Catalytic hydrogenolysis of lignin: the influence of minor units and saccharides

This chapter is based on:
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Abstract

The precise elucidation of native lignin structures plays a vital role for the development of “lignin first” strategies such as reductive catalytic fractionation. The structure of lignin and composition of the starting material has a major impact on the product yield and distribution. Here, the differences in structure of lignin from birch, pine, reed, and walnut shell were investigated by combining detailed analysis of the whole cell wall material, residual enzyme lignin, and milled wood lignin. The results of the 2D heteronuclear single quantum coherence NMR analysis could be correlated to the product from Ru/C catalyzed hydrogenolysis if monomeric products from ferulate and p-coumaryl and its analogous units were also appropriately considered. Notably, residual polysaccharide constituents seemed to influence the selectivity towards hydroxy-containing monomers. The results reinforced the importance of adequate structural characterization and compositional analysis of the starting materials as well as distinct (dis)advantages of specific types of structural characterization and isolation methods for guiding valorization potential of different biomass feedstocks.
3.1. Introduction

With the inevitable limits to the consumption of fossil fuels, lignocellulosic biomass is the most promising feedstock for development of a sustainable chemical and fuel supply. Among the main building blocks of biomass, lignin is the only polymer that nearly exclusively consists of aromatic units, and considerable attention has gone into achieving high-value products from lignin. Polymeric lignin is constructed mainly from three monolignols known as p-coumaryl alcohol (H), coniferyl alcohol (G), and sinapyl alcohol (S) that are biosynthesized from phenylalanine (Phe) (Figure 1a) and subsequently undergo radical coupling (Fig. 3.1b), favoring the formation of the aryl ether (β-O-4) linkage among other linkages such as resins (β-β) and phenylcoumaran (β-5) (Fig. 3.1c).

H, ferulate (FA), and p-coumarate (pCA) units are less abundant units, but their presence can have a significant impact on the lignin structure. For example, the lower unpaired electron density on the phenolic oxygen gives p-coumaryl alcohol the highest possibility to serve as a starting site for lignin polymerization, which means an increasing amount of H units (yellow in Fig. 3.1a, c) can lead to overall lower-molecular-weight lignin. Additionally, such minor units such as FA (blue in Fig. 3.1a, c) and pCA (red in Fig. 3.1a, c), often found in herbaceous biomasses, can serve as terminal groups and pendant chains, which make the quantification and elucidation of H units of lignin a challenge. FA and pCA have also been claimed to serve as constituents of hemicellulose (or as bridging unit between lignin

![Fig. 3.1. Overview of the fate of aromatic components in biomass upon reductive fractionation or hydrogenolysis with specific focus on H, pCA and FA units. (a) Simplified monolignol biosynthesis pathways; (b) radical and radical transformation after enzymatic oxidation of the monolignols; (c) representative structures of lignin and hemicellulose in the cell wall; (d) monomer products released from reductive fractionation or hydrogenolysis.](image-url)
and hemicellulose, Fig. 3.1c). These non-homologous building blocks and complex linkages increase the challenge of refining and structural elucidation and the valorization of lignin, thus understanding their influence is pivotal for maximization of biomass utilization. Several promising lignin conversion technologies have been studied such as pyrolysis, hydroconversion, and selective oxidation of hydroxyls for modification and degradation. Among these, reductive catalytic fractionation (RCF) or catalytic hydrogenolysis of isolated lignin have been extensively studied as a lignin-first bio-refinery strategy with the potential to produce valuable alkyl phenolic monomers from lignin. Various catalytic systems with metal-based catalysts have been explored to produce functional aromatic monomers from different biomasses, and the product portfolio was shown to be tunable by the choice of catalyst and reaction conditions. The monomers can be converted to marketable added-value chemicals by catalytic funneling. These monomers are mainly obtained by the cleavage of the β-O-4 linkage and thus monomer yields closely correlate with the structure and composition of the starting material. Studies have shown that the structure and quality of lignin are significantly important for the lignin-first oriented RCF or the hydrogenolysis of isolated lignins and are easily influenced by lignocellulosic species and extraction or isolation methods. Therefore, prior to reductive fractionation, a robust and comprehensive method for analyzing the main structure of lignin to guide valorization potential is desired.

Many approaches have been applied to analyze the structure of lignin such as thioacidolysis (TA), pyrolysis, nitrobenzene oxidation, and derivatization followed by reductive cleavage (DFRC), all in combination with GC-MS, as well as carbon-13 cross-polarization/magic angle spinning (CP/MAS) solid-state NMR (CPMAS SS NMR) and infrared (IR) spectroscopy. Proportions of units of the original lignin can roughly be estimated by quantification of the different monomers from reductive hydrogenolysis and vice versa as well as for the identification of difficult linkages such as the 4-O-5. Precise analysis of lignin by extrapolating degraded fragments needs careful consideration of products derived from units originally connected by C-C bond in the lignin. Currently, there is still limited knowledge on influence of minor units on the product distribution from lignin hydrogenolysis, although this is important for biomass sources with significant quantities of these, such as walnut shell and reed. This is hampered by challenges in their accurate quantification due to overestimation. Therefore, a good biomass analysis protocol that can deal with such challenges can give improved insight in the highly desired prediction of the expected product portfolio from specific biomass sources. Multi-dimensional NMR techniques such as heteronuclear single quantum coherence (HSQC) can provide detailed structural information. Whole-cell-wall (WCW) gel-state NMR spectroscopy can be used to directly analyze the lignin in plant cells but yields very complex spectra. Alternatively, mild enzymatic hydrolysis can achieve removal of a majority of polysaccharides providing isolated residual
enzyme lignin (REL), although intensive ball milling treatment has to be applied to enhance the enzyme activity.\textsuperscript{43} Furthermore, signals from residual proteins introduced by the enzyme treatment can overlap with those of H units in lignin, which restricts estimation of the H content.\textsuperscript{44} [44] Mildly extracted milled wood lignin (MWL) gives cleaner well-solvated lignin fragments for 2D HSQC NMR analysis, which can partly represent the larger lignin polymeric structure.\textsuperscript{43,45–47}

In this study we aim to get insight in the value of different sample preparation techniques and analysis methods to correlate to lignin hydrogenolysis product mixtures with emphasis on the minor units. For this purpose, four representative biomasses were selected for the WCW analysis, REL isolation, and MWL extraction, and the results were correlated to obtained Ru/C RCF product mixtures. The lignin substrate samples were subjected to a detailed 2D HSQC NMR study complemented with Fourier-transform (FT)IR spectroscopy, scanning electron microscopy (SEM), thermal gravimetric analysis and first-derivative thermogravimetric (TGA/DTG), and gel permeation chromatography (GPC) analyses. Product mixture correlation was further extended to catalytic hydrogenolysis of isolated lignins samples. The difference in the lignin structures in the different biomass samples and those of REL and MWL were comprehensively analyzed in order to understand which lignin analysis protocol can provide what insight on the expected hydrogenolysis product mixtures, in particular for elucidation of the minor H units and its analogous units in terms of overestimation, monomer origin, and influence of the presence of polysaccharides in the substrate.

3.2. Experimental section

3.2.1. General remarks

All the chemicals were used as received and purchased from Sigma-Aldrich or Fluorochem unless otherwise noted. Birch wood, pine, reed, and walnut shells were picked up from local sources. The catalyst used for RCF/hydrogenolysis was 5\% Ru on carbon and aluminum oxide (Sigma). \textit{p}-Coumureic acid (pCCA), ferulic acid (FAA), xylan from birch, and Avicel \textup{*PH-101} cellulose from cotton were also purchased from Sigma-Aldrich. Solvents were supplied by Fisher Scientific. Some standard chemicals including 4-(3-hydroxypropyl)-2,6-dimethoxyphenol (S1), 2,6-dimethoxy-4-propylphenol (S2), 4-ethyl-2,6-dimethoxyphenol (S3), methyl 3-(4-hydroxy-3-methoxyphenyl)propanoate (FMA), methyl (E)-3-(4-hydroxy-3-methoxyphenyl) acrylate (FAME), methyl 3-(4-hydroxyphenyl)propanoate (PMA), hydroxycyclohexyl)propanoate (OHP), and methyl (E)-3-(4- hydroxyphenyl)acrylate (pCAMe) were synthesized and isolated for this study (S3.1.5). More detailed information on the synthetic and analytical procedures can be found in the Supporting Information.
3.2.2. Lignocellulose sample pretreatment

Biomass samples were cut into small sections and grounded in a Wiley mill with a 20-mesh screen and extracted with 1:2 (v/v) ethanol/toluene for 8 h in a Soxhlet apparatus. After volatilizing the solvents, the extract-free samples were dried at 65°C for 16 h and stored in a valve bag before use. Ball milling was conducted in a planetary ball milling instrument (Fritsch GmbH, Idar-Oberstein, Germany) in the presence of 250 mL ZrO$_2$ jar and balls (5 × 15 mm, 10 × 5 mm) and a program with 450 rpm rotation, 10 min milling, and 15 min pausing were used for all the samples, and the time displayed throughout the manuscript only represents the active milling time, excluding the time for pauses in between. The 50 mM acetate buffer was prepared by directly dissolving the precalculated sodium acetate and adjusting the pH to 5.5 by adding acetic acid. Tetracycline chloride was added into the buffer to make a concentration of 0.8 mM based on the volume of the buffer to inhibit the growth of bacteria. All the enzymatic hydrolysis reactions were conducted at 50 °C in a VWR incubating orbital shaker (Model 3500L) at 250 rpm for 72 h with a solid/liquid ratio of 25.

3.2.3. Whole cell wall sample preparation and NMR experiments

The sample for WCW analysis was prepared according to a published procedure with minor modifications. Ball milling for walnut shells started from around 30 g biomass, and 5 g sample was taken from the jar after milling 6, 12, and 24 h, and birch, pine, and reed sample were milled 24 h from a 10 g scale, and the swelling was conducted by submerging 80 mg milled biomass sample in premixed 0.7 mL DMSO-$d_6$/pyridine-$d_5$ solvent and sonicated for 1–5 h until a gel-state sample appeared.

3.2.4. Residual enzyme lignin isolation

The REL was isolated by following our previous publication. $^{49}$ 10 g biomass with 6 h ball-milled sample was used for the first enzymatic hydrolysis, after the treatment, the solid product was separated by centrifugation and freeze-dried using a lyophilizer (ALPHA 2-4 LD, Appropriate Technical Resources), and then the dried residue was further milled for 6 h before the second enzymatic hydrolysis treatment. The enzyme (Ctec2, Novozymes) loading for hydrolysis is 0.5 mL (g substrate)$^{-1}$. Accordingly, the solids obtained from the second treatment were noted as samples with a prefix of REL.

3.2.5. Extraction of milled wood lignin

The milled wood lignin was extracted by following published steps. $^{50}$ 20 g wood powder obtained from 6 h ball-milling described above was suspended in 400 mL dioxane/water (v/v, 96: 4) and stirred for 24 h under dark, and residue was collected by centrifugation and further extracted by fresh solvent for another 24 h. The liquid was combined and condensed to approximately 30 mL, and further precipitated in 3 volume times 96% ethanol. After separation with centrifugation, the ethanol phase
was concentrated to around 30 mL (this step was repeated) and the lignin was precipitated in 10 volume times acid water (pH=2). The lignin was isolated by filtration and further washed by acid water and freeze dried.

**3.2.6. Reductive catalytic fractionation/hydrogenolysis**

RCF/hydrogenolysis was performed by using optimized parameters from a published paper with minor modification, with a Ru/C catalysis in methanol. For each reaction, a 10 mL homemade batch reactor was loaded with 250 mg of lignocellulose or 50 mg isolated lignin (for MWL, 20 mg), 4 mL methanol (for MWL, 2 mL methanol), 25 mg of 5 wt% Ru/C (for MWL, 10 mg of Ru/C) and a magnetic stirring bar. The reactor was closed, and 60 bar H₂ was charged at room temperature before heating. After 30 minutes, once the pressure of H₂ constantly keep at 60 bar, the reactor was put into a preheated oil bath with a temperature of 250°C at a 300 rpm stirring. 3 h later, the reaction was terminated by washing the reactor with cooling tap water. 4 mL methanol (for MWL, 2 mL) with a concentration of 0.004 mmol mL⁻¹ octadecane was added to the reactor before filtration for quantitation and dilution. 1 mL was transferred to a GC vial and filtered using a 0.45 μm polytetrafluoroethylene (PTFE) filter before injection into GC-flame ionization detector (FID). GC-FID used for the quantitation of monomers was an Agilent 8860 with HP5 colum (30 m × 0.25 mm × 0.25 μm). The following operating conditions were used: injection volume: 1 μL, injection temperature: 280 °C, column temperature program: 40°C (5 min), 10 °C min⁻¹ to 320 °C, 320 °C (5 min), detection temperature 320°C. The calibration was built by corresponding standard monomers with octadecane as reference (see S3.1.6), and curves were attached in the our published paper.

**3.3. Results and discussion**

The structure of lignin is inevitably altered during the isolation or extraction process, which hampers the establishment of adequate correlations between the native lignin structure and the products obtained from lignin-first methods such as RCF. Therefore, several methods that are known to give insight into the structure of native-like lignin are combined in this study. Firstly, a gel-state 2D HSQC NMR spectrum was collected in order to illustrate the as-close-to-native structure of lignin in the cell wall material. This was done by directly swelling the ball milled WCW material in DMSO-d₆/pyridine-d₅ (4 : 1, v/v). Secondly, REL was isolated by milder enzymatic hydrolysis of polysaccharides via double round sequential treatment using optimized parameters from our previous publication. Thirdly, MWL was isolated by multiple neutral 1,4-dioxane extractions. The REL and MWL samples served as typical isolated native-like lignins and were used for the comparison to investigate the difference in structural information that could be obtained and the limitation to each of these analysis procedures. The unit constituents of lignin depend on the biomass species. Guaiacyl (G) units and minor amounts of p-
hydroxyphenyl (H) units typically comprise gymnosperms lignins, whereas angiosperm dicot lignin consists of syringyl (S) and G units with even lower amounts of H units. As mentioned in the introduction, walnut shells contain a particularly high amount of H units, and grasses typically have high amounts of H derivatives such as pCA. Therefore, the selection of different biomass species is significant for the outcome of lignin-first methodologies, and several significantly different biomass samples were selected to represent hardwood, softwood, grass, and nutshell lignocellulosic biomass. Birch wood was selected to represent a hardwood, as used in some current biorefineries.\(^{21,22}\) Pine wood was selected as the most universal gymnosperm and used to represent a typical softwood. Reed, a fast-growing and widely distributed grass, was used here as a typical grassy lignocellulose sample. As walnut shell contains a relatively high amount of lignin and more than 5.5 megatons walnuts (with shells) were produced in 2016,\(^{52}\) it was set as a non-wood material to study lignin that is not a specific hard- or softwood. After successfully obtaining these samples, analysis of the yield, carbohydrate analysis, thermal stability, and molecular distribution was conducted. (see S3.2, S3.4, S3.5, and S3.7 in the Supporting Information) before turning to analysis by 2D HSQC NMR spectroscopy.

3.3.1. Qualitative 2D HSQC NMR analysis

As far as we are aware, walnut shell and reed gel-state 2D HSQC NMR spectra have not been previously reported. Therefore, we aimed to find the parameters for optimal formation of a gel sample for NMR spectroscopy in DMSO-\(_d_6\)/pyridine-\(_d_5\). This was achieved for walnut shell, which is the hardest of the four materials, by a time course of ball milling time over 24 h and with around 4 h sonication (detailed study see S3.1.2). The 2D HSQC NMR spectra of all the four WCW gel-state samples were collected under the same optimized conditions (Fig. 3.2). Although the signals of the 2D HSQC NMR from WCW samples showed complexity and overlap, most signals from linkages and aromatic rings of lignin could be well distinguished after careful analysis, which showed valuable information about the saccharide and lignin structures (assignment was attached in our published paper\(^{51}\) according to previous papers).\(^{10,48,53,54}\)

Looking at the aromatic region (AR, Fig. 3.2), WCW_Birch showed predominantly S signals, and the signal of α-ketone structures of S units was relatively strong compared to the other samples. G unit signals dominated the spectrum of the WCW_Pine, while the intensity of G was similar to S units for walnut shell and reed. As expected, a considerable strong H signal only appeared on the spectrum of WCW_Walnut. Both p-coumarate (pCA) and ferulate (FA) were clearly more abundant in WCW_Reed
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Fig. 3.2. 2D HSQC NMR spectra of the WCW from four biomasses (DMSO-d_6:pyridine-d_5, 4:1, v/v). AR, aromatic region, AL, aliphatic region. The common polysaccharide and lignin label system is used. The structure and corresponding signals in the spectra were color-coded with each other.

when compared to the other biomasses. Signals from polysaccharides crowded in the (oxygenated)
aliphatic region (AL, Fig. 3.2), which overlapped with the signals of the linkage motifs of lignin, such as the overlap between β-O-4a and 2,3-di-O-AC-β-D-Xylp, as well as γ position of β-O-4 and β-5 with C-I6 and X-I5, which increased the challenge for assigning the signals of lignin and relative comparison by directly using WCW 2D HSQC NMR spectra (detailed discussion see S3.6 in the Supporting Information).10

A lot of signals could still be assigned by using the available literature; nevertheless, 2D HSQC NMR analysis of REL samples can be significantly easier due to clearer signals and less overlap. This can be observed in Fig. 3.3, where many more signals could be assigned with increased confidence, in particular the (oxygenated) aliphatic region (AL, Fig. 3.3) due to reduced interference of carbohydrate signals. A definite signal of acylated aryl ethers (β-O-4') resulting from the acylation of aryl ethers (β-O-4) via pCA or FA appeared in REL_ Reed.55 This is a possible bridge for connecting lignin and saccharides, in line with the discussion of high amount of saccharide impurities and pCA and FA in REL_Reed. In addition, signals for tricin, one member of the flavonoid family found in some grass biomasses, were now clearly observed in the aromatic region (AR, Fig. 3.3) of REL_Reed.8 For all

Fig. 3.3. 2D HSQC NMR spectra of REL from four biomasses (DMSO-d6:pyridine-d5, 4:1, v/v). AR, aromatic region, AL, aliphatic region. The common polysaccharide and lignin label system is used. The structure to the corresponding signals in the spectra were shown in Fig. 3.2.
samples the different signals in the aromatic units for H-type units and derivatives became more clearly visible in the REL spectra. The exception is the H$_{2,6}$ signal in REL_Walnut, which was similar but less intense compared to that of WCW. The H units seldom reaches more than 5% except for some genetically modified plants; however, our analysis indeed showed that walnut shell contains an unusually high content of H units (see below) making it still clearly visible in the WCW samples.

Dibenzodioxocin (D) units, from coupling between a monolignol and biphenyl unit, in REL_Birch showed the weakest signal among all the four REL samples, as the biphenyl units are solely formed from coupling of G or H units. REL_Pine displayed a similar type of G lignin to that of WCW_Pine, except some signals from end groups. For instance, the cinnamyl alcohol end group (X1) and the cinnamaldehyde end group (X2) were clearly visible. The units of D and 4-O-5 are normally present in low levels and hard to be detected, but evident cluster signals of D and 4-O-5 were observed in REL_Pine around 83.97/4.99 ppm (D$_{α}$), 86.35/4.01 ppm (D$_{β}$), and 104.80/6.81 ppm (4-O-5$_{α}$), respectively. These two minor units are traditionally thought of as cross-linking sites in lignin but have recently been considered as end groups of lignin with phenolic groups. A clear disadvantage of the 2D HSQC NMR analysis of REL samples is that the aromatic region (AR) of all the spectra was
contaminated by signals from protein residues, such as Phe and Tyr originating from the enzyme
cocktail used for the carbohydrate removal and possibly also from residual protein left behind from
the biomass substrate.

MWL can alleviate the contaminations originating from carbohydrates and residual proteins, giving
cleaner spectra (Fig. 3.4), but at the expense of some signals for minor linkages. For instance, no clear
signals of 4-O-5 and end group were observed for MWL_Pine in contrast to the corresponding REL
spectrum. A well-isolated signal of H units appeared, which was also here strongest for MWL_Walnut
compared to MWL from other biomasses. The spectra of MWL_Reed showed similar aromatic signals
as those found in WCW_Reed and REL_Reed. Clearer signals for tricin were found in the MWL_Reed
spectrum, which is likely because tricin is involved in initializing a polymer chain, and thus acts as an
end group.[8] Such end groups are enriched due to the 1,4-dioxane extraction as part of the MWL
procedure that favors extraction of shorter oligomeric chains that are more soluble. The molecular
weight (MW) distribution of MWLs and RELs clearly revealed that RELs have a high molecular weight
(Fig. S3.7) with weight-average molecular weight much higher than that of their corresponding MWL
samples (Table S3.5), showing that indeed MWL just contains shorter oligomers released from the
pendent part of lignin or cleaved from the high-molecular part of lignin during ball milling. Moreover,
for REL spectra of these four biomasses, stronger H units are observed for lignin with lower MW. For
instance, the MW sequence of RELs followed REL_Walnut<REL_Reed<REL_Pine<REL_Birch, while the
relative intensity of the signals from H in the 2D HSQC NMR spectra followed a sequence of
REL_Walnut>REL_Reed>REL_Pine>REL_Birch (detailed discussion see S.7). It seemed that the MW was
associated with the percentage of H unit in lignin, which might be due to H units having a tendency to
serve as starting sites during lignin biosynthesis.11

Overall, qualitative analysis of WCW samples not only clearly revealed the main signals of aromatic
rings of lignin but also was a great way to study the saccharide part of the biomasses, but had
limitations on revealing minor linking motifs and minor aromatic units of lignin. REL could well avoid
the limitations of WCW analysis. However, the protein contamination seriously influenced the
estimation of the H units fin REL samples. MWL isolation could overcome these adverse effects of these
contaminations but led to overrepresentation of signals from end groups due to MWL consisting of
shorter extracted lignin chains.

3.3.2. Semi-quantification of 2D HSQC NMR spectra

It is a challenge to absolutely quantify the linkages and pendant units of lignin by 2D HSQC NMR spectra,
as signal integrations can be influenced by many parameters, such as $T_1$ and $T_2$ relaxation rates, carbon
pulse offset effects, and multiplicity and magnitude of coupling constants.59 Pendant units, such as H
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units, p-hydroxybenzoates (pB), and pCA can be easily overestimated as the difference of relaxation time between the interior structure of lignin and these terminal groups. Nevertheless, semi-quantification of the spectra based on signals that represent C9 units still provides valuable information such as relative reliable S/G ratios and relative amounts of the main linking units of lignin from similar resources.

To demonstrate the difference in information that can be extracted from these different types of lignin analyses, semiquantitative analysis was performed by signal integrations (Table 3.1). The semi-quantification revealed that structural information of lignin extracted from WCW_Reed, REL_Reed, and MWL_Reed had the highest concordance among the four biomasses (apart from FA quantities, see discussion below), while for the other biomasses some differences could clearly be observed between the differently prepared lignin NMR samples. Examples are the unusual proportion of H units in walnut shell samples and variation of S/G ratio for birch samples as well as quite big difference of the β-O-4 linkage quantification for the pine samples.

Roughly, S/G ratios of WCW and REL were higher than that of MWL, which is explained as S units are harder to be released from biomass in organic extraction of lignin as S units always present in the main

Table 3.1. Composition of the lignin aromatic units, S/G ratio, and relative abundance of main inter unit linkages of whole cell wall (WCW), milled wood lignin (MWL), residual enzyme lignin (REL).

<table>
<thead>
<tr>
<th>Sample</th>
<th>S°</th>
<th>S'°</th>
<th>G°</th>
<th>G'°</th>
<th>H° b</th>
<th>FA c</th>
<th>β-O-4 a</th>
<th>β-S°</th>
<th>β-β a</th>
<th>S/G a</th>
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<tr>
<td>WCW_Birch</td>
<td>65</td>
<td>13</td>
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<td>&lt;1</td>
<td>1</td>
<td>57</td>
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<td>1</td>
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<td>&lt;1</td>
<td>53</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
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<td>91</td>
<td>2</td>
<td>7</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>34</td>
<td>11</td>
<td>2</td>
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<tr>
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<td>93</td>
<td>5</td>
<td>2c</td>
<td>&lt;1</td>
<td>&lt;1</td>
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<tr>
<td>WCW_Reed</td>
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<td>56</td>
<td>0</td>
<td>6</td>
<td>12</td>
<td>24</td>
<td>49</td>
<td>9</td>
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<tr>
<td>REL_Reed</td>
<td>40</td>
<td>3</td>
<td>52</td>
<td>0</td>
<td>6c</td>
<td>7.5</td>
<td>23</td>
<td>48</td>
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<td>53</td>
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<tr>
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<td>&lt;1</td>
<td>48</td>
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S, syringyl units, S', syringyl with an α-ketone structures, G, guaiacyl structure, G', guaiacyl units with an α-ketone structures, H, p-hydroxylphenyl units, FA, ferulate, pCA, para-coumarate, β-O-4, aryl ether linkage, β-β, resinols, β-5, phenylcoumaran; The data was calculated by semi-quantitative method and based on C9 units, and the molar percentage was based on the integration of the signal at α position of the linkage, and divided it by the total integration of S (1/2 S°,6), S' (1/2 S'°,6), G (G°), G' (G'°), H (1/2 H°,6), S/G ratio obtained by (1/2 S°,6 + 1/2 S'°,6)/(G° + G'°), (See Figure 1 for structures).

b As residue enzyme lignin were contaminated during enzyme treatment, and the signals of H2'6 units were overlapped with phenylalanine from residue protein, so the integration of H units was based on the overlapped signals. The integration values for the H units might be slightly larger than their actual H units because of the overlap of Phe3,5, in particular for Birch_REL and Pine_REL.
back bone of the lignin and high steric hindrance. In addition, higher proportions of H units were found in WCW compared to that of corresponding REL and MWL samples, confirming overestimation of H units by the method of gel-state WCW. Higher levels (>10%) H units, with a relatively lower percentage of G units, were determined in WCW, REL, and MWL of walnut shell, indicating that walnut shell-derived lignin indeed contains a higher proportion of H units. This unusually high H content was observed before in specifically extracted walnut shell lignin that was shown to contain nearly 30% H units. The discrepancy for the lignins was also found for the semi-quantification of pCA and FA for reed samples. The percentage of pCA was similar among WCW, REL, and MWL from reed, while FA in WCW_Reed was much higher than the corresponding lignins. This is likely because FA is part of the hemicellulose structure and actually might only be part of the lignin as bridge between lignin and hemicellulose. pCA incorporates in lignin as a pendant or end group. Therefore, FA could be removed during the enzyme treatment and hardly extracted as it connected with saccharide, and pCA was easily released together with lignin during organic extraction and conserved during enzyme treatment.

All the samples obtained by different protocols revealed a similar proportion of β-O-4 linkages. As expected, birch with a higher S content showed the highest amount of β-O-4 linkages among all the WCW samples. By contrast, the abundance of β-O-4 linkages in WCW_Pine was determined as the lowest, as it only contains G units with minor H units. In general, the sequence of β-O-4 followed the order of birch > reed > pine, which is consistent with order of the percentage of S units. This observation was also found in semi-quantification of REL and MWL for these three biomasses. However, in the case of walnut shell, some abnormalities were observed. For instance, in contrast to the reed samples, the data of WCW and MWL from walnut shell have higher level of S units, but it did not result in a higher content of β-O-4. This may be due to the unusually high amount of H units that have a stronger tendency to form other linkages such as S-S linkages; this can also be confirmed by comparing the intensity of dimers for reed and walnut shell samples from reductive hydrogenolysis (see the Supporting Information S3, Fig. S12-15 and 16, Table S3.3, and discussion below). Relatively high amounts of β-5 and β-β linkages were found in MWL compared to their corresponding WCW and REL samples for all the four biomasses, which showed the limitation of MWL to represent native constituents of lignin. These are relatively stable structures having a higher possibility to be well conserved during extraction and also might be further enriched as they occur more in shorter extractable lignin chains. The influence of higher proportion of S units was also shown in the abundance of β-5 linkages, but the order followed the opposite trend as S units cannot form β-5 linkages. However, walnut shell contained a much lower content of G units than that of reed, but β-5 linkage content was similar for the two biomasses and lower than that of lignin from pine. Also, this might be because walnut shell contained a higher amount of H units and the p-coumaryl alcohol
favoring the production of C - C linkages.\textsuperscript{63} For instance, the amount of β-5 linkages of MWL_Walnut (13\%) was far more than that of corresponding REL (7\%), supporting our view that the higher amount of H units leads to more stable C - C linkages such as the β-5 linkages more easily extracted by organic extraction. Samples from reed contained the lowest amount of β-β linkages among the four biomass samples, which might be due to the fact that a certain amount of γ-hydroxy groups of the β-O-4 units was acetylated as revealed by the evident signals of the acylated units (β-O-4′γ) in the spectra. This is the result of an acylated precursor in the biosynthesis (Fig. 3.1a),\textsuperscript{64,65} which also decreases the possibility for them to radically couple to form a β-β linkage.\textsuperscript{66} Therefore, lignin fragments with higher percentage of β-O-4′γ can indeed be expected to also contain a relatively lower content of β-β linkages, as is indeed observed when comparing the ratio of β- O-4γ and β-O-4′γ of WCW_Reed, REL_Reed, and MWL_Reed (9.7, 5.2, and 5.6, respectively, based on C9 units) with the β-β linkages in these samples.

\textbf{3.3.3 Reductive catalytic fractionation}

The information that can be gained from structural analysis of the feedstock should preferably guide expected product formation upon its conversion by RCF. The aim here is to find distinct correlations between the detailed 2D HSQC NMR structural analysis and to link this to the products obtained from RCF. For this purpose, RCF of the lignocellulosic materials was performed using a commercially available Ru/C (5\%) catalyst at published optimized parameters for lignin monomer release,\textsuperscript{21,22} and the main released monomers such as 4-n-propylsyringol (S1) and -guaiacol (G1), and 4-n-propanolsyringol (S2) and -guaiacol (G2), as well as 4-n-ethylsyringol (S3) and -guaiacol (G3) were quantified (Fig. 3.5a and Table S3.2). The relative ratio between G- and S-type monomers was generally higher than the value determined by 2D HSQC NMR spectroscopy. This was in particular observed for lignin with a higher amount of β-5 linkages, which is due to the presence of more β-5 and 5–5 type linkage-connected G units. Units with these C - C bonds are released as dimers or oligomers instead of monomers.

The highest monomer yield was achieved by RCF of birch wood, and pine showed the highest recalcitrance with a relative lower monomer yield. These overall monomer yields corresponded well to the relative proportion of β-O-4 linkage determined by 2D HSQC NMR spectroscopy for these lignins (WCW and REL samples), and the yield was consistent with previously reported values.\textsuperscript{21,22} Walnut shell gave somewhat lower amounts of total monomers compared to reed than expected based on the β-O-4 linkage content, which is likely due to FA and pCA groups in reed (see below) combined with the higher H content in walnut shells linked to more stable C – C bonds. The latter could not be correlated to the amount of dimers and trimers in the product oil as determined by GPC analysis (Fig. S3.8), and this effect might thus be minor compared to the effect of the monomers related to FA and pCA. The
Fig. 3.5. Monomers from hydrogenolysis of WCW, REL and MWL, and the data of lignin was corrected based on the carbohydrates analysis. (a) Data obtained from whole cell wall materials; (b) H related monomers in detail from whole cell wall samples; (c) data from lignin samples; (d) H related monomers in detail from lignin samples. The chemical structure was colored and their yields have corresponding color in the figure. Monomer includes 4-(3-hydroxypropyl)-2,6-dimethoxyphenol (S1), 2,6-dimethoxy-4-propylphenol (S2), 4-ethyl-2,6-dimethoxyphenol (S3), 4-(3-hydroxypropyl)-2-methoxyphenol (G1), 2-methoxy-4-propylphenol (G2), 4-ethyl-2-methoxyphenol (G3), 4-(3-hydroxypropyl)phenol (H1), 4-propylphenol (H2), and 4-ethylphenol (H3). The products obtained from hydrogenation and methyl esterification include methyl 3-(4-hydroxyphenyl)propionate (PMA), methyl 3-(4-hydroxy-3-methoxyphenyl)propionate (FMA) and methyl 3-(4-hydroxycyclohexyl)propionate (OHP). Reaction condition: 250 mg biomass (50 mg for REL, 25 mg for MWL), 25 mg Ru/C (10 mg for MWL), 4 mL methanol, 250 °C, 3 h and 60 bar H₂. The monomer yield of WCW materials was based on Klason lignin.
yield of methyl-3-(4-hydroxyl-3-methoxyphenyl) propionate (FMA) derived from methanol transesterification and double bond hydrogenation of FA was only observed in the product oil of reed, which was correlated to the presence of FA.

For the rest, a significant amount of products, in particular for reed, did not fall in the regular class of monomers with S and G aromatic ring substitution patterns. Therefore, we also carefully quantified the amounts of minor monomers. Monomers with H substitution patterns, such as 4-propylphenol (H1), 4-propylphenol (H2), and 4-ethylphenol (H3) were found, as well as products that are likely associated with pCA such as methyl 3-(4-hydroxyphenyl) propionate (PMA) and methyl 3-(4-hydroxy cyclohexyl)propanoate (OHP) (Fig. 3.5b, WCW samples). Walnut shell was shown to contain the highest amounts of H units by 2D HSQC NMR analysis, which also revealed the total monomer yield when only taking into account H1 and H2. This led us to conclude that H1 and H2 are the main products of H units that are part of the lignin chain originating from the p-coumaryl alcohol precursor in the lignin biosynthesis. The products from reed revealed overall the highest amounts of these minor phenolic compounds when other components such as H3, OHP, and PMA were considered (nearly 10% of the 35% total monomer yield). This indicates that these monomers are likely derived from pCA units that are abundant in reed. These results show that the nature of the H and its related units (pCA) significantly affect the products distribution from RCF of lignocellulosic biomass rich in such units, which can readily be deduced from 2D HSQC NMR analysis of the whole biomass. Nevertheless, the fact that the total amount of H1 and H2 in all the product oils were less than 2%, means that lower amount of these products was produced than expected based on the 2D HSQC NMR results (up to 24% for walnut), indicating that their quantities in WCW samples are indeed likely significantly overestimated by 2D HSQC NMR spectroscopy.

3.3.4. Hydrogenolysis of isolated lignins

To further investigate the role of the H units and derivatives, hydrogenolysis of the different isolated lignins was performed (Fig. 3.5c, d, REL and MWL). Overall, the amount of monomers was typically somewhat lower, but higher for REL than MWL, which overall showed a larger deviation from the reductive fractionation, in particular for birch. A similar distribution between S and G monomers was found that related well to the S/G ratio determined by 2D HSQC NMR spectroscopy. The only curious observation were the increased amounts of S1, G1 and H1 relative to S2, G2 and H2 (the MW distribution also clearly revealed this difference, as shown in Fig. S3.8). As the main difference between RCF and the hydrogenolysis of isolated lignin is the presence of saccharides, we performed control reactions where cellulose (Avicel® PH-101) and xylan (birch) were added to REL samples followed by hydrogenolysis. Interestingly, the presence of birch xylan had a negative effect on the overall yield,
which could be due to the presence of impurities introduced during its isolation. Nevertheless, the same trend in the product distribution was observed in terms of increased formation of S1, G1, and H1 relative to S2, G2, and H2 (Fig. 3.6a). This led us to believe that the presence of carbohydrates significantly influences the catalyst selectivity leading to a different product distribution.

Regarding the minor units, also the distinct difference between reed and walnut shell is revealed. For walnut shell again the main products are H1 and H2, while reed samples contain more H3, OHP, and in particular PMA. The amount H3 decreased significantly for all samples going from RCF to hydrogenolysis of isolated lignins. H3 was highly suspected to be linked to pCA based on our initial analysis, even though the isolated lignins still seemed to contain pCA according to 2D HSQC NMR spectroscopy. So it seems that some other constituents or pCA linked to particular lignin or polysaccharide motifs in the raw biomass contribute to the production of H3.

3.3.5. Analysis of the dimer fractions

G units, but also H units, are expected to have more tendency to form C - C bonds such as 5,5 and β-5 linkages during biosynthesis, and thus their presence should lead to higher amounts of dimers, trimers, and oligomers. As mentioned above, GPC analysis of the oils did not really lead to any conclusive results (Fig. S3.8), and therefore we further derivatized the oil obtained from hydrogenolysis of REL and MWL and performed a more in-depth GC-MS analysis (see Fig. S3.12-S15 for the spectra of signals of identified dimers with the aid of previous dedicated publications). Dimers derived from S and G units with linkage of β-β, β-5, and β-1 could be readily identified (see the original spectra in our published paper). To correlate this analysis to the structure of the different lignins, a rough percentage of peak area of dimer correlated to different linkages was calculated (Tables S3.3 and S3.4). β-β and β-5 were the most readily linked to specific dimers and were thus most abundant, but their relative distribution between the biomass samples correlated excellently with the values obtained from 2D HSQC NMR analysis. As the H-derived monomer products are always lower than 2%, the identification of specific H derived dimers was expected to be very challenging. Nevertheless, a significant attempt was made to find signals that corresponded to H units, and one H - G dimer derived from a β - β linkage could be assigned. This dimer had the highest relative abundance in the MWL_pine and MWL_walnut samples, which also had the highest combination of G and H units. Looking at the total amounts of dimers, it seems that in particular the pine-derived oils as well as the REL_walnut-derived oil showed significant amounts of dimers, which correlates well to the lower amounts of monomers. This fits the lower amounts of cleavable β-O-4 linkages in pine as well the significant presence of H and G units in these samples.
3.3.6. Monomers derived from \( p \)-CA and FA

To gain further insight into the expected RCF product portfolio from \( p \)-CA and FA, \( p \)-coumaric acid (\( p \)CCA), ferulic acid (FAA), and their methylated counterparts were converted under RCF conditions, and the products were analyzed (Fig. 3.6b and Fig. S3.9a). Both FAA and its methyl ester (FAME) resulted in same product FME, which is the result of the side chain hydrogenation (and esterification for FAA). No G3, G2, or G1 were observed in the reaction of FAA and FAME, indicating that these components do not contribute to the main lignin hydrogenolysis products. However, FMA was found in the reed samples, and the relative amount (WCW > REL > MWL) matched the amounts of FA determined by 2D HSQC NMR spectroscopy of the different samples and also likely corresponded to associated hemicellulose residues, which were significantly lower in the isolated lignins. For \( p \)CCA and \( p \)CAMe a similar product (PMA) was found in 25% yield corresponding to the hydrogenation of the double bond and for \( p \)CCA methyl esterification. Moreover, the yield of PMA for WCW, REL, and MWL were similar to each other and all less than 5%, which correlated with the similar percentages of \( p \)CA quantified by 2D HSQC NMR spectroscopy in WCW, REL and MWL. But, the yield of PMA is much lower than might be expected based on the semi-quantification (more than 20% for all the reed samples), which further indicated the overestimation of these end groups by 2D HSQC NMR spectroscopy. Additionally, here also significant amounts (>65%) of the ring hydrogenation products (OHP) were

![Fig. 3.6. Monomers from hydrogenolysis of model compounds and the influence of polysaccharide addition for the monomer distribution. (a) Hydrogenolysis of model compounds with and without polysaccharide addition, and model compounds includes \( p \)-coumaric acid (\( p \)CA), methyl (E)-3-(4-hydroxyphenyl)acrylate (\( p \)CAMe). Reaction condition: ‘Ru/C and Ru/Alumina’ 20 mg start material, 10 mg Ru/C or Ru/Alumina, 4 mL methanol, 250 °C, 3h, 60 bar H\textsubscript{2}, ‘Ru/C+Xyl+Glu’, 20 mg start material, 10 mg Ru/C or Ru/Alumina, 20 mg cellulose, 10 mg xylan, 4 mL methanol, 250 °C, 3h, 60 bar H\textsubscript{2}.(b) The influence of polysaccharides for monomer distribution of residual enzyme lignin from birch. Reaction condition: ‘REL+Xyl’, 50 mg Birch\textsubscript{REL}, 60 mg xylan from birch, 25 mg Ru/C, 4 mL methanol, 250 °C, 3h, 60 bar H\textsubscript{2}, ‘REL+Glu’ 50 mg Birch\textsubscript{REL},110 mg cellulose from cotton, 25 mg Ru/C, 4 mL methanol, 250 °C, 3h, 60 bar H\textsubscript{2}, ‘REL+Xyl+Glu’ 50 mg Birch\textsubscript{REL}, 60 mg xylan from birch, 110 mg cellulose from cotton, 25 mg Ru/C, 4 mL methanol, 250 °C, 3h, 60 bar H\textsubscript{2}. The chemical structure was colored and their yields have the some corresponding color in the Fig. 3.5.](image-url)
found. These products were also formed in significant amounts in the reed samples in the order WCW < REL < MWL, matching the amount of carbohydrate residues in the samples. Indeed, control reactions with \( p\text{-CCA} \) and \( p\text{-CAMe} \) in the presence of cellulose and xylan showed also lower amounts of OHP, indicating that the formation of such over-hydrogenated products is inhibited by the presence of saccharides (Fig. 3.6b). In total, through control experiments by the addition of carbohydrates to the isolated lignins and model compounds, it was found that the polysaccharide constituents inhibit dehydroxylation of the side chains of the aromatic monomers as well as over-hydrogenation of the aromatic rings.

Thus far, all products found from RCF could be traced back to individual structural units in the original lignins, however only the origin of \( H3 \) remained a bit of a mystery. For \( p\text{-CCA} \) around 5% was degraded into \( H3 \) but none of the other model compound reactions gave any \( H3 \), which would be the result of a decarboxylation reaction. Changing the catalyst (Fig. 3.6b), adding carbohydrates or changing to the corresponding ester \( p\text{-CAMe} \) did suppress the formation of this small amount of \( H3 \). It was reported that alkoxide species play a very important role in surface interaction of compounds on catalysts and decarboxylation reactions,\(^{68,69}\) and thus the free acid might still be partly responsible for the formation of \( H3 \). In addition, hemicellulose constituents of \( p\text{-CAA} \) with free carboxylic acid end group have been reported\(^{70}\) but under reaction conditions are likely quickly esterified as the model compound reactions revealed. We further used a hemicellulose extracted from mixed grasses and rich in \( p\text{-CAA} \) (Fig. S3.10) for an additional control reaction. The main products were derived from \( p\text{-CAA} \) and \( FA \), and only trace amounts of \( H \) monomers were monitored (Fig. S3.9c and S3.11). Finally, the stability of \( H1 \), \( H2 \), and \( H3 \) in this reductive treatment was investigated (Fig. S9b), revealing trace amounts of \( H3 \) that was produced from reductive treatment of \( H1 \), which indicated that \( H3 \) could also be formed by further reactivity of these components in solution, but the difference in yield of \( H3 \) from the different biomasses could not fully traced back to the specific structures of the original biomass samples, and it is likely that an unidentified effect or biomass constituent also plays a role in the formation of \( H3 \).

### 3.4. Conclusion

Overall, in this study, lignins in four representative biomasses were comparable evaluated by whole cell wall (WCW), residual enzyme lignin (REL), and milled wood lignin (MWL), and the reliability and concordance of lignin analyses based on the three protocols was discussed. Gel-state 2D heteronuclear single quantum coherence (HSQC) NMR spectroscopy provided detailed structural information of the WCW after milling, but the overestimation of end group was an issue. REL was more representative than MWL to reveal the integrated structure of lignin in terms of larger molecular weight and much detail in linking units. Comparing the 2D HSQC NMR analyses, relatively big differences were observed
for birch and walnut shell in terms proportion of units and linkage content, with the WCW and REL providing more similar results. When relating these analyses to monomer yield and distribution obtained from reductive catalytic fractionation (RCF) led to a clear correlation between monomer distribution and main linkages and aromatic unit constituents of the original lignin component of the biomass as determined by 2D HSQC NMR samples. Overall, comparing the monomer yield and similar distribution with that of WCW indicated that the product of REL hydrogenolysis is more representative than that of MWL to reveal the actual behavior of lignin during reductive fractionation. One disadvantage of REL analysis is that ferulic acid units linked to hemicellulose are removed, which will end up in the phenolic product mixture and thus contribute to the total monomer yield. The presence of polysaccharide constituents such as xylan and cellulose significantly influences the aromatic monomer selectivity and inhibits over-hydrogenation of the aromatic ring as well as dehydroxylation to give more propanol-substituted aromatic monomers. Furthermore, the RCF products from minor units significantly impacted product distributions. For instance, a higher amount of H units predominantly led to increasing amount of H1 and H2 especially for the reed and walnut shell biomass. In particular, p-coumaric acid units in reed contributed nearly 10% of the 35% of the monomeric product mixture. Indepth analysis of the monomer distribution derived from minor units indeed enhanced understanding of their original state and structure in lignin and biomass. The relative quantities of these units were more readily accurately quantified by 2D HSQC NMR spectroscopy of REL samples compared to ge-state 2D HSQC NMR of the WCW due to overestimation of these pendant units. In total, this study shows how different analyses of the lignin structure of the original biomass can provide good prediction to the obtained monomer portfolio from RCF of different biomass and the effects of minor units and carbohydrate impurities. This information can guide studies on the viability of “lignin-first” related biorefinery efforts.
Supplementary Material - Chapter 3

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S3.2. Yield and carbohydrate analysis
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S3.1. Experimental support section:

S3.1.1. General remarks

The Silicagel (60 Å 230-400 micron) was provided by Sigma-Aldrich.

Thin layer chromatography was performed on pre-coated aluminum plates (60/kieselguhr F₂₅₄ Merk) and visualized by staining with KMnO₄.

S3.1.2. General characterization

The samples after enzymatic hydrolysis were analyzed by SEM with an accelerating voltage of 5.0 kV at ZEISS EVO 18 (Carl Zeiss, Inc, Oberkochen, Germany); FT-IR were collected on a Nicolet iN10 FT-IR spectrophotometer (Thermo Scientific, USA) with an MCT detector. The spectra were recorded in the region of 4000-400 cm⁻¹ at a resolution of 16 cm⁻¹. TGA and DTG was obtained on a simultaneous thermal analyzer (TGA 4000, PerkinElmer, USA) with a 20 mL/min flow of nitrogen at a heating rate of 10 °C/min. GPC was performed in DMF (containing 0.01 M LiBr) on a Viscotek GPC max equipped with model 302 TDA detectors, two columns (Agilent Technologies-PolarGel-L and M, 8 µm 30 cm) at a flow rate of 1.0 mL·min⁻¹. The columns and detectors were held at 50 °C. Data acquisition and calculations were performed using Viscotek OmniSec software version 5.0. Molecular weights were determined based on a conventional calibration curve generated from narrow dispersity polymethylmethacrylate standards (Agilent and PSS, MW from 550 to 1.190.000 g/mol). The samples were dissolved in 0.2 µm PTFE filter prior to injection, and a 200 µL sample with a concentration of 2.0mg/mL was injected. The determination of X-ray diffraction (XRD) was conducted by a D8 ADVANCE Brochures instrument (Bruker, Germany) with Ni-filtered Cu Ka radiation source (k = 0.1542 nm) generated at 40 kV and 30 mA. The scattering angle (2θ) ranged from 5 ° to 40 ° using the reflection method at a scanning speed of 2 °/min. GC-MS determination was performed on an Agilent 6890 series GC system equipped with a HP973 mass detector with helium as carrier gas.

S3.1.3. Chemical composition analysis

The carbohydrate composition analysis was conducted according National Renewable Energy Laboratory (NREL) with minor modification, with a 0.3 g scale for the analysis of WCW and a scale of 10 mg for lignin. Samples were hydrolyzed by 72% sulfuric acid at 30 °C for 1 h and further diluted to 4% at 121 °C for another 1 h. Monosaccharides were determined by HPLC (1200 Agilent Technologies, USA) with a refractive index detector (RID). An aminex column HPX-87H (Bio-Rad, USA) was used to analyze the monosaccharides at 50 °C with 5 mM sulfuric acid at a flow rate of 0.6 mL/min. In these conditions, xylose, mannose and galactose were eluted at the same retention time, so they were integrated at a single peak. An external standard calibration curve was built by the individual monosaccharide, and the constant of xylose was selected to calculate the total amount of xylose, mannose, and galactose.

S3.1.4. NMR analysis

¹H and ¹³C spectra were collected from a 400 MHz NMR spectrometer (A400a, Agilent, BASIC PROBHD) instrument or 600 MHz Bruker Biospin (Rheinstetten, Germany, BASIC PROBHD) with solvent peak as the reference to correct the chemical shift. Multiplicities was showed by the following abbreviations: s = singlet, d = doublet, t = triplet, q = quartet and m = multiplet and j coupling in Hz. 2D HSQC NNR spectra of the gel-samples were collected on a 600 MHz Bruker Biospin (Rheinstetten, Germany, BASIC PROBHD) instrument. The signal of DMSO solvent was used as internal reference (⁹C 39.5, ⁶H 2.49 ppm). Bruker standard pulse sequence ‘hsqcetgpsisp.2’ was used for the ¹³C-¹H correlation experiment. A reported parameters with minor modification was used for the analysis: spectra use 2048 data points from 11 to 0 ppm in F2 (¹H) (acquisition time 130 ms), 160 to 0 ppm in F1 (¹³C) with 256 increments (acquisition time 6 ms) of 64 scans with 500 ms internal delay; the d² delay was set to 86 ms. The total acquiring time is 3.54 h. ²D NMR spectra of residue enzyme lignin were collected by parameter with that of the whole cell wall sample with some modification, and the d1 was set 1s. ²D NMR of MWL was collected on a 400 MHz NMR spectrometer (A400a, Agilent, BASIC PROBHD) instrument. The signal of DMSO solvent was used as internal reference (⁶C 39.5, ⁶H 2.49 ppm). A reported parameters with minor modification was used for the analysis: spectra use 2048 data points from 11 to 0 ppm in F2 (¹H).
(acquisition time 150 ms), 160 to 0 ppm in F1 (13C) with 256 increments of 32 scans with 1.0 s internal delay. Volume integration of the contours in HSQC spectra was performed by MestReNova (12.0.4), and the integration was used for relative comparisons, and the data was based on the total integration of S, G and H.

S3.1.5. Synthesis of authentic standards

(3-hydroxypropyl)-2,6-dimethoxyphenol (S1):

S1 was synthesized by slight modification of a reported procedure.22 A mixture of (E)-3-(4-hydroxy-3,5-dimethoxyphenyl) acrylic acid (5 g, 22.32 mmol) and 5% Pd/C (0.1 g) in THF (80 mL) were placed in a high pressure Parr autoclave. The reactor was sealed, purged 3 times with H2, pressurized with H2 (40 bar) and stirred at room temperature for 18 h. After the reaction was completed and the pressure released the reaction liquid was filtered through a Celite plug and the solvent was evaporated under reduced pressure to provide 5 g of 3-(4-hydroxy-3,5-dimethoxyphenyl) propanoic acid (22.12 mmol, 99% yield), which was used without further purification. The product was added to a rapidly stirred suspension of LiAlH4 (1.26 g, 33.18 mmol) in 50 mL of THF at 0 °C. After the addition was complete, the reaction mixture was refluxed for 60 min, and then poured into ice water, which was then poured into a solution of 5% HCl cooled using an ice bath, and extracted with once with diethyl ether (100 mL) and twice with 50 mL portions of EtOAc. The combined organic extract was washed with a saturated solution of NaHCO3 (50 mL) and brine (100 mL), dried over anhydrous MgSO4 and the solvent was evaporated under reduced pressure to give a 81% yield of S1.

1H NMR (600 MHz, CDCl3) δ 6.43 (s, 2H), 3.88 (s, 6H), 3.69 (t, J = 6.4 Hz, 2H), 2.67 – 2.62 (m, 2H), 1.91 – 1.85 (m, 2H).

2,6-dimethoxy-4-propylphenol (S2):

S2 was synthesized according to a published procedure with minor modification.23 A mixture of 4-allyl-2,6-dimethoxyphenyl (100 mg, 0.5 mmol) and 10% Pd/C (20 mg) in ethanol (5 mL) were placed in a homemade stainless steel high pressure mini-reactor. After sealing off the reactor, it was charged with 20 bar H2 and then stirred overnight at room temperature. A colorless oil was obtained in 89% yield after filtering off the catalyst and removing the solvent under reduced pressure.

1H NMR (400 MHz, CDCl3) δ 6.40 (s, 2H), 5.35 (s, 1H), 3.87 (d, J = 1.2 Hz, 6H), 2.51 (t, J = 7.7 Hz, 2H), 1.66 – 1.56 (m, 2H), 0.99 – 0.90 (m, 3H).

4-ethyl-2,6-dimethoxyphenol (S3):

S3 was synthesized according to a published procedure with a minor modification.24 A mixture of 1-(4-hydroxy-3,5-dimethoxyphenyl)ethan-1-one (100 mg, 0.55 mmol) and Raney Ni (wet, 50 mg) in iso-propanol (5 mL) was placed in a round flask and refluxed for 2 h. The product was purified by column chromatography (SiO2, Pentane/EtOAc 90:10 to 70:30) after removing the catalyst by filtration and the solvent under reduced pressure. The yield of the final product was 70%.

1H NMR (400 MHz, CDCl3) δ 6.42 (s, 4H), 5.36 (s, 2H), 3.88 (s, 12H), 2.58 (q, J = 7.6 Hz, 4H), 2.01 – 1.92 (m, 1H), 1.77 (d, J = 12.9 Hz, 1H), 1.22 (t, J = 7.6 Hz, 8H), 1.00 – 0.83 (m, 2H).

Methyl 3-(4-hydroxy-3-methoxyphenyl)propanoate (FMA):

FMA was separated from the product mixture of a reductive hydrogenolysis reaction under standard conditions using ferulic acid as substrate. A mixture of ferulic acid (100 mg, 0.48 mmol), 5 % Ru/C (40 mg) in 4 mL methanol was placed in homemade stainless steel minireactor. After sealing off the reactor, it was charged with 60 bar H2 and then was stirred for 3 h at 250 °C. The product was purified by column chromatography (SiO2, Pentane/EtOAc 90:10 to 60:40) after removing the catalyst by filtration and the solvent under reduced pressure. The yield of the final product was 20%.
Catalytic hydrogenolysis of lignin: the influence of minor units and saccharides

$^1$H NMR (400 MHz, CDCl$_3$) δ 7.09 – 7.02 (m, 2H), 6.79 – 6.71 (m, 2H), 5.12 (s, 1H), 3.67 (s, 3H), 2.88 (t, J = 7.7 Hz, 2H), 2.60 (dd, J = 8.3, 7.2 Hz, 2H).

Methyl (E)-3-(4-hydroxy-3-methoxyphenyl)acrylate (FAME):

FAME was synthesized by dissolving 0.97 g (5 mmol) ferulic acid in 25 mL methanol, and 3 drops of concentrated sulfuric acid were added. The reaction was refluxed overnight, and after cooling down, the solution was neutralized by adding a saturated solution of sodium bicarbonate (10 mL) over 30 min while stirring. The mixture was then extracted by ethyl acetate (3 x 30 mL) and washed with brine (10 mL). The combined organic phases were dried over magnesium sulfate. After filtration, a light yellow oil was obtained, which was used for the reductive hydrogenolysis without any purification. The yield of final product was 96%.

$^1$H NMR (600 MHz, CDCl$_3$) δ 7.62 (d, J = 15.9 Hz, 1H), 7.07 (ddd, J = 8.2, 2.0, 0.5 Hz, 1H), 7.02 (d, J = 1.9 Hz, 1H), 6.92 (d, J = 8.2 Hz, 1H), 3.92 (s, 3H), 3.79 (s, 3H).

Methyl 3-(4-hydroxyphenyl)propanoate (PMA):

PMA was separated from the product mixture of a reductive hydrogenolysis reaction under standard conditions using p-coumaric acid as substrate. A mixture of p-coumaric acid (100 mg, 0.55 mmol), 5% Ru/C (40 mg) in 4 mL methanol was placed in a homemade stainless steel mini-reactor. After sealing off the reactor, it was charged with 60 bar H2 and stirred for 3 h at 250 °C. The product was purified by column chromatography (SiO$_2$, Pentane/EtOAc 90:10 to 60:40) after removing the catalyst by filtration and the solvent under reduced pressure. The yield of final product was around 40%.

$^1$H NMR (400 MHz, CDCl$_3$) δ 6.83 (d, J = 7.9 Hz, 1H), 6.73 – 6.65 (m, 2H), 5.51 (s, 1H), 3.87 (s, 3H), 3.67 (s, 3H), 2.88 (t, J = 7.9 Hz, 2H), 2.60 (dd, J = 8.4, 7.1 Hz, 2H)

Methyl 3-(4-hydroxycyclohexyl)propanoate (OHP):

OHP was separated with the separation of PMA by column chromatography from a reductive hydrogenolysis reaction starting from p-coumaric acid described above. The yield of this product was around 30%.

$^1$H NMR (600 MHz, Chloroform-d) δ 3.96 (q, J = 3.8 Hz, 1H), 3.66 (d, J = 2.1 Hz, 3H), 2.32 (qd, J = 7.6, 2.0 Hz, 2H), 1.97 (t, J = 1.2 Hz, 2H), 1.07 – 0.91 (m, 1H).

$^{13}$C NMR (151 MHz, CDCl$_3$) δ 174.46, 70.95, 66.86, 51.55, 51.53, 36.21, 35.86, 35.37, 32.17, 31.89, 31.74, 31.52, 31.07, 30.87, 26.60.

Methyl (E)-3-(4-hydroxyphenyl)acrylate (pCAMe):

pCAMe was synthesized according to a published procedure with a minor modification. 0.82 g (5 mmol) p-coumaric acid was dissolved in 25 mL methanol and 3 drops of concentrated sulfuric acid were added. The reaction was refluxed overnight, and after cooling down, the solution was neutralized by the addition of saturated sodium bicarbonate (10 mL) over 30 min while stirring. The mixture was then extracted with ethyl acetate (3 x 30 mL) and washed with brine (10 mL). The combined organic phases were dried by magnesium sulfate. After filtration, a light yellow solid was obtained, which was used for the reductive hydrogenolysis without any purification. The yield of final product was 94%.

$^1$H NMR (600 MHz, CDCl$_3$) δ 7.64 (d, J = 16.0 Hz, 1H), 7.45 – 7.39 (m, 2H), 6.88 – 6.83 (m, 2H), 6.30 (d, J = 16.0 Hz, 1H), 3.80 (s, 3H).

S3.1.6. Hemicellulose extraction from mixed grass

The extraction of the hemicellulose was extracted from published paper with minor modification. Twenty grass samples were collected from the Groningen city area, and the grass samples were freeze-dried and smashed by...
a Retsch ZM 200 mill equipped with a 1.0 mm sieve. 10 g the grass powder was suspended in 200 mL 1 M KOH containing 0.26 M NaBH₄ in a 250 mL bottle for 24 h at room temperature. The mixture was separated by centrifugation and the solid pellet was further extracted by 200 mL 1 M KOH containing 0.26 M NaBH₄ in a 250 mL bottle for 24 h. The combined supernatant was neutralized by acetic acid and concentrated by rotary evaporator. The supernatant was further dialyzed by spectra/Por® and freeze dried.

**S3.1.6. Monomer quantification and yield calculation from reductive fractionation**

\[
RF_c = \frac{A_c}{C_c}, \quad RF_is = \frac{A_is}{C_is}\]
\[
RRF = \frac{RF_c}{RF_is} = \frac{(A_c / C_c)}{(A_is / C_is)} = \left(\frac{A_c}{C_c}\right) * \left(\frac{C_is}{A_is}\right)
\]
\[
C_c = (A_c / RRF) * \left(\frac{C_is}{A_is}\right) = \left(\frac{A_c}{A_is}\right) * \left(\frac{1}{RRF}\right) * C_is
\]
\[
Y_{monomer} = \frac{C_c * V * MW_{monomers}}{W_{lignin or W_{Klason lignin}}}
\]

In the equations: \(A_c\), the integration of monomers; \(C_c\), the mole concentration of monomer (mmol/mL); \(A_is\), the integration of monomers; \(C_is\), the mole concentration of monomer (mmol/mL); \(V\), the total volume after dilution (mL); \(MW\), mole weight of monomer (mg); \(W_{lignin}\) or \(W_{Klason lignin}\), the weight of lignin or the weight of Klasson lignin; \(Y_{monomer}\), the yield of the monomer based on the weight of lignin or Klasson lignin.

The monomer yields of REL and MWL were calculated on the weight of lignin after correction of the weight by the result of the compositional analysis. The monomer yields of raw material were calculated based on the determined Klasson lignin content after removal of ash content without counting acid soluble lignin. The total monomers showed in the Fig.3.5 was all the monomers quantified from GC-FID, and all the monomer yield was based on lignin. The parts with solid line border was from the reductive catalytic depolymerization of syringyl and guaiacyl units, and the parts with dash border was un-identical whether them are from lignin or lignin analogous compounds.

**S3.1.7. Derivatization and GC-MS procedure**

The derivatization was performed according to a published paper. Oil obtained from hydrogenolysis was dissolved in dichloromethane to make around 10 mg mL⁻¹ solution, and then 600 μL oil solution was added to a 2 mL GC vial. 50 μL pyridine and 100 μL of silylating agent [(N-O-Bis(trimethylsilyl)trifluoroacetamide) with 1% trimethylchlorosilane] was added. The mixture was further heated in an oven at 50 °C for 1 h before injection on the GC-Mass. A split ratio of 10:1 and a split flow of 12 mL min⁻¹. The inlet temperature was 280 °C and the oven was set at 5°C min⁻¹ between 150 and 300 °C and held at 300 °C for 18 min. The spectra was collected from a GC-MASS (Shimadzu GC-MS, QP2010 Ultra) system with an HP-5ms Ultra Inert 30 m × 250 μm × 0.25 μm. 5 min was used to prevent overloading the detector with the monomers. The GC-MS spectra were identified by comparing with the spectra published in the literature and prediction structure with unknown MS spectra.
Supplementary text:

S3.2. Yield and carbohydrate analysis

Lignocellulosic biomass mainly consists of cellulose, hemicellulose and lignin, and the three main constituents were also influenced by the biomass species, age, growing areas, environment, and even different part of biomass. Lignin is covalently intertwined and possibly covalently linked with polysaccharides in biomass, which limits its yield (Table S3.1), purity and application. The first aim was to get insight into the relative composition of the specific biomasses used in this study and evaluate the recovery yield and purity of the isolated lignin. For this purpose compositional analysis of MWL, REL and WCW was conducted (Table S3.1) and these values corrected for their isolated yield are presented in Fig. S3.1. Only the saccharides content was determined. The Klasson lignin was determined for the raw materials (Table S3.1).

The difference of composition was clearly observed among the four biomasses. Reed contains the highest amount of cellulose and hemicellulose, and walnut shell has the lowest proportion of the two sorts of polysaccharides. Birch and pine have a similar carbohydrate quantity. The difference of the main constituents in the raw material were also revealed by the TGA/DTG curves (Fig. S3.3), in which each raw biomass had an individual distinctive DTG curve, especially in the temperature range of 200 to 400 °C where saccharides are paralyzed. As expected, the yield of MWL was much lower than that of REL. Theoretically, the enzymatic removal of the polysaccharides allows for the quantitative isolation of lignin from biomass. However, some weight loss of REL was observed by comparing the yield of REL with the lignin ratio of WCW, which was due to that some lignin fragments were lost during washing steps. The result also exhibited some residual saccharide impurities in REL (Table S3.1). Though walnut shell contains less polysaccharides in the raw material, the carbohydrate impurity was the highest among the four REL samples, which might be due to that walnut shell has a higher hardness, and a high proportion of lignin in walnut shell could exhibit a higher recalcitrance towards ball milling and enzyme hydrolysis. However, REL_Walnut isolated from the softest raw material, also contained a higher proportion of carbohydrate impurity which was only slightly less than that of REL_Walnut. This could be caused by a relatively high proportion of lignin-carbohydrate linkages in reed. MWL had a higher purity, as the isolated MWL was further purified by ethanol and water precipitation but at the cost of yield compared to that of REL or more dedicated organosolv extractions. Moreover, All the REL samples had a higher mass loss rate than that of MWL in temperature range of 200 to 400 °C as the isolated MWL (Fig. S3.3), and different of peaks of C=O of cellulose (1034 cm⁻¹ of birch, walnut and reed, and 1026 cm⁻¹ of pine) as well as the peak of acetyl group of hemicellulose (1736 cm⁻¹ of birch, pine and walnut, 1720 cm⁻¹ of reed) on the FT-IR (Fig. S3.2) from these samples, further indicating the higher saccharide impurities of the RELs compared to MWLs.

S3.3. WCW swelling

We were firstly interested to get as much information as possible by analysis of the WCW material. 2D HSQC NMR is less straightforward for whole biomass compared to isolated lignins as the quality of the spectra depends on the swelling properties of the samples to obtain a suitable “gel-state”. Intensive physical ball milling is an essential prerequisite for the gel formation. As gel-state NMR was to our knowledge not reported previously for walnut shell, walnut shell firstly selected to monitor the gel formation as a function of ball milling. Walnut shell after 6 h, 12 h, 24 h milling were used to prepare the gel samples. Around 4 h sonication was necessary to collect ideal state of the gel sample, using for collecting 2D HSQC NMR. As shown in the Fig. S3.4, with increase of the ball milling time, the sample showed significantly improved swelling in the mixture of DMSO-d₅/pyridine-d₅, which corresponded to increased intensity of the signals for β-O-4, β-β and β-5 linkages. In order to explain why intensive ball milling enhanced swelling WCW. SEM graphs of the milled samples were collected. From these graphs (Fig. S3.5), it can be deduced that the predominant particle size of sample obtained after 6 h of milling was almost the same as that of the sample obtained after 24 h of milling, which was correlate to our previous study in which the particle size of walnut shell even slightly increased under longer ball milling time as a result of aggregating under the harsh ball milling condition. It was reported that the predominant size of biomass particles less than 5 μm is required for optimal gel formation, however, the main particle size of walnut shell had reached around 5 μm after 6 h milling. Nevertheless, the quality of the gel sample was not satisfactory, indicating that particle size was not the only parameter to influence the gel formation. Previous work reported that crystalline cellulose is hard to swell in DMSO-d₅/pyridine-d₅, which might correlate with the poor swelling...
properties of samples obtained from the shorter milling time\textsuperscript{10}. In order to gain the sight of crystal structure of cellulose after ball milling, XRD spectra of these sample were collected. The spectra (Fig. S3.6) of samples obtained after 24 h of milling had no clear sharp peaks in contrast to the spectra of raw material, indicating almost all the crystalline cellulose disappeared, and the relative crystallinity value further confirm this alteration. It seemed that extra-long time milling could alter the structure of micro crystallite of cellulose and increase the interplanar distance from the observation of the shift and broadening of typical peaks of crystalline cellulose, which resulted in enhancing the penetration of solvent and formation of gel-state sample. This may explain why no difference was observed in terms of particle size from different milling times, but a longer milling time could achieve a better gel sample and higher quality spectra.

S3.4. Thermal-stability of lignin and biomass

The pyrolysis behavior of polymer depends on the structure and composition. Therefore, the pyrolysis curve of polymer could reveal some information about the structure and composition of the polymer. Thermos-stability of the biomass and isolated lignin were tested by TGA/DTG and depicted in the Fig. S3.3. Three steps was clearly observed from the curve of WCW, the weight lost occurred within 150 °C, hemicellulose was deposed between 200 °C and 300 °C, followed by cellulose degrading between 300 °C and 350 °C, lignin was depolymerized at a temperature range of 300 °C to 500 °C, which is corresponded to previous report.\textsuperscript{81} Lignin samples exhibited a higher stability, and MWL showed a similar TGA curve with that of DREL, but DREL lignin showed a higher weight loss at a lower temperature. Except Pine_WCW (347 °C), two peaks were observed from the DTG curves of WCW_Birch (304 °C, 341 °C), WCW_Walnut (294 °C, 339 °C) and WCW_Reed (296 °C, 341 °C), but WCW_Pine had the highest weight loss rate (0.53 mg/s).The first peak of the DTG curve was assigned to the degradation of hemicellulose and the second was ascribed to the depolymerization of cellulose, the tails of the curve was due to decomposition of remained lignin. The difference of mass loss rate between cellulose and hemicellulose of the four WCWs was followed by the order of reed > birch > walnut. For the sample of REL all the samples showed a similar TGA curve, and a peak was observed on the DTG curve for all the REL samples that corresponded to the hemicellulose impurities. The temperature was 341 °C, 313 °C, 300 °C, 294 °C for REL_Reed, REL_Pine, REL_Walnut and REL_Birch, respectively, when the maximum mass loss was reached, which could partially reveal the thermal stability of the corresponding samples. The remaining tails of DTG of the REL were similar with corresponding MWL.

S3.5. FT-IR analysis

In order to analyze the structure difference between the WCW and two lignin samples, FT-IR spectra were further collected and depicted in Fig. S3.2. The assignment of the peaks was followed by published papers.\textsuperscript{82,83} A broad peak around 3400 cm\textsuperscript{-1} was ascribed to the stretching of -OH, and a broad peak with a shoulder peak was followed attributing to the stretching of -CH. Both of the two peaks were observed on all the spectra. The peak at 1736 cm\textsuperscript{-1} only observed on the spectra of raw material was mainly caused by the C=O of hemicellulose, which confirms the high purity of the lignin samples. Peaks of 1651 cm\textsuperscript{-1}, 1597 cm\textsuperscript{-1}, 1505 cm\textsuperscript{-1} and 1419 cm\textsuperscript{-1} were the peaks from the skeletal vibration of aromatic rings. Only MWL_Pine, a typical G lignin, showed distinctive peaks at 1134 cm\textsuperscript{-1}. The spectra of REL were all similar to its corresponding MWL, which indicated the relative high purity of the isolated residual enzyme lignin.

S3.6. Analysis of polysaccharides

The assignment of the 2D HSQC NMR data of lignin by overlapping with model compounds was intensively studied.\textsuperscript{53,84} However, limited information about assignment of polysaccharides was reported, which increased the difficulty of structure interpretation. Almost all the peaks of saccharides appeared in the range of 2.5-5.5 ppm (\textsuperscript{1}H) and 50-110 ppm (\textsuperscript{13}C) in the spectra (Fig. 3.2), which showed the backbone and branch structure signals of polysaccharides. Acetylation of substituents of hemicellulose was widely spread in the lignocellulosic biomass, the signals could be found in all spectra, for example, peaks at 99.5/4.6 ppm (C1/H1) and 73.3/4.7 ppm (C2/H2) were attributed to the 2-O-Ac-β-D-Xylp, and a 3-O-Ac-β-D-Xylp showed correlation at 101.7/4.5 ppm (C1/H1) and 74.9/5.0 ppm (C3/H3). The acetyl group is an ideal peak for evaluating carbohydrate removal as the strong signal was easily distinguished in 2D HSQC NMR and FT-IR spectra (Fig. S3.2). The signals of reducing-end group and non-reducing end group of cellulose and xylan could be recognized in the spectra, although some of these peaks overlapped with those of internal units of the polysaccharides. 4-O-methyl-α-D-glucuronic acid (4-O-MeGlcA) was also a ubiquitous component in the four samples, which showed typical signal around 97.5/5.4 ppm (C1/H1).
Catalytic hydrogenolysis of lignin: the influence of minor units and saccharides

The peaks of mannan (Manp) only appeared in the spectra of WCW_Pine, although O-acetylated glucomannan had also been found in aspen, poplar, kenaf bast and corn.\textsuperscript{48,54} These structures could be used for the study of structure changes of lignin and removal of saccharide.

S3.7. Molecular weight analysis

The molecular weight distribution is a lignin property that cannot be easily distinguished by 2D HSQC NMR. Nevertheless, it is an important property to explain the discrepancies observed in the 2D HSQC NMR spectra between the different types of samples. The determination of molecular weight of samples was conducted by directly dissolving lignins in DMF without further derivatization, and MWL could be entirely solvated, whereas, REL only partially dissolved, therefore, their data only represent the soluble faction. The molecular weight distribution of REL and MWL were shown in Fig. S3.7 and listed in Table S4. The order of weight molecular of MWL followed by birch > pine > walnut shell > reed, and the H units and its analogous units also followed a similar trend, which indicated that H related units of raw material seemed to be a key factor to influence the molecular weight of MWL. It has been discussed that lignin with higher content of H units have short chain and a better solubility in some organic solvents.\textsuperscript{85} From the curves, the MWL had a lower polydispersity in contrast to REL, which may be due to that only a certain range of molecular lignin is extracted and precipitated. In addition, the weight molecular weight of REL were much higher than that of MWL even without full dissolution. This confirms that the MWL was small oligomers and released from pendent part of lignin or depolymerized part of lignin during ball milling process, and the milder neutral dioxane/water has limited capability to extract high molecular range lignin.
Fig. S3.1. Recovery yield and compositional analysis of WCW, REL, MWL from the four selected biomasses. MWL, REL and WCW represent milled wood lignin, residue enzyme lignin obtained from two treatment rounds of a sequential milling and enzymatic treatment. Others was calculated by subtracting the content of cellulose and hemicellulose; AIL, acid insoluble lignin; ASL, acid soluble lignin; PL, lignin after removing polysaccharide impurities.

Fig. S3.2. FT-IR spectra of residual enzyme lignin (REL), milled wood lignin (MWL) and whole cell wall (WCW). birch, (a), pine, (b), reed, (c), walnut, (d).
Fig. S3.3. TGA/DTG of residual enzyme lignin (REL), milled wood lignin (MWL) and whole cell wall (WCW). (a) Birch_TGA, (a1) Birch_DTG, (b) Pine_TGA, (b1) Pine_DTG, (c) Reed_TGA, (c1) Reed_DTG, (d) Walnut_TGA, (d), and Walnut_DTG, (d1).
Fig. S3.4. 2D HSQC NMR spectra of whole cell wall of walnut shell obtained from milling 6 h (a), 12 h (b) and 24 h (c). The common polysaccharide and lignin label system is used. The structure was color-coded and showed in the Fig. 3.2 Milling condition: 500 mL ZrO$_2$ jar, balls (5.15 mm, 10, 5 mm), 450 rpm rotation, 10 min milling and 15 min pausing, 30 g walnut shell. 5 g sample was taken once the effective milling time reaching 6 h and 12 h.

Fig. S3.5. SEM micrographs of walnut shell powder obtained from milling 6 h, 12 h, 24 h. Milling condition: 500 mL ZrO$_2$ jar, balls (5.15 mm, 10, 5 mm), 450 rpm rotation, 10 min milling and 15 min pausing, 30 g walnut shell. 5 g sample was taken once the effective milling time reaching 6 h and 12 h.
Fig. S3.6. XRD spectra of walnut shell powder and walnut powder after milling 6, 12, 24h (Walnut_Raw, the raw material without ball milling, Walnut_6h, Walnut_12h, Walnut_24h, the walnut powder obtained from ball milling 6 h, 12 h, 24 h). The relative degree of crystallinity were calculated from the intensity ratio of $[I(22^\circ)-IAM]$ and $I(22^\circ)$, in which IAM is the minimum intensity between $22^\circ$ and $16.4^\circ$.

Fig. S3.7. Molecular weight distribution of residual enzyme lignin (REL) and milled wood lignin (MWL), and data was determined by GPC with a polymethylmethacrylate as internal standard and DMF as mobile phase. birch, (a), pine, (b), reed, (c), walnut, (d).
Fig. S3.8. GPC chromatograms and molecular weight for the oil obtained with Ru/C.

Fig. S3.9. Products yield and distribution from model compounds. (a), Reductive treatment of FAA and FAME under Ru/C and Ru/Al₂O₃ (5%) alumina; reaction condition: 20 mg model compound, 10 mg Ru/C or Ru/Al₂O₃, 250 °C, 3h, 60 bar at room temperature, 4 mL methanol; (b), Blank control study of H related products under reductive hydrogenolysis, over hydrogenation products was observed; reaction conditions: 20 mg start material, 10 mg Ru/C (5%), 250 °C, 3h, 60 bar H₂ at room temperature, 4 mL methanol; (c), Reductive treatment of hemicellulose; reaction condition: 50 mg hemicellulose, 25 mg Ru/C, 250 °C, 3 h, H₂ 60 bar at room temperature. Model compounds includes ferulic acid (FAA), methyl (E)-3-(4-hydroxy-3-methoxyphenyl)acrylate (FAME). The products obtained from hydrogenation and methyl esterification of FAA was methyl 3-(4-Hydroxy-3-methoxyphenyl)propionate) (FMA). OHP is the over hydrogenation of PMA.
Hemicellulose extracted from mixed grass

Fig. S3.10. 2D HSQC NMR spectra of hemicellulose from extracted grass. pCA, p-coumarate, FA, ferulate.

Fig. S3.11. GC-FID chromatograms obtained from hydrogenation of hemicellulose extracted from the mixed grasses.
Chapter 3

Fig. S3.12. GC_MASS spectra of the product oil of REL and MWL from birch after derivatization. See Fig S3.16 below for structure of dimers.

Fig. S3.13. GC_MASS spectra of the product oil of REL and MWL from pine after derivatization. See Fig S3.16 below for structure of dimers.
Fig. S3.14. GC_MASS spectra of the product oil of REL and MWL from reed after derivatization. See Fig S3.16 below for structure of dimers.

Fig. S3.15. GC_MASS spectra of the product oil of REL and MWL from walnut shell after derivatization. See Fig S3.16 below for structure of dimers.
Fig. S3.16. Dimers identified from the analysis of the mass spectra (see the raw spectra in our published paper) of lignin oil from hydrogenolysis.
**Supplementary Tables**

Table S3.1. Carbohydrate analysis whole cell wall (WCW), residual enzyme lignin (DREL) and milled wood lignin (MWL) from four biomasses.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cellulose</th>
<th>Hemicellulose</th>
<th>Acid soluble lignin</th>
<th>Acid insoluble lignin</th>
<th>Ash</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birch_WCW</td>
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<td>24.4</td>
<td>6.5</td>
<td>18.4</td>
<td>0.5</td>
<td>Nd</td>
</tr>
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<td>Birch_REL</td>
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<td>4.4</td>
<td>Nd</td>
<td>Nd</td>
<td>Nd</td>
<td>22.0</td>
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<tr>
<td>Birch_MWL</td>
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<td>Nd</td>
<td>Nd</td>
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</tr>
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<td>Pine_WCW</td>
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<td>25.8</td>
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<tr>
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<td>Nd</td>
<td>Nd</td>
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<tr>
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<td>1.4</td>
<td>20.1</td>
<td>2.5</td>
<td>Nd</td>
</tr>
<tr>
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<td>Nd</td>
<td>Nd</td>
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<tr>
<td>Reed_MWL</td>
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<td>Nd</td>
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<tr>
<td>Walnut_WCW</td>
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<td>5.5</td>
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<td>Nd</td>
</tr>
<tr>
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<td>Nd</td>
<td>Nd</td>
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</tr>
<tr>
<td>MWL_Walnut</td>
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<td>Nd</td>
<td>Nd</td>
<td>1.1</td>
</tr>
</tbody>
</table>

a Data was duplicated collected and the error less than 3%.

b The cellulose was represented by glucan.

c Hemicellulose was represented by the total amount of xylan and arabinan.

d Acid soluble lignin was calculated by absorbance of UV light at recommended wavelength.

e Acid insoluble lignin was calculated by the moss difference after acidic hydrolysis.

f Ash content was calculated by the moss difference after calcination.

g Yield was the calculated by dividing the weight of the products to the weight of the starting material.

Nd, not determination.
Table S3.2. Monomer constituents obtained from hydrogenolysis of WCW, DREL, MWL, and material loading, actual lignin, catalyst loading as well as proportion of different units and ratio of syringyl units with guaiacyl units.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$^{a}H_3$</th>
<th>$^{a}H_2$</th>
<th>$^{a}H_1$</th>
<th>$^{a}G_3$</th>
<th>$^{a}G_2$</th>
<th>$^{a}G_1$</th>
<th>$^{a}S_3$</th>
<th>$^{a}S_2$</th>
<th>$^{a}S_1$</th>
<th>$^{b}$total (%)</th>
<th>PMA</th>
<th>FMA</th>
<th>$^{c}$SMT (mg)</th>
<th>$^{d}$LL (mg)</th>
<th>Catylst (mg)</th>
<th>S/G</th>
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<td>0.12</td>
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<td>6.04</td>
<td>1.74</td>
<td>0.38</td>
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<td>1.46</td>
<td>10.59</td>
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<td>0.47</td>
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<td>250</td>
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<td>25</td>
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<td>1.08</td>
<td>0.07</td>
<td>8.03</td>
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<td>8.71</td>
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<td>0.11</td>
<td>20</td>
<td>19.56</td>
<td>10</td>
<td>2.91</td>
</tr>
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<td>0.00</td>
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<td>0.00</td>
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<td>0.07</td>
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<td>0.64</td>
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<td>8.02</td>
<td>2.38</td>
<td>3.56</td>
<td>26.20</td>
<td>6.18</td>
<td>3.59</td>
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<td>43.82</td>
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<td>0.45</td>
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<td>0.61</td>
<td>0.45</td>
<td>5.55</td>
<td>5.30</td>
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<td>24.14</td>
<td>6.23</td>
<td>0.66</td>
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<td>19.89</td>
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<td>1.17</td>
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<td>10.05</td>
<td>1.87</td>
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<td>26.67</td>
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<td>0.02</td>
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<td>0.25</td>
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<td>0.82</td>
<td>0.68</td>
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<td>3.24</td>
<td>8.52</td>
<td>26.26</td>
<td>0.21</td>
<td>0.03</td>
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<td>42.50</td>
<td>25</td>
<td>2.16</td>
</tr>
<tr>
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<td>0.03</td>
<td>20</td>
<td>19.79</td>
<td>10</td>
<td>1.19</td>
</tr>
</tbody>
</table>

$^a$ H$_3$, ethylphenol, H$_2$, propylphenol, H$_1$, propanolphenol, G$_3$, ethylguaiacol, G$_2$, propylguaiacol, G$_1$, propannolguaiacol, S$_3$, ethylsyringol, S$_2$, propylsyringol, S$_1$, propannolsyringol, PMA, methyl 3-(4-hydroxyphenyl)propionate, FMA, methyl 3-(4-hydroxy-3-methoxyphenyl)propionate; $^b$ the total monomer yield; $^c$ The loading of starting material, SMT, starting material; $^d$ the real load of lignin from Klason lignin or after sugar correction.
Table S3.3. The linkage type origin and the percentage distribution of individual assigned dimers found in the product oil from hydrogenolysis of REL and MWL from different biomasses.

<table>
<thead>
<tr>
<th>Peaks&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Linkage&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Type&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Birch REL&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Birch MWL&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Pine REL&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Pine MWL&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Reed REL&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Reed MWL&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Walnut shell REL&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Walnut shell MWL&lt;sup&gt;d&lt;/sup&gt;</th>
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<tbody>
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<td>a</td>
<td>β-β</td>
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<td>1,9</td>
<td>2,1</td>
<td>7,1</td>
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<td>0,7</td>
<td>5,9</td>
</tr>
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<td>G-G</td>
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<td>7,6</td>
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<td>G-G</td>
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<td>2,1</td>
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<tr>
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<td>S-G</td>
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<td>G-G</td>
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<td>G-G</td>
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<td>S-S</td>
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<td>3,0</td>
<td>2,4</td>
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<tr>
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<td>β-5</td>
<td>G-S</td>
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<td>2,7</td>
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<td>1,9</td>
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<tr>
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<td>8,7</td>
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<td>G-G</td>
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<td>21,7</td>
<td>7,7</td>
<td>4,8</td>
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<td>14,1</td>
<td>5,9</td>
<td>9,3</td>
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<tr>
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<td>S-S</td>
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<td>5,0</td>
<td>0,0</td>
<td>0,0</td>
<td>6,4</td>
<td>17,0</td>
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<td>G-S</td>
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<td>10,3</td>
<td>11,1</td>
<td>12,5</td>
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<tr>
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<td>G-S</td>
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<td>7,6</td>
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<td>1,8</td>
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<td>3,5</td>
<td>23,8</td>
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</table>

<sup>a</sup> Peaks identified from the GC-MASS spectra, <sup>b</sup> The original linkage type that corresponds to the assigned dimer, <sup>c</sup>, the dimer aromatic units, <sup>d</sup>, oil from residual enzyme lignin (REL), oil from milled wood lignin (MWL), data was the percentage of the peak area in the total area of the identified peaks.
Table S3.4. The relative distribution linkages based on the assigned dimers found in the product oil from hydrogenolysis of REL and MWL from different biomasses.

<table>
<thead>
<tr>
<th>Totala</th>
<th>RELb</th>
<th>MWLb</th>
<th>REL</th>
<th>MWL</th>
<th>REL</th>
<th>MWL</th>
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<td>11,9</td>
<td>5,0</td>
<td>7,6</td>
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<tr>
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<td>16,2</td>
<td>9,3</td>
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</table>

a β-β= a+7+17+23+24, 5-5=1+16, β-1=3+4+5+9, 4-O-5=2, β-5=6+11+12+13+14+18+22, oil from residual enzyme lignin (REL), oil from milled wood lignin (MWL), data was calculated from Table S3.6.

Table S3.5. Molecular distribution of milled wood lignin (MWL), double round residue enzyme lignin (DREL).

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<th>Samples</th>
<th>Mw</th>
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<tr>
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<tr>
<td>MWL_Walnut</td>
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</tr>
<tr>
<td>WL_Reed</td>
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<tr>
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<tr>
<td>REL_Pine</td>
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<tr>
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<tr>
<td>REL_Reed</td>
<td>19700</td>
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</tbody>
</table>

a Data was determined by GPC with a polymethylmethacrylate as calibration standard and DMF as mobile phase.
References

Chapter 3


