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Xylooligosaccharides Production, Quantification, and Characterization in Context of Lignocellulosic Biomass Pretreatment

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19.1 Introduction

19.1.1 Definition of Oligosaccharides

Oligosaccharides, also termed sugar oligomers, refer to short-chain polymers of monosaccharide units connected by α and/or β glycosidic bonds. In structure, oligosaccharides represent a class of carbohydrates between polysaccharides and monosaccharides, but the range of degree of polymerization (DP, chain length) spanned by oligosaccharides has not been consistently defined. For example, the Medical Subject Headings (MeSH) database of the US National Library of Medicine defines oligosaccharides as carbohydrates consisting of 2–10 monosaccharide units; in other literature, sugar polymers with DPs of up to 30–40 have been included as oligosaccharides [1–3].

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Table 19.1 *Lignocellulosic feedstocks that have heteroxylans as dominant hemicellulose types.*

Plant group	Examples	Wall type	
		Primary cell wall	Secondary cell wall
Hardwood	Poplar	Xyloglucan	4-O-methyl-glucuronoxylan
Energy grasses	Switchgrass	Glucuronoarabinoxylan	Glucuronoarabinoxylan
Agricultural residues	Corn stover	Glucuronoarabinoxylan	Glucuronoarabinoxylan

19.1.2 Types of Oligosaccharides Released during Lignocellulosic Biomass Pretreatment

Oligosaccharides exist naturally in plant tissues, but their amounts are small compared to cell-wall structural polysaccharides, such as cellulose and hemicellulose [4]. During pretreatment of lignocellulosic biomass, most of the insoluble hemicellulose is removed from the surface of cellulose microfibrils and broken into various soluble oligosaccharides. However, the amounts and structures vary with pretreatment types and severity. The majority of oligosaccharides released during lignocellulosic biomass pretreatment are hydrolysis products of hemicellulose, and the types of oligosaccharides (composition, DP, and substitution) depend on the structure and composition of the corresponding hemicellulose.

Hemicellulose refers to several amorphous polysaccharides found in the plant cell-wall matrix that have β-(1–4)-linked backbones with an equatorial configuration [5], which are commonly categorized into several groups such as xyloglucans, heteroxylans, (galacto) glucomannans, and arabinogalactans [6–8]. For example, glucuronoarabinoxylan refers to one type of heteroxylans which have a backbone of β-(1–4)-xylosyl residues with a few short side chains that mainly contain arabinosyl residues and glucuronic acid residues, but could also contain other sugars or sugar acid residues [4]. The number of side chains and the side-chain residues composition vary with biomass and cell-wall types and life stage of the same plant. Dominant forms of hemicellulose polysaccharides in major lignocellulosic biomass feedstocks, except softwoods, are xyloglucans and "heteroxylans" as listed in Table 19.1. In lignocellulosic biomass feedstocks, the mass fraction of secondary cell walls based on total plant dry weight is much greater than that of primary cell walls [4]. Thus, xylooligosaccharides (XOs) from heteroxylans hydrolysis are the predominant type of oligosaccharides released during pretreatment.

19.1.3 The Importance of Measuring Xylooligosaccharides

Understand Plant Cell-wall Structure and its Role in Biomass Recalcitrance

In general, plant cell walls represent an enormous source of complex polysaccharides that can be broken down to monosaccharides for potential conversion into biofuels and chemicals. The framework of plant cell walls is cellulose, a highly ordered, water-excluding natural crystalline polymer of glucose molecules joined by β-(1–4)-glycosidic bonds, with its chains connected by many intra/inter-chain hydrogen bonds. Outside the framework, cellulose microfibrils and hemicellulose are intimately interlocked with one another and often with lignin, both covalently and non-covalently [5]. The hydrophobic association of cell-wall polysaccharides and lignin, termed the lignin-carbohydrate complex (LCC), is an important part of plant cell-wall defense and has been recognized as the main barrier for economic deconstruction of cell-wall polysaccharides [5,9–11]. Such collective resistance, which plants and plant materials pose to deconstruction by microbes and enzymes, is defined as "biomass recalcitrance" [5,12]. Although the aspect(s) most responsible for biomass recalcitrance to conversion are not clear, a better understanding of cell-wall polysaccharides compositions and structures would greatly facilitate advanced process designs that achieve more effective breaking of such defenses with lower cost, as well as aid in production of less recalcitrant plants using genetic tools. For example, through comparison of glucuronoxylan (GX) structures

in poplar wood, Lee *et al.* found transgenic reduction of GX in secondary cell wall reduced recalcitrance of wood to cellulase digestion [13].

Unfortunately, direct characterization of cell-wall polysaccharides is difficult because of the heterogeneous and complex nature of cell walls. Thus, using either enzymes or chemicals to break down cell-wall polysaccharides followed by characterizing the corresponding oligosaccharides and monomers has been an effective way to study cell-wall polysaccharides structures and their possible roles in biomass recalcitrance. Effective structural studies normally contain two parts. First, optimized enzymatic or chemical treatment methods are applied to extract certain types of polysaccharides from the insoluble cell wall in which they are held. For example, heteroxylans are typically extracted by 4% KOH whereas heteroglucans may require 24% KOH [5,14]. The isolated polysaccharides or fragments are then purified and broken down into oligosaccharides for detailed characterization. Important structural information about hemicellulose polysaccharides can be determined, such as the glycosyl residue composition, the glycosyl linkage composition, the sequence of glycosyl residues in both the backbone and side chains, and non-carbohydrate substituents through characterizing hemicellulose oligosaccharides [4].

Engineer Reaction Pathways for Economic Deconstruction of Structural Cell-wall Polysaccharides

As a feedstock for fuels and chemicals production, lignocellulosic biomass has many benefits such as not competing for food and feed supply, low production costs, and wide availability over a range of locations and climates [15,16]. Utilization of cell-wall carbohydrates makes lignocellulosic biomass a promising renewable feedstock for large-scale conversion into liquid fuels and organic chemicals. Different reaction pathways have been devised to break down cell-wall polysaccharides in lignocellulosic biomass into monomeric sugars: thermal, chemical, biological, and/or a combination of these. In lignocellulosic ethanol production for example, cellulase (which is a synergistic combination of several proteins) in combination with hemicellulases and other accessory enzymes degrades cellulose and residual hemicellulose into glucose and xylose. However, pretreatments have proven to be essential to open up the rigid biomass structure through removing or altering hemicellulose and lignin and loosening the structure of cellulose, enhancing access of enzymes to their respective substrates. Hemicellulose polysaccharide chains can be broken into oligosaccharides and then further hydrolyzed to monosaccharides, especially during low to neutral pH pretreatments, which in turn can react to degradation products as described in the following:

$$\text{Polysaccharides}_{(\text{solid})} \rightarrow \text{Hemicellulose Oligosaccharides}_{(\text{aq})} \rightarrow \text{Monosaccharides}_{(\text{aq})} \rightarrow \text{Degradation products}_{(\text{aq})}$$

Employing harsh pretreatment conditions can reduce macro-barriers to enzymes reaching cellulose and improve micro-accessibility of cellulose to enzymes through changes in its crystal structure and degree of polymerization and result in better conversion to sugars [17–19]. However, such conditions also degrade xylooligosaccharides and xylose into by-products such as furfural [20,21], resulting in sugar losses and formation of inhibitors to enzymes and microbes for sugars fermentation [22,23]. Pathway optimization is therefore needed to achieve the highest sugar recovery for economical processing. For that reason, qualitative and quantitative measurements of xylooligosaccharides are important because they are essential for detailed studies of hemicellulose hydrolysis kinetics and degradation mechanisms. Such studies can also play a key role in engineering effective pretreatment technologies to achieve high sugar recovery with good economics.

It is also important to note that xylooligosaccharides have recently been shown to have a strong negative effect on cellulase activity in decomposing cell-wall polysaccharides into fermentable sugars [24,25]. Quantitative analysis and characterization of xylooligosaccharides, including improved purification and characterization techniques, facilitate the understanding of xylooligosaccharide inhibition mechanisms and development of strategies for reducing inhibition.

Oligosaccharides for High-value-added Products

Xylooligosaccharides have been shown to have important prebiotic properties and thus great potential for use in medicinal, food, and health products [26]. Xylooligosaccharides for such uses are mainly derived from lignocellulosic biomass by enzymatic and/or chemical hydrolysis to remove hemicellulose polysaccharides (mainly heteroxylans in the case of cellulosic biomass) from the surface of cellulose and break them into water-soluble xylooligosaccharides. Separation technologies then isolate and purify these xylooligosaccharides into desired DP ranges for prebiotic applications. This fast-growing market for xylooligosaccharides creates great opportunities to process xylan-rich pretreatment hydrolyzates in cellulosic biorefineries into high-value products which could improve conversion economics.

19.2 Xylooligosaccharides Production

Xylooligosaccharides are usually produced from xylan-rich lignocellulosic materials (LCM) by autohydrolysis from heating in water or steam, chemical treatments in dilute aqueous solutions of mineral acids [27,28], direct enzymatic hydrolysis of susceptible lignocellulosic materials [29–31], or chemical fractionation of a suitable LCM to isolate (or solubilize) xylan with further enzymatic hydrolysis to XOs [32]. Typical raw materials for XOs production are hardwoods (e.g., birchwood, beechwood), corn cobs, straws, bagasse, rice hulls, malt cakes, and bran [26]. In recent years, the fast-growing functional food market and the increasing number of other industrial applications are encouraging identification of renewable and cheap xylan sources instead of hardwood xylan for XOs production. As a result, agricultural residues such as cotton stalks, tobacco stalks, and wheat straw have also been intensively studied [27].

19.2.1 Thermochemical Production of XOs

Thermochemical production of XOs is usually accomplished by steam, dilute mineral acids, or dilute alkaline solutions. The single-step production of XOs by reaction with steam or water through hydronium-catalyzed degradation of xylan is known as autohydrolysis, hydrothermolysis, or water prehydrolysis [26]. Autohydrolysis takes place at slightly acidic ($pH \leq 4$) conditions created by acetic acid released by partial cleavage of acetyl groups in the plant cell wall. A considerable fraction of acetyl and uronic acid groups remain attached to the oligosaccharides, giving them distinctive characteristics like very high solubility in water [33]. In autohydrolysis treatment, XOs behave as typical reaction intermediates whose concentration depends mainly on the tradeoff between breakdown of polymeric hemicellulose in biomass to XOs and their further decomposition to monomeric xylose. Therefore, reaction severity (R_o) influences the concentrations of total XOs as well as of monomeric xylose that could be achieved in hydrolysate and is often represented by a single parameter that combines temperature, time, and reaction pH [34]:

$$R_o = t \exp\left(\frac{T - 100}{14.75}\right) - pH$$

Medium-severity conditions are usually preferred to balance formation of oligosaccharides against their degradation and maximize XO concentration [35]. However, the degree of polymerization DP (or molecular weight) distribution in XOs mixtures generally depends on not only the treatment severity but also on the substrate and its concentration during treatment [33]. In a study by Nabarlatz *et al.*, comparative assessment of six agricultural residues of different botanic origin showed that characteristics of the raw material played a major role in the yield and composition of XOs. However, their yield also depended on the initial content of acetyl groups since their cleavage liberated acetic acid, which in turn catalyzed xylan depolymerization

into XOs [33]. In the initial stage of autohydrolysis, hydronium ions were generated through autoionization (dissociation) of water under high temperature or pressure. However, as the reaction proceeded, cleavage of acetyl groups from the xylan backbone formed acetic acid and was believed to contribute more hydronium ions. Although adding acids beyond that released naturally from biomass can facilitate xylan or hemicellulose degradation, XO yields will generally be reduced by generating more monomeric xylose than without added acid. Controlling the temperature and reaction time can also influence XO characteristics such as the acetyl content and the molar mass distribution [36], although the nature of the raw material plays a significant role [33].

Autohydrolysis has the advantage of eliminating corrosive chemicals for extraction and hydrolysis of xylan, but requires equipment that can be operated at temperatures and pressures as high as or higher than acid or alkali treatments. Besides xylan degradation, several concurrent processes occur including extractives removal, solubilization of acid-soluble lignin, and solubilization of ash, all of which contribute to undesired non-saccharide compounds in liquors from autohydrolysis processing. The molecular weight distribution of XOs produced by autohydrolysis after solvent extraction contains a large proportion of high-DP compounds (MW 1000–3000 g/mol) and a much smaller fraction of low-DP compounds (MW $<$ 300 g/mol) [33]. In addition, autohydrolysis at mild temperatures does not modify cellulose and lignin substantially, allowing their recovery for further processing and utilization.

XOs can also be produced by hydrolytic processes either in basic or dilute acidic media. Dilute sulfuric acid (0.1–0.5 M) is most commonly used for acid production of XOs. The DP distribution of the XOs depends on acid concentration, temperature, and reaction time, but the yield of monosaccharides also depends on the structure and composition of xylan [27]. A major disadvantage of acid hydrolysis is low yields of oligomers compared to monomers in addition to production of furfural and other degradation products. However, this disadvantage could be controlled by shortening the reaction time, reducing acid concentration, or removing these by-products by adsorption chromatography and membrane separation. A major advantage of acid hydrolysis is simple, rapid kinetics; for example, dilute acid hydrolysis requires much less reaction time compared to enzymatic hydrolysis to achieve the same xylan to XOs conversion (a few minutes compared to several hours) [27].

Figure 19.1 summarizes the xylan reaction pathway. The depolymerization of xylan has been described as combined reactions of fast-reacting and slow-reacting fractions, which are first decomposed into high-molecular-weight XOs [28]. As the hydrolytic degradation reaction proceeds, high-molecular-weight XOs

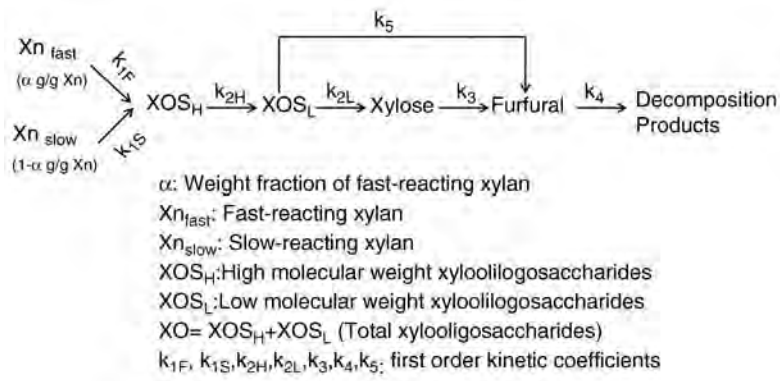

Figure 19.1 *Xylan reaction pathway in autohydrolysis to oligomers, xylose, furfural, and degradation products. (Adapted from Parajo* et al. *[28] © 2004, Elsevier).*

are converted into lower-molecular-weight XOs which are further depolymerized to xylose; xylose is then degraded to furfural and many unidentified degradation products. In some cases, low-molecular-weight XOs can be directly degraded to furfural or other degradation products [21,28]. First-order kinetics with Arrhenius-type dependence on temperature are usually adequate to describe reaction rates profiles, with the weight fraction of fast-reacting xylan, the pre-exponential factors of the kinetic coefficients involved in the reaction, and the corresponding activation energies determined by fitting the data to the kinetic model. In the study by Kumar and Wyman with purified xylooligosaccharides degradation at different pH values, they showed that all the XOs disappeared at higher rates compared to monomeric xylose, and the ratio of XOs disappearance rate constants to xylose degradation rate constant increased with decreasing pH. In addition, the direct degradation of low-DP XOs (mainly DP 2 and 3) to undesired products was significant for hydrothermal reactions but could be minimized by adding acid [21].

Alternatively, depolymerized hemicellulose may be extracted from lignocellulosic materials by strong alkali solutions (for example, a solution of KOH, NaOH, $Ca(OH)_2$, ammonia, or a mixture of these compounds). However, the extractability of depolymerized hemicellulose varies with the alkali type and isolation conditions used for different plants. In general, alkaline treatment disrupts the cell wall of lignocellulosic materials by dissolving hemicelluloses and lignin, hydrolyzing uronic and acetic esters, swelling the cellulose, decreasing cellulose crystallinity, and cleaving the α-ether linkages between lignin and hemicelluloses as well as the ester bonds between lignin and/or hemicelluloses and hydroxycinnamic acids, such as *p*-coumaric and ferulic acids. The depolymerized xylan therefore loses acetyl groups and uronic acids by saponification during extraction and has very limited solubility in neutral aqueous solutions [37]. Alkali processing of xylan-containing materials is favored by the pH stability of this polymer, and solubilized xylan and xylan degradation products can be recovered by precipitation with organic compounds (including acids, alcohols or ketones) [26]. However, xylan or soluble XOs obtained from alkali extraction require dilute acid or enzymatic treatment to break them down further to lower-DP XOs [26].

19.2.2 Production of XOs by Enzymatic Hydrolysis

XOs can also be produced by enzymatic hydrolysis of xylan-containing materials. However, because the xylan-lignin complex is naturally resistant to enzyme attack, current commercial processes are usually carried out in a two-stage sequence: (1) alkaline extraction followed by (2) enzymatic hydrolysis. In most plant materials, xylan is a heteropolymer with homopolymeric backbone composed of β-1, 4-linked xylose units and various branching units including L-arabinose, D-glucuronic acid, 4-O-methyl glucuronic acid, D-galacturonic acid, ferulic acid, coumaric, and acetic acid residues and, to a lesser extent, L-rhamnose, L-fucose, and various O-methylated neutral sugars [38]. Consequently, synergistic action of different enzymes is needed to completely hydrolyze these complex xylan structures. Generally, endo-β-1, 4-xylanases degrade xylan by attacking the β-1, 4-bonds between xylose units to produce XOs, and β-xylosidase converts lower-DP XOs into monomeric xylose. In order to maximize production of XOs and minimize xylose production, enzyme mixtures with low endoxylanase and/or β-xylosidase activity are desirable. Debranching enzymes such as α-L-arabinofuranosidase, α-glucuronidase, and several esterases are needed to cleave xylan side groups [39,40] and can be dissolved in the reaction media or immobilized. They can also be produced *in situ* by microorganisms such as fungi and bacteria that make multiple endoxylanase isoenzymes, reflecting the need for xylanases with specificities that are capable of acting on different substrates [41].

In contrast to autohydrolysis and chemical treatment methods, enzymatic hydrolysis avoids production of undesirable by-products or high amounts of monosaccharides, or require high-pressure or high-temperature equipment. However, enzymatic methods usually require much longer reaction times than acid hydrolysis or autohydrolysis. In addition, xylanase with different substrate specificities produces different

hydrolysis end-products, and control of production of XOs with a desired DP range can be more difficult. On the other hand, acid hydrolysis of xylan randomly hydrolyzes glycosidic bonds between adjacent xylose units. Acid hydrolysis is therefore more practical for production of XOs in the DP range of 2–15 [42]. A study of the hydrolysis patterns of purified endoxylanase on birchwood, beechwood, and oat spelt xylans indicated that xylotriose (X3) is the shortest XOs released by xylanase [41]. Xylotriose and xylotetraose (X4) fragments are believed to be inaccessible to xylanase enzymes, probably due to substitution with arabinosyl residues. Commercial xylanase preparations are often low in β-xylosidase activity, resulting in xylobiose accumulation (X_2) [43]. Similarly, commercial cellulase preparations are usually low in β-xylosidase activity; that deficiency, coupled with the high inhibition of cellulase by xylooligosaccharides, has recently been shown to be an important contributor to reduced hydrolysis of xylooligosaccharides to xylose [43] as well as cellulose to glucose [25].

19.3 Xylooligosaccharides Separation and Purification

XOs from thermochemical or enzymatic treatment usually contain a wide DP range of oligomers and possibly other compounds as stated in the previous section. To produce more pure XO fractions used in food or pharmaceutical industries, the hydrolysis liquor must be refined by removing monosaccharides or non-saccharide compounds to obtain the highest possible XO content or a given DP range. Purification and separation of XOs from autohydrolysis liquor is complicated and may require multistage processing for reaction and/or fractionation. Depending on the degree of purity desired, a sequence of several physicochemical treatments may be needed [44].

19.3.1 Solvent Extraction

Solvent extraction is frequently applied to recover XOs and also applied to pre-extract interfering components before chemical or enzymatic treatment to simplify purification. Vacuum evaporation may be applied first to concentrate the crude XOs solution produced by hydrothermal processing and remove volatile components. Then, as shown in Figure 19.2, solvent extraction can remove non-saccharide compounds to yield both a refined aqueous phase and a solvent-soluble fraction that mainly contains most of the phenolics and extractive-derived compounds. The recovery yields and the degree of purification depend on the solvent employed for extraction, with ethanol, acetone, and 2-propanol the most common choices to refine crude XOs solution [44,46,47]. However, lignocellulosic materials used for XOs production may contain stabilizing non-saccharide components, especially comparatively high proportions of uronic groups and/or compounds in autohydrolysis liquors that are influenced by the XOs substitution pattern [44]. A study of solvent extraction of freeze-dried solids by 2-propanol, acetone, and ethanol showed that the highest purities were achieved with ethanol, although at the expense of lower recovery yields [44].

19.3.2 Adsorption by Surface Active Materials

Adsorption by surface active materials has been used in combination with other treatment steps to separate oligosaccharides from monosaccharides or remove other undesired compounds. The most widely used adsorbents for purification of XOs liquors include activated charcoal, acid clay, bentonite, diatomaceous earth, aluminum hydroxide or oxide, titanium, silica, and porous synthetic materials [26]. For example, Pellerin *et al.* used activated charcoal followed by elution with ethanol to fractionate XOs based on their molecular weight [48]. In the first stage, XOs were retained by activated charcoal and then released according to DP by changing the ethanol concentration during elution [48]. Zhu *et al.* [49] employed the same approach to purify oligosaccharides from aqueous-ammonia-pretreated corn stover and cobs. In this case,

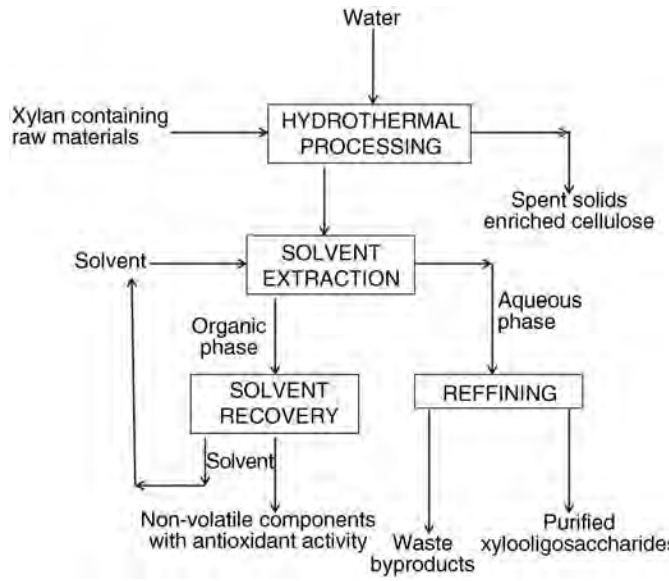

Figure 19.2 *Hydrothermal treatment coupled with solvent extraction for production of purified XOs from xylan-containing lignocellulosic materials. (Adapted from Moure* et al. *[45] © 2006, Elsevier).*

1–10% w/w activated carbon was added to the supernatant containing oligosaccharides, and the mixture was subsequently eluted with a solution containing 0–50% ethanol in water. The highest XOs yield was achieved for elution with 15–30% ethanol, but only the total oligosaccharides concentration was measured by traditional post-hydrolysis with 4% sulfuric acid at 121 °C for 1 hour and not the concentrations of each oligosaccharide DP fraction [50].

Montane *et al.* proposed that activated carbon treatment of raw XOs solutions obtained by autohydrolysis of lignocellulosic materials is feasible for removal of extractives, lignin-derived compounds, and carbohydrate degradation products [51]. Selective adsorption of lignin products compared to carbohydrates was favored by three commercial activated carbons at slightly acidic pH. The results also showed that selectivity towards lignin adsorption was higher when the carbon used was highly microporous and had smaller mesopore diameters, a low volume of mesopores, and a low concentration of basic surface groups to favor adsorption of lignin derivatives [51].

19.3.3 Chromatographic Separation Techniques

Although all the methods outlined above could be used to refine and concentrate XOs solutions, the resulting XOs solution may not be sufficiently pure. On the other hand, high-purity XO fractions have been produced at the analytical scale by chromatographic separations. For example, samples from hydrothermally treated lignocellulosic materials were fractionated by anion-exchange or size-exclusion chromatography [52–54]. However, techniques such as ^{13}C NMR [55], matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF), and nanospray mass spectrometry have usually been employed for refining samples before structural characterization of XOs [53]. Katapodis *et al.* employed size-exclusion chromatography (SEC) in combination with other techniques for purification of feruloylated oligosaccharides [56]. Jacobs *et al*. purified hemicellulose-derived products from hydrothermal microwave treatments of flax shive by employing ion-exchange chromatography and/or SEC in combination with enzymatic processing [57].

Industrially, charcoal chromatography is preferred for sugar purifications due to its higher loading capacity than for other separation methods. However, it is difficult to separate XOs with high DPs, and acidic oligosaccharides (XOs with uronic acid substituents) would overlap with simpler XOs on the chromatograph. As a result, Dowex 1-X4 anion-exchange resin in the acetate form was used before charcoal chromatography to avoid overlapping. Due to its low efficiency and time-consuming operation, this method is less satisfactory in separation of high-purity XOs. In addition, continuous operation was not applied because of the gradient elution mode used and requirement for column regeneration.

Gel permeation chromatography (GPC) with cross-linked polyacrylamide and cross-linked dextran beads has been successfully applied for fractionating oligosaccharides since the 1960s. Sugars from mannose through mannoheptose were separated from each other using Sephadex G-25, but longer oligomers were not well resolved [58].

Unlike Sephadex, Bio-Gel is composed of polyacrylamide which is not susceptible to microbial degradation and does not leak carbohydrates during elution. Pontis applied Bio-Gel P-2 to separate sucrose through the heptaoligosaccharide of fructosan but, once again, larger fractosans were not separated well [59]. Havlicek and Samuelson applied a Bio-Gel P-2 column to separate XOs with DP ranging from 2 to 18 from acid-pretreated birchwood xylan hydrolysate after removing acidic saccharides with ion-exchange resin [60]. Korner *et al.* [61] fractionated XOs up to DP 7 using a Bio-Gel P-4 column operated at 40 °C with 0.05 M Tris/HCl buffer (pH 7.8) at a flow rate of 30 mL/h. Under such conditions, the series of XOs were eluted according to size exclusion principles, whereas acidic saccharides composed of xylose and uronic acids were separated according to partition principles, resulting in xyloheptose being superimposed on glucuronosylxylose. Because Bio-Gel and Toyopearl gels are known to be resistant to the permeation of acidic saccharides into pores of the gel particles, acidic saccharides are eluted near the void fraction with distilled water as eluent. Distilled water eliminates the need to remove buffer salts after separation, and the XOs fractions collected after separation could be easily concentrated by evaporation. Furthermore, columns filled with Bio-Gel with different pore sizes could be combined in series to maximize separation purity. Sun *et al.* [42] used three combinations of two columns connected in series to isolate xylose and XOs with DPs ranging from 2 to 15. Bio-Gel P-4 and Toyopearl HW-40F columns provided good resolution, and chromatography with Bio-Gel P-4 and P-2 columns also achieved separation of XOs up to DP 15. However, the resolution of the latter was slightly lower than that of Bio-Gel P-4 and Toyopearl HW-40 columns. In contrast, Toyopearl HW-50 and HW-40 columns connected in series could only separate XOs up to DP 8 (Figure 19.3).

Gel-permeation chromatography is a widely used separation technique that can be easily adapted for an auto-preparative system using an auto-sampler (or injection pump), a refractive index detector, and an automatic fraction collector that responds to the detector signal. Depending on the Bio-Gel pore size, relatively high-purity XOs fractions with different DP ranges can be collected, and more columns can be connected in series to further improve separation performance. The main disadvantage of GPC for separation of oligosaccharides is its relatively high cost. Thus, although GPC purification is frequently used to obtain fractions of XOs for structural characterization and the degree of purification of the different DP fractions is relatively good, GPC does not tend to be cost effective for large-scale production of XOs.

19.3.4 Membrane Separation

Membrane technology, mainly ultrafiltration and nanofiltration, is currently seen as the most promising downstream strategy for industrial manufacture of high-purity and concentrated oligosaccharides. Ultrafiltration separates oligosaccharides from higher-molecular-weight compounds or fractionates oligosaccharides of different DP. On the other hand, nanofiltration can concentrate liquors and/or remove undesired

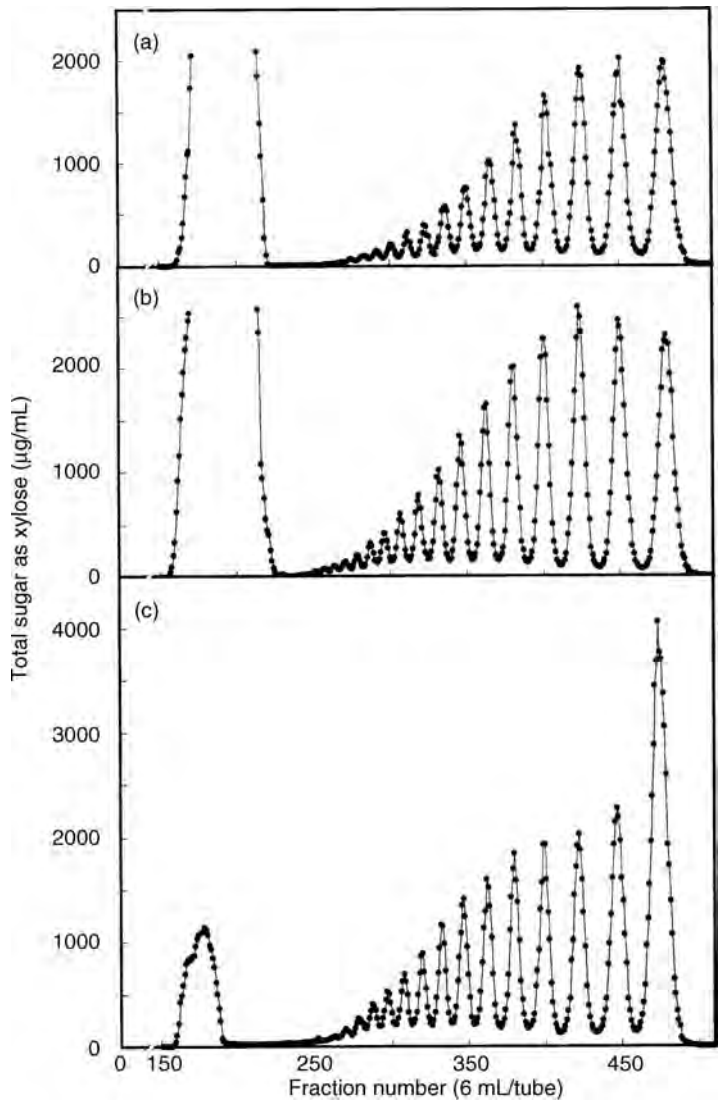

Figure 19.3 *Elution profiles of hydrolysis products of three kinds of xylans on BioGel P-4 and Toyopearl HW-40F columns connected in series: (a) cottonseed xylan; (b) birchwood xylan; and (c) oat spelt xylan. (Adapted from Sun* et al. *[42] © 2002, Elsevier).*

low-molecular-weight compounds such as monosaccharides or phenolics, enabling purification of oligosaccharide mixtures [62]. Compared to the other purification methods discussed above, membrane separation has a number of advantages including low energy requirements, easily manipulated critical operational variables, and relatively easy scale-up [63,64]. Certain operational variables including pressure, temperature, feed flow rate, and agitation can impact properties of the solute and membrane and physical aspects directly related to diffusion and convection of the solute, which in turn affect the overall process efficiency. In addition, oligosaccharide inhibition of enzymatic reactions can be reduced by continuous or semi-continuous

product removal by membranes [62]. However, membrane separation performance can be affected by structural characteristics of oligosaccharides, including the types of monosaccharides, substitutions and linkages in oligomer structure, and the final molecular weight and extent of branching. Furthermore, oligosaccharide solubility has a major impact on membrane separation performance [62].

Membrane separations have been used for preparing various concentrations of several oligosaccharides, including fructose oligosaccharides [65], maltooligosaccharides [66], soybean oligosaccharides [67], pecticoligosaccharides [68], and chitooligosaccharides [69]. However, applications of membranes to refining XOs-containing solutions are limited in the literature; some publications have dealt with the processing of solutions/slurry resulting from hydrolytic treatment followed by enzymatic reaction. Recently, some studies successfully applied membranes to XOs produced by enzymatic hydrolysis or autohydrolysis of xylan-containing materials [47,70]. Yuan *et al*. employed nanofiltration membranes for concentrating XOs obtained by enzymatic hydrolysis of xylan from steamed corn cobs, whereas concentration and fractionation of XOs by sequential membrane-based steps has been employed in multistage purification processes [71].

Although microfiltration and ultrafiltration are well-established separation processes for purifying oligosaccharides from high-molecular-weight enzymes and polysaccharides, commercial streams often contain low-molecular-weight sugars that are undesirable or do not contribute to beneficial properties of the higher-molecular-weight oligosaccharides. In a study by Akpinar *et al.* [31], ultrafiltration was used to separate and purify XOs from hydrolysate generated by enzymatic hydrolysis of cotton stalk xylan. The hydrolysate was first filtered through a 10 kDa molecular weight cut-off membrane to remove high-molecular-weight polysaccharides and enzymes, followed by filtration through a 1–3 kDa molecular weight cut-off membrane to further fractionate the XOs. Permeate from the 1 and 3 kDa membranes contained mixtures of different DPs, with 43.3% and 81.6% reported to have DPs higher than 5, respectively. Although chromatography is still the principal choice, Leiva and Guzman reported that nanofiltration membranes can concentrate or purify oligosaccharide mixtures [72] as an alternative to more expensive chromatographic techniques. The molecular weight cut-off of nanofiltration membranes is in the range of 200–1000 Da, combining ultrafiltration and reverse osmosis separation properties. However, despite its promise for industrial-scale purification and concentration of oligosaccharide mixtures, its performance for fractionation of oligosaccharide mixtures has not yet been convincingly proven.

19.3.5 Centrifugal Partition Chromatography

Centrifugal purification chromatography (CPC), a method based on countercurrent chromatography, has recently been proposed for XOs purification [73]. Separation is based on the differences in partitioning behavior of components between two immiscible liquids. CPC uses a so-called “hydrostatic mode” resulting from constant centrifugal force intensity and direction for separation. Therefore, the mobile phase penetrates the stationary phase either by forming droplets or by jets stuck to the channel walls, broken jets, or atomization. The intensity of agitation of both phases depends on centrifugal force intensity, mobile phase flow rate, and solvent physical properties. Compared to countercurrent chromatography, stationary phase retention is less sensitive to physical properties of the solvent systems such as viscosity, density, and interfacial tension [74]. Similar to other chromatographic techniques, the CPC method is able to separate compounds with a broad range of molecular weights. In addition, samples can be recovered by flushing the system since the stationary phase is also a liquid. In contrast to other chromatographic techniques such as GPC, CPC could be used for preparative separation or purification because of its large stationary phase volume [75].

CPC has been widely used as an efficient purification and separation tool for many compounds including flavonoids, flavonolignans, and macrolide antibiotics [75]. Shibusawa *et al.* [76] employed CPC to purify apple-derived catechin oligosaccharides by operating in an ascending mode with a solvent system

containing equal volumes of hexane, methyl acetate, acetonitrile, and water. Apple catechin oligosaccharides up to DP 10 were successfully fractionated by this method. However, the total mass and corresponding purity of each DP fraction were not reported.

Lau *et al.* [75] used CPC to separate and purify xylan-derived oligosaccharides from birchwood xylan. A CPC solvent system containing dimethyl sulfoxide (DMSO), tetrahydrofuran (THF), and water in a 1:6:3 volumetric ratio, respectively, was chosen for its ability to dissolve XOs of different DPs. Monomeric xylose and XOs up to DP 5 (xylopentose) were collected with this separation system with relatively high purity for DP 1 and 2 (higher than 85%) and relatively low purity for other DPs (lower than 55%).

19.4 Characterization and Quantification of Xylooligosaccharides

19.4.1 Measuring Xylooligosaccharides by Quantification of Reducing Ends

With few exceptions, each oligosaccharide chain has a reducing end on its terminal sugar residue. Because the aldehyde or ketone group of this terminal sugar residue is not fixed into a ring structure, it is free to undergo oxidation-reduction reactions with chemical reagents to form products that can be detected by colorimetric methods. By measuring the number of reducing ends in a sample, the total number of oligosaccharide chains can be determined. Colorimetric methods to measure monosaccharides as well as oligosaccharides employ a UV-Vis spectrophotometer, a simple and inexpensive instrument. Although these methods are still used today, different types of sugars cannot be differentiated.

The most widely used method for colorometric quantification of reducing ends is the dinitrosalicylic acid (DNS) assay, which was first developed to determine the concentration of monosaccharides [77–79] and then applied to quantify the total numbers of oligosaccharide chains in aqueous solution [80,81]. In the DNS assay, 3, 5-dinitrosalicylic acid reacts with sugar reducing ends to form red-brown 3-amino-5-nitrosalicylate, quantified by comparison of its absorbance at 560 nm or above [77–79] to that with pure sugar calibration standards. The volumetric concentration of reducing ends can therefore be calculated by determining the intensity of color formation of 3-amino-5-nitrosalicylate. However, the equivalence between amino-nitrosalicylate produced and the number of reducing ends varies for different sugars, suggesting that the DNS assay can only be accurate for evaluation of a single sugar [77,82]. Other methods in this category, such as the arsenomolybdate (ARS; also known as Nelson-Somogyi assay) assay [83], the p-hydroxybenzoic acid hydrazide (PAHBAH) assay [84,85], and the phenol-sulfuric acid assay [86,87] are also used to measure reducing sugars but have similar response variance issues with different sugars as the DNS assay. When these colorimetric methods were applied to measure reducing ends of xylooligosaccharides, they responded differently to xylooligosaccharides of different DPs. For example, the ARS assay showed less reactivity to higher-DP xylooligosaccharides, while the DNS assay showed the opposite trend [81]. The reason, however, is not well understood.

19.4.2 Characterizing Xylooligosaccharides Composition

The determination of structure of oligosaccharides released from biomass hydrolysis first requires knowledge of what monosaccharide components are present and in what amounts. This can be achieved by enzymatic or chemical decomposition of oligosaccharides into their monosaccharide building blocks followed by identification and quantification of each component by gas chromatography (GC) or high-performance liquid chromatography (HPLC) [4]. GC methods require multistep formation of volatile derivatives of monosaccharides prior to analysis, with two derivatization methods routinely used: formation of alditol acetates or TMS methyl glycosides [4].

Although GC methods have the advantage of baseline sugar resolution [50], HPLC is more widely used for analysis because sugar derivatization is not required. In standard biomass analytical procedures developed by the National Renewable Energy Laboratory (NREL), HPLC employing a refractive index (RI) detector is the default tool for determining total component monosaccharides released from post hydrolysis of oligosaccharides with 4 wt% sulfuric acid at 121 °C for 1 hour [88]. Two columns, both from Bio-Rad, are commonly used in this application. The HPX-87P column can separate all common biomass sugars (cellobiose, glucose, xylose, galactose, arabinose, and mannose) with high resolution. However, retention times (RT) for xylose, mannose, and galactose on the HPX-87H column are close (within 0.1 min); this often results in a single peak, depending on column conditions. Considering that heteroxylans are the dominant form of hemicellulose in most lignocellulosic feedstocks (refer to Section 19.1.2), the amounts of galactose and mannose in oligosaccharides released from biomass pretreatment are low. The HPX-87H column is therefore widely used to measure the glycosyl composition of xylooligosaccharides because it provides stable and near-baseline resolution of glucose, xylose, and arabinose.

19.4.3 Direct Characterization of Different DP Xylooligosaccharides

As discussed in Section 19.1.3, there is a significant and increasing demand for reproducible, fast, and simple methods to characterize and quantify XOs released from biomass pretreatment to better understand the decomposition mechanisms of hemicellulose in major lignocellulosic feedstocks. To date, several methodologies for qualitative and quantitative analysis of XOs have been developed, which can be grouped into the following categories: HPLC, high-performance anion-exchange chromatography (HPAEC), and capillary electrophoresis (CE).

HPLC

Li *et al.* [89] quantitatively analyzed XOs derived from hydrothermal pretreatment of oat spelt xylan at 200 °C for 15 min with a 5 wt% solid loading. A Waters model 717 chromatography system, equipped with a RI detector and a Bio-Rad Aminex HPX-42A ion-moderated partition (IMP) column was used. At a flow rate of 0.2 mL/min and a column temperature of 85 °C, xylooligosaccharides up to DP 10 were separated, but the baseline for the IMP chromatogram was difficult to resolve, especially for DP higher than 5 as shown in Figure 19.4. Commercial low-DP XOs standards (xylobiose, xylotriose, xylotetraose, and xylopentaose) were used to calibrate the IMP system for quantification of xylooligosaccharides with DPs in that same range of 2–5. It was also shown that xylooligosaccharides of DP 2–5 could be quantified by taking the ratio of peak heights of each XO to the peak height for xylose and multiplying this ratio by the concentration of the latter. These results confirmed that peak height followed concentrations closely for xylooligosaccharides with DP less than 5 for an RI detector. This approach was extended to quantifying XOs with higher DPs from 6 to 10; however, accuracy could not be confirmed due to the lack of standards.

Ohara *et al.* [90] used a cation-exchange column (Sugar KS-802; Showa Denko, Tokyo) with RI detector to characterize xylooligosaccharides up to DP 6, which were prepared by enzymatic hydrolysis of birchwood xylan with endoxylanase. The column temperature was 60 °C and the mobile phase was water with a flow rate of 0.6 mL/min.

For HPLC systems, an evaporative light scattering detector (ELSD) was reported to be more sensitive and thus provide better baseline stability than RI-based detectors for measuring oligosaccharides [91]. Yu and Wu [92] identified gluco-oligosaccharides from DP 2 to DP 6 using a Prevail carbohydrate ES column with an ELSD 3300 detector from Alltech. Other literature on specific characterization of xylooligosaccharides using ELSD, however, is scarce.

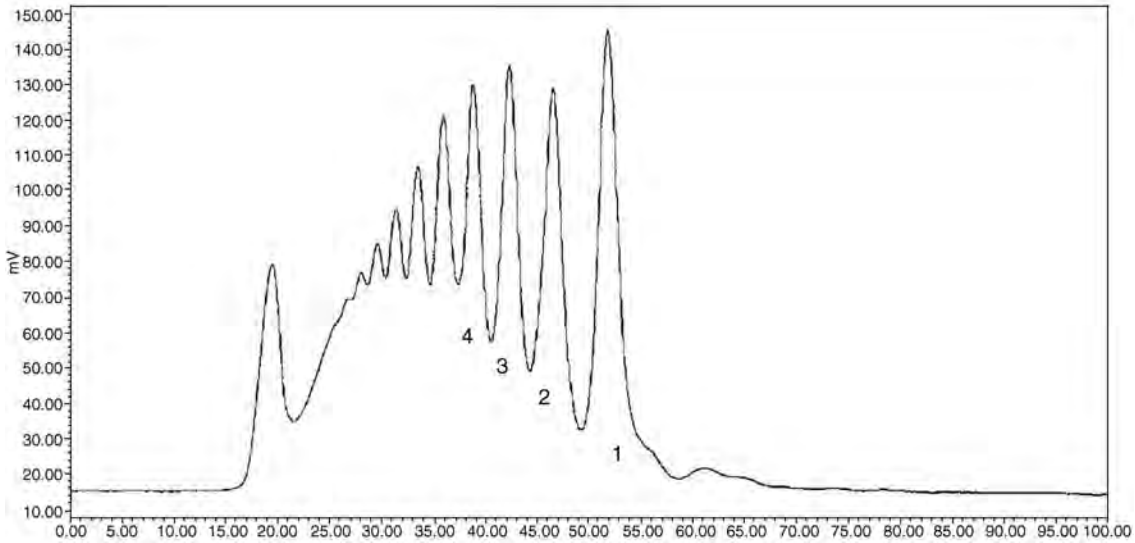

Figure 19.4 *IMP chromatogram of xylooligosaccharides derived from hydrothermal pretreatment of oat spelt xylan. 1: DP1; 2: DP2; 3: DP3; 4: DP4. (From Li* et al. *[89] with permission from Springer).*

Recently, a Waters Acquity ultrahigh-performance liquid chromatography (UPLC) equipped with a BEH HILIC (unbonded ethylene bridged hybrid efficient hydrophilic interaction chromatography) column and 4000 QTrap MS detector was applied to characterize xylooligosaccharides by Tomkins *et al.* [93]. As the chromatogram in Figure 19.5 shows, the approach was very sensitive, with detection of xylooligosaccharides at concentrations of about 1 pmol and very fast within only 2 min needed to separate xylooligosaccharides of DP up to 6.

HPAEC

The advent of HPAEC featuring pulsed amperometric detection (HPAEC-PAD) in the 1980s provided a highly sensitive and selective tool for separation and detection of complex carbohydrates without derivatization. The recognition by Johnson in 1986 that oligosaccharides could be detected by PAD greatly enhanced the popularity of HPAEC [94]. HPAE-CPAD is often classified as an HPLC method. However, HPAEC-PAD technology is discussed in some detail here because of its novel ability to analyze and characterize XOs over a wide DP range. The unique advantages of HPAEC were first described in the paper by Rocklin and Pohl in 1983 [95]. The oligosaccharides were separated in strong alkaline eluents ($pH > 13$), where their hydroxyl groups were deprotonated and thus rendered anionic. The number of hydroxyl groups in a single oligosaccharide molecule varies with DP, resulting in various weakly acidic properties which the HPAEC uses to separate oligosaccharides.

The PAD detection mechanism consists primarily of a three-step potential waveform with a frequency of 1–2 Hz. When analyte molecules are absorbed on the oxidation-free surface of the gold working electrode illustrated in Figure 19.6 [96], a detection potential (E_1) appropriate for the analyte properties as well as oxidation mechanism is applied first, and then the analyte molecules are oxidized. Thus, the anodic signal current can be measured in this step. Following detection, the electrode surface is usually oxidatively cleaned by a positive potential (E_2) and then reactivated by a negative

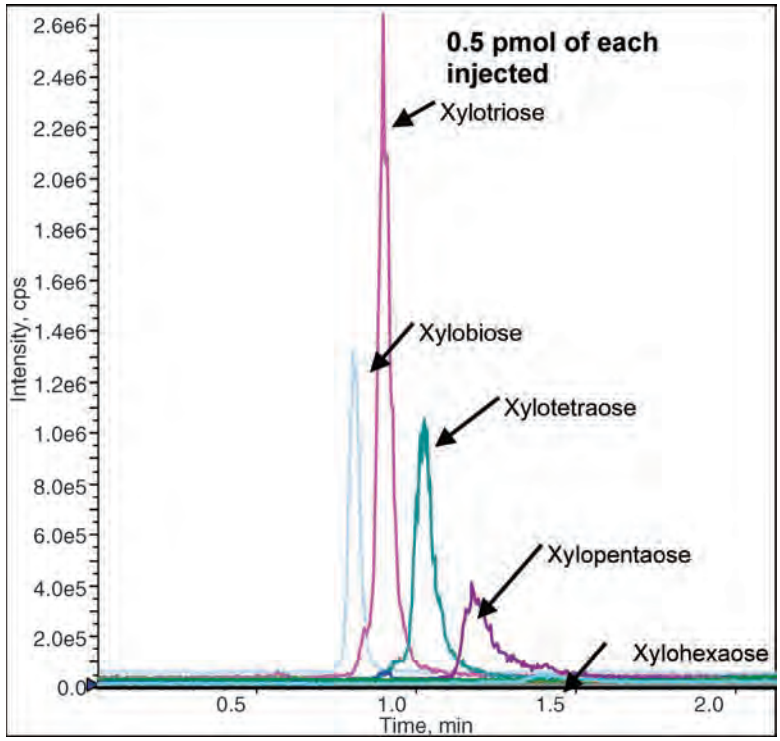

Figure 19.5 *UPLC chromatogram of xylooligosaccharides of DP 2–6. (From Tomkins* et al. *[93] with permission of the authors).*

potential (E_3). Alternatively, the cleaning potential (E_2) could be negative as demonstrated for effectively minimizing electrode wear [97,98]. In particular, a waveform suggested by Dionex Technical Note 21 has been widely used for characterizing monosaccharides and oligosaccharides, as shown in Table 19.2 [97].

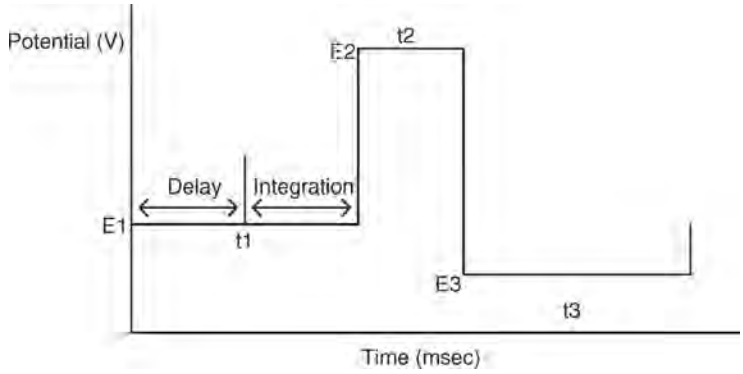

Figure 19.6 *Diagram of the pulse sequence for carbohydrate detection on a PAD detector. (Reproduced with permission of Dionex Corporation).*

Table 19.2 *Waveform of PAD for carbohydrates analysis using the Dionex IC system. (Reproduced with permission of Dionex Corporation).*

Time (ms)	Potential (V)	Integration
0	+0.1	
200	+0.1	Begin
400	+0.1	End
410	−2.0	
420	−2.0	
430	+0.6	
440	−0.1	
500	−0.1	

Dionex Corporation recently advanced its oligosaccharides profiling using HPAEC-PAD with its CarboPac PA-100 and CarboPac PA-200 columns. Several publications have successfully profiled the DP distribution of the oligosaccharides amylopectin, maltodextrin, and inulin up to DP 60 [3,99–103]. However, HPAEC-PAD characterization of xylooligosaccharides derived from hemicellulose in lignocellulosic biomass is difficult due to their low solubility at room temperature and resulting precipitation of higher-DP oligosaccharides [104]. In addition, the heterogeneous glycosyl residue composition of side chains and different linkage substitutions also limit HPAEC-PAD. Yang and Wyman [2] successfully separated XOs released from hydrothermal pretreatment of corn stover using a Dionex DX-600 module with a CarboPac PA100 column. The mobile phase was operated in a gradient mode (50–450 mM of sodium acetate, NaAc) through 150 mM NaOH [103] with the same waveform shown in Table 19.2. As the chromatogram shows in Figure 19.7, xylooligosaccharides with DPs up to 13 were separated well with near baseline resolution. Peaks suspected to represent higher-DP xylooligosaccharides could also be detected, but the separation was relatively poor.

The PAD response of these xylooligosaccharides is believed to depend on the size and spatial structure of analyte molecules [105,106] and vary with DP in this situation. Koch *et al.* showed that relative

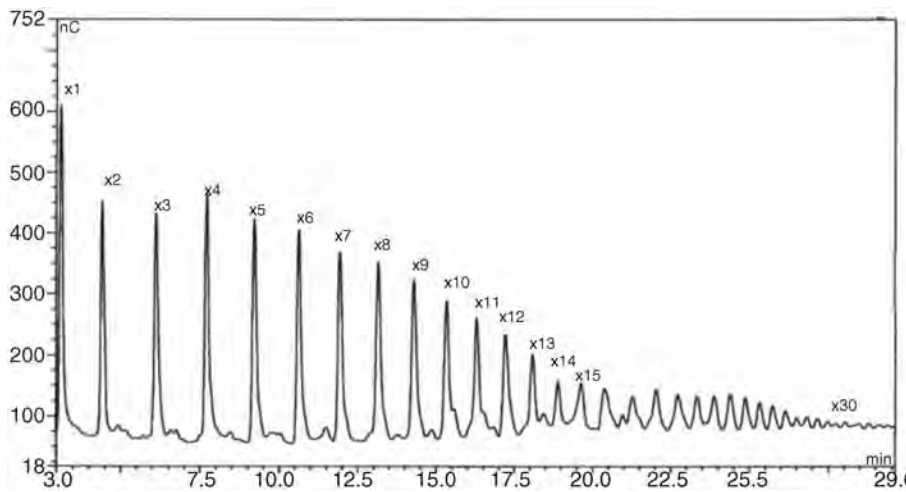

Figure 19.7 *Dionex IC chromatogram of xylooligosaccharides released from hydrothermal pretreatment of corn stover. (From Yang and Wyman [2] with permission of Elsevier).*

electrochemical responses of amylopectin oligosaccharides increased with DP based on molar concentrations but decreased with DP based on mass concentrations [100]. The variable detection behavior is one of the disadvantages of this technique and results in the need for sugar standards for accurate quantification of each DP fraction. However, standards of xylooligosaccharides are only available for DPs below 6 (Megazyme International Ireland Ltd., Ireland) and even then are very expensive.

In the case of HPAEC-PAD for oligosaccharides with the same glycosidic linkages, smaller DP molecules elute first followed by larger ones; however, the order can change when different linkage variants are mixed. For example, Morales *et al.* showed that isomaltohexaose eluted before maltotriose [107]. In fact, factors like charge, molecular size, sugar composition, and glycosidic linkages can impact chromatographic separation [108]. The effects of these factors therefore must be considered when measuring oligosaccharides released by lignocellulosic biomass, particularly for biomass with highly heterogeneous cell-wall polysaccharides. In this situation, additional analytical techniques following HPAEC-PAD are required, such as mass spectroscopy (MS) or nuclear magnetic resonance spectroscopy (NMR).

Capillary Electrophoresis

Capillary electrophoresis (CE) has been successfully applied to separate a wide range of xylooligosaccharide compounds. Since the mid-1990s, application of CE to characterize xylooligosaccharides released from plant cell-wall polysaccharides has been reported in several publications [109–112]. However, due to the lack of charged groups and chromophores, applications to separation of important oligosaccharides by CE are limited [113,114]. Kabel *et al.* applied CE-LIF (laser-induced fluorescence detector) successfully to separate xylooligosaccharides derived from hydrothermally treated *Eucalyptus* woods [115]. In this case, xylooligosaccharides were derivatized with 9-aminopyrene-1,4,6-trisulfonate (APTS), which attached to the reducing end of oligosaccharide molecules to provide a fluorescent APTS tag as well as three negative charges. As shown by the LIF-electropherograms in Figure 19.8, a series of xylooligosaccharides up to DP

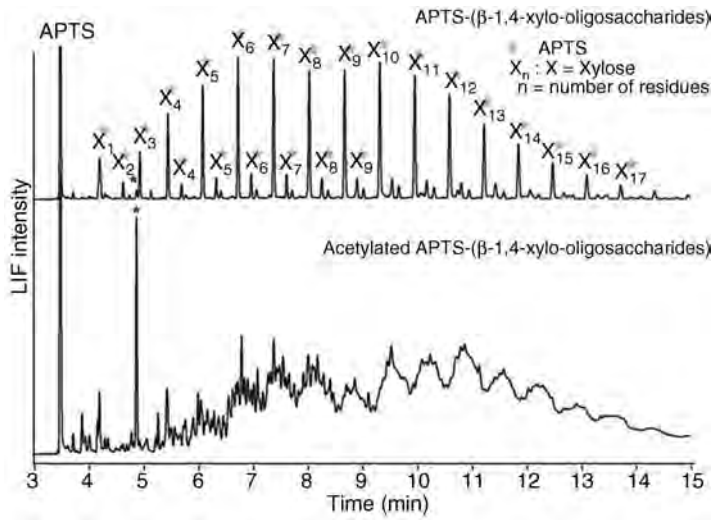

Figure 19.8 *LIF-electropherograms of APTS derivatized* β*-(1, 4)-xylooligosaccharides (top) and (less diluted)* O*-acetylated* β*-(1, 4)-xylooligosaccharides (bottom) obtained from hydrothermally treated* Eucalyptus *wood (* is maltose internal standard). (From Kabel* et al. *[115] with permission from Elsevier).*

17 that had been derivatized with APTS were separated with very high resolution and much better than similar xylooligosaccharide samples that were not derivatized. Coupled with MS, the minor peaks between the major peaks were identified as linear 1, 4-β-xylooligosaccharides with a different structure.

Although derivatization leads to improved sensitivity and resolution with CE, different reactivity of derivatizing reagents and formation of several by-products result in control problems for consistent preparation of analytes [116]. High-pH buffer and other detection techniques have been used to avoid derivatization, but successful application of oligosaccharides profiling has not been shown.

19.4.4 Determining Detailed Structures of Oligosaccharides by MS and NMR

The analytical techniques reviewed in Sections 19.4.1–19.4.3 are effective for characterizing glycosyl residue compositions for oligosaccharides as well as DP profiling of oligosaccharides with the same type of glycosyl linkages. However, they cannot provide detailed structural information for oligosaccharides such as glycosyl linkage composition, the sequence of glycosyl residues, and the anomeric configuration. MS and NMR are needed to characterize such structural features for oligosaccharides.

MS has proven to be valuable for several aspects of structural characterization of oligosaccharides. With different combinations of ionizations and analyzers, MS is often coupled to chromatography techniques such as GC-MS, HPLC-MS, and HPAEC-MS, but many challenges remain. For example, HPAEC could provide good separation of oligosaccharides without the need for derivatization, but the eluent used in HPAEC contains a high concentration of salt which limits use with MS [115]. Regardless of whether MS is online or offline, good separation of oligosaccharides prior to MS analysis will always facilitate structural characterization. Thus, preparative columns such as size exclusion [52] and ion exchange are also used to isolate oligosaccharides into different fractions for offline MS analysis. Currently, electrospray ionization (ESI) and matrix-assisted laser desorption (MALDI) [52] are the most common ionization sources used for xylooligosaccharides characterization in combination with tandem MS analyzers [117–122]. Usually, one type of MS analysis is better for a particular type of oligosaccharide; for example, MALDI-TOF (time-of-flight) MS allows routine determination of the molecular weight of oligosaccharides containing more than 10 glycosyl residues [4]. As the MALDI-TOF mass spectra in Figure 19.9 show, xylooligosaccharides from *Eucalyptus* wood with different chain lengths were characterized with additional information on the degree of acetylation [123].

NMR has proven valuable for understanding oligosaccharide structures. The most-used isotopes in oligosaccharides characterization are ^{1}H and ^{13}C. For example, ^{1}H-NMR can identify the anomeric configuration of glucosyl residues in an oligosaccharide fragment [124–126], and the glycosyl sequence of oligosaccharides can be determined by both 1D and 2D NMR [4,127]. NMR provides an effective method for quick and accurate characterization of specified molecular structures or chemical bonds when the corresponding chemical shifts have been previously defined. In most cases, NMR spectra are insufficient to analyze an unknown structure, and structural information from MS and other analytical techniques must be also used.

19.5 Concluding Remarks

Characterizing oligosaccharides released during biomass pretreatment or enzymatic hydrolysis can reveal important structural information about hemicellulose polysaccharides in plant cell walls and how they change during deconstruction to form sugars or other products. XOs also have excellent potential for applications in pharmaceutical, agriculture, and food industries. XOs can be produced by chemical or enzymatic methods on an industrial scale from lignocellulosic materials. Chemical methods are preferred to produce XO mixtures with a wide DP range, while enzymatic methods are preferred in the food or pharmaceutical

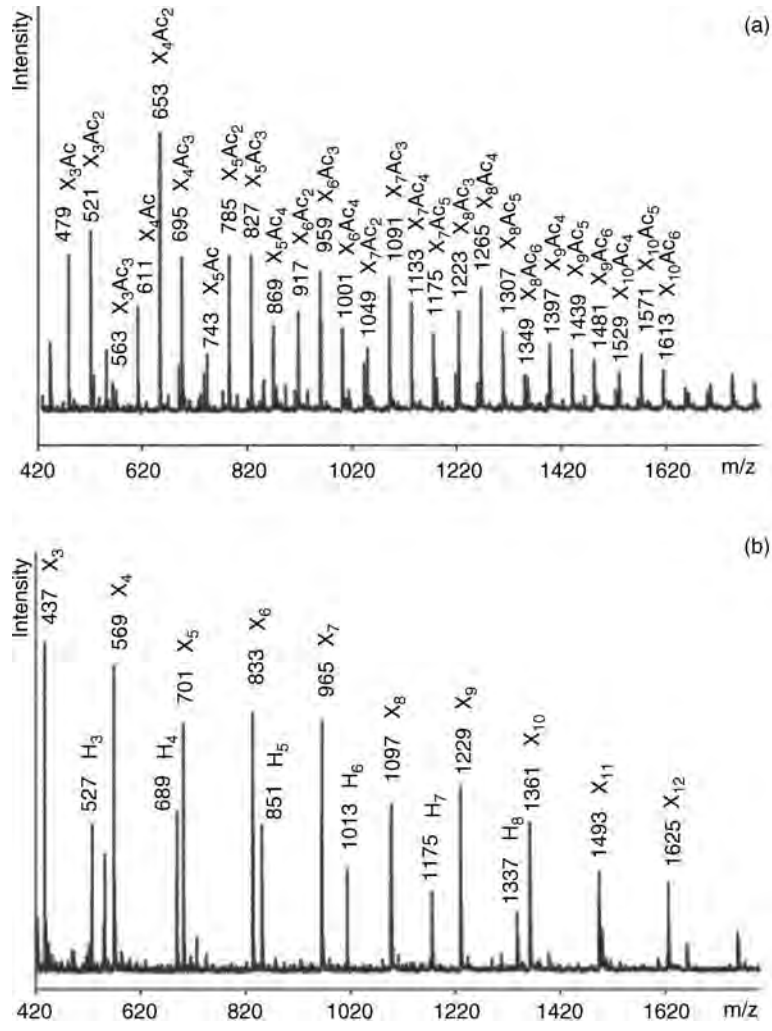

Figure 19.9 *MALDI-TOF mass spectra of the neutral xylooligosaccharides obtained from* Eucalyptus *wood hydrolysate (a) before and (b) after saponification (X = xylose; Ac = acetyl-group; H = hexose). (From Kabel* et al. *[123] with permission from Elsevier).*

industries to reduce formation of degradation products. With the growing importance of making fuels from cellulosic biomass and the increasing demand for xylooligosaccharides, more opportunities are emerging to process xylan-rich pretreatment hydrolyzate in a cellulosic biorefinery into high-value products that could further lower the cost of cellulosic biofuels. However, separation technologies are needed to produce high-purity XO fractions that span desired DP ranges for industry applications or characterization.

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