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Published in:
Biotechnology Advances

DOI:
10.1016/j.biotechadv.2023.108230

Publication date:
2023

Biotechnology Advances 68 (2023) 108230

Contents lists available at ScienceDirect

Biotechnology Advances

journal homepage: www.elsevier.com/locate/biotechadv

Research review paper

The isolation of lignin with native-like structure

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ARTICLE INFO

Keywords:
Lignocellulosic biomass
Lignin
Native-like structure
Isolation
Fractionation
Extraction
Characterization

ABSTRACT

Searching for renewable alternatives for fossil carbon resources to produce chemicals, fuels and materials is essential for the development of a sustainable society. Lignin, a major component of lignocellulosic biomass, is an abundant renewable source of aromatics and is currently underutilized as it is often burned as an undesired side stream in the production of paper and bioethanol. This lignin harbors great potential as source of high value aromatic chemicals and materials. Biorefinery schemes focused on lignin are currently under development with aim of acquiring added value from lignin. However, the performance of these novel lignin-focused biorefineries is closely linked with the quality of extracted lignin in terms of the level of degradation and modification. Thus, the reactivity including the degradation pathways of the native lignin contained in the plant material needs to be understood in detail to potentially achieve higher value from lignin. Undegraded native-like lignin with an as close as possible structure to native lignin contained in the lignocellulosic plant material serves as a promising model lignin to support detailed studies on the structure and reactivity of native lignin, yielding key understanding for the development of lignin-focused biorefineries. The aim of this review is to highlight the different methods to attain "native-like" lignins that can be valuable for such studies. This is done by giving a basic introduction on what is known about the native lignin structure and the techniques and methods used to analyze it followed by an overview of the fractionation and isolation methods to isolate native-like lignin. Finally, a perspective on the isolation and use of native-like lignin is provided, showing the great potential that this type of lignin brings for understanding the effect of different biomass treatments on the native lignin structure.

1. Introduction

As the most abundant natural aromatic polymer, lignin possesses enormous potential as a renewable and sustainable starting material for production of valuable aromatic chemicals and materials (Collinson and Thielemans, 2010; Zakzeski et al., 2010). However, most lignin streams produced from industry are by-products from polysaccharide-orienting processes, and normally burned to provide energy or recover chemical reagents. Many approaches towards reaching added value from lignin have been undertaken. Examples are the selective chemical modification of lignin for use in functional materials, the (*in-situ*) selective catalytic depolymerization of lignin for the production of aromatic chemicals and bioengineering to attain lignin that can be easier to process by genetic modification (Rinaldi et al., 2016; Upton and Kasko, 2016; Zakzeski et al., 2010). However, the intrinsic heterogenous structure and various units bring many challenges for the utilization of lignin. One of the greatest challenges is the detailed structural elucidation of the total structure of native lignin which is important for understanding its reactivity. This is fundamental for all the ways of lignin conversion described above, and restricted by analytics of lignin as part of the lignocellulosic matrix. Although sophisticated synthetic model substrates have been developed and have been used to provide detailed insight on lignin reactivity they also are limited in the full structural resemblance to the native lignin structure (Deuss and Barta, 2016; Lahive et al., 2020). For example, these will not cover the full extent of the presence of lesser lignin linking motifs, rare monomeric units and size distributions present in lignocellulosic lignin. This leads to significant differences in structure and properties causing for example very different solubility profiles. To get around this problem, researchers have been trying to isolate lignin from the lignocellulosic matrix without chemical changes. However, each method for the isolation of so-called "native-like lignin" has its perks and flaws. Common limitations are that certain structural units are more easily released, and the loss of linking motifs that are easily cleaved. This means that also here the

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https://doi.org/10.1016/j.biotechadv.2023.108230
Received 25 April 2023; Received in revised form 3 August 2023; Accepted 4 August 2023
Available online 7 August 2023

obtained lignin is not necessarily fully representative of the total lignocellulosic lignin structure. Moreover, the closer the lignin to native-state the more challenging it is to process it in general solvent systems, which significantly hinders their structural elucidation and limits their application in lignin reactivity studies. Developing better methods by which lignin is isolated and handled in a state as close as its native structure can thus be of great significance. With enough purity, yield and representativeness of high-quality native-like lignin, more information, conversion pathways and relevant chemistry about the actual native lignin structure can be extracted and allowing their application for studying conversion strategies both new or well-developed. This will help find novel or unknow lignin constitutional units, structures and chemical reactivity as well as understanding on the interactions with other lignocellulose components. Using state of the art analytical techniques these native-like lignins can help to provide more reliable correlation between lignin chemistry that applies to lignocellulosic biomass processing, which can aid increasing the efficacy of the utilization of lignin. For this purpose, this review aims to provide an overview of current methods to isolate native-like lignin for structural elucidation and reactivity studies, discussing their advantages and disadvantages in terms of their methodologies and the structure and properties of the native-like lignin products. This is supported by a concise introduction on what is known about native lignin structure as well as key analytical techniques employed in its structural characterization. A detailed review is provided with special focus on the content in relation to native-like lignin by following a general categorization of methods including organic fractionation, isolation by upcoming solvent systems, the use of flow-through reactors, and via enzyme treatment. Native-like lignin obtained from these methods have different physiochemical features, which endow diverse challenges for their application. Therefore, some recommendations are provided based on our own experience to help researchers select suitable methods of native-like lignin isolation for their studies.

1.1. An introduction to the diversity of constituents and linkages found in native lignin

Lignin biosynthesis mainly starts from phenylalanine and tyrosine derived from the pentose pathway followed by the shikimate pathway (Fig. 1a), and these amino acids are transferred to cytosol to be further hydroxylated and methylated by series enzymes (Boerjan et al., 2003; Mottiar et al., 2016). In this process, the building blocks such as the three precursors, *viz. p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol are used for producing the three main units of lignin (Fig. 1b), *viz. p*-hydroxyphenyl units (H), guaiacyl units (G) and syringyl units (S), respectively. Additionally, other potential building blocks are synthesized in this process such as monolignol conjugates, incompletely methylated monolignols, hydroxycinnamaldehyde and hydroxycinnamate esters (Vanholme et al., 2019). Phenolic monomers beyond canonical phenylpropanoid are also described to serve as lignin monomers and can be incorporated into the lignin structure (Del Río et al., 2012). These structures include flavonoids, hydroxystibenes and hydroxycinnamic amides. After their biosynthesis, these compounds are delivered to the cell wall and oxidized by laccases and peroxidases to produce reactive radical intermediates that undergo polymerization to form native lignin (Brunow et al., 1998; Freudenberg and Neish, 1968; Harkin, 1967). The reactive radicals can be transferred to various positions on the monolignols via kinetically controlled radical coupling creating different types of bonding motifs (Fig. 1b and c) (Gani et al., 2019; Ralph et al., 1999b). Thus, the result is that lignin contains a variety of linkages in different quantities. For instance, the most abundant aryl ether linkage (β-O-4) and two kinds of carbon-carbon linkage such as linkages represented in the resinol (β-β) and phenylcomaran (β-5) motifs are often found in lignin. But some other rarer linkages can also be detected in lignin, such as the biphenyl (5–5), the biphenyl ether (4-O-5) and the spirodienone (β-1).

1.2. Factors influencing the structure of lignin

The diverse units and combinatorial coupling of radicals imbue lignin with highly variable chemical structure and physicochemical properties. Moreover, the quantity, units composition, and structural features of lignin are quite diverse and vary across plant species, growing environment and stages, cell types and wall layers (Campbell and Sederoff, 1996; Vanholme et al., 2019). For instance, the lack of ferulate 5-hydroxylase (F5H) in the biosynthesis gives gymnosperm (softwood) lignin primarily G units with abundant stable carbon-carbon bonds such as the β-5, 5–5 and β-β linkages and relatively lower abundance of the labile cleavable β-O-4 linkage (Fig. 1b, middle). This increases the recalcitrance of softwood lignin towards chemical degradation (Humphreys et al., 1999; Osakabe et al., 1999). Typical lignin from dicotyledonous angiosperm (hardwood) contains a high proportion of both S and G units and is richer in β-O-4 linkages (Fig. 1b, left). Lignin from monocots (grass) has the most complicated unit constituents. In grass lignin S and G units are still the main units, but these lignin structures also contain relatively higher proportion of H units. In addition, hydroxycinnamate (*p*CA) units are present in grass lignins, which serves as a pedant part of the polymer or as the anchor points for lignin to accumulate in the cell wall via ester bonds (Fig. 1c, right). Ferulate (or ferulic acid) is also found in larger amounts in grass lignins and is suggested to serve as the linking motif to connect the main lignin backbone with polysaccharides (Giummarella et al., 2019). Apart from these regular patterns, the unit composition can be highly variable in some particular biomass materials. For example, normally the H unit level in the natural plant material is hardly higher than 5%, but a quite high H unit was found in the some nutshell materials such as walnut shell (Kim et al., 2017; Ralph et al., 2019). Recently, a novel lignin made up from caffeyl alcohol by β-O-4 coupling was isolated from the seed coats of vanilla (*Vanilla planifolia*) and characterized. This so called C-lignin provides great potential to produce catechol monomers upon depolymerization (Chen et al., 2012, 2013; Li et al., 2018; Wang et al., 2021a). Lignin content and structure are significantly influenced by the plant's geographical distribution. This has been widely investigated for understanding the influence of lignin content and structure on the fermentable sugar release under enzymatic treatment and monomer release from depolymerization as well as from genetic mapping (Anderson et al., 2019; Muchero et al., 2015; Studer et al., 2011). The distribution and structure of lignin in the different cell layers and types also vary. In general, the middle lamella and cell corners have a higher density of lignin with more H units (Fengel and Wegener, 2011; Önnerud, 2003). Moreover, the comparison of lignin obtained from different tissues of the same plant clearly reveals the difference in terms of amount, unit ratio and composition (Ralph et al., 1994; Wen et al., 2013b).

2. Key characterization techniques and methods for studying the lignin structure

The development of new analytical methods is of significance for exploring the variability of the lignin structure and its influence on reactivity. In particular, finding methods that provide reliable, accurate and reproducible data on the linkage and unit composition is essential. The selection of methods that can be applied is influenced by the lignin properties, which is for example totally different for isolated lignin compared to lignin as part of the lignocellulosic biomass. This is because the other components of biomass interact with lignin via complicated physiochemical interactions and sometimes covalent bonds. Furthermore, any way to individually separate the biomass components tends to more or less change the lignin structure providing completely different solubilities and physical appearances. There are many sample treatment methods and analytical instruments available for the analysis of lignin composition and structure, but no single method can reveal all the structural and compositional features of lignin samples. Thus, the

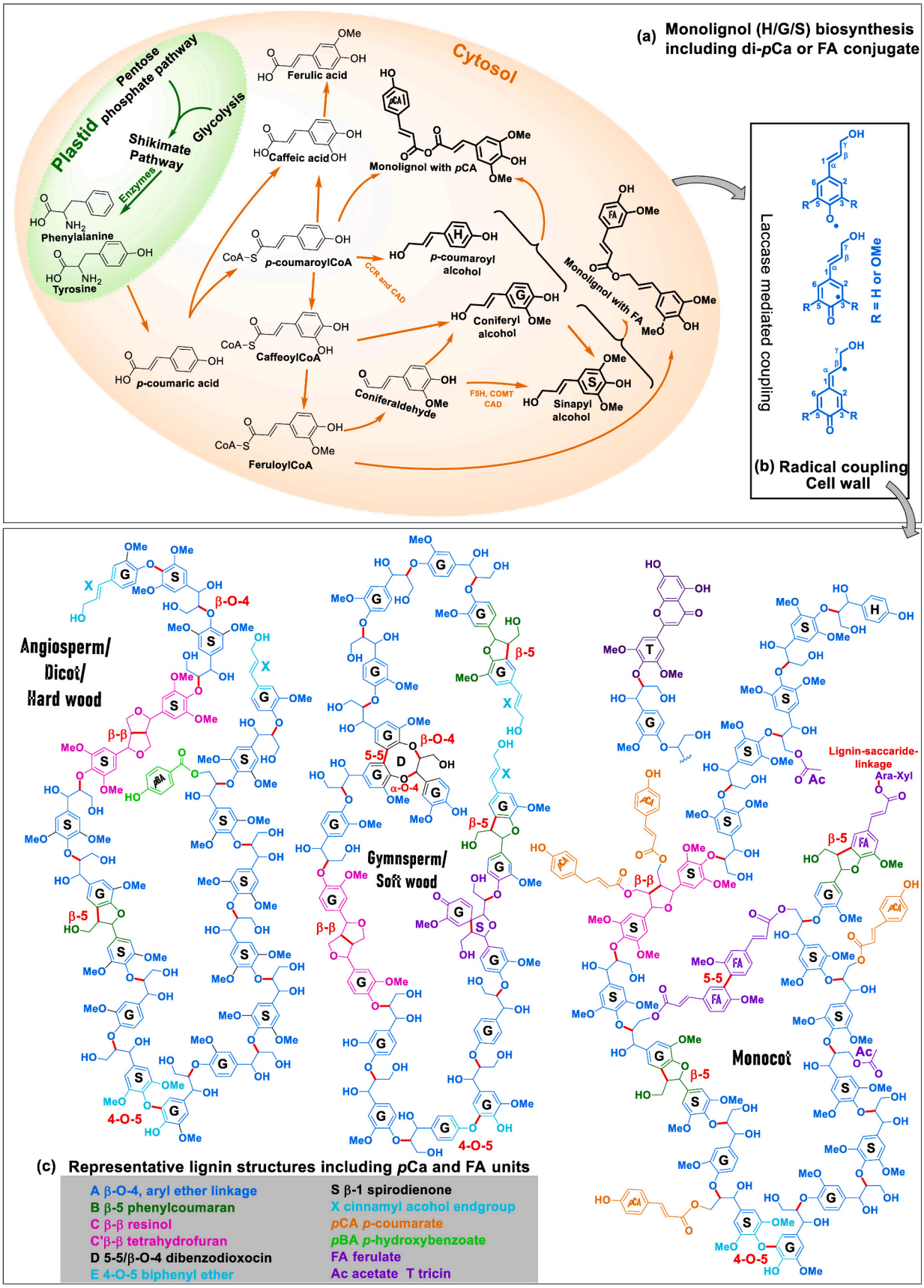


Fig. 1. Overview of the biosynthesis of representative monolignols and model structures of lignin from different biomasses. (a) simplified monolignol biosynthesis pathways; based on literature descriptions (Mottiar et al., 2016) (b) radical species formed upon enzymatic oxidation of the monolignols; based on literature description (Mottiar et al., 2016) (c) representative structures of lignin based on representative examples in literature (Ralph et al., 2019).

combination of different methods is an integral part of studying the lignin structure. Therefore, knowledge of the most important methods and technologies is a prerequisite for researchers working on lignin valorization. In general, for analytical methods, two main categories can be distinguished: degradative and non-degradative methods (Fig. 2).

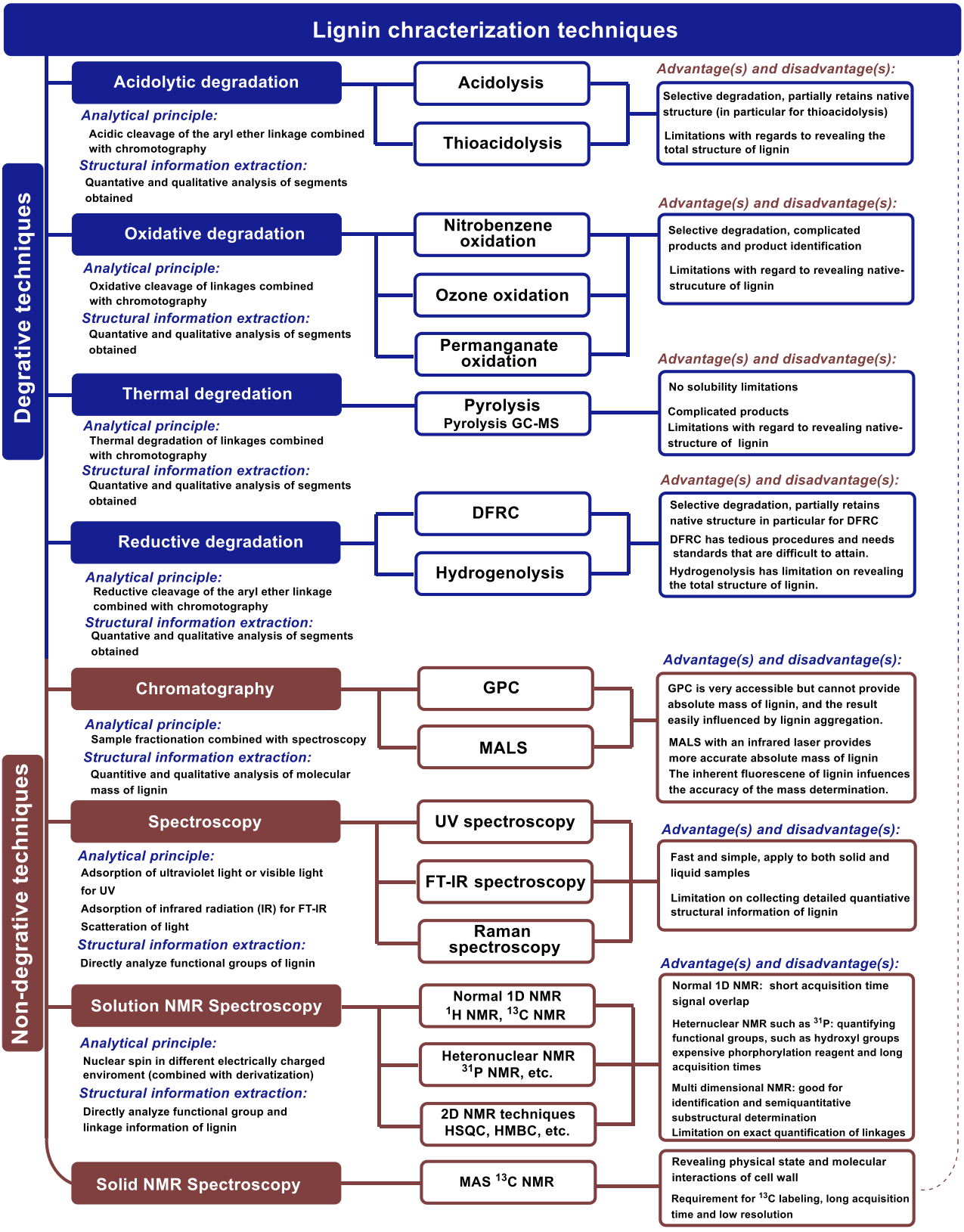


Fig. 2. Overview of a selection of important techniques used for the structural characterization of lignin.

2.1. Structural information extracted from degradative methods

Labile aryl ether linkages provide the possibility to, after cleavage, selectively release lignin fragments that are suitable for general quantification of the main linkage composition and the identification of rare and novel lignin units and linkages. Degradative methods reveal this information by extrapolating the identification and quantification of degradation products released from chemical and/or thermal treatments. These treatments include acidolysis methods (acidolysis, thioacidolysis), high temperature base, oxidation (nitrobenzene oxidation, ozone oxidation, permanganate oxidation, etc.), pyrolysis (pyrolysis GC-MS), and hydrogenolysis, which all provide indirect information about the structure and composition (Grabber et al., 2000; Lin and Dence, 2012; Lu et al., 2015; Lu and Ralph, 2014, 1999; Ralph and Hatfield, 1991; Ralph and Lu, 1998; Wilkerson et al., 2014). After treatment, degradation products are typically identified and quantified using GC-MS with authentic standards, but also more advanced techniques like GC×GC-FID and GC×GC/TOF-MS can be used (Figueiredo et al., 2020; Salvachúa et al., 2015; Yang et al., 2023a, 2023b). Among these methods, lignin thioacidolysis and hydrogenolysis are the most commonly used methods, since in these treatment processes can selectively cleave alkyl aryl ether linkages to release degradation products retaining part of the native structure.

Both as degradative analytic methods for lignin, acidolysis and thioacidolysis provide information for lignin via acidic cleavage of the alkyl aryl ether linkages. Information such as the quantity of uncondensed alkyl aryl ether structures is extracted by analyzing segments released from this process (Adler et al., 1957; Rolando et al., 1992). Compared with acidolysis, thioacidolysis produces less complicated degradation products (Lapierre et al., 1985). Therefore, for example, only caffeyl alcohol was observed from the thioacidolysis of C-lignin, indicating that this lignin was entirely composed by this unit (Chen et al., 2012). In addition, through the usage of marker compounds, rare units and structure of lignin can be effectively tracked. For example, this has been used in the identification and quantification of the constitution of hydroxylcinnamate or hydroxycinnamyl aldehydes in lignin (Fournand et al., 2003; Kim et al., 2002; Ralph et al., 2008). Detailed study of the dimeric products provides insights of various interunit-linkage distribution of lignin such as more precise quantification of carbon-carbon linkages with the help of authentic dimeric model compounds (Yue et al., 2017).

Lignin hydrogenolysis is another method used and under developing for lignin structure characterization. Derivatization followed by reductive cleavage (DFRC) reported by Lu and Ralph is robust and widely used for studying the structure of lignin (Lu and Ralph, 1997). In contrast to thioacidolysis, DFRC has some unique advantages. It can not only break the ether linkage but also preserve some other linkages in the form of released dimers, significantly enhancing the ability to study the lignin structure but also pathways in the lignin biosynthesis with the help of marker compounds (Lu et al., 2015; Lu and Ralph, 2014, 1999; Ralph and Lu, 1998; Wilkerson et al., 2014). For instance, the occurrence of natural *p*-hydroxybenzoylated lignin was confirmed by the acetylated coniferyl and sinapyl *p*-hydroxybenzoate products released from DFRC treatment (Lu et al., 2015). The exact percentage of H units in the natural lignin is wildly debated. Since, quantification of H units via the current normal 2D HSQC NMR (See next section), is often exaggerated by slower relaxation of terminal end units including H units, *p*-hydroxbenoates (*p*B), *p*-coumarates (*p*CA) etc (Mansfield et al., 2012). Moreover, *pB* and *pCA* are analogous H units, and can influence the direct quantification of H units due to signal overlap. A more precise quantification of H units was achieved via DFRC after elimination of undesirable protein residues. This revealed that H units solely derived from *p*-coumaryl alcohol comprise rarely higher than 5% of the monomer composition (Kim et al., 2017). However, this method also involves tedious steps and incomplete alkyl aryl ether linkage cleavage. Recently, direct catalytic hydrogenolysis of lignin as component of lignocellulosic biomass samples under reductive conditions is proved to be advantageous for lignin structure analysis. In particular, the quantification of alkyl aryl ether linkages and rare units were shown to be readily extractable from GC-MS analysis of the product mixture. For instance, direct hydrogenolysis of lignin in biomass almost quantitatively cleaves the alkyl aryl ether linkages and results in near theoretical amounts of monomers (Parsell et al., 2015; Pepper and Lee, 1969; Shuai et al., 2016; Van Den Bosch et al., 2015; Yan et al., 2008). This means that by accurately quantifying the total degradation products based on a reliable fragment data library precise quantification of the alkyl aryl ether linkage content of lignin as part of the lignocellulosic matrix is possible. Also, information about special or rare lignin monomer and linking units can be extracted with this method. For instance, our work on lignin hydrogenolysis supported by isolated native-like lignins built a detailed correlation between monomeric product distributions and several rare units of the lignin, confirming that some other units analogous with *p*-hydroxyphenyl units (H units) also contributed to the monomeric products derived from H units (Wang and Deuss, 2021).

2.2. Structural information extracted from non-degradative methods

Non-degradative analytical methods can directly reveal information about the structure of lignin without the need to decompose it. Spectroscopy has been wildly employed in lignin structural studies. For instance, UV, FT-IR and Raman spectra are commonly used for collecting information of functional groups. Using anti-stroke Raman scattering and simulated Raman scattering, the distribution of polysaccharide and lignin can be collected. This is done by semi-quantification of the typical Raman absorption wavelength of these constituents, which is an effective method to track the lignin extrusion or degradation under different physicochemical treatments (Boeriu et al., 2004; Friese and Banerjee, 1992; Gärtner et al., 1999; Saar et al., 2010; Saka et al., 1982; Zeng et al., 2010).

Once lignin is isolated, pretreated, degraded or chemically modified, molecular weight and distribution are often key parameters to be tracked. For this purpose, gel permeation chromatography (GPC) is most widely applied. GPC uses the physical porous structure to separate lignin fragments with different structural volume, and then refractive index or UV response of these fractions can be used to calculate the molecular weight distribution based on synthetic standards (typically polystyrenes). Nevertheless, aggregation of lignin during such analysis can significantly influence the precise determination of the actual molecular weight of lignin, and therefore widely varying numbers are reported due to different solvent systems and setups (Crestini et al., 2011). The analysis of the absolute molar mass of lignin is being explored with the development of state-of-the-art techniques. For instance, matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) or electrospray ionization mass spectrometry (ESI-MS) are employed on molar mass determination of lignin (Jacobs and Dahlman, 2000; Mattinen et al., 2008; Richel et al., 2012; Rönnols et al., 2017). However, it is only applicable to lignin with small and narrow fragments (Zinovyev et al., 2018). Multi-angle scattering (MALS) combined with an infrared (IR) laser to suppress the influence of inherent fluorescence of lignin was found to provide more accurate molar mass determinations of lignin (Zinovyev et al., 2018).

Nuclear magnetic resonance (NMR) is one of the most powerful analytical techniques in term of determining different features of the chemical structure of lignin. NMR can reveal the linkage quantity and qualitative information but also allow the identification of lignin subunits such as dibenzodioxocine, spirodienone and acetylated units that are hard to be analyzed by alternative analytical methods (Karhunen et al., 1995, 1999; Shawn D. Mansfield et al., 2012; Zhang et al., 2006). Therefore, NMR and in particular multi-dimensional NMR techniques are gaining more attention in the structure elucidation of lignin and serves as a routine method to monitor the structural changes during various physicochemical or biological treatments of lignocellulosic

biomass (Lu and Ralph, 2011; Ralph and Landucci, 2010).

2.2.1. NMR and whole cell wall analysis (WCW)

NMR techniques have some unique advantages for the structural elucidation of lignin in terms of revealing for example multiple types and quantities of linkages and direct quantification of the ratio between S and G units (Fig. 3). NMR spectra can be collected in the solid and solution state, and both types have been applied in lignin research. Solid state NMR is especially important for studying lignin substrates that are insoluble in general deuterated organic solvents used in solution state NMR. However, the requirement of ^{13}C labeling, long acquisition time and low resolution hamper the wide utilization of this technique for structural determination of lignin. In general, solid-state NMR is more regularly employed in structural studies of cellulose (Foston, 2014; Hall and Wooten, 1998; Isogai et al., 1989). Recently, the utilization of solid state ^{13}C-multidimensional-NMR also revealed detailed information on the xylan conformation and spatial interaction between polysaccharide and lignin, which provided valuable information for cell wall 3D structure and nanocellulose manufacturing (Kang et al., 2019; Simmons et al., 2016; Terrett et al., 2019).

To date, solution state NMR is still the most accessible form of NMR for studying the structure of lignin (Wen et al., 2013a, 2013b). Even though, typical ^{1}H and ^{13}C NMR can provide some valuable structural information of lignin, signal overlap typically hinders its assignment and quantification by signal integration. Higher dimensional NMR enhanced by pulsed field gradients and inverse detection can overcome these issues (Ralph et al., 1999a; Wen et al., 2013a). Amongst the multiple dimensional NMR, in particular the heteronuclear single-quantum coherence (HSQC), a short ^{1}H-^{13}C correlation experiment, has become a routine practice for the structural characterization of lignin and relative quantification of different chemical motifs (Heitner et al., 2016). Apart from qualitative information, 2D HSQC, quantitative ^{13}C NMR or the combination of both provide rough estimation of units and linkages of lignin using selected aromatic units as internal standard. Normally, a cluster of signals that represent all the phenyl propanoid units (C9 units) is picked up for the quantification (% of linkages discussed in the following section is all based on C9 units) (Capanema et al., 2004; Heikkinen et al., 2003; Martínez et al., 2008; Wen et al., 2013a; Wen et al., 2012). More precise quantification of lignin structure via ^{13}C NMR can be obtained by using internal reference compounds such as 1,3,4-trioxane and pentafluorobenzene (Xia et al., 2001). Gradient-selective $HSQC_0$ provides more accurate quantitative results compared to regular HSQC via reduction of T_1 noise and extrapolation to a zero-relaxation time (Amiri et al., 2019) Heteronuclear NMR such as ^{31}P after derivatization of lignin samples with a phosphorylation agent, provide excellent information related to the presence of specific types and quantities of structural groups present in the parent lignin. (Pu et al., 2011; Wang et al., 2022b). For fundamental study and analysis of lignin structure and transformation, combination of multiple types of NMR is necessary to provide accurate information.

As lignin intertwines and links with polysaccharides in biomass, and any form extraction and isolation alter the integrity of the whole

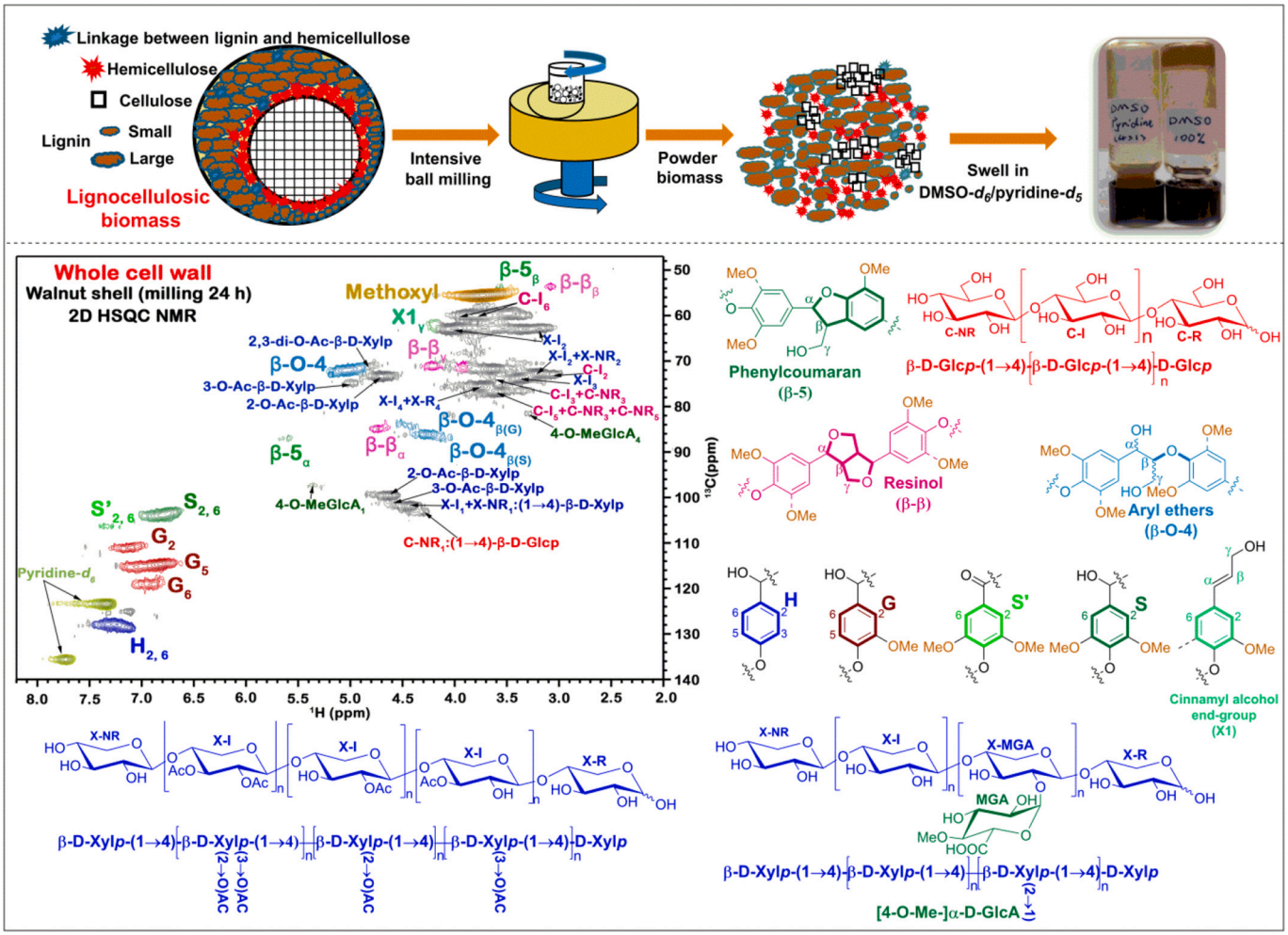
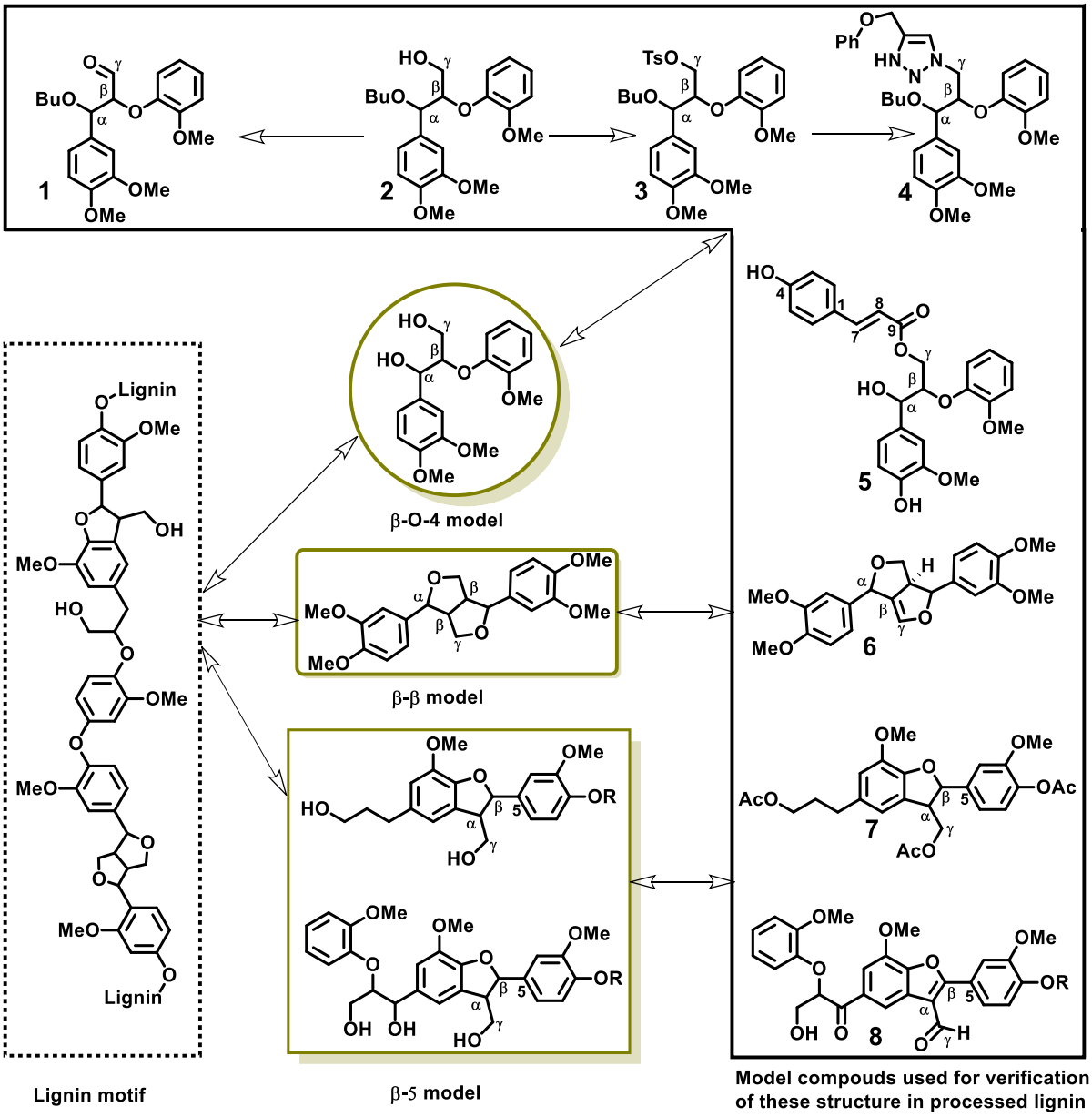


Fig. 3. A schematic presentation for preparation of a gel-state whole cell wall sample and a representative 2D HSQC NMR spectrum with assignment (walnut shell) from Wang and Deuss, 2021, which was obtained by following a published procedure by Kim and Ralph, 2014.

structure, direct analysis of lignin in plant cells has great potential to investigate lignin in its native state (Mansfield et al., 2012). However, the challenge for collecting high resolution NMR spectra is the low solubility and swelling capacity of the whole cell wall material (WCW) in common deuterated solvents. In addition, complete dissolution of WCW without any structural alteration is not an easy task, as typically intensive (mechanical) pretreatment is necessary before dissolution can be achieved. Nevertheless, directly swelling cell wall samples provides the possibility to analyze all the acylated groups in lignin as well as the polysaccharide structures. Moreover, the structural changes to all main components of biomass are more precisely monitored by this method. The low solubility and high viscosity can be improved by specific mixtures of different solvents. Deuterated ionic liquids and DMSO/LiCl can be used for direct dissolution of WCW for NMR analysis (Çetinkol et al., 2010; Hedenström et al., 2009; Jiang et al., 2009; Lu and Ralph, 2011, 2003; Wang et al., 2010, 2009). Alternatives with enhanced swelling and mobility of the WCW samples are conducted by simply swelling ball-milled biomass in DMSO-d_6/pyridine-d_5 or DMSO-d_6/hexamethylphosphoramide-d_{18} (4:1, *v*/v), and they give the possibility to rapidly analyze WCW structure and exhibits detailed information and excellent reproducibility (See Fig. 3) (Kim et al., 2008; Mansfield et al., 2012; Yoo et al., 2016). Some native lignin structural motifs that normally are hard to analyze by alternative methods can be tracked by this way. For instance, gel-state WCW analysis of pine in DMSO-d_6/pyridine-d_5 achieved a better resolution of alkyl aryl ether correlation than an acetylated sample of pine (Lu and Ralph, 2010; Mansfield et al., 2012). Similarly, some cell wall-associated compounds, such as ferulates, *p*-coumarates and hydroxybenzoates can be discerned in this way (Mansfield et al., 2012). Another example is fast quantification of S and G ratio for lignin by NMR, as when this is measured by chemical degradation followed with relative quantification of the fragments via GC or GC-MS the results are influenced by the incomplete degradation and missing quantification of dimers or oligomer products. WCW NMR analysis has been exploited in lignin engineering studies (Marita et al., 2016; Van Acker



Scheme 1. Representative lignin native structure with an array of synthetic lignin model compounds used to study its structure and reactivity (Lahive, 2018; Panovic et al., 2019b, 2017; Ralph et al., 1994; Tran et al., 2015).

et al., 2017; Yelle et al., 2008), lignin degradation and modification (Christopher S Lancefield et al., 2017; Luterbacher et al., 2015). For example, a combination of derivatization by AcBr/AcOH and WCW 2D HSQC NMR allows one to avoid intensive ball milling and can provide excellent structural information which was used to compare structural alterations of components before and after alcohol pretreatment (Lancefield et al., 2017). Also, over or down regulation of specific enzymes can result in obvious alterations of the lignin that are visible by WCW 2D HSQC NMR (Petrik et al., 2014; Smith et al., 2015).

2.3. Lignin structural motif identification by the use of model compounds

Synthetic model compounds are essential in lignin research. Such model compounds are not only of significance for assignment and identification of the unambiguous structure of lignin, but also serve as simplified monomers, dimers and even oligomers with representative lignin linking motifs that can be exploited for studying the reactivity of lignin (Deuss and Barta, 2016; Lahive et al., 2016, 2020; Lancefield and Westwood, 2015; Ralph et al., 2004b). For instance, benzylic alkoxylation of lignin by butanol (**2** in Scheme 1) and its subsequent oxidation of lignin were confirmed by butoxylated β-O-4 and γ oxydized model compounds (**1** in Scheme 1) (Panovic et al., 2019a; Zhang et al., 2020). Additionally, NMR spectra of technical lignin obtained from a sequential modification by butanol alkoxylation, followed by tosylation, azidation, and copper-catalyzed azidealkyne triazole had a clear overlap with the corresponding synthesized model compounds (**3** and **4** in Scheme 1) (Panovic et al., 2017). Studies using model compounds can significantly simplify the identification and analysis of the products and speed up the further development of strategies to address lignin conversion. For instance, the occurrence and incorporation of *p*-coumarate monolignol in maize lignin was clearly revealed by comparison of the 2D HSQC NMR spectra of this lignin and of the corresponding *p*-hydroxycinnanmate ester model compounds (**5** in Scheme 1) (Ralph et al., 1994). Additionally, the chemical reaction induced by DDQ treatment of Kraft lignin was clearly revealed by the model compounds with β-β linkage (**6** in Scheme 1) (Tran et al., 2015). The application of more complex compounds representing the β-5 linkage motif (**7** and **8** in Scheme 1) in lignin was able guide and identify the oxidation of the corresponding units by DDQ in isolated lignin (Lahive et al., 2018). The structural changes that occur during physicochemical treatment of lignin can also be elucidated by model compounds. For example, Hibbert ketone model compounds enabled clear assignment of these products released from acidolysis of lignin and also to track their occurrence in acidolysis fractionation or in acidolysis treatment of isolated lignin systems (Miles-barrett et al., 2016). However, in quite a few circumstances, simplified monomer or dimers cannot represent the complex linkage or large molecular size feature of lignin, which leads to a failure to transfer model compound results to actual lignin. For instance, a large amount of valuable information can be gained from model monomers and dimers for electrochemical depolymerization, there are limitations to their correlation to raw lignin degradation (Garedew et al., 2021). Moreover, synthesized lignin polymers seldom included linkages of α-O-4 and 4-O-5, which has limitation on clarifying structures involving on these linkages, such as whether these linkages serve as crosslinking site of lignin (Lancefield and Westwood, 2015; Ralph et al., 2019; Yue et al., 2016). Therefore, advanced model lignin polymers are developed, which can mimic not only the diversity of the subunits but also the complex linkage (2D environment) and potentially the 3D environment in terms of interchain interactions, which gained substantial attention for applications to study lignin depolymerization (Graham Forsythe et al., 2013; Klinger et al., 2020; Lancefield and Westwood, 2015). Note that the more complex the model compound, the lower the advantage gained from the simplified analytics. Furthermore, model compounds need to be updated based on new insights such as those discussed in the next section (Mottiar et al., 2016; Ralph et al., 2019; Vanholme et al., 2019).

2.4. Novel lignin structural elucidation approaches

The veil that hangs over the whole complex structure of lignin is still being lifted, with insight being continuously updated with the development of the new state of art analysis methodologies. New promoters incorporating in lignin via radical coupling were confirmed not too long ago (Vanholme et al., 2019). For instance, the combination of isolated lignin samples and model compounds used in two dimensional NMR studies indicated that the *p*-coumarate of lignin is synthesized from the monolignol pre-acylated with *p*-coumaric acid (Lu and Ralph, 2005; Ralph and Hatfield, 1991). Early work speculated that diferulates might be subunits of lignin and by now the incorporation of diferulate in 5–5, β-5, β-O-4 and β-β linkages in lignin has been confirmed by an *in situ* study with feruloylated maize walls (Grabber et al., 2002, 2000, 1995; Hartley and Jones, 1976). The release of γ-acetylated syringyl β-β-linked de-hydrodimers from DFRC treatment of kenaf indicates that sinapyl acetate can serve as a lignin initiator for the radical polymerization (Lu and Ralph, 2002).

Below a few more recent developments are highlighted to showcase further new insights that are still being unveiled. For instance, the analysis of the products released from lignin of palm oil empty fruit bunch by DFRC suggests that the natural *p*-hydroxybenzoyl containing lignin structures are biosynthesized via a γ-hydroxybenzoylated monolignol (Lu et al., 2015). Recently, natural enzymes involved in incorporation of monolignols esterified with ferulate groups into the lignin backbone biosynthesis were found in wild type plants, even though the incorporation of the monolignol ferulate (Fig. 1a) into the lignin had previously only been achieved by biosynthesis pathway perturbations and engineering (Karlen et al., 2016; Wilkerson et al., 2014). More evidence shows that the role of some pedant units of lignin such as *p*-coumarate, acetates and *p*-hydroxybenoylates serve as radical transfer intermediates to other monolignols for lignin polymer growth (Hatfield et al., 2008; Lu et al., 2015; Ralph et al., 2004a). Additionally, they have relatively lower activity for radical formation, making them normally present but less abundance in lignin. Whether the structure of lignin contains crosslinking sites is still in dispute, since no solid evidence has been found for branching structures in native-lignin and it is difficult to distinguish between structures that are definitely present in the native lignin or are the result of the sample processing. This is even though, the 5–5, 4-O-5 and β-1 linkage of lignin can potentially serve as the crosslinking sites. Moreover, analysis of terminal groups and inter-unit linkage of lignin gives opposite conclusions on whether lignin is a linear polymer (Balakshin et al., 2020; Crestini et al., 2011). Studies showed that the units such as dibenzodioxocin, biphenyl ether and spirodienone only have phenolic end site without further connection to other lignin units (Karhunen et al., 1995; Ralph et al., 2019; Zhang et al., 2006). Thus, the cross linking state of lignin needs further detailed study. Apart from the structure of lignin, linking complex between lignin and hemicellulose are also under intensive study and structural debate. Covalent bonds such as glycosides, ethers, esters and acetal have been proposed as the connection between lignin and hemicellulose (Giummarella et al., 2019). However, only very limited direct evidence has been obtained (Nishimura et al., 2018). Again, this is hampered by the fact that observed crosslinks can often also be the result of sample treatment. The library of the fundamental structure of lignin is being enriched with the further development of lignin structure elucidation. Other recently discovered structures include tricin derived from a non-monolignol biosynthetic pathway normally presenting in grasses (Del Río et al., 2012; Lan et al., 2016), spirodienone derived from β-1 coupling between a new monolignol and preformed alkyl aryl ether units (Zhang and Gellerstedt, 2001), hydroxy-stilbenes such as piceatannol and resveratrol(Carlos del Río et al., 2017) and benzodioxanes in catechyl lignin (Chen et al., 2013, 2012; Li et al., 2018). These studies exemplify how more and more is being understood of the lignin structure and its formation.

3. Native-like lignin fractionation and isolation

The quality of isolated lignin significantly influences its potential for various applications to study the reactivity of (native) lignin. The benchmark to evaluate the quality of lignin is based on the similarity with native or protype lignin. In concept, native lignin or prototype lignin is defined as the lignin inside the lignocellulosic biomass in its native structure and state. No available method can achieve isolation of lignin completely in its native state, since such a native state has close interaction with the other components in lignocellulosic biomass, and any isolation or extraction protocol will change the lignin structure to some extent. The intact matrix of lignocellulosic biomass has strong recalcitrance towards physicochemical treatment, which significantly restricts the efficient fractionation of native high molecular weight lignin. Nevertheless, there are a range of distinct isolation methods that come close, but these are very different from methods typically used for larger scale lignin isolation. Normally, intensive chemical treatment is necessary in order to completely release lignin from lignocellulosic biomass in the form of smaller soluble fragments. Examples of this are treatments used to obtain kraft, soda and lignosulfonate lignin (technical lignins) in cellulose fibre production. The lignin obtained from these processes has undergone serious structural alteration such as formation of stable C-C linkage, which significantly suppresses their downstream upgrading (Fig. 4). In contrast, native-like lignin has lower phenolic content, is abundant in labile linkages and has more uniform reactive functional groups. Various methods are developed to extract and isolate more native-like lignin. This is achieved by careful selection of pre-treatments or extraction mediums with precise control of the extraction conditions. Examples of methods are the use of alternative solvents, biochemical treatments or the application of dedicated flow-through setups. Below short descriptions of relevant methods for the extraction of native-like lignin are provided using chemical techniques, which in the next section 4 will be extended with methods using enzymatic carbohydrate degradation steps.

3.1. Organic solvent extraction

In contrast to the other biomass components, the aromatic constituents and diverse functional groups make it possible to extract lignin using organic solvents. This has been noticed by pioneering researchers who have been working on lignin from an early stage up to nowadays (Adler et al., 1955; Brauns, 1939; Klason, 1911; Schuerch, 1952; Vermaas et al., 2020). Here are several identified challenges: 1) The interactions between saccharide and lignin significantly hamper the release of lignin during organic solvent extraction; 2) The high molecular weight of proto-lignin in biomass inherently hard to dissolve in most common solvents and thus often extensive fragmentation is required to allow for efficient extraction. Thus, in order to isolate lignin without too much structural alteration, solvents and the extraction conditions need to be carefully considered and optimized. Extraction can be performed by either solvent with high solubility of lignin aided by thermal or autocatalytic fragmentation, or assisted by acids or bases. The challenge of these methods is to find the proper balance between extraction efficiency indicated by percentile lignin removal from the biomass and the structural integrity of the lignin indicated by the retention of β-O-4 units and other labile linking motifs and the extend of formation of condensed structures.

3.1.1. Organic solvent extraction by neutral organic solvent

Organic extraction without any acidic or basic catalysts under milder conditions provides a possibility to isolate lignin with minor structural alterations. This method has been used for isolating native-like lignin for a long time, but typically with very low extraction efficiency (<10%). A historical standard is a native-like lignin named Brauns' lignin that is isolated from biomass after Wiley mill grounding in the presence of ethanolwith long extraction times (8–10 days) at room temperature (F. E. Brauns, 1939; Brauns and Brauns, 1952; Buchanan et al., 1949). Due to the mild extraction conditions and compact cell wall structure, the yield of lignin from applying this method is limited even under the assistance of ultrasound (Su et al., 2022). Later, Björkman employed intensive ball milling and a variety of organic solvents for the extraction of native-like lignin at room temperature (Björkman, 1954). Lignin extracted via 1,4-dioxane is often used as a native-like lignin reference material in recent times and is referred to as (ball) milled wood lignin (MWL). In this method, the lignin yield is closely linked with the actual energy input by ball milling. The more energy transferred by the milling

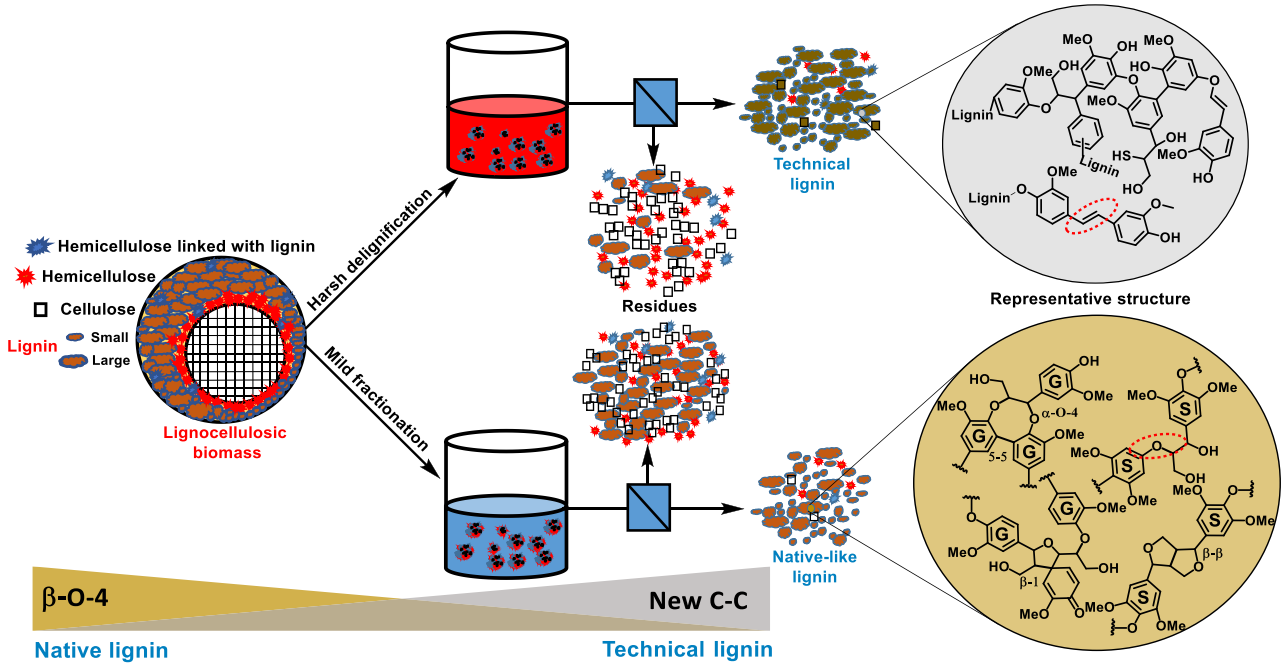


Fig. 4. Native-like lignin vs technical lignin. The structure of technical lignin was redrawn based on a suggested literature structure (Zakzeski et al., 2010).

process means more physicochemical structural alterations of the lignocellulosic biomass material, resulting in higher effective extraction potential and lower physical recalcitrance. This process breaks some cleavable linkages and decreases the size of lignin to facilitate an increase in the extraction yield of lignin fragments. Several works have shown that these methods only released small fragments of the whole lignin that could dissolve in organic solvents, which brings some limitations in revealing the full picture of the total heterogeneity in native lignin (Chang et al., 1975; Holtman et al., 2007; Ikeda et al., 2002).

3.1.2. Organic solvent extraction with different acids

The addition of acids is the most common way to facilitate the release of extractable lignin fragments for organic solvent extraction of lignin. Hydrochloric acid (HCl) is the most widely used and has been employed for lignin studies from an early stage. Already in the 1950's, Pepper et al. evaluated different solvent systems with 0.2 M HCl at a temperature between 90 °C and 95 °C with different extraction times (Pepper et al., 1959). Lignin recovered from 1,4-dioxane:water (9:1) gave the highest yield and no benzylic alkoxylation. Among them, aspen lignin obtained from this mixed solvent gave similar methoxy content to that reported aspen Braun's lignin. 2D HSQC NMR revealed a 45% β-O-4 content of lignin from walnut shell extracted by following these early established methods (Deuss et al., 2015). Mixture of different alcohols/water and 1,4-dioxane/ethanol after carefully acidified by HCl isolated lignin in relatively high β-O-4 (60%–67%) (Pals et al., 2022; Zijlstra et al., 2020). Sulfuric acid (H_2SO_4) is another inorganic acid that is widely employed for lignin isolation. The Dumesic group showed a highly efficient system in which more than 70% lignin could be recovered from corn stover by fractionation using 80 wt% γ-valerolactone (GVL) and 20% water with minor sulfuric acid (150 mM) dosage at 145 °C. No exact percentage of alkyl aryl ether content was provided, but the native structure was claimed based on a monomer content from hydrogenolysis [37.7% from GVL lignin vs 42.1% from CEL (see section 4.1)] (Luterbacher et al., 2015). This system is widely used for lignin application-oriented studies with the demand of lignin isolation in high yield and high quality in terms of alkyl aryl ether percentage. As high as 75% (based on Kalson lignin) yield of lignin from corn stover was extracted via this system, and a percentage of 81% of the total linkage content (calculated from 2D

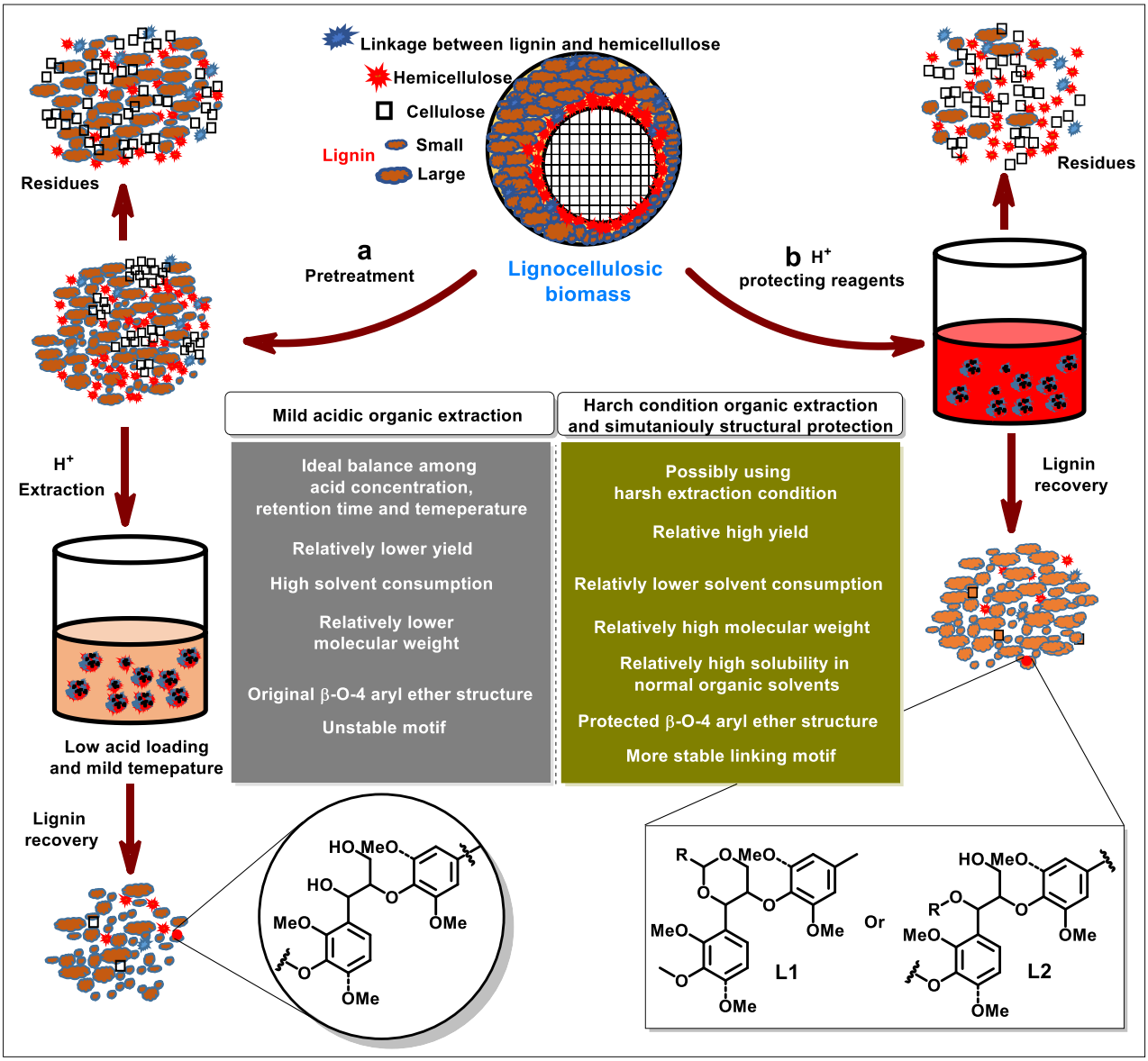


Fig. 5. Schematic illustration of acidic organic extraction of native-like lignin, **L1**, lignin with aldehyde protection (Lan et al., 2018; Shuai et al., 2016), **L2**, lignin with benzylic alkoxlation(Lancefield et al., 2017; Liu et al., 2021; Wang et al., 2022b; Zijlstra et al., 2020)

HSQC NMR) was β-O-4 linkage (Timokhin et al., 2020). The isolated lignin was subjected for specifically production of *p*-coumaric acid in a crude and purified yield of 8.3% and 4.8%, respectively, which further confirmed the close-to-native state of the obtained lignin. From above discussed cases, several suggestions and dis/advantages for using acidic organic solvents to extract native-like lignin are proposed in path **a** of Fig. 5.

Organic acids also show high efficacy for enhancing lignin extraction and can serve as solvent. Formic acid and acetic acid are two of the most studied organic acids for lignin isolation without serious structural alteration. Li et al. evaluated fractionation of lignin from bamboo by boiling 88% formic acid for 2 h. Lignin recovered under these conditions showed slight decrease of the β-O-4 linkage content (64%) compared to the corresponding MWL (74%) (Li et al., 2012). It seems that lignin isolated from acidified 90% acetic acid also had relatively high structural integrity (Li et al., 2012). Reagents used for the acidification, physical assistance such as microwave significantly influence the native state of lignin (Avelino et al., 2018; Pan and Sano, 2000). Among them, the cation hardness of the acid played a dominant role on lignin yield and structure integrity.

Acids, a double-edged sword in lignin isolation, enhance not only lignin liberation but also depolymerization and condensation, which make it hard to reach a balance between high yield lignin and conservation of native-like structure. Thus, systems in which the acidic depolymerization and condensation can be circumvented will significantly increase balance towards high yield and conservation of β-O-4 linking motif (path **b** in Fig. 5). Many efforts via structure protection strategy have been made to achieve this target. For instance, lignin extraction with simultaneously benzylic alkoxylation not only hampers lignin condensation but also increases its solubility, which provide the possibility to isolate high β-O-4 lignin under high temperature and strong acidity (Lancefield et al., 2017; Liu et al., 2021; Wang et al., 2022b; Zijlstra et al., 2020). This is further verified by lignin extraction from different alcohols acidified by HCl (Zijlstra et al., 2020). Yield of MWL isolated by methanol significantly increased from 2% to 24.2% via methanol alkoxylation under HCl (1.37 mol/L), and at the same time, almost all the β-O-4 linkages (42.6% including 20.3% alkoxylated by methanol) were preserved (Chen et al., 2022). Another strategy also achieved high β-O-4 lignin isolation in high yield by *in-situ* aldehyde protection. In this process, β-O-4 linking motif is preserved by production of a 1,3-dioxolane-structure between the aldehyde and α, γ-diol of the β-O-4 linking motif (Lan et al., 2019, 2018; Shuai et al., 2016). Through this way, 1.2 g lignin was isolated from 5.0 g birch with propionaldehyde as protection reagents, and an intact aromatic region on the 2D HSQC NMR was clearly observed (Lan et al., 2019).

3.1.3. Organic solvent extraction with different alkalis

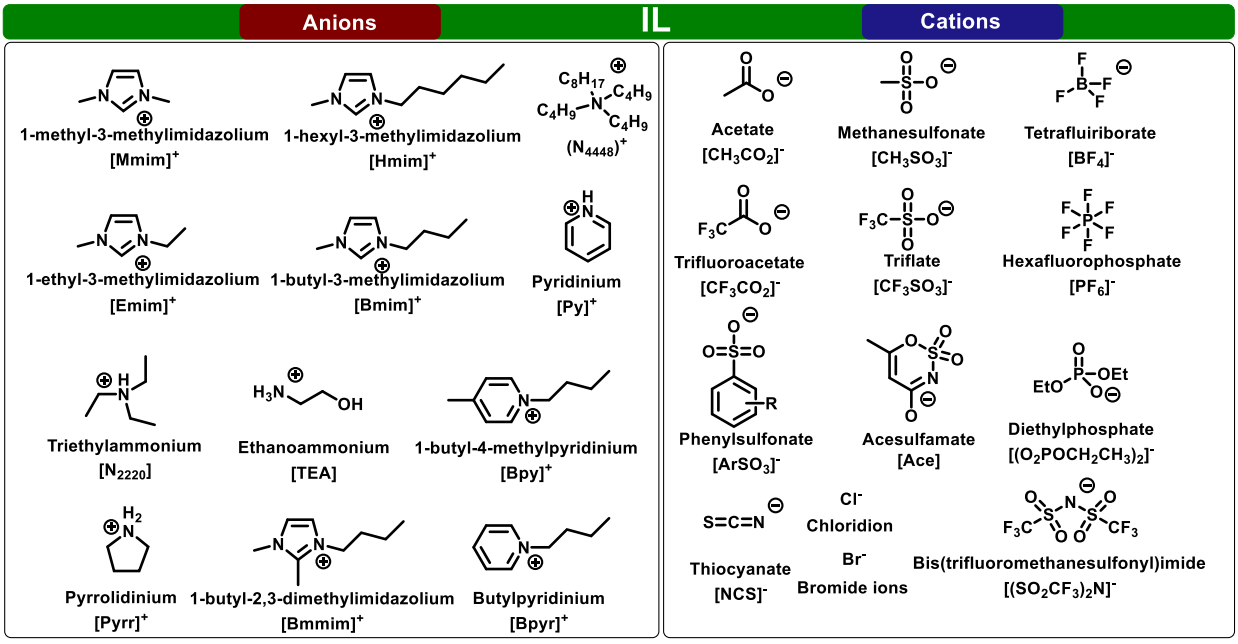
Alkaline conditions can also facilitate the hydrolysis of labile lignin linkages and disrupt bonds between lignin and polysaccharides to further decrease the recalcitrance towards organic extraction. Serious cleavage of alkyl aryl ether linkages and condensation are typically associated with intensive alkaline treatment. Therefore, in order to conserve the native-like structure of the lignin, the alkaline extractions conditions have to be kept very mild. Only limited research employed alkaline organic solvent for extracting native-like lignin. Despite the scarcity of the data, lignin obtained from mild alkaline organic extraction can in some cases exhibit high β-O-4 linkage content. For instance, Chen et al. extracted ball milled *Eucalyptus* by aqueous alkaline 1,4-dioxane (1,4-dioxane: water, 80:20, with 0.05 M NaOH), and lignin recovered from this extraction had a much higher yield and purity than that of MWL (Chen et al., 2018). They further studied the molecular weight and main linkages of lignin precipitated under pH 2, 4 and 6, respectively. All these lignins obtained from their work exhibited similar structural features with differences in terms of molecular weight, sugar impurities and relative percentage of main linkages. The β-O-4 linkage content quantified by 2D HSQC NMR of these samples were all higher than corresponding MWL (53%). Pre-swelling *Eucalyptus* chips with mixture of ionic liquid and 1,4-dioxane significant increases the yield of lignin via ethanol (70%) basified by 1 M NaOH (Sun et al., 2013). A maximum lignin yield of 23.3% was obtained with percentage of β-O-4 linkage being 79% quantified by 2D HSQC NMR (Sun et al., 2013). 60% ethanol basified by *n*-propylamine could isolate more than 70% lignin out of the residue obtained from hot-water treatment. Even a relative harsh (180 °C, 30 min) condition was applied. The isolated lignin still have around 24% β-O-4 linkages in contrast to 38% for native-like residue enzyme lignin (REL, see section 4.2) (Chen et al., 2017b).

3.2. Fractionation by ionic liquids (ILs)

Ionic liquids consist of a cation and anion ion pair (Scheme 2). Among them, the room temperature ionic liquids (RTILs) are widely studied for applications like synthesis and extraction (Kuchenbuch and Giernoth, 2015; Marsh et al., 2004). In general, RTILs are composed of large asymmetric organic cations and inorganic or organic anions providing a mixture with a melting temperature lower than 100 °C. The neglectable vapor pressure, tunable polarity, viscosity and density give ILs different miscibility with other solvents and different dissolution capability towards chemicals. The application of ILs as an extraction medium for lignin has gained increased attention (Glas et al., 2015; Hart et al., 2015; Lee et al., 2009; Pu et al., 2007; Wang et al., 2014). The solubility of lignin in ILs can be tuned by the cation and ion selection. For instance, ILs with $[MeSO_4]^-$ and $[CF_3SO_3]^-$ as anions showed promising capability for dissolution of lignin (Hart et al., 2015; Pu et al., 2007). In order to isolate native-like lignin, the acidity and alkalinity also need to be taken into account. ILs with mild acidity have been confirmed to allow isolation of lignin with high yields in a well preserved state (Saha et al., 2018; Wen et al., 2014; Xu et al., 2015). Among them, 1-ethyl-3-methyllimidazolium acetate ([Bmim]-[OAC]) is widely applied in lignin isolation (Espinoza-Acosta et al., 2014). Kim et al. compared lignin isolated from poplar using [Bmim]-[OAC] at 110 °C for 16 h with MWLby DFRC and ^{13}C NMR analysis. The results from elemental analysis, functional groups and C9 formula indicated that these two lignin had similar structures, except a slight difference in molecular weight and S/G ratio (Kim et al., 2011). Later, Wen et al. comprehensively evaluated lignin isolated from [Bmim]-[OAC] under a temperature range of 110–170 °C for 1–16 h, and their data clearly showed that lignin recovered under lower temperature (110 °C and 16 h) and short retention time (130 °C and 3 h) had similar β-O-4 linkage (52.9% and 53.1%, respectively) and distribution to that of native-like lignin (55.8%) isolated by milder alkaline aqueous extraction (Wen et al., 2014). Lignin extraction in [Bmim]-[OAC] under microwave-assisted treatment could increase lignin yield from 34.7% to 43.3% (Based on Klason lignin) but decreased the β-O-4 linkage content from 58.5% to 48.0% (Sun et al., 2019a, 2019b). We designed a protic ionic liquid formed by triflic acid (HOTf) and triethylamine, and by simply acidifying the IL with the same acid and further mixing with ethene glycol (EG), we could achieve a 7.4% yield (based on Klason lignin) of EG benzylic-alkoxylated lignin with a total of 61.2% aryl ether linkage at milder condition (50 °C, 1.5 h) (Wang et al., 2022b). The partially delignification boosts enzymatic hydrolysis of the polysaccharide residue to result a 21.3% (based on starting biomass) yield of residual enzyme lignin with also benzylic alkoxylation and a total of 76.2% aryl ether linkage, indicating a potential for an integrated strategy for native-like lignin isolation. These examples show that a proper balance between the extraction intensity and acidity is necessary for native-like lignin isolation using acidic IL, even for IL with lower acidity.

3.3. Fractionation by deep eutectic solvents (DESs)

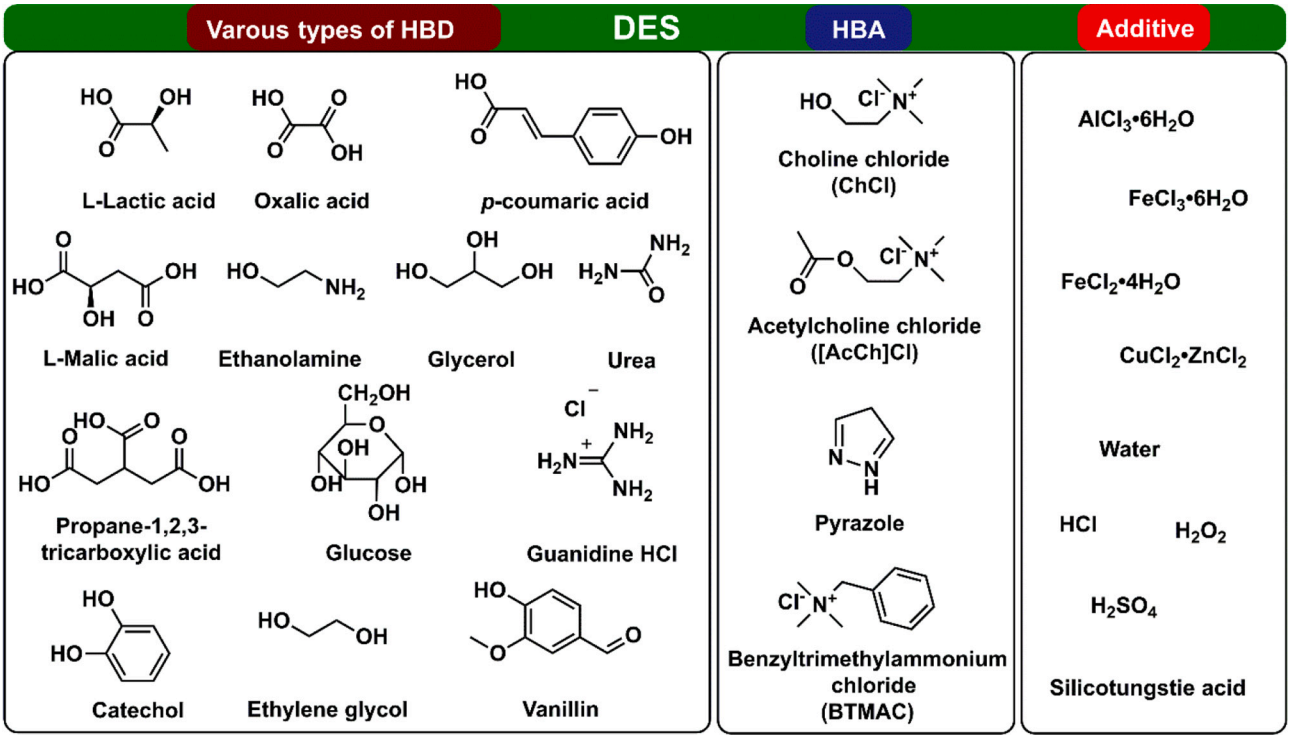
DESs used in lignin fractionation are generally formed by a variety of quaternary ammonium salts with halide metal salts or carboxylic acids, alcohols and amides with hydrogen bonding capabilities. Melting points



Scheme 2. Examples of chemical constituents of ionic liquids used for lignin extraction.

of DESs are sometimes significantly lower than those of the individual compounds as a result of lower lattice energy or the charge delocalization from the occurrence of hydrogen bonding (Scheme 3) (Abbott et al., 2004; Hong et al., 2020; Smith et al., 2014). DESs consisting of choline chloride (ChCl) and compounds with hydrogen bonding networks overcome the drawback of ILs in terms of high cost, tedious synthesis, toxicological properties and high disposal cost. They exhibit promising performance as an alternative to IL for lignin fractionation and pulp production. Both acidic and basic DES systems have been employed for the fractionation of lignin, but also here the lignin is easily seriously degraded under harsher fractionation conditions (Yue et al., 2020). Therefore in order to conserve the native-like structure of lignin, the constitutions and extraction conditions of DES have to be carefully studied and optimized.

Neutral DESs can in theory isolate lignin with minimal structure alteration, but the yield is normally much lower than that of DES systems with acidic or alkaline catalysts, additives or constituents (Xia et al., 2018). So far, there is little available data to show the structural and chemical transformation of lignin obtained from neutral DES extraction. Plenty of acidic DES or neutral DES with added acid have been employed



Scheme 3. Examples of chemical constituents of DES mixtures used for lignin extraction

for lignin fractionation with reasonable retention of the native lignin structure. For instance, more than 50% lignin was recovered in a ChCl/EG (1:2) with 1.0% H_2SO_4 within 1 h fractionation of switchgrass. The lignin recovered from this systems showed some similar structural properties with that of MWL (Chen et al., 2018a). The affinity between DES and lignin plays an important role in its isolation. For instance, DES prepared from pyrazole and lactic acid showed high affinity with lignin and had excellent performance on isolating lignin from wheat straw with a high yield of 80% and a high retention of alkyl aryl ether linkage content (39% from DES vs 58% from MWL) (Lin et al., 2023). The DESs using polyol as hydrogen donor showed excellent performance on lignin fractionation, and the benzylic alkoxylation of lignin significantly increased the capability of lignin to handle harsh extraction conditions (Chen et al., 2018b; Guo et al., 2020). For instance, the group of Barta reported a tunable and functional ternary ChCl/ethylene glycol/oxalic acid system which could recover 40% benzylic alkoxylated lignin (Based on Klason lignin) with high total β-O-4 linkage (53%) conservation under 100 °C for 24 h, which is only somewhat lower than the corresponding MWL (63%) (Liu et al., 2021). The effect of this acidic DES treatment on residual enzyme lignin (REL, see section 4.2) was studied, and results showed that β-O-4 linkage was more sensitive toward the increase of temperature than acidity (Wang et al., 2022a). Most basic DES systems focus on pretreatments of lignocellulosic biomass oriented for reducing the recalcitrance towards high fermentable sugar production (Chen et al., 2020; Wang and Lee, 2021). Several works reported the delignification of biomass via basic DES systems such as ChCl/urea and ChCl/amidazole, but did not provide enough characterization to analyze the quality of the lignin (Procentese et al., 2015). Recently, detailed structural information was provided for the lignin obtained by following the conditions used for ChCl/urea system. In their work, under the harshest condition (150 °C, 15 h), around 60% lignin was removed from Populus, and the obtained lignin had high purity (>94.4%) and alkyl aryl ether retention (72.2% based on the result from 2D HSQC NMR)(Li et al., 2021a, 2021b). An alkaline DES composed by ChCl and monoethanolamine achieved more than 90% lignin isolation from industrial xylose residue after 1 h treatment at 80 °C, and the obtained lignin contained 31.6% alkyl aryl ether linkage content that is similar to that (40.5%) of corresponding the double enzymatic lignin (DEL, one method that will discuss in section **4.2** normally isolated lignin in high structural integrity) (Ma et al., 2022). Additionally, a special so-called alkaline DES fabricated from glycerol and K_2CO_3 showed excellent performance on isolating lignin from wheat straw in relatively high purity (73%–91%) and yield (15.6% based on starting biomass) as well as high alkyl aryl ether linkage content (61.7%–78.2%). This indicates the promising potential of alkaline DES on highly efficient lignin isolation (Yue et al., 2022), Therefore, the application of basic DES on fractionation of native-like lignin requires further study (Hong et al., 2020; Li et al., 2021a, 2021b).

3.4. Alternative solvent systems for fractionation

Water can also be used for fractionation of lignin under relatively high temperature or with base addition at lower temperature. The lignin isolated from hot water extraction (145 °C–230 °C) presents low yield and a condensed structure caused by released organic acids and thus this method is seldomly used for lignin isolation, let alone native-like lignin isolation (Machmudah et al., 2015; Nagardeolekar et al., 2020; Wang et al., 2016). However, lignin shows better stability under alkaline aqueous conditions at moderate temperature (< 80 °C). A combination of water and base with precise control of the extraction conditions can give a high yield of lignin with a near native-like structure. The alkaline conditions deprotonate phenol groups to significantly increase lignin solubility and also partially cleave the aryl ether linkage resulting in a lower molecular weight. The latter also releases additional phenolic end groups providing further increased solubility after deprotonation. For example, Sun et al. systematically evaluated extraction of lignin from wheat straw by 1.5% potassium and lithium hydroxide under near room temperature for various extraction times (Sun et al., 1996). The ^{13}C NMR spectra clearly revealed uncondensed S and G units. Later, the lignin of *Eucalyptus* was isolated by following these optimized parameters, and the 2D HSQC NMR further confirmed that the lignin had similar β-O-4 linkage (55.5%) to that of (55.3%) corresponding CEL (Cellulolytic enzyme lignin), a type of native-like lignin discussed below (CEL, section 4.1) (Wen et al., 2015). Additionally, it is reported that different acidity of the supernatant used for inducing lignin precipitation significantly influences the distribution of the alkyl aryl ether linkage (Zhang et al., 2022). Lignin precipitated under a pH of 9 had the higheer alkyl aryl ether linkage range (45%–76%) in this study. In order to increase the extraction efficiency, various pretreatment methods are combined to boost extraction efficacy (Sun et al., 2019a). Ammonia pretreatment can significantly enhance the lignin recovery yield without serious degradation in aqueous NaOH extraction at room temperature (Mittal et al., 2017). The combination of ionic liquid pre-dissolution and followed with alkaline extraction is also reported to isolate lignin with high β-O-4 linkage retention (less than 7% decrease) (Yang et al., 2013; Yuan et al., 2013). Recently, hydrotropes solutions like *p*-toluenesulfonic acid (*p*-TsOH) were shown to have excellent delignification performance under mild conditions (Chen et al., 2017a; Mikulski and Kłosowski, 2022). The aggregation of acid can fractionate lignin in high yield at lower temperature (<80 °C) and short extraction time (< 60 min), which can preserve lignin from intensive degradation. 69.4% poplar lignin was recovered from a treatment with 70% aqueous *p*-TsOH at 50 °C and 20 min, and the lignin was visually showed a similar β-O-4 linkage structure compared to that of the corresponding native lignin as determined by analysis of the WCW material (Chen et al., 2017a). Fractionation of lignin mixture of polyol and oxalic acid also showed potential to isolate high aryl ether linkage (56% in contrast to 57% of CEL) and yield (53%) lignin under milder condition (90 °C) (Yang et al., 2023b).

3.5. Fractionation with rapid flow-through reactors

In batch reactors (Fig. 6a), a relative prolongation of extraction time is necessary in order to increase the degree of lignin release. But the long retention time induces secondary reaction such as condensation and repolymerization of extracted fragments, which seriously affects the quality of the obtained lignin. Therefore, if lignin can be rapidly separated from the extraction liquor at reaction conditions, degradation can be significantly suppressed. Flow-through extraction of lignin in a fixed biomass-bed extractor (Fig. 6b) can dramatically decrease the retention time of lignin in the extraction medium, and thus preserve lignin from further degradation and condensation. Flow-through reactors were shown to better preserve the structure of the extracted lignin compared to a batch reactor under similar delignification of biomass (Xu et al., 2021; Zhou et al., 2019; Zijlstra et al., 2019). This concept was used in our group to develop a very efficient butanol-based organic extraction protocol that showed excellent lignin recovery (>90%) and high β-O-4 linkage retention from different biomasses (Zijlstra et al., 2021). Zhou et al. compared the performance of flow-through reactor for the extraction of lignin from poplar by 72% formic acid at 130 °C and 10 min, and more than 50% (Based on Klason lignin) lignin was isolated in a 45.9% β-O-4 linkage that is close to that (46.1%) of corresponding MWL. Also, *p*-TsOH can achieve fractionation of lignin under mild conditions, and this system also can be expanded in flow-through systems (Wang et al., 2019b). 22% yield of lignin obtained from this system at 80 °C and 60 min exhibited a 54.4% β-O-4 linkage that is similar to 57.8% of corresponding MWL. Recently, the Beckham group reported a highly efficient tandem flow-through reactors which could achieve lignin release with methanol at 225 °C and then direct hydrogenolysis was applied on the extraction liquor to give high monomer yield (Brandner et al., 2021). The lignin obtained from *ex situ* flow-through solvolysis liquor exhibited a high β-O-4 structure as revealed from the 2D HSQC NMR.

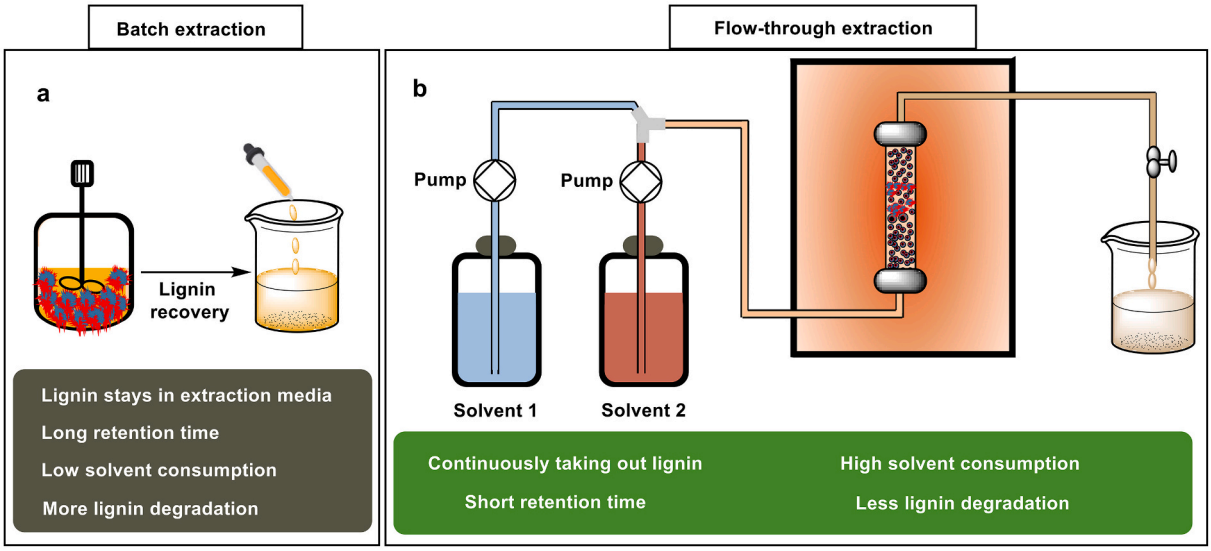


Fig. 6. Schematic representation of batch and flow-through extraction indicating advantages of the latter, although high solvent consumption is often reported for flow system, and this is not necessarily true (Zijlstra et al., 2019).

4. Fractionation involving enzymatic treatments

Milder operational conditions are accessible via the aid of biocatalysis. Enzymatic treatments are thus attractive to facilitate lignin isolation without significant structural alterations. Treatment with enzyme cocktails specializing at hydrolyzing polysaccharides of lignocellulosic biomass can enhance subsequent lignin extraction efficiency or leave lignin as a residue (Guerra et al., 2006). The methods developed with the enzymatic treatment are named and categorized by the stage at which the enzymatic treatment is performed and at which state lignin is isolated (see Fig. 7). The most used methods are cellulolytic enzyme lignin (CEL, see path **a** in Fig. 7), enzymatic mild acidolysis lignin (EMAL, see path **b** in Fig. 7) and residual enzyme lignin (REL, see path **c** Fig. 7). Among them, CEL and EMAL consist of lignin fragments that can be extracted by organic solvents with and without acids, while REL is the method that leaves lignin as the residue from enzymatic carbohydrate removal. These methods are discussed in more detail below.

4.1. Cellulolytic enzyme lignin (CEL) isolation and enzymatic mild acidolysis lignin (EMAL)

In these methods, ball milled biomass is treated by commercial enzyme cocktails to partially remove and alter the polysaccharide fractions, resulting in a lignin-rich residue. From this residue, lignin is

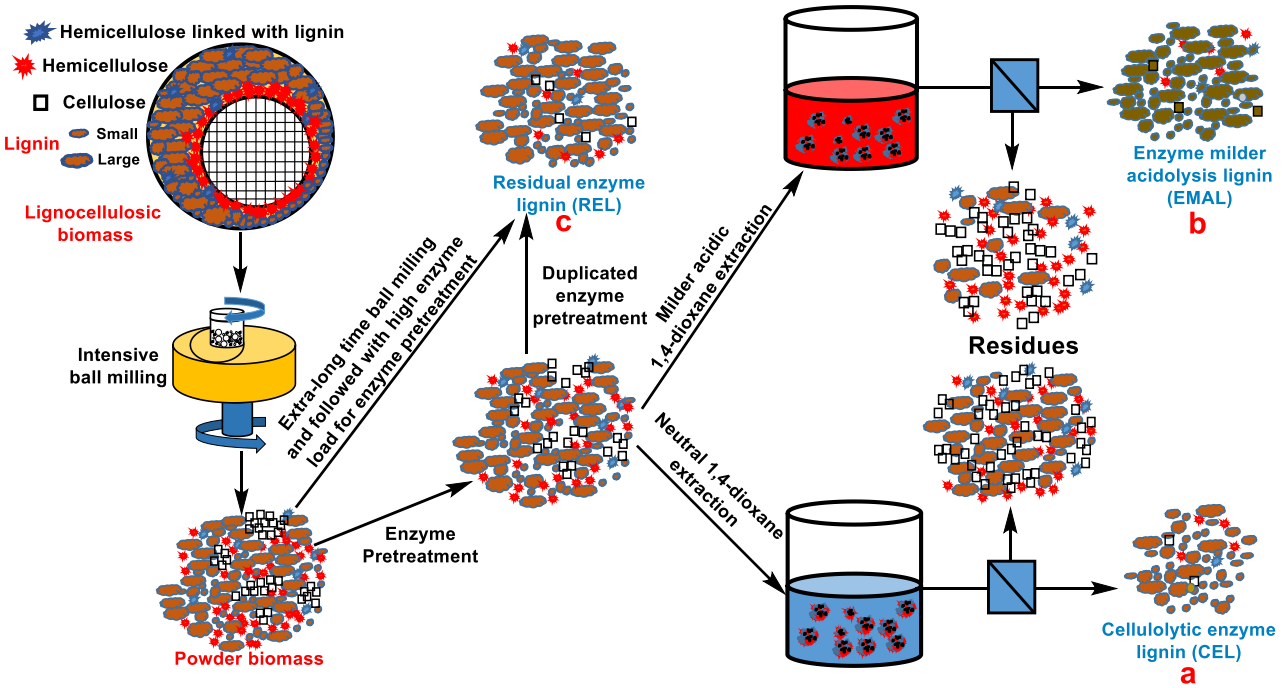


Fig. 7. Overview of native-like lignin obtained by enzymatic treatment. **a**, the isolation of cellulolytic enzyme lignin by for **c** example Chang et al., 1975; **b**, the isolation of enzyme milder acidolysis lignin by for example Wu and Argyropoulos, 2003;, the isolation of residual enzyme lignin by for example Pew, 1957 and Wang et al., 2021b.

then isolated by organic solvent extraction (path **a** in Fig. 7). This is similar to typical MWL isolation but with a slight increase of the yield. The final organic extraction with purification steps gives lignin with lower amounts of impurities. In 1975, Chang et al. firstly reported the CEL method (Chang et al., 1975). In this lignin isolation approach, spruce wood was subjected to a sequential of ball milling and enzymatic treatment, and followed by extraction with 1,4-dioxane/water at room temperature. Here, mixtures with both 96% and 50% 1,4-dioxane led to lignin isolation yields that were higher than those obtained from MWL from sapwood of sweetgum and spruce. Zhang et al. further developed this method through pretreating ball milled wood meal with dimethyl sulfoxide/N-methylimidazole and found that CEL yield of loblolly pine and basswood improved after dissolution of ball-milled wood and subsequent regeneration of the wood by precipitation of the slurry in ethyl acetate (Zhang et al., 2010). Other works modified the isolation method by starting extracting CEL after extraction of MWL lignin. Holtman et al., 2007; Ikeda et al., 2002). In general, CEL has a somewhat higher yield than MWL for isolation of similar native-like lignin structures. CEL has been used for lignin structural elucidation, tracking chemical changes, modifications during various physicochemical treatments.

The residue obtained from the cellulase treatment still shows a strong recalcitrance towards the following organic solvent extraction. Therefore, EMAL was developed where a small amount of acid is added during the organic solvent extraction to decrease the recalcitrance and partially fragment the lignin. This method achieves a 2–6 times increase of yield for lignin with negligible structure alteration when compared to CEL and MWL from different biomasses (path **b** in Fig. 7) (Wu and Argyropoulos, 2003). Argyropoulos et al. reported this method to isolate lignin from residual pulp for investigating the effect of the pulping process on structural properties of the lignin (Argyropoulos et al., 2002; Wu and Argyropoulos, 2003) Anderson et al. comprehensively evaluated MWL, CEL (modified method) and EMAL from different biomass species, and the results confirmed that EMAL isolation had a higher yield than the other two isolation methods and because this actually yielded lignin with more diverse structural properties (Guerra et al., 2006).

The high lignin recovery and minor structural alterations make EMAL widely applicable for serving as a typical native-like model lignin for fundamental studies comprising lignin such as synthesis of lignin-based materials and optimization of functionalized aromatic monomers from lignin degradation. For example, Anderson et al. employed EMAL from *Eucalyptus* as a model lignin to compare the monomer products from DFRC and thioacidolysis (Guerra et al., 2006). Zhang et al. studied the EMAL lignin structural alteration of corncob after acid catalyzed steam explosion, which showed that acid steam-explosion caused lignin degradation (Zhang et al., 2019).

4.2. Residual enzyme lignin (REL) isolation

REL isolated by enzymatic hydrolysis of polysaccharide leaves lignin as a residue after the released sugars are washed away (path **c** in Fig. 7). This treatment has unique advantages than CEL and EMAL to study the whole native structure of lignin. As mentioned above, pretreatments and in particular chemical treatments always cause undesired structural alteration. In contrast, physical treatments under proper control of the severity can circumvent this to a certain extent. Pretreatment by ball milling combined with enzymatic treatment to isolate lignin not only avoids serious structural alteration but also has almost quantitative yield of the lignin. This has been employed for lignin structure elucidation from an early period. In 1957, Pew had achieved a 95% polysaccharide removal of spruce wood meal by glucosidase treatment after grinding for 5–8 h in a vibratory ball mill (Pew, 1957). Later, sweetgum wood was used to isolate REL, and almost all the polysaccharide could be digested, but no detailed characterization was conducted because of the low solubility of the lignin in common organic solvent (Chang et al., 1975). This is a major disadvantage of REL, as the native structure and high molecular weight make it generally insoluble in common organic solvents. With the development of new analytical techniques and proper selection of biomass, several works have revealed more of the structure of REL. For example, recently, Dou et al. compared the structural difference of lignin in the bark and wood from willow. REL of bark and wood were isolated and analyzed by wet chemical methods and 2D HSQC NMR in a DMSO-d_6/pyridine-d_5 solvent, and they found that lignin in bark contained higher S units and β-5 linkages (Dou et al., 2018). Methods used to isolate REL are still under development. For instance, double enzymatic lignin (DEL) isolated via optimized two or more cycles of ball milling and enzyme treatment shortens the isolation time and avoids extra structural alteration that arise from ball milling (Chen et al., 2017a; Wang et al., 2017; Wang et al., 2021a, 2021b). Impregnation of biomass with dilute acid solutions (acetic acid and hydrochloric acid) can further decrease the ball milling time to 1 h without losing the valuable alkyl aryl ether linkage of the obtained REL (Wang et al., 2018).

REL isolation has been gaining attention for its application in fundamental studies on lignin (Sun, 2020). REL has helped to reveal lignin distribution in the cell wall and reveal structural changes induced by other processing methods. For instance, structure analysis of the REL isolated from residue after extraction of CEL showed that the extracted CEL is not representative of the complete lignin structure (Hu et al., 2006). Detailed analysis of the DEL isolated from different growth stages revealed that more S units and aryl ether linkage were found in the poplar collected from later growth stages (Chen et al., 2017a; Wang et al., 2019a, 2019b). REL has a high yield and can better conserve the original state of lignin compared to other methods, which make it ideal for studying the influence of various pretreatments on the structure of lignin (Jääskeläinen et al., 2003; Jensen et al., 2017; Wang et al., 2021a, 2021b; Wen et al., 2015; Yamasaki, 1981).

5. The challenges associated with the use native-like lignins

From previous illustration and discussion, it is found that no perfect method can provide ideal native-like lignin owning all required properties in terms of purity, yield, structural retention, representativeness of native lignin and a material that is easy to handle and analyze. The disadvantages and advantages of different methods used for native-like lignin isolation are summarized in Fig. 8. Regarding the use of neutral organic solvents, lignin extracted from these mild systems have less structural alteration except slight oxidation induced by the intensive long-ball milling. The mild conditions limit the release of lignin and thus representativeness of total lignin due to being limited to the extraction of low Mw fragments. Long time ball milling and tedious purification steps increase the solvent and energy consumption, which make large scale production impossible. However, for a lab trial intending for studying lignin structure or concept-proof of lignin modification and degradation, lignin isolated from this method in particular MWL totally satisfy the role as these are also often readily dissolved in common solvents. Acid and base significantly enhance the efficiency of organic extraction to achieve native-like lignin isolation in large scale. Finding the balance between native-structure retention and yield is necessary for these systems, otherwise unstable labile linkages are extensively cleaved. Simultaneous protection during extraction can push balance towards high yield and β-O-4 linkage retention. Lignin obtained from this method is suitable for application-orienting studies that normally need large quantity of lignin as starting material. Recyclable and relatively environment-friendly IL and DES are excellent alternative systems to replace organic solvent and have similar performance with acidic/basic organic extraction on isolation of native-like lignin. Both of the two systems are applicable on flow-through system to further enhance lignin removal and retention of native traits. Native-like lignin such as REL, CEL and EMAL, isolated under the assistance of enzyme treatment have well preserved structure, and possess many advantages for studying lignin reactivity and develop new lignin-based products, which is an important part of developing lignin-focused biorefineries. The native-like state of these lignins provides not only the advantages such as

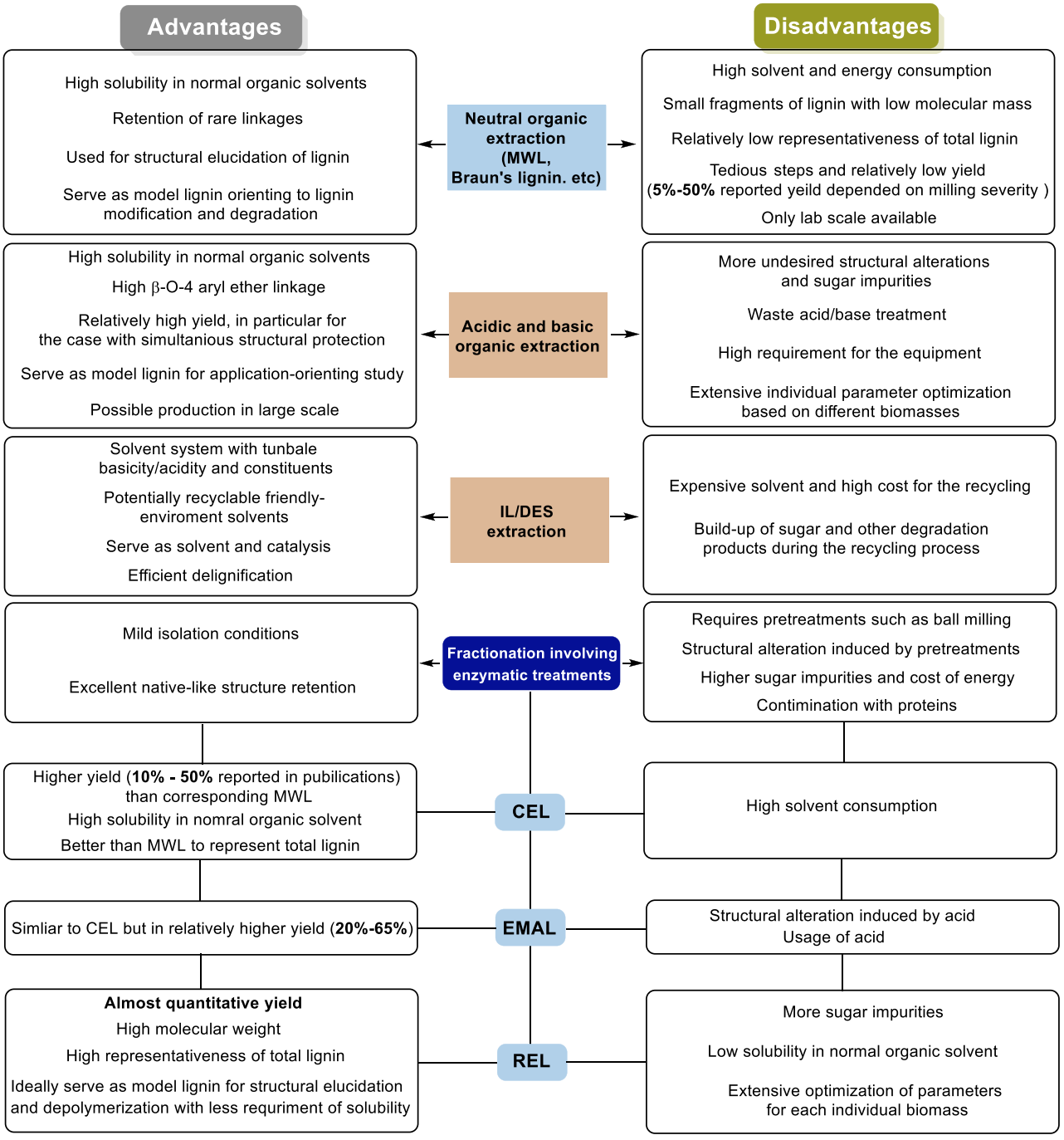


Fig. 8. Overview of dis/advantages of different methods for isolating native-like lignin.

abundant native structure in high molecular weight, but also some impediments including complex and diverse structure as well as the high molecular weight. The native-state lignin has a high molecular weight and often saccharide impurities arising from the tight interconnection of these biomass components. Therefore, the more native-like lignin is close to proto-type lignin, the more challenging it is to isolate and handle. MWL and CEL allow for the isolation of lignin with native-like features in relatively high purity, but as these lignins have lower MW, thus these are somewhat less representative and the lower recovery yields leave their scale-up even more impractical. Native-like lignin such as EMAL isolated by acidic and basic solvent extraction provides lignin with higher yield and purity, but the recycling of solvents and partial loss the native-like structure limit its wide-spread application. The isolation principle of REL renders it to have the closest structure to proto-type lignin, but the high molecular weight and remaining linkages with polysaccharide make it even harder to dissolve in some powerful solvents, which significantly restrict their application on studying lignin reactivity. In addition, the contamination of protein residues either from the enzymatic hydrolysis process or the inherent residues left from the bio-synthesis process can influence lignin characterization and quantification (Kim et al., 2008, 2017; Rencoret et al., 2011; Wang et al., 2021b). Since then, several works have tried to alleviate such problems by further treating the lignin with proteases. Recently, Kim et al. precisely identified and characterized amino acid residues by using pure compounds of tyrosine, phenylalanine, tryptophan, and tyramine in WCW, MWL, CEL and REL. REL was picked up and treated by protease K,

resulting in better quantification of H units and the Klason lignin content (Kim et al., 2017). Although REL has the highest β-O-4 linkage content, the energy-consuming ball milling and relatively high proportion of saccharides impurities give challenges for large scale production and application. Incorporating the isolation of REL into the utilization of the polysaccharides might address these challenges. For instance, REL isolated from mildly dilute acid impregnation had high alkyl aryl ether bond retention to potentially allow for utilization in many high value applications but also achieved efficient hydrolyzing of polysaccharides into valuable monosaccharides, which is significant for overall cost efficiency for such processes (Wang et al., 2018). Such holistic studies will be important to evaluate overall economic compatibility for scaling of native-like lignin isolation strategies. Upcoming strategies might focus on combination of organic extraction, IL and DES extraction with a lignin protecting strategy such as benzylic alkoxylation, acetal protection with aldehydes during the extraction process. By these strategies, the released and protected lignin is more stable, which can significantly improve the yield of lignin and quality at the same time. Such strategies that achieve high delignification are often a prerequisite for efficient carbohydrate utilization. The strategies can further benefit from the implementation of flow-through reactors to unlock possibilities to produce native-like lignin in large scale.

6. Conclusion and perspective

In order to collect the maximum profit from lignocellulosic biomass, lignin should be given adequate attention in upcoming lignin-focused biorefining concepts. In order to transform lignin into value-added products, plenty of fundamental reactivity studies are urgently needed. In these studies, lignin models with representative structures compared to proto-lignin that is part of the plant material are significant for developing effective and innovative methodologies to bio-refineries. No proper method can isolate lignin in a pristine proto-state. The existing methods for native-like lignin isolation are trying to separate lignin from the biomass matrix with as close as possible to the native state. For all the different methods, the key factor that influences the quality of the obtained lignin is to precisely control treatment severity to build a balance between quality and yield. The diversity of the lignin isolation methods makes that all these different lignins have different features. The methodologies being studied for lignin-focused bio-refineries also have different requirements for required model lignins and thus different requirements to the obtained native-like lignin for examples, in terms of yield, purity and solubility. Therefore, a comprehensive overview on the methods for native-like lignin isolation and understanding the features of the corresponding lignin is significant for choosing the best method for lignin isolation. Thus, the selection of native-like lignin for the study of lignin-focused biorefineries needs a carefully consideration of the representativeness of lignin for different biomasses and actual research target. Detailed structural information such as the content of alkyl aryl ether linkage, distribution of the three building blocks, molecular weight should be investigated. Native-like REL is quite suitable as a lignin model in reactivity studies in which solid biomass can be directly used as the starting material. Examples are its use for studying lignin solubility in different novel solvents systems (IL, DES, GVL etc.), RCF of lignin in particular for flow-through RCF, pyrolysis of lignin, evaluation of novel lignin fractionation methods, as well as structure elucidation of the whole lignin native-structure in combination with powerful characterization techniques. Additionally, REL is valuable for lignin structural elucidation studies involving bio-engineering of plants oriented for producing lignin with new liable-degradation structures or components in minor proportion, as well as the studies focusing on the minor units of lignin such as spirodienone, dibenzodioxin, diary ether, biphenyls and tricin. For studies using native-like lignin as a starting material with proper modification for novel materials exploration, the lignin with more alkyl aryl ether linkages and phenolic end groups in high yield and purity are needed, since these studies require more reactive functional groups, and the fundamental characterization for materials needs a high quantity to have clear results. EMAL, and lignins obtained from milder acidic and basic organic extraction are strongly recommended for more generic studies, since these methods can provide lignin with high yield without losing too much native-like structure and provide material that is relatively well solubilized in common solvents. In the cases with requirements for high solubility of lignin such as some milder acidolysis of lignin under homogenous catalytic system, lignin with smaller molecular size and high native-like structure preservation can serve as a fundamental starting lignin to show the maximum potential of the system on lignin depolymerization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

Z.W. acknowledges the China Scholarship Council for funding (grant number 201706300138). P.J.D acknowledges funding from NWO-XL project WOODLIG with a grant number OCENW.XL21.XL21.059, which is financed by the Dutch Research Council (NWO).

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