already presented the $^{1}$H-chemical shifts of 2-*O*- and 3-*O*-acetylated internal xylosyl residues and indicated the influence of the *O*-acetyl substituent on neighbouring xyloses.

A disadvantage of LC-HPLC-NMR is that still relatively high amounts of sample are needed for the NMR analysis (50-150 µg per RP-HPLC peak applied to NMR), while often only very low amounts of pure material are available. Furthermore, the amount of sample applied to RP-HPLC is limited because of the risk of overloading the column. However, the development of nano-technology is on-going and most likely a reduction in the amount of sample needed for HPLC-NMR will be reached in the near future [*46,51*].

## 7.4 Structural characteristics of xylo-oligosaccharides present in brewery's spent grain and *Eucalyptus* wood hydrolysates

### *7.4.1 Arabinoxylo-oligosaccharides from barley are O-acetylated*

Fractionation of the hydrothermally treated brewery's spent grain resulted in three pools of which two (1A and B; Fig. 7.1) contained relatively high molecular weight xylan-fragments, singly and doubly branched with arabinose ($X_nA_m$), separated from a pool of XOS (DP < 50) less branched with arabinose (1C; Fig. 7.1) In all pools obtained the arabinosyl residues were located at the 3-*O*- and/ or 2-*O*-positions of the xyloses present (Chapter 3).

Interestingly, endoxylanase I-digestions [*26,52*] of all three brewery's spent grain-pools (1A-C) resulted in oligomers, which contained *O*-acetyl substituents. The content of *O*-acetyl substituents present in pool 1A, B and C was 0.7, 1 and 3 % (w/w) respectively. In the original brewery's spent grain hydrolysate this content was 0.9 % (w/w). A MALDI-TOF mass spectrum of endoxylanase I-treated pool 1B, also representative for similarly treated pool 1A and 1C, is shown in figure 7.5. To confirm the presence of the *O*-acetyl substituents the endoxylanase I-digested material was treated with alkali, which removes alkali-labile *O*-acetyl esters. By MALDI-TOF MS it was indeed confirmed that after treatment with alkali the masses of the *O*-acetylated XOS indicated in figure 7.5 lacked, leaving only masses of a series of non-acetylated pentoses (not shown).

In the MALDI-TOF mass spectra of the original hydrolysate of brewery's spent grain used for fractionation the masses corresponding with *O*-acetylated XOS were hardly present. Most likely, if *O*-acetylated XOS (DP < 20) were present in the hydrolysate their concentration was too low to detect by MALDI-TOF MS. Additionally, *O*-acetylated xylan-fragment having a DP higher than 20 are difficult to detect by MALDI-TOF MS.

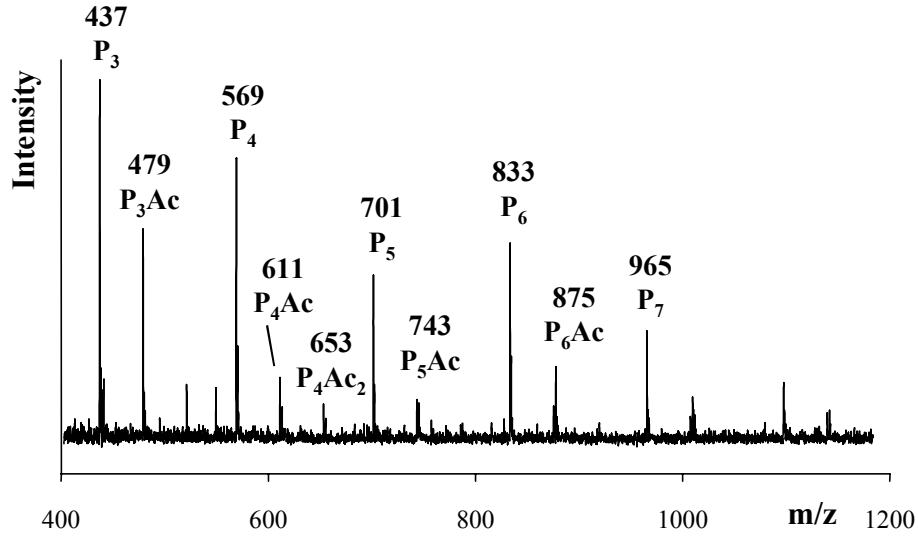


**Figure 7.5** MALDI-TOF mass spectrum of endoxylanase I-digested brewery's spent grain pool B. Oligosaccharides are analysed as sodium-adducts (P = pentose; Ac = *O*-acetyl).

The precise location of the *O*-acetyl substituents within the (arabino-)xylo-oligosaccharides has not been established yet. To our knowledge only few publications reported the presence of *O*-acetyl substituents in xylans from grasses [*53,54*] and is it for the first time that *O*-acetyl substituents are presented to occur in xylan-fragments obtained from cereals. A reason to explain the fact that the *O*-acetyl substituents are overseen in general can be that studies regarding the structural features of xylans normally include alkali-extractions to purify the xylans prior to characterisation. This way, information about the presence of ester-linked substituents (e.g. *O*-acetyl) is lost.

Purification of the hydrothermally treated brewery's spent grain resulted in a pool containing charged xylan-material (pool II; Fig.7.1). Most likely, the recovered xylan-fragments were still partly substituted with (4-*O*-methyl-)glucuronic acid residues. However, the charged pool represented less than 1 % of the material obtained after hydrothermal treatment of brewery's spent grain and was not studied in more detail.

### *7.4.2 O-acetylated and/ or 4-O-methylglucuronic acid containing xylo-oligosaccharides*

The fractionation of the hydrothermally treated *Eucalyptus* wood resulted in a neutral pool, mainly consisting of *O*-acetylated XOS (N1; Fig. 7.1), and three pools of charged 4-*O*-methylglucuronosyl containing xylan-fragments (Chapter 3). Two of these pools contained a series of (*O*-acetylated) XOS carrying *one* 4-*O*-methylglucuronic acid (AII and AIII; Fig. 7.1), while the third charged pool contained (*O*-acetylated) XOS and xylan-fragments substituted with *two* 4-*O*-methylglucuronic acids (AIV; Fig. 7.1). Additionally, a series of XOS containing both 4-*O*-methylglucuronic acid and a hexose, most likely galactose, was

detected in the pools AII, AIII and AIV. The linkage 2-*O*-α-galactopyronosyl-4-*O*-methyl-α-D-glucuronic acid is described for xylan extracted from *Eucalyptus globulus* Labill as well [*16*].

The neutral *O*-acetylated XOS were characterised in more detail by using a combination of RP-HPLC, MS and NMR. Knowing the positions of the *O*-acetyl substituents of the released XOS might be helpful in understanding the mechanisms occurring during hydrothermal treatment. The precise position of the *O*-acetyl substituent within 6 xylo-tetramers and 4 xylo-trimers was determined (§7.3.2; Chapter 5).

To our knowledge for the first time *O*-acetyl migration was proven to occur in xylo-oligosaccharides (§7.3.2). Mainly *O*-acetyl migration within the same xylosyl residue was observed. An equilibrium was established between the 2-*O*- and 3-*O*-acetyl substituted xylo-oligomers in favour of the 3-O-acetylated compound, regardless the original position (2-*O*- or 3-*O*-) of the *O*-acetyl substituent. Based on these observations, it is expected that *O*-acetyl migration already might have occurred during hydrothermal treatment of the *Eucalyptus* wood. Therefore, the position and distribution of the *O*-acetyl substituents in the xylo-oligomers analysed could not be extrapolated directly to the xylan-structures originally present and does therefore not allow conclusions about the mechanisms of degradation during hydrothermal treatment. It even could be possible that *O*-acetylation in native xylans occurs at only one position (2-*O*- or 3-*O*-) of the xylosyl residues and that external circumstances influences migration and the distribution of the *O*-acetyl substituents further on.

A more detailed characterisation of the (*O*-acetylated) charged XOS in order to determine the exact location of the 4-*O*-methylglucuronic acid and *O*-acetyl substituents present was not performed, mainly due to a lack of time.

## 7.5 Biological activity of xylo-oligosaccharides

Part of the aim of the European-project was to study possible uses for the different XOS obtained during the treatments described in food and pharmaceutical industries. Therefore, some physiological properties of the variously substituted XOS obtained from hydrothermally treated brewery's spent grain and *Eucalyptus* wood were tested. Both fermentability by the human intestinal flora present in faecal inocula and anti-ulcer activity were studied.

### *7.5.1 Fermentability of XOS*

One of the strategies to increase the number of health promoting bacteria in the colon, mainly *Bifidobacterium* and *Lactobacillus*, is to supply these bacteria with specific non-digestible oligosaccharides (NDO), which are not degraded in the upper gastrointestinal tract and are less assimilated by undesirable flora present [*55*]. To study whether the XOS obtained during hydrothermal treatment could be assimilated by the human intestinal flora in general and

whether substituents present might influence fermentation, XOS with various substituents were fermented *in vitro* by faecal inocula (FI) (Chapter 6). Generally, FI are accepted to be a representative sample from the intestinal flora [*56*]. Furthermore, the production of short-chain fatty acids (SCFA) and lactate was studied. The type of bacteria grown were not analysed, since the aim of our study was to analyse the correlation between the fermentation and structural features of substituted XOS mainly based on degradation patterns rather than performing a complete microbiological study.

Non-substituted XOS (nXOS) and arabino-XOS (AXOS) were fermented faster than the more complex structures of acetylated XOS (AcXOS) and XOS containing an 4-*O*-methylglucuronic acid group ($GlcA_{me}XOS$). However, after an adaptation time the $GlcA_{me}XOS$ were fermented in the same rate as the nXOS and AXOS. The XOS containing the substituent 4-*O*-methylglucuronic acid-hexose were hardly fermented at all.

Studying the fermentation of AcXOS and $GlcA_{me}XOS$ by MALDI-TOF MS in more detail, mainly masses were present representing substituted XOS and no masses were observed representing a series of linear XOS. Therefore, it was concluded that during fermentation of the substituted XOS (partial) release of the substituents was immediately followed by rapid fermentation of the remaining xylo-oligomers. Similar observations were described by Englyst et al. for time-course measurements of fermentations of several polysaccharides by mixed populations of human faecal bacteria [*57*]. They reported that arabinose side-chains of pectin, xylan and arabinogalactan were co-utilised with the backbone sugars. These results might point at the suggestion that the kind and amount of substituents per oligomer present influences the rate of fermentation.

The importance to be able to distinguish between differently substituted (xylo-) oligosaccharides was also recently indicated by Van Laere et al. [*28*], who included linear XOS and arabino-xylo-oligosaccharides (AXOS) in a fermentation study of a range of (complex) plant cell wall derived oligosaccharides. In this study it was found that linear XOS were fermented by more intestinal strains tested as compared to the branched AXOS.

In the first stage (0-40 hours) of the fermentations of nXOS and AXOS mainly acetate and lactate were formed. Lactic acid bacteria (e.g. *Lactobacillus*, *Bifidobacterium* and *Streptococcu*s species) may play an important role in this part of the fermentation, as they do not produce butyrate nor propionate, but they do produce acetate and lactate [*58*]. The fermentations of AcXOS- and $GlcA_{me}XOS$ resulted in a lower lactate production, while the concentration of propionate and butyrate increased. A high concentration of acids formed might be desirable since, by a decrease in pH, the growth of potentially pathogenic micro-organisms and growth of putrefactive bacteria will be inhibited [*55,59*].

Mechanisms responsible for the health effects as a result of microbial growth in the human intestine are not clearly established yet. In order to understand such fermentation-mechanisms more precise detailed structural elucidation of NDO's in general is important. The latter is emphasised by the results described in Chapter 6 obtained for the differently substituted XOS.

### *7.5.2 Anti-ulcer properties of XOS*

Plant-derived polysaccharides as well as oligosaccharides can stimulate the operation of immune defence as reviewed by Yamada et al. [*60*] or act as specific ligands attached to host cell surface glycoconjugates that inhibits the pathogen cell adherence [*61*]. Other pharmaceutical applications have been reported, such as the reduction of the incidence of otitis media in humans by orally ingesting oligosaccharides [*62*] or meningitis prevention through the use of vaccines containing poliribosyl ribitol phosphate oligosaccharide [*63*].

Within the frame of the European-project, of which the research described in this thesis was also part, the anti-ulcer biological activity of different XOS series extracted from hydrothermally treated *Eucalyptus* wood, brewery's spent grain and corn cobs was studied.

**Material and methods (by Corvo et al., INETI, Portugal)**

Male wistar rats weighting 250-300g were obtained from CRIFFA (Barcelona, Spain). Animals were fed with standard laboratory food and water *ad libitum*. All animal experiments were carried out with the permission of the local animal ethical committee, and in accordance with the Declaration of Helsinki.

The preparation of gram-quantities of XOS from hydrothermally treated *Eucalyptus* wood (DP2-50) and of XOS from hydrothermally treated brewery's spent grain and corn cobs (DP 3-12) were collected from the hydrolysates by performing preparative size-exclusion chromatography (Chapter 3; Superdex 30 column (Amersham); 25 ml/min).

Fifteen minutes before the administration of ethanol (ulcer-inducing agent) to overnight fasted male Wistar rats, the XOS series were orally administered (po) with 2 animals per group. After one hour of ethanol administration, animals were sacrificed and gastric ulceration was scored subjectively concerning degree of haemorrhage and severity of ulcerative lesions. Inhibition of gastric ulcers by more than 50 percent suggests cytoprotective activity. Atropine at a dose of 50 mg/kg of body weight was used as reference agent. To evaluate the cytoprotective activity, a subjective score scale was elaborated as following: score 0 - no ulceration (no inflammation); score 2-3 - Inhibition of gastric ulcers by 50 percent; score 5 - Control animals (maximum of ulceration observed). The statistical analysis was performed with ANOVA test with a significance interval of 95 %.

The results obtained in studying the anti-ulcer activities of the structurally different XOS are presented in Table 7.2.

Both XOS from hydrolysates of corn cobs and brewery's spent grain (500 mg/kg) have shown to have some cytoprotective effect, since the stomachs analysed showed signs of inflammation without severe lesions (score 1, table 7.2). However, the stomachs analysed after the administration of XOS from hydrothermally treated corn cobs showed gas production as side effect. XOS obtained from hydrolysates of *Eucalyptus* wood were shown to be the best cytoprotector (score 0.5; table 7.2) of all XOS tested at similar doses (500 mg/kg). The tested XOS series compared favourably with a commercial XOS, at a dose of 500 mg/kg, since less anti-ulcer activity was obtained (score 2.5). For all tested XOS series, the anti-ulcer activity was dose-dependent.

**Table 7.2** Anti-ulcer activities of XOS from hydrothermally treated corn cobs, brewery's spent grain (BSG) and *Eucalyptus* wood in rats provoked by ethanol (ulcer-inducing agent). NU, number of ulcers; I, signs of inflammation; MI, minor inflammation; G, stomach gas production.

| Group | Dose (mg XOS/ kg bodyweight) | Observation | Score |
|---|---|---|---|
| Normal animals (no ethanol administration) | - | normal stomach | 0 |
| Negative control (only ethanol administration) | - | NU > 10 | 5 |
| **Commercial XOS** | **500** | **NU = 3-7; I** | **2.5** |
| XOS from corn cobs | 300 | NU = 3-6; I, G | 2.5 |
| **XOS from corn cobs** | **500** | **NU = 0-2; I, G** | **1** |
| XOS from BSG | 300 | NU = 3-5; I | 2.5 |
| **XOS from BSG** | **500** | **NU = 0-2; I** | **1** |
| XOS from *Eucalyptus* wood | 300 | NU = 0-5; I | 2 |
| **XOS from *Eucalyptus* wood** | **500** | **NU = 0-1; MI** | **0.5** |

The mechanisms responsible for the differences in anti-ulcer activity between XOS with different substituents have to be investigates further. However, the remarkable activity showed for the XOS obtained from the *Eucalyptus* wood hydrolysates could be the effect of the high amount of *O*-acetyl substituents and/ or the 4-*O*-methylglucuronic acids present lacking in the other XOS tested. Therefore, in further research it would be of interest to make a distinction between *O*-acetylated and 4-*O*-methylglucuronic acid containing XOS in anti-ulcer studies to be able to study the effect of these substituents on the occurrence of ulcers in the stomach of rats.

## 7.6 Conclusions

From this research it was concluded that by performing hydrothermal treatments of the xylan-rich by-products a variety of differently substituted XOS (DP 2-50) and xylan-fragments (DP >50) can be obtained. These XOS included linear XOS, xylan-fragments substituted with arabinoses, *O*-acetylated XOS and (*O*-acetylated) XOS substituted with one or two 4-*O*-methylglucuronic acid(s).

HPAEC, RP-HPLC, RP-HPLC-MS, MS, RP-HPLC-NMR and NMR spectroscopy showed to be very useful for the separation and characterisation of the detailed structures of the substituted XOS. Furthermore, the use of these methods resulted in the recognition of

some structural features, which have not been reported before to our knowledge. *O*-acetyl substituents were found to occur not only in hard wood xylans as shown in literature, but also in xylan-fragments obtained from cereals (brewery's spent grain). Additionally, 2-*O*- or 3-*O*-acetyl substituted terminal xylosyl residues were analysed by NMR to occur in XOS present in the *Eucalyptus* wood hydrolysates. The complete structural characterisation of several *O*-acetylated XOS obtained from *Eucalyptus* wood hydrolysates was established by using (RP-HPLC-)NMR. To our knowledge for the first time *O*-acetyl migration was proven to occur in xylo-oligosaccharides.

Preparative anion-exchange and size exclusion chromatography enabled the separation of the structurally different XOS to be used in bioactivity assays. Both fermentability by the human intestinal flora present in faecal inocula, and anti-ulcer activity of several series of differently substituted XOS were studied. The rate of fermentation of the XOS obtained depended on the kind of substituents present. Also, in the anti-ulcer tests the presence of substituents seemed to influence the number of ulcers formed in the stomachs of rats. These results put emphasis on the necessity to be able to perform a detailed elucidation of the structural features of oligosaccharides in general to understand their mechanisms in biological activity tests more precisely.

## 7.7 References

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## SUMMARY

The subject of the research described in this thesis was to characterise hydrolysates of four hydrothermally treated xylan rich by-products. An environmental friendly hydrothermal treatment was performed in order to obtain xylose and xylo-oligosaccharides (XOS) from agricultural by-products for use in the pharmaceutical and food industries.

In a general introduction (chapter 1) the by-products used are described as well as some general structural features of xylans, hydrothermal treatment, techniques used for separation and identification of oligosaccharides and fermentability of oligosaccharides.

Chapter 2 describes the characterisation of four xylan rich by-products (wheat bran, brewery's spent grain, corn cobs and *Eucalyptus* wood) followed by a mild hydrothermal treatment of these by-products. The chemical characterisation of the feedstock materials, with emphasis on alkali extracted xylan fractions, resulted in rather detailed pictures of the xylans present. Depending on the feedstock material studied, the xylan present was substituted with arabinose, 4-*O*-methylglucuronic acid and/ or *O*-acetyl groups. During the hydrothermal treatment, arabinose was rather easily removed from the xylan-backbone (wheat bran, brewery's spent grain and corn cobs). The *O*-acetyl and uronic acid substituents were partly released from the feedstocks, becoming available to depolymerise the xylan by acid catalysis (corn cobs and *Eucalyptus* wood). Due to this partial release of the substituents and depolymerisation of the xylan a wide variety of XOS and xylan-fragments with different structural features corresponding to the xylan-structure of the original feedstock were obtained.

Hydrolysates from the hydrothermally treated *Eucalyptus* wood and brewery's spent grain were studied in more detail (Chapter 3). Hereto, they were fractionated by preparative anion-exchange and size-exclusion chromatography. Several pools were obtained and their sugar composition was determined. Additionally, the oligosaccharides in the pools described were further identified. The fractionation of the brewery's spent grain hydrolysate resulted in two pools containing relatively high molecular weight xylan-fragments with xylopyranosyl moieties, singly and doubly branched with arabinose, separated from a pool of XOS less branched with arabinose. In all three pools arabinoxylan-fragments containing *O*-acetyl substituents were present, suggesting the presence of *O*-acetyl in cereal arabinoxylans. The fractionation of the hydrothermally treated *Eucalyptus* wood resulted in a neutral pool, consisting of a variety of *O*-acetylated XOS. Furthermore, charged pools containing a series of (*O*-acetylated) XOS carrying either *one* or *two* 4-*O*-methylglucuronic acid(s) were obtained. Additionally, a series of XOS containing both 4-*O*-methylglucuronic acid and a hexose, most likely galactose, was detected in the charged pools.

In order to be able to separate and structurally characterise the XOS in the hydrolysates of the different by-products several methods were studied. In chapter 2 and 3 the use of preparative chromatography and analytical high performance size-exclusion

chromatography (HPSEC), high-performance anion-exchange chromatography (HPAEC) and matrix-assisted laser desorption/ ionisation time-of-flight (MALDI-TOF) mass spectrometry (MS) is described. Chapter 4 describes the off-line coupling of HPAEC to MALDI-TOF MS. Hereby, molecular mass information was obtained overcoming the rather unpredictable HPAEC elution behavior of (unknown) oligosaccharides. For MALDI-TOF MS on-line desalting of the HPAEC eluent was performed using an anion self regenerating suppressor (ASRS) in series with a cation self regenerating suppressor (CSRS). This way the oligosaccharides separated by HPAEC were obtained in pure water. Following, automated fractionation after HPAEC separation and computer controlled MALDI-TOF MS sample preparation using a robot are applied as well.

A combination of techniques was used to localise the *O*-acetyl substituents in XOS, which were present in hydrolysates of *Eucalyptus* wood (Chapter 5). Reversed phase (RP)-high performance liquid chromatography (HPLC) coupled on-line to both a mass spectrometer and an evaporating light scattering (ELS) detector provided data about the order of elution of the various *O*-acetylated oligomers. Additionally, by using standards and comparing similar fractions ELS detection also allows quantification of oligosaccharides in different mixtures. The retention of the oligomers on the RP-HPLC column depended on the number *and* position of the *O*-acetyl substituents within the XOS. One dimensional (1D)- and two dimensional (2D)-$^{1}$H NMR spectroscopy were used to study the structural features of several xylotetramers, each having one *O*-acetyl substituent. However, for each 'pure' *O*-acetylated xylotetramer a mixture of 2-*O*- and 3-*O*-acetyl substituted xylotetramers was found. *O*-acetyl migration was proven to have occurred in these XOS. To avoid *O*-acetyl migration RP-HPLC-NMR was performed. The precise location of the 2-*O*- or 3-*O*-acetyl substituent in 6 xylotetramers and 4 xylotrimers was established. Taking in account these observations, it was expected that *O*-acetyl migration already might have occurred during hydrothermal treatment of the *Eucalyptus* wood. Therefore, the position and distribution of the *O*-acetyl substituents in the xylo-oligomers analysed could not be extrapolated directly to the xylan-structures originally present and does therefore not allow conclusions about the mechanisms of degradation during hydrothermal treatment.

One of the strategies to apply XOS in the food-industry is the use as non-digestible oligosaccharides (NDO) or prebiotics to promote the growth of beneficial bacteria in the human intestine. To study whether the XOS obtained during hydrothermal treatment could be fermented by the human intestinal flora present in faecal inocula in general and whether the different types of substituents present might influence this fermentation, *in vitro* fermentation studies with faecal inocula (FI) were performed (Chapter 6). By all FI used the degradation patterns were different for linear XOS, arabinose substituted XOS, *O*-acetylated XOS and XOS containing a 4-*O*-methylglucuronic acid group. Furthermore, differences were observed in the fermentation-products (short-chain fatty acids and lactate) depending on the kind of substituent present. These results put emphasis on the necessity to be able to perform a

detailed elucidation of the structural features of oligosaccharides in general to understand their mechanisms in bioactivity tests more precisely. Also, anti-ulcer tests showed that different substituents in XOS influence the number of ulcers formed in the stomachs of rats.

Finally, in chapter 7 an overview of the main results of this thesis work and some final results are given. The hydrothermal treatments performed and the use of the resulting variety of structurally different XOS are discussed. Additionally, it was shown that (a combination of) techniques like chromatography, MS and NMR are powerful analytical tools in the separation and characterisation of the variety of XOS obtained.

## SAMENVATTING

In dit proefschrift is de karakterisering van hydrolysaten van vier hittebehandelde xylaan rijke bijproducten beschreven. Een milieu-vriendelijke hittebehandeling werd uitgevoerd om xylose en xylo-oligosacchariden (XOS) uit agrarische bijproducten te verkrijgen voor gebruik in de farmaceutische en levensmiddelen industrie.

In een algemene introductie (hoofdstuk 1) zijn de bestudeerde bijproducten beschreven, als ook algemene xylaan structuur kenmerken, karakteristieken van de hittebehandeling, scheidings- en identificatie technieken van oligosacchariden en fermenteerbaarheid van oligosacchariden.

De vier xylaan rijke bijproducten (tarwe zemelen, bierbostel, maïs kolven en *Eucalyptus* hout) zijn onderworpen aan een hittebehandeling om het aanwezige xylaan uit de bijproducten te verkrijgen en gedeeltelijk af te breken (Hoofdstuk 2). Om een indruk te krijgen van de structuren van het aanwezige xylaan zijn de bijproducten chemische gekarakteriseerd met nadruk op de aanwezige xylanen. Afhankelijk van het soort bijproduct waaruit het xylaan geëxtraheerd was, bleek het xylaan vertakt te zijn met arabinose, 4-*O*-methylglucuronzuur en/ of *O*-acetyl groepen. Tijdens de hittebehandeling werden de arabinoses redelijk eenvoudig afgesplitst van de xylaan-hoofdketen (tarwe zemelen, bierbostel en maïs kolven). *O*-acetyl en uronzuur substituenten werden afgesplitst, wat bijdroeg aan de hydrolyse van het xylaan (maïs kolven en *Eucalyptus* hout). Door deze afsplitsing van zijgroepen en hydrolyse van de xylaan-hoofdketen ontstond een grote variëteit aan XOS met verschillende structuren afhankelijk van de structuur van het oorspronkelijk aanwezige xylaan.

Hydrolysaten van *Eucalyptus* hout and bierbostel zijn gedetailleerder bestudeerd in hoofdstuk 3. Hiervoor zijn ze gefractioneerd met behulp van preparatieve anionenwisselings en gel-permeatie chromatografie. Van de verkregen fracties is de suikersamenstelling bepaald. Vervolgens zijn de in de fracties aanwezige oligosacchariden verder geïdentificeerd. De fractionering van het hittebehandelde bierbostel resulteerde in drie fracties waarvan er twee xylaan-fragmenten bevatten met een relatief hoog molecuulgewicht en xylosyl bouwstenen, die enkel en dubbel vertakt waren met arabinose, gescheiden van een fractie met laag vertakte XOS. In alle drie de fracties zijn *O*-acetyl substituenten aangetoond, wat de aanwezigheid van *O*-acetyl in tarwe arabinoxylanen suggereert. De fractionering van het hittebehandelde *Eucalyptus* hout resulteerde in een neutrale fractie, die een variëteit aan *O*-geacetyleerde XOS bevatte. Ook werden verschillende geladen fracties verkregen, die meerdere series van (*O*-geacetyleerde) XOS gesubstitueerd met *één* of *twee* 4-*O*-methylglucuronzu(u)r(en) bevatten. In de geladen fracties werd tevens een serie XOS gedetecteerd, die een 4-*O*-methylglucuronzuur en een hexose, waarschijnlijk galactose, bevatte.

Verscheidene methoden voor de scheiding en karakterisering van de verschillende XOS zijn bestudeerd. In hoofdstuk 2 en 3 is het gebruik van preparatieve chromatografie en

analytische high performance size-exclusion chromatografie (HPSEC), high performance anion-exchange chromatografie (HPAEC) en matrix-assisted laser desorption/ ionisation time-of-flight (MALDI-TOF) massa spectrometrie (MS) beschreven. Hoofdstuk 4 beschrijft de koppeling van HPAEC met MALDI-TOF MS. Het elutie gedrag van HPAEC is over het algemeen onvoorspelbaar en daarom is identificatie van onbekende oligosacchariden aan de hand van HPAEC-elutiepatronen moeilijk. De koppeling van HPAEC met MALDI-TOF MS leverde echter informatie over de massa's van de HPAEC geëlueerde (onbekende) oligosacchariden welke behulpzaam was bij de identificatie van deze oligosacchariden. Het HPAEC-eluent is on-line ontzout voor MALDI-TOF MS met behulp van een anionen en cationen suppressor membraan, waardoor de HPAEC gescheiden oligosacchariden werden verkregen in puur water. Vervolgens werd het HPAEC-eluent in fracties opgevangen en met behulp van een robot automatisch op een MALDI-TOF meetplaat gebracht en gemeten.

Een combinatie aan technieken is gebruikt om de plaats van *O*-acetyl substituenten in XOS uit hittebehandeld *Eucalyptus* hout te bepalen (Hoofdstuk 5). Reversed phase (RP)-high performance liquid chromatografie (HPLC) gekoppeld aan een massa spectrometer en een ELS (evaporating light scattering) detector verschafte informatie over de volgorde van elutie van verscheidene *O*-geacetyleerde oligomeren. De vergelijking van ELS-signalen van verwante oligomeren geeft een indruk van de hoeveelheden van die oligomeren. De scheiding van de XOS met behulp van RP-HPLC was afhankelijk van het aantal en positie van de *O*-acetyl substituenten in de XOS. Eén dimensionaal (1D)- en twee dimensionaal (2D)- $^{1}$H NMR spectroscopie werd gebruikt om de structuren te bestuderen van verschillende xylotetrameren, welke allen met één *O*-acetyl gesubstitueerd waren. Echter, voor iedere 'zuivere' *O*-geacetyleerde xylotetrameer bleek een mengsel van 2-*O*- en 3-*O*-acetyl gesubstitueerde xylotetrameren aanwezig te zijn. Dit was het bewijs dat *O*-acetyl migratie was opgetreden binnen de bestudeerde oligomeren. Om *O*-acetyl migratie te voorkomen tijdens het bepalen van de *O*-acetyl plaats werd gebruik gemaakt van RP-HPLC direct gekoppeld aan NMR. Hiermee werd de precieze plaats van de 2-*O*- of 3-*O*-acetyl substituent in 6 xylotetrameren en 4 xylotrimeren bepaald. Rekening houdend met deze waarnemingen wordt verwacht dat *O*-acetyl migratie ook al tijdens de hittebehandeling had plaatsgevonden. De positie en verdeling van de *O*-acetyl substituenten in de bestudeerde XOS zegt daarom waarschijnlijk weinig over de verdeling van de *O*-acetyl substituenten in het oorspronkelijk aanwezige xylaan en kan daarom niet gebruikt worden om conclusies te trekken over het mechanisme van de hydrolyse tijdens hittebehandeling.

Een van de mogelijke strategieën om XOS toe te passen in de levensmiddelenindustrie is hun gebruik als non-digestible oligosacchariden (NDO) als prebiotica om de groei van gezondheidsbevorderende darmbacteriën stimuleren. Om te bekijken of de verkregen XOS gefermenteerd konden worden door de menselijke darmflora in het algemeen en of de aanwezige substituenten de fermentatie zouden beïnvloeden, werden verschillende gesubstitueerde XOS *in vitro* blootgesteld aan fermentatie door faeces monsters (Hoofdstuk

6). De afbraakpatronen waren verschillend voor lineaire XOS, XOS gesubstitueerd met arabinose, *O*-geacetyleerde XOS en 4-*O*-methylglucuronzuur bevattende XOS. Tevens werden verschillen aangetoond in de fermentatieproducten (kortketenige vetzuren en lactaat) afhankelijk van de aanwezigheid en soort substituent. Deze resultaten benadrukken de opheldering van de precieze structuren van oligosacchariden in het algemeen, zodat hun werkingsmechanismen in bio-activiteitstesten beter worden begrepen. Ook uit testen naar de vorming van maagzweren bleek dat de verschillende substituenten aan XOS een effect hadden op de vorming van maagzweren bij ratten.

In hoofdstuk 7 wordt een overzicht gegeven van de belangrijkste resultaten uit dit proefschrift en enkele aanvullende resultaten. De hittebehandelingen als ook de structuren en de potentiële toepassing van de verkregen verschillend vertakte XOS worden bediscussieerd. Verscheidene (combinaties van) methoden, zoals analytische chromatografie, MS en NMR spectroscopie, bleken zeer waardevol voor de scheiding en karakterisering van de variëteit aan verkregen XOS.

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*Mirjam*

## CURRICULUM VITAE

Mirjam Kabel werd geboren op 24 augustus 1974 te 's Gravenhage. Na het behalen van haar Gymnasium diploma in 1992 aan het Gymnasium Camphusianum te Gorinchem, begon zij in september van dat jaar met de studie Levensmiddelentechnologie aan de toenmalige Landbouwuniversiteit te Wageningen. In het kader van deze studie deed ze tijdens de doctoraalfase haar afstudeeronderzoek bij de leerstoelgroep Levensmiddelenchemie. Daarnaast liep ze stage bij het toenmalige Gist-Brocades (DSM) in Delft, Nederland. In maart 1998 behaalde zij het doctoraal diploma.

Van mei 1998 tot mei 2002 deed zij een promotieonderzoek bij het Laboratorium voor Levensmiddelenchemie aan de Wageningen Universiteit, onder begeleiding van prof. dr. ir. A.G.J. Voragen en dr. ing. H.A. Schols. Het onderzoek dat in deze periode werd uitgevoerd staat beschreven in dit proefschrift.

Sinds juli 2002 is zij werkzaam als postdoc bij het Laboratorium voor Levensmiddelenchemie.

## ADDENDUM

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Cover by S.H.D. Hulleman (www.insight-art.nl): Xylose, crystallised from water, microscopy (2.5 x 10 x 3), with 90° crossed polaroid filters.

**Stellingen**

1. De fermenteerbaarheid van xylaan-oligosacchariden wordt sterk beïnvloed door de aanwezigheid van substituenten.
   *dit proefschrift*

2. Arabinosylering is een beter criterium voor het onderscheiden van xylanen uit monocotylen en dicotylen dan acetylering.
   *dit proefschrift*

3. Migratie van *O*-acetyl substituenten bemoeilijkt het vaststellen van hun oorspronkelijke positie aanzienlijk.
   *dit proefschrift*

4. Een koffie-automaat nabij de werkplek vermindert RSI-klachten.

5. De verschillen in Euro-munten weerspiegelen de wens naar eensgezindheid binnen de Europese unie.

6. Verkleining van de veestapel zal veevoer tot voedsel maken.

7. Als een onderzoeker in staat is zijn 'vrijdagmiddag'-proefjes te reproduceren hangt er een publicatie in de lucht.

8. De weg naar het genieten is genieten van de weg.
   *H.H. Bolck; vrij naar B. Hoff, Tao van Poeh*

9. Roeien en promoveren hebben beide baat bij een snelle start, stug doorhalen in het middenstuk met tijdige tussensprints en een niet te verwaarlozen eindsprint.

Stellingen behorende bij het proefschrift

*Characterisation of complex xylo-oligosaccharides from xylan rich by-products*

M.A. Kabel

Wageningen, maandag 30 september 2002