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THE LIGNIN CONCEPT

Lignin, the second most abundant organic substance on earth, is one of the three structural polymers (cellulose, hemicellulose and lignin) present in all woody plants. In contrast to cellulose, which is formed by all plants, the formation of lignified cell walls is essentially unique to vascular plants adapted to life on land. The lack of knowledge of the structural composition of lignin polymers is certainly the core of most of the problems encountered in lignin chemistry and, consequently, it is of fundamental importance to construct a more detailed picture of lignin.

Without lignin, plants would never have moved from the aquatic to the terrestrial environment during evolution. Indeed, it is lignin that stiffens the plant stem to withstand the forces of gravity and wind; lignin seals the water conducting system against the hydraulic pressure drop produced by the transport of water from the soil to the leaves; lignin provides plants with a protective barrier against the attack by micro-organism. Lignin plays also an important role in the natural ecology cycle: it serves the soil as a complexation agent for minerals and as a moisture-retention aid.

The chemical pulping of wood, one of the ten largest industrial activities in North America, involves the removal of lignin from wood. For this reason there is a great effort in the scientific field to understand the modification, depolymerisation and solubilization of lignin during the pulping processes.

The utilization of this by-product of the pulp and paper manufacturing industry as a renewable resource has numerous possibilities but, due to the competitively low price of fossil fuel as starting material, only few of them are commercially exploited.

Over the past 150 years of research, lignin has proven to be quite an intractable macromolecule and many questions still exist about its formation, structure, occurrence and commercial utilization. These uncertainties continue to be addressed with new scientific methodologies.

The multifaceted task of understanding the formation and structure of lignin, its reactions and modifications during pulping and bleaching processes, continues to attract the attention of scientists in all disciplines.

Occurrence and formation of lignin

The composite nature of wood was first recognised in 1838 when Payen [1], a wealthy chemical manufacturer in France, identified a

The article is taken in part from the PhD Thesis "Mass Spectrometry in the Study of Lignin Biosynthesis and Degradation" by Samantha Reale

fibrous substance, cellulose, which could be isolated after the treatment of wood with nitric acid. The encrusting material, in which cellulose in wood was embedded, was named lignin by Schultze in 1865 [2]. This discovery was made around half a century after the French Revolution. Since that time, thousands of scientific papers and patents, and hundreds of books have been published concerning these two most important natural polymers. To date about 10,000-12,000 scientific papers have been published relating to lignins alone [3].

In spite of the great interest in studying lignin, progress towards understanding its occurrence, structure and biodegradation has been so poor until the last decades of the past century. K. Freudenberg, in fact, in 1968 in his review on lignin chemistry said that "for almost 30 years the progress we made on the lignin problem was depressingly slow" [4].

The building-up of the lignin macromolecules by plants comprises complicated biological, biochemical and chemical systems, which have been extensively studied and repeatedly reviewed [2, 4-6]. The general term "lignin" does not consider differences in the composition of this cell wall component. In fact lignin heterogeneity was proved for numerous plants of different botanical classes, orders and even species as well as for different tissues, cells and even cell wall layers [5]. On the other hand, numerous studies with radioactive carbon (^{14}C) confirmed that three cinnamyl alcohols, namely *p*-hydroxycinnamyl alcohol, coniferyl alcohol and sinapyl alcohol are the primary precursors and building units of all lignins. A detailed understanding of the metabolic pathways leading to the synthesis of the lignin precursor monomers and the accompanying enzymatic steps has also been achieved.

The biosynthesis of lignin starts with glucose deriving from photosynthesis. Glucose is converted to scikimic acid, the most important intermediate substance of the so-called scikimic acid pathway. The two aromatic amino acids L-phenylalanine and L-tyrosine are formed as the final compounds of this pathway by reductive amination of phenyl piruvic acid, through the intermediate prephenic acid. On the other hand they are the starting substances ("amino acid pool") for the enzymatic phenylpropanoid metabolism (cinnamic acid pathway) leading not only to the three cinnamyl alcohols via activated cinnamic acid derivatives, but also to extractive components like flavonoids or stilbenes. Indeed the aminoacids are deaminated by deaminases (for instance PAL, phenylalanine ammonia lyase) to their corresponding cinnamic acid. The dominant further steps are hydroxylation of the aromatic nuclei followed by partial methylation leading to *p*-coumaric acid, caffeic acid, ferulic acid, 5-hydroxy-ferulic acid, and sinapic acid. The cinnamyl alcohols are finally formed by enzymatic activation and reduction of the corresponding acids *via* coenzyme-A thioester and aldehyde intermediates.

The biosynthesis of lignin from the monomeric phenylpropane units can be generally described as a dehydrogenative polymerization. Freudenberg and co-workers elaborated the original ideas on this pathway. They were the first to produce an *in-vitro* lignin called DHP (dehydrogenative polymerizate) by treating coniferyl alcohol with a fungal laccase or with horseradish peroxidase and hydrogen peroxide [2, 4, 5].

In this generally accepted scheme, the first step in lignin polymerization is dehydrogenation of the phenolic hydroxyl group of the *p*-hydroxycinnamyl alcohol precursor derivatives, which is initiated by hydrogen peroxide and catalysed by peroxidase. The complexity

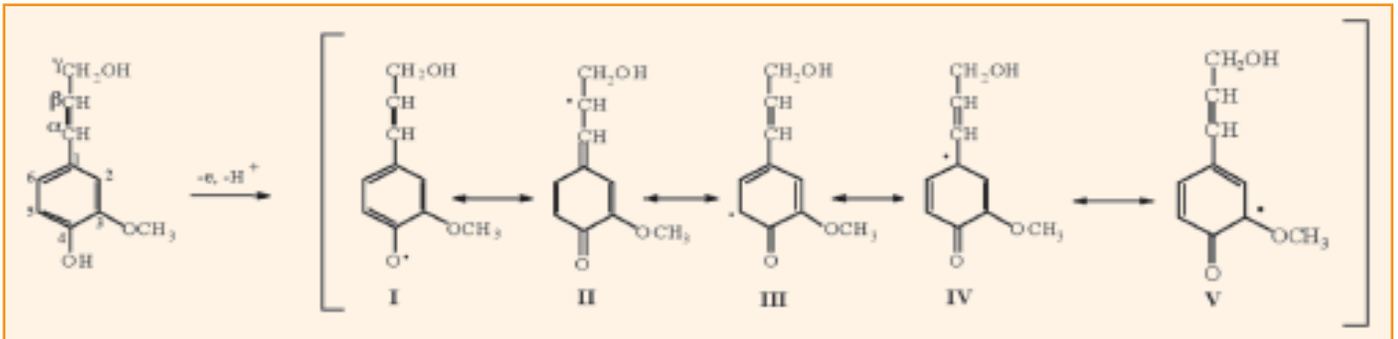

Fig. 1 - Enzymatic dehydrogenation of coniferyl alcohol and related phenoxy radicals

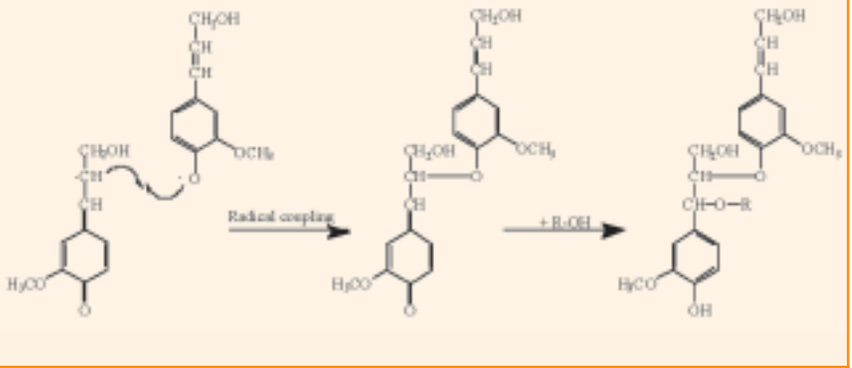

Fig. 2 - Formation by radical coupling of a β-O-4 quinone methide followed by ionic addition of a R-OH molecule

of the evolving dehydrogenative polymer results from the coexistence of five mesomeric forms of the phenoxy radical, as illustrated in Figure 1 in the case of coniferyl alcohol dehydrogenation. Coupling of these radical forms leads to the formation of a statistical polymer in which the frequency of the inter-unit linkages depends on the electron spin densities of the reactive sites.

Actually only four phenoxy radicals (I-IV) are involved in lignin biosynthesis, as V is sterically hindered and thermodynamically disfavoured [6] The principal coupling modes of the radical are shown in the Table.

The relative electron densities determine the frequency of the different sites involved in the coupling reactions. From quantum mechanical calculations it has been deduced that all phenoxy radicals have the highest π-electron densities at the phenolic oxygen atom thus favouring the formation of aryl ether linkages such as the β-*O*-4 linkage, the most frequent type of bond in softwood and hardwood lignins [6].

A non-radical ionic coupling reaction in lignin formation is the addition of transient quinone methide (Fig. 2) to water or phenolic groups [4].

Schematic models describing lignin as a heterogeneous polymer formed by random dehydrogenative polymerization, have been proposed by Nimz [7], Adler [2], Glasser *et al.* [8], Sakakibara [9], Hwang *et al.* [10]. However, many questionable points still exist in this radical polymerization mechanism and several reports have been published, suggesting an enzymatic controlled polymerization process giving a more ordered lignin structure [11, 12].

As a matter of fact, as reviewed by Goring [13], some characteristics of lignins are compatible with the paradigm of a random three-dimensional network polymer (such as non-cristallinity, insolubility, conformation of the macromolecule in solution) while others fit the concept of a more ordered structure. For example the molecular weight distribution of lignins, with several maxima, suggests a repetitive pattern of weak links, which allows the polymer to be degraded into separate families of molecules. Even the structural differences of lignins depending on the cell region (middle lamella *vs.* secondary wall) suggest a difference in the biosynthetic sequence of lignification in plant tissue. This evidence is not compatible with the paradigm of an infinitive random three-dimensional network polymer, which implies a uniform chemistry throughout.

The lignified cell wall is formed by the successive deposition of pectin, cellulose, hemicellulose and lignin to form a composite material where the three polymers are physically and chemically associated in a biochemically regulated manner. This process has been compared to the formation of reinforced concrete because it forms rigid cell walls by combining the tensile strength of cellulose with the compressive strength of lignin.

Terashima *et al.* [14] postulated a comprehensive structural model of cell wall lignification where the individual cell controls the deposition of polysaccharides and lignin. The primary cell wall is the first layer deposited and consists of cellulose microfibrils and hemicelluloses. Subsequently, secondary thickening inside the primary wall forms the secondary cell wall. X-ray crystallography studies revealed that the orientation of the cellulose microfibrils appears to be random in the primary cell wall but fixed in the outer (S_1), middle (S_2), and inner (S_3) layers of the secondary cell wall [15]. The intercellular layer, which separates the walls of adjoining cells, is

Principal coupling modes in the coniferyl alcohol dimers formation

	I	II	III	IV
I	Unstable peroxide	β-O-4	4-O-5	1-O-4
II	β-O-4	β - β	β - 5	β - 1
III	4-O-5	β - 5	5 -5	1-5
IV	1-O-4	β - 1	1-5	1-1

called the middle lamella (ML).

The first stage of lignification starts at the cell corners and middle lamella after the deposition of carbohydrates in the primary cell wall and just before the formation of S_1 begins [16]. The thickness of the middle lamella is more than 100 nm and, according to Goring, the lignin formed in this region is a three-dimensional gel which is non-crystalline and optically inactive [15]. During the second stage, cellulose microfibrils are deposited in the S_2 layer and subsequently lignification proceeds while the S_3 layer is formed. Because of geometric constrains imposed by the cellulose microfibrils, lignin in the secondary wall is probably a non-random two-dimensional network polymer of about 2 nm thickness [15]. The deposition of precursor monomers changes with type, age and morphological region of the cell.

The lignin macromolecule cannot be described by a simple combination of one or a few monomeric units linked by one or a few types of linkages as it is in the case of cellulose or polyoses: it follows that its structure is still a matter of models. The first lignin model was proposed by Freudenberg in 1968 [4]: it is based on the dehydrogenative polymerization concept and fulfils all the analytical data available at that time. This scheme, described for spruce lignin, represents 18 phenylpropane units as a section of the total molecule, which was assumed to consist of more than 100 units in the native state (Fig. 3). Later on, Adler proposed a structural scheme for spruce lignin comprising 16 prominent C_9-units, mainly derived from the results of oxidative degradation experiments (Fig. 4) [2].

In such a fragmentary scheme it is unavoidable that certain structural units and linkages cannot be accounted for exactly. Thus, for example, the appearance of one syringyl unit (nucleus “13” in Fig. 4) is not quantitative, and the pinoresinol unit (nucleus “10” in Fig. 4) probably overemphasizes this structural element.

Apart from the lignin models describing portions of the lignin macromolecule, obtained by random manual coupling of substructures and functional groups based on experimentally identified linkages, the largest softwood lignin structural model was evaluated by simulating lignin formation by computer assisted design and calculation. The original model reported by Glasser and Glasser [16] was mainly based on simulation of radical coupling reactions of the *p*-hydroxycinnamyl alcohols, and was then extended and improved in detail [17].

All the structural models of lignin are based on a wide array of analytical information obtained from investigation both on isolated natural lignins and *in vitro* biosynthetic lignin models (DHPs). Among the methods involved in the structural description there are elemental analysis, sugar and ash determination, ^{1}H-NMR spectroscopy for the determination of functional groups, oxidation experiments followed by GC/MS, and gel permeation chromatography (GPC). In general structural analysis of the lignin polymer at the molecular level is mostly performed by destructive analysis methods in which the isolated lignins are depolymerized to pro-

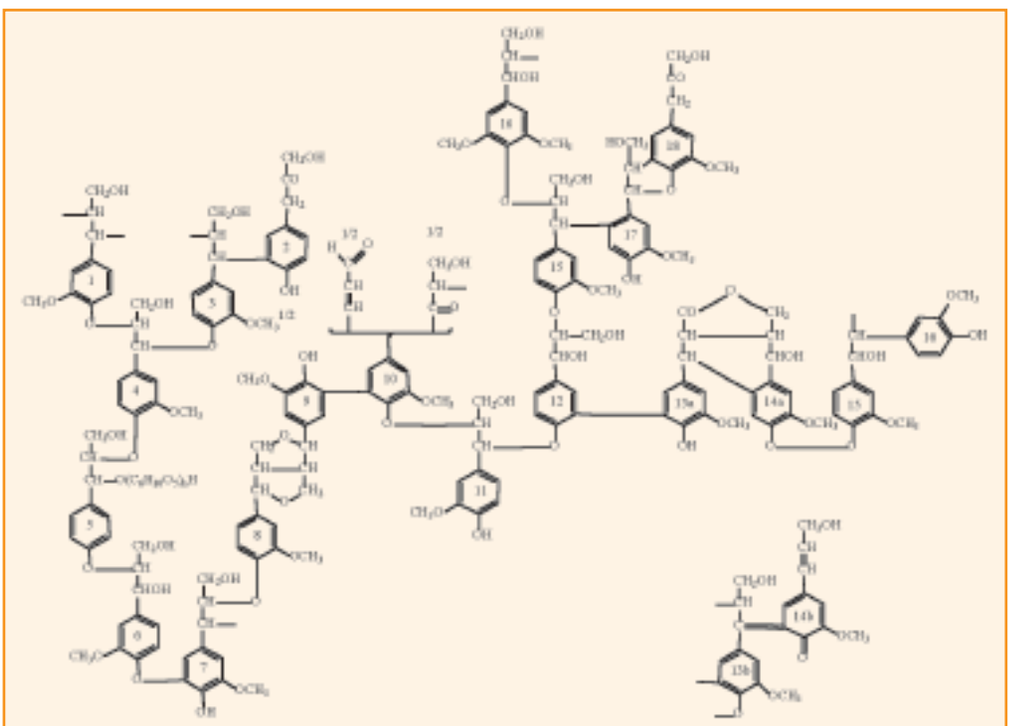

Fig. 3 - Proposed structural model of spruce lignin by Freudenberg (modified from [4])

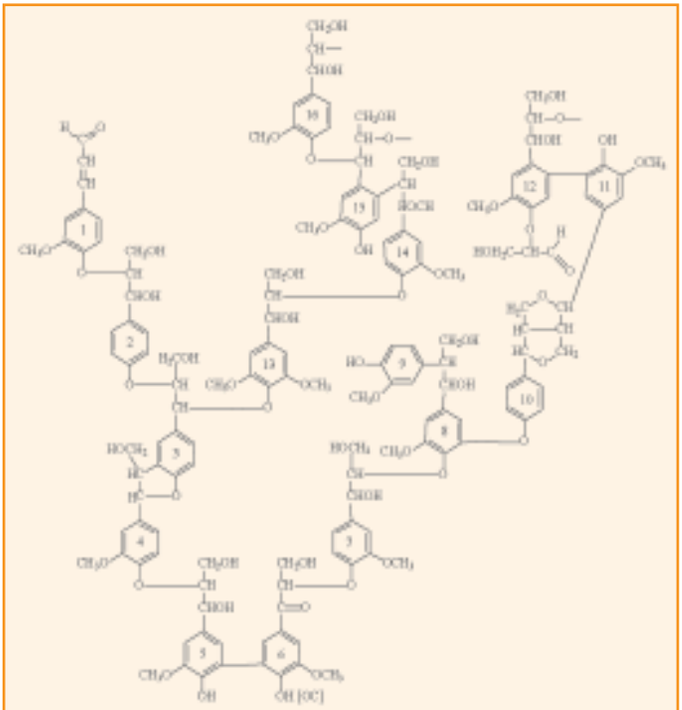

Fig. 4 - Structural scheme of a spruce lignin according to Adler (modified from [2])

duce polymer fragments that provide structural information on the original polymer structure.

One of the main problems in the study of lignin is the difficulty in its isolation from the other components of wood without damaging its structure. In fact, due to the properties of lignin associated with its molecular structure and localization within the cell wall, the isolation of unchanged lignin and its thorough structural determination have not yet been obtained. All isolation methods have the disadvantage of changing fundamentally the native structure of lignin; isolation by acid hydrolysis gives, for instance, a structurally modified lignin. Also the classical method of quantifying the lignin content in wood, which is based on carbohydrate hydrolysis with 72% sulfuric acid giving lignin as a residue (Klason lignin) [18], is only limited to the quantitative determination of the lignin content, since the structural integrity of the lignin polymer is completely destroyed. On the other hand, solvent extraction gives only portions of the lignin macromolecule, even if relatively unchanged.

The methods able to afford an essentially chemically unchanged lignin are only three:

1) extraction with organic solvents, suggested by Brauns [19, 20], to give the so-called “Brauns lignins” (low yields);

2) solvent extraction after an extensive grinding, introduced by Björkmann in 1956 [21], to give the so-called “Milled Wood Lignin (MWL)”;

3) to increase the amount of solubilized lignin, the finely ground wood meal can be treated with hydrolytic enzymes for removal of associated polysaccharides prior to solvent extraction: in this way it is possible to extract the so-called cellulolytic enzyme lignins (CEL) [22].

The main analytical approaches in lignin characterization are essentially two; both of them make an extensive use of mass spectrometry [23]:

- degradative studies, where the macromolecule is fragmented (either thermally or chemically) to its constituent building blocks, and the resulting products are analyzed by gas chromatography (GC) and/or MS. In that approach, MS has a prominent role in the identification of the degradation products. As a first observation, it is important to outline that thermal (pyrolysis) and chemical (CuO oxidation) degradation studies do not cause any scrambling rearrangement of the carbon skeleton within the lignin degradation products. It has been demonstrated in fact, by the isotope-ratio monitoring (IRM)-GC-MS technique that, by using a ^{13}C-labelled coniferyl alcohol incorporated into *Ginkgo biloba L.* lignin, the label is retained in its original position on the side chain, after the pyrolytic or oxidative treatment [24]. That study gives strong support to analytical pyrolysis and chemolysis as viable techniques for structural investigations on lignins. Conversely, it should be pointed out that uncertainties abound, concerning the confidence on the degradation products as representative of the actual building blocks of the macromolecule. In particular the possibility that secondary reactions occur, which involve rearrangements and further degradation of primary products, has been demonstrated [24];
- a series of investigations where lignins have been analyzed without previous degradation. These studies have been performed classically by means of NMR spectroscopy and, in the last years, by means of soft-ionization mass spectrometric techniques which enable structural information to be derived from the “intact” macromolecule. Solution ^{1}H- and ^{13}C-NMR have been extensively used to characterize the soluble part of lignin polymers [25, 26]. Solid-state ^{13}C-NMR has been shown able to provide quantitative analysis of some of the key structural features of lignin polymers in plant tissues

and isolated lignin samples [27, 28], but the interpretation of the ^{13}C-NMR spectra depends entirely on the availability of lignin model compounds. Even if NMR spectroscopy allows non-destructive characterization of the lignin macromolecule, this technique is hindered by the difficulty in making unequivocal assignments to each signal, due to the extensive overlapping of lignin and carbohydrate resonances and, in the case of ^{13}C solid-state NMR, to low sensitivity. In addition, NMR spectroscopy does not allow MW determination. On the other hand, soft-ionization mass spectrometric techniques enable, in principle, MW determination of the whole lignin molecules and, by collision-induced fragmentation, their structural characterization.

Lignin molecular weight

Molecular weight determination of lignin is still a questionable matter. This is caused by several factors such as multiplicity of lignin isolation procedures, degradation of the macromolecule during isolation, condensation effects (especially under acidic conditions), pronounced polidispersity of all solubilized lignins, insufficient determination methods with regard to the polydisperse character of isolated lignins, uncertainties about the behaviour of lignins in solution; all these factors contribute to complicate the calibration systems.

Björkman [21, 29] was the first to determine a $\bar{M}w$ of 11,000 Da for spruce milled wood lignin (*Picea abies*). A comparable $\bar{M}w$ value of 11,000 Da ($\bar{M}w/\bar{M}n$ = 3.1) was then reported for pine MWL (*Pinus taeda*) [30]. For softwood milled wood lignin higher molecular weights of 15,000 Da and 21,000 Da have been reported [31, 32]. The highest $\bar{M}w$ values are reported for analytical lignins: 77,000 Da for an enzymatically isolated MWL of Eastern hemlock (*Tsuga canadensis*) and 85,000 Da for a dioxane spruce lignin fraction (*Picea glauca*) [33].

By means of improved column chromatographic systems and calibration techniques more detailed information have been obtained with regard to lignin molecular weight and size distribution, clearly demonstrating the typical polidispersity of all types of lignins [34]. Also, bimodal patterns of the molecular weight distributions covering large ranges are typical of most of the investigated lignins.

The question as to whether association effects between lignin fragments, the elution solvent and the column bed-material influence the elution profiles, still remains not clarified. In fact, complex interaction phenomena between lignin molecules or between lignin and the column gel can cause problems in Size Exclusion Chromatography (SEC) [35].

It has been found that the intermolecular associations of lignins are small when tetrahydrofuran (THF) is used as the mobile phase [36]. The lignin interactions with the styrene-divinylbenzene copolymer gels can, however, still be strong when THF is used. In order to overcome the problem, recently an ion-pair SEC method was suggested for lignin analysis. This method employed styrene-divinylbenzene gel columns and a quaternary amine in THF as the mobile phase [37].

Another problem associated with the SEC analysis of lignins is the choice of the calibration substances. Calibration in SEC consists of obtaining an expression that correlates the elution time to the molecular weight distribution curve. This curve is normally obtained using standards chemically similar to the analyte. In the case of lignins it is still questionable if the conventionally used linear polystyrene standards have similar elution behaviour as lignins themselves. Indeed, molar mass determination of an unknown lignin sample dimensionally calibrated by using, as reference, lignins structurally determined by low angle laser light scattering (LALLS) analysis was found to deviate considerably from the results obtained by THF-based SEC-system, calibrated by using polystyrene standards [38].

The use of a LALLS detector coupled on line with a SEC column has been suggested for lignin analysis [39, 40]. However, the UV-absorptivity and fluorescence of lignins complicates the molar mass determination by LALLS [41].

More recently, Metzger *et al*. [42] showed that individual lignin species could be identified and quantified up to a molecular weight of as high as 50,000 Da by the use of MALDI mass spectrometry. Also in the case of the mass spectrometric analysis of lignin, its polidispersity gives origin to many troubles: actually, only for narrow lignin fractions ($\bar{M}w/\bar{M}n$ from 1.01 to 1.07) it is possible to evaluate the absolute molar mass by MALDI-TOF MS. It follows that for wide lignin distributions the analysis by MALDI-TOF/MS requires a previous separation of the lignin fraction by off-line size-exclusion chromatography [43].

As already pointed out, there are no uniform values for molecular weight of both analytical and technical lignins due to their heterogeneity, depending on the isolation or pulping processes; however, the multitude of results reveal that the broad molecular weight distributions of lignins are not an effect of separation methods but essentially they are a demonstration of the polydisperse character of isolated lignins.

Lignin utilization

Lignin is the most abundant aromatic biopolymer on earth and therefore represents a potential renewable resource for the preparation of industrially important chemicals.

Worldwide, about $50 \cdot 10^6$ tons/year of modified lignin polymers are produced as by-products from wood at pulp mills. However up to now, conversion procedures aimed at producing value-added products from lignin are not considered economically competitive with processes for the same chemicals based on petroleum. It is estimated that only 2% of lignin produced by the pulp industry is recovered for by-product purposes: the remainder is discarded or incinerated [44].

Commercial lignin is currently produced as a secondary product of the paper industry, being separated from wood by a chemical pulping process. Lignosulfonates (also called lignin sulfonates or sulphite lignins) are products of the sulphite pulping process [45]; in particular Kraft lignins (also called sulphate lignins) are obtained by the "kraft" pulping process. The usefulness of commercial lignosulfonates products comes from their dispersing, binding, complexing and emulsifying properties [46]. Other delignification technologies make use of organic solvents or a high-pressure steam treatment to remove lignins from plants.

Industry first began to use lignins in the 1880s, when lignosulfonates were used in leather tanning and dye baths. Since then, lignosulfonates have found applications even in food products, serving as emulsifiers in animal feed and as raw materials in the production of vanillin. Lignin uses have expanded into literally hundreds of applications, impacting on many facets of our daily lives. For instance lignosulfonates are very effective and economical adhesives, acting as a binding agents or "glue" in pellets or compressed materials. Lignosulfonates used on unpaved roads reduce environmental concerns from airborne dust particles and stabilize the road surface. Their binding ability make them useful components of coal briquettes, ceramics, fertilizer and herbicides, dust suppressants, animal feed pellets, fibreglass insulators, soil stabilizers and so on.

Moreover lignosulfonates can act as dispersants, preventing clumping and settling of undissolved particles in suspension. Their dispersing property also make them useful in cement mixes, clay and ceramics, dyes and pigments, leather tanning, etc. Also, lignosulfonates stabilize the emulsion of immiscible liquids, making them highly resistant to breaking. Additionally they are at work as emulsifiers in asphalt emulsions, pigment and dyes, pesticides and wax emulsions. Lignosulfonates can tie up metal ions keeping them in solution, thus preventing them from reacting with other compounds and becoming insoluble, eventually. In agriculture, metal ions sequestered by lignosulfonates remain available to plants. Finally, lignosulfonates are used in micronutrients systems, cleaning compounds, water treatment for boilers and cooling systems.

As we have just seen most lignin used today for non-fuel applications is used in the form of chemically modified copolymers in adhesives and binders, dispersants and soil applications. The main negative factors involved in the chemical utilization of lignin include its molecular mass heterogeneity and the three-dimensional structure, which cause high fractionation and modification costs to make them amenable to industrial applications.

At the moment lignin from biomass is still an attractive renewable raw material for the future. The ever increasing environmental restrictions and the depletion of fossil fuels will gradually promote the use of lignin and lignin derivatives as raw materials at a large industrial scale.

Il paradigma lignina

ABSTRACT

La lignina, dopo la cellulosa, è la più abbondante sostanza naturale di origine polimerica presente nelle piante. Nella cellula vegetale essa ricopre ruoli fondamentali nel trasporto di acqua e nutrienti, assicurando al tempo stesso alla parete cellulare rigidità e robustezza. Nonostante la sua importanza nella fisiologia vegetale e la sua grande disponibilità in quanto prodotto secondario nell'industria della carta, la struttura del polimero lignina risulta, ancora per molti punti, del tutto oscura. La struttura della lignina non è stata ancora descritta nei dettagli soprattutto a causa delle difficoltà che si incontrano nell'isolare la lignina dal legno. Le procedure di estrazione e di purificazione portano, infatti, inevitabilmente alla degradazione del polimero nativo; è inoltre da sottolineare come le caratteristiche strutturali delle lignine isolate dipendano fortemente dai protocolli di estrazione. Anche le complesse vie biosintetiche, che la formazione della lignina segue nelle piante, risultano al momento poco note.

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