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Research Article

Fractionation and Absolute Molecular Weight Determination of Organosolv Lignin and Its Fractions: Analysis by a Novel Acetone-Based SEC—MALS Method

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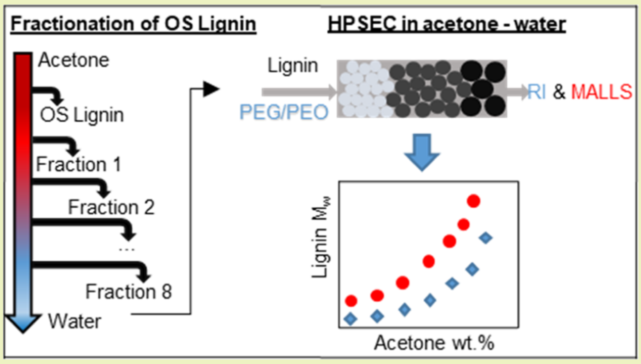

ABSTRACT: Accurate and reliable structural characterization of technical lignins is still challenging and inhibits industrial utilization as only poor understanding of structure−property relationship is available. Especially, molar mass analysis of technical lignins is of paramount interest; however, the usage of conventional size exclusion chromatography (SEC) and synthetic polymer standards as a consequence of inaccessible lignin standards is predominantly found in academia. This leads to a huge discrepancy in molecular weight analyses of lignins between different laboratories and consequently to fragile comparability. In this study, organosolv (OS) lignin from beech wood was exemplarily used to develop and evaluate a new acetone−water-based SEC method with the ability to be coupled to an electrospray ionization-mass spectrometer (ESI-MS) or to a multi-angle light scattering detector (MALLS or short MALS). The eluent system used shows very good solubility for the lignins investigated and hence lowers the probability for molecular aggregation of lignin molecules in solution. Solvent fractionation in acetone−water mixtures was conducted to acquire molecular weight classes. The starting lignin (parent lignin) was initially dissolved in 80 wt % acetone−water. Various fractions of the parent lignin were produced by a stepwise reduction of the acetone content. Molecular weights based on narrow polyethylene glycol (PEG)/polyethylene oxide (PEO) standards and absolute molar masses by coupling SEC with MALS were obtained, and the drawback of using polymer standards is discussed in detail. At a lower acetone content, low-molecular-weight fractions were found. Additionally, the specific refractive index increments (d*n*/d*c*) were determined for the parent lignin and its fractions. The impact of d*n*/d*c* on the final molecular weight (M_W) was evaluated considering the chemical composition obtained by nuclear magnetic resonance spectroscopy (NMR) analysis. Furthermore, light scattering revealed that the absorption behavior for this OS lignin is low and neglectable. This article proposes a new acetone-based analytical method for direct determination of absolute molar masses of OS lignin molecules.

KEYWORDS: *size exclusion chromatography, molecular weight distribution, organosolv lignin, light scattering, absolute molecular weight, fractionation, green feedstock*

INTRODUCTION

Along with cellulose, lignin is one of the most common organic compounds in the world and is an important building block of plant structures. This renewable biopolymer contributes almost 30 wt % of lignocellulosic biomass [lignified material (LCB)].[1−4] Besides the pulp and paper industry, several biorefineries process LCB into the key components cellulose, hemicellulose, and lignin. Hereby, the organosolv (OS) is widely considered a promising pretreatment process in biorefineries, which is based on the delignification of biomass with hot organic solvent−water mixtures, such as ethanol− or acetone−water.[5−7] The combination of organic solvent mixtures and a low amount of an acid or base catalyst in the OS pulping is relatively mild and enables the production of high-quality lignin in comparison to other technical lignins.[8,9] In particular, lignins isolated from the acetone OS Fabiola process show relatively high purities paired with an overall energy-efficient process.[7,9,10]

From a chemical perspective, lignin is a highly complex three-dimensional biopolymer based on three cinnamyl alcohols as shown in Figure 1.[6,11]

The heterogeneity of lignin biopolymers arises from the molecular composition and linkage types between the hydroxycinnamyl alcohols, so called monolignols. These

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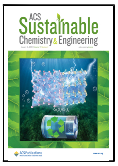

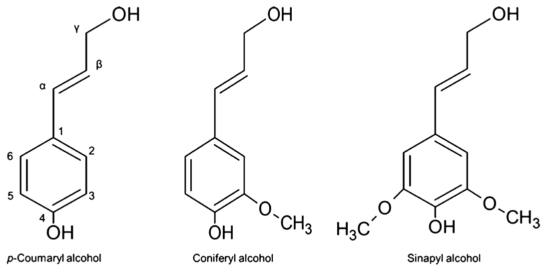

Figure 1. Major monomer structures of lignin.

monomers in lignin are named *p*-hydroxyphenyl, guaiacyl, and syringyl and different linkages among these monomer units are found, such as β-O-4′, β- β', β-5′, β-1′, 5−5′, and 4-O-5′of that the content of the β-O-4′ linkage is over half whole linkages in lignin.[3,12]

Technical lignins are still not sufficiently utilized even though they hold considerable potential for production of renewable chemicals and materials.[1,13] The direct material usage is still hindered by a number of barriers, prominently by the lignin heterogeneity caused by a high molecular weight (MW) dispersity, various interunit linkages, and impurities.[14−17] Fractionation of technical lignins by preparative size exclusion chromatography (SEC), solvent, or membrane fractionation has been revealed to provide narrower fractions of MW classes, which further showed enhanced performance in specific applications such as bio-based polyurethane foams or carbon fibers.[10,18,19] In particular, solvent fractionation, with multiple solvents in a certain order, classifies technical lignins in size.[20] However, the solvent fractionation method, used for this work, in only acetone−water mixtures is much more process- and cost-efficient in regard to scale-up. Due to acetone's low enthalpy of vaporization, it requires less energy for recycling than water or ethanol and does not lead to azeotropic mixtures with water. Furthermore, lower process costs, for example, for tanks, are needed using only acetone−water mixtures. Additionally, in the literature, it is reported that the acetone−water system has been successfully used to obtain monodisperse and homogeneous lignin nanoparticles for Kraft lignin,[21] which underlines its wider applicability.

The MW analysis can give significant structural insights to understand the reactivity and physicochemical properties of lignin. Furthermore, the width and shape of MW distributions (MWDs) can decisively impact the physical properties of polymeric materials.[22] However, current methods for molar mass analysis of polymeric lignin suffer from accuracy and provide poor reliable structure−property−application correlations. Thus, accurate and absolute characterization of MWD and related molar mass-dependent parameters is of paramount importance.[14]

Since the beginning of this century, the capability to determine the molecular weight (M_W) of native and technical lignins has been primarily focused on SEC approaches. SEC ideally separates polymers solely in terms of their size in solution rather than by their absolute M_W and requires, therefore, the use of a previous calibration.[23] Technical lignin molecules are challenging solutes in SEC since their solubility is hampered in common solvents, and usually, derivatization is necessary for full solubilization.[15,24] As no narrow lignin standards are available, the conventional molar mass analysis is based on narrow synthetic polymer standards. However, applying this calibration method is not sufficiently accurate for lignin, and obtained molecular weights remain questionable.[14] Numerous studies reported this issue in the literature, as isolated lignin structures entirely differ from linear polymer standards.[14,23,25−28]

This obstacle is further complexed by lignin species obtained from different raw materials with various phenylpropanoid monomer unit ratios, resulting essentially in a cluster of copolymers.[29] Additionally, interactions of lignin molecules with the stationary phase are almost inevitable due to its three-dimensional and intrinsic branched structure, which can prolong the elution time and consequently lead to misleading molar masses.[15,30] In this context, the aggregation of lignin molecules by intermolecular forces, especially initiated by hydrogen bonding of hydroxyl groups, has been discussed earlier.[31] Furthermore, it has been reported that clustering of lignin aggregates occurs due to the $\pi-\pi$ interaction between the aromatics.[32] Suppression of this severe associative behavior of lignin molecules in solution can be diminished by the addition of salts.[31] Due to the aforementioned factors, comparing results from different research groups is challenging and remains under discussion until today. Baumberger and others marked the demand for standardized SEC analysis and evaluated lignin samples by using different column configurations and eluents and reported that the number-average molecular weight (M_n) seemed to be more reproducible within laboratories than the weight-average molecular weight M_w.[33,34]

To tackle this challenge, absolute molar mass determination techniques are of huge interest since they are independent of the separation method and calibrations. To get closer to the absolute molar masses of (technical) lignins, mass spectrometry (MS) approaches for lignin have been tested. Jacobs et al. investigated molar masses of lignin with SEC in combination with offline matrix-assisted laser desorption ionization (MALDI) MS.[35] Fractions of lignin were collected by SEC, and absolute MWDs were determined by MALDI MS for hard and softwood lignin.[35,36] Presently, MALDI is considered the only approachable technique for the determination of MWD in synthetic polymers, proteins, and polysaccharides. However, due to partial fragmentation, it is challenging to differentiate between small and large molecule fragments and to interpret the m/z patterns precisely.

This issue could be overcome by coupling liquid chromatography (LC) to MS, but due to sample preparation for MALDI analysis, MS detection with MALDI is limited as the ion source cannot be used for online analysis with the LC eluent.[37,38] Furthermore, MALDI ionization reveals predominantly formation of single-charged species, which leads to suppression of the ionization of high-MW species.[35] Electrospray ionization (ESI) high-resolution time-of-flight (TOF) MS was developed by Andrianova et al. for Kraft lignin.[39,40] The method was optimized regarding ion formation and enabled a detection range of lignin species between 150 and 9000 g mol^{-1}.[39] The accurate analysis of lignin molecules, especially for larger molecules, is so far limited due to the above-mentioned reasons.

Alternatively, the absolute molar mass can be determined without any calibration by multi-angle laser light scattering detector (MALS) detection. In comparison to (calibrated) SEC, where correct calibration for the polymer of interest and also the pure SEC separation mechanism are obligatory, MALS provides correct Mw even if non-SEC mechanisms occur.[14,23,41] Briefly speaking, the molar mass is determined from the intensity of scattered light at different angles extrapolated to a zero scattering angle.

The fundamental light scattering equation describes the angular and concentration dependence of the intensity of scattered light:[41]

$$\frac{R_\theta}{K^* \times c} = M_w P(\theta) - 2A_2 c M^2 P^2(\theta) + \ldots \tag{1}$$

where R_θ is the excess Rayleigh ratio, c is the concentration of the polymer in solution, M_w is the weight-average molecular weight, A_2 is the second virial coefficient, K^* is the optical constant, and $P(\theta)$ is the particle scattering function. The Rayleigh ratio R_θ represents the ratio of the scattered and incident light intensities at one measured angle θ. The optical constant K^* is further described by

$$K^* = \frac{4\pi n_0^2}{\lambda_0^4 N_A} \times \left(\frac{\mathrm{d}n}{\mathrm{d}c}\right)^2 \tag{2}$$

where n_0 is the refractive index (RI) of the solvent, λ the wavelength of the incident light, N_A the Avogadro number, and dn/dc the specific RI increment.[41]

In case of online SEC-MALS, the second term of eq 1 ($2A_2cM \ll 1$) can be neglected since typical values for A_2 and c lie in a magnitude of 10^{-4} and 10^{-3}. It becomes visible that M_w is inversely proportional to the square of dn/dc.[42,43] This demonstrates the significant and critical knowledge of dn/dc values for the determination of MWs of a polymer. For a periodically ordered polymer (homopolymer), it is well accepted that the specific RI increment value is primarily dependent on the monomer and minimally dependent on the MW. However, for copolymers, a single value of dn/dc cannot always be assumed since the composition may change with increasing MW. If the composition of a copolymer varies by increasing weight, the specific RI increment will change, and ultimately, uncertainties in the MW determination will occur.[43]

Lignin is certainly not a homopolymer and is much more complex due to its variety of inter-unit linkages and cross-linkings.[1] Classifying the size and chemistry by (solvent) fractionation leads to classes of more homogeneous lignin molecules, which will most likely influence the dn/dc value. Additionally, technical lignins can exhibit absorption (and fluorescence) behavior.[15,41] Once the sample absorbs incident light, this can cause a change in the scattered intensity and lead to misleading calculation in the MW. In a recent work, Zinovyev et al. discussed the dependency of the dn/dc value on the MW and the absorption phenomena for Kraft lignin and fractions. The latter could be compensated by installing band pass filters on the detectors and correcting the absorbance using the forward monitors.[14,26,31] Furthermore, they reported a dn/dc trend behavior of Kraft lignin fractions (up to 9000 g mol^{-1}) as that found for homopolymers.[14]

Currently, to the best of the author's knowledge, no acetone-based SEC method for the MW analysis of lignin has been reported. The aim of this study was to implement and establish a versatile SEC-ESI–MS method for the absolute molar mass determination of (OS) lignin, where no modification of the isolated lignin (i.e., derivatization steps) is necessary. However, in the course of the investigations, the focus was switched to a more robust and handy SEC-MALS technique because of unpredictable uncertainties in the ESI-MS method. In addition, we discuss the acquired MW differences based on the relative SEC and absolute MALS method by investigating fractions obtained by solvent fractionation in acetone–water mixtures. Moreover, we stress the influence and importance of the correct dn/dc value for each lignin MW class and attempt to visualize the range of accuracy regarding the MW analysis depending on these parameters.

■ MATERIALS AND METHODS

Chemicals and Solvents. The parent lignin used in this work is obtained from the lignocellulose biorefinery pilot plant at Fraunhofer CBP in Leuna, Germany. Beech wood lignin is processed with a mild acetone OS process (Fabiola) and subsequently precipitated by the LigniSep process[7,44] from the pulping liquor. Further details of the process conditions can be found in the literature.[9] Lignin samples are dried and stored at room temperature prior to use. Acetone >99.9% was purchased from VWR International (CAS: 67-65-1), formic acid from Fisher Scientific (CAS: 64-18-6), and polyethylene glycol/polyethylene oxide (PEG/PEO) standards from Agilent Technologies, and tap water was purified using a Millipore filter system [resistivity 18.2 MΩ cm, total organic carbon (TOC) 3 ppb].

Size Exclusion Chromatography. For the implementation of acetone–water as an eluent, completely newly developed polymeric GPC columns were installed. Three AppliChrom Acetone-AQ-Phil-P analytical columns [each 8 mm × 300 mm, Acetone-AQ-Phil-P 350/250/150 (pore sizes 350 Å/250 Å/150 Å)] and a guard column (8 mm × 50 mm) were connected in series to separate lignin molecules by size in a range of 100–10^6 g mol^{-1}. SEC measurements were carried out using an Agilent HPLC 1200 system equipped with an RI detector, a two-channel degasser, a quaternary pump, a column thermostat, and an autosampler, followed by a MALS detector (DAWN HELEOS, Wyatt Technology) or ESI-MS (Accurate Mass Q-ToF LC/MS 6530, Agilent Technologies) for absolute molar mass determination. The following SEC conditions were derived after rigorous optimization: Acetone, deionized water, and traces of formic acid were used as the eluent (79.2/19.8/1 vol %). The flow rate was set at 1 ml min^{-1} and the column oven controlled at 35 °C. An injection volume of 100 μL and a sample concentration of 1–5 mg mL^{-1} were chosen. A series of 12 narrow single PEG/PEO standards in the molar mass range from 640 to 538,000 g mol^{-1} were used for column calibration.

SEC Preparation and Data Evaluation. The trace of formic acid in the above-mentioned eluent is included to suppress possible self-association behavior of lignin molecules and to enhance ionization of the molecules for the SEC-ESI-MS measurement. This specific solvent system has been chosen since the acetone OS lignin obtained by the Fabiola process showed very good solubility in preliminary experiments, which is a sign of high solvent–lignin interactions, leading to the assumption of minimal lignin–lignin interactions and thus low molecular lignin aggregation in solution. Furthermore, the eluent allows potential SEC-ESI-MS measurements due to its volatile nature. The eluent is filtered (nylon filter, 0.45 μm, Agilent Technologies) and further degassed for 15 min with helium before being connected to SEC. Prior to analysis, lignin samples and PEG/PEO standards were dissolved in the eluent for 24 h at room temperature using an overhead shaker (Reax 2, Heidolph) to complete dissolution. All lignin and calibration standard samples were fully soluble in the eluent (no solid residues observed) and were filtered using a 0.2 μm syringe filter (ROTILABO, PA 0.2 μm, Carl Roth) prior to injection. Software WinGPC (PSS Perfect Separation Solutions) was used for data acquisition and evaluation of the MWD by conventional analysis (SEC + RI), and software ASTRA 8.1.1 (Wyatt Technologies) was used for data interpretation and evaluation of absolute molar mass analysis (SEC + RI + MALS). The baseline and integration limits (~ $t_{\text{retention}}$ 18–31.8 min) are set in a way that all lignin peaks, shoulders, and potential agglomerates eluted from the columns are included.

Determination of the Specific Refractive Index Increment d*n*/d*c*. The dn/dc for lignin samples and its fractions were determined by using the above-mentioned RI detector. Four different concentrations of the samples (0.1–2.0 mg mL^{-1}) were prepared in the eluent and filtered prior to direct manual injection into the RI detector. The analysis and evaluation were done by using software ASTRA 8.1.1 (Wyatt Technology).

Determination of the Amount of Hydroxyl Groups by ^{31}P NMR. NMR spectroscopy was performed on a Bruker 600 MHz spectrometer. ^{31}P NMR was measured after derivatization of the samples with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (95%, Sigma-Aldrich) in pyridine/$CDCl_3$ (1.6:1 v/v), in the presence of cholesterol as the internal standard, according to the standard protocol.[45,46] Hydroxyl content analyses were performed using a quantitative procedure as published by Meng et al.[46]

Fractionation of Beech Wood Organosolv Lignin. 5 g of the parent lignin was added to 80 wt % acetone (45 g) in 250 mL centrifuge tubes. The tubes were set in an overhead shaker (Reax 2, Heidolph) for 2 h to allow phase equilibration. Afterward, the solution is transferred into a centrifuge (Rotanta 460, Hettichlab) and run at 4600 rpm at 23 °C for 30 min. The undissolved solid fraction is separated and defined as non-soluble lignin and named F_0. Subsequently, water was added in each fractionation step to reduce the acetone content of each supernatant from roughly 80 to 60, 60−40, 40−35, 30, 25, 20, and 10 wt % acetone to receive the lignin fractions F_1−F_8, respectively, after equilibration for 60 min in the overhead shaker before centrifugation of the sample under the condition described above. The acetone content of each liquid phase (supernatant) was determined using an offline beforehand calibrated FT-IR spectrometer (ALPHA II, Bruker). The final remaining soluble lignin in ∼10 wt % acetone was obtained by solvent evaporation with a rotary evaporator. Each obtained fraction was dried prior to analysis in a vacuum oven at 40 °C and <10 mbara for 24 h or until stable weight was reached.

ESI-MS Analysis. An Agilent 6530 ESI Q-TOF LC/MS system was used for the initial development of the absolute molar mass determination method for OS lignin. A flow rate of 0.1 mL min^{-1} into the ionization chamber of MS was guaranteed facilitated using a T−splitter behind the SEC setup. The flow rate was set by adjusting the length of the capillary between the T-splitter and ESI chamber resulting in a constant backpressure.

A preliminary study of LC−MS conditions (sample concentration, injection volume, ionization polarity, and fragmentor potential) was tested. MassHunter software package B.06.00 was used for data processing. The spectra of OS lignin and PEG standards (1000, 3350, and 8000 Da) were recorded, and possible multiply charged ions were deconvoluted using a built-in software tool.

■ RESULTS AND DISCUSSION

SEC-ESI-MS Method Development. The solvent system (79 vol % acetone +1 vol % formic acid) used in this work not only displays high solubility toward OS lignin but is also suited due to its volatile nature for the injection in a series-connected ESI-MS. The ionization of the molecules of interest is one of the critical steps in the ESI-MS method. The usage of formic acid in the solvent system can increase the ionization efficiency of lignin molecules. Here, we have found that a positive ionization increases the signal to noise ratio more than a negative ionization mode does. This observation agrees with the reports of Andrianova et al. They reported that a positive ionization mode was found to be more effective, especially for methoxy-substituted arenes and polyphenols. Furthermore, they reported an effective formation of multiply charged species for Kraft lignin by the usage of 100 mmol L^{-1} formic acid in their system.[39] On top of this reason, we acetified the solvent system with formic acid to avoid any potential lignin cluster in the solvent system. Sodium, magnesium, or other metal traces in the sample exhibit ionic bonds with phenolates, which indicate a more hydrophilic character than that of the more hydrophobic solvent system. The samples fully dissolve; however, not inevitably every lignin molecule is completely surrounded by a solvation shell. To avoid any micelle formed by phenolates at any concentration, the stronger formic acid is used to minimize the amount of phenolates by protonation. Kubatova et al. have reported a similar assumption. They reported an unfolding of lignin polymers by the usage of 100 to 200 mm formic acid.[40]

For the SEC-ESI-MS analysis, we kept a flow rate of 0.1 mL min^{-1} and the positive ionization mode constant and varied primarily the injection volume and the fragmentor potential (Table S1) to observe any differences in the quality of the obtained spectra. Based on the signal to noise ratio, the setting with an injection volume of 10 μL and a fragmentor voltage of 200 V showed the best results. However, we observed during data processing missing charge (z) values for most masses which does not lead to a complete representative deconvolution spectrum for the OS sample (Figure S1). Due to the extensive number of peaks in the mass spectrum, partially resulted by fragments, the reading of the isotopic pattern becomes challenging for the deconvolution tool. Nevertheless, solely the 1000 Da PEG sample displays a clear distributed spectrum around the expected mass (Figure S2). At higher molecular weights, we found difficulties in interpreting the spectra due to missing charges. Within the PEG 3350 Da spectrum, we observed three main peaks, of which one lies around 3350 Da and the two others are fragmentation peaks at lower masses (around 1000 and 2000 Da). However, toward higher MW (3350 and 8000 Da), the interpretations of the obtained deconvoluted spectra become very weak (Figures S3 and S4). In comparison to the 3350 Da PEG sample, the 8000 Da PEG sample displays another peak around 2500 Da. Other than this, the spectra are very similar. Thus, a similar fragmentation by these MS conditions can be concluded for the analyzed higher-MW PEG samples. Detailed information regarding the ESI conditions and the deconvoluted spectra is available in the Supporting Information (Table S1 and Figures S1−S4). The dispersity of a sample plays a critical role during the ionization process as suppression of bigger molecules can occur. Considering a polymer sample with a low dispersity (<1.03) and a repetitive chemical identity as the PEG standard (1000 Da), the analysis by ESI-MS seems elegant and robust. However, by increasing the MW, the MS conditions need to be adjusted and experimentally optimized. Hence, considering lignin as a sample distributed in size and functionality, we decided to move toward a more applicable and more robust absolute analysis method by SEC-MALS.

Fractionation of OS Beech Wood Lignin. Eight dilution steps were followed to obtain fractions from the parent lignin. Figure 2 displays the precipitated amount for each fraction during the sequential fractionation. The non-soluble part F_0 contains around 1.87 wt % of the starting OS lignin. In the first diluting step, a marginal amount of 1.09 wt % for F_1 was precipitated. F_2 shows the highest yield of 27.4 wt %. F_3, obtained at an acetone content of 34.3 wt %, leads to a yield of 20.38 wt %. Lowering the acetone content to 29.3 wt % depicts a yield of 12.72 wt % for F_4. Starting from F_5 to F_8, similar yields from 9.39 to 9.12 wt % could be observed.

The amount of precipitated lignin in F_1 is very low, probably due to the similar solubility in this acetone range. Sadeghifar et al. reported for switch grass and pine OS lignin full solubility between 60 and 90 wt % acetone.[10,13] In addition, Duval et al. reported recently a high solubility of isolated OS lignin in acetone based on the evaluation of Hansen and Kamlet−Taft solubility parameters.[10] The solubility decreases, mainly starting from 60 wt % acetone. F_2 shows the highest yield

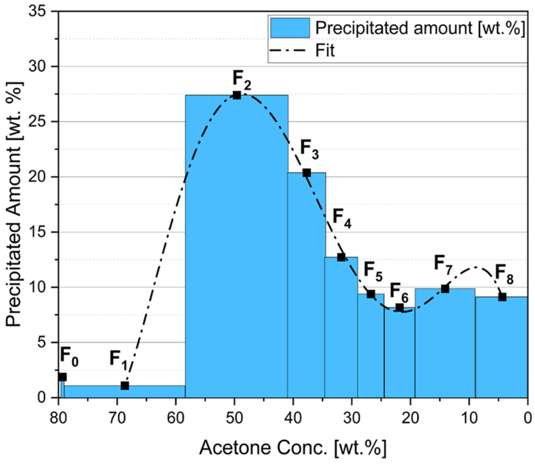

Figure 2. Precipitated amount for obtained fractions in sequential dilution fractionation.

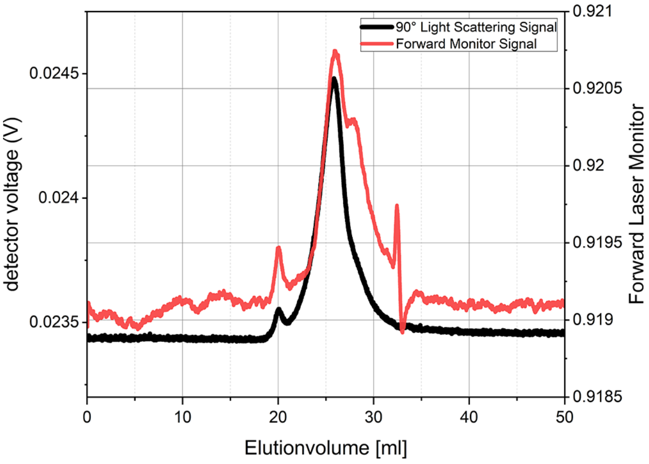

Figure 3. Diagnosis of absorption behavior of the lignin sample by using the FM signal.

due to the greater acetone decreasing step from 58.1 to 41.1 wt %. The overall mass recovery lies over 99%. The distribution results clearly prove that the acetone concentration has a major influence on the solubility and consequently on the fractionation process. However, the obtained yields do not display a relationship with different acetone concentrations. The obtained fractions were dried and subsequently analyzed by SEC-MALS. The elution profiles of the parent lignin and the fractions are shown in Figure 7 later. For the sake of simplicity, F_1 is not displayed in the upcoming figures since the yield is relatively low, and obtained MWs and the dn/dc value of F_1 are in the comparable range of the parent lignin (see Tables 2 and 3). Therefore, we strongly assume that fraction 1 majorly consists of unfractionated parent lignin.

Influence of Absorption and Fluorescence Behavior of Lignin in Light Scattering. Analyzing the molar mass of lignin by MALS brings the question of how greatly the absorption and fluorescence phenomena occur on the incident light. Technical lignins, especially Kraft lignin, have been reported to show absorption of light in the ultraviolet and the visible range, which is often accompanied by fluorescence behavior.[26,47] To diagnose, if the sample significantly absorbs light at a certain wavelength, the signal obtained by the forward monitor (FM) is taken into account. The laser monitor (LM) only displays the intensity of the incident laser, whereas the FM displays the intensity of the incident laser light after the flow cell. Once a sample shows absorption behavior, the FM signals appear to be an inverted or flipped copy of the light scattering signal. The wavelength of the MALS detector was 664 nm.

In Figure 3, the light scattering signal at 90° and the FM signal for the parent lignin are displayed. The FM signal does not mirror the light scattering signal. Furthermore, the voltage of the FM does not drop or change drastically. The FM can correct sample absorbance as long as the FM signal does not change by more than approximately 20%.

The OS lignin used in this work only shows a change of 0.15% in the signal intensity, which represents marginal absorption behavior. Due to the very low, almost neglectable amount of absorption, no further considerations (higher wavelength, bandpass filters, or correction) have been taken regarding the MALS setup.

SEC Calibration and Analysis. Prior to MW analysis of the parent lignin and fractions, the new acetone-based SEC columns were primarily calibrated with narrow PEG/PEO standards. Figure 4 shows the narrow distributed standards (a) eluted from the three columns connected in series. An elution window of 13.61 mL covers the necessary MW range. By using WinGPC software, a calibration curve [sigmoidal course (b)] could be obtained and fitted with a third-degree polynomial function (Figure 4b) (R^2 = 0.999).

Analysis and Determination of d*n*/d*c* Values for Beech Wood Lignin and Its Fractions. The specific RI increments of the parent lignin and its fractions were determined by batch measurements beforehand. Figure 5 shows the RI detector response plotted against the injected mass. By using a linear fit (R = 0.999), a specific RI increment of 0.1865 mL g^{-1} could be obtained for the parent lignin. This value lies in the comparable range for OS lignins.[14]

Molecular Weight Distribution and Analysis by SEC and SEC-MALS. MWD and weight-average molecular weights were obtained by the relative calibration based on PEG/PEO standards. The starting parent lignin shows an M_w of 11,063 g mol^{-1}. The eight produced fractions exhibit higher (e.g., 21,000 to 11,400 g mol^{-1}) and lower (9600 to 2800 g mol^{-1}) M_w than the parent lignin. In Figure 6, the weight (blue)- and number (green)-average MWs are displayed for F_2 to F_8.

A tendency to lower MW classes toward a higher water content can be derived from the figure. Through the polarity change in the stepwise fractionation, lignin solubility decreases with the change of the acetone concentration. By increasing the polarity, larger lignin molecules, which exhibit more of an unpolar character, tend to precipitate. This could explain the tendency toward smaller MW distributions by increasing the water content. Similar trends of MW classification have been reported earlier.[10,13,48] Sain et al. have also reported that the solubility of lignin in an organic solvent depends primarily on the MW and on the functionality as the aliphatic hydroxyl number of the lignin.[49]

Furthermore, the dispersity (ratio of M_w and M_n) reduces during the course of the fractionation as represented in Table 1. The highest irregularity with a dispersity of 3.08 is found in F_2.

Fraction 7 shows the lowest value of 1.40. This is most likely explainable by the range of the acetone content. F_2 has been obtained with a bigger acetone step (∼20 wt %) than that of F_7 (∼5 wt %), and hence, the dispersity is lower in smaller

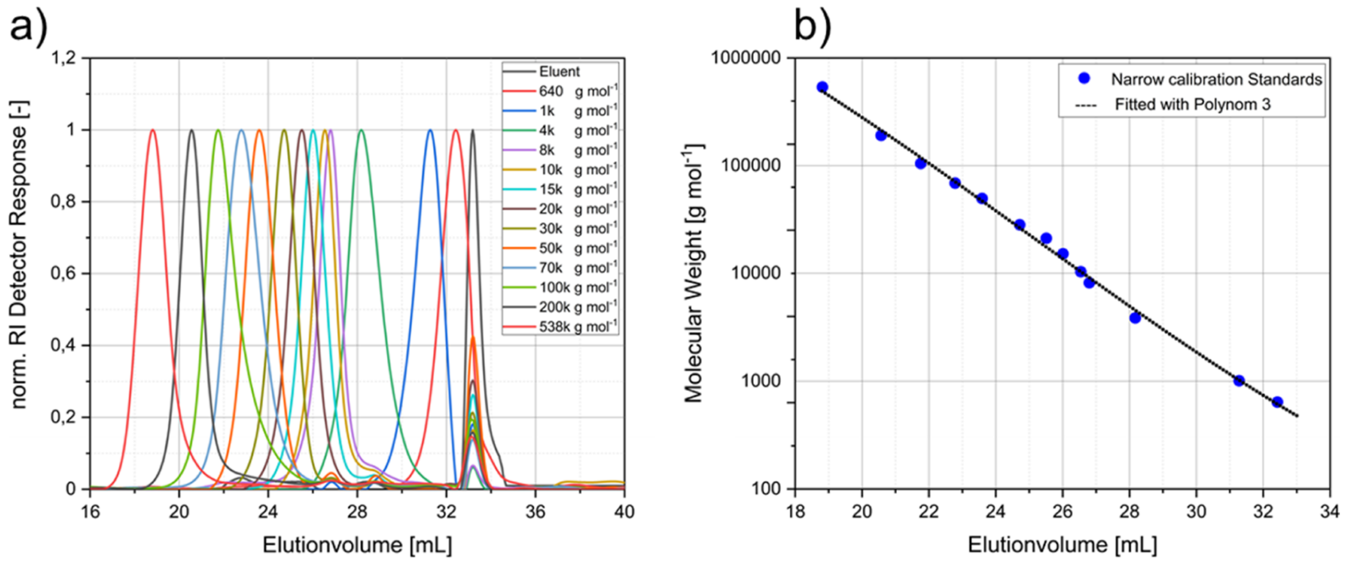

Figure 4. Narrow elution profiles of PEG/PEO standards (a) and column calibration curve with PEG/PEO in 80 wt % acetone following a sigmoidal function (b).

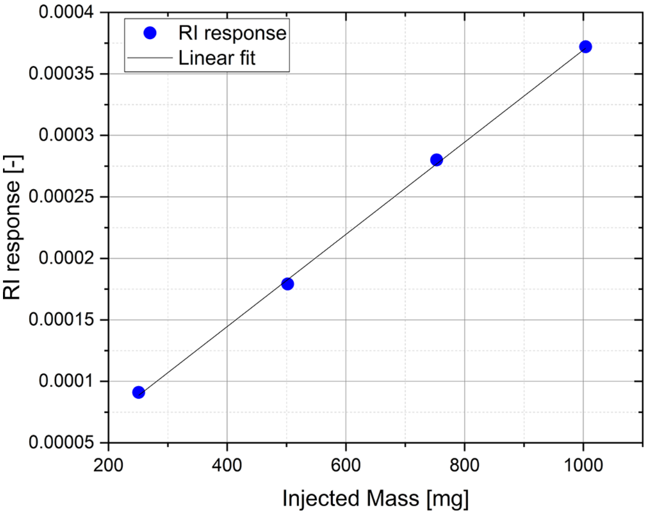

Figure 5. d*n*/d*c* determination of beech wood lignin in 80 wt % acetone.

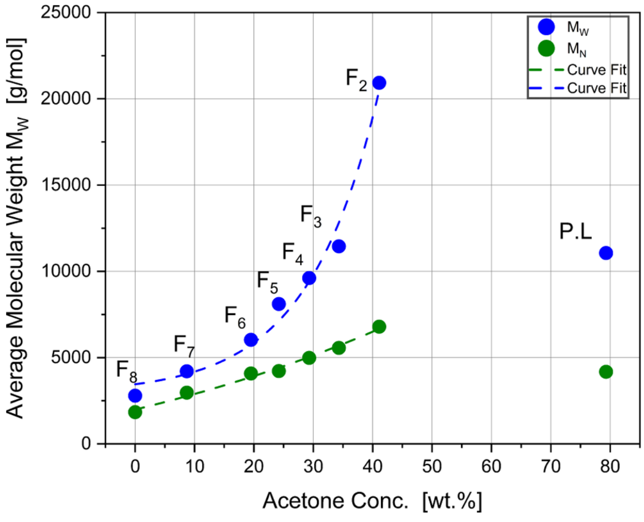

Figure 6. MWs of lignin and its fractions based on PEG/PEO standards.

acetone steps. Both the average MWs and dispersity lie distributed around the parent lignin as summarized in Table 1.

For SEC-MALS analysis, the dn/dc of the parent lignin and of each fraction has been included. In Figure 7, the differential MWD is displayed for the parent lignin and each fraction. The MWDs of the fractions do overlap with each other to some extent; however, the peak maxima (M_P) of the fractions are clearly distributed around the parent lignin. All samples display at least bimodal behavior, which is likely explainable by the wide analytical separation range of 90 cm. It is noticeable that the parent lignin covers the complete molar mass range and thus displays the biggest dispersity (see Table 2). F_2 is represented by the higher-MW fraction; however, due to the bigger acetone jump of ~20 wt %, F_2 shows the highest dispersity among the fractions.

The MWD of F_3 and F_4 (brown dashed and rose lines) are mostly overlying, representing a similar average MW. Furthermore, F_3 and F_4 only depict a minor shoulder beside a narrow distribution, which leads to similar lower dispersity values. The lowest dispersity value is found for F_7 even though it represents two major peaks. However, the range of MW in F_7 is lower than that in F_8, and therefore, F_8 displays a slightly higher dispersity. From F_5 to F_8, a stronger shift to lower M_p

Table 1. Summarized Values for M_n, M_w, and Dispersity Obtained by Relative Calibration[a]

sample	M_n [g mol^{-1}]	M_w [g mol^{-1}]	dispersity [−]
Parent Lignin	4200	11,000	2.62
F_1	4000	10,000	2.50
F_2	6800	21,000	3.08
F_3	5600	11,400	2.03
F_4	5000	9600	1.92
F_5	4200	8100	1.93
F_6	4100	6000	1.46
F_7	3000	4200	1.40
F_8	1800	2800	1.56

[a] Maximal standard uncertainties are U(M_n) = 223 g mol^{-1}, U(M_w) = 843 g mol^{-1}, and U(D) = 0.08 (for the 0.95 level of confidence).

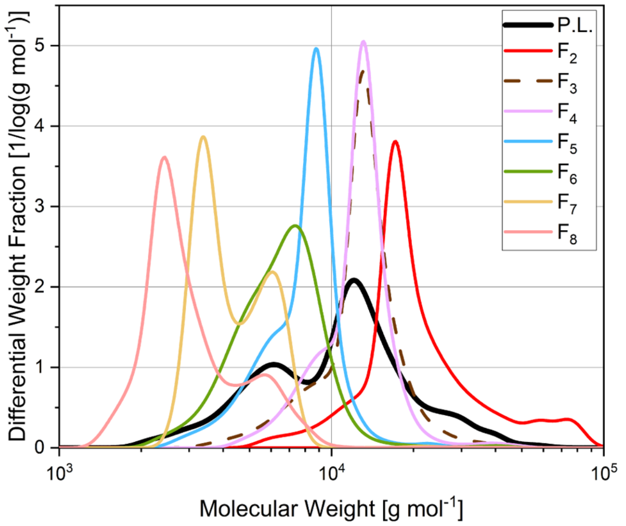

Figure 7. MW distribution of beech wood lignin and its fractions determined by SEC-MALS.

Table 2. Summarized Values for Mn, Mw, and Dispersity Obtained by Absolute Molar Mass Analysis

sample	M_n [g mol^{-1}]	M_w [g mol^{-1}]	dispersity [−]
Parent Lignin	8700	12,600	1.45
F_1	9300	13,000	1.40
F_2	18,400	23,900	1.30
F_3	11,800	13,300	1.13
F_4	11,700	12,800	1.10
F_5	7300	8100	1.11
F_6	5900	6800	1.15
F_7	4100	4500	1.10
F_8	2900	3400	1.17

can be observed. Statistical moments as average M_w can be consequently derived from the MWD and are summarized in Table 2. A similar trend for the molar masses by the fractionation is obtained via SEC-MALS analysis. Nevertheless, Figure 8 clearly indicates that absolute values (blue dots) for the average MWs are slightly higher than those obtained by the conventional method (green dots). The biggest discrepancy in MW is found for F_4, where a correction factor of 1.33 would be needed to obtain the absolute value based on the relative analysis. All other values lie within a range from 1.00 to 1.21. Additionally, the absolute dispersity values are much lower than those obtained by the relative method.

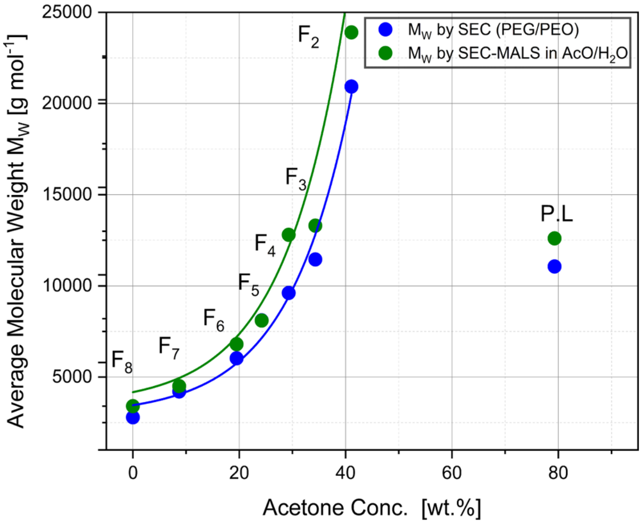

Figure 8. Average MWs, Mw, obtained by SEC (blue dots) and SEC-MALS (green dots).

The underestimation of the lignin molar mass obtained by the relative calibration has been reported in the literature.[26,47] To understand the discrepancy in MW analysis obtained by the relative method, the PEG/PEO standards have been analyzed via SEC-MALS. In Figure 9, the molar mass course for the parent lignin (black) and the PEG/PEO standards (green) is displayed. Furthermore, the RI detector and light scattering signal (blue and red, respectively) are shown. The signals of the detectors do not overlap due to the polydispersity of lignin.

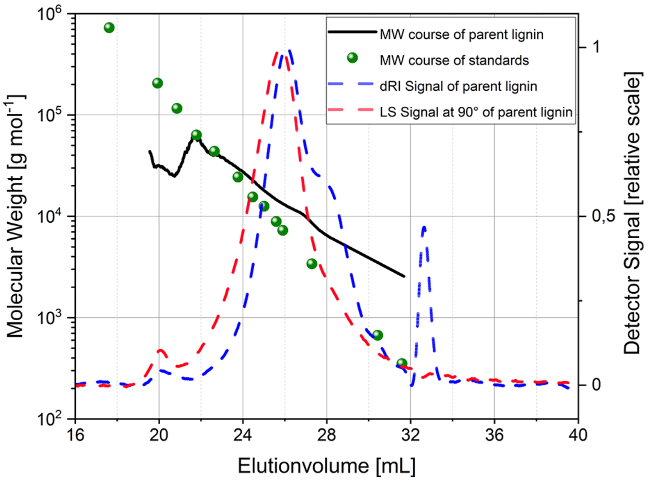

Figure 9. MW course plotted against the elution volume for the parent lignin (black) and standards (green dots).

An amount of less than 1 wt % of the injected sample is eluted at a retention time (t_r) of 20 mL. This amount is taken into account for the full molar mass analysis and most probably represents agglomerated lignin molecules due to anchoring effects and not a perfect SEC separation mechanism. The major amount of lignin starts eluting at t_r = 22 min. The MW decreases for the parent lignin as shown by the black line in Figure 9. In the major separation window (t_r = 22 to 32 min), we observe that each of the PEG/PEO standards (green dots) shows lower MW than the parent lignin. Since SEC separates not by MW but only by hydrodynamic volume, this observation supports strongly the hypothesis that lignin molecules (likely branched and folded due to intra-molecular interactions) are more compact than (linear) PEG/PEO standards in this solvent system. This leads to the earlier shown mismatch and underestimation of lignin MWs by considering the relative method.

This is in agreement with previously found differences in the literature. Fredheim et al. found for Kraft lignin absolute values, which are 1.7 times higher than those obtained by the conventional method. Wang et al. even showed differences of a factor between 6.2 and 6.5 between the absolute and relative method for acetylated hardwood Kraft lignin.[26,47] Zinovyev et al. reported an M_w of 10,000 g mol^{-1} for an OS hardwood sample analyzed in their SEC-MALS system.[14] The absolute weight-average MW for the OS beech wood lignin used in this work lies in a similar range with 12600 g mol^{-1}.

Influence of the Specific Refractive Index Increment (d*n*/d*c*) on the Molecular Weight Determination. The d*n*/d*c* value is a crucial parameter affecting the molar masses determined by SEC-MALS and needs particular consideration. The dn/dc value is a discrete number for a given polymer in a given solvent system at a certain wavelength. Lignin is not a periodically ordered biopolymer, and therefore, specific incremental indices for each fraction were determined. The structural dependency of technical lignins is not only based on three monomer units and their various binding types but also on the type of lignin, the isolation conditions, and the fractionation method. Hence, every lignin and fraction display a unique distribution of various molecule sizes and a cluster of functional activities as described in the introduction.[23,41] In Table 3, d*n*/d*c* values determined for the different fractions are collected. It is noticeable that the values for the parent lignin and for F_1 are in the same range, which underlines the hypothesis of mostly undissolved parent lignin remaining in F_1 derived above.

Table 3. d*n*/d*c* Values for Parent Lignin and Its Fractions

sample	d*n*/d*c* [mL g^{-1}]	R^2
parent lignin (P.L.)	0.1865 ± 0.0081	0.999
F_1	0.1836 ± 0.0065	0.999
F_2	0.2131 ± 0.0021	0.999
F_3	0.2097 ± 0.0035	0.999
F_4	0.1826 ± 0.0085	0.998
F_5	0.2219 ± 0.0070	0.999
F_6	0.2168 ± 0.0020	0.999
F_7	0.2135 ± 0.0009	1
F_8	0.1632 ± 0.0011	1

In the lower MW range from 3400 to 8100 g mol^{-1}, represented by fractions F_8 to F_5, an increase in the dn/dc value from 0.1638 to 0.22 mL g^{-1} is observed. For the fractions with higher MWs, starting from F_4 to F_2, an additional asymptotic course starting from 0.1832 to 0.2176 mL g is exposed. The results are illustrated in Figure 10a revealing two trends for the d*n*/d*c* values obtained by the solvent fractionation. For comparison, Zinovyev et al. reported d*n*/d*c* values for Kraft lignin and its fractions obtained by an ultrafiltration process. They observed a significant increase in d*n*/d*c* up to 2000 g mol^{-1} and a constant value above up to 9000 g mol^{-1}.[14]

As described earlier, the d*n*/d*c* values change significantly for (bio)polymers based on different monomers by the MW. In this connection, phosphorous NMR (^{31}P NMR) can exhibit, based on the amount of hydroxyl groups, structural insights into lignin molecules regarding the chemical composition. Lignin molecules are polymerized of three phenylpropanoid monomer units, which consist of a polyphenolic skeleton with nine carbon atoms and only differ in the number of methoxyl groups (Figure 1). In Figure 10b, the determined amount of hydroxyl groups present in the phenolic (red), aliphatic (green), and carboxyl (blue) region is plotted against the weight-average MW for the parent lignin (stars) and its fractions (dots). A significant decrease in phenolic OH and a slight decrease for aliphatic and carboxylic OH contents with increasing MW are observed. Thus, by the solvent fractionation, not only a classification by MW appears but additionally on the chemical composition, here displayed for hydroxyl groups.

The lower the average MW, the fewer the amounts of bindings between the monomers are formed, which consequently leads to a higher amount of free functional groups as hydroxyl endings. This trend is clearly noticeable for the fractions in Figure 10b. However, potentially two linear trends with different slopes can be observed. The slope from F_8 to F_5 is much higher for all three hydroxyl regions than the slope from F_4 to F_2 and may explain the drop in the d*n*/d*c* values found between F_4 and F_5.

The parent lignin and F_4 represent almost the same M_w (Table 2). Furthermore, the analysis by ^{31}P NMR reveals an approximately similar amount of hydroxyl groups in all regions for the parent lignin and F_4. Both sets of information have an impact on the specific RI increment and result in comparable d*n*/d*c* values (see Table 3). Thus, we may have found a description for the chemical composition by the amount of OH groups for the lignin molecules. This hypothesis is further

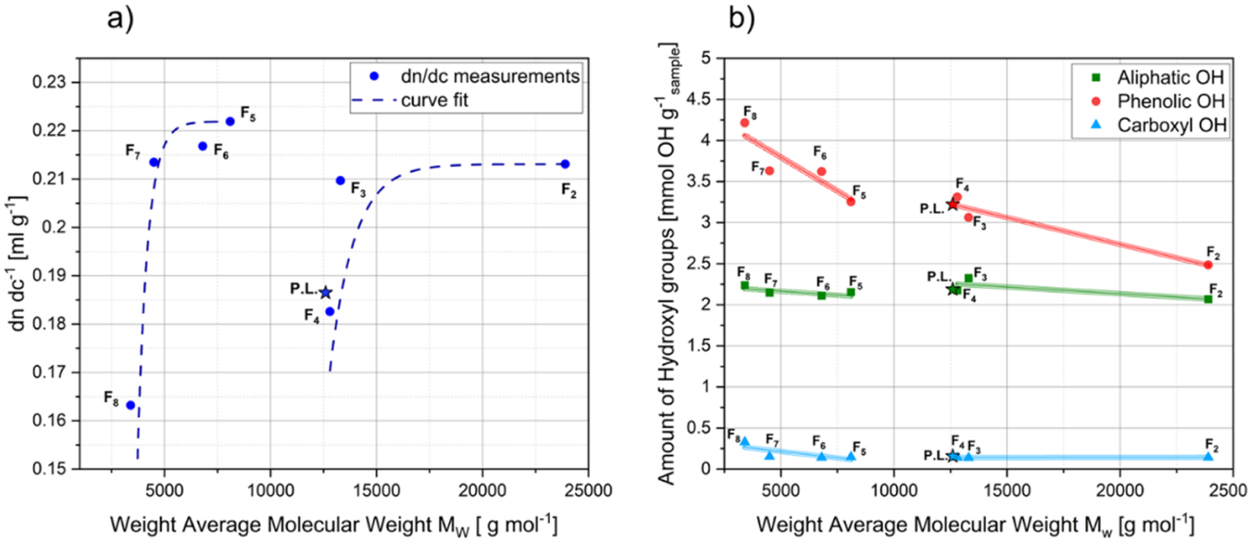

Figure 10. Obtained dn/dc values (a) and amount of OH groups (b) plotted against M_w for the parent lignin (stars) and its fractions (dots).

strengthened by the fact that the main linkage type in lignin samples is represented by β-O-4 bindings obtained via phenolic OH groups. The influence of the main linkages in lignin on the functionality in the fractions can be further understood and investigated by, for example, $^{1}H-^{13}C$ NMR. The amount of β-O-4 binding most likely decreases with the course of the fractionation. Wang et al. reported a similar trend, where a decrease in the 5−5′ interunit was observed by decreasing MW.[50]

To the best of the author's knowledge, a split in the d*n*/d*c* trend for fractions obtained of a technical lignin has not been reported before. This split indicates that by the fractionation, not only the molar mass is classified, but also, most probably, a non-linear clustering of the chemical composition especially by functional groups within the fractions occurs.

In Figure 11, the weight-average MW is plotted for the parent lignin and its fractions obtained by the relative method (blue curve, analogous to Figure 8), the absolute method by considering only the d*n*/d*c* value for the parent lignin (red curve), and the absolute method by considering the d*n*/d*c* value for each sample (black curve).

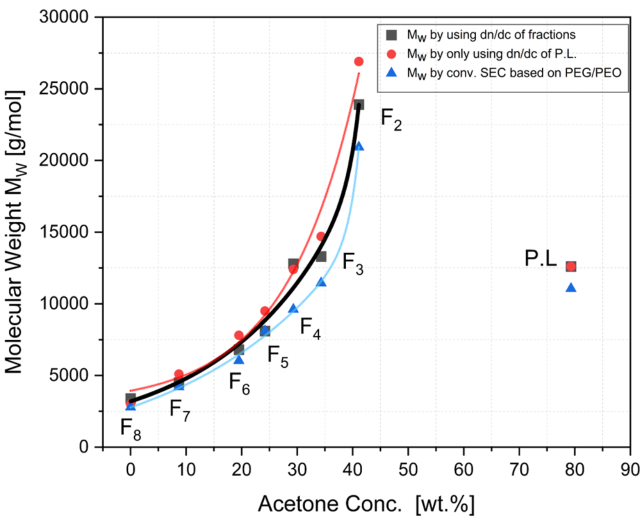

Figure 11. Influence of dn/dc and relative calibration on lignin MW.

The structural difference between calibration standards and technical lignin leads often to an underestimation by only considering the relative method via SEC. An overestimation of the MWs occurs by considering only the dn/dc value of the parent lignin. Using the d*n*/d*c* values for the individual fractions shows that the real absolute MWs for the samples are found in-between as shown in Figure 11 (black curve). These results clearly show that the d*n*/d*c* value for each lignin type plays a significant part in the MW determination and can lead to misleading analysis if not addressed. Therefore, we emphasize for the accurate MW analysis of lignin by MALS the consideration of individually determined dn/dc values.

CONCLUSIONS

A new aqueous acetone-based SEC−MALS method has been developed for the analysis of OS lignin to provide insights into the relative and absolute molar mass distribution and MWs. This unique analytic method provides elegantly the analysis of molar mass distribution and average MWs without any further derivatization steps of the sample. In comparison to other widely used solvent systems (e.g., DMSO or NaOH) in SEC for lignin, this new solvent system can be easily recycled, and instruments as MS (not suitable with DMSO) or the RI detector (not long-term suitable with NaOH) are less prone to get damaged, which leads toward a more practical and sustainable approach. Furthermore, this SEC method allows the coupling of ESI-MS for additional absolute characterization of lignin molecules, especially of narrow fractions in the lower-MW range. However, in this work, ESI-MS was found to be not suitable for samples >1 kDa due to dispersity, not optimal MS conditions, and poor deconvolution reasons.

OS lignin from beech wood produced by the Fabiola process has been fractionated by a stepwise decrease in the solvent content to obtain more homogeneous fractions. Both analytical methods (calibrated SEC and absolute SEC-MALS) reveal the classification in MW by the fractionation. Hereby, a trend toward lower MW by increasing the water content has been identified.

Technical lignins such as Kraft lignin often display high absorption and fluorescence behavior. Contrarily, the processed lignin in this work shows almost no absorption behavior probably due to its prior relatively mild extraction process conditions. This work further addresses the unreliability due to structural difference in molecular conformation between lignin and PEG/PEO standards used to determine the MW. The analysis of calibration standards in solution by SEC-MALS reveals lower molar masses than those of lignin molecules at equal elution time, which leads to the strong assumption of a less compact molecular structure for the synthetic standards in this solvent system. In cases of heterogeneous polymers, the most significant errors in molar mass can be observed when the parent homopolymers are of notable different d*n*/d*c*. We investigated the d*n*/d*c* for each fraction and found expected differences but also surprisingly two distinguishable d*n*/d*c* trends for two groups of lignin fractions. A difference in the abundance of OH groups between those two lignin fraction groups was found to be a possible explanation of the unsteady d*n*/d*c* trend with increasing molar mass.

The further characterization of physicochemical properties for understanding the effect of the fractionation on the molar mass−structure-−property relationships is addressed in current studies.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssuschemeng.2c06309.

Additional experimental details regarding the ESI-MS conditions and ESI-MS spectra of lignin, PEG 1000, PEG 3350, and PEG 8000 Da (PDF)

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