Current Trends in Lignocellulosic Analysis with Chromatography

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Abstract

The conversion of lignocellulosic biomass into biofuel and biomaterial is promising for the substitution of fossil resources in energy and material applications. Given the complexity of plant cell wall, the main challenge is to obtain lignocelluloses with high yield and purity. For a better understanding of lignocellulosic biomass, chromatography stands out as a powerful separation method that can support the lab directed research and pilot scale production of biomaterial and biochemical. This paper provides a review on the characterization of cellulose, hemicellulose and lignin along with their derivatives and decomposed sugar monomers, in particular their isolation and purification methods using various specific types of chromatography. Methods with various specific types of chromatography. This review also summarizes different chromatographic methods for obtaining the molecular weights of cellulose, hemicellulose and lignin that have been used in recent years, and highlights future opportunities for the application of those biopolymers.

Lignocellulosic Feedstock for Bioenergy and Biomaterial

The rising global demand for energy and material from unstable and expensive petroleum resources plus concerns about the economy and global warming call for the development of renewable energy sources to replace fossil fuels [1,2]. Since the first-generation biofuels produced from sugar, starch, and oil-seed-based feedstocks have generated serious competition and a debate for food resources. Therefore, second-generation biofuels expanded the feedstocks to lignocellulosic biomass, including agricultural residues, agro-industrial wastes, forestry and energy crops, which are abundant, renewable, and cost-effective non-food resources [3]. Certain oils produced by plants and algae can be used directly as fuel or chemically transesterified to biodiesel. Hydrogen gas can be produced by not only photosynthetic algae and cyanobacteria under certain nutrient- and/or oxygen-depleted conditions but also bacteria and archaea utilizing organic substrates under anaerobic conditions. Other biofuels such as bioethanol and biobutanol can also be produced as organic substrates fermented by microbes under anaerobic conditions [1].

First-generation biofuels can impact the environment, the economy, water overconsumption and pollution, deforestation, biodiversity loss, and social conflict [4]. Most authorities such as the EU-Renewable Energy Directive and Food and Agriculture Organization of the United Nations have become aware of these effects and issued regulations on the production of first-generation biofuels [4,5]. To date, one of the most important liquid forms of biofuel from carbohydrates is bioethanol. The United States became the world's largest producer of ethanol fuel in 2005, the production of which had increased from 1.63 billion gallons in 2000 to 13.2 billion U.S. liquid gallons (49.2 billion liters) in 2010 and subsequently to 13.9 billion U.S. liquid gallons (52.6 billion liters) in 2011 [7]. Moreover, the minimum volumes of different types of biofuels that must be included in the United States' supply of fuel for transportation by the Renewable Fuel Standard (RFS) is intended to rise to 36 billion gallons by 2022 [5-7]. Recently, technological advances have boosted the production of cellulosic ethanol and ethanol from plant waste (e.g., corn stover) on a commercial scale with the opening of a \$275 million, 25-million gallon per year cellulosic ethanol plant in Emmetsburg, Iowa-a joint venture between POET, an American biofuels company, and the Dutch firm Royal DSM [8]. Poet-DSM will produce cellulosic ethanol from corn cobs, leaves, husks, and corn stalks harvested by farmers located within a 30- to 40-mile radius of the plant [9].

In addition to the biofuel production, application of biomaterials from lignocellulosic biomass is also essential to the functioning of industrial societies and critical to the development of a sustainable global economy. Although wood and paper products have already played important roles in the evolution of civilization, there is increasing interest in the improvement of the quality and manufacturing efficiency of high performance lignocellulosic composites. As main biopolymers in plant cell wall, cellulose and hemicellulose along with their derivatives such as cellulose esters, silyl celluloses, cellulose sulfonates, aminocelluloses, cellulose nanowhiskers, have been widely and successfully applied to the practical products including novel film, foam, coating systems membranes, pharmaceuticals, cosmetics, and food [10-12]. However, lignin as the second most

abundant biopolymer on earth is still underutilized in related fuel and material projects [13]. Although lignin combustion helps the papermaking chemical recovery process, as a fuel it is very inefficient, processing less than $\frac{1}{4}$ as much energy per pound as middle distillate fuels [14].

Lignocellulosic biomass is mostly cell wall material mainly composed of three biopolymers: cellulose (35-50%), hemicellulose (20-32%) and lignin (10-35%) [15,16]. The sources can generally be grouped into six categories: energy crops, agricultural residues, logging residues, mill residues, forest resources, and urban waste. Forest-derived resources include forest residues from harvesting or land conversion, unused mills from wood or pulp processing, waste from urban wood, and debris from construction/demolition. The energy crop category includes lignocellulosic crops such as herbaceous switch grass and woody crop poplar [17]. Cellulose is a linear homopolysaccharide composed of β -D-glucopyranosyl units linked by 1 $\rightarrow$ 4 glycosidic bonds with cellobiose as the repeating unit [18]. Cellulose also has a strong tendency to form intra- and intermolecular hydrogen bonds that result in unique ultrastructure of native cellulose in plant. Some of these H-bonds contribute to stiffening the straight chain and promoting aggregation in the crystalline structure (highly ordered regions); the others could form less-ordered (amorphous) regions [13] that result in the change of cellulose crystallinity in lignocellulosic materials according to their origin and acquisition process. Crystalline cellulose displays six various allomorphs (I, II, III, IIII, IVI, and IVII) with the possibility of conversion from one form to another [19]. Hemicellulose is a collective term used to present a family of polysaccharides found in the plant cell wall with various compositions and structures. Different from cellulose, hemicelluloses are composed of combinations of pentose and/or hexoses, such as xylose, arabinose, mannose, galactose, and glucose, with acetylated groups, uronic acid and 4-O-methyl ethers [20,21].

The hemicelluloses are broadly classified into four general classes of polysaccharide types based on structural differences in the cell wall: xylans, mannans, β -glucans with mixed linkages, and xyloglucans. The main hemicelluloses in softwood are galactoglucomannans and arabinoglucuronoxylan while that in hardwood is glucuronoxylan [20,21]. Highly branched structures and acetyl groups on the polymer chain result in a lack of crystalline structure in hemicellulose. The most abundant sugar monomer in the softwood hemicelluloses is mannose; correspondingly, the most abundant sugar monomer in hardwood hemicellulose is xylose. Xylan is a heteropolysaccharide with a homopolymeric backbone chain of 1,4-linked β -D-xylopyranosyl units. The branches of xylan, which may contain arabinose, glucuronic acid, or the 4-O-methyl ether, acetic, ferulic, and *p*-coumaric acids, vary depending on their origins [22]. For example, poplar normally contains ~20% hemicelluloses in which O-acetylated 4-O-methylglucuronic acid xylan or glucuronoxylan is the main component. Numerous studies revealed that xylans (15.9 to 22.4%) are the major hemicellulose in all the poplar species in their study, followed by mannans (0.9 to 3.4%). 4-O-methyl-glucuronic acid (4-O-MeGlcA; 2.2 to 2.8%), galacturonic acid (2.3 to 2.8%) and minor amounts of glucuronic acid (0.1 to 0.3%) have been identified as the uronic acids present in poplar [23]. Galactoglucomannan is comprised of (1 $\rightarrow$ 4)-linked β -D-glucopyranosyl and D-mannopyranosyl units that are partially acetylated at the C2-OH and C3-OH and partly substituted by (1 $\rightarrow$ 6)-linked α -D-galactopyranosyl units [24]. Figure 1

summarizes the monosaccharide of several lignocellulosic biomasses from literature [25-30].

Figure 1: Content of monosaccharides for several lignocellulosic biomasses.

Lignin is the second most abundant biomass component and the primary renewable aromatic resource in nature [13]. Lignin fills the spaces between cellulose and hemicelluloses acting as a resin that bonds the lignocelluloses matrix together. Distinctly different from cellulose and hemicelluloses, lignin is one of the most complex natural polymers synthesized by enzymatic dehydrogenative polymerization of 4-hydroxyphenyl propanoid units [31]. Lignin is composed predominantly of three phenylpropane monomers: *p*-hydroxyphenyl (H, from *p*-coumaryl alcohol), guaiacyl (G, from coniferyl alcohol), and syringyl (S, from sinapyl alcohol) units [32]. The composition of lignin varies depending on its origins, [33,34] softwood (i.e., gymnosperm, Scots pine) lignin is predominantly derived from G-type monolignol, in contrast, hardwood (i.e., angiosperm, *Populus*) lignin is mainly derived from G- and S-type monolignols with trace of H-type monolignol [35]. Softwood generally contains about 25-35% of lignin and hardwood 18-25% of lignin. Grass lignin (e.g., switchgrass) is derived mostly from G- and S-type monolignols with significant amount of H-type monolignol, along with significant levels of *p*-coumaric and ferulic acid that is involved in cross-linking to lignin and hemicelluloses complex [36]. In addition, hemicelluloses are covalently linked by relative hydrophobic lignin and thereby form those cross-links that are also called Lignin-Carbohydrate Complexes (LCCs) with heterogeneous structures. LCCs, consisted of phenyl glycoside bonds, esters, and benzyl ethers, are presumed to exist in higher molecular weight lignin fractions which are water insoluble. LCCs in hardwood and grass are composed in part from 4-O-methylglucuronoxylan and arabinoglucuronoxylan, respectively [37-39]. In contrast, carbohydrate portions are mainly composed of galactomannan, arabinoglucuronoxylan, and arabinogalactan, all of which linked to lignin at benzyl positions for LCCs in softwood [40,41].

An integrated biorefinery application of the three main biopolymers along with their derivatives and hydrolysates calls for related separation, purification and analytic methods. Chromatographic method is one of the most powerful analytical techniques for the analysis of the type and concentration of carbohydrates and lignin. Thin layer chromatography (TLC), gas chromatography (GC), high performance liquid chromatography (HPLC) are commonly used to separate and identify carbohydrates and lignin, which are separated on the basis of their differential adsorption characteristics, such as their partition coefficients, polarities or sizes, by passing the solution to be analyzed through a

column. HPLC is currently the most important chromatographic method for analyzing biopolymers, such as size-exclusion chromatography (e.g. gel-permeation chromatography) and ion exchange chromatography (e.g. ion-exchange chromatography and ligand-exchange chromatography), because of its rapid, specific, sensitive and precise measurements [42].

Molecular Weight Analysis of Cellulose, Hemicelluloses and Lignin

The knowledge of molecular weight and its distribution for lignocellulosic biopolymers are necessary to not only monitor the chemical reactions of polymeric materials including repolymerization and/or depolymerization, but also indicate the changes of their physiochemical properties during various treatments and estimate their process ability and basic utility properties.

Cellulose isolation and determination of degree of polymerization

Many properties of cellulose depend on its degree of polymerization (DP), such as the effect of pretreatment technologies on the DP and its subsequent influence on the enzymatic hydrolysis of cellulose for the purpose of producing fermentable sugars [43,44]. DP can be defined in terms of the number-average DP (DP_n), weight-average DP (DP_w) and viscosity-average DP (DP_v) according to the following equations [45]:

$$DP_n = \frac{M_n}{MW_{glu}} = \frac{\sum N_i M_i}{\sum N_i} / MW_{glu}$$

$$DP_w = \frac{M_w}{MW_{glu}} = \frac{\sum N_i M_i^2}{\sum N_i} / MW_{glu}$$

$$DP_v = \frac{M_v}{MW_{glu}} = \frac{\sum N_i \eta}{\sum N_i} / MW_{glu}, \text{ where } \eta = K_m M_i^{\alpha+1}$$

where N_i is the number of moles of a given fraction i with molecular weight M_i , M_n is the number-average molecular weight, M_w is the weight-average molecular weight, M_v is the viscosity-average molecular weight, MW_{glu} is the molecular weight of an hydroglucose (162 g/mol), η is viscosity, K_m is a constant, and the values of α for cellulose and cellulose derivatives range from 0.75 to 1 [45].

Isolation and purification of cellulose without chain alteration prior to the analysis of cellulose DP normally consist of Soxhlet extraction of native biomass, delignification by using glacial acetic acid and sodium chlorite and/or peracetic acid to generate the holocellulose, and extraction with concentrated sodium hydroxide according to the TAPPI T-203 cm-09 method [46,47]. Most commonly used techniques of measuring cellulose DP are viscometry and gel permeation chromatography (GPC) methods [48]. Although the viscometry method provides cellulose DP_v relatively quickly and conveniently, it has several limitations, including non-absolute average M_v values, which depend greatly on solvent and temperature conditions, no clear information concerning the molar mass distribution, and complex metal solutions used with the testing method that can degrade cellulose [49]. In contrast, GPC as a type of size exclusion chromatography (SEC) can provide more detailed information, including DP_n , DP_w with all three molecular weights (M_n , M_w , M_v), and the polydispersity index ($PDI = M_w/M_n$), which is used to measure the broadness of the molecular weight distribution of a polymer. The relative cellulose molecular weights

that GPC provides are based on the molecular weight of well-defined polystyrene standards with varying molecular weights [50]. A list of the DPs of cellulose determined by the nitration method followed by the measurement of viscosity is summarized in Figure 2 [51-55]. Nitration was carried out in a mixture of $HNO_3:H_3PO_4:P_2O_5$ (64:26:10, w/w), and the final product was then used for a nitrogen assay and for the viscosity measurement after fractionation. The specific viscosity of each fraction was calculated from the measurement of the efflux times at decreasing concentrations in a viscometer at 25°C. The intrinsic viscosity was calculated by extrapolation to zero of a plot of specific viscosity over concentration. The average molecular weight of each fraction was estimated by the Mark-Houwink equation [56]. Thus, the distribution pattern of the DP of the cellulose could be determined. This method does not require pre-isolation of cellulose through holocellulose pulping and the base-catalyzed hydrolysis of the hemicellulose [50]. However, the nitration method is rarely used because of uncertainty arising from the possible acid hydrolysis of the cellulose chain during derivatization along with the instability of the derivative. Nitric and phosphoric acids are very dramatic, resulting in the over-hydrolysis of cellulose chains, which has been confirmed by several studies that have shown a significant reduction in the DP of cotton and aspen cellulose by the nitration method [48-50].

Figure 2: DP of types of cellulose after nitration by the viscometric method.

To date, cellulose tricarbanilate (CTC) has been a commonly used cellulose derivative for DP measurement by GPC [57]. Cellulose tricarbanilation is commonly performed by the reaction of cellulose with phenyl isocyanate in pyridine as the solvent. The unreacted phenyl isocyanate is quenched by methanol that is then added to the mixture. Afterwards, the mixture is poured into a 3:7 water-methanol mixture to precipitate the cellulose tricarbanilate. The derivatized cellulose is finally purified and dissolved in tetrahydrofuran (THF) for GPC measurement [47,57]. When CTC facilitates the study of cellulose DP by GPC, it has several advantages, including complete substitution, no de-polymerization during derivatization, the stability of the derivative, and solubility and stability in the THF [58]. However, this testing method is only applied to the pure cellulose isolated from samples that are native biomass and biomass after various treatments. Figure 3 summarizes the DP values of several native cellulose samples based on the viscometry technique, [59] which is more adequate for analyzing cellulose DP because of the complexity of the lignocellulosic biomass [59-63]. Cellulose DP ranges from around 1,500 to 4,500, depending on the various origins of biomass such as hardwoods (e.g., poplar and aspen) with a cellulose DP of 3,500 and 4,500, and agricultural residues, which vary within a range of 1,800 to 4,000.

Furthermore, for the molecular weight analysis without cellulose derivatization there are two basic types of direct SEC

Figure 3: DP of native woody and non-woody cellulose.

methodologies based on the solvent used: (1) aqueous SEC, and (2) SEC in aprotic solvents, such as Dimethylsulfoxide (DMSO) and N,N-dimethylacetamide/lithium chloride (DMAc/LiCl). Viscometry method as described above to determine the cellulose DP is based on the aqueous SEC, in which stable solvents for cellulose are employed in the initial attempts to elaborate a SEC methodology for the direct analysis of underivatized cellulose. Cadoxen, a solution of CdO in aqueous ethylenediamine as a clear, colorless and stable solvent for cellulose was widely used to investigate the cellulose that can be dissolved in aqueous sodium hydroxide with the DP no more than 400-500. However, several drawbacks, such as the aggressive property to destroy the packing of the column and the time-consuming preparation, hindered the further use for the undissolved cellulose with longer molecular chain length [64,65]. For the SEC in aprotic solvents, DMAc/LiCl was widely used as the solvent of choice for direct analysis of cellulose in the study of wood pulps of different origin [66]. However, studies found that DMAc/LiCl caused (1) aggregation of cellulose in the solution, which is dependent on the concentration of cellulose or LiCl; (2) incomplete dissolution of certain cellulose samples, such as tunicate cellulose and soft wood bleached kraft pulps; and (3) detrimental degradation of cellulose upon heating during dissolution [67,68]. The application of lithium chloride/1,3-dimethyl-2-imidazolidinone (LiCl/DMI) has exhibited great advantages over DMAc/LiCl, such as stability of cellulose and solubility of tunicate cellulose and kraft pulps [68-72]. Figure 4 summarizes some of the samples that are dissolved in 8% LiCl/DMI and analyzed by SEC-MALLS with their respective DP values. Recently, light scattering techniques are usually applied to obtain absolute molecular weight of cellulose and DP. SEC coupled with a multiangle laser light scattering (MALLS) and a refractive index (RI) detector is the system typically used for the light scattering measurement. New chromatographic method based on GPC analysis with a MALLS detector for cellulose

Figure 4: DP of cellulose-rich samples dissolved in LiCl/DMI with the SEC-MALLS method. SW: softwood, HW: hardwood.

molecular weight analysis employing novel solvent mixture of dimethylformamide (DMF) containing 10-20% (v/v) 1-ethyl-3-methylimidazolium acetate (EMIM Ac) has also been developed as cellulose solvent and eluent that eliminates time intensive sample preparation and allows to measure larger sample numbers necessary for in-depth understanding of enzymatic cellulose hydrolysis. This mixture dissolves cellulose and elutes cellulose from the GPC column to evaluate three commonly used cellulose types Avicel, α -cellulose and Sigmacell 101, showing great advantages, such as no requirement of any prior cellulose swelling, activation, or derivatization, reducing the sample preparation time from several days to a few hours [73]. This method can potentially be extended for the measurement of lignin and wood, similar to earlier approaches working with ionic liquids [74,75]. Moreover, in order to obtain the molecular weight distributions of the eluted cellulose, either the system of the column set will be calibrated or the use of a molecular weight sensitive detector will be required. The simplest and widely accepted way to perform the column calibration is to use a relative calibration based on a set of well characterized polymer standards with a narrow distribution. Therefore, poly (ethylene oxides), dextrans and the pullulans have been widely developed and used as standards for the aqueous SEC, whereas polystyrene and pullulan standards are widely applied for the organic SEC analysis [76,77].

In brief, viscometry and GPC are the two commonly used techniques to measure DP of cellulose, by which the viscosity average DP (DP_v), the number average (DP_n) and weight average DP (DP_w) can be provided respectively. However, given the complex structure of lignocellulosic materials, the future DP measurement techniques need to meet requirements that can both fractionate biomass so that cellulose can be extracted, dissolved, and analyzed for DP values without derivatization, and minimize alteration of the native structure of cellulose [78,79].

Fractional purification and DP of hemicelluloses

The isolation of hemicellulose typically begins with a dignified holocellulose followed by an aqueous alkaline extraction [80]. In order to determine the detailed structure without tedious wet-chemistry fractional precipitation, the extracted hemicelluloses can be further fractionated and purified by using chromatographic techniques, including the size-exclusion chromatography and anion-exchange chromatography. Alumina, carbon, cellulose, diethylaminethyl (DEAE) cellulose, and ion-exchange resins are the common column packings to fractionate the hemicelluloses, of which DEAE derivatized column has been successfully used to fractionate the water- and alkali-soluble hemicelluloses from dewaxed sugarcane bagasse [81]. Compared with cellulose, hemicelluloses are more easily fractionated by SEC according to molar mass or more correctly according to hydrodynamic volume due to their good solubility either in aqueous and aprotic organic solvents. Most hemicelluloses have relatively low molecular weight with a DP of 50~300 in wood that could contain 15-30% of hemicellulose in hardwood or softwood, summarized in Table 1 [23,82-84].

Lignin isolation and molecular weight analysis

Characterization of lignin molecular weight has been studied using a pool of instruments, including vapor pressure osmometry (VPO), ultra filtration, light scattering (static and dynamic), mass

spectrometry, and gel permeation chromatography (GPC), [85-90] of which GPC stands out due to its great advantages, such as short sample processing times, tiny quantities of sample required for testing, good tolerance of synthetic and naturally occurring polymers, and the broad range of molecular weight detection [91]. Derivatization is typically needed through methylation, acetylation, or silylation to enhance lignin's solubility in an organic solvent (e.g. tetrahydrofuran THF) with the aid of polystyrene standards that are used to construct the calibration curve for GPC measurements. After derivatization and dissolution, the lignins are then introduced to a porous gel column to accomplish the size separation. The hydrodynamic radius of lignin dictates whether a lignin molecule is included or excluded from a pore in the gel. The lignin then elutes from the column at a volume related to its size and the dimensions of the pores in the gel. The elution volume is coupled with a standard to determine relative molecular size and weight [92]. Various types of lignin polymerization pathways result in a highly branched macromolecule with weight average of molecular weight (M_w) ~20,000 in softwood and slightly low molar mass in hardwood, along with polydispersity of 2.5 and 3.5 respectively [93]. The molecular weights of various lignins isolated using enzymatic mild acidolysis from different resources are summarized in Figure 5 [94,95].

Figure 5: Weight-average molecular weight (M_w) and number-average molecular weight (M_n) of various lignins isolated using enzymatic mild acidolysis from various biomasses.

Carbohydrate Analysis and Sugar Monomers Separation

The most abundant sugars in nature consist of D-glucose, D-fructose, D-galactose, and D-mannose (hexoses), as well as D-xylose, L-arabinose, and D-ribose (pentoses) [96]. Sugar production from acid and enzymatic hydrolysis has been investigated for centuries [2]. There are several steps required to obtain pure sugars after produced from biomass through an acidic hydrolysis pathway, including neutralization of sugar-containing hydrolysate, filtration of formed salt if mineral acids were used as catalysts, and/or evaporation of organic acid if used. Thereafter, the monomeric sugars can be separated to different sugar fractions with the aid of chromatographic techniques, in which different types of anionic or cationic exchanger resins facilitate the separation. Cellulose hydrolysis with concentrated mineral acids generates abundance of salts formed during the neutralization step along with high cost of equipment maintenance due to the corrosive problem by strong acids at high temperatures. Diluted acids were used to release most of the sugars from hemicelluloses in order to keep most of cellulose and lignin intact, since too high temperatures resulted in the side reaction and the dissolution of cellulose and lignin. One of the best methods to achieve quantitative saccharification of wood that is also used for

Table 1: Composition and DP of Hemicellulose in lignocellulosic feedstocks.

Biomass	Hemicellulose	Carbohydrate composition of the hemicellulose		
		Sugar residues	Molar ratio	DP
Aspen	Glucuronoxylan	4-O-MeGlcA:Xyl	9:100	101-122
Birch	Glucuronoxylan	4-O-MeGlcA:Xyl	5:100	101-122
Poplar	Glucuronoxylan	4-O-MeGlcA:Xyl	12.5~13.8:100	50-300
Larch	Arabinoglucuronoxylan	Ara: 4-O-MeGlcA:Xyl	10:12:100	107-145
	Galactoglucomannan	Gal:Glu:Man	8:26:100	118-132
Spruce	Arabinoglucuronoxylan	Ara: 4-O-MeGlcA:Xyl	6:13:100	107-145
	Galactoglucomannan	Gal:Glu:Man	16:24:100	118-132
Pine	Arabinoglucuronoxylan	Ara: 4-O-MeGlcA:Xyl	10:16:100	107-145
	Galactoglucomannan	Gal:Glu:Man	9:22:100	118-132

*, weight ratio.

the analysis of carbohydrate and Klason lignin is a two-step method to saccharify biomass as follows: acid hydrolysis with 72 wt% sulfuric acid at 30°C for 1 h, followed by a secondary hydrolysis using 4 wt% sulfuric acid at 121°C for 1 h in autoclave. Oligosaccharides were formed at the first stage, whereas at the second stage, monomeric sugars were obtained as products. Alternatively the second hydrolysis can also be performed with 1 wt% sulfuric acid to produce xylose and arabinose, which are more sensitive to further reactions [97].

With the development of technology, current bio-processes for producing lignocellulosic ethanol are typically divided into several steps: size reduction of biomass, pretreatment, enzymatic cellulose hydrolysis (saccharification), fermentation, and distillation [98], of which various sugar monomers are generated after the enzymatic hydrolysis of cellulose and hemicelluloses. Methodologies for analysis and separation of woody and non-woody sugars have undergone rapid advances in recent years. The older methods used to determine carbohydrate composition of wood and pulp were paper and thin-layer chromatography, which were initially replaced by gas chromatography-mass spectrometry (GC-MS) following derivatization, and later by cation-exchange HPLC coupled with refractive index detection. Recently anion-exchange HPLC with pulsed amperometric detection (PAD) has been found to provide rapid and versatile methods for carbohydrate analysis with great advantages of high sensibility, selectivity and specificity, and easier sample preparation [43,99] PAD is based on the oxidation of the carbohydrates in multistep potential waveforms applied to Au electrodes in miniature flow-through cells. The separation of sugar monomers is based on the weakly acidic properties of carbohydrates (Table 2) in alkaline solutions. Neutral and acidic carbohydrates are partially or completely ionized at high pH and retained on the column. This technique is also applicable to monosaccharides, disaccharides and oligosaccharides. Furthermore, an improved separation method of carbohydrates utilizing a carbonate-modified anion exchange column was also developed to give an improved separation of monosaccharide with higher accuracy, shorter instrument analysis time, and minimal sample preparation [100]. In this study, a commercially available anion-exchange column that is modified with carbonate prior to analysis and pulsed amperometric detection was developed to alleviate stability concerns common to HPAEC separations. The modified column is capable of providing near-baseline resolution of monomeric sugars and sucrose (with isocratic elution) in approximately 5 minutes. Cellobiose and maltose can also be accommodated with a minimal increase in run time

Table 2: Dissociation Constants of Some Common Carbohydrates (in water at 25°C).

Sugar	pKa
Fructose	12.03
Mannose	12.08
Xylose	12.15
Glucose	12.28
Galactose	12.39
Dulcitol	13.43
Sorbitol	13.60

(ca. 3 and 6 minutes respectively). The new approach offers a 3- to 4-fold reduction in analysis time, compared to current practice, with no significant compromise in chromatographic resolution [100].

Conclusion and Outlook

This review has outlined various chromatographic methods for the molecular weight determination of lignocellulosic biopolymers and fractionation of carbohydrate hydrolysates. The key parameter in the methods to determine molecular weight of cellulose and lignin is that the isolation and derivatization/dissolution techniques must not significantly alter their native properties. GPC is one of the best techniques to use for detailed analysis on the molecular weight and/or DP of cellulose, hemicellulose and lignin because it allows analyzing the distribution of the molecular weights and acquiring more insights on the nature of the bipolymer chains. In an effort to resolve the main limitation of all methods- biopolymer solubility, new solvents, like the ionic liquids that were introduced recently in cellulose and lignin chemistry, are also of great interest in the analytical field but still with limited level of application in SEC. Comparative studies of different methods for determining molecular weight and sugar fractionation are still rare. Besides, developing a molecular weight measurement technique that can minimize alteration of the native structure of cellulose and lignin is a major future need in the field of biofuel and biomaterial.

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References

- Drapcho CM, Nhuan NP, Walker TH. Biofuels engineering process technology. McGraw-Hill New York, NY, USA. 2008.
- Ragauskas AJ, Williams CK, Davison BH, Britovsek G, Cairney J. The path forward for biofuels and biomaterials. *Science*. 2006; 311: 484-489.
- Munasinghe PC, Khanal SK. Biomass-derived syngas fermentation into biofuels: Opportunities and challenges. *Bioresour Technol*. 2010; 101: 5013-5022.
- Gasparatos A, Stromberg P, Takeuchi K. Sustainability impacts of first-generation biofuels. *Animal Frontiers*. 2013; 3: 12-26.
- International Energy Agency. From First- to Second-Generation Biofuel Technologies: An Overview of Current Industry and RD&D Activities. Paris: OECD/IEA. 2008.
- Association RF. Accelerating industry innovation: ethanol industry outlook. Renewable Fuels Association. 2012.
- Lichts F. Industry statistics: 2010 world fuel ethanol production. Renewable Fuels Association. 2010.
- Service RF. Renewable Energy. Cellulosic ethanol at last? *Science*. 2014; 345: 1111.
- <http://poet-dsm.com/>
- Habibi Y, Lucia LA, Rojas OJ. Cellulose nanocrystals: chemistry, self-assembly, and applications. *Chem Rev*. 2010; 110: 3479-3500.
- Eichhorn SJ. Cellulose nano whiskers: promising materials for advanced applications. *Soft Matter*. 2011; 7: 303-315;
- Hansen NM, Plackett D. Sustainable films and coatings from hemicelluloses: a review. *Biomacromolecules*. 2008; 9: 1493-1505.
- Ragauskas AJ, Beckham GT, Biddy MJ, Chandra R, Chen F. Lignin valorization: improving lignin processing in the biorefinery. *Science*. 2014; 344: 1246843.
- Kadla J, Kubo S, Venditti R, Gilbert R, Compere A, Griffith W. Lignin-based carbon fibers for composite fiber applications. *Carbon*. 2002; 40: 2913-2920.
- Kumar P, Barrett DM, Delwiche MJ, Stroeve P. Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. *Ind Eng Chem Res*. 2009; 48: 3713-3729.
- Zhao XB, Zhang LH, Liu DH. Biomass recalcitrance. Part I: the chemical compositions and physical structures affecting the enzymatic hydrolysis of lignocellulose. *Biofuels Bioprod. Biorefining*. 2012; 6: 465-482.
- Gronowska M, Joshi S, MacLean HL. A review of US and Canadian biomass supply studies. *BioResources* 2008; 4: 341-369.
- Klemm D, Heublein B, Fink HP, Bohn A. Cellulose: fascinating biopolymer and sustainable raw material. *Angew Chem Int Ed Engl*. 2005; 44: 3358-3393.
- Klemm D, Schmauder HP, Heinze T. Cellulose. Wiley-VCH Verlag GmbH & Co. KGaA. 2005.
- Pu YQ, Zhang, DC, Singh PM, Ragauskas AJ. The new forestry biofuels sector. *Biofuels Bioprod. Biorefining*. 2008; 2: 58-73.
- Ebringerova A, Hromadkova Z, Heinze T. Hemicellulose. In *Polysaccharides*. i Springer. 2005; 1-67.
- Saha BC. Hemicellulose bioconversion. *J Ind Microbiol Biotechnol*. 2003; 30: 279-291.
- Willfor S, Sundberg A, Pranovich A, Holmbom B. Polysaccharides in some industrially important hardwood species. *Wood Science and Technology*. 2005; 39: 601-617.
- Willfor S, Sundberg A, Hemming J, Holmbom B. Polysaccharides in some industrially important softwood species. *Wood Science and Technology*. 2005; 39: 245-257.
- Huang F, Singh PM, Ragauskas AJ. Characterization of milled wood lignin (MWL) in Loblolly pine stem wood, residue, and bark. *J Agric Food Chem*. 2011; 59: 12910-12916.
- Hu Z, Sykes R, Davis MF, Charles Brummer E, Ragauskas AJ. Chemical profiles of switchgrass. *Bioresour Technol*. 2010; 101: 3253-3257.
- De Vrije T, De Haas G, Tan G, Keijsers E, Claassen P. Pretreatment of Miscanthus for hydrogen production by Thermotoga elfii. *International journal of hydrogen energy*. 2002; 27: 1381-1390.
- Templeton D, Sluiter A, Hayward T, Hames B, Thomas S. Assessing corn stover composition and sources of variability via NIRS. *Cellulose*. 2009; 16: 621-639.
- Sun J, Mao F, Sun X, Sun R. Comparative study of hemicelluloses isolated with alkaline peroxide from lignocellulosic materials. *Journal of wood chemistry and technology*. 2005; 24: 239-262.
- Silverstein RA, Chen Y, Sharma-Shivappa RR, Boyette MD, Osborne J. A comparison of chemical pretreatment methods for improving saccharification of cotton stalks. *Bioresour Technol*. 2007; 98: 3000-3011.
- Ralph J, Lundquist K, Brunow G, Lu F, Kim H, Schatz PF, et al. Lignins: natural polymers from oxidative coupling of 4-hydroxyphenyl-propanoids. *Phytochemistry Reviews*. 2004; 3: 29-60.

32. Pu Y, Hu F, Huang F, Davison BH, Ragauskas AJ. Assessing the molecular structure basis for biomass recalcitrance during dilute acid and hydrothermal pretreatments. *Biotechnol Biofuels*. 2013; 6: 15.
33. Walker GM. *Bioethanol: Science and technology of fuel alcohol*. Bookboon. 2010;
34. Hallac BB. *Fundamental understanding of the biochemical conversion of Buddleja davidii to fermentable sugars*. 2011.
35. Boerjan W, Ralph J, Baucher M. Lignin biosynthesis. *Annu Rev Plant Biol*. 2003; 54: 519-546.
36. Baucher M, Monties B, Montagu MV, Boerjan W. Biosynthesis and Genetic Engineering of Lignin. *Critical Reviews in Plant Sciences*. 1998; 17: 125-197.
37. Yuan TQ, Sun SN, Xu F, Sun RC. Characterization of lignin structures and lignin-carbohydrate complex (LCC) linkages by quantitative ¹³C and 2D HSQC NMR spectroscopy. *J Agric Food Chem*. 2011; 59: 10604-10614.
38. Lawoko M, Henriksson G, Gellerstedt G. Structural differences between the lignin-carbohydrate complexes present in wood and in chemical pulps. *Biomacromolecules*. 2005; 6: 3467-3473.
39. Lawoko M, Henriksson G, Gellerstedt G. Characterisation of Lignin-Carbohydrate Complexes (LCCs) of spruce wood (*Picea abies* L.) isolated with two methods. *Holzforschung*. 2006; 60: 156-161.
40. Azuma J, Takahashi N, Koshijima T. Isolation and Characterization of Lignin-Carbohydrate Complexes from the Milled-Wood Lignin Fraction of *Pinus-Densiflora* Sieb Et Zucc. *Carbohydr Res*. 1981; 93: 91-104.
41. Mukoyoshi SI, Azuma JI, Koshijima T. Lignin-Carbohydrate Complexes from Compression Wood of *Pinus-Densiflora* Sieb Et Zucc. *Holzforschung*. 1981; 35: 233-240.
42. Irgum K. High-Performance Liquid Chromatography of Biological Macromolecules. *Encyclopedia of Analytical Chemistry*. 2000.
43. Sun Q, Foston M, Sawada D, Pingali SV, O'Neill HM, Li H, et al. Comparison of changes in cellulose ultrastructure during different pretreatments of poplar. *Cellulose*. 2014; 21: 2419-2431.
44. Sun Q, Foston M, Meng X, Sawada D, Pingali SV, O'Neill HM, et al. Effect of lignin content on changes occurring in poplar cellulose ultrastructure during dilute acid pretreatment. *Biotechnology for biofuels*. 2014; 7: 150.
45. Zhang YH, Lynd LR. Toward an aggregated understanding of enzymatic hydrolysis of cellulose: noncomplexed cellulase systems. *Biotechnol Bioeng*. 2004; 88: 797-824.
46. Browning B. The isolation and determination of cellulose. *Methods in Wood Chemistry*. 1967; 387-414.
47. Foston M, Ragauskas AJ. Changes in lignocellulosic supramolecular and ultrastructure during dilute acid pretreatment of *Populus* and switchgrass. *Biomass & Bioenergy*. 2010; 34: 1885-1895.
48. Sjöholm E. Size exclusion chromatography of cellulose and cellulose derivatives. *Handbook of Size Exclusion Chromatography and Related Techniques: Revised and Expanded*. 2003; 91: 311.
49. Dupont AL, Mortha G. Comparative evaluation of size-exclusion chromatography and viscometry for the characterisation of cellulose. *J Chromatogr A*. 2004; 1026: 129-141.
50. Hallac BB, Ragauskas AJ. Analyzing cellulose degree of polymerization and its relevancy to cellulosic ethanol. *Biofuels, Bioproducts and Biorefining*. 2011; 5: 215-225.
51. Puri VP. Effect of crystallinity and degree of polymerization of cellulose on enzymatic saccharification. *Biotechnol Bioeng*. 1984; 26: 1219-1222.
52. Snyder J, Timell T. Molecular properties of native balsam fir cellulose. *Sven papperstidn*. 1955; 58: 851-859.
53. Timmel T. Chain length distributions of native white spruce cellulose. *Pulp Paper Mag. Canada* 1955; 10: 104-117.
54. Timell T. Molecular properties of seven native wood celluloses. *Tappi Journal*. 1957; 40: 25.
55. Timell T. Molecular weight and polymolecularity of white birch celluloses. *Sven papperstidn*. 1956; 59: 1-11.
56. Blaschek W, Koehler H, Semler U, Franz G. Molecular weight distribution of cellulose in primary cell walls: Investigations with regenerating protoplasts, suspension cultured cells and mesophyll of tobacco. *Planta*. 1982; 154: 550-555.
57. Cohen R, Jensen KA, Houtman CJ, Hammel KE. Significant levels of extracellular reactive oxygen species produced by brown rot basidiomycetes on cellulose. *FEBS Lett*. 2002; 531: 483-488.
58. KlemanLeyer KM, SiikaAho M, Teeri TT, Kirk TK. The cellulases endoglucanase I and cellobiohydrolase II of *Trichoderma reesei* act synergistically to solubilize native cotton cellulose but not to decrease its molecular size. *Appl. Environ Microbiol*. 1996; 62: 2883-2887.
59. Kumar R, Mago G, Balan V, Wyman CE. Physical and chemical characterizations of corn stover and poplar solids resulting from leading pretreatment technologies. *Bioresource Technology*. 2009; 100: 3948-3962.
60. Jahan MS, Mun SP. Studies on the macromolecular components of nonwood available in Bangladesh. *Industrial Crops and Products*. 2009; 30: 344-350.
61. Sweet MS, Winandy JE. Influence of degree of polymerization of cellulose and hemicellulose on strength loss in fire-retardant-treated southern pine. *Holzforschung*. 1999; 53: 311-317.
62. Jahan MS, Mun SP. Effect of tree age on the cellulose structure of *Nalita* wood (*Trema orientalis*). *Wood science and technology*. 2005; 39: 367-373.
63. Xu W, Reddy N, Yang Y. Extraction, characterization and potential applications of cellulose in corn kernels and Distillers' Dried Grains with Solubles (DDGS). *Carbohydrate polymers*. 2009; 76: 521-527.
64. Bao YT, Bose A, Ladisch MR, Tsao GT. New approach to aqueous gel permeation chromatography of nonderivatized cellulose. *Journal of Applied Polymer Science*. 1980; 25: 263-275.
65. Eremeeva T, Khinoverova O, Treimanis A. Possibilities of SEC method utilisation for direct determination of molecular-mass distribution of cellulose. *Methods of cellulose investigation, Inst. Wood Chem, Riga*. 1988; 190-192.
66. Sjöholm E, Gustafsson K, Berthold F, Colmsjö A. Influence of the carbohydrate composition on the molecular weight distribution of kraft pulps. *Carbohydrate polymers*. 2000; 41: 1-7.
67. Sjöholm E, Gustafsson K, Eriksson B, Brown W, Colmsjö A. Aggregation of cellulose in lithium chloride/N, N-dimethylacetamide. *Carbohydrate Polymers*. 2000; 41: 153-161.
68. Yanagisawa M, Isogai A. SEC-MALS-QELS study on the molecular conformation of cellulose in LiCl/amide solutions. *Biomacromolecules*. 2005; 6: 1258-1265.
69. Röder T, Morgenstern B, Schelosky N, Glatter O. Solutions of cellulose in N, N-dimethylacetamide/lithium chloride studied by light scattering methods. *Polymer*. 2001; 42: 6765-6773.
70. Tamai N, Tatsumi D, Matsumoto T. Rheological properties and molecular structure of tunicate cellulose in LiCl/1,3-dimethyl-2-imidazolidinone. *Biomacromolecules*. 2004; 5: 422-432.
71. Potthast A, Rosenau T, Sixta H, Kosma P. Degradation of cellulosic materials by heating in DMAc/LiCl. *Tetrahedron Lett*. 2002; 43: 7757-7759.
72. Yanagisawa M, Isogai A. Size exclusion chromatographic and UV-VIS absorption analyses of unbleached and bleached softwood kraft pulps using

- LiCl/1,3-dimethyl-2-imidazolidinone as a solvent. *Holzforschung*. 2007; 61: 236-241.
73. Engel P, Hein L, Spiess AC. Derivatization-free gel permeation chromatography elucidates enzymatic cellulose hydrolysis. *Biotechnol Biofuels*. 2012; 5: 77.
74. Guerra A, Mendonça R, Ferraz A. Molecular weight distribution of wood components extracted from *Pinus taeda* biotreated by *Ceriporiopsis subvermispora*. *Enzyme Microb. Technol.* 2003; 33: 12-18.
75. Zoia L, King AW, Argyropoulos DS. Molecular weight distributions and linkages in lignocellulosic materials derivatized from ionic liquid media. *J Agric Food Chem*. 2011; 59: 829-838.
76. Timpa JD. Application of universal calibration in gel permeation chromatography for molecular weight determinations of plant cell wall polymers: cotton fiber. *J Agric Food Chem*. 1991; 39: 270-275.
77. Striuk S, Wolf BA. Fractional dissolution of "solid" unsubstituted cellulose. *Macromol Chem Phys*. 2000; 201: 1946-1949.
78. Yanagisawa M, Shibata I, Isogai A. SEC-MALLS analysis of softwood kraft pulp using LiCl/1,3-dimethyl-2-imidazolidinone as an eluent. *Cellulose*. 2005; 12: 151-158.
79. Isogai T, Yanagisawa M, Isogai A. Degrees of Polymerization (DP) and DP distribution of dilute acid-hydrolyzed products of alkali-treated native and regenerated celluloses. *Cellulose*. 2008; 15: 815-823.
80. Sun J, Sun X, Sun R, Su Y. Fractional extraction and structural characterization of sugarcane bagasse hemicelluloses. *Carbohydrate Polymers*. 2004; 56: 195-204.
81. Peng F, Ren JL, Xu F, Bian J, Peng P, Sun RC. Comparative studies on the physico-chemical properties of hemicelluloses obtained by DEAE-cellulose-52 chromatography from sugarcane bagasse. *Food research international*. 2010; 43: 683-693.
82. Jacobs A, Dahlman O. Characterization of the molar masses of hemicelluloses from wood and pulps employing size exclusion chromatography and matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *Biomacromolecules*. 2001; 2: 894-905.
83. Sannigrahi P, Ragauskas AJ, Tuskan GA. Poplar as a feedstock for biofuels: a review of compositional characteristics. *Biofuels, Bioproducts and Biorefining*. 2010; 4: 209-226.
84. Gabrieli I, Gatenholm P, Glasser W, Jain R, Kenne L. Separation, characterization and hydrogel-formation of hemicellulose from aspen wood. *Carbohydrate Polymers*. 2000; 43: 367-374.
85. Gosselink R, Abächerli A, Semke H, Malherbe R, Käuper P, Nadif A, et al. Analytical protocols for characterisation of sulphur-free lignin. *Industrial Crops and Products*. 2004; 19: 271-281.
86. Jönsson AS, Nordin AK, Wallberg O. Concentration and purification of lignin in hardwood kraft pulping liquor by ultrafiltration and nanofiltration. *Chemical Engineering Research and Design*. 2008; 86: 1271-1280.
87. Gidh AV, Decker SR, See CH, Himmel ME, Williford CW. Characterization of lignin using multi-angle laser light scattering and atomic force microscopy. *Anal Chim Acta*. 2006; 555: 250-258.
88. Evtuguin D, Domingues P, Amado F, Neto CP, Correia A. Electrospray ionization mass spectrometry as a tool for lignins molecular weight and structural characterisation. *Holzforschung*. 1999; 53: 525-528.
89. Gidh AV, Decker SR, Vinzant TB, Himmel ME, Williford C. Determination of lignin by size exclusion chromatography using multi angle laser light scattering. *J Chromatogr A*. 2006; 1114: 102-110.
90. Baumberger S, Abächerli A, Fasching M, Gellerstedt G, Gosselink R, Hortling B, et al. Molar mass determination of lignins by size-exclusion chromatography: towards standardisation of the method. *Holzforschung*. 2007; 61: 459-468.
91. Seidel A. Characterization and analysis of polymers. John Wiley & Sons. 2008.
92. Brunow G. Methods to reveal the structure of lignin. *Biopolymers Online*. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany. 2005; 89-99.
93. Sjöström E. Wood chemistry: fundamentals and applications. Gulf Professional Publishing. 1993.
94. Baumberger S, Dole P, Lapierre C. Using transgenic poplars to elucidate the relationship between the structure and the thermal properties of lignins. *J Agric Food Chem*. 2002; 50: 2450-2453.
95. Guerra A, Lucia LA, Argyropoulos DS. Isolation and characterization of lignins from *Eucalyptus grandis* Hill ex Maiden and *Eucalyptus globulus* Labill. by Enzymatic Mild Acidolysis (EMAL). *Holzforschung* 2008; 62: 24-30.
96. Lee CC, Shallenberger R, Vittum M. Free sugars in fruits and vegetables. 1970; 1: 1.
97. Moxley G, Zhang YHP. More accurate determination of acid-labile carbohydrates in lignocellulose by modified quantitative saccharification. *Energy & Fuels*. 2007; 21: 3684-3688.
98. Luo L, van der Voet E, Huppel G. Biorefining of lignocellulosic feedstock-- Technical, economic and environmental considerations. *Bioresour Technol*. 2010; 101: 5023-5032.
99. Foston M, Ragauskas AJ. Biomass Characterization: Recent Progress in Understanding Biomass Recalcitrance. *Industrial biotechnology*. 2012; 8: 191-208.
100. Sevcik RS, Mowery RA, Becker C, Chambliss CK. Rapid analysis of carbohydrates in aqueous extracts and hydrolysates of biomass using a carbonate-modified anion-exchange column. *J Chromatogr A*. 2011; 1218: 1236-1243.