

Al Pianeta Terra

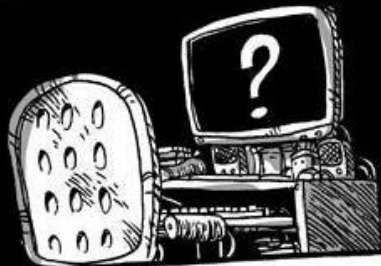
“Chi dice che è impossibile non dovrebbe disturbare chi ce la sta facendo”

(Albert Einstein)

“Se in principio l'idea non è assurda, non ha speranza”

(Albert Einstein)

LA FASCIA ORARIA DELLE BERMUDE È QUELL'ARCO DI TEMPO TRA LE NOVE DI SERA E LE DUE DI NOTTE IN CUI PERSONE ALL'APPARENZA NORMALI SI SIEDONO DAVANTI AL COMPUTER ED IN UN BATTITO DI CIGLIA SI ACCORGONO CHE SONO INSPIEGABILMENTE TRASCORSE CINQUE ORE, INGHIOTTITE IN UNA FITTA COLTRE DI NEBBIA ED OBLIO, CHE PREGIUDICA CONSEGNE, ESAMI, SALUTE...



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DIPARTIMENTO DI SCIENZE ECOLOGICHE E BIOLOGICHE

DOTTORATO DI RICERCA IN BIOTECNOLOGIE VEGETALI

XXV CICLO

**BIOTECHNOLOGICAL PROCESSES FOR
PLANT POLYPHENOLS UPGRADING**

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Dottoranda: FEDERICA MELONE

Coordinatore: Prof.ssa Stefania Masci

Tutor: Prof. Raffaele Saladino

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1. INTRODUCTION

1.1 GREEN CHEMISTRY: SUSTAINABILITY AS FUNDAMENTAL PRINCIPLE

After the Second World War mankind watched a substantial growth of consumption. The upgrading of chemical industries, developed in the beginning of the last century, flooded the market with a multitude of products that improved the quality of life, from the pharmaceutical field and food processing to every-day items as well as the highest technologies. The rapid advances in most parts of the different research fields had negative environmental effects over time, however, and chemical industry became the symbol of the pollution caused by human activities. Because of a series of accidents – 1976, Seveso (Italy); 1978, Love Canal (USA); 1984, Bophal (India) – in the last 30 years the first “environmental laws” were introduced to protect human health and the environment.

During the new century, “sustainable development” became the keystone of technological advances, the chemical research put its efforts into the replacement of old technologies by new “eco-friendly” processes.

Since 1991, Paul Anastas, professor at Yale University and theorist of the “green chemistry” philosophy, claimed that the goal of “green chemistry” is the development of new and radically changed methodologies for a safe, efficient and eco-sustainable production cycle, ranging from synthesis to waste management.

His theories were included in his “12 principles of green chemistry” which became the manifest for eco-friendly chemistry, and which significantly modified the landscape of chemical industries around the world.

The 12 principles are here summarized:

- 1: It is better to prevent waste than to treat or clean up waste after it is formed.
- 2: Synthetic methods should be designed to maximize the incorporation of all materials used in the process into the final product.
- 3: Wherever practicable, synthetic methodologies should be designed to use and generate substances that possess little or no toxicity to human health and the environment.

- 4: Chemical products should be designed to preserve efficacy of function while reducing toxicity.
- 5: The use of auxiliary substances (e.g. solvents, separation agents, etc.) should be made unnecessary wherever possible, and innocuous when used.
- 6: Energy requirements should be recognized for their environmental and economic impacts and should be minimized. Synthetic methods should be conducted at ambient temperature and pressure.
- 7: A raw material or feedstock should be renewable rather than depleting wherever technically and economically practicable.
- 8: Reduce derivatives - Unnecessary derivatization (blocking group, protection/deprotection, temporary modification) should be avoided whenever possible.
- 9: Catalytic reagents (as selective as possible) are superior to stoichiometric reagents.
- 10: Chemical products should be designed so that at the end of their function they do not persist in the environment and break down into innocuous degradation products.
- 11: Analytical methodologies need to be further developed to allow for real-time, in-process monitoring and control prior to the formation of hazardous substances.
- 12: Substances, and the form of a substance used in a chemical process, should be chosen to minimize potential for chemical accidents, including releases, explosions, and fires.

Applying these green chemistry principles, an organic solvent should be removed or replaced with an aqueous solution; biomasses should be used as starting materials instead of petrochemical by-products; catalytic procedures should replace stoichiometric reagents; all efforts should be focused on the synthesis of non-toxic and biodegradable compounds that maintain the same suitable properties of natural products.

In particular, sustainable development was defined as 'Development that meets the needs of the present without compromising the ability of future generations to meet their own needs'.¹

Many people think that applying the twelve principles is a necessary, but not sufficient condition for the "greening" of the chemical industry; to realize a new, totally

ecological system it would be necessary to modify the situation deep down...But this would be a political matter.

1.2 BIOTECHNOLOGY AND BIOCATALYSIS: ENZYMES IN INDUSTRIAL APPLICATIONS

The most important subject finely integrated into green chemistry principles is biotechnology. Biotechnology is a multi-disciplinary field, with roots in the areas of chemistry, engineering and a wide part of biology, including microbiology and immunology.

Among the biotechnologies, biocatalysis certainly plays a predominant role.

Biocatalysis, that can be commonly defined as the use of biological molecules - usually enzymes - as catalysts, has many attractive features with respect to green chemistry, and its impact is thus expected to grow.

The use of enzymes absolutely concurs with the “greening” of the industrial processes since they are natural catalysts present in every living organism that carry out a wide variety of chemical reaction. These molecules work in aqueous systems and under mild conditions of pH, temperature and pressure; moreover, they do not produce secondary toxic metabolites and by-products.

More and more chemical companies are looking into biocatalysis to improve the sustainability of their manufacturing; for example, the pharmaceutical industry is quickly replacing old processes with new and sustainable strategies including enzymatic catalysis to reduce the large amount of solvents, the number of steps, and the extensive purifications required in drug synthesis.

Enzymes are proteins having a catalytic function; they have the capability to increase the reaction rate, up to a million times. Just as conventional catalysts, they cause a lowering of the activation energy ($\Delta G^\ddagger$) (Figure 1.1) stabilizing the transition state of the reaction or providing an alternative reaction pathway characterized by a lower energy consumption.²

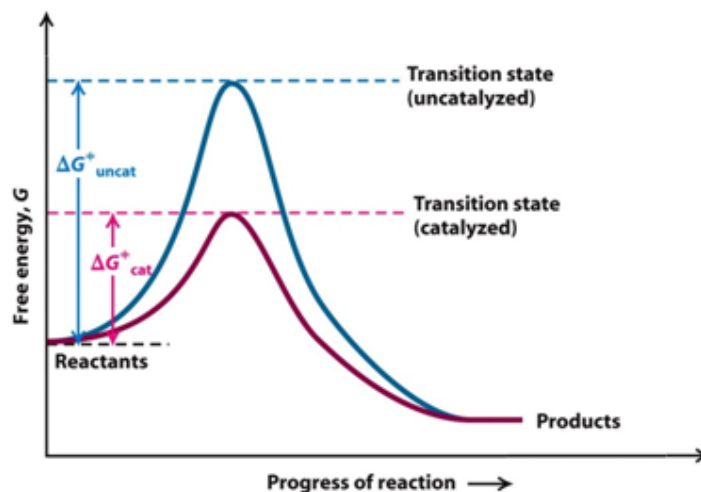


Figure 1.1: Lowering of activation energy caused by catalysts.

The efficiency of enzymes, their specificity, high catalytic activity, and the opportunity of operating in eco-friendly conditions, makes enzymes a precious biotechnological tool to replace conventional catalysts.

Biocatalysis, or, in other words, the employment of enzymes, is not a new technology as such, but it is a tool used for millennia in the production of beer, wine, vinegar, yoghurt and cheese: the Egyptians, Sumerians and Babylonians, for example, produced alcoholic beverages from barley.³

Nowadays enzymes are widely employed by chemical industry for several industrial applications.

Laundry detergents contain proteinases, lipases, amylases and cellulases for the digestion of oils and fats and to remove resistant residues; starch industry uses amylases, amyloglucosidases, glucoamylases and glucose isomerase to convert starch into glucose, and other sugars.⁴ Dairy industry employs lipases and lactases in the manufacture of cheese, to convert lactose to glucose and galactose, while textile industries need amylases to remove starch from woven fabrics. Baking industry needs α -amylase, β -xylanase and proteinases in the manufacture of bread to reduce the protein content in flours and to enhance the breakdown of starch in flours; pulp and paper industry employs β -xylanases, ligninases and cellulases to enhance pulp-bleaching and to degrade lignin and starch.

Nevertheless, the enzymes used for industrial processing do not have long-term stabilities under the reaction conditions employed; their recovery and reuse are often difficult; and together with the costs of isolation and purification, in combination with their sensitivity to environmental conditions, thus represent serious drawbacks on the way to a widespread industrial use of enzymes.

1.3 IMMOBILIZATION TECHNIQUES: METHODS AND ADVANTAGES

An approach to overcome some of these constraints is the use of immobilized enzymes. The immobilization of an enzyme is provided by “confining” it onto the surface or inside an inert matrix in order to obtain insoluble particles.⁵

Immobilization provides several advantages:

- increase of enzyme stability
- increase of enzyme resistance to reaction conditions and environmental changes
- modulation of catalytic properties
- protection from microbial or protein contamination
- easier separation of the product and recovery of the catalyst
- opportunity of multiple reuses of the enzymatic catalyst.

One of the first and well-known application of an immobilized enzyme in an industrial processes was the production of 6-aminopenicillanic acid (6-APA) by means of hydrolysis of penicillin G: the production yield was 600 kg of 6-APA per kg of immobilized enzyme.⁶

The opportunity to work with enzymes firmly bound to an inert matrix allows an efficient separation of the catalyst from the reaction cocktail, avoiding undesired contaminations of the product.

Nevertheless, the immobilization can compromise enzyme activity: this alteration results from possible structural changes of the native form during the immobilization procedure that inhibit or even inactivate the biocatalyst.⁷

In spite of these disadvantages, the creation of a microenvironment may allow the enzyme to remain active at different temperatures or pHs than would be predicted for the native enzyme, increasing the application possibilities.⁸

Because of the variety of enzymes and their different chemical features, since 1980 several strategies for enzymes immobilization have been developed. They can be classified in physical and chemical strategies on the basis of the type of bond between the enzyme and the matrix. The physical strategies allow the enzyme to be linked to the matrix by means of weak interactions (van der Waals interactions, hydrogen bonds). In the chemical methods, the enzyme is covalently linked to an insoluble matrix: the linkage can occur directly on the support (if it has suitable functionalities) or, otherwise, by means of multifunctional linkers that act as a bridge between the matrix and the biocatalyst⁹.

Generally, for each enzyme there is an opportune methodology that does not modify the chemical structure, preserving its enzymatic activity at best. The choice of the inert support to bind the enzyme plays an important role in retaining of the tertiary structure that is critical for the activity, as well as for the thermal stability of the biocatalyst. Moreover, the anionic or cationic nature of the inert matrix can provoke a sensitive shift of the optimum pH of the enzyme, extending or modifying the pH range in which the enzyme can work effectively.¹⁰

1.3.1 PHYSICAL METHODS

The common physical methods are based on encapsulation, entrapment or adsorption of the biocatalyst into, or onto the inert matrix.

ENCAPSULATION:

This method serves to cover the enzyme by a semi-permeable coating that shields it from the environmental conditions, allowing its catalytic functions to be fully retained.¹¹ (Figure 1.2) The most commonly used materials for enzyme encapsulation are amino polymers such as polyethyleneimine. Unfortunately, physical encapsulation is not suitable for drastic reaction conditions.

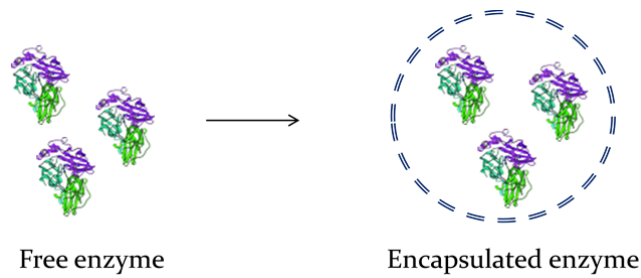


Figure 1.2: Encapsulation method.

ENTRAPMENT:

This method incorporates the enzyme into a porous matrix,¹² usually a polymeric network, that can be organic, inorganic, or a membrane device such as a hollow fiber (Figure 1.3). The network structure and the polymer porosity of the matrix can be adjusted modifying the polymerization conditions.¹³

Although this method prevents direct contact between the enzyme and the potentially denaturing environment, it confers only a weak bonding to the biocatalyst, thus frequently causing an unavoidable leakage.¹⁴

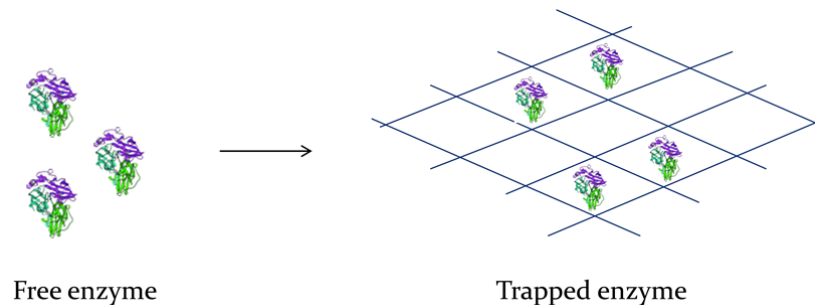


Figure 1.3: Entrapment method.

ADSORPTION:

This relatively simple method lies in the immersion of the matrix in a solution of the enzyme for a sufficient time, allowing the physical adsorption of the enzyme onto the surface.⁹ (Figure 1.4)

Although simple and cheap, this method is not so advantageous because the environmental conditions make the matrix-enzyme interaction reversible.¹⁵

Physical methods are generally advantageous because they do not induce structural modification of the enzyme, thus not interfering with its activity, but they are unstable under drastic reaction conditions.

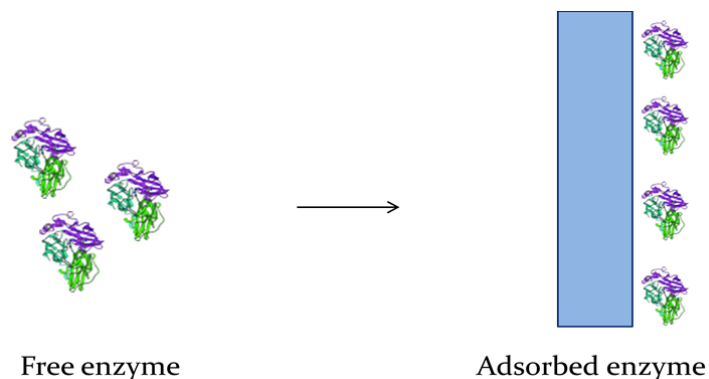


Figure 1.4: Adsorption method.

1.3.2 CHEMICAL METHODS

These methods are characterized by chemical linkages, covalent or ionic, between the enzyme and the matrix:

COVALENT LINKAGE:

This method commonly uses a water insoluble matrix, which is characterized by reactive functionalities that link the enzyme without compromising its catalytic activity. Usually the linkage is established via the amino groups of particular aminoacids on the surface of the protein; typically lysine residues react preferentially (Figure 1.5). Eupergit is an example for a matrix that can bind directly the free amino groups of the enzyme by means of its oxyranlyl functionalities.^{16, 17}

It is possible to have a loss of the catalytic activity when the covalent binding provokes significant alteration of the conformational structure of the enzyme.

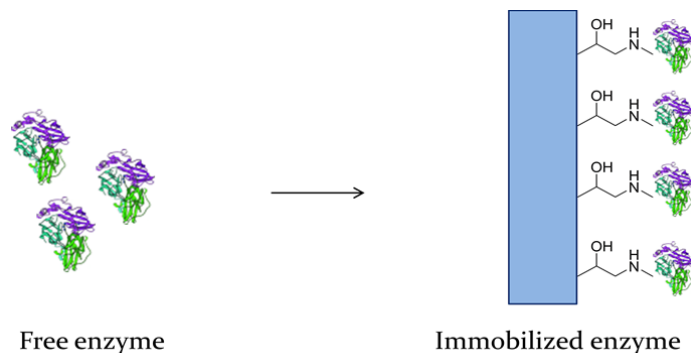


Figure 1.5: Covalent linkage method.

IONIC LINKAGE:

Unlike the covalent binding, the ionic interaction between the enzyme and the water-insoluble matrix does not modify the conformational structure of the catalyst, thus more likely preserving enzyme activity (Figure 1.6). This kind of linkage is weaker than the covalent binding but it is stronger than the adsorption within a physical interaction. The matrices commonly used for this strategy are chitosan, agarose and dextran.¹⁸

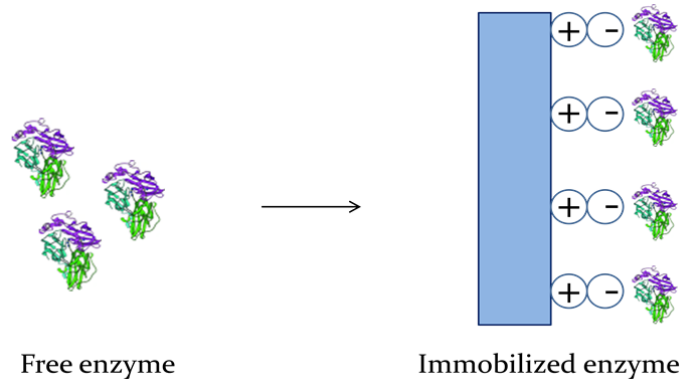


Figure 1.6: Ionic linkage method.

CROSS-LINKING STRATEGY:

When the water insoluble matrix has not opportune reactive functionalities to bind the enzyme, the immobilization takes place thanks to a multifunctional linker that act as a bridge between the matrix and the enzyme. A common linker widely used in the

immobilization strategies targeting enzymes is glutaraldehyde, employed to connect the enzyme to alumina particles prefunctionalised with an amino group carrying siloxane (Figure 1.7).¹⁹

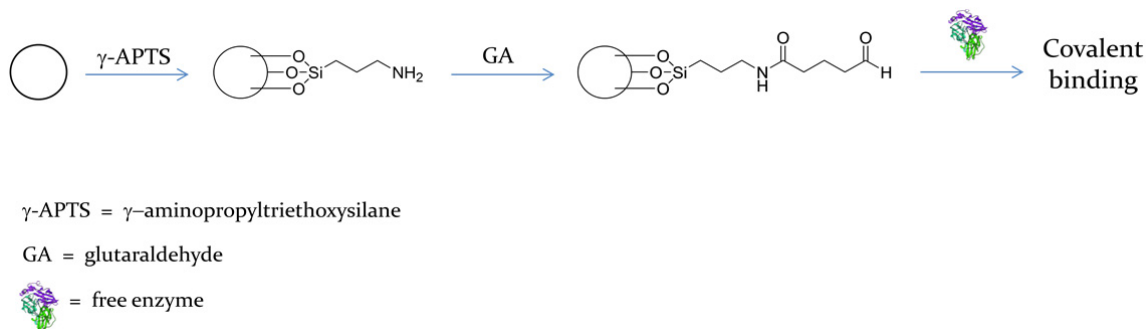


Figure 1.7: Cross-linking method.

1.3.3 THE LAYER-BY-LAYER (LbL) TECHNIQUE

The layer-by-layer (LbL) technique, developed by Decher *et al.*²⁰ in the early 90's, consists in the stratification of ultrathin films of alternatively charged polyelectrolytes onto a solid charged surface. It offers the opportunity to deposit in individual multilayers a wide variety of materials, including polyions, metals, ceramics, nanoparticles, and biological molecules, preserving the deposition sequence.²¹

In particular the deposition of the polyions layers occurs dipping the charged matrix into a solution of the polyelectrolyte, alternatively charged, for few minutes. The consecutive deposition of polyion layers can be repeated several times, washing the matrix after each deposition in order to eliminate the excess of polyelectrolyte (Figure 1.8).

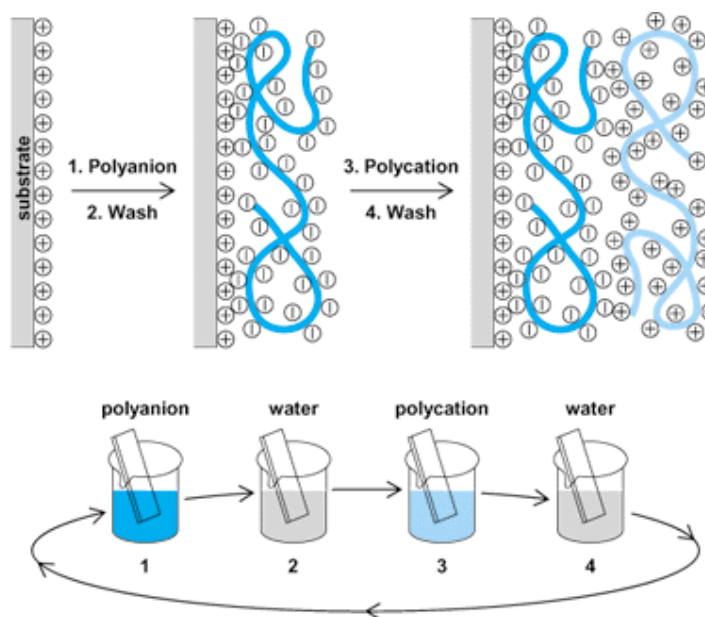


Figure 1.8: Layer-by-layer (LbL) deposition. [(Courtesy of Gero Decher) Copyright © McGraw-Hill Education. All rights reserved]

This technique has become an important tool for enzyme immobilization and finds applications both in physical and chemical methods. In fact, the resulting complex multilayer coating has the ability to protect the enzyme from high-molecular-weight denaturing agents or bacteria, while not preventing the substrate from reaching the catalytic site.²² Moreover, it preserves the protein from high temperature and drastic pH, and avoids the desorption from the support.^{23,24}

Beside applications in biocatalysis, the LbL technique is nowadays employed in electrochemistry, for sensing/biosensing and in electrochromic devices.^{25,26,27,28,29,30}

1.4 FOCUS ON ENZYMES

In the frame of the development of new biotechnological processes, the use of new immobilized enzymes has a pivotal importance for setting up specific functionalization/oxidation processes.

This PhD was focused on the development and study of different families of immobilized and co-immobilized enzymes for the valorization of plant polyphenols, particularly lignins and tannins. More specifically the attention was focused on two

different classes of oxidative enzymes for lignin treatment, namely laccase and peroxidase, and on a hydrolytic enzyme for the valorization of tannins, namely tannase.

1.4.1 LACCASE

OCCURRENCE

Laccases, also called bezenediol: oxygen oxidoreductase (EC 1.10.3.2), are copper-containing enzymes responsible of the oxidation of a great variety of organic compounds, as phenols, polyphenols, methoxy-substituted phenols and aromatic amines, but not tyrosine, with the concomitant reduction of molecular oxygen to water. They were discovered in 1883 by Yoshida during his studies on the exudates of *Rhus vernicifera* (the Japanese lacquer tree), but only 13 years later Bertrand and Laborde demonstrated they were fungal enzymes.³¹ More recently, proteins having typical laccases features have also been found in insects and prokaryotes.^{32,33} In the plant kingdom, laccases have been identified in trees, vegetables, fruits and fungi: among trees, the most studied and common laccases are from *Rhus vernicifera*; laccases from *Acer pseudoplatanus*,^{34,35} *Pinus taeda*,³⁶ *Populus euroamericana*³⁷ and *Nicotiana tobacco*³⁸ are only partially characterized. Among vegetables and fruits they were found in cabbages, asparagus, potatoes, apples, pears, peaches and various others. The majority of laccases, however, are isolated from fungi such as ascomycetes, deuteromycetes and basidiomycetes. Up to date dozens different laccases have been purified from the wood rotting fungi belonging to the genera *Cerrena*, *Coriolopsis*, *Lentinus*, *Pleurotus*, and *Trametes*.³⁹

In the plant kingdom these enzymes have more than one role: plant laccases are involved in the radical-based mechanisms of lignin polymer formation,^{40,41} while fungi-based laccases are involved in morphogenesis, pathogenesis⁴² and lignin degradation.⁴³ Because of the properties of their substrates, fungi laccases are mainly extracellular, but in some species, such as *Phanerochaete chrysosporium*⁴⁴ and *Suillus granulatus*⁴⁵ intracellular laccase activity was also detected. In some species of white-rot basidiomycetes, such as *Irpex lacteus*,⁴⁶ laccase activity is almost exclusively associated with the cell wall. Most probably, the localization of laccases is related to its physiological function, and determines the range of substrates available to the enzyme.

MOLECULAR PROPERTIES

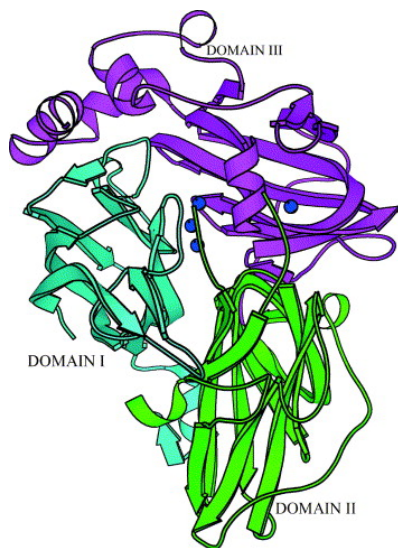


Figure 1. 9: Typical laccase, 3D model.

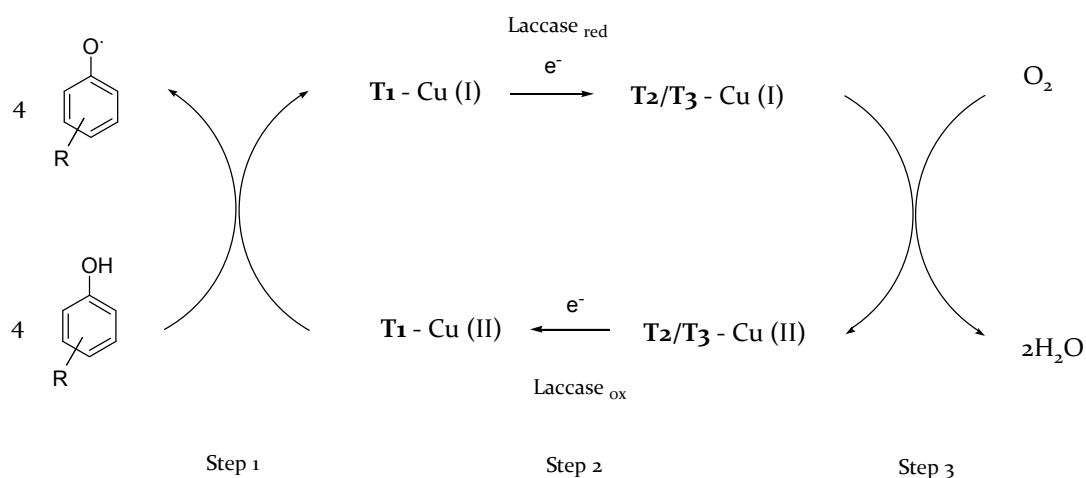
The typical laccase exists as a 50-70 kDa polypeptide containing of about 500.- 600 amino acids. It is characterized by carbohydrates covalently linked to the polypeptide backbone, and that confer the high stability to the enzyme.⁴⁷ The 500 amino acids are assembled in 3 cupredoxin domains, arranged in three spectroscopically different catalytic sites containing copper-types 1 (T₁), 2 (T₂), and 3 (T₃). T₁ is a mononuclear copper centre, paramagnetic, characterized by a strong absorption at 600 nm, and thus responsible for the blue color of the protein. As T₁, T₂ is a mononuclear copper centre, paramagnetic, but it does not show absorption (it is a “non-blue” copper). T₃ is a dinuclear copper centre, EPR silent because of the diamagnetic spin-coupled pair of copper atoms.⁴⁸

Although the majority of laccases is characterized by the described multicopper system, some purified laccases do not show these typical features. Laccases isolated from fungal cultures are not typically blue, but yellow-pale brown because of an altered oxidation state of the copper in the catalytic centre.³¹

CATALYTIC PROPERTIES

Laccases catalyze the oxidation of a great variety of organic substrates with the concomitant reduction of molecular oxygen to water.

During the oxidation process, a sequential transfer of electrons occurs, from the substrate to the T₁ copper, then to the T₂/T₃ systems and finally to molecular oxygen, which results in its reduction to water. The catalytic cycle, divided in these three steps, is shown in (Scheme 1.1).^{49,50,51}



Scheme 1.1: Laccase catalytic cycle.

In the first step the substrate transfers the electrons to the copper in T₁ giving rise to the radical form of the substrate and the concomitant reduction of the metallic centre [T₁-Cu(II) → T₁-Cu(I)]. In the second step the electrons are transferred from the reduced copper T₁ to the trinuclear copper system T₂/T₃. Finally, in the third and last step, oxygen is bound by the T₂/T₃ system, which transfers the electrons causing the reduction of oxygen to water.

The efficiency of the oxidation depends on the difference of potentials between the copper T₁ of the enzyme and the substrate. Both the redox potentials of the substrate and the laccase are sensitive to the pH condition: at high pH values the differences in redox potentials between laccase and the phenolic substrates can increase, but the hydroxide ions would hinder the internal electron transfer between the T₁ and the

T₂/T₃ centre, causing the inhibition of laccase activity.⁵² In general, fungal laccases perform best at pH-values in the range between 3.5 to 5.0, when the substrates are hydrogen atom donor compounds.

APPLICATIONS IN INDUSTRIAL PROCESSES

In the last decades laccases proved to be an important and precious tool for many industrial applications, thanks to their capability to oxidize both phenolic and non-phenolic compounds as well as pollutants. They are widely employed in the pulp and paper, in the textile and in the food and drink industry. They are also used in the medical field, as well as for the detoxification of several aromatic pollutants found in industrial waste..

The paper industry removed lignin from wood by means of chemical or mechanical pulping. The first method degrades lignin structure while the second one consists in physically tearing the fibres apart. Mechanical pulping is cheaper than chemical pulping; however, mechanical pulping, however, yields a lower quality paper ⁵³. A precious alternative process is the “biological” pulping based on the employment of ligninolytic enzymes such as laccases. The use of enzymes in the pretreatment of wood chips reduces the energy requirement. Moreover, in the same process, they can be used as bio-bleaching agents, replacing chlorine and oxygen-based oxidant during the delignification and bleaching of the paper pulp.

In the textile industry laccases, combined with an appropriate mediator, are used for bleaching the indigo dye in denim: this biotechnological process has considerably reduced the water and energy consumption, and has replaced the common and not safe oxidants as ipochlorite.⁵⁴

In the food and drink industry laccase is widely used for wine stabilization in order to prevent the color and flavor alterations. It has the capability of oxidize the phenolic compounds (as polyvinylpyrrolidone) responsible of oxidative reactions both in musts and wines.⁵⁵ In the production of fruit juices, it replaces the conventional treatment of the browning processes based on the employment of ascorbic acid and sulfites.

Laccases have found application also in the medical field: laccase-iodide salt overcomes the direct iodine application in water sterilization (swimming pool) or disinfection of small wounds.

1.4.2 HORSERADISH PEROXIDASE

OCCURRENCE

Peroxidases (EC 1.11.1.x) are oxidoreductases that are able to catalyze the oxidization of a large variety of substances through the reaction with hydrogen peroxide.

Nowadays, there are 15 different EC numbers related to peroxidases, from EC 1.11.1.1 to EC 1.11.1.16, but there are also families with dual enzymatic domains classified with the numbers EC 1.13. 11.44, EC 1.14.99.1, EC 1.6.3.1.⁵⁶ However, they are divided in heme and non-heme proteins, distributed between 11 superfamilies and about 60 subfamilies.

The heme peroxidases can be classified into two big different classes, the animal peroxidases class and the plant peroxidases class, on the basis of the occurrence. The plant peroxidases can be further divided in three subclasses:

Class I, the class of intracellular enzymes as ascorbate peroxidase, catalase and cytochrome c peroxidase;⁵⁷

Class II, the class of fungal peroxidases as lignin peroxidase (LiP) and manganese peroxidase that play an important role in lignin degradation;

Class III, the class of secretory plant peroxidases as horseradish peroxidase, involved in plant cell wall formation and lignification.

Among all peroxidases, horseradish peroxidase (HRP) has received a special attention because of its commercial use and its several applications, above all in medicinal field as component of clinical diagnostic and for immunoassays.^{58,59} It is extracted from horseradish (*Armoracia rusticana*), a perennial plant native to western Asia and south eastern Europe, for its white long roots that, once grated, produce mustard oil. The root of the plant contains a large number of peroxidase isoenzymes among which the isoenzyme C (HRP C) is the most abundant.⁶⁰ The isoenzymes have many functions in plant physiology such as crosslinking of cell wall polymers, lignification and resistance to intracellular infections.

MOLECULAR PROPERTIES

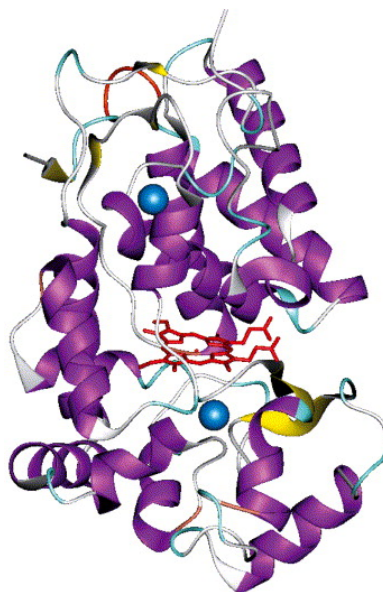


Figure 1.10: Typical peroxidase, 3D model.

Horseradish peroxidase isoenzyme C (HRP C) is a single polypeptide composed of 308 amino acid residues; the total carbohydrates content of HRP C depends on the source from which it is extracted but the typical values of glycosylation ranges from 18 and 22% m/m.⁶¹ The three dimensional structure of the enzyme is mainly composed of α -helical and small region of β -sheet organized in two domains, the distal and the proximal.⁶⁰

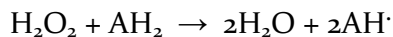
The enzyme contains two different metal centres: one iron protoporphyrin IX and two calcium atoms. The heme group is located between the distal and the proximal domains and is composed of four pyrrole rings, organized in a planar structure, with a five-coordinated iron atom held in the middle.

The open bonding site in axial position is occupied by the imidazole side chain of the proximal histidine residue (His 170); the remaining axial coordination site is vacant during the resting state of the enzyme but is open to attach hydrogen peroxide during the activation.⁶² The bonding of the hydrogen peroxide to the iron atom gives rise to an octahedral configuration, considered the active geometry of the catalytic site.

The two calcium centres, located in distal and proximal positions, are linked to the heme-binding region by a network of hydrogen bonds.⁶³ Both the iron and the calcium centres are essential for the structural and functional integrity of the enzyme.⁶⁴

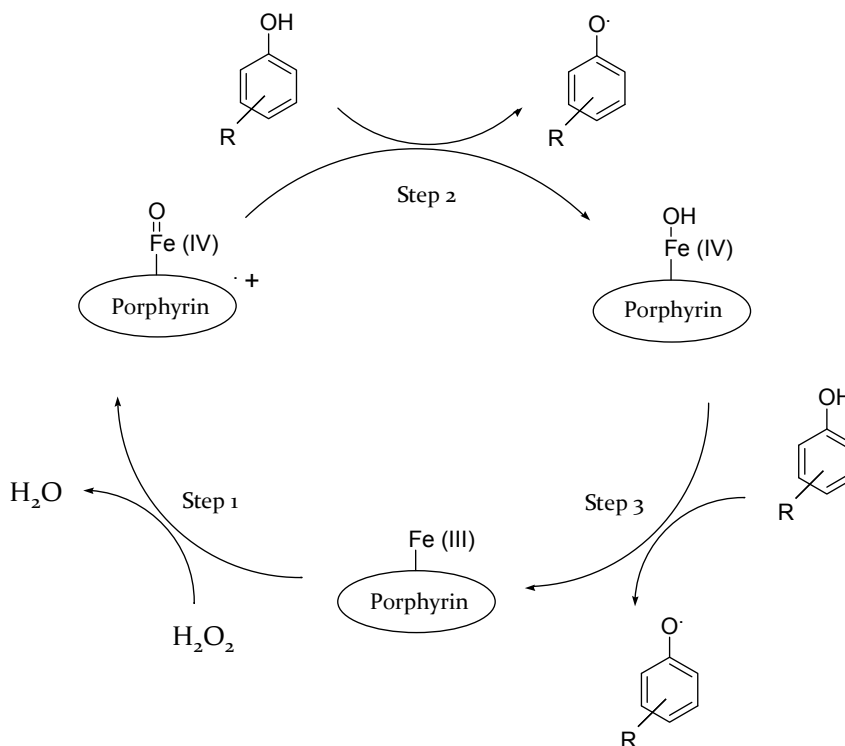
CATALYTIC PROPERTIES

The reaction catalysed by HRP isoenzyme C can be expressed as following:



where AH_2 is a phenol or a phenolic acid, an amine, indole or sulfonate, that is subjected to oxidation by HRP, yielding $\text{AH}^\cdot$.

The catalytic cycle can be divided into three steps, as shown in Scheme 1.2.



Scheme 1.2: Catalytic cycle underlying porphyrin activity.

In the first step the Fe(III) resting state is oxidized by H_2O_2 : two electrons are removed for the reduction to water, one from Fe(III) and one from the porphyrin, producing a Fe(IV) centre and a porphyrin cation radical. In the second step the substrate reacts

with the catalytic centre reducing the porphyrin only. The enzyme returns to the resting state in the third step, after the reaction with a second molecule of the reducing substrate that turns the Fe(IV) to Fe(III).

Noteworthy, the catalytic cycle of classical peroxidases shows a pathway different from the heme monooxygenases: while they are believed to insert the ferryl oxygen directly into the substrate, the classical peroxidases bind the substrate near to the heme edge, transferring the electron.⁶⁵

APPLICATIONS IN INDUSTRIAL PROCESSES

Peroxidases are widely used for applications in many different areas, especially diagnostic, in biosensors and immunodetections. In particular, horseradish peroxidase is used as a label for immunoassay, used to detect antigens and antibodies. Its extensively employment as enzyme-linked immunosorbent assays (ELISA) over the three most popular enzyme labels (HRP, alkaline phosphatase, and B-galactosidase) is due to its high stability and to the reduced dimension.^{66,67}

Moreover, the availability of substrates for colorimetric, fluorimetric and chemiluminescent assays provide several detection options.^{68,69,70}

Thanks to its capability of reducing H₂O₂ and other organic peroxides, HRP-based biosensors can be used to monitor these peroxides, in pharmaceutical, environmental and dairy industries,⁷¹ in textile and paper industries that operate bleaching processes.⁷² It is employed also for the removal of carcinogenic aromatic amines from water⁷³ and for the treatment of many other industrial wastewaters.⁷⁴ In fact, during the oxidation of aromatic amine and phenols, HRP generates free radicals that undergo to polymerization. Then, since the polyaromatic products are nearly water-insoluble, they can be easily removed from the solution by coagulation and sedimentation.⁷⁵ Finally, HRP finds application also in organic synthesis, especially for enantioselective oxidations.⁷⁶

1.4.3 TANNASE

OCCURRENCE

Tannin acyl hydrolase, also known as tannase (E.C.3.1.1.20), is an enzyme capable of hydrolyzing tannins, that represent the main class of natural anti-microbials occurring in the plants. This enzyme was unintentionally discovered by Tieghem⁷⁷ during an experiment targeting the production of gallic acid in an aqueous solution of tannins, in which two fungal species grew that were identified later.

In nature, the main producers of tannase are fungi, yeasts and bacteria. In the last years, however, tannase was also found to be produced by few animals.⁷⁸ The most part of research works use fungal tannase: *Aspergillus* (*awamori*, *niger*, *oryzae*, *versicolor*) and *Penicillium* (*notatum*, *glaucom*) are the most widely used species of fungi exploited for tannase production. Only very few reports deal with the enzyme production from yeasts.; however, the main producers of tannase belong to the *Candida* species.⁷⁹ The production of tannase from bacteria has been almost unknown before the 1980's, but in the last 25 years more than 100 reports about bacterial tannase have been published, and about 25 tannase positive bacteria have been isolated.⁸⁰ Bacterial sources of tannase are provided by *Bacillus cereus*, *Bacillus plumilus*, *Lactobacillus plantarum*, *pentosus* and *acidilactici*, *Pseudomonas aeruginosa*.

The production of tannase from these organisms strongly depends on the fermentation system used.⁷⁹ It can be carried out through different methodologies as liquid surface, submerged and solid-state fermentation. The production by submerged culture mainly yields intracellular enzyme, that is further secreted to the culture medium, while the production by solid state yields extracellular enzyme, that do not require expensive extraction methods. Although tannase has several important applications in food, chemical, and pharmaceutical industries, the practical use of this enzyme is still limited because of the insufficient knowledge about its properties and optimal expression.

However, in the last decade the efforts have been directed to the search for new tannase sources,⁸¹ to develop new fermentation systems,⁸² and to optimize the culture conditions,⁸³ improving the production, the recovery and the purification processes of the enzyme.

MOLECULAR PROPERTIES

Up-to-date, no crystal structure of a tannase has been published. Thanks to circular dichroism analysis, tannase was found to be a globular protein mainly composed of β -sheet structures.^{84, 85}

The most part of fungal tannases were found to be multimeric proteins, formed by 2 up to 8 subunits, having a molecular weight between 50 and 320 kDa depending on the source of extraction. In the middle of 1990's Hatamoto *et al.*⁸⁶ carried out several studies on *Aspergillus oryzae* and concluded that the native tannase consisted of four pairs of two subunits, forming a hetero-octamer with a molecular weight of about 30 kDa.

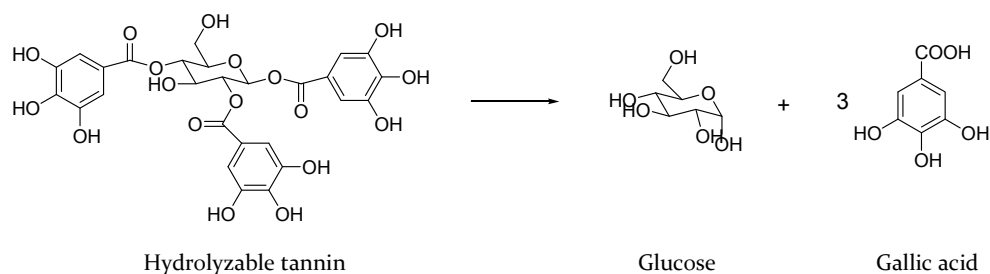
The isoelectric point, as well as the pH and temperature range of activity and stability also depend on the source of extraction: for example, tannases of *Aspergillus* exhibits an activity and stability ranges from pH 5 to pH 6, and from pH 3,5 to pH 8, respectively, at an optimum temperature of 35-40° C.⁸⁷ In 2003, Ramirez-Coronel *et al.* produced by solid state culture a single tannase in two different forms, a monomeric and a dimeric form, having molecular masses of 90 and 180 kDa respectively. The mixture of the two enzymes shows an isoelectric point of 3,8, a temperature optimum of 60-70°C, and a pH optimum of 6.⁸⁸

Tannase was found to be a glycoprotein. The fungal and yeasts tannases have a carbohydrate content that range from 5,4 to 64% m/m.^{85,89,90}

Probably the biological function of the bound carbohydrates lies in the protection of the enzyme from the substrate itself (tannins), that show a denaturing action. It was supposed that the tannin does not bind directly to the protein, but probably binding occurs via the carbohydrate chains. This hypothesis is supported by the comparison between tannase and other tannin-resistant proteins that were found to have a high carbohydrate content.⁹¹

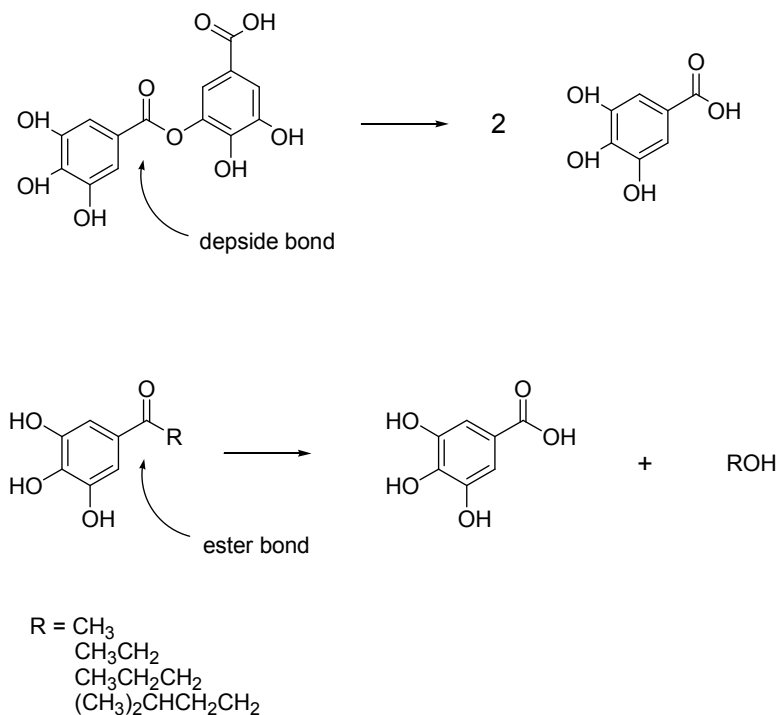
CATALYTIC PROPERTIES

Tannase catalyzes the hydrolysis of the ester bonds in hydrolyzable tannins to yield gallic acid and glucose (Scheme 1.3).



Scheme 1.3: Hydrolysis of hydrolysable tannins yields gallic acid and glucose.

Tannase was found to hydrolyze both the simple galloyl esters of an alcohol moiety and the galloyl esters of gallic acid. For this reason, Toth and Barsony⁹² proposed that tannase activity could be composed of two separated enzymes: a “depsidase” that hydrolyzes the depside bonds, typical of galloyl esters of gallic acid (Scheme 1.4), and an “esterase” that catalyzes the cleavage of simple galloyl esters, such as methyl gallate, ethyl gallate, *n*-propylgallate, as well as *n*- and *i*-amyl gallate.⁹³

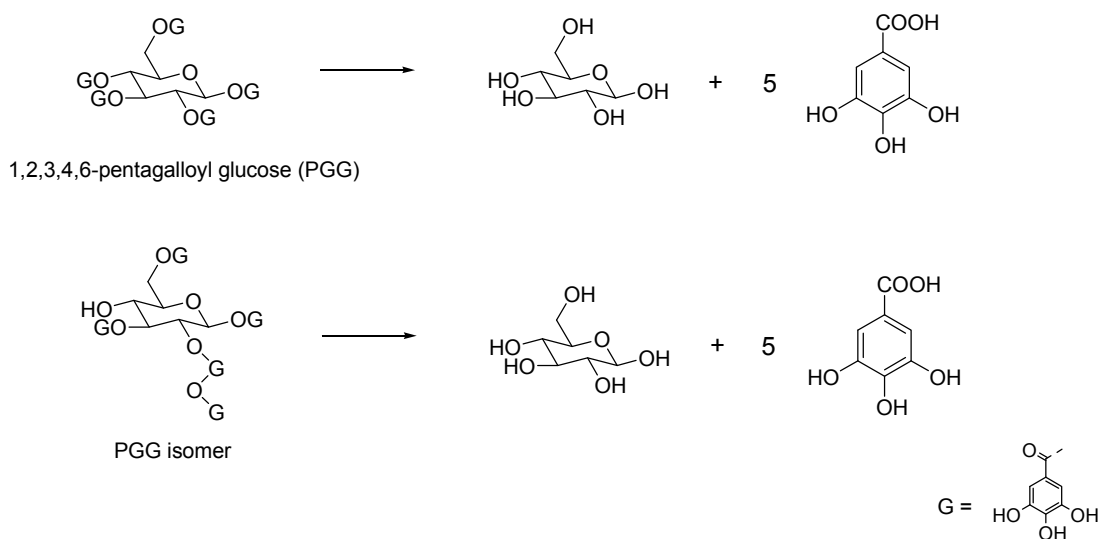


Scheme 1.4: Depside and ester bonds in tannic acid.

Up-to-date, there are no reliable informations about the structure of the catalytic site of tannase. In 1971, Adachi *et al.* proposed that the active site of tannase from *Aspergillus flavus* contained an active serine residue.⁹⁴ This was confirmed only at the beginning of 2000's, after several studies on enzyme inhibition provided significant information both on the general structure of the enzyme and on its active site.⁹⁵

Mata-Gómez *et al.* found an high inhibition of *Aspergillus niger* tannase by ferric ions, while Cu^{2+} and Zn^{2+} only showed a mild inhibitory effect.⁹⁶ Aguilar *et al.* and Battestin *et al.* studied the behavior of *Aspergillus niger* and *Paecilomyces variotii*, respectively, and discovered a considerable inhibition after the addition of cysteine or 2-mercaptoethanol to the reaction medium.^{95,97} The inhibition of tannase activity by cysteine and 2-mercaptoethanol suggested the presence of sulphur containing aminoacids in the active site of the enzyme, probably a methionine or a cysteine residue.

The main expression of tannase activity takes place on gallotannins, that belong to the class of hydrolyzable tannins: the hydrolysis of the ester bonds yields gallic acid and glucose (Scheme 1.5).



Scheme 1.5: Common gallotannins – hydrolysis of depside and ester bonds.

Tannase acts also on ellagitannins (Figure 1.11 A, B, C), but the biochemical mechanism is not completely understood because of the chemical complexity and diversity of the substrates.⁹⁸ However, it is known that the selective hydrolysis of galloyl groups of the ellagitannin phyllanemblinin (Figure 1.11 C) is catalyzed by a particular species of tannase.⁹⁹

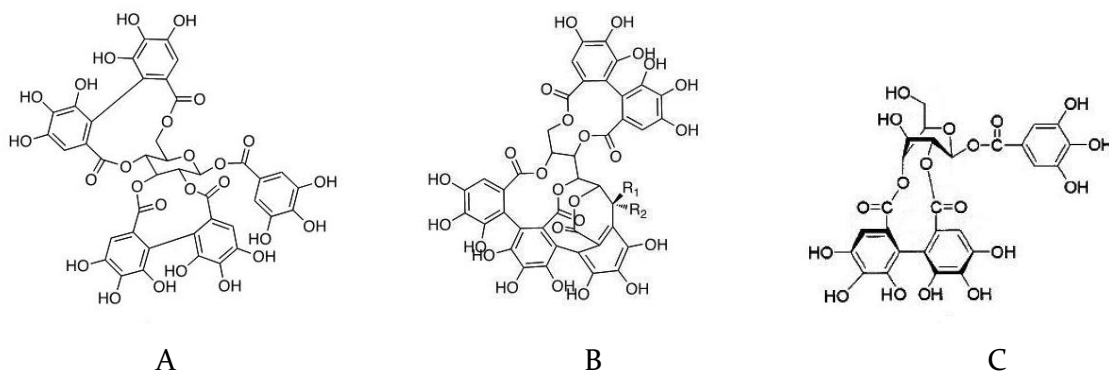


Figure 1.11: A): Casuarictin; B): R₁=H, R₂=OH Castalgin, R₁=OH, R₂=H Vescalgin; C): Phyllanemblinin.

In any case, the substrate of tannase has to be an ester compound of gallic acid, whatever is the alcohol that forms the ester bond and, probably, esters and carboxylic acids cannot be hydrolyzed by the enzyme unless they have phenolic hydroxyls.¹⁰⁰

APPLICATIONS IN INDUSTRIAL PROCESSES

Tannase is nowadays exploited for several industrial applications, above all in chemical, pharmaceutical, food and beverage industries. The pharmaceutical and chemical companies employ tannase for the production of gallic acid whose synthesis is known to be very expensive and not always selective. Gallic acid is used as intermediate for chemical and enzymatic synthesis of pyrogallols and gallic acid esters, as propyl gallate, which finds application as antioxidant in fats and oils, as well as in beverage industries. Moreover, gallic acid is employed for the manufacture of trimethoprim, a strong antibacterial drug.¹⁰¹

On the other hand, tannase is widely used in food and beverage industrial products mainly to remove the undesirable effects of tannins. Tannins are responsible of the

turbidity of wines, beers and fruit juices because of the formation of insoluble tannin-protein complexes; moreover, they confer them bitterness and astringency. Therefore, tannase is employed as clarifying agent against the beverage (wine, beer and coffee flavored soft drink) turbidity and finds application in fruit juice debittering.¹⁰²

1.5 LIGNINS BIOPROCESSING BY MEANS OXIDATIVE ENZYMES: LACCASE AND HORSERADISH PEROXIDASE

Nowadays' energy production is highly related to the exploitation of fossil-based fuels. In the last years the efforts have been directed to the replacement of fossil-based fuels with alternative and renewable sources of energy, in order to reduce the strong reliance on oil demand. The most sustainable and renewable resource for energy production is represented by biomasses. The term biomass is related to non-fossil biological materials and it is composed of forestry and agricultural wastes or waste materials from pulp and paper industries and from the food and beverages ones. Hence, it could be exploited not only as source of fuel but also for the production of a wide set of compounds for industrial processes.¹⁰³

Among the biomasses, the lignocellulosic biomass represents the most precious and sophisticated energy store in fact the amount of CO₂ produced by biomass-powered industries is extremely close to the amount of CO₂ stored by the biomass during its growth. The almost neutral CO₂ balance represents therefore an invaluable aspect for the sustainability of the industrial processes.

The most part of lignocellulosic materials are exploited by pulp and paper industry and for bioethanol production; the residual waste of these processes is mainly composed of lignin, that constitutes up to 30% of wood. It is estimated that 140 million tons of cellulose and pulp annually yield 50 million tons of waste lignin, that is exploited for thermo-valorization or for other low added value applications.

The complexity of this biopolymer represents the highest barrier to its use; its polyphenolic structure could be exploited, for example, for the production of raw materials and fine chemicals or for the synthesis of new polymers. The lack of a repetitive sequence, specific subunits or interunit bondings, makes the lignin

upgrading a challenging task for the chemists. The possible strategies for its valorisation are based on two different treatments, namely its selective functionalisation in order to improve its compatibility in copolymer materials, or its oxidative depolymerization to get polyfunctional monomeric compounds to be used as an alternative to fossil fuels derived building blocks.

In nature, the selective oxidation of lignin is carried out by white-rot basidiomycetes, that produce a pool of extracellular enzymes composed of laccase, Mn peroxidase (MnP) and lignin peroxidase (LiP)^{104,105} It is important to note that white rot fungi have the capability to degrade not only lignin but also a great variety of pollutants, such as chlorinated and heterocyclic aromatic compounds, dyes and synthetic high polymers^{106,107,108,109} Thanks to the strong oxidative activity and the low substrate specificity of their ligninolytic enzymes, white rot fungi has proven to be a precious tool for several industrial processes and for the detoxification of several aromatic pollutants found in industrial waste and in contaminated soil and water. Laccase finds application in the textile, food and drink industries and, above all, in the pulp and paper one.^{110,111} However, its efficiency in bleaching pulps was found to be inadequate, therefore, in the last years, a new laccase-mediator system have been developed: in presence of a natural or synthetic radical mediator, usually a low molecular weight phenol or a N-hydroxy derivative (as 1-hydroxybenzotriazole), a sensible increase of its activity has been shown.^{112,113,114}

So, the new laccase-mediator system have been exploited for a wide set of applications, as pulp delignification, oxidation of organic pollutants and development of biosensors or biofuel cells.^{115,116} The two class of peroxidases produced by white rot fungi, that is Mn peroxidase (MnP) and lignin peroxidase (LiP), have not found wide application in industrial processes because of their low stability: the disadvantage of LiP is the inactivation by excess H_2O_2 and high concentrations of aromatic substrates.¹¹⁷ Moreover, their high redox potential and their optimum pH, that is near 2,¹¹⁸ contributes to limit their exploitation. HRP, that follows the same reaction pathway of LiP in catalyzing the oxidation of substrate by H_2O_2 , is one of the most exploited peroxidases in industrial processes thanks to its stability and to its mild optimal pH. It finds application in different area, in analytical, environmental and clinical fields.¹¹⁹ The use of fungi and of their enzymes has proven to be a precious tool at industrial

level because of the sensible reduction of manufacturing costs as well as the pollution, contributing to the use of eco-friendly processes. However, although the enzymes are characterized by a high value of catalytic constant, a low substrate specificity, and mild operating conditions, their stability and reactivity are strongly influenced by the environmental conditions of the industrial process in which they are involved.¹²⁰ It represents, perhaps, the bottleneck of current enzyme applications at industrial level. An approach to overcome these constraints is the use of immobilized enzymes. The immobilisation of an enzyme is achieved by “confining” it on the surface or inside an inert matrix. With respect to free enzymes, they are more robust and resistant to environmental changes, moreover they allow to recover easily both the product and the biocatalyst for multiple reuse and for a continuous enzymatic process, in a great variety of bioreactor designs.

Literature extensively reports examples of immobilized laccase,^{121,122,123,124} and HRP.^{125,126,127}

In the last years, immobilization methods were combined with layer-by-layer (LbL) technique, developed by Decher.²⁰ The highest enzymatic activity is shown when 3 thin layers of polyelectrolites are adsorbed; applying more layers results in a decreased enzyme activity, probably because the multi-film acts as a barrier for substrates to reach the enzyme.¹²⁰

In the first part of my Ph.D. project I directed my efforts on the development of novel multi-enzyme biocatalyst based on the co-immobilization of laccase and horseradish peroxidase (HRP) on a single matrix, with the aim of evaluating the potential synergy of the two enzymes for lignin degradation. The enzymes were also singularly immobilised and used for lignin oxidation in order to evaluate the possibility of different behaviour from the co-immobilized system. Moreover, I investigated the efficiency and the reaction pathway of both laccase and HRP running some experiments with native enzyme and using, only for laccase-catalysed oxidations, a chemical mediator (1-hydroxybenzotriazole).

In particular, laccase and HRP was chemically immobilized on alumina pellets (3 mm diameter), suitably silanised and activated by glutaraldehyde treatment. The singularly immobilized and the co-immobilized catalyst were protected applying the LbL

technique, in order to avoid the denaturation, to improve their stability and resistance to reaction conditions, and to develop processes suitable for scale up.

The whole set of oxidations was carried out using wheat straw lignin as substrate. Choosing a single substrate for the experiments, we focused our attention on the possibility of different reaction pathways between the soluble and immobilized laccase and HRP, used singularly or in mixture. The ultimate aim was the comparison of the immobilized multi-catalyst activity respect to the singularly immobilized one: the possible occurrence of cascade reaction in the multi-catalyst system would give rise to a valuable synergy in lignin oxidative modifications.

Lignin structural modifications were determined using phosphorous magnetic resonance technique (^{31}P -NMR) and gel permeation chromatography (GPC).

1.6 TANNIN BIOPROCESSING BY MEANS OF HYDROLYTIC ENZYMES: TANNASE

Tannins, widely distributed occurring plant polyphenols, have a controversial role in the industrial field. To one hand, they constitute important additives for wine refining, they are a powerful tool in the hands of winemakers to refine the taste, the colour intensity and the stability of their products.^{128,129,130} As well, they are widely used in soft drinks or juices to modify taste.¹³¹

On the other hand, the presence of tannins in soils represents a hazard for the micro-environment. Their wide employment in the tanning industries to convert skins into a stable material has rised environmental problems because of the discharge of effluents directly into bodies of water. The effluents still contain unreacted tanning compounds that are known to inhibit the growth of important microorganisms such as the methanogens bacteria,^{132, 133} that offer an effective means to pollution reduction.

In order to control the damage that water soluble tannins provoke to the environment, tanneries developed preliminary treatments of the raw materials to avoid the massive use of tannins and other tanning compounds. It is calculated that the raw skin has 30% loss of organic material during the working cycle:¹³⁴ the organic pollutants, such as superficial epidermic matter including hair that are not transformed into leather, are

removed during a pretreatment step.¹³⁵ Another approach that avoids the surplus of tanning substances in the tanneries baths is the removal of the subcutaneous adipose layer, that gives rise to undesirable phenomena such as hardness to touch, loss of physical strength and dyeing imperfections. For what concerning the treatment of wastewaters, tanneries support the chemical-physical treatment of tanning effluents such as the separation of the biomass from effluents by means of membranes in order to partially recycle the sludges. In some cases, the treatments of wastewater with chemical methods turn out to be insufficient or unsuitable: an example is provided by the employment of an extra high dose of metal coagulant for the precipitation and recovery of the active sludge, which results in an additional metal pollution. Therefore, replacing a chemical relief with a biological treatment of tannin-containing wastewaters might be the only eco-friendly solution; this biotechnological remedy represents a valuable and sustainable way to clean up contaminated environments.

The enzymatic treatments might be directed to modify the structural features of tannins: the oxidations or hydrolysis carried out by biocatalysts might provide a valuable tool to achieve tannins structural modifications with the aim of limiting their inhibiting activity towards bacteria.

On the basis of our previous results on lignin oxidative coupling carried out by laccase,^{136,137} since tannins have a polyphenolic structure as well as lignin, we applied the same enzymatic treatment on different tannin samples to induce coupling reactions among tannin molecules, with the aim of obtaining a polymeric compound. The product thus yielded would have been basically insoluble in water, allowing an easier recover. As supposed and somehow expected, tannins inactivated laccase giving rise to a highly insoluble and dark product, certainly caused by the formation of a tannin-enzyme complex, as described in chapter 2. (2.1.1 - DEFINITION AND OCCURRENCE).

The enzyme that plays a key role in tannins degradation, and that has the capability to lead structural modification on this family of polyphenols without being inactivated, is tannase, or Tannin Acyl Hydrolase (EC 3.1.1.20), that catalyzes the hydrolysis of the ester bonds in hydrolyzable tannins to yield gallic acid and glucose.

The treatment of tannin-containing wastewater with tannase would represent a precious and sustainable alternative to the chemical relief. Subjected to tannase,

tannins are hydrolyzed into sugars and gallic acid that can be easily removed or neutralized. The use of tannase absolutely concurs with the “greening” of the industrial processes since enzymes work in aqueous moiety and with mild conditions of pH, temperature and pressure; moreover, they do not produce secondary toxic metabolites and by-products.

The hydrolytic action of tannase can be also exploited for the production of gallic acid in industrial scale. The synthesis of gallic acid is known to be expensive but it represents a compound of great interest to both pharmaceutical and chemical industries¹³⁸ because of its antiviral, analgesic and anti-apoptotic activities. Moreover, it is the substrate for the synthesis of the propylgallate, a potent antioxidant used in food and beverage industry, and an important intermediary compound in the synthesis of the antibacterial drug, trimetoprim.^{139,140} Conventionally, gallic acid is produced by acidic hydrolysis of tannins, but this process releases a large amount of toxic effluent that causes environmental hazards.¹⁴¹

Thus, biotechnological production of gallic acid by enzymatic hydrolysis should be preferred.

Making a hypothesis on the development of a treatment on industrial scale or in a flow chemistry set up, both for the treatment of tanneries wastewater and for the synthesis of gallic acid, it is important to consider the disadvantages or constraints that characterize the employment of enzymes. In fact, the enzymes used for industrial processing have no long-term stability towards the reaction conditions; moreover, their recover from the batch for a potential reuse is often difficult. An additional constraint is given by the cost of isolation and purification of the biocatalyst that can seriously limit the industrial applications.

An approach to overcome these constraints is the use of special techniques of enzyme immobilization: the procedure facilitates the recovery and the reuse of costly enzymes, minimizing or avoiding also the contamination of the product. Additionally, the procedure increases the enzyme stability and resistance towards the environmental condition, allowing the repeated reuse of the biocatalyst.

Several attempts have been done to immobilize the tannase, investigating among a wide variety of suitable matrix and methodologies. Among them physical adsorption on aminoalkylsilane-alumina, covalent binding on chitosan and chitin¹⁴² and

entrapment on polyacrylamide and Ca-alginate have been tested, reporting different results.¹⁴³ According to Abdel-Naby results,¹⁴⁴ the immobilized enzyme prepared by covalent binding to chitosan showed the highest immobilized activity and the highest immobilization yield (26,6%) with respect to the other tested matrix, under the same reaction condition.

The most convenient and common method is the entrapment in Ca-alginate beads: this technique was shown to be simple and cheap, providing transparent, non-toxic and stable particles.^{145,146,147} Beside it, there is no information in literature about tannase immobilization by means of a Layer-by-Layer technique. Therefore, in the second part of my PhD project I directed my efforts on the development of a novel strategy for the immobilization of tannase, based on the deposition of ultrathin layers of polyelectrolites onto the enzyme, previously immobilized through chemical method on eupergit C 250L. Eupergit C 250L is a carrier consisting of macroporous beads made by copolymerization of N,N'-methylene-bis-(methacrylamide), glycidyl methacrylate, allyl glycidyl ether and methacrylamide. Because of its structure, Eupergit is stable, both chemically and mechanically, over a pH range from 0 to 14, and does not swell or shrink even upon drastic pH changes in this range, showing a high mechanical stability. It binds proteins via its oxirane-groups which react with the amino groups of the protein molecules to form covalent bonds which are long-term stable within a pH range 1 to 12.¹⁴⁸

The deposition of a multi-layer of polyelectrolites onto the binded enzyme allows to protect the enzyme from high-molecular-weight denaturing agents or bacteria and to preserve the protein from drastic pH, avoiding the desorption from the support.

The activity of the novel immobilized tannase was tested at first on tannic acid and then on two different samples of hydrolizable tannins provided by Dal Cin S.p.A., an oenological company located in the north of Italy. The hydrolytic process was followed in the course of time by HPLC analysis.

In order to have further details on the process and to evaluate the structural modifications caused in the tannins, we developed a novel analytical method based on ³¹P NMR spectroscopy, that is able to quantitatively characterize different classes of tannins.

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2. BIO-SUBSTRATE FOR BIO-TECHNOLOGICAL PROCESSES

2.1 THE PLANT KINGDOM: FROM SIMPLE TO POLYMERIC PHENOLIC COMPOUNDS

Phenolic compounds seem to be universally distributed in plants. As stated by Harborne, the term "phenol" or "polyphenol" is related to a substance which possesses an aromatic ring having one (phenol) or more (polyphenol) hydroxyl substituents, including functional derivatives (esters, methyl ethers, glycosides, etc)¹ biogenetically from the shikimate-phenylpropanoids-flavonoids pathways.

Phenolic compounds are essential for the growth, reproduction and pigmentation of plants, and the majority of them are produced as protection from herbivory, insects and parasites in order to prevent plague tissues, as well as protecting the plant from ultraviolet radiation and oxidants.^{2,3}

They are usually accumulated in the central vacuoles of guard cells and epidermal cells as well as subepidermal cells of leaves and shoots.⁴

To date, more than 8000 phenolic compounds have been identified in various plant species,⁵ characterized by a large range of structure: the monomeric and dimeric phenols can be polymerized into larger molecules such as the proanthocyanidins (condensed tannins) and lignins. On the basis of their skeleton the phenolic compounds have been classified in several groups:^{6,7,8,9}

Table 2.1: Classification of phenolic compounds.

C6	simple phenol, benzoquinones
C6-C1	phenolic acid
C6-C2	acetophenone, phenylacetic acid
C6-C3	hydroxycinnamic acids, coumarins, phenylpropanes, chromones
C6-C4	Naphthoquinones
C6-C1-C6	Xanthenes
C6-C2-C6	stilbenes, anthraquinones
C6-C3-C6	flavonoids, isoflavonoids
(C6-C3) ₂	lignans, neolignans
(C6-C3-C6) ₂	Biflavonoids
(C6-C3) _n	Lignins
(C6) _n	Catechols, melanins
(C6-C3-C6) _n	condensed tannins

Although plants synthesize a great variety of secondary metabolites, bacteria, fungi, algae and generically animals, are poor of these compounds. This discrepancy could be explained on the basis of the different system that animals and plants employ to protect themselves from predators and diseases; in fact, animals are enable to avoid danger thanks to a developed nervous and immune systems while plants synthesize a wider class of secondary metabolites having antibiotic, antinutritional or unpalatable properties because they cannot rely on physical mobility to escape to their predators.¹⁰

In the last years phenols achieved resounding success as antioxidant, finding a great variety of applications in industrial processes, from the food and beverage industries to the agricultural, cosmetic and medical ones. Their excellent antioxidant capability have been exploited by the food industry to replace the chemical antioxidants as BHT (butylhydroxytoluene) and BHA (butylhydroxyanisol), moreover, they have gained worldwide interest in human health. The exploitation of these compounds by industrial scientists started in the 19th century when they found applications in the synthesis of dyes, aspirin, and one of the first high explosives, picric acid. As early as in 1872, it was found that phenol could be condensed with aldehydes (for example formaldehyde) to make resinous compounds, a process still in use today. Phenol-

formaldehyde resins are the basis of the oldest plastics, still used to make low cost thermosetting plastics such as melamine and bakelite used in electrical equipment. These resins are also used extensively as bonding agents in manufacturing wood products such as plywood.¹¹

Among the antioxidants it is certain that tannins play a fundamental role in many industrial applications, in particular in wine and beer refining, thanks to their antioxidant, antiviral, and anticancer properties (see above and below).^{12,13}

These metabolites accumulated by the plants in roots, bark, leaves and fruits, are responsible of the dry and pucker feeling in the mouth, caused by the precipitation of the salivary proline-rich proteins.

Every winemaker knows the important role played by these polyphenols in the gustative quality of red wines; they widely employ tannins to improve not only the colour intensity and the stability of their products but also to refine the taste and the roundness, satisfying the increasing demand of the consumers.^{14,15} However, the use of oenological tannins in wine refinery is treated with great care since a wrong formulation may cause the lost of equilibrium of their products.¹⁶

Their capability of precipitate proteins, minerals and other macromolecules has been exploited also for tanning for thousands of years.¹⁷ Tanning is the process which matches the protein of the raw hide or skin into a stable material which will not putrefy and is suitable for a wide variety of end applications. In the vegetable tannage, the collagen chains of the skins are reticulated through hydrogen bonds between phenolic groups of tannins and NH-CO groups of the collagen: it makes the collagen fibers extremely stable and no longer biodegradable.¹⁸ Another valuable polyphenol widely distributed in the plant kingdom is represented by lignin. Lignin constitutes up to 30% of wood and it is the second most abundant polymer in nature. Although its chemical heterogeneity prevents its valorization, it was found to be an excellent raw material for many applications, in particular it is employed as dispersant in high performance cement application, as additive in agricultural chemicals, as raw material for the production of fine chemicals as vanillin,¹⁹ DMSO, and syringaldehyde.²⁰ More recently, lignin has been successfully used to produce expanded polyurethane foam. It is also an excellent fuel, since lignin yields more energy than cellulose when burned.

However, up to now lignin has been exploited only for low added-value applications but, actually, its antioxidant, anticancer, antiviral and antibacterial properties make lignin a precious raw material for the development of new products for food, cosmetic and medical applications while its highly functionalized structure makes it an excellent and economic building block for the synthesis of new materials.

During my PhD project I focused my attention on tannins and lignins as substrate for biotechnological applications since they are widely distributed in nature, and they have valuable properties that, up to now, have not been thoroughly exploited. Lignin, in particular, represents a waste product of paper industry and of modern saccharification processes and clears the way for the developing of interesting strategies for its upgrading and valorization.

2.2 TANNINS

2.2.1 DEFINITION AND OCCURRENCE

As alkaloids and terpenes, tannins are naturally occurring plant polyphenols. They are located in vacuoles or surface wax of various parts of the plant as buds, roots, seeds, leaves and stems. They have several biological activities, from toxicity to hormonal mimicry, and play a crucial role in protecting plants from herbivores and disease. The accumulation of tannins in roots, leaves and stems, into the layer between epidermis and cortex, assures a barrier against pathogens penetration and colonization, preventing the microbial activity.

Their antimicrobial activity, as well as their defence against herbivores, is related to their ability to precipitate proteins.²¹ This capability is responsible both to the inhibition of extracellular microbial enzymes²² and to the complexation of the salivary proteins of mammals respectively, provoking the dry, bitter and unpleasant feeling in the mouth. Moreover, they are potential metal ion chelators.^{23,24} For this reason they are defined as antinutrient of plants.²⁵ The same astringent taste is encountered when we partake of wine or unripe fruits.

The salivary proteins synthesized by mammals are unusually rich of proline (the so-called salivary proline-rich proteins) and they constitute the best “defence

mechanism” against possible toxic effects of dietary tannins²⁶ since they constitute the preferred bond site.

However, the detailed chemistry of tannin-protein interaction is only partially understood. It is clear that the type and the strength of interactions are influenced by both tannin and protein chemistry, by the temperature, pH, solvent composition and tannin:protein ratio.³⁹

Tannin-protein interactions are most frequently based on hydrophobic and hydrogen bonding since the phenolic group in tannins is an excellent hydrogen donor that forms strong bonds with the protein's carboxyl group. Ionic and covalent bonding occur less frequently.²⁷ To have high protein affinity, tannins must be small enough to penetrate interfibrillar region of protein molecules but large enough to crosslink peptide chains at more than one point.

With respect to their “molecular dimension”, in 1962 Bate-Smith and Swain defined tannins as “water-soluble phenolic compounds having molecular weights between 500 and 3000”²⁸ but, however, this definition does not include all tannins, since, recently, molecules with a molar mass of up to 20 kDa have been discovered,²⁹ which should also be classified as tannins on the basis of their molecular structures. Nowadays the term tannin is related to any large polyphenolic compound plant extract containing sufficient hydroxyls and other suitable groups (such as carboxyls) to form strong complexes with proteins and other macromolecules.

They are widely distributed in the plant kingdom, both in gymnosperms as well as angiosperms, but, according to a study on 180 families of dicotyledons and 44 families of monocotyledons carried out by Mole,³⁰ most families of dicotyledons contain tannin-free species (tested by their ability to precipitate proteins). The best known families of which all species tested contain tannin are: *Aceraceae*, *Actinidiaceae*, *Anacardiaceae*, *Bixaceae*, *Burseraceae*, *Combretaceae*, *Dipterocarpaceae*, *Ericaceae*, *Grossulariaceae*, *Myricaceae* for dicotyledons and *Najadaceae* and *Typhaceae* in Monocotyledons. To the family of the oak, *Fagaceae*, 73% of the species tested contain tannin; for those of acacias, *Mimosaceae*, only 39% of the species tested contain tannins. Some families like the *Boraginaceae*, *Cucurbitaceae*, *Papaveraceae* contain no tannin-rich species.

2.2.2 CLASSIFICATION

The tannins appear as pale yellow or brownish powders. Due to the enormous structural diversity of the tannins, a systematic classification system based on specific structural characteristics and chemical properties has been established.

Tannins are classified in two groups, namely hydrolysable and condensed tannins.

Hydrolysable tannins are esters of sugars and phenolic acids or their derivatives; the sugar is usually glucose, but in some cases polysaccharides have been identified.³¹ Acidic or basic hydrolysis often occurs spontaneously during extraction or purification.³²

Hydrolysable tannins are further subdivided into gallotannins and ellagitannins.^{33,34}

Gallotannins are considered to be hydrolysable tannins since they yield gallic acid if submitted to hydrolysis. The early works on this topic proved that they are polygalloyl glucose derivatives.³⁵

The simplest gallotannin is pentagalloyl glucose (β -1,2,3,4,6-pentagalloyl-*O*-*D*-glucopyranose) (PGG) (Figure 2.1 A), but PGG is present in different isomers in which one aliphatic hydroxyl group is free and other present a digalloyl ester (Figure 2.1 B).

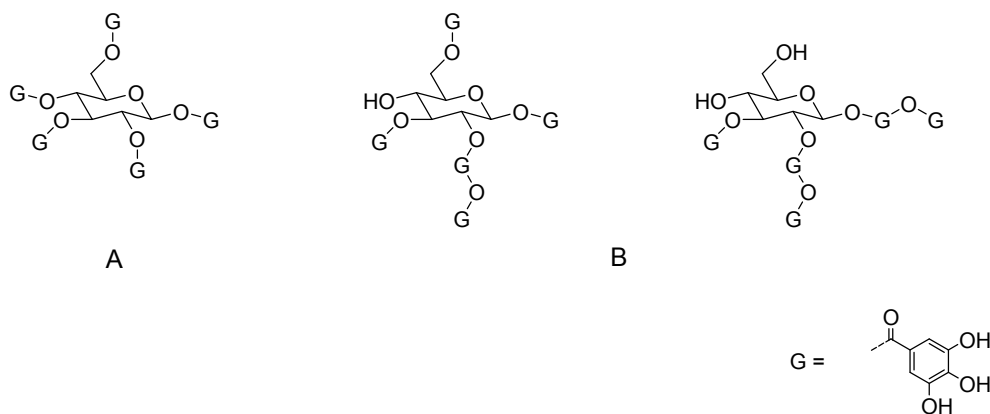


Figure 2.1: A: PGG (β -1,2,3,4,6-pentagalloyl-*O*-*D*-glucopyranose); B: PGG isomers.

Polygalloyl ester chains are formed by a *meta*- or *para*-depside bond (Figure 2.2), involving a phenolic hydroxyl rather than an aliphatic hydroxyl group.³⁶

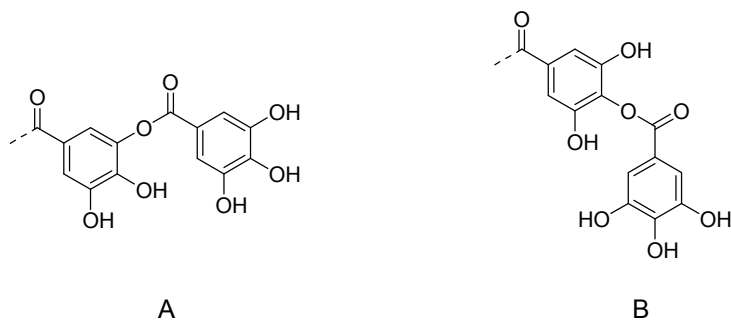


Figure 2.2: *meta*-Depside bond (A) and *para*-depside bond (B).

In nature a great variety of gallotannins exist; their diversity is given by the different degree of esterification of the core sugar and by the nature of the core itself, usually glucose but also glucitol, hammamelose, quinic acid and quercitol (Figure 2.3).

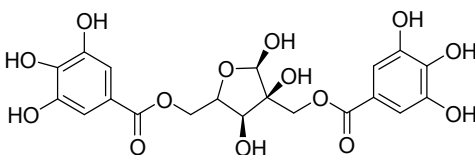


Figure 2.3: Hammamelitanin. Galloyl ester of hammamelose.

A simple gallotannin having up to 12 esterified galloyl groups and a core *D*-glucose is called tannic acid (Figure 2.4).

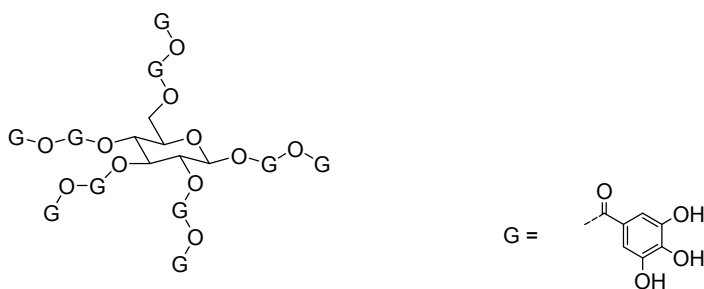


Figure 2.4: Tannic acid.

Although commercial sources provide an exact molecular weight for tannic acid (1294 g/mol), the compound is actually a mixture of different isomers and partially galloylated glucose. For this reason, tannic acid is not an appropriate standard for analysis.

The most important sources of gallotannins are Sumac (*Rhus semialata*) galls or Sumac (*Rhus coriaria*) leaves and Aleppo oak (*Quercus infectoria*) galls.

Ellagitannins are esters of hexahydrodiphenic acid (HHDP) (Figure 2.5 A) generated by oxidative coupling of two vicinal galloyl groups in a galloyl *D*-glucose ester. If submitted to hydrolysis the HHDP spontaneously lactonizes to ellagic acid (Figure 2.5 B).

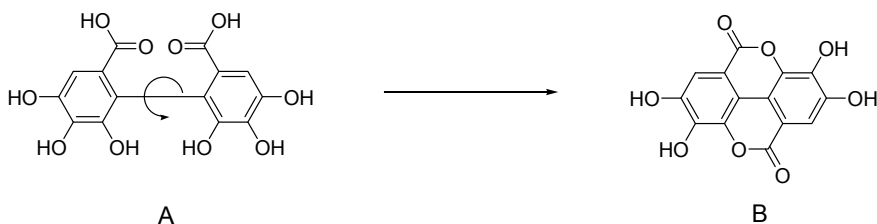


Figure 2.5: Lactonization of HHDP (A) yields ellagic acid (B).

The oxidative coupling occurs usually between the position C₄/C₆, as in the eugeniin (Figure 2.6 A), and between the position C₂/C₃, as in the casuarictin (Figure 2.6 B) but, in some plants, it occurs between the position C₃/C₆, C₂/C₄ or C₁/C₆, as in corilagin (Figure 2.6 C), geraniin (Figure 2.6 D) and davidiin (Figure 2.6 E) respectively.³⁷

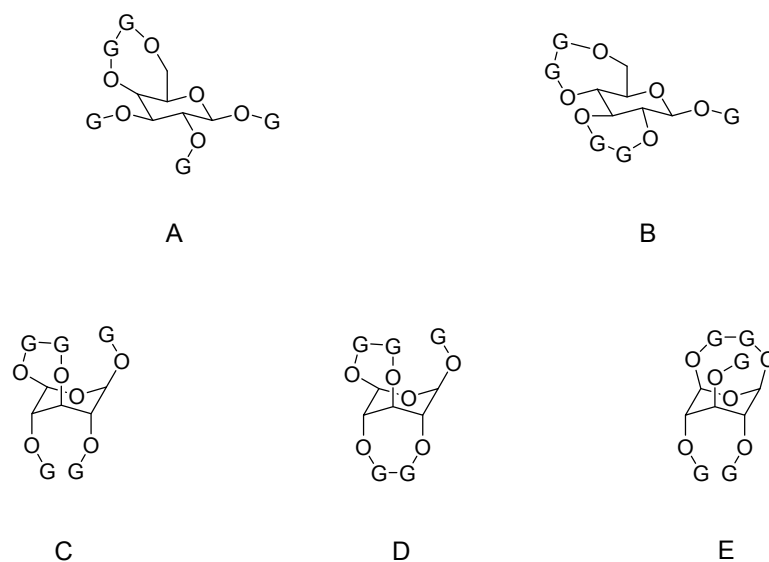


Figure 2.6: Ellagitannins: Eugeniin (A), Casuarictin (B), Corilagin (C), Geraniin (D), Davidiin (E).

In contrast to the rather limited distribution of gallotannins in nature, ellagitannins form the largest group of known tannins with more than 500 natural products characterized.²⁹ Their enormous structural variability is due to the different possibilities for the linkage of HHDP residues with the glucose moiety, and in particular it is due to their strong tendency to form dimeric and oligomeric derivatives. The polymerization process would lead to insoluble compounds, as well as the ellagic acid yielded from the hydrolysis and the further lactonization of the HHDP.³⁸ In some plants, including oak and chestnut, the ellagitannins are further elaborated via ring opening, the pyranose ring of glucose opens giving rise to another family of ellagitannins (Figure 2.7).³⁹

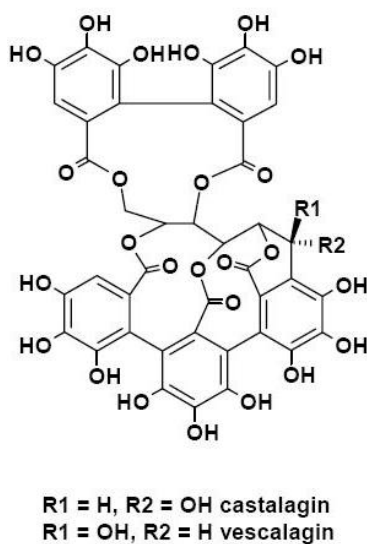


Figure 2.7: Ring opening. Castalagin and Vescalagin.

The occurrence of ellagitannins in common foodstuffs is limited to a few fruit and nut species. Some fruits especially are rich sources of ellagitannins and ellagic acid, in particular pomegranates, black raspberries, raspberries, strawberries, walnuts and almonds.^{40,41}

Much less is known about condensed tannins, their structure and many aspects are yet to be elucidated.³⁵ Condensed tannins are polymers of flavan-3-ol units, namely catechin and epicatechin (Figures 2.8 A, B).

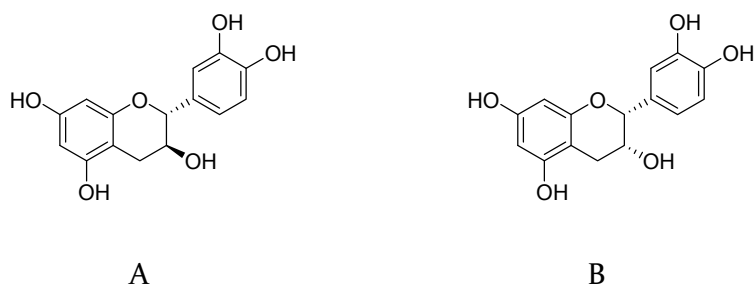


Figure 2.8: Flavan-3-ol units: catechin (A) and epicatechin (B).

Biosynthetically, the condensed tannins are formed by the successive condensation of the single flavan-3-ol units, with a degree of polymerization between two and greater than fifty blocks being reached. Oxidative coupling between the monomers occurs

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most commonly between positions 4 and 8, but may also involve positions 4 and 6 of the monomer (Figure 2.9).⁴²

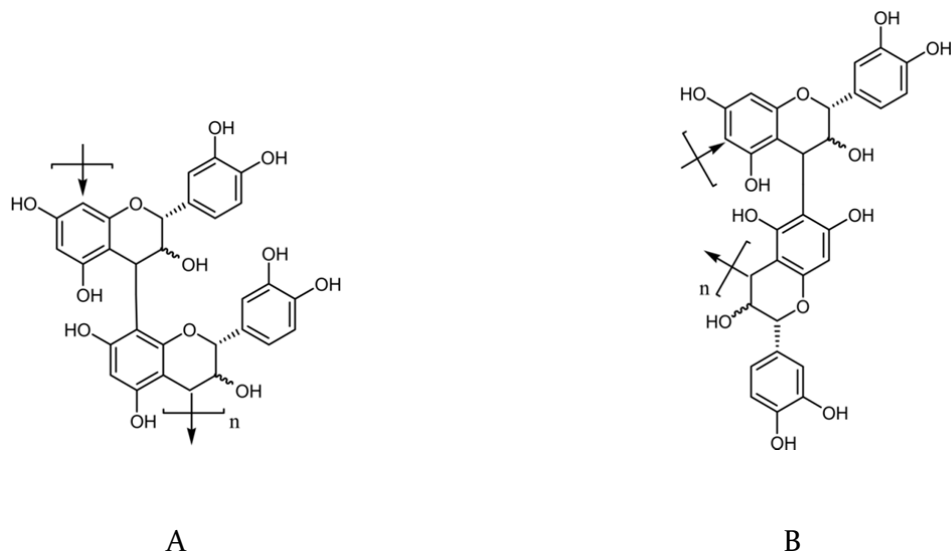


Figure 2.9: C₄/C₈ linkage (A); C₄/C₆ linkage (B).

The polymerization can yield C₄/C₈ polymers, as in Sorghum procyanidin (Figure 2.10), while polymers with both C₄/C₈ and C₄/C₆ linkages are less common.³⁹

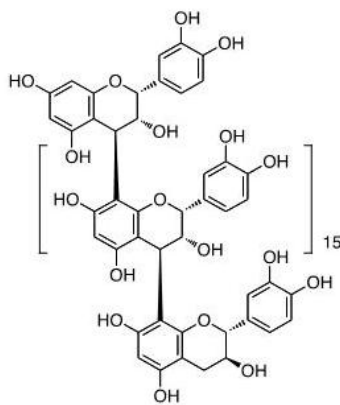


Figure 2.10: Sorghum procyanidin – epicatechin-[(4β->8)-epicatechin]₁₅-(4β->8)-catechin.

Upon oxidative cleavage, these compounds yield anthocyanidin pigments (Figure 2.11). In turn they constitute the basis of the analytical method for the quantification of

leucoanthocyanidins and proanthocyanidins to estimate the degree of polymerization of condensed tannins.^{43,44}

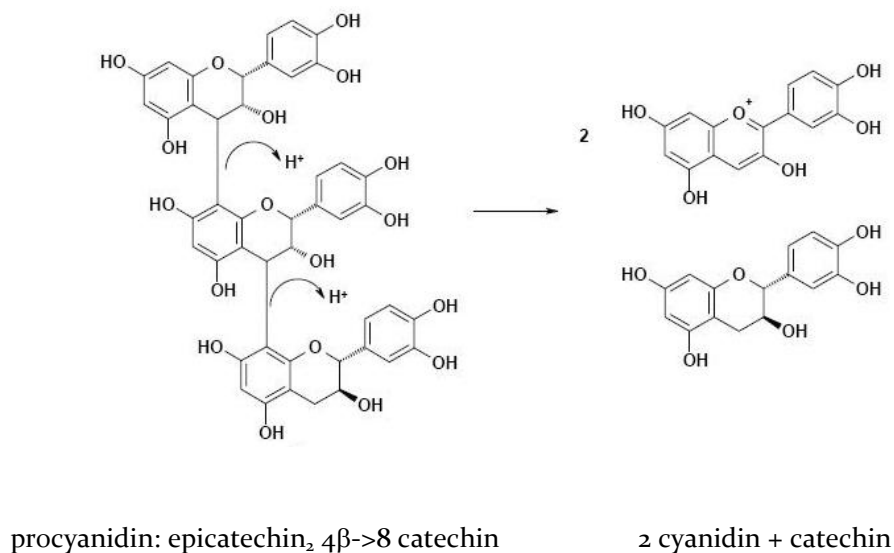


Figure 2.11: Oxidative cleavage of condensed tannins.

Addition of a third phenolic group on the B-ring yields epigallocatechin and gallocatechin, while flavan-3-ols with a single phenolic group on the B ring are less common (Figure 2.12).⁴⁵

Tropical shrub legumes and tea leaves⁴⁶ are rich in catechin tannins that combine the flavonoid and gallic moieties.⁴⁷

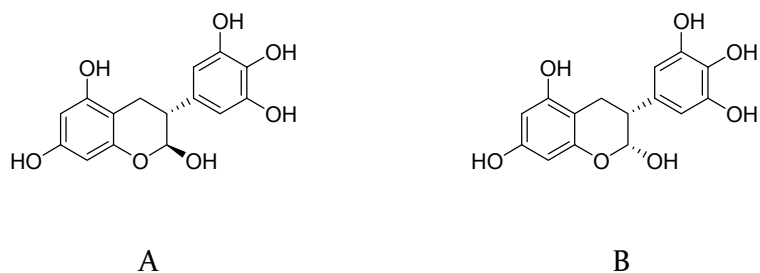


Figure 2.12: Gallocatechin (A) and epigallocatechin (B).

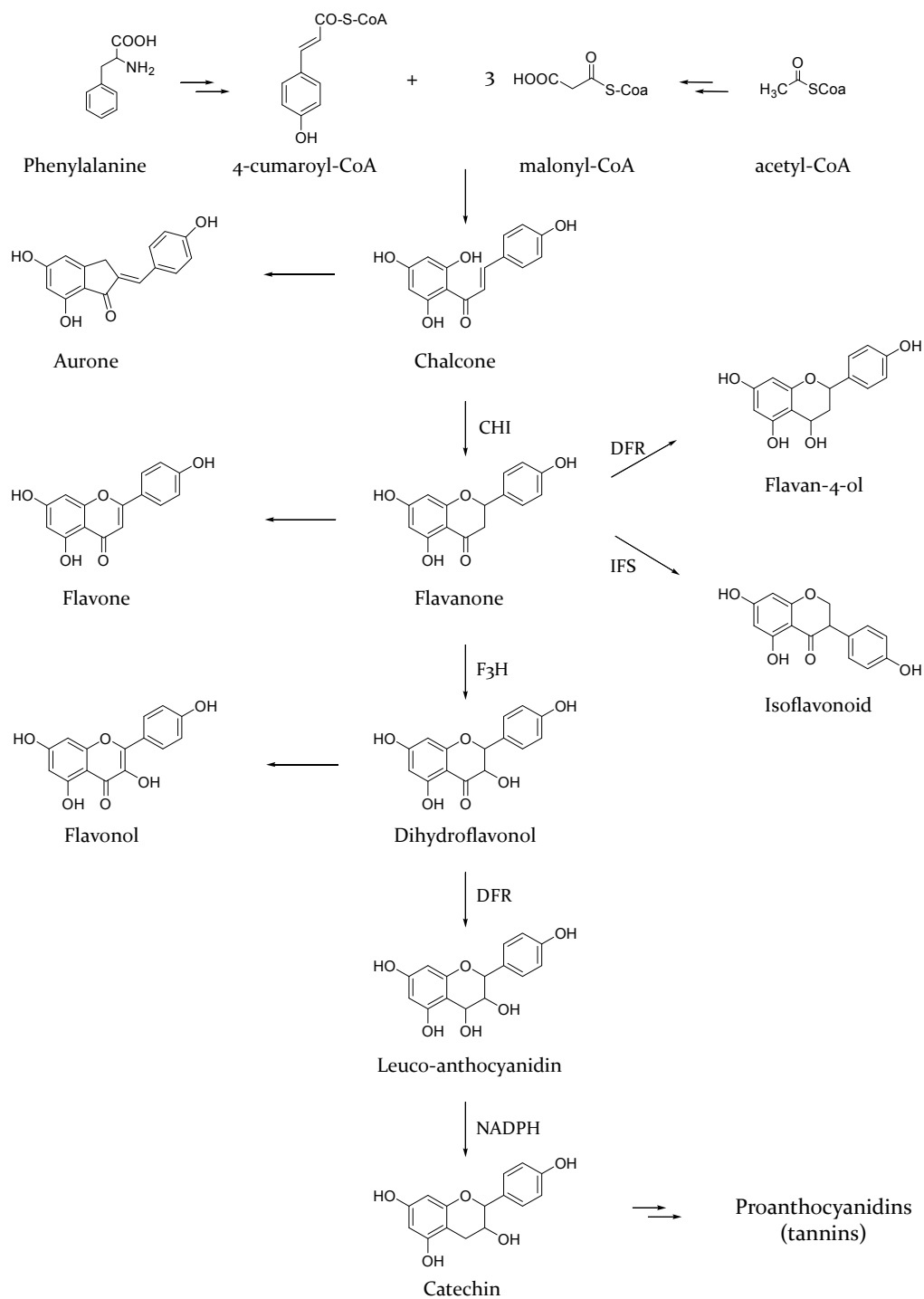
However, there is a great variety of condensed tannins in the plant kingdom, whose diversity is given by the different hydroxylation patterns of the aromatic rings and different configurations at the chiral centers C₂ and C₃.

Traditionally, most commercial sources of condensed tannins are heartwood of quebracho, bark of wattle. They are commonly found in fruits and seeds such as grapes, apple, olives, beans, sorghum grains, carob pods, cocoa & coffee, besides tree bark & heart wood.⁴⁸

2.2.3 BIOSYNTHESIS

The shikimate pathway plays a pivotal role in the production of aromatic amino acids and phenolic compounds. Flavonoids constitute a relatively wide family of aromatic molecules that are derived from *L*-phenylalanine and malonyl-coenzyme A (CoA; via the fatty acid pathway). These compounds include six major subgroups that are found in most higher plants: the chalcones, flavones, flavonols, flavandiols, anthocyanins, and the more complex condensed tannins (or proanthocyanidins).⁴⁹

Flavonoid synthesis starts with the condensation of one molecule of 4-coumaroyl-CoA and three molecules of malonyl-CoA yielding naringenin, a chalcone (Scheme 2.1). Coumaroyl-CoA is synthesized from the amino acid *L*-Phenylalanine by three enzymatic steps while Malonyl-CoA is synthesized by carboxylation of acetyl-CoA, a central intermediate in the Krebs tricarboxylic acid cycle.⁵⁰



Scheme 2.1: Biosynthetic pathway of condensed tannins arising from the biosynthesis of flavonoids.

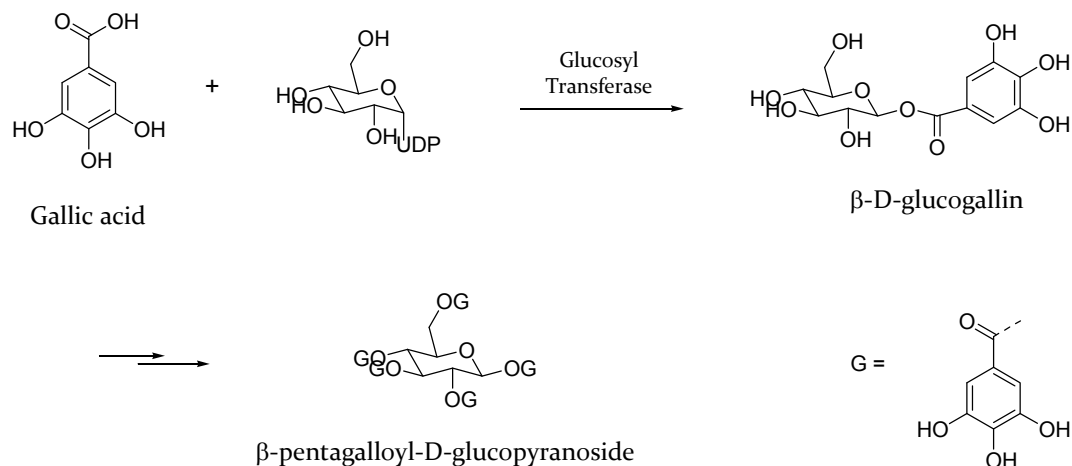
The chalcone is subsequently isomerised by the enzyme chalcone flavanone isomerase (CHI) to yield a flavanone. From these central intermediates the pathway diverges into

several side branches, each yielding a different class of flavonoids, as shown in Scheme 2.1.

The dihydroflavonol 4-reductase (DFR) converts the flavanone in flavan-4-ol while the isoflavonoid syntase (IFS) gives rise to the family of isoflavonoid. The enzyme flavanone 3 β -hydroxylase (F₃H) converts the flavanone in dihydroflavonol, that will yield a leuco-anthocyanidin after a reduction. The further reduction with NADPH give rise to catechins, the basic units of condensed tannins (Scheme 2.1)

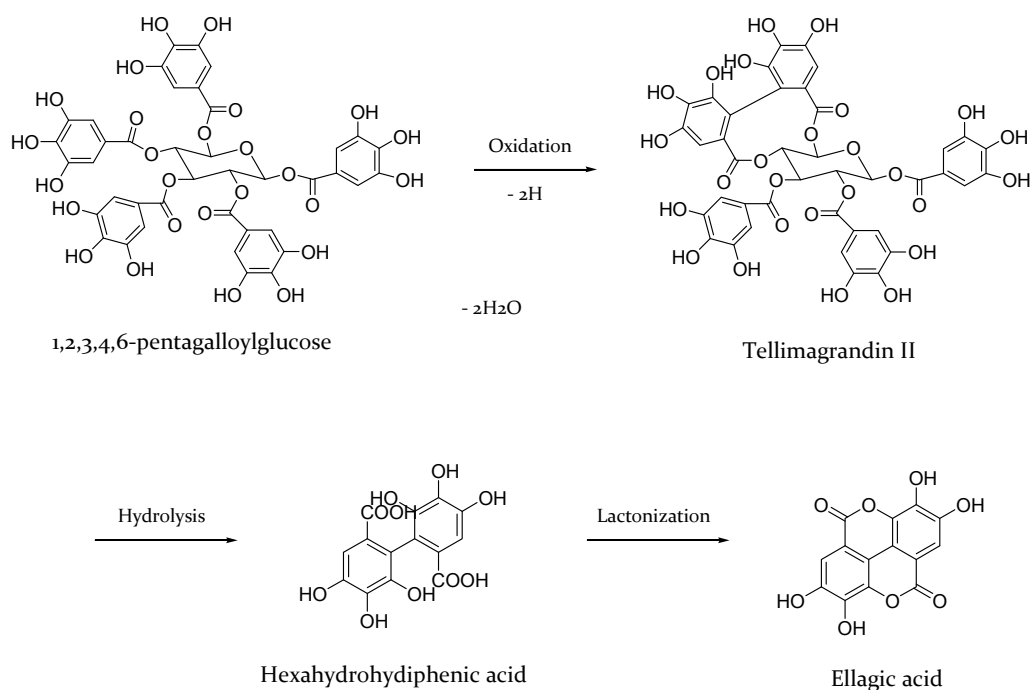
Contrary to the synthesis of condensed tannin through the flavanoids, the biosynthesis of hydrolyzable tannins in higher plants probably does not proceed via *L*-phenylalanine. The results show that gallic acid, the common precursor of hydrolyzable tannins, is formed from an intermediate compound of the shikimate pathway, the dehydroshikimic acid (Scheme 2.2).⁵¹

The dehydroshikimic acid arises from eritrose-4-phosphate and phosphoenolpyruvate after reduction and dehydration steps. The further dehydrogenation and rearrangement of the structure yields gallic acid. Subsequently the glucosyl transferase carries out the reaction between gallic acid and glucose yielding from mono- (β -glucogallin) to penta-galloylglucose (1,2,3,4,6-penta-*O*-galloyl- β -D-glucopyranose).^{52, 53}



Scheme 2.2: Biosynthesis of 1,2,3,4,6-pentagalloylglucose towards more complex hydrolyzable tannins.

Pentagalloylglucose was found to have a crucial role as immediate precursor of two different routes: the first one leads to the formation of more complex gallotannins by the addition of further galloyl residues to the pentagalloylglucose; the second one gives rise to the family of ellagitannins by the oxidative coupling between adjacent galloyl groups of pentagalloylglucose.⁴⁸ The reaction, carried out by the action of polyphenoloxidase enzyme,⁵⁴ gives rise to the formation of hexahydroxydiphenic acid (HHDP), the typical unit of ellagitannins. The primary ellagitannin metabolite is the Tellimagrandin II that can be subjected to further oxidation reaction (Scheme 2.3). However, it is necessary for further studies to elucidate the routes and the mechanism of ellagitannins biosynthesis, since the information is not well understood.



Scheme 2.3: Oxidation of pentagalloylglucose to ellagic acid.

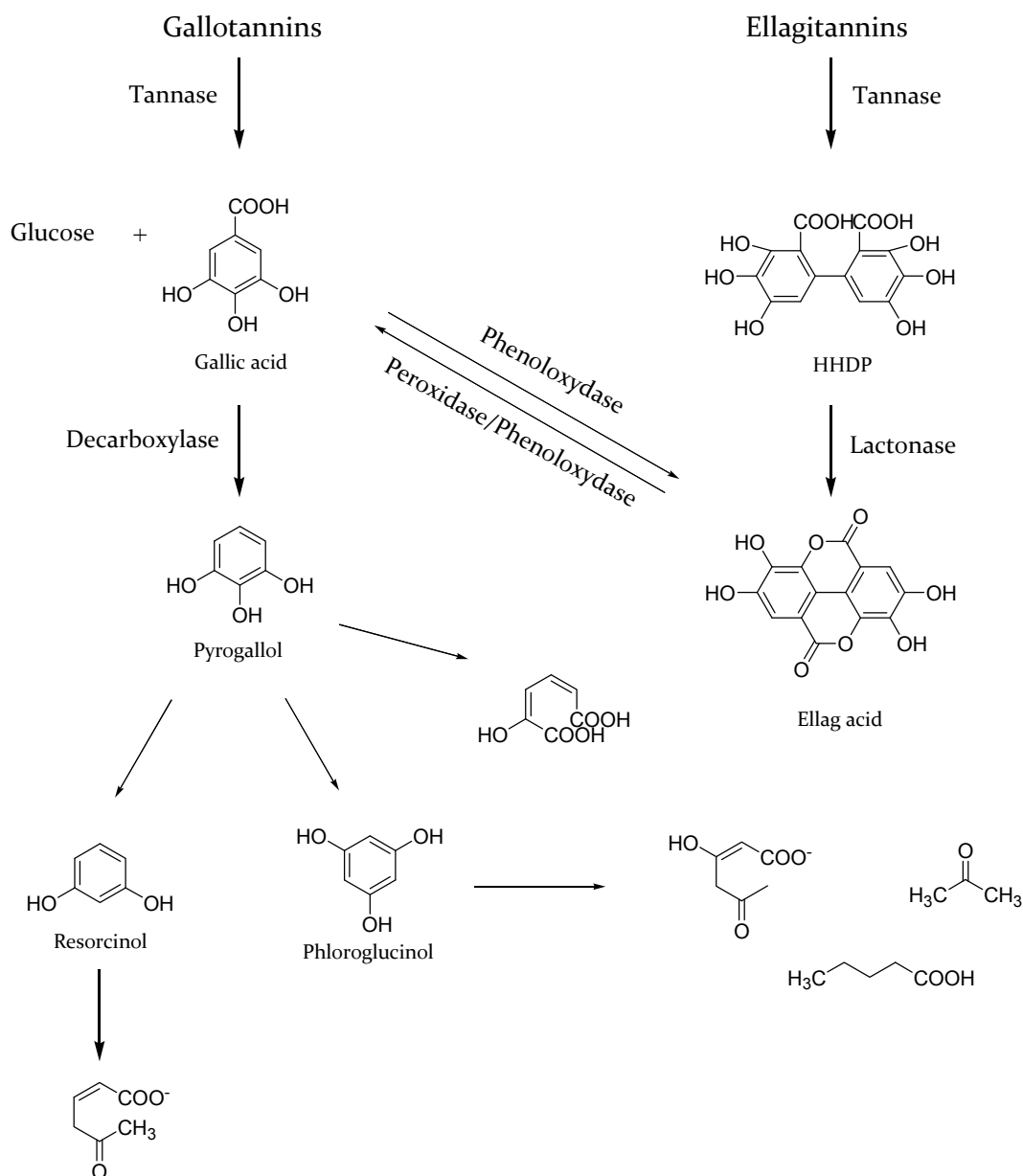
2.2.4 BIODEGRADATION

Hydrolyzable tannins are readily cleaved by micro-organisms and for this reason most of the reports available deal with the degradation of hydrolysable tannin.

Fungi, bacteria and yeasts have different resistance to tannins and show different mechanisms of tannin degradation.⁵⁵ It is shown that both hydrolysis and oxidation

occur in the degradation of gallotannins and ellagitannins with different enzymes, namely tannase, peroxidase or polyphenoloxidase and decarboxylase.

Tannase has both pronounced esterase and depsidase activities and it is responsible of hydrolytic cleavage (see above),^{56,57} to give gallic acid and glucose from gallotannin. Decarboxylase carries out the conversion of gallic acid to pyrogallol⁵⁸ whose aromatic ring will be cleaved into several side branches to yield several simple aliphatic acids (Scheme 2.4).



Scheme 2.4: Pathways for the biodegradation of gallotannins and ellagitannins.

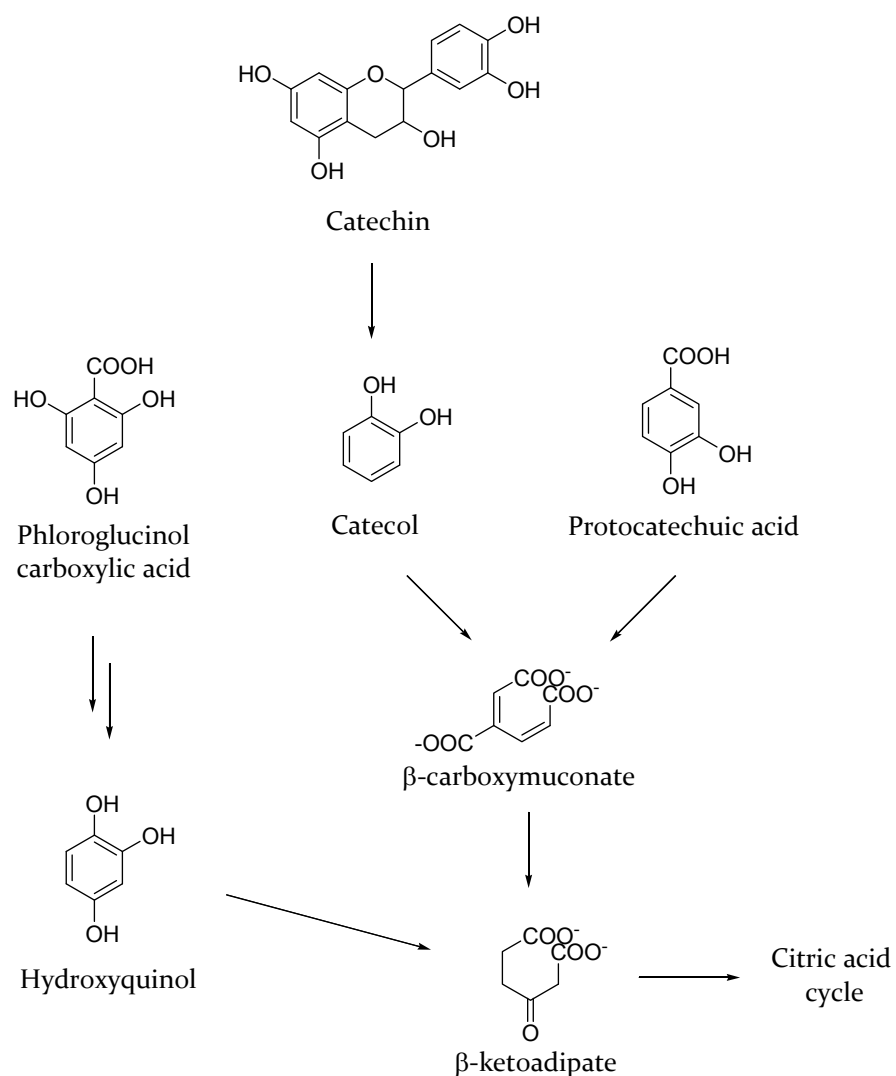
Ellagitannins are subjected to tannase activity, too (only few microorganism produce tannases able to cleave HHDP moieties). After the cleavage of the ester bonds HHDP acid is yielded. A spontaneous lactonization yields ellagic acid, subsequently converted in gallic acid by means of peroxidase or phenoloxidase.⁵⁹ The degradation cycle continues with the degradation of gallic acid (Scheme 2.4).

Fungal tannases (from *Aspergillus*, *Fusarium*, *Penicillium*, *Sporotrichum*, *Rhizoctonia*, *Cylindrocarpon* and *Trichoderma* spp) are versatile for degrading different types of hydrolysable tannins.⁶⁰ They degrade in particular gallotannins, and for this reason they have been used for biodegradation of tannery effluent.⁵⁵ On the other hand, there are only a few reports on tannin-degrading yeasts.

Yeast tannases are effective only in decomposing tannic acid and weakly degrade natural tannins.^{61,62} Bacterial tannases (from *Achromobacter*, *Bacillus*, *Corynebacterium*, *Klebsiella*, and *Citrobacter* spp.) have found to have the capability to degrade tannic acid as well as natural tannins like chestnut, oak and myrobalan tannins.^{63,64}

Compared to gallotannins, ellagitannins are much more difficult to be degraded by microbes because of their complex structure with the further coupling C-C. Some bacteria, fungi and yeasts can only hydrolyze the galloyl residues in ellagitannins, but some other bacteria and fungi from can produce highly active tannase to hydrolyze hexahydroxydiphenoyl and other residues of ellagitannins.⁶⁵ Usually the microbial degradation of condensed tannins is to an extent less than that of gallotannins and ellagitannins. Because of the complexity and heterogeneity of their structure, there have been few studies on the biodegradation of condensed tannins; however, it has been shown that some bacteria and fungi are able to degrade these polyphenolic compounds, with a nearly identical mechanism.⁵⁵ Although some differences have been found, the final products of biodegradation of condensed tannins are acid metabolites, such as β -keto adipate, a simple aliphatic acid that will undergo the citric acid cycle. Tannase is completely extraneous to the biodegradation that is rather carried out by a series of enzymes, properly oxygenases, that prepare the aromatic compounds for ring cleavage and for subsequent ring fission for the generation of tricarboxylic cycle intermediates.

The following scheme shows a general biodegradation pathway: catechin, the basic unit of condensed tannins, is degraded to phloroglucinol carboxylic acid, protocatechuic acid (PCA) and catechol by means of catechin oxygenase. Each of them is subsequently degraded in a multiple step that ends with the cleavage of the aromatic ring (Scheme 2.5).^{66,67}



2.5: General biodegradative pathway of catechin.

2.2.5 INDUSTRIAL APPLICATIONS

As already briefly mentioned above, tannins play an important role as a natural ingredient for a large number of industrial applications. Particularly they are employed in tanning industry, to convert the raw hide or skin into a stable material which will not putrefy; in the oenological sector, as clarifying agents for the production of wine and beer; in cosmetics and pharmaceutical industries for their antioxidant and anticancer properties, and in the textile industry as efficient natural dyes.

The leather tannin industry is one of the most ancient processes still in use. Hide protein, mainly collagen, are rendered insoluble and less susceptible to biological degradation or other attacks. For the manufacture of soft leather the main tanning products used today are acid salts of trivalent chromium but for the manufacture of heavy, rigid and hard leathers (shoe soles, belts) natural vegetable tannins are preferred. They have a strong astringent effect that gives hardness and toughness to the leather produced thanks to the reticulation between phenolic groups of tannins and NH-CO groups of the collagen through hydrogen bonds. The combination with synthetic resins gives light colored leathers having a high resistance to light-induced degradation.⁶⁸

Tannins are unquestionably powerful tools in the hands of the winemaker. Every winemaker knows the important role played by these polyphenols in the gustative quality, colour intensity and stability of their products. One of the most important characteristics of red wine quality is astringency. The term astringency refers to the drying and a puckering sensation in the mouth⁶⁹ when we partake of wine or unripe fruits. It influences the quality of red wine^{70,71} Therefore the knowledge of the structures of astringent compounds in a wine and their relative impact on its sensory properties can be a crucial aspect of winemaking.

Red wine astringency has been mostly associated with condensed tannins or proanthocyanidins, particularly flavan-3-ol polymers, which come mainly from grape seeds and skins.⁷² Hydrolysable tannins, a chemically different family of tannins, also contribute to wine astringency. These tannins come mostly from oak barrels or from other commercial products that are used during wine ageing.⁷³ Previous reports have shown that different tannins vary in the intensity of the astringency response they elicit.^{74,75}

The exact mechanisms of astringency are not well understood but many factors are known to contribute this sensation, including the interactions between tannins and oral epithelial proteins.⁷⁶

The loss of saliva lubricity is thought to result from the interaction of tannins with salivary proteins that has been shown to reduce the lubricity of saliva by increasing friction in the oral cavity.⁷⁷

However, astringency is influenced not only by the quantity of tannins in wine, but also by the presence of macromolecules such as polysaccharides⁷⁸ and residual sugars,⁷⁹ the concentration of smaller molecules such as anthocyanins and catechin monomers,⁸⁰ the acidity⁸¹ and ethanol concentration.⁸² The understanding of how different wine constituents contribute to astringency will enable the winemakers to increase control over the characteristics of the produced wine, in order to satisfy the increasing demand of the consumers.

Tannins are used in a similar fashion in soft drinks or juices to modify taste. They are used to mask undesired taste components of artificial sweeteners or to impart the typical mouth feel, dryness or astringency typically associated with tannins. Moreover, they are employed as colorants for food applications,⁸³ for example they are used to prevent the migration of artificial colorants to adjacent parts of the product.

With the increasing awareness of the environmental degradation and toxicity associated with some synthetic dyes, the consumers' preference shifted to natural products, particularly biologically produced, as substitutes for synthetic substances.

In the traditional natural dyeing of textiles an important part of red/yellow dyes was formed by extraction of anthocyanin/flavonoid dyes from fruits and vegetables.

Tannin dyes directly the natural and synthetic fibers and shows different colors when metals are added. They are commonly employed to color wool, nylon and silk fiber and in the case of nylon fibers, tannic and tartaric acid are commonly used for color fixation.⁸⁴

Tannins have shown also potential antiviral,⁸⁵ antibacterial⁸⁶ and antiparasitic effects.⁸⁷ Although both hydrolyzable and condensed tannins have been used to treat diseases in traditional medicine, the hydrolyzable tannins are generally considered as officinal in Europe and North America. They have been included in many

pharmacopoeias, in the older editions in particular, and are specifically referred to as "acidum tannicum" or tannic acid.⁸⁸

Few accounts with respect to the use of condensed tannins originate from China, where plant extracts containing these tannins as their major constituents are also applied as medicinal agents for the treatment of burns.^{89,90} The treatment of burn wounds was started by Davidson in 1925⁹¹ on the assumption that they reduce the systemic reaction due to the absorption of toxic substances on the burnt skin. The use of tannins on the treatment of burn wounds was abandoned in the middle of 1940's due to the availability of topical antibacterial agents. However, the use of tannins as adjuvant therapy for burn wounds has regained interest.⁹²

Finally, tannins, and particularly hydrolyzable tannins, are precious raw material for the production of gallic acid, produced at the industrial scale by the enzymatic hydrolysis of tannic acid.⁹³ Gallic acid possesses wide range of biological activities such as antibacterial, antiviral, analgesic and anti-apoptotic activities; because of its several unique biological properties, gallic acid is a compound of great interest to both pharmaceutical and chemical industries⁹⁴ It is a substrate for the chemical or enzymatic synthesis of the propylgallate, a potent antioxidant used in food and beverage industry, moreover, it represents an important intermediary compound in the synthesis of the antibacterial drug, trimetoprim, used in the pharmaceutical industry.^{95,96}

It is mainly produced by tannic substrates, namely a mixture of gallotannin, extracted by a great variety of plant, usually using tannin acil hydrolase.^{97,98,99} Therefore, tannins represent a valuable and endless source of a series of product and subproducts having a wide range of exploitable beneficial properties.

2.2.6 TANNINS AS POLLUTANTS

As briefly mentioned earlier, leather industry has been always considered one of the most polluting industries characterized by low technological level of its operations. In fact, only part of the tannins present in the initial tanning solution reacts with the skins; the residual quantity remains in the exhausted bath with other non-tannin substances.¹⁰⁰ The direct discharge of effluents from tanneries into water has grown

environmental problems because of the presence of sulfide, ammonia, phenol, and chromium in the wastewater.¹⁰¹ Therefore, the treatment of exhausted tanning liquid before its discharge is mandatory, not only to recover the unreacted tannin, but also to mitigate environmental problems. Besides chromium tanning compounds, that were found to be carcinogenic and that are mainly recovered by chemical precipitation, coagulation, membrane process and ion exchange,^{102,103,104} also tannins represent a pollutant since they inhibit the growth of microorganisms and therefore, are toxic to activated sludge. In a more detailed view, tannins are thought to directly inhibit methanogens, that are bacteria capable of producing energy through the reduction of CO₂ to methane ($\text{CO}_2 + 4\text{H}_2 \rightarrow \text{CH}_4 + 2\text{H}_2\text{O}$).¹⁰⁵

Methanogens bacteria play a vital ecological role in anaerobic environments of removing excess hydrogen and fermentation products (CO₂) that have been produced by other forms of anaerobic respiration. These bacteria inhabit soil, the slime of ponds and the lakes and are key agents of remineralization of organic carbon in continental margin sediments and other aquatic sediments with high rates of sedimentation and high sediment organic matter.

Although H₂ and CO₂ are the main substrates available in the natural environment and the preferred substrates of these bacteria, also formate, methanol, methylamines, and CO are converted to CH₄.¹⁰⁶ It means that these anaerobes play an important role not only in establishing a stable environment at various stages of methane fermentation, but also in offering an effective means of pollution reduction. Moreover, most of the natural gas and fossil fuels found on the planet is a direct result of its long-term metabolism in favorable environments.

Tannins were found to be strong inhibitor of methanogens because of the occurrence of hydrogen-bondings with either bacterial enzymes or free extracellular enzymes.

Particularly, according to "The tannin theory of methanogenic toxicity,"¹⁰⁷ oligomeric tannins, that are the most commonly employed by the tanneries since they penetrate into the interfiber spacings of the hide proteins, proved to have an enhanced ability to penetrate the bacterial barrier and to form multiple H-bonds^{108, 109,110} with the bacterial proteins.

In order to control the damage that water soluble tannins provoke to the environment, their concentration in tanneries wastewater should be limited. Their precipitation and

recovery would require an extra high dose of metal coagulant that will cause an additional metal pollution. Therefore replacement of a chemical relief with a biological treatment of tannin-containing wastewater might be a more reasonable solution.

2.2.7 ISOLATION METHODS

Extraction process of tannins from natural matrix is nowadays performed by empirical methods, and industrially no optimization of extraction process has to date been carried out.

Water is the most extensively studied solvent for the tannins extraction, but also organic solvents such as methanol, ethanol, ethylacetate or acetone and aqueous solutions of the same organic compounds are employed. However, the extraction method should be planned carefully since the results, particularly the percentage of recovery and the selectivity, are affected by the extraction steps.

Tannin can be extracted from fresh, frozen, lyophilized or dried plant samples since the treatment does not affect the extractability.^{111,112}

Conventional methods typically involve reducing the plant tissues into smaller particles using liquid nitrogen and a mortar and pestle. The pulverized plant material is then extracted in a solvent or in a mixture of them.

Three solvents are commonly used to extract tannins from plant samples: boiling aqueous methanol, aqueous acetone, or acidic methanol.

Boiling aqueous methanol is thought to be the most effective solvent for condensed tannins but the recovery of tannins is estimated to be as low as 30% for some tissues.¹¹³ Since aromatic ester (depside) bonds are hydrolyzed by aqueous alcohols, they are thought to be unsuitable for extraction of hydrolyzable tannins.¹¹⁴ Aqueous acetone is routinely used to extract hydrolyzable tannins,¹¹⁵ but no quantitative estimates of recovery are available. Some authors believe condensed tannins are extracted quite efficiently with aqueous acetone^{116,117,118} but others have found that condensed tannins are recovered in low yields when aqueous acetone is employed.¹¹⁹ Acidic methanol is the best solvent for extracting the condensed tannins from some varieties of sorghum.¹²⁰ It is hypothesized that the tannin in those varieties is chemically unique, perhaps covalently attached by an acid-labile bond to some component of the grain. Although

aqueous methanol (pH 5-6) causes the methanolysis of depside bonds in hydrolyzable tannins, at more acidic pH values (pH<3) methanolysis does not occur.¹¹⁴ Thus acidic methanol should be appropriate for extraction of hydrolyzable tannin.

In some cases, the extraction is carried out by using a sonicator, that “force” the solvent to penetrate in unwettable materials,¹²¹ or by an homogenizer. The superiority of the homogenizer is partially due to its efficacy in breaking down the cell-walls, in a way in which the sonicating bath cannot.¹²²

The extraction of plant tissues yields a mixture of phenolic and polyphenolic compounds so the separation of tannins from non-tannins phenolics represents the subsequent step.

The mixture is purified on a sephadex column equilibrated with 80% ethanol in water. Sephadex is a cross-linked dextrane gel normally manufactured in a bead form and most commonly used for gel filtration columns.

The plant extract, dissolved in a minimum aliquot of the extracting solvent is applied on the column and a first elution with ethanol separates the non-tannin phenolics. Following exhaustive washing with ethanol, the tannins that remain on the top of the column as a brownish band are eluted with 50% aqueous acetone.³⁹

Separation of tannins from non-tannins is based, therefore, upon the finding that tannins were adsorbed to Sephadex LH-20 in 95% ethanol.¹²³ An amount of ascorbic acid (0,1 %) is in some cases added to the extractive before the purification on sephadex, in order to prevent the oxidation of tannins during the purification procedures.^{124,125}

2.2.8 CHARACTERIZATION METHODS

The wide presence of tannins with variable structures makes the development of analytical methods of structural characterization a challenging and scientific relevant task.

The most diffused analysis methods are based on the general evaluation of the phenolic groups content, on the overall condensed or hydrolysable tannins content by specific functional groups assays and on protein precipitable methods.

The protein precipitable methods are based on the characteristic capability of tannins to precipitate proteins. Therefore, it is a general method to recognize the presence of

“tannic compounds”. There are several methods for determining tannins which take advantage from the interaction between tannins and proteins, among them the Radial Diffusion Assay,¹²⁶ the Protein-Precipitable Phenolics method¹²⁷ and the Blue BSA method¹²⁸ are the most common and convenient.

They measure the amount of condensed and hydrolyzable tannins precipitated by a standard protein, the bovine serum albumin (BSA). In some cases, the tannin-protein complex is colored in presence of a reagent (FeCl_3 in Protein-Precipitable Phenolics Assay; Prussian Blue reagent in Modified Radial Diffusion Assay; Remazol dye in Blue BSA method) and determined spectrophotometrically.

The functional groups methods are dependent to the chemical reactivity of the functional groups of a given type of tannin and provide both qualitative and quantitative information.

Basically, these methods exploit the characteristic reaction of phenols, above all their tendency to be oxidized. There are methods for determining the amount of total phenols, such as the Prussian Blue method,¹²⁹ but specific methods for both for hydrolyzable and condensed tannins have been also described.

The Prussian Blue method is a colorimetric assay based on the oxidation of the phenolic analyte with the subsequent reduction of the reagent to form a chromophore. This method provides neither a method to distinguish tannins from non-tannins, nor to identify a specific type of tannin (hydrolyzable or condensed).

Among the methods for determining the amount of condensed tannins (proanthocyanidins), Acid Butanol Assay,¹³⁰ Vanillin Assay¹³¹ and Phloroglucinol Assay¹³² are the most common. The Acid Butanol method involves the HCl catalyzed depolymerization of condensed tannins in butanol to yield a red anthocyanidin product that can be detected spectrophotometrically. The Vanillin Assay involves the reaction between the aldehydic function of vanillin and the meta-substituted ring of flavanol to yield a red adduct can be detected spectrophotometrically. Since vanillin reacts only with the meta-substituted flavanoids, the 5-deoxy proanthocyanidins (as quebracho) do not produce colored species.³⁹ The Phloroglucinol Assay is also based on the cleavage of the interflavan bonds to yield the monomers, that are allowed to react with phloroglucinol to form the phloroglucinol adduct, spectofotometrically detected.

Among the method for determining the amount of hydrolyzable tannins the Rhodanine method,¹³³ the Potassium Iodate method¹³⁴ and the Nitrous Acid method¹³⁵ are the most common.

They are based on the determination of gallic or ellagic acid released by the hydrolysis of gallotannins and ellagitannins respectively.

In general, the hydrolysis is carried out using sulfuric acid and the assay is based on the spectrophotometric determination of the chromogen formed between free gallic/ellagic acid and a specific reagent, namely rhodanine or potassium iodate for the detection of gallotannin, and nitrous acid for the detection of ellagitannins.

Although these assays are often standardized with simple phenolics, such as gallic acid, complex polyphenols may have a different response with respect to the simple standard. For this reason, the results are expressed in “gallic acid equivalents” (GAE) rather than as absolute weight.

Because of their heterogeneity and their complex structure, tannins analysis has represented a challenge for researchers. The most diffused analysis are based on the general evaluation of phenolic group content, distinguishing between hydrolysable and condensed tannins, but they do not provide information about the fundamental structural features, such as the degree of polymerization and the regiochemistry of condensed tannins or the degree of esterification and the regiochemistry of hydrolysable tannins. Moreover, the common tannin assays based on the functional groups are affected by the presence of impurities, they often require the pretreatment of the analyte in order to avoid interferences.

2.3 LIGNINS

2.3.1 OCCURRENCE

Lignin derives from the Latin term “lignum”, which means wood.¹³⁶ Actually it is one of the three structural polymers that compose wood, constituting about 30% of its structure, and represents the most abundant polymer, after cellulose, of the plant kingdom.

In 1838 Anselme Payen recognized in the wood an “encrusting material” that embedded cellulose on the inside, later (1985) defined by Schulze as “lignin”.

It is an integral part of the secondary cell walls of every plant species, including also some algae,¹³⁷ and its amount ranges from 20 to 40% among the species. The less lignified plants was found to be the monocotyledons,¹³⁸ that are one of the two major groups of flowering plants, or angiosperms.

This biopolymer plays a crucial role in plants since it provides a mechanical support without which plants would never moved from the aquatic to the terrestrial environment during Carboniferous.¹³⁹

In the higher plants, gymnosperms and angiosperms, it seals the water conducting system against the hydraulic pressure drop produced by the transport of water from the soil to the leaves and needles. It also plays an important role as protection from destructive enzymes that are responsible of the cell wall degradation.¹⁴⁰ Its distribution within the cell wall is not uniform but the high concentration was found in the lowest, highest and inner parts of the stem.¹⁴¹ This complex polymer has a significant role in the carbon cycle. Its slow decomposition gives rise to the most part of the material that becomes humus as it decomposes. The humification, that is degradation of the organic matter, is an important processes that gives not only precious structural and chemical properties to the soil but also permits the full recycling of carbon in the environment, ensuring closure of the carbon cycle.¹⁴²

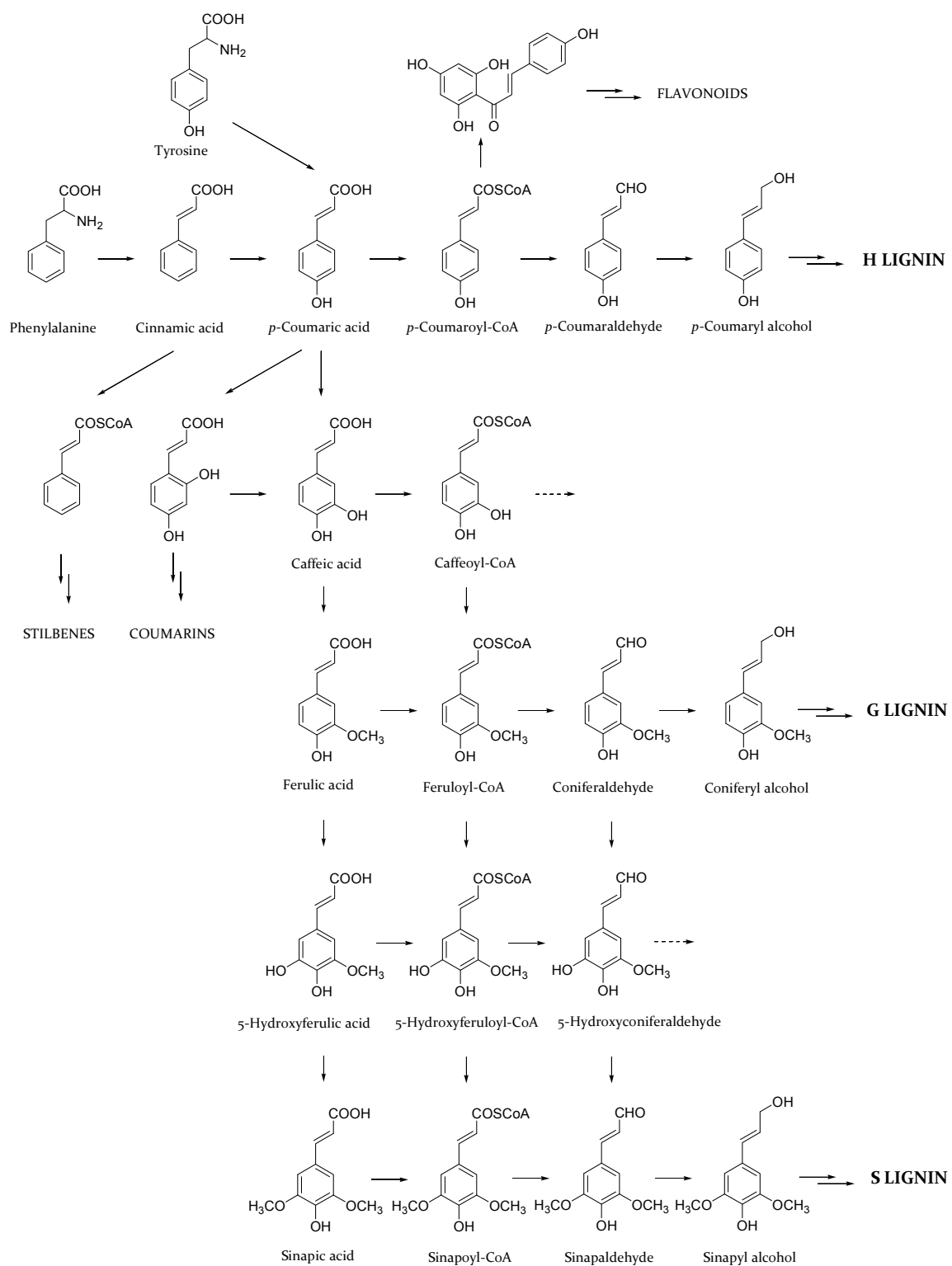
2.3.2 BIOSYNTHESIS

In the biosynthesis of lignin, the three monomeric phenylpropanoic units that compose lignin, coniferyl, sinapyl and p-hydroxycinnamyl alcohols, undergo an

enzyme-mediated dehydrogenative polymerization, arising to a complex and amorphous polymer.¹⁴⁰

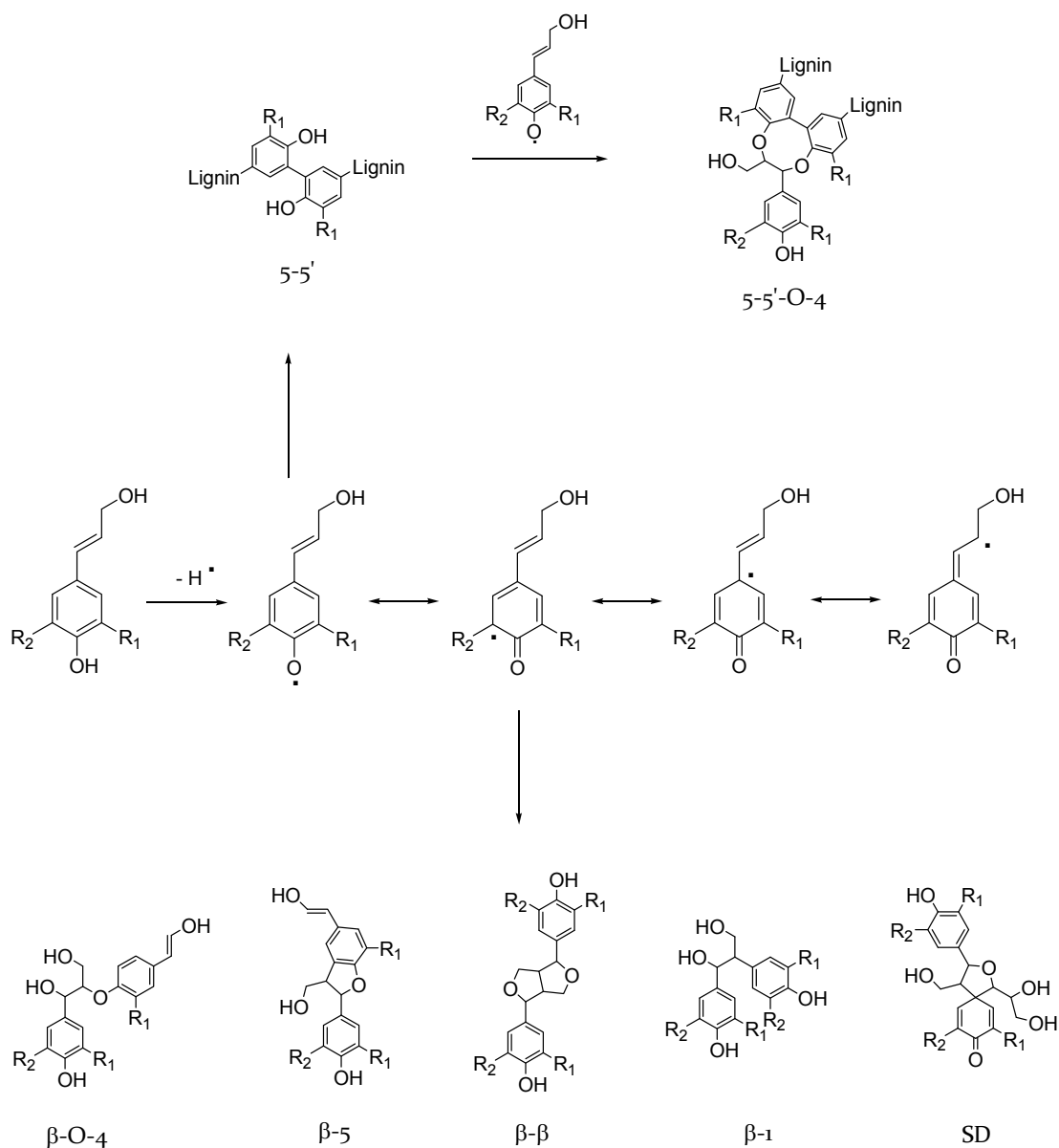
On the basis of the relative content of these three monomeric precursors, it is possible to classify lignin in three different types: guaiacyl lignins (or G-lignins), guaiacyl-syringyl lignins (or GS-lignins) and guaiacyl-syringyl-*p*-hydroxyphenyl lignins (or GSH-lignins).¹⁴³ G-lignins are predominantly composed of coniferyl alcohol (guaiacyl alcohol) and are distinctive of gymnosperms (softwood); GS-lignins are characterized by a large amount of coniferyl and synapyl alcohol are typical of hardwood; GSH-lignins represent the most heterogeneous lignins, containing the same amount of the three monomers, and are distinctive of grass.

The biosynthesis of lignin starts with the conversion of glucose to shikimic acid, that represents the most important intermediate substance of the so called “shikimic acid pathway”.¹⁴⁴ The final products of the shikimic acid pathway, the amino acids L-phenylalanine and L-tyrosine, represent the starting compounds for the enzymatic phenylpropanoid metabolism, also called “cinnamic acid pathway”. It yields not only the three cinnamyl alcohols via activated cinnamic acid derivatives, but also to extractive components like flavonoids or stilbenes. The hydroxylation of the aromatic rings followed by the partial methylation yield *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 (Scheme 2.6).



Scheme 2.6: Lignin biosynthetic pathway.

In the further steps the cinnamyl alcohols undergo to randomic radical coupling reactions, followed by the addition of water or of primary, secondary and phenolic hydroxyl groups to quinonemethide intermediates, without following a regular mechanism (Scheme 2.7).



Scheme 2.7: Lignin biosynthesis. Coupling mode for monolignols and oligomeric lignin chains.

The randomic coupling of the lignols yield a three dimensional and non linear polymer. For this reason, lignin is not considered a defined compound but as a

composite of physically and chemically heterogeneous materials whose structure may be represented by models.

In plants this complex biopolymer is located in the space between the polysaccharidic fibrillar elements in the cell wall. X-ray crystallography studies revealed that in the primary cell wall the deposited microfibrils of cellulose and hemicelluloses are randomly disposed while in the secondary cell wall, constituted of three internal layers (S_1 , S_2 , S_3), the orientation of the cellulose microfibrils appears fixed (Figure 2.13).

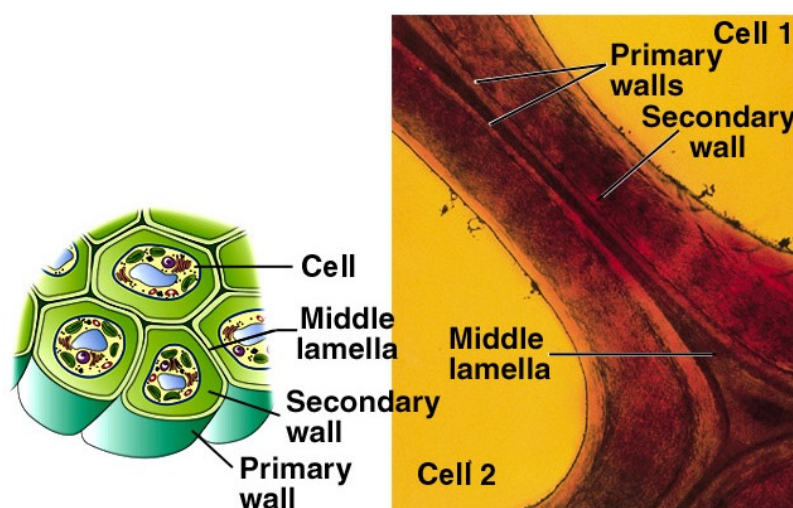


Figure 2.13: Schematic representation of cell wall. Copyright © The McGraw-Hill Companies, Inc.

The lignifications process starts with the deposition of carbohydrates in the primary cell wall before the formation of S_1 layer of the secondary wall.¹⁴⁵ As soon as the S_1 layer is formed, the cellulose microfibrils are deposited in the S_2 layer with the contemporary lignifications. The lignifications process proceeds up to the internal S_3 layer.

Because of geometric constrains imposed by the cellulose microfibrils, lignin in the secondary wall is probably not completely randomic but it results somehow organized in two-dimensional network polymer of about 2 nm thick.¹⁴⁶ The deposition of precursor monomers changes with type, age and morphological region of the cell, generally in the order of p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol.

It is known that lignin is not loosely sitting in the cell wall, but that it is associated with the polysaccharidic wall itself. The phenomenon of the intimate association between the polysaccharide and lignin is described by the terms Lignin-Polysaccharide Complex (LCC). The covalent linkages existing between lignin and polysaccharides cannot be easily cleaved by selective chemical treatments or special purification techniques, even in highly purified cellulose where usually remains some residual lignin. The suggested bond types between lignin and the polysaccharidic microfibrils were highlighted from degradation experiments, mostly performed by means of mild alkaline, acid or enzymatic hydrolysis: most frequently they are ether linkages, ester linkages and glycosidic bonds, as reported in Figure 2.14.^{147,148}

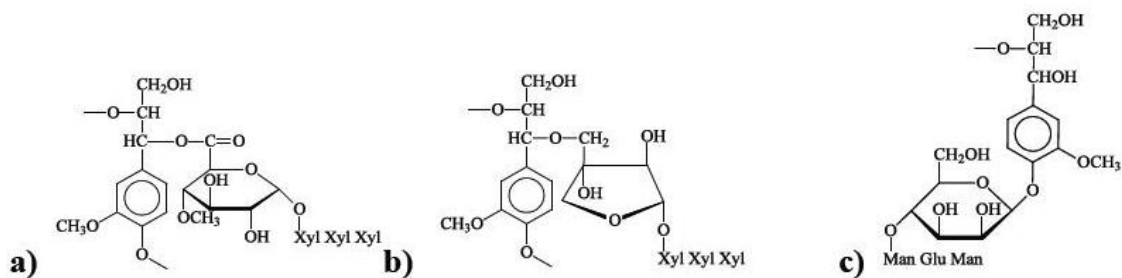


Figure 2.14: Lignin a): glycosidic bonds: benzyl ester linkage; b): benzyl ether linkage; c): phenyl glycosidic linkage.

2.3.3 STRUCTURE

After many years of study, the structure of native lignin still remains unclear because of its complex structure and heterogeneity. Up to now, several analytical studies of model compounds, synthetic lignins and isolated lignins were necessary to obtain a representative structure for this complex biopolymer. Lignin suffers from the impossibility to obtain a reproducible product from wood because of the carbohydrate impurities and the degradation effects. Finally it is still questionable whether carefully isolated lignins are representative of the total lignin in wood.¹⁴¹ However, the numerous studies on model compounds, synthetic and isolated lignins proved to be fundamental to elucidate structural features such as the dominant linkages between

the basic monomeric units as well as the abundance and frequency of functional groups. In any case, lignin cannot be described as a simple combination of few monomeric units by few types of linkages.

Commonly, lignin is described as a phenyl-propanoid (C_9) polyphenol composed of three monomeric precursors, coniferyl, sinapyl and *p*-hydroxycinnamyl alcohols (Figure 2.15) mainly linked between them by arylglycerol ether bonds.¹⁴⁹

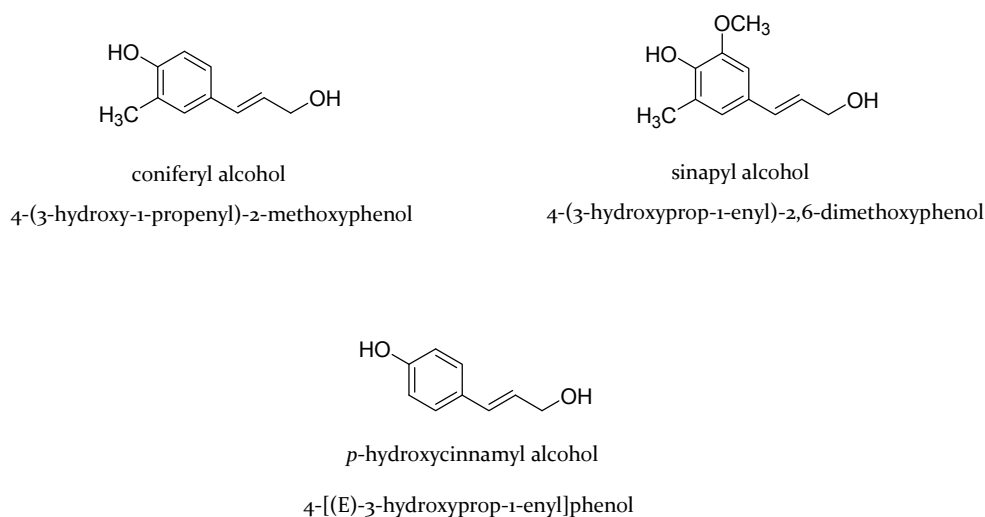


Figure 2.15: Monomeric phenyl propanoic precursors.

Over the years several researchers gave their own contribution in the elucidation of lignin structure;¹⁴³ in 1968 Freudenberg designed the first lignin model based on the dehydrogenative polymerization concept, later Adler gave a structural scheme for spruce lignin carrying out oxidative degradation experiments,¹⁵⁰ but, however, the most part of lignin models were obtained by computational simulations, as Glasser reported.¹⁵¹ By means of a simulation of radical coupling reactions of the *p*-hydroxycinnamyl alcohols, he obtained a complex structure composed of 94 units corresponding to a total molecular weight of more than 17 kDa.¹⁵²

Later on, Brunow proposed a new structural scheme for softwood lignin, that was characterized by a widely branched backbone.^{153,154,155}

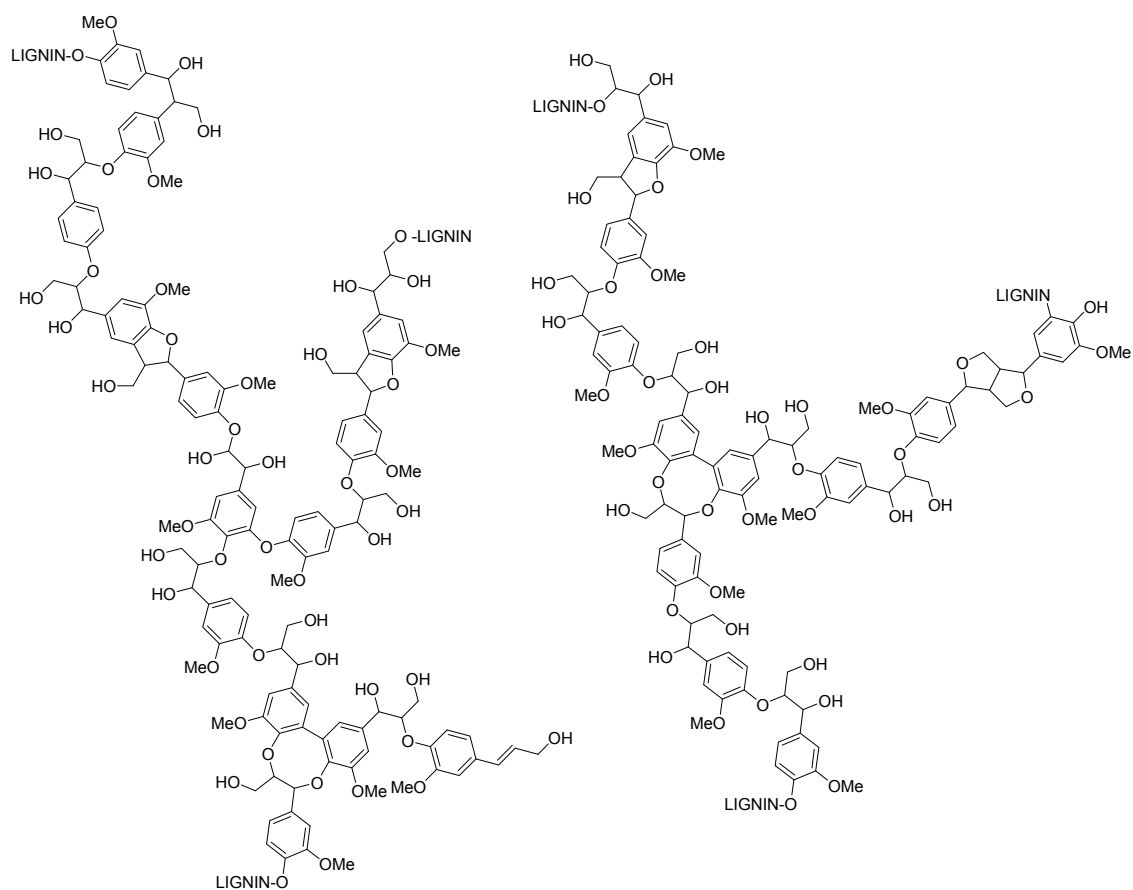


Fig 2.16: Scheme of lignin structure proposed by Brunow.

Although lignin seems to have not repetitive pattern of units and linkages, several studies carried out on softwood lignin highlighted some of common linkages that characterize its structure (Figure 2.17).

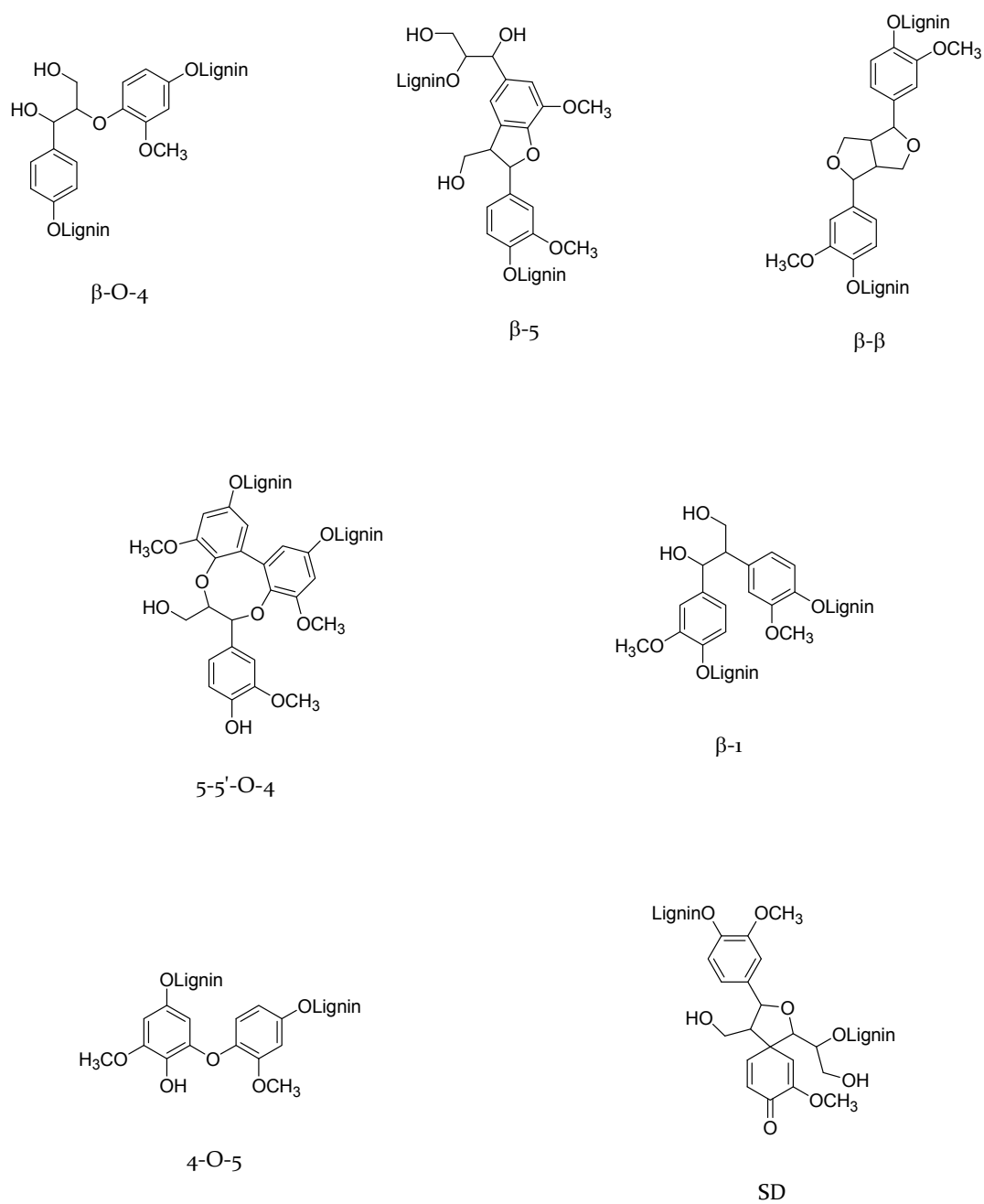


Figure 2.17: Common linkages in lignin.

The abundance of these linkages in lignin structure is reported in Table 2.2 while Table 2.3 shows the frequency of some common functional groups.¹⁵⁵

Table 2.2: Abundance of linkages in softwood and hardwood lignins.

Lignin interunit bond	Abundance (n° per 100 C ₉ units)
β -O-4	44,7 $\pm$ 0,9
β -5	10,6 $\pm$ 2,0
β - β	3,19 $\pm$ 0,01
β -1	1,25 $\pm$ 0,34
5-5'-O-4	3,07 $\pm$ 0,05
SD	0,24 $\pm$ 0,05

Table 2.3: Abundance of functional groups in softwood lignin.

Functional group	Abundance (n° per 100 C ₉ units)
Free phenolic hydroxyl	15 $\div$ 30
Methoxyl	92 $\div$ 96
Benzyl alcohol	15 $\div$ 20
Carbonyl	10 $\div$ 15

However, the extreme heterogeneity of lignin prevents the acquisition of a satisfying knowledge of its structure; for this reason its quantitative structural characterization is still an open debate.

2.3.4 CHARACTERIZATION METHODS

Many of the methods used for the investigation of lignin structure and reactivity proved to be inadequate so that researchers are still directing their efforts towards the development of a new method capable of dealing with the heterogeneity of lignins.

The study of lignin structure is still carried out following two different approaches: the degradative and the non-degradative methods. The first approach is based on lignin modification through derivatization or chemical degradation of the sample. The second approach is based on the analysis of the sample as it is, in solid state or in

solution. It is also possible to combine the two approaches in order to obtain a more complete and clear information.¹⁵⁶

The crucial constraint of the degradative methods (acidolysis, thioacidolysis, hydrogenolysis, oxidation with nitrobenzene, cupric oxide and permanganate) lies in the lack of a complete information about lignin structural features since all these methods liberate only a fraction of the polymer for analysis. The analysis of the fragments obtained from the cleavage of lignin backbones provides partial data due to the specificity of the treatments. Using permanganate oxidation for cleavage the side-chains are degraded to carboxyl groups attached to the aromatic ring. The structure of the products reveal the substitution pattern of the aromatic rings. The structural information obtained by permanganate oxidation is mostly qualitative since the low yields of degradation acids make it difficult to estimate the quantitative distribution of structural units.¹⁵⁷

During thioacidolysis arylglycerol- β -aryl ethers are selectively cleaved by a treatment with boron trifluoride etherate and ethanthiol. Monomeric products substituted with thioethyl groups are formed and can be analysed by gas chromatography. Dimeric products can be analyzed after removal of sulfur substituents by reduction with Raney-Nickel.¹⁵⁸

An important limitation of thioacidolysis is that it can only detect structural units bound by arylglycerol- β -ether bonds. Another selective β -arylether cleavage is provided by the DFRC protocol (Derivatization Followed by Reductive Cleavage), that consist into bromination of the benzylic positions and concomitant acetylation of free hydroxyl groups by acetyl bromide followed by the reductive cleavage of the brominated intermediate by zinc metal reduction and acetylation.¹⁵⁹ Quantification of products is carried out by gas chromatography. However the presence of elemental bromine, combined with the existence of intact β -O-4 interunit linkages and the high average relative molecular weight of the treated lignin samples indicates that the DFRC method does not completely, or not efficiently enough degrade the lignin polymer.¹⁶⁰

Thus to date there is not any available method that allows the contemporary identification and quantification of all the interunit bondings in lignin. Furthermore, different analytical methods applied to same lignin samples often provide significantly different results that are not directly comparable. In a more detailed view, the

abundance of the β -1 linkage (Figure 2.18) has been estimated to range from 1 to 15% in spruce lignins according to the analytical method used.

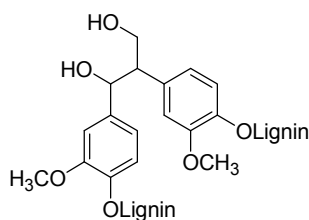


Figure 2.18: β -1 linkage.

Such discrepancies have been explained suggesting that the precursor of the β -1 structure, a spirodienone (Figure 2.19), might be present in native lignins and form β -1 moieties upon hydrolysis or thioacidolysis.¹⁵⁵

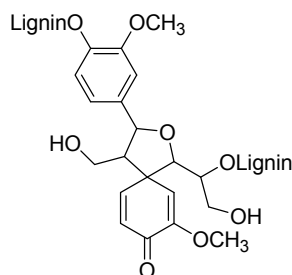


Figure 2.19: Spirodienone.

However, more clear details about lignin structure were revealed only during recent years thanks to NMR techniques.

NMR as a powerful structural elucidation technique has been employed in lignin characterization both in solution and in solid state.**Error! Bookmark not defined.**¹⁶¹

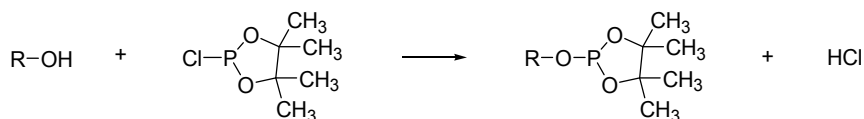
Unfortunately, conventional ^1H proton and ^{13}C -NMR spectra of lignins do not allow detailed assignment and quantification of individual signals due to broad and overlapping NMR signals resulting in low resolution spectra. ^1H - ^{13}C -correlated (HSQC, HMQC) spectroscopy, which combines the sensitivity of ^1H NMR with the higher resolution of ^{13}C NMR, proved to be the best method to reveal the frequencies of the different lignin units and the interunit bonding patterns and has allowed their use as a

valid analytical technique in the analysis of complex samples.^{162,163,164} HSQC experiments allow the identification of lignin structural features that cannot be characterized by alternative structural analytical techniques.¹⁶⁵ Nevertheless, the standard NMR experiments cannot be used to determine the molecular weight of lignin samples; there are many analytical methods able to elucidate the distribution of the molecular weights in the sample: among them Gel Permeation Chromatography (GPC), Light Scattering, Vapor Pressure Osmometry (VPO) and Ultrafiltration are the most common.

In my PhD studies the elucidation of lignin structure and the detection of its structural modifications was carried out basically through the ³¹P Nuclear Magnetic Resonance (³¹P NMR), 2D heterocorrelated NMR spectroscopy (HSQC) and the Gel Permeation Chromatography technique (GPC).

³¹P-NMR

Since lignin has not phosphorous nuclei included in its structure, the analysis requires the derivatization of lignin functional groups with an appropriate phosphitylation reagent. The technique, that will be described in detail later, allows the determination of hydroxyl aliphatic groups, discerning the primary to the secondary OH,^{155,166} the determination of the carboxylic groups content and the discrimination between the different phenolic hydroxyl groups contents (syringyl, guaiacyl and p-hydroxyphenyl); moreover, it gives the possibility to distinguish between the condensed 4-O-5', 5-5' and diphenylmethoxy forms.^{167,168} One of the most common phosphitylation reagent widely employed in our studies was 2-chloro-4,4',5,5'-tetramethyl-1,3,2-dioxaphospholane. The phosphitylation reaction is reported in Scheme 2.8.



Scheme 2.8: Phosphitylation reaction of hydroxyl groups.

This reagent allows to determine the overall distribution of hydroxyl groups present in lignin, discerning the aliphatic OH from the phenolic and the acid ones. In particular, it allows to distinguish among the phenols the different degree of substitution.

The analysis is carried out in presence of an internal standard that gives the possibility to quantify the different hydroxyl groups.¹⁶⁹

Thanks to the ³¹P natural abundance, its sensitivity, the availability of the derivatizing reagents and the ease of obtaining quantitative derivatization under mild conditions, the phosphorous quantitative NMR proven to be a precious analytical tool for the analysis of complex biopolymer.

On the basis of the numerous studies reported in literature about lignin structure, it was possible to assign ranges of chemical shift for phosphorus nuclei labeling hydroxyl groups in different environments in lignin (Table 2.4).

Table 2.4: ³¹P-NMR attribution (from Argyropoulos et al.).

OH groups	Chemical shift
Aliphatic	149,0-146,0
Diphenylmethane	144,27-142,78
4-O-5'	142,78-141,24
5-5'	141,72-140,24
Guaiacylic	140,24-138,8
p-OH phenylic	138,8-137,4
COOH	135,5-134,0

The typical lignin profile is shown in Figure 2.19.

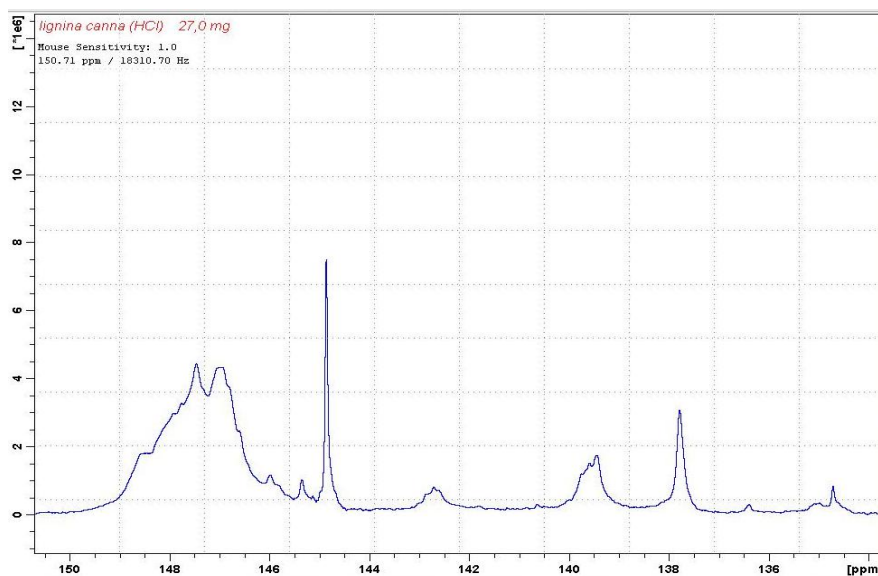


Figure 2.19: Typical lignin profile.

QUANTITATIVE HSQC MEASUREMENTS: GENERAL ASPECTS

Generally, the bidimensional HSQC technique allows good resolution of the NMR signals since the resonances are dispersed along two dimensions belonging to different nuclei, in this case proton and carbon. However several problems prevent the use of standard HSQC for quantification studies. The first constraint is caused by a non-optimal polarization transfer: as a consequence of the fixed INEPT transfer delay used, the polarization transfer proves to be optimal only for a specific J_{CH} coupling, so it reduces the volume of cross peaks belonging to CH groups with different couplings.

Another constraint is caused by the so-called offset problem: the great dispersion of carbon resonances is positive in terms of resolution but often 90° and 180° carbon pulses do not uniformly cover the wide spectral width of a carbon spectrum. Finally relaxation occurs during the delays.

In order to overcome these constraints, Heikkinen *et al.*¹⁷⁰ developed an upgraded 2D HSQC technique that accomplished a uniform polarization transfer over a range of J_{CH} couplings by collecting four HSQC with fixed delays periods corresponding to different J_{CH} evolution lines (namely 2.94, 2.94, 2.94 and 5.92 ms). In this way a wider range of J_{CH} couplings is selected after summation of the four experiments. However, the resulting

experiment, called quantitative HSQC (Q-HSQC) has the disadvantage that a fourfold experimental time is required.

The problem of long acquisition times was elegantly solved by Peterson and Loening.¹⁷¹ They developed an experiment called Quick Quantitative HSQC (QQ-HSQC) in which the problems associated with different J_{CH} couplings were solved with the introduction of slice selective adiabatic pulses in which three quarter of the region experiences the lower delay (2.94 ms) and one quarter the higher delay (5.92 ms). Since this happens within a single scan there is no longer the need for 4 experiments, retaining the main feature of Q-HSQC.

Zhang and Gellerstedt contributed to the improvement of this technique reporting an extensive study on the integration. Comparing the integrals of CH signals having comparable T_2 values with the corresponding integrations of the of 1D NMR spectra, they proved that any of these signals can be used as an internal standard, since different resonances from the same type of macromolecule having similar structural features always have similar T_2 profiles.¹⁷² The use a low molecular weight internal standard would not be suitable for quantification because the effects caused by different T_2 relaxation could not be corrected.

This technique was finally used to determine the amount of interunit bonding of lignin.

The table below summarizes the results obtained by the QQ-HSQC determination of Norway spruce milled wood lignin and compares them with previously reported data.^{173, 174}

Table 2.5: Amount of interunit bonding in Noerway spruce milled wood lignin as evaluated by QQ-HSQC and from previous literature data.

Interunit bonding	QQ-HSQC *	Zhang ¹⁷³ (NMR method)*, ^a	Adler ¹⁷⁴ (wet chemistry method)*, ^a
β -O-4	44,7 $\pm$ 0,9	40-43	48
β -5	10,6 $\pm$ 2,0	10-12	9-12
β - β	3,19 $\pm$ 0,01	3,5	2
β -1	1,25 $\pm$ 0,34	2	2
5-5'-O-4	3,07 $\pm$ 0,05	5	-
SD	0,24 $\pm$ 0,05	1,2-2	-

*: Number per 100 C₉ units

^a: the ranges here reported are not experimental errors, but resul from different literature reports that have been presented without evaluation of the pertinent experimental errors

The QQ-HSQC NMR pulse sequence can be successfully applied to lignin structural elucidation. It provides good resolution and signal-to-noise ratio in a reasonable analysis time. The standard deviations of the results were found ranging from 0.01 to 2 bondings per 100 C₉ units.

DETERMINATION OF THE MOLECULAR WEIGHT DISTRIBUTION IN LIGNINS

Several analytical techniques have been employed in order to characterize lignin average molecular weight, ranging from vapor osmometry, crioscopy, ultrafiltration, to GPC and light scattering analysis; however, different analytical techniques yield discordant results.

The solvent system used to determine the molecular weight distribution has crucial importance since non-hydrogen bonding solvents such as dioxane and THF can undergo large self-aggregation in lignin, and hence the results are highly dependent on concentration. These techniques suffer from the disadvantage of being affected by supramolecular aggregation processes, lignin in fact shows a high tendency to aggregate and the results of such analyses are strongly dependent upon the freshness of the lignin preparation.^{175,176,177}

Gel Permeation Chromatrography is the most widespread analytical method currently in use to determine lignin molecular weight distribution due to its ease of use.

It is a kind of size-exclusion chromatography (SEC) that separates the analytes on the basis of the size or hydrodynamic volume.¹⁷⁸ Before analyzing an unknown sample, the calibration of the system is required using, usually, a solution of monodispersed polystyrene in THF as standard. Once determined the retention time of the standard polymer, a calibration curve can be obtained by plotting the logarithm of the molecular weight versus the retention time. However, since the commercially available standards for the calibration curves construction are synthetic polymers, usually polystyrene, they might have a different behavior with respect to lignin polymers, and thus might represent a poor source for obtaining the necessary standard curve. The stationary phase in the column is a gel, usually sephadex, having different pores sizes; molecules of low molecular weight are able to penetrate into the gel particle pores but large molecules are excluded from the pores and pass directly through the column. Consequently, the largest molecules elute first and the smallest last. For the GPC analysis, the sample should be completely soluble in THF, so in the case of lignin, the derivatization of the sample, by means of acetylation or methylation, is mandatory (see below).

The acetylation is based on the overnight reaction of lignin with pyridine-acetic anhydride at RT and allows the complete recovery of the sample with a minimum of added impurities. For the methylation, the addition of diazomethane is required after the solubilization of the sample in a mixture of dioxane-methanol.

Another procedure that can be used in order to make lignin samples soluble in THF is the acetobromination. It is based on the reaction of lignin with a mixture of acetic acid and acetyl bromide (98:2 volume ratio) for two hours at room temperature. Before analyzing the sample, the solvent is removed under reduced pressure and the acetylated product is dissolved in THF. According to this procedure the primary alcoholic and the phenolic hydroxyl groups are acetylated, while the benzylic α -hydroxyls are displaced by bromide.¹⁵⁸

This procedure has the important advantage to avoid the handling of pyridine, for this reason it was chosen as exclusive acetylation method for our studies. Moreover, the brominated lignin proven to be more soluble in THF.

In Figure 2.20 is shown a typical lignin GPC profile obtained after acetobromination:

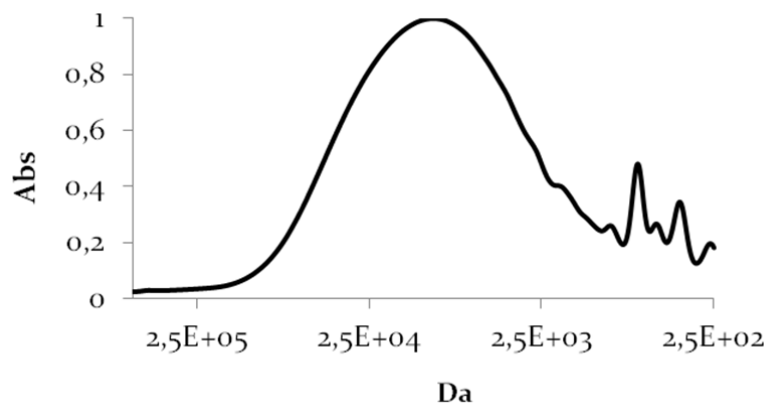


Figure 2.20: Typical lignin GPC profile after acetobromination.

Characteristic molecular weight numbers – usually M_n (number average molecular weight) and M_w (weight average molecular weight – for the lignin polymer can be determined by standard table calculations from this curve.

Although GPC represents the most widespread analytical method to determine lignin molecular weight distribution, it is potentially affected the phenomena of supramolecular aggregation.

However, although the analysis cannot provide absolute results, it is currently employed to correlate data belonging to the same set of experiments. In my PhD studies GPC analysis has been used not to have absolute information about lignin molecular weight distribution, but mainly to detect the modification of its structure after treatments, by correlation with the starting material.

2.3.5 BIODEGRADATION

It is known that lignin plays an important function in plant natural defence against parasites, preventing its degradation.

In nature only few organisms are able to degrade it but there is a variety of fungus that digests moist wood, causing it to rot. Wood-decay fungi can be classified according to the type of decay that they cause: the best-known types are brown rot, soft rot, and white. While the brown and the soft rot break down the cellulose or hemicelluloses provoking the discoloration and the crack of the wood, white rot fungi (Figure 2.21 A) preferentially attack lignin more readily than hemicellulose and cellulose in wood tissue, leaving the lighter-colored cellulose behind (Figure 2.21 B).



A



B

Figure 2.21: A) White-rot fungus; B) delignification of wood caused by white-rot fungi.

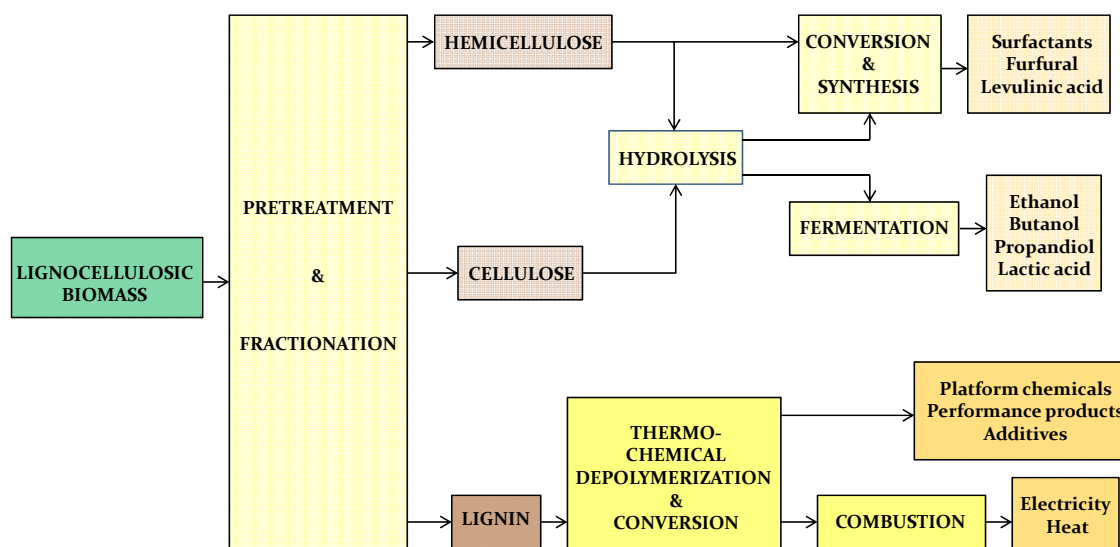
Many white-rot fungi, however, exhibit a pattern of simultaneous decay characterized by degradation of all cell wall components with formation of radial cavities. The degrading action is carried out by a pool of oxidative enzymes produced by the fungi that causes the degradation of the cell wall. The four major groups of enzymes for the degradation of lignin are lignin peroxidase (EC 1.11.1.14), manganese dependent peroxidase (EC 1.11.1.13), versatile peroxidase (EC 1.11.1.16) and laccase (EC 1.10.3.2). Examples of this group of fungi include *Trametes versicolor*, *Heterobasidium annosum* and *Irpex lacteus*.¹⁷⁹ These oxidative enzymes have opened the way to several industrial applications of these fungi because many uses of wood involve preferentially the removal of lignin, such as in biopulping.¹⁸⁰

2.3.6 LIGNIN AS A BIORESOURCE

Nowadays the replacement of fuels with alternative and renewable materials is becoming increasingly important because of the fossil fuel depletion and its escalating cost.

Cellulosic biomass represents a low cost and abundant resource potentially exploitable for the large-scale production of fuels. The amount of lignocellulosic material has been calculated to be 170 billion metric tons,¹⁸¹ derived from agricultural and forestry residues or various industrial wastes.

The facility that carries out biomass conversion processes for the production of fuels, power, heat and value-added chemicals is defined biorefinery.¹⁸² From this point of view, the biomass is separated into a number of compounds in order to take advantage from the various fractions, reducing as much as possible the waste and the by-products (Scheme 2.9).



Scheme 2.9: The biorefinery process.

The most common technologies for biomass fractionation include enzymatic treatment by cellulases and chemical hydrolysis by hot water treatment, steam explosion, ammonia fiber explosion, dilute or concentrated acid hydrolysis, alkaline treatment and organosolv processes.

Lignin is an important constituent of the biomass and it is exceeded in natural abundance only by cellulose. However, only a small amount (ca. 1-2%) is isolated and employed for other applications than combustion.¹⁸³ Its use is still limited to thermal valorisation processes, as filler in composites, component in binders and coatings^{184,185} in polyurethane foams¹⁸⁶ or, at a lower extent, surfactant/dispersant additives. Actually, its potential as a source of valuable phenols in the production of high value-added biopolymers in alternative to petrol chemistry is largely unexploited. Lignin from lignocellulosic biomass should be a high promising renewable material for polymers that are used as component for plastic and resins¹⁸⁷ However, lignin produced as a by-product of pulp and paper processing or of advanced saccharification processes for the bioethanol production, is not immediately suitable as a direct replacement petrochemicals, it needs to be further refined and functionally modified before its use. The main obstacle for its exploitation is represented by its structural heterogeneity that makes it recalcitrant to a great variety of treatments.

The researchers, whatever, are directing their efforts to the development of new processing methods to extend the role of lignin for future biomass and biofuel applications.

The possible strategies of lignin valorisation are focused into two main directions, namely the selective functionalisation of the lignin polymer in order to improve its compatibility and performance in composite and copolymer materials, or, in alternative, its oxidative depolymerization to get polyfunctional monomeric compounds to be used as feed-stocks for polymer industry as an alternative to fossil fuels derived building blocks.¹⁸⁸

2.3.7 ISOLATION METHODS

The heterogeneity of lignin structure has always represented an obstacle for its isolation; up to now researchers challenged to develop suitable methods for isolating a

compound as similar as possible to the “natural” polymer. Thanks to the numerous studies carried out on the isolated lignins, the researchers obtained important information about its structure and the mechanism of microbial degradation.

Lignin can be isolated according to different methodologies but each isolation processes yield an extractive lignin with slightly different features. The most common preparation are acidolysis lignin, cellulase enzyme lignin, milled wood lignin, Braun’s native lignin, Klason lignin and Kraft lignin & lignosulfonates:¹⁵⁵

Acidolysis lignin: It is a lignin characterized by a small amount of carbohydrate impurities; it is extracted from plant tissues by using a mild acidic conditions (0,2 M HCl in aqueous dioxane) at RT.

Cellulase enzyme lignin (CEL): It is a lignin characterized by 12-14% of carbohydrate impurities; the milled wood is treated with a mixture of cellulase-hemicellulase commercially available in order to remove the polysaccharides.¹⁸⁹

Milled wood lignin (MWL): This lignin is obtained following Björkman’s procedure: wood is at first extracted with organic solvent (a mixture of water and acetone) in order to remove low molecular weight phenols, and then it is finely milled. The purification from the aqueous dioxane extract of finely milled wood yields a lignin considered to be representative of the original lignin.¹⁹⁰ Despite this, wood lignin is not considered to be representative of whole lignin in wood, it originates primarily from the secondary wall of the plant cell tissue.

Braun’s native lignin: The lignin is prepared by the extraction of lignocellulosic material using 95% solution of ethanol in water. The yield is very low because the most part of the lignin is insoluble in ethanol and only a small portion is soluble in ethanol-water.

Lignin isolated following this method is generally considered not representative of the bulk of lignin in the extracted tissues. Except for its molecular weight, the structure of Braun’s native lignin is similar to that of MWL and therefore may be used as the preliminar lignin substrates.¹⁹⁰

Klason lignin: It is a highly condensed and resinous lignn recovered after the 72% sulfuric acid treatment of lignocellulosic material. The method is used to analyze quantitatively the acid-insoluble lignin content of lignocellulose material.

Kraft lignin and lignosulfonates: These lignin are the residue of chemical pulping processes in paper production. Kraft lignin is a low molecular weight lignin, highly modified, characterized by a higher phenolic content and a lower methoxyl content with respect to the other preparations (Figure 2.22). Lignosulfonate is the sulfonated lignin removed from wood by sulfite pulping and has a higher molecular weight than kraft lignin (Figure 2.23).

Although these lignins are very important as industrial byproducts because of their prompt bioconversion,¹⁹⁰ they are not considered representative of native lignin.

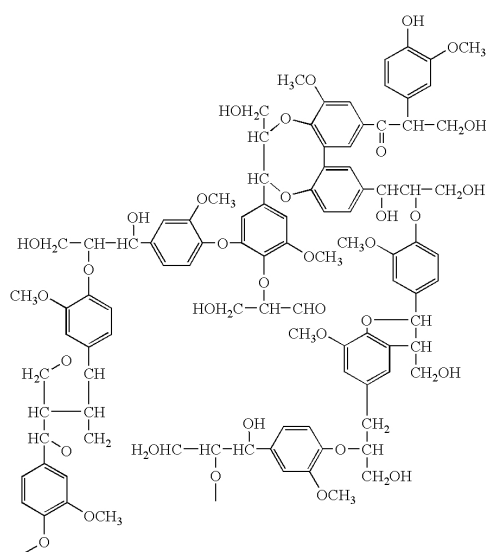


Figure 2.22: Kraft lignin.

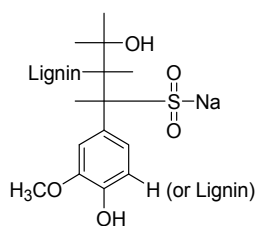


Figure 2.23: Lignosulfonate.

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3. DEVELOPMENT OF A NEW ANALYTICAL METHOD FOR STRUCTURAL CHARACTERIZATION OF TANNINS

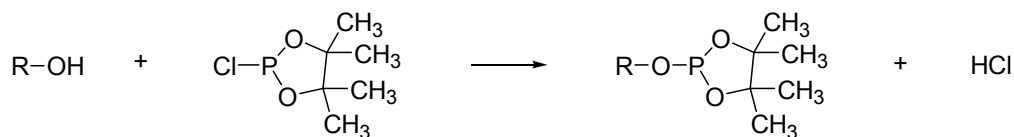
The most diffused analyses on tannins are based on protein precipitable methods or on evaluation of phenolic group content, with the capability of distinguishing between hydrolysable and condensed tannins. (Chapter 2 – Tannins – Characterization method) None of them, however, is completely satisfactory because they do not provide information about the fundamental structural features, particularly they do not elucidate regiochemical patterns of the aromatic rings or the degree of esterification or polymerization. Moreover, the common assays based on the functional groups are affected by the presence of impurities; therefore they often require the pretreatment of the analyte in order to avoid interferences. On the basis of known magnetic resonance technique applied on polyphenolic polymers,^{1,2,3} my effort have been directed on the development of a novel analytical method for the structural elucidation of tannins from different sources, that overcomes the pretreatment constraint.

3.1 ANALYSIS OF TANNINS MODEL COMPOUNDS

The development of an analytical method able to clarify the features of a complex samples requires the preventive analysis of simple and known compounds representative of the analyte. At the beginning, structural differences in tannins were evaluated taking into consideration the aromatic rings substitution patterns, namely catecholic, *o*-substituted and *o*-disubstituted phenolic groups. The early objective of the work was to develop a simple, reliable and quantitative technique which would provide structural information on the fundamental functional groups, namely the phenolic, the carboxylic and the aliphatic hydroxyl groups.

The intent was to extend the common NMR-based method for the analysis of polymers to tannins. On the basis of previously reported results^{1,2,3} we developed a new ³¹P-heteronuclear correlated NMR spectroscopic technique able to detect and to quantitatively evaluate all functional hydroxyl groups in tannins, distinguishing the aliphatic from the phenolic and the acidic ones, after quantitative functionalization with a suitable phosphitylating reagent.

In a more detailed view, we selected an array of different tannins model compounds and we submitted them to functionalization with 2-chloro-4,4',5,5'-tetramethyl-1,3,2-dioxaphospholane (Cl-TMDP), which reacts with all hydroxylic labile proton according to the following scheme (Scheme 3.1):



Scheme 3.1: Phosphitylation of hydroxyl groups with 2-chloro-4,4',5,5'-tetramethyl-1,3,2-dioxaphospholane (Cl-TMDP).

The oxygens surrounding the phosphorus atom ensured to obtain singlet signals containing no coupling information.

In order to evaluate the amount of different functional groups a suitable internal standard is needed. In this respect the choice was on cholesterol, whose Cl-TMDP-derivatized form has a chemical shift (144,82 ppm) that does not overlap with other tannins functional groups signals. The presence of an internal standard allowed the comparison of the areas of the NMR peaks due to the different functional groups.

The panel of models we selected can be divided into three subclasses:

- Hydrolyzable tannin model compounds
- Condensed tannin model compounds
- Complex tannin model compounds

In all cases derivatization of tannins models was found quantitative.

3.1.1 HYDROLIZABLE TANNINS MODEL COMPOUNDS

Gallic acid, methyl gallate, ellagic acid and pentagalloyl glucose (Figure 3.1) were selected as representative hydrolysable tannins model compounds.

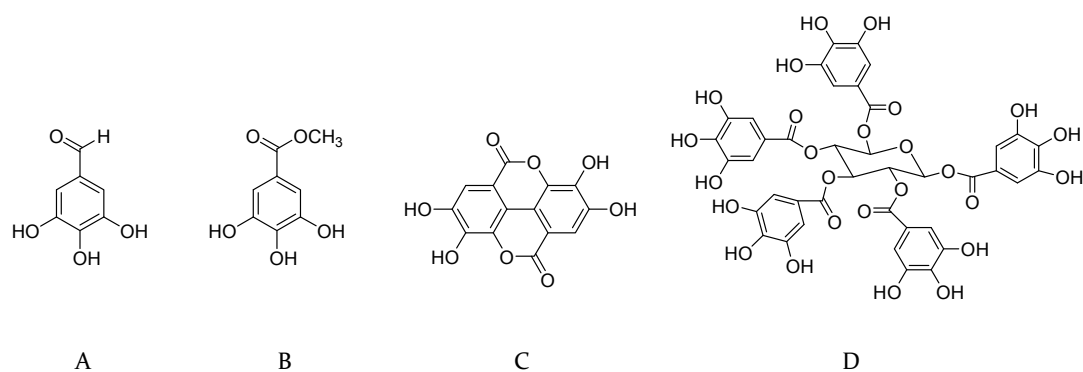


Figure 3.1: Gallic acid (A), methyl gallate (B), ellagic acid (C), pentagalloyl glucose (D) structures.

Their ^{31}P NMR spectra after phosphorylation are reported in Figure 3.2 A, B, C and D respectively.

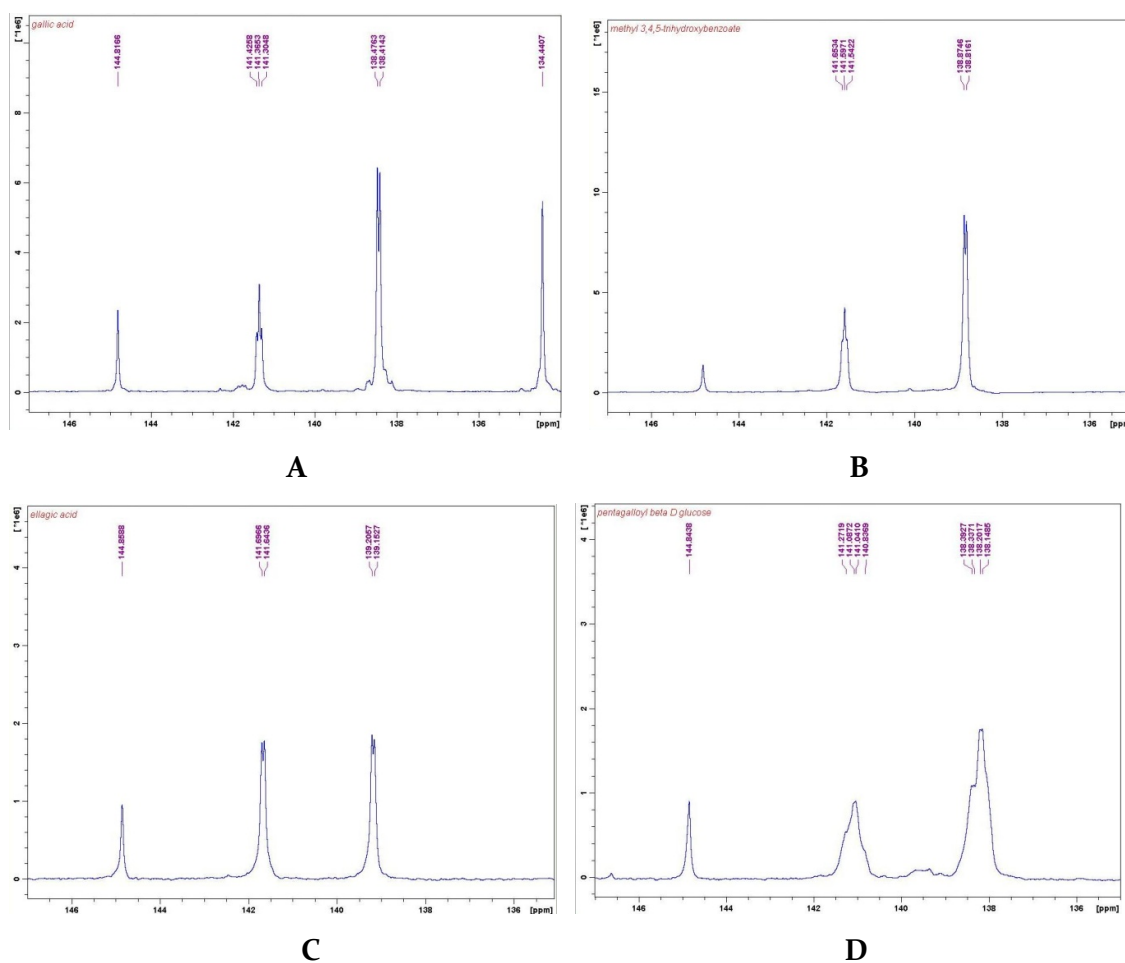


Figure 3.2: ^{31}P NMR spectra of gallic acid (A), methyl gallate (B), ellagic acid (C), pentagalloyl glucose (D) after derivatization with Cl-TMDP.

Table 3.1 shows the assignment on the basis of the different classes of hydroxyl group and the respective chemical shifts. Among the phenols, o-disubstituted, o-substituted and o-unsubstituted have been recognized.

Table 3.1: ^{31}P NMR data of hydrolyzable tannins model compounds with the assignments and the respective chemical shifts.

Entry	Model	Aliphatic OH	o-disubstituted phenols	o-substituted phenols	o-unsubstituted phenols	Acidic OH
1	Gallic acid	-	141.36	138.44	-	134.45
2	Methyl gallate	-	141.59	138.84	-	-
3	Ellagic acid	-	141.67	139.17	-	-
4	Pentagalloyl glucose	-	141.06	138.21	-	-

Gallic acid spectrum (Figure 3.2 A) shown three signals: the first, attributed to the o-disubstituted phenolic OH at 141.36 ppm, the second, that accounted for 2 catecholic signals with a double intensity at 138.44 ppm and the third, due to the carboxylic moiety was found at 134.45 ppm. A long range ^{31}P -coupling was evident since the downfield peak was a triplet while the catecholic signal a doublet. The absorbance regions were found in accordance with previous assignment of different phenolic and carboxylic moieties of lignin model compounds previously studied in our laboratory. In the same fashion methyl gallate shown the disubstituted phenolic OH absorbance at 141.59 ppm and the catecholic signals at 138.84 ppm. As expected, a carboxylic acid signal was missing (Figure 3.2 B). Ellagic acid shown only two signals of equal intensity assigned to the o-disubstituted and to the catecholic OH, at 141.67 and 139.17 ppm respectively (Figure 3.2 C). In both case the presence of a rigid structure deshielded the signals with respect to those of gallic acid.

The more complex pentagalloyl glucose shown two sets of signals attributed to the o-disubstituted phenolic at 141.06 ppm and to the two catecholic moieties per gallate residue 138.21 ppm, respectively. The esterification to the polyol shifted upfield all the phenolic signals of about 0.5ppm with respect to those of the free acid. The absence of

carboxylic and aliphatic OH absorbances shows the purity of the sample that is both completely esterified and does not contain free gallic acid.

3.1.2 CONDENSED TANNINS MODEL COMPOUNDS

Catechin, epicatechin, quercetin, gallocatechin and epigallocatechin (Figure 3.3) were selected as representative condensed tannins model compounds.

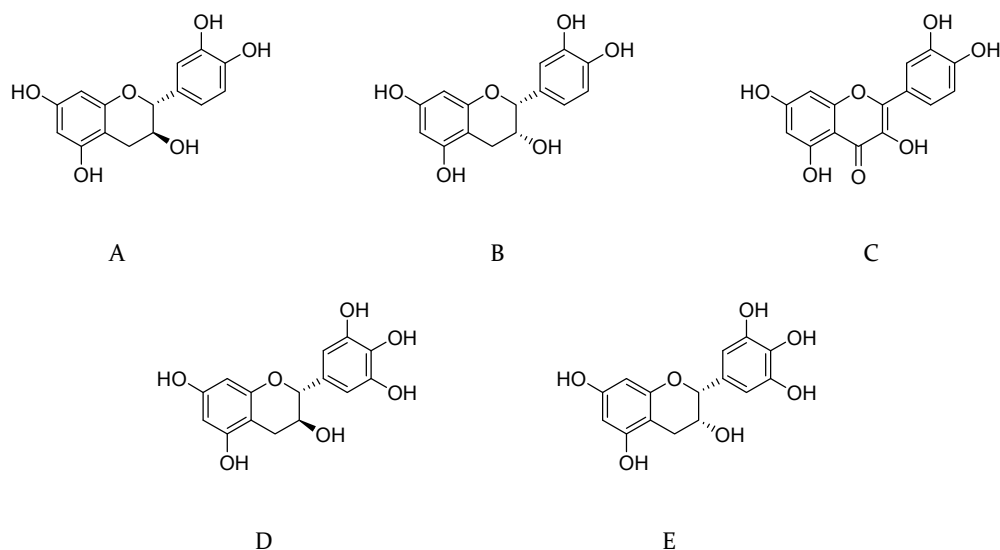
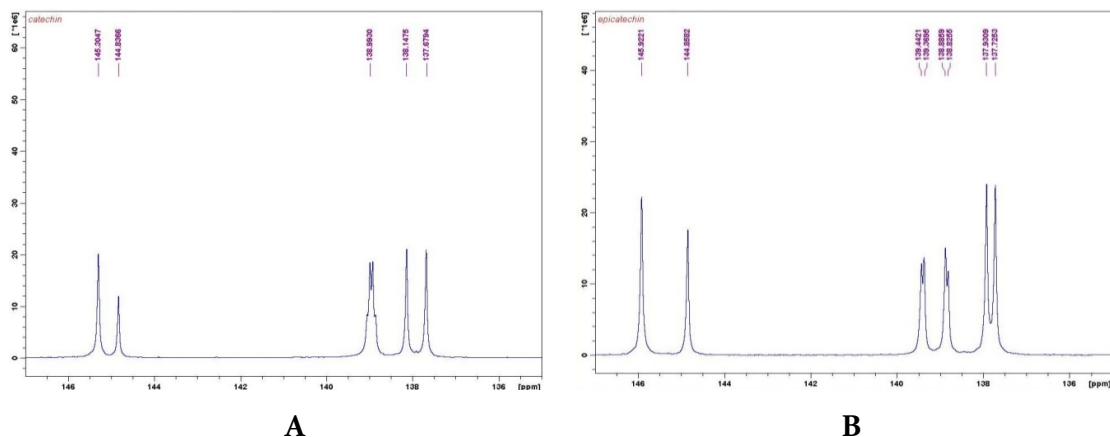


Figure 3.3: Catechin (A), epicatechin (B), quercetin (C), gallocatechin (D), epigallocatechin (E) structures.

Their ^{31}P NMR spectra after phosphorylation with Cl-TMDP are reported below in Figure 3.4 A, B, C,D and E respectively.



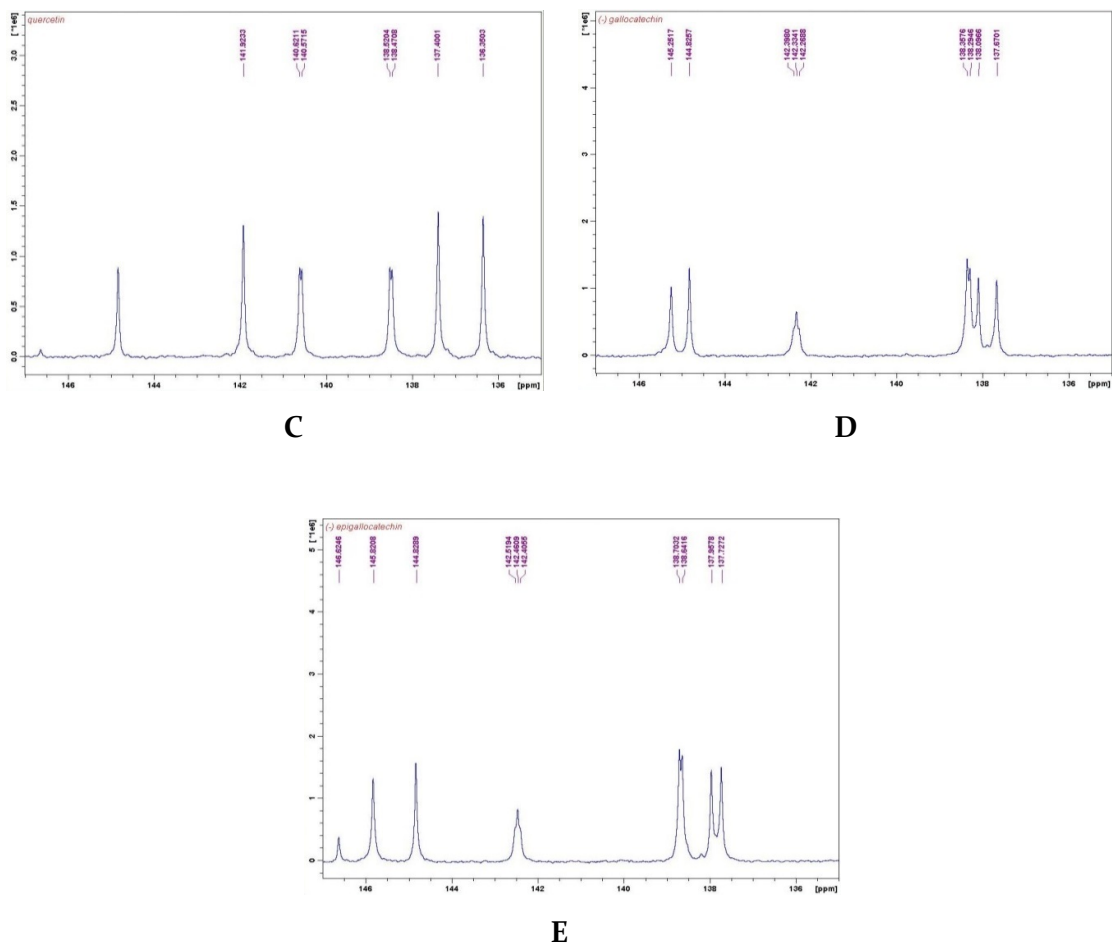


Figure 3.4: ^{31}P NMR spectra of catechin (A), epicatechin (B), quercetin (C), gallicocatechin (D) and epigallocatechin (E) after derivatization with Cl-TMDP.

Table 3.2 shows the assignment on the basis of the different classes of hydroxyl group and on the different substitution patterns of the aromatic ring, with the respective chemical shifts.

Table 3.2: ^{31}P NMR data of condensed tannins model compounds with the assignments and the respective chemical shifts.

Entry	Model	Aliphatic OH	o-disubstituted phenols	o-substituted phenols	o-unsubstituted phenols	Acidic OH
1	Catechin	145.29	-	139.00 138.87 138.07	137.65	-
2	Epicatechin	145.94	-	139.44-139.37 138.89-138.83 137.89	137.67	-
3	Quercetin	-	141.92	140.60 138.50	137.40	136.35 (H-bonded phenolic OH)
4	Gallocatechin	145.25	142.33	138.32 138.10	137.67	-
5	Epigallocatechin	145.82	142.46	138.7 137.95	137.72	-

^{31}P NMR spectra of the phosphitylated models (Figure 3.4) showed an interesting stereochemistry derived behavior. In fact epicatechin and epigallocatechin showed downfield signals with respect to the corresponding catechin omologues (Table 3.2, entry 2, 5 vs entry 1, 4).

Quercetin is not properly a tannin model, since it presents the flavanol ring oxidized to the corresponding flavonol. This structure is of high interest since oxidized proanthocyanidin substructures are easily formed in condensed tannins.

The occurrence of hydrogen bonding between the keto group on the flavonol moiety (C_4) and the hydroxyl group in the C_5 position might give rise to the formation of a stable six membered ring (Figure 3.5).

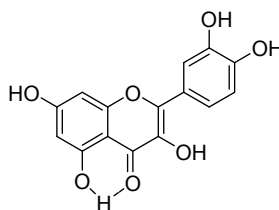


Figure 3.5: Occurrence of hydrogen bonding between the keto group on the flavonol moiety and the hydroxyl group in the C_5 in quercetin.

In this case the phosphorylation of the phenolic group in the C₅ position gave rise to a upfield shielded signal. This is possibly due to the increase of acidity of the phenolic OH upon hydrogen bonding with the keto moiety in the position C₄ (Table 3.2, entry 3). The spectrum (Figure 3.4 C) contains five different signals due to the catecholic OH groups (positions 3' and 4' of ring B respectively) the C₃ OH of ring C, the o-unsubstituted OH in the position 7 of the A ring, while the signal of the C₅ phenolic group occurs at 137.4 ppm.

3.1.3 COMPLEX TANNIN MODEL COMPOUNDS

Catechin gallate, epicatechin gallate, gallo catechin gallate and epigallocatechin gallate were selected as complex tannins model compound, having the characteristic flavonoid-gallate structure (Figure 3.6).

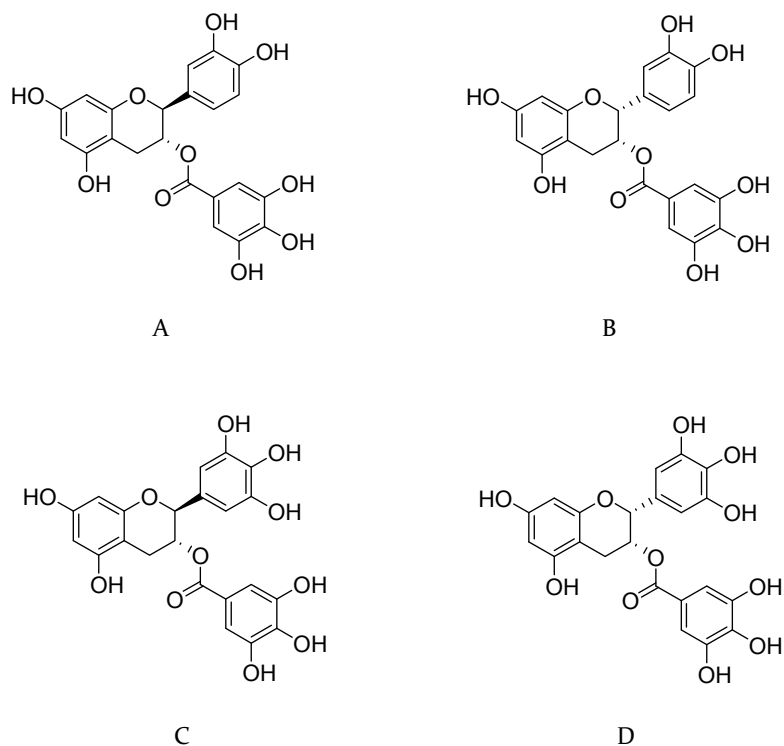


Figure 3.6: Catechin gallate (A), epicatechin gallate (B), gallo catechin gallate (C) and epigallocatechin gallate (D), structures.

Their ^{31}P NMR spectra recorded after phosphorylation of the models with Cl-TMDP are shown in Figure 3.7

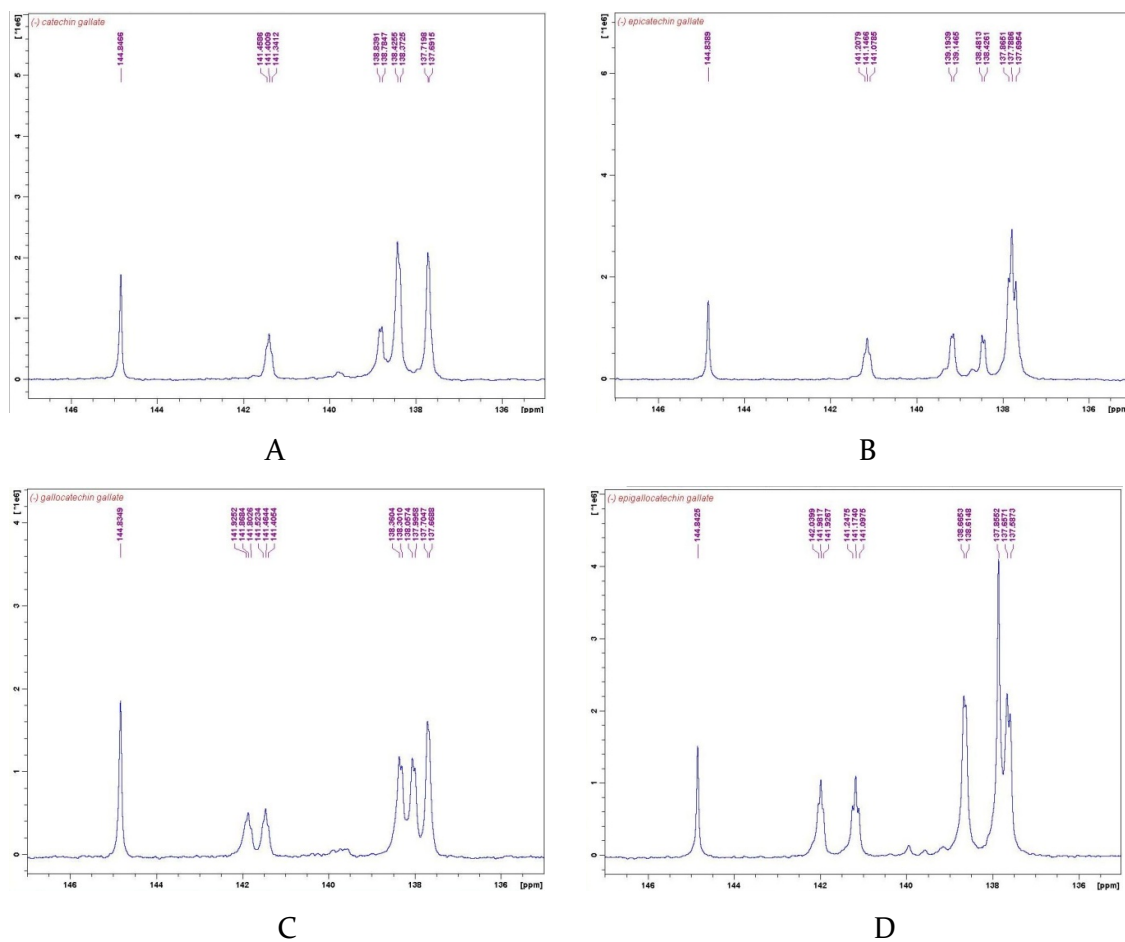


Figure 3.7: ^{31}P NMR spectra of catechin gallate (A), epicatechin gallate (B), gallo catechin gallate (C) and epigallocatechin gallate (D), after derivatization with Cl-TMDP.

As for hydrolysable and condensed tannins model compounds, Table 3.3 shows the assignment on the basis of the different classes of hydroxyl group, distinguishing among the different substitution patterns of the aromatic ring.

Table 3.3: ^{31}P NMR data of complex tannins model compounds with the assignments and the respective chemical shifts.

Entry	Model	Aliphatic OH	o-disubstituted phenols	o-substituted phenols	o-unsubstituted phenols	Acidic OH
1	Catechin gallate	-	141.41	138.43 138.42-138.80 137.72	137.72	-
2	Epicatechin gallate	-	141.15	139.19-139.16 138.42-138.47 137.78 137.69	137.70	-
3	Gallocatechin gallate	-	141.47 141.87	138.37-138.32 138.07-138.00 137.70	137.66	-
4	Epigallocatechin gallate	-	141.99 141.17	138.66 137.85 137.65	137.58	-

On the basis of the results obtained from the hydrolyzable and condensed tannins models, the assignment of signals due both to the gallate and to the flavonoid structures appeared straightforward. In all cases the introduction of a galloyl group in the position C_5 caused the lack of the aliphatic OH signal. In the catechin gallate and epicatechin gallate spectra (Figure 3.7 A, B) it is noteworthy the presence of a signal at 141.41 and 141.15 ppm respectively, arisen from the o-disubstituted phenolic group on the galloyl moiety. The catecholic groups on the galloyl moiety overlapped the catecholic group of the flavonoid ring. In the gallocatechin gallate and epigallocatechin gallate spectra (Figure 3.7 C, D) the two signals in the o-disubstituted region (Table 3.3 entry 3, 4) belong to the two o-disubstituted phenols, the first on the galloyl moiety and the second on the flavonoid ring. In the o-substituted region the catechol belonging to the galloyl moiety overlapped those belonging to the flavonoid ring.

3.1.4 CONCLUSION

Quantitative *in situ* phosphorous labeling of a wide panel of tannins model compound allows the definition of specific ranges of chemical shift typical of each aliphatic, phenolic and carboxylic OH groups present in tannin samples. Moreover, it allows to distinguish the different regiochemistry of the aromatic ring, namely the degree of substitution of phenolic OH.

The presence of a suitable internal standard allowed to evaluate the extent of labeling and to establish the completeness of the reactions.

Table 3.4 summarizes the chemical shift of tannins model compounds.

Table 3.4: ^{31}P NMR signal assignments and chemical shift of tannins model compounds.

Signal	Chemical shift (ppm)	
Aliphatic OH	145.94-145.25	
o-disubstituted OH	142.46-141.06	142.46-141.87
		gallo/epigallocatechin
		141.47-141.06 gallate
o-substituted OH	140.60-137.59	140.2-138.3
		Catechols
		138.8-137.6 non catechols
o-unsubstituted OH	137.72-137.40	
COOH	135.5-134.0	
Total phenolic OH	144.0-137.0	

3.2 ANALYSIS OF COMMERCIAL TANNINS

The study of different tannin model compounds and the elucidation of their substructures allowed for the first time to clarify the composition of complex tannin preparations and to evaluate their purity degree.

On the basis of the results we obtained with the development of a novel analysis protocol we evaluated the different structural features of an array of hydrolysable tannins and proanthocyanidines (condensed tannins). In a more detailed view, we selected five samples of hydrolyzable tannins, two gallotannins and two ellagitannin, and one sample of condensed tannin (proanthocyanidine), as explained below:

- Tannic acid from Chinese nut galls
- Tannic acid Turkish oak galls
- Ellagitannin from chestnut wood

- Ellagitannin from oak wood
- Condensed tannin from grape peel

All of them were submitted to ^{31}P NMR analysis and to Gel Permeation Chromatography (GPC) analysis, which gave the possibility to evaluate the distribution of the molecular weight of the analytes.

3.2.1 GALLOTANNINS FROM CHINESE NUT GALLS AND TURKISH OAK GALLS

A sample of tannic acid extracted from Chinese nut galls was submitted to ^{31}P NMR analysis after phosphorylation in situ with Cl-TMDP (Figure 3.8).

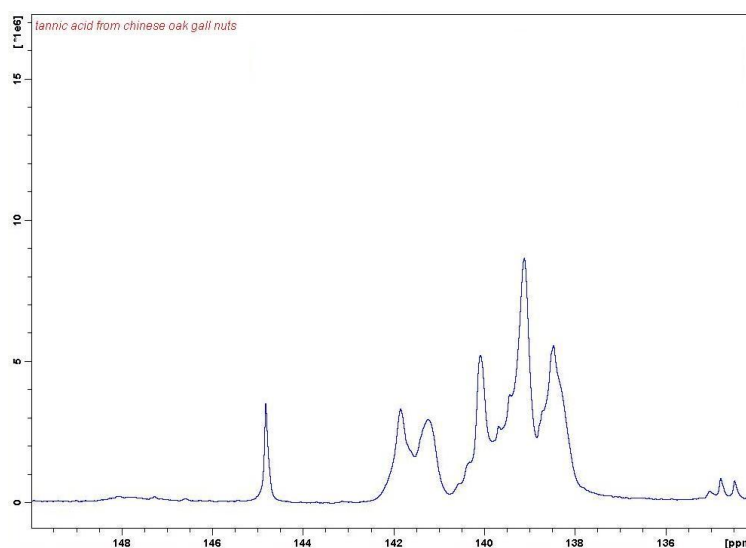


Figure 3.8: ^{31}P NMR of tannic acid extracted from natural Chinese nut gall after phosphorylation with Cl-TMDP.

The spectrum shows multiple signals in the acid region, meaning that the sample contained free acids, among which gallic acid. Two regions of signals were identified, attributed to *o*-disubstituted and *o*-substituted phenolic OH, from 142.2 to 141.00 ppm, and from 141.00 to 138.0 ppm, respectively.

The disubstituted phenolics show two distinct peaks. According to the terminal gallate chemical shift shown by pentagalloyl glucose (*vide* Figure 3.2 D) it was possible to

assign signals at 141.2 ppm to the phenolic OH onto the terminal gallate moieties (terminal disubstituted OH) and signal at 141.9 ppm to internal o-disubstituted phenolics. (Figure 3.9)

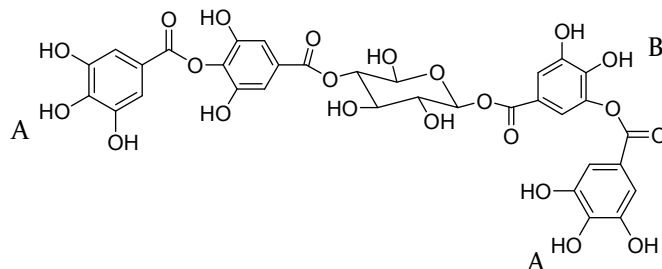


Figure 3.9: Terminal (A) and internal (B) o-disubstituted phenolic OH in a common gallotannin.

The integrals of the single peak clusters allowed further insight into the tannin structure. In fact the ratio between the o-disubstituted phenolics provides quantitative information about the regiochemistry of the depside bond. More specifically if the tannin has a meta-depside regiochemistry, the amount of terminal and internal disubstituted phenolics would be the same (Figure 3.10).

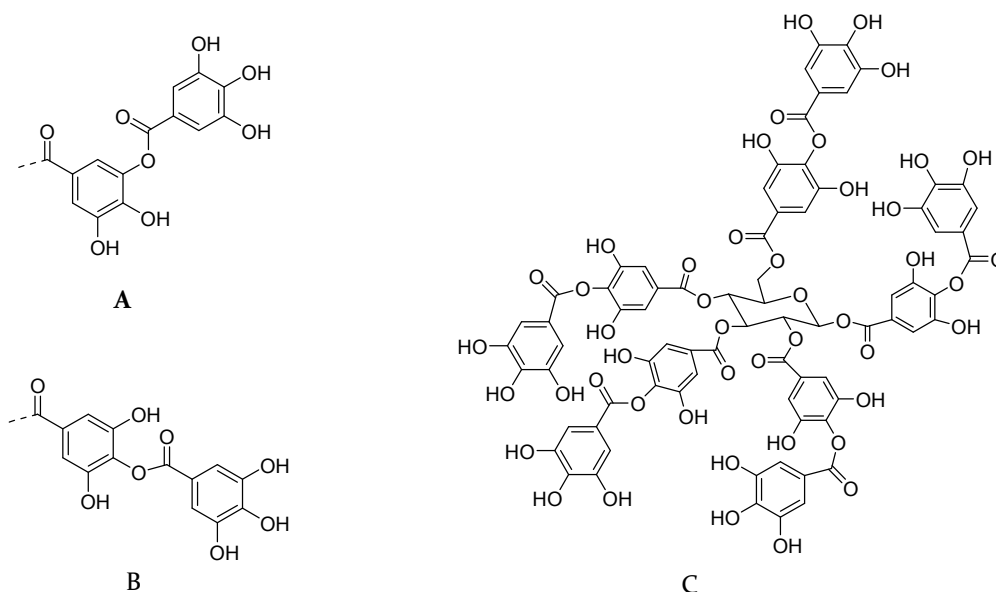


Figure 3.10: *meta*-Depside (A) and *para*-depside (B) bonds; tannic acid structure in fully *para*-depside fashion (C).

In a fully meta-depside array we would expect 5 internal and 5 terminal disubstituted OH per mol. On the contrary when para-depside bonds are present, the internal o-disubstituted phenolics are not present in the tannin (Figure 3.10 C). In this specific case, the integrals showed a 4 to 5 ratio between the internal and external o-disubstituted phenols. This implies that one para-depside bond is present every five terminal gallate units.

This is also confirmed by the integration in the o-substituted region. In fact here we found the presence of overall 16 phenolic OH groups, exactly as expected by the presence of one para-depside bonding per tannic acid molecule.

The phosphorylation in situ with Cl-TMDP of a different sample of tannic acid, extracted from Turkish oak galls, provided a different spectrum (Figure 3.11).

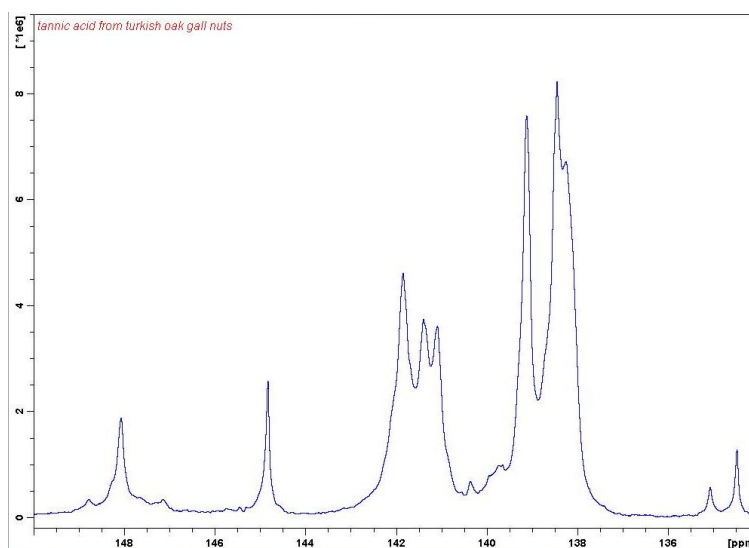


Figure 3.11: ^{31}P NMR of tannic acid from Turkish oak galls after phosphorylation with Cl-TMDP.

Also in this case it was possible to identify the two clusters of signals due to the terminal and internal o-disubstituted phenolics at the same chemical shift as before. Moreover, the spectrum showed a signal in the aliphatic region, indicating that pentagalloylglucose (PGG) is not the only compound of this extract. More specifically

the integration of the signals showed that the average number of galloylgallate units per tannic acid molecule is 4.

The integration of the two *o*-disubstituted signals showed a 3.5 to 4 ratio. This suggested the occurrence of one para-depside bond every 8 galloylgallate units, meaning that the average frequency of the para-depside bond is one every 2 tannin molecules. This is confirmed by the integration of the *o*-substituted OH region with an integral of 8.5.

It was also possible to determine the purity of the sample. Besides the presence of aliphatic OH signals that highlighted the partial esterification of the polyol core, the presence of acidic OH attributable to free gallic acid confirmed the degradation of the sample.

The two samples of gallotannin were then submitted to GPC analysis, after derivatization with acetylbromide, according to a procedure described in literature.⁴ The GPC profile elucidated the presence of an array of products with different molecular weight, thus confirming the heterogeneous nature of tannic acid preparations.

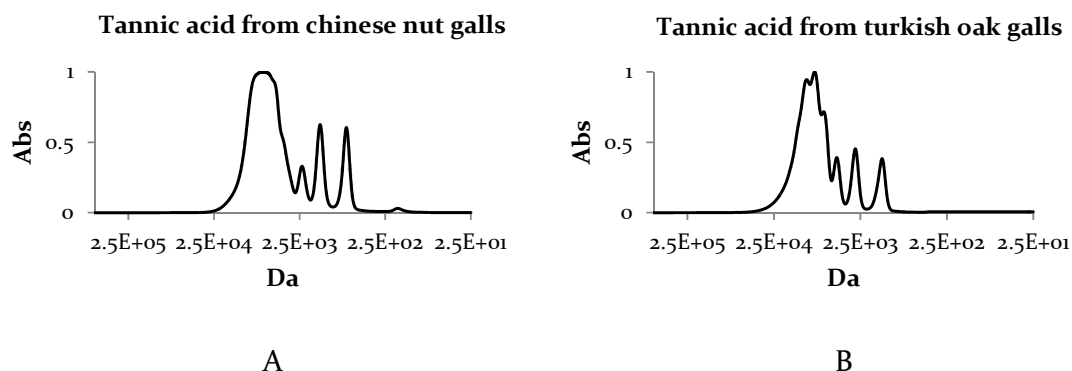


Figure 3.12: GPC analysis of tannic acid extracted from natural Chinese oak gall, (A) and natural Turkish oak gall, (B).

3.2.2 ELLAGITANNINS FROM CHESTNUT AND OAK WOOD

Samples of ellagitannins extracted from different woods were submitted to ^{31}P NMR analysis after phosphorylation in situ with Cl-TMDP.

Although derived from different plant species, the respective spectra appeared similar (Figure 3.13).

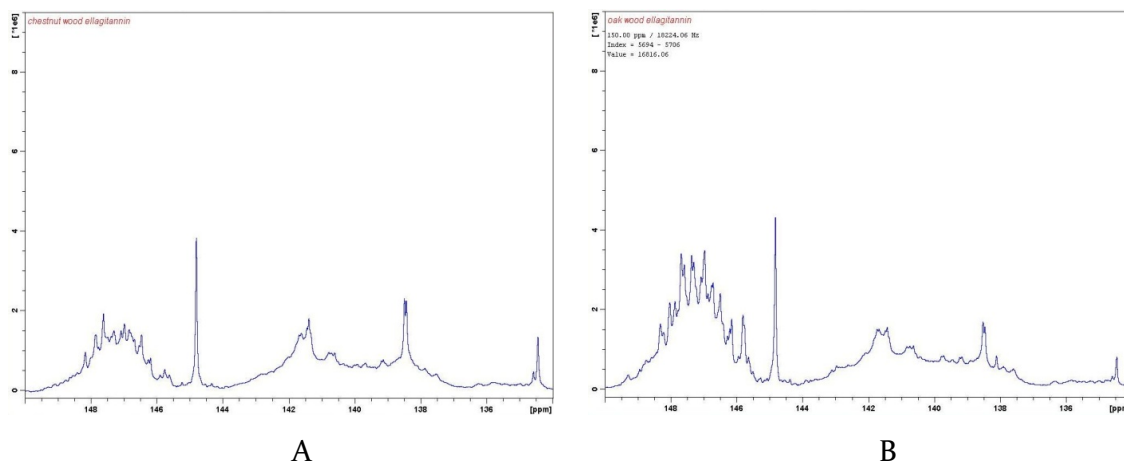


Figure 3.13: ^{31}P NMR of ellagitannins extracted from chestnut (A) and oak (B) wood after phosphorylation with Cl-TMDP.

The phenolic region showed broad absorbancies in the o-disubstituted, catecholic and o-substituted phenolic region, in agreement with the highly complex ellagitannins structures reported in literature. Particularly, both the spectra showed peaks at 141.4 and 138.4 ppm attributable at the hexahydroxydiphenic moieties, typical of ellagitannins (Figure 3.14).

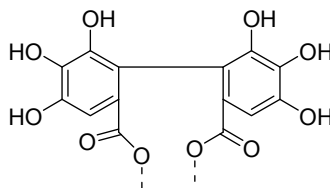


Figure 3.14: Hexahydroxydiphenic residue.

Despite of this, they contained free gallic acid impurities, as highlighted by the presence of a signal in the acidic region (134.4 ppm). Moreover, the spectra revealed intense aliphatic OH signals, indicative of the presence of carbohydrates. The relative amount of aliphatic OH groups is a criterion for the evaluation of the tannin purity. Also the GPC profiles of the two different ellagitannins showed similar molecular weight distributions (Figure 3.15). The multiplicity of signals confirmed the heterogeneous nature of both the sample.

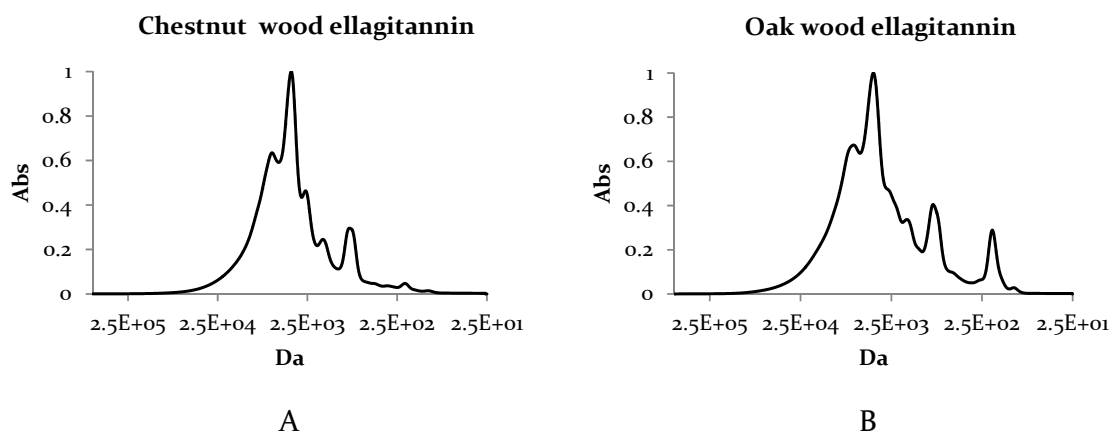


Figure 3.15: GPC analysis of ellagitannins extracted from chestnut (A) and oak wood (B).

3.2.3 CONDENSED TANNIN FROM GRAPE PEEL.

A sample of condensed tannin extracted from grape peel were submitted to ^{31}P NMR analysis after phosphitylation in situ with Cl-TMDP (Figure 3.16).

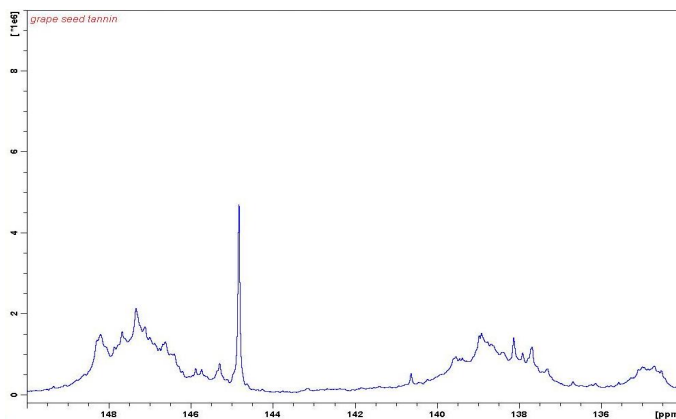


Figure 3.16: ^{31}P NMR of condensed tannin extracted from grape peel after phosphitylation with Cl-TMDP.

The spectrum showed catecholic, *o*-substituted and *o*-unsubstituted signals, as expected from the catechin and epicatechin substructure. The absence of signals in the *o*-disubstituted region was noteworthy. In fact, it provided crucial information about the regiochemistry of polymerization of the analyte. In a more detailed view, the polymerization of flavonol units between the position 4 and 8 does not give rise to *o*-disubstituted phenols while the occurrence of regiochemistry 4-6 would imply the presence of *o*-disubstituted OH groups (Figure 3.17).

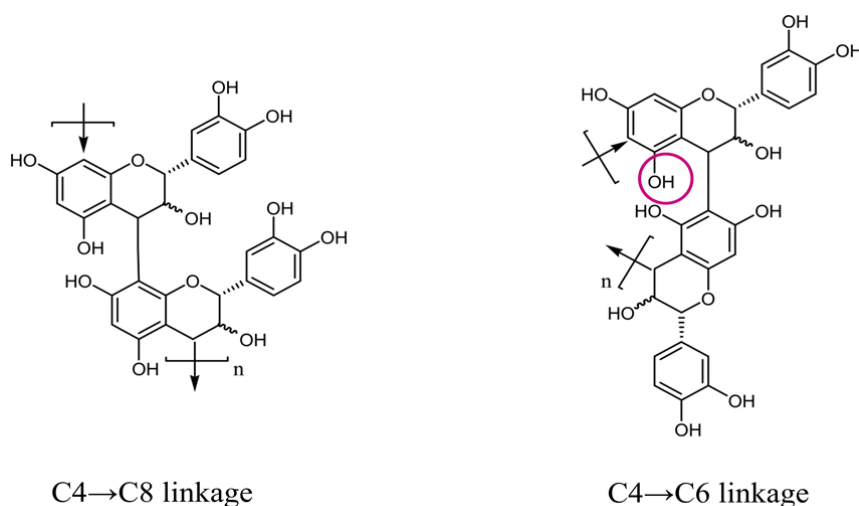


Figure 3.17: C₄→C₈ linkage (A) and C₄→C₆ linkage (B). The regiochemistry 4-6 implies the presence of *o*-disubstituted phenols.

The total lack of signals in the *o*-disubstituted region attested that the regiochemistry of polymerization of extracted proanthocyanidine was characterized by a fully C₄→C₈ fashion.

A significant cluster of aliphatic OH groups was also present. It would be imputable to the presence of carbohydrates impurities in the sample. Despite of this, it was possible to notice in the same region the signals belonging to the aliphatic functionalities of both catechin and epicatechin.

The GPC profile showed a wide distribution a molecular weight, but unlike the much more heterogeneous gallotannins, it could be imputable to C₄→C₈ oligomers characterized by different degree o polymerization (Figure 3.18).

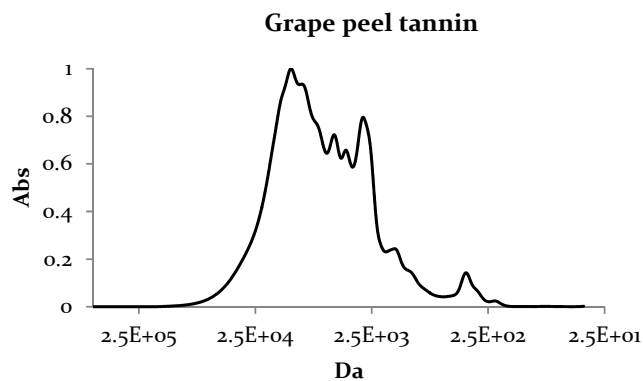


Figure 3.18: GPC analysis of condensed tannin extracted from grape peel.

3.3 ANALYSIS OF GRAPE STALKS

Tannins were also extracted from *Vitis vinifera* wood (grape stalks) in order to compare laboratory preparations with commercial samples.

The extraction procedure of grape stalks according to literature standard procedures^{5,6} yielded a mixture of polyphenols classifiable into two species: tannins and non-tannins. The ³¹P NMR spectrum after the phosphorylation of the sample is shown below (Figure 3.19):

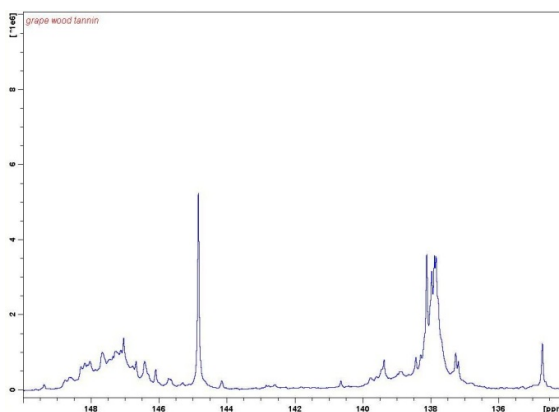


Figure 3.19: ³¹P NMR of polyphenols mixture extracted from grape stalks after phosphorylation with Cl-TMDP.

The profile was basically analogous to the spectrum of grape peel (Figure 3.16), showing a fully $C_4 \rightarrow C_8$ polymer and an evident cluster of aliphatic OH groups.

In order to understand if the aliphatic OH signals were caused by glycosylate tannins or to a contamination of the sample, the mixture of polyphenols was submitted to purification by chromatography on sephadex⁷ in order to separate tannins from non-tannic compounds. Figure 3.20 shows the spectrum of the sample after purification.

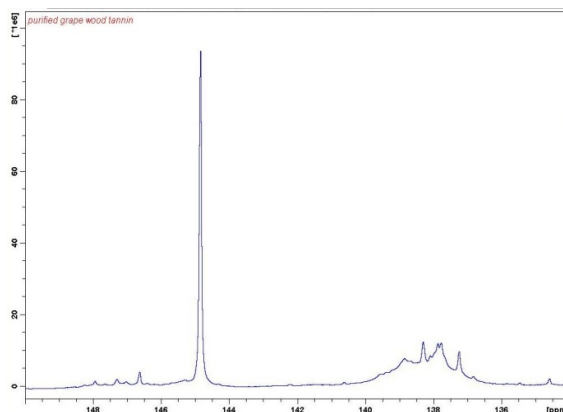


Figure 3.20: ^{31}P NMR of purified grape stalks tannin after phosphitylation with Cl-TMDP.

After the purification, the carbohydrate fraction was efficiently removed by purification, thus it was not chemically bound to the tannin.

Figure 3.21 shows the GPC profile of grape wood tannin before and after purification. The high molecular weight profile was not significantly changed while the low molecular weight fractions were removed.

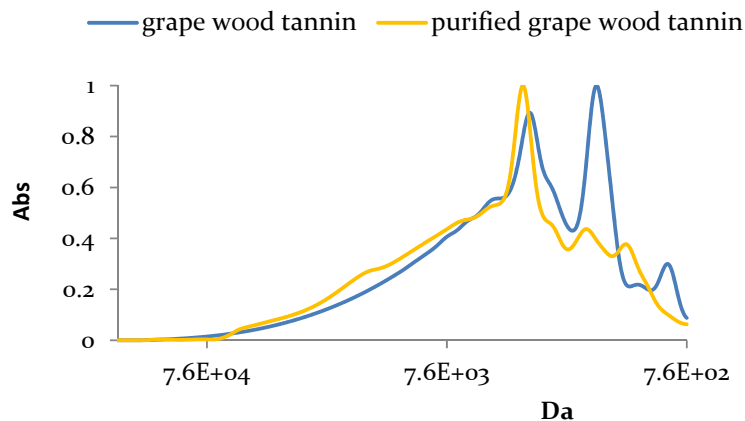


Figure 3.21: GPC analysis of grape wood tannin before and after purification.

3.4 TANNINS TOTAL PHENOLIC CONTENT

The total phenolic content of tannins was evaluated by integration of the ^{31}P NMR spectra of ^{31}P -labeled samples. In order to compare the above reported method of analysis with the available analytical techniques, the phenolic content was also evaluated by the traditional Folin-Ciocalteu method.⁸ Table 3.5 shows the relative results found by the two methods.

Table 3.5. Comparison of the total phenolic content of tannins samples as evaluated by the Folin-Ciocalteu method and by the integration of the ^{31}P NMR spectra.

Tannin sample	mg GAE/100mg ^a	phenolic OH (mmol/100mg) ^b
Tannic acid from chinese oak gall	73,9	147
Tannic acid from Turkish nut gall	111	200
Chestnut wood ellagitannin	75,5	84,9
Oak wood ellagitannin	62,9	55,0
Grape peel tannin	69,0	48,4

a: mg of gallic acid equivalent per 100mg of tannin sample.

b: mmoles of phenolic OH per 100 mg of tannin sample.

While the general trend of total phenolic groups content is confirmed, the correlation degree between the two methods is not high ($R^2=0,79$). This is due to the fact that the Folin-Ciocalteu method expresses the results in gallic acid equivalents rather than by total amount of phenolic groups. In the case of proanthocyanidins, this approach is less suitable, since proanthocyanidins are not structurally related to gallic acid. Moreover, while it has been demonstrated that the phosphorylation and ^{31}P NMR procedure is quantitative for a wide array of tannin model compounds, it has been widely reported that the response to the Folin Ciocalteu method is not linear for all the tannins.⁹

The contemporary quantification of the aliphatic OH groups, of the carboxylic acids and of the different classes of phenolic groups allowed the evaluation of the sample purity.

Table 3.6 shows the different aliphatic, phenolic OH and carboxylic acid content of the different samples studied.

Table 3.6: Aliphatic, phenolic OH and carboxylic acid content of the different tannins samples as evaluated by ^{31}P NMR analysis.

Sample	Aliphatic OH*	o-disubstituted OH*	o-substituted OH*	o-unsubstituted OH*	COOH*
Gallic acid	-	5,35	10,8	-	5,36
Ellagic acid	-	6,62	6,63	-	-
Chinese oak gall (Tannic acid)	-	5,29	9,4	-	0,31
Turkish oak gall (Tannic acid)	0,96	5,40	9,15	-	0,32
Chestnut wood ellagitannin	4,412	3,602	1,443	1,762	0,452
Oak wood ellagitannin	5,885	2,579	0,996	0,97	0,277
Grape seeds tannin	4,336	0,333	0,871	2,215	0,563
Grape wood polyphenols	2,11	0,12	0,50	2,22	0,16
Purified grape wood tannin	0,75	0,72	1,24	3,76	-

* mmol OH/gram of sample.

3.5 CONCLUSION

The *in situ*-phosphorous labeling of the sample with 2-chloro-4,4',5,5'-tetramethyl-1,3,2-dioxaphospholane (Cl-TMDP) allowed to distinguish different regions of absorbance, assigned on the basis of the corresponding signals displayed by model compounds. The presence of a suitable internal standard allowed quantifying such groups.

The method gave the possibility to establish the nature of a tannin sample, distinguishing gallotannins from ellagitannins and proanthocyanidines on the basis of the specific signals attributable to their related substructures. Moreover, the phosphorylation provided for each sample a specific fingerprint, which can be used as a first step for quality control, namely to evaluate the impurity. It is noteworthy that the analytical method elucidated and quantified for the first time tannins structural features in a singular analysis. Particularly, it was possible to unambiguously assign the degree of esterification of gallotannins and the related regiochemistry; as well, it was possible to clarify the regiochemistry of proanthocyanidines polymerization.

The capability of clarifying a wide array of structural peculiarities makes of ^{31}P NMR analysis a precious analytical tool for the detection and the quantification of any other elusive polyphenolic compound.

3.6 EXPERIMENTAL SECTION

3.6.1 QUANTITATIVE ^{31}P -NMR PROCEDURE

About 7-10 mg of tannin sample accurately weighed were dissolved in 400 ml of a solvent mixture composed of pyridine and deuterated chloroform, 1.6:1.0 (v/v) ratio. 100 ml of 2-chloro-4,4',5,5'-tetramethyl-1,3,2-dioxaphospholane were added, followed by 100 ml of the internal standard solution (cholesterol 0.1 M + 5g/L of Cr(III) acetyl acetate as relaxation agent). The reaction cocktail was allowed to stir at room temperature for two hours. The NMR spectra were recorded on a Bruker 300 NMR spectrometer using previously published methods.^{1,10} To obtain a good resolution of the spectra, a total of 256 scans were acquired. The maximum standard deviation of the reported data was 2×10^{-2} mmol/g, while the maximum standard error was 1×10^{-2} mmol/g.

3.6.2 GEL PERMEATION CHROMATOGRAPHY ANALYSIS

7-10 mg of tannin was suspended in 2 ml of a mixture of acetic acid/acetyl bromide 92:8 v/v and stirred at room temperature during 2 hours. The solvent was evaporated under reduced pressure and the residue was dissolved in 5 ml of THF. 20 μ l were injected into a Shimadzu LC 20AT liquid chromatography equipped with a system of columns connected in series (Varian PL gel MIXED-D 5 μ m, 1-40K and PL gel MIXED-D 5 μ m, MW 500-20K) and with a SPD M20A ultraviolet diode array (UV) detector. The analysis was carried out using THF as eluent at a flow rate of 0.50 ml min⁻¹ and the absorbance was recorded at 280 nm. The GPC system has been calibrated against polystyrene standards (molecular weight range of 890 – 1.86 x 10⁶ g mol⁻¹).

3.6.3 POLYPHENOLS EXTRACTION FROM GRAPE WOOD

Wood chips were defatted by shaking with diethyl ether. The dried defatted wood was then extracted with 30% aqueous acetone in soxhlet overnight.¹¹ The extractive was finally evaporated under reduced pressure and then freeze-dried.

3.6.4 PURIFICATION OF TANNINS

About 100 mg of the dried extractive was purified on a column of Sephadex LH-20 equilibrated with ethanol. It was dissolved in a sufficient aliquot of ethanol (a drop of water could be necessary to solubilize it), loaded on the column and eluted with ethanol thoroughly. The ethanol soluble fraction was collected and evaporated under reduced pressure. The column was then eluted with 50% aqueous acetone. The collected tannin fraction was evaporated and freeze-dried.⁷

3.6.5 FOLIN-CIOCALTEAU ASSAY: ANALYSIS OF THE TOTAL PHENOLIC CONTENT⁸

CALIBRATION CURVE:

500 mg of gallic acid were dissolved in 10 ml of ethanol and diluted with water in a 100 ml volumetric flask. A solution of gallic acid 0, 50, 100, 150, 250 and 500 mg/L were prepared dissolving 0, 1, 2, 3, 5 and 10 ml of the previous stock solution into a 100 ml

volumetric flask and diluting to volume with water. 20 µl of each gallic acid solution were pipetted into a cuvette with 1,580 ml of water, then 100 µl of the Folin-Ciocalteu reagent were added. After 2 min 300 µl of a sodium carbonate solution (1g/10mL) were added. The reactions were allowed under stirring at 40°C for 30 min, and then the absorbance was read at 765 nm against the blank for each solution. The calibration curve was obtained plotting the absorbances versus the concentrations.

ASSAY:

Solutions 50 mg/L of different tannins were prepared. As for the calibration curve, 20 µl of each sample were pipetted into a cuvette with 1,580 ml of water. After the addition of 100 µl Folin-Ciocalteu reagent and 300 µl of the sodium carbonate solution, the reaction was allowed to stir at 40°C during 30 min. The absorbance was recorded at 765 nm against the blank. Results were reported in Gallic Acid Equivalent (GAE).

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4. DETERMINATION OF LIGNIN DEGREE OF POLYMERIZATION: A NEW METHOD TO EVALUATE THE MOLECULAR WEIGHT DISTRIBUTION

The lack of suitable methods for the evaluation of lignin molecular weight distribution directed our effort on the development of a novel method able to properly establish lignin degree of polymerization (DP), overcoming the constraint of supramolecular aggregations.

An analytical method suitable for determination of degree of polymerization of oligomers and small polymers is the end groups titration. It consists in the identification and quantification of a specific polymer end groups. We decided to focus our attention on phenolic end units since the aliphatic ones are less characterized and more widespread in an array of different structures, i.e., aldehydes, COOH, and cinnamyl and aliphatic OH groups that cannot be correctly quantified.¹

Once evaluated the phenolic end groups, it is possible to calculate the average DP of the polymer as the ratio between the monomeric units and the end groups.

Because of lignin heterogeneity, it is not possible to identify a specific monomer formula weight. However, it is possible to obtain a C₉-based molecular formula since the monomeric constituents of lignin, the so-called C₉ unit (Figure 4.1), can be easily determined by elemental analysis joined with the determination of the methoxy groups content.

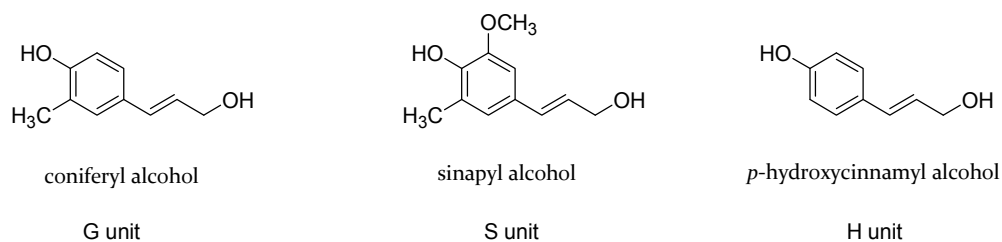


Figure 4.1: Lignin C₉ monomers

4.1 EVALUATION OF LIGNIN PHENOLIC END GROUPS: THEORETICAL ASPECTS

If lignin were a linear polymer simply connected by β -O-4 aryl ether and phenyl coumaran (β -5') interunit linkages, (figure 4.2) each polymer chain would contain a single phenolic unit and the amount of phenolic OH would reflect directly the amount of polymer chains.

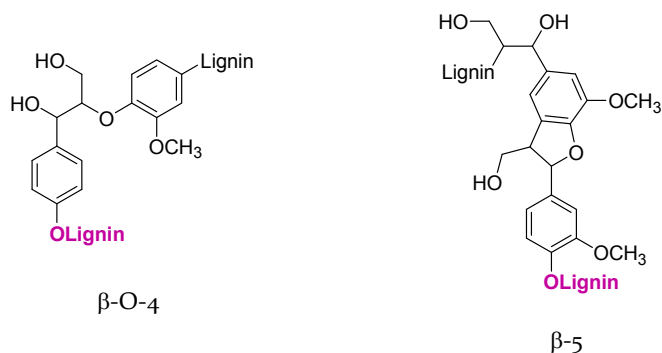


Figure 4.2: β -O-4 aryl ether and phenyl coumaran (β -5') interunit linkages.

We had indeed to consider an accepted paradigm: lignin branching. According to this paradigm, lignin is a three dimensional polymer characterized by branches, associated with diaryl ether and diphenyl subunits, respectively 4-O-5 and 5-5' bondings (Figure 4.3).²

Figure 4.3: Diaryl ether (4-O-5) and diphenyl (5-5') subunits cause lignin branching.

For this reason, the end units of lignin chains have been determined taking into consideration all the relevant interunit bondings (Figure 4.4).

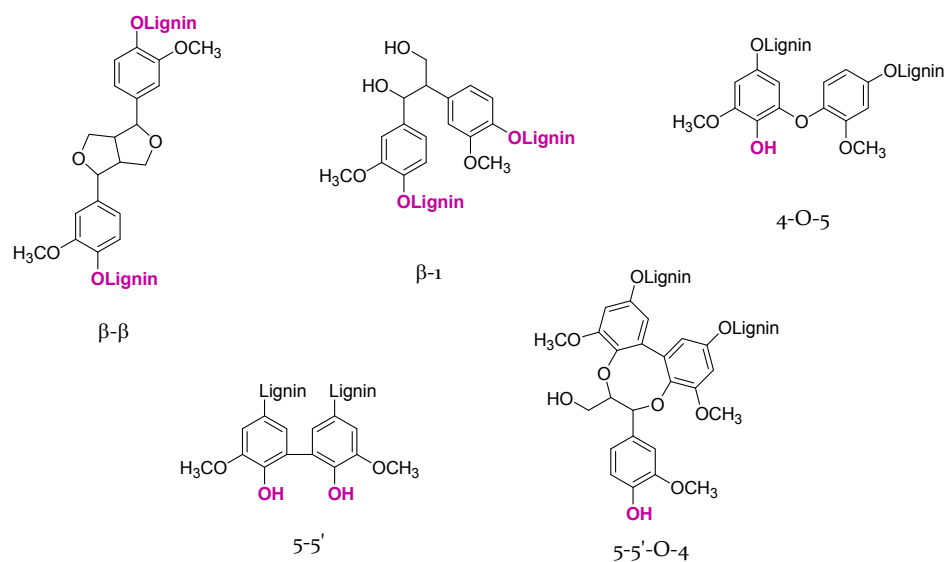


Figure 4.4: Other lignin interunit bonding.

Apart from β -O-4 aryl ether and phenyl coumaran (β -5') interunit linkages, that generate one single phenolic OH end unit per polymeric chain, 4-O-5' and 5-5'-O-4 bondings generate in principle a branching point in lignin that does not increase the number of phenolic OH groups per chain, since the branching is in the direction of the aliphatic end. β - β and β -1 bondings induce an inversion in the chain polymerization direction since they are generated by a bonding between two side chains. Moreover, each β - β and β -1 bonding in lignin induces an increase of one phenolic OH group. It is noteworthy that in a linear lignin chain only one β - β and β -1 bonding can occur. 1 Phenolic 5-5' units cause an increase in the phenolic end of the lignin chain. Taking into account the above considerations, the average number of lignin chains and so the quantification of phenolic end groups, can be calculated according to the equation below:

$$\text{Lignin chains} = \text{phenolic end groups} = \text{Total phenolic amount} - [\beta\text{-}\beta + \beta\text{-}1 + 4\text{-O-}5 + 5\text{-}5']$$

Equation 1: Quantification of phenolic end groups.

The total amount of phenolic OH groups can be quantitatively determined by ^{31}P NMR,³ while β - β and β -1 can now be readily estimated by QQ-HSQC analysis of lignin⁴.

The quantitative determination of phenolic 4-O-5 and 5-5' units can be efficiently carried out by ^{31}P NMR ³ since they provide signals at 142 and 141 ppm respectively.

4.1.1 THE QUESTION OF LIGNIN BRANCHING: THE DFRC TREATMENT

Lignin, and particularly softwood lignin, has been for a long time considered as a cross-linked network polymer. Originally the main branching units in lignin were considered diaryl ethers and diphenyl units (Figure 4.5).²

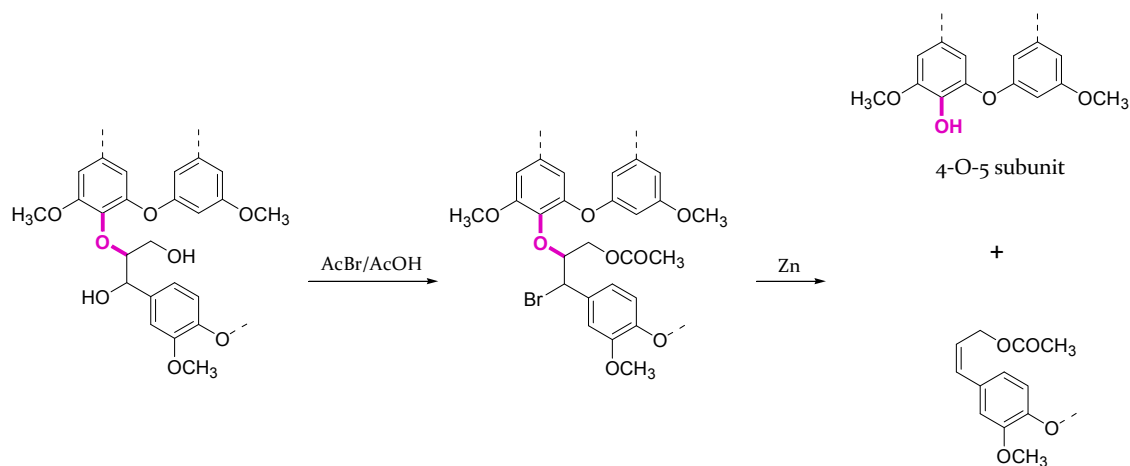


Figure 4.6: DFRC procedure.

After the treatment, the resulting fragments from the selective β -aryl ethers cleavage were submitted to ^{31}P NMR analysis.^{6,7} The quantitative determination of the non-phenolic (branching point) 4-O-5, 5-5' and 5-5'-O-4 units can be efficiently carried out by ^{31}P NMR since they provide signals from 141 to 142 ppm.

The presence of the considered subunits in a phenolic fashion did not interfere with the following ^{31}P NMR determination since all the phenolic groups were protected by acetylation step.

The combination of the DFRC procedure with the quantitative ^{31}P NMR analysis proven to have significant potential for the determination of lignin structure, providing the possibility to quantitatively integrate the amount of end groups and monomers of the polymer in question.

4.2 DETERMINATION OF SOFTWOOD LIGNINS DEGREE OF POLYMERIZATION

The HSQC/DFRC determination of lignin average degree of polymerization was carried out on three different softwood lignin samples: Norway spruce MWL (NS-MWL), an enzymatic mild acidolysis lignin from spruce (NS-EMAL) and a hardwood beech milled wood lignin (Beech MWL). The lignin samples were submitted to ^{31}P NMR and QQ-HSQC experiments. Table 1 shows the relevant experimental data collected. More specifically, β -1, β - β , phenolic 5-5' lignin subunits, and overall phenolic OH groups were measured (Table 4.1, entries 2–5).

Table 4.1: Lignin interunit bondings and phenolic OH groups/100C₉ units.

entry	Interunit bonding	NS-MWL	NS-EMAL	Beech-MWL
1	C ₉ MW	198	199,7	220
2	β -1/100 C ₉ ^a	1.5 ± 1	2.6 ± 1	35 ± 1
3	β - β /100 C ₉ ^a	3.0 ± 1	1.6 ± 1	8.0 ± 1
4	phenolic OH/100 C ₉ ^b	18.6 ± 0.5	19.8 ± 0.5	20.3 ± 0.5
5	phenolic 5-5'/100 C ₉ ^b	3.4 ± 0.5	1.6 ± 0.5	50 ± 5

^a Evaluated from QQ-HSQC experiments. Maximum error: 1/100C₉.

^b Evaluated from ^{31}P NMR of suitably phosphorylated samples. Maximum error: 0.5/100C₉.

Quantitative analysis of β -1, β - β , and 5-5'-O-4 (DBDO) lignin subunits were carried out using the aromatic C₂-H signal as internal standard for the C₉ units and comparing the peak volume with those of the corresponding β -1/ β , β - β / β , and DBDO/ β CH signals, respectively. Figure 4.7 shows the obtained 2D spectrum. Figure 4.8 shows the quantitative ^{31}P NMR spectrum of Norway spruce MWL after phosphorylation.

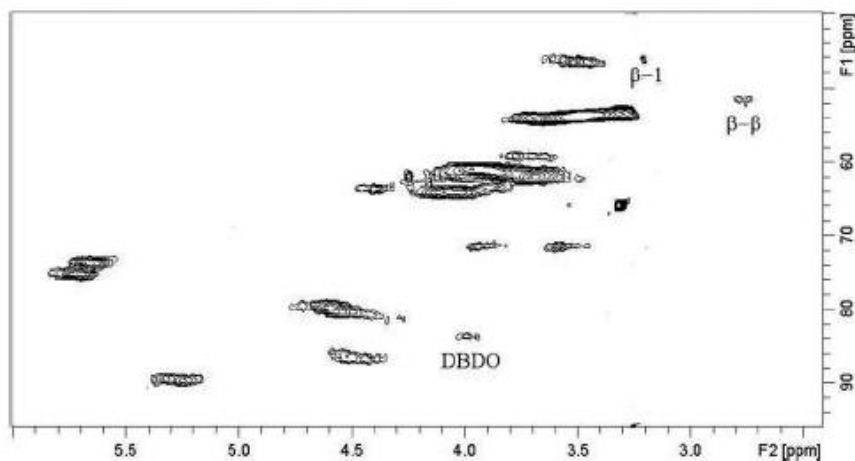


Figure 4.7: QQ-HSQC of Norway spruce MWL

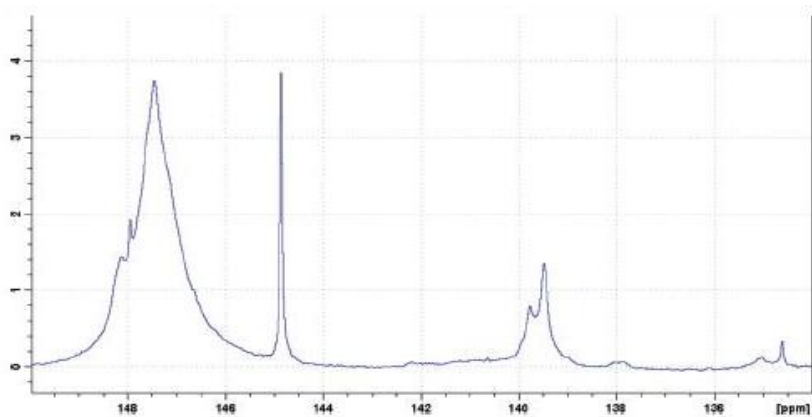


Figure 4.8: ^{31}P NMR of Norway spruce MWL

On the basis of the data in Table 6.2.1, after applying Equation 1, it was possible to calculate the number of lignin chains and thus the average degree of polymerization (DP). The results are summarized in Table 4.2.

Table 4.2: Average degree of polymerization of different MWL samples as evaluated from QQ-HSQC and ^{31}P NMR analysis.

Lignin sample	DP	DP max error
NS-MWL	9.4	2.2
NS-EMAL	7.2	2.0
Beech-MWL	12.1	5.9

Although the maximum error associated with the DP determinations is rather high in percentage, these data are extremely significant because they imply that in the case of the softwood lignin samples analyzed the average DP cannot exceed 11, while in the case of hardwood MWL it cannot be higher than.⁸

The so obtained data were compared with those provided by the GPC analysis. Each lignin sample was then derivatized according to literature methodologies⁹ and submitted to GPC analysis, known to be affected by supramolecular aggregation phenomena.

Table 4.3 shows the M_n values as obtained by NMR end units titration and GPC analysis.

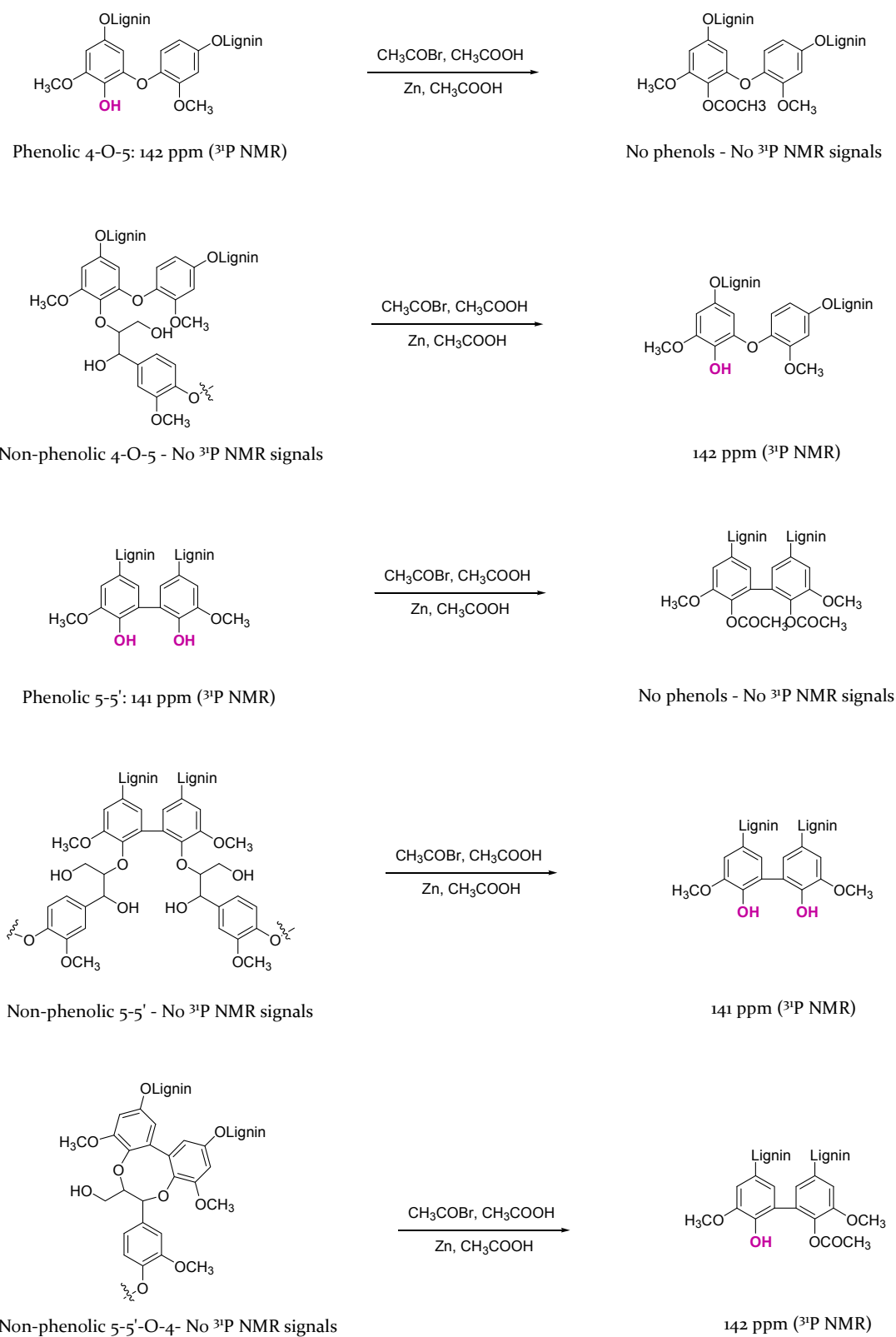
Table 4.3: Number-Average molecular weight (M_n) determined for different MWL samples by GPC and NMR end-groups titration.

Lignin sample	M_n (GPC)	M_n (end-group titration)
NS-MWL	14200	1800
NS-EMAL	7300	1400
Beech-MWL	9600	2600

It is evident that in all the considered cases the degree of polymerization obtained by end-groups titration was lower than from GPC analysis. The higher M_n found by GPC analysis is highly dependent on sample concentration, solvent system, and pH that can induce extensive self-aggregation in lignin chains,⁹ although the use of acetylation procedures and THF as solvent is preferred for reducing such problems. It is noteworthy that from end-group titration measurements the different MWL samples emerge as oligomeric systems rather than polymers. Such oligomeric structure of milled wood lignins is confirmed by earlier studies on lignin molecular weight determination carried out by vapor osmometry^{10,11} and, more recently, by MS methods that show relatively little dependence on aggregation.¹²

The attention was then focused on the softwood lignin branches, in order to have additional structural details. For this reason the three possible lignin branching subunits (Figure 4.5) were considered.

Lignin samples were analyzed by means QQ-HSQC and ^{31}P NMR before and after the DFRC treatment in order to clarify the amount of both phenolic and non-phenolic branching substructures. Scheme 6.2.1 shows the expected reaction pathways after the DFRC treatment, and the relative ^{31}P NMR absorbance peaks.



Scheme 4.1: Fragments released upon DFRC treatment from lignin condensed subunits.

Focusing the attention on Scheme 4.1 it was possible to make some evaluations:

- Phenolic 4-O-5' subunits are acetylated during DFRC treatment and will not interfere with the following ^{31}P NMR determination since all the phenolic groups are protected;
- Etherified 4-O-5' and 5-5' subunits are expected to react under DFRC conditions releasing phenolic 4-O-5' and 5-5' fragments respectively;
- Monoacetylated 5-5' and 4-O-5' fragments show ^{31}P NMR overlapping absorbance peaks centered at 142 ppm;
- β -etherified 5-5'-O-4 units undergo cleavage to monoacetylated 5-5' fragments.

Table 4.4 summarizes the quantitative results obtained after the analyses.

Table 4.4: Lignin subunits amount evaluated by ^{31}P NMR, QQ-HSQC, and ^{31}P NMR after DFRC.

entry	interunit bonding/ C ₉ unit	NS-MWL*	NS-EMAL*	Beech MWL*
1	phenolic 5-5' ^b	0.034 ± 0.005	0.057 ± 0.005	0.005
2	phenolic 4-O-5' ^b	0.022 ± 0.005	0.016 ± 0.005	<0.005
3	5-5'-O-4 ^a	0.033 ± 0.01	0.038 ± 0.01	<0.005
4	non-phenolic 4-O-5' + 5-5'-O-4 ^c	0.038 ± 0.005	0.041 ± 0.005	<0.005
5	etherified 5-5' ^c	<0.005	<0.005	<0.005

* mmol/g of sample

^a Evaluated from QQ-HSQC experiments.

^b Evaluated from ^{31}P NMR of suitably phosphorylated samples.

^c Evaluated from ^{31}P NMR of suitably phosphorylated samples after DFRC treatment.

The peak integrated at 142 ppm after DFRC treatment accounts for the sum of etherified 4-O-5' and 5-5'-O-4 units (Scheme 4.1). This value was always found to agree, within experimental errors, with the amount of 5-5'-O-4 revealed by QQ-HSQC (Table 4.4, entry 4 vs entry 3). This could imply that 4-O-5' etherified subunits are not present in the MWL samples studied.

Phenolic 5-5' units are acetylated under the DFRC procedure and will not appear in the ensuing ^{31}P NMR spectrum (Scheme 4.1).

β -Etherified 5-5' units are expected to react in the DFRC protocol releasing phenolic 5-5' fragments with ^{31}P NMR absorbance of the phosphitylated sample centered at 141 ppm (Scheme 4.1).

However after DFRC no signals were revealed in this region, thus indicating the absence of 5-5' β -etherified subunits (Table 4.4, entry 5). Thus, it is possible to conclude that neither 4-O-5' nor 5-5' β -etherified units are present in MWL.

Etherified 5-5'-O-4 is thus the only remaining potential branching unit in lignin. Despite the fact that direct evidence for its selective quantification is not available, HMBC experiments recently carried out by Ralph *et al.*¹³ showed the lack of correlation peaks due to such lignin substructures in MWL samples. This implies that all 5-5'-O-4 fragments are terminal phenolic units in lignin.

On the basis of the present data and recent literature reports, all the potential branching units in milled wood lignin are terminal, and thus they constitute merely chain inversion points in lignin polymerization direction. The branching in lignin is therefore negligible or absent. The absence of branching units supports the idea of lignin as a supramolecular aggregates of oligomers (Figure 4.9).¹⁴

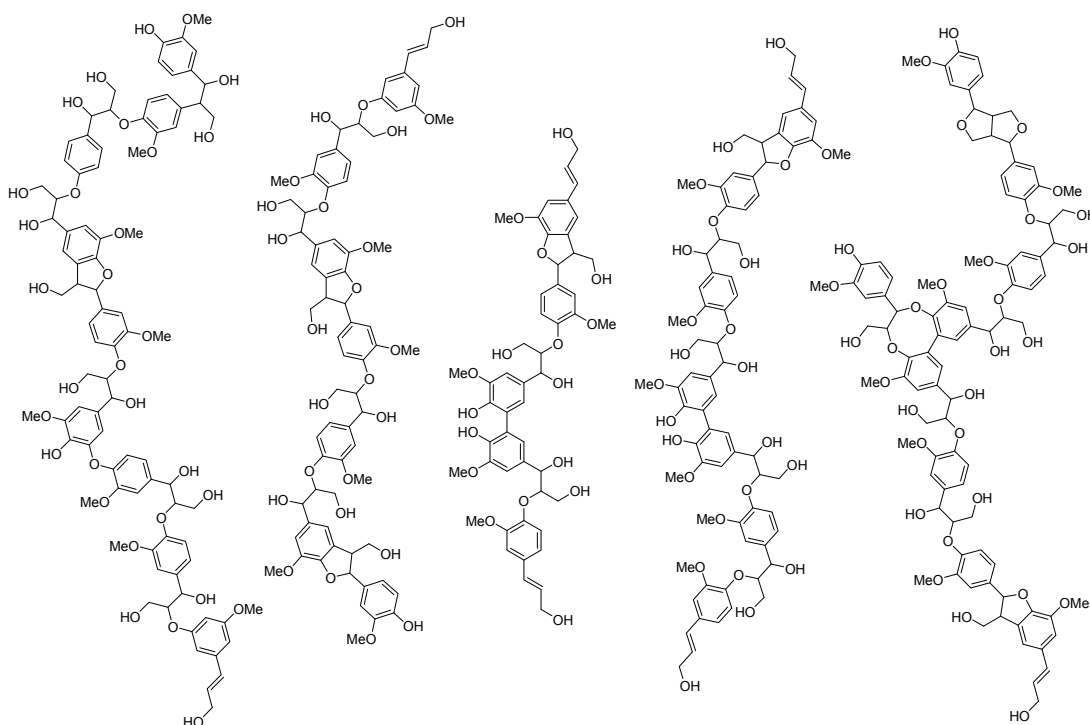


Figure 6.3.1: Schematic representation of softwood milled wood lignin after the last results.

4.3 CONCLUSION

The joint use of quantitative ^{31}P NMR and QQ-HSQC analysis allowed us to develop a new analytical tool for the determination of the average degree of polymerization of lignin. The spectroscopic techniques provided the knowledge of monomer formula weight and the possibility to quantitatively integrate the amount of end groups and monomers of the polymer studied. The developed experimental protocol showed the absence of branching units in the lignin samples under study, consequently, we propose the view that milled wood lignin is a linear oligomer rather than a polymeric network.

Although the method is not suitable to characterize high molecular weight polymers, it is completely independent of supramolecular association phenomena and leads to unequivocal determination of average DP and M_n values.

4.4 EXPERIMENTAL SECTION

4.4.1 LIGNINS

Milled wood lignin was isolated from spruce (*Picea abies* and *Picea mariana*) and beech (*Fagus*) wood by slight modification of the Bjorkman method.¹⁵

The enzyme mild acidolysis milled wood lignin sample was prepared and distributed by Prof. D. S. Argyropoulos using Norway spruce in a round robin effort in the frame of the cost Action E 41 Analytical Tools with Applications for Wood and Pulp Chemistry according to literature reports.⁹

4.4.2 LIGNIN ACETILATION

Lignin acetylation was carried out as previously reported in the literature with pyridine/acetic anhydride (1:1) at 25 °C for 48 h. HCl (4 h) was then added to obtain pH 3, and the mixture was stirred 12 h. The residue was centrifuged, washed with water, centrifuged again, and freeze-dried.¹⁶

4.4.3 ^{31}P NMR ANALYSIS

The procedure of lignin derivatization is widely described in literature.^{17, 18}

About 25 mg of lignin accurately weighed were dissolved in 400 ml of a solvent mixture composed of pyridine and deuterated chloroform, 1.6:1.0 (v/v) ratio. 100 ml of 2-chloro-4,4',5,5'-tetramethyl-1,3,2-dioxaphospholane were added, followed by 100 ml of the internal standard solution (cholesterol 0,1 M + 5g/L of Cr(III) acetyl acetonate as relaxation agent). The reaction cocktail was allowed to stir at room temperature for two hours. The NMR spectra were recorded on a Bruker 300 NMR spectrometer using previously published methods.¹⁹ To obtain a good resolution of the spectra, a total of 256 scans was acquired. The maximum standard deviation of the reported data was 2 E-2 mmol/g, while the maximum standard error was 1 E-2 mmol/g.

4.4.4 QQ-HSQC SPECTROSCOPY

All spectra were acquired at 303 K with a Bruker Avance 600 spectrometer equipped with cryoprobe. The QQ-HSQC sequence of Peterson and Loenig was used without further modifications using special attention to pulse calibration.²⁰ The sample consisted of 80 mg of lignin dissolved in 600 μL of DMSO. A matrix consisting of 400 $\times$ 2048 points was obtained using 8 scans. Pulse widths of 8.35 and 16.00 μs were used for protons and carbons, respectively. Data were processed with MestreNova (Mestrelab Research) using a 60° shifted square sine-bell apodization window; after Fourier transform and phase correction a baseline correction was applied in both dimensions. The final matrix consisted of 1024 $\times$ 1024 points and cross-peaks were integrated with the same software that allows taking into account the typical shape of peaks present in the spectrum.²¹ Errors analysis was performed by acquiring the same experiment three times on the same sample.

4.4.5 DFRC TREATMENT

Acetyl bromide in acetic acid (1:9 v/v; 12.5 mL) was added to 50 mg of softwood milled wood lignin. The reaction mixture was stirred at 50 °C for 3 h. The solvent was then evaporated to dryness under reduced pressure. The above residue was dissolved

in the acidic reduction solvent (dioxane/acetic acid/water 5:4:1 v/v/v, 12.5 mL). Zinc dust (250 mg) was added, and the mixture was stirred at room temperature for 30 min. The reaction mixture was then quantitatively transferred to a saturated ammonium chloride solution (50 mL) in a separatory funnel using methylene chloride (20 mL). The aqueous layer was extracted with further methylene chloride (2 × 10 mL). The combined extracts were evaporated to dryness under reduced pressure and placed into the desiccator for 24 h. The final acetylation step of the standard DFRC protocol was not carried out so that the released phenols could be determined by quantitative ^{31}P NMR.⁶⁷

4.4.6 GPC ANALYSIS

Lignin samples were submitted to acetobromination prior to GPC analysis. Acetobromination was carried out following the procedure described previously.⁹ 10 mg of lignin was suspended in acetic acid glacial/acetyl bromide mixture (2.5 mL of 92:8 v/v) and stirred at room temperature. After 2 h the solvent is evaporated under reduced pressure and then the residue is dissolved in 5 mL of THF. The GPC analyses were performed using a Shimadzu LC 20AT liquid chromatography with a SPD M20A ultraviolet diode array (UV) detector set at 280 nm. The sample (20 μL) is injected into a system of columns connected in series (Varian PL gel MIXEDD 5 μm , 1–40K and PL gel MIXED-D 5 μm , MW 500–20K), and the analysis is carried out using THF as eluent at a flow rate of 0.50 mL min⁻¹. The GPC system was calibrated against polystyrene standards

(molecular weight range of 890–1.86 × 10⁶ g mol⁻¹) and lignin monomers and model dimers. In particular, apocynol and (3-methoxy-4-ethoxy-2-phenyl)-2-oxoacetaldehyde were synthesized according to literature procedures and used as monomer and dimer lignin standards, respectively.

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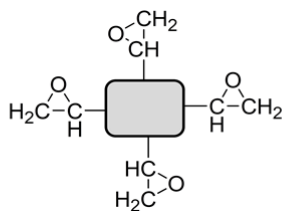
5. BIOPROCESSING OF TANNINS BY MEANS OF A HYDROLYTIC ENZYME: IMMOBILIZED TANNASE

5.1 TANNASE IMMOBILIZATION AND COATING

Tannase from *Aspergillus Ficum* was at first immobilized onto alumina particles, previously functionalized with γ -aminopropyltriethoxysilane and glutaraldehyde. The chemical immobilization onto functionalized alumina was chosen on the basis of the results obtained by my group of research for the immobilization of other enzymes, especially for the high yield of immobilization (90%) and high retained enzymatic activity.^{1,2} Moreover the mechanical resistance of alumina at high values of pH and temperature is well known.

Unfortunately, the immobilization onto alumina particle failed, the Bradford assay³ executed on the reaction cocktail showed that the enzyme did not bind the matrix at all.

The enzyme was then chemically immobilized onto eupergit C 250L, a carrier consisting of macroporous beads made by copolymerization of N,N'-methylene-bis-(methacrylamide), glycidyl methacrylate, allyl glycidyl ether and methacrylamide, characterized by highly reactive oxirane-groups (Figure 5.1).



Eupergit

 = beads made by copolymerization of N,N'-methylene-bis-(methacrylamide), glycidyl methacrylate, allyl glycidyl ether and methacrylamide

Figure 5.1: Eupergit C.

The new matrix was chosen because of its chemical and mechanical stability over a pH range from 0 to 14; moreover, it forms long-term covalent bonds stable within a pH range from 1 to 12.⁴

The immobilization onto eupergit did not require any previously functionalization because the reactive epoxy groups bind directly the amino groups of the protein molecules to form covalent bonds.

This procedure yielded 100% of loaded enzyme respect to the soluble enzyme employed and 97% of retained activity. The yield of immobilization was evaluated spectrophotometrically by the Bradford assay,³ analyzing the residual enzymatic content in the waste water after the immobilization reaction and successive washing. The retained activity of the immobilized enzyme was evaluated according to the rhodanine assay, based on the formation of chromogen between rhodanine and the gallic acid released by the hydrolysis of the substrate (methylgallate).⁵

This result was comparable with previously reported results for the chemical immobilization of tannase.

Abdel-Naby *et al.*⁶ immobilized tannase on various carriers by different methods. The covalent binding provided the highest yield of immobilization with respect to the physical adsorption, entrapment and ionic binding. Particularly, the covalent immobilization on chitosan, previously functionalized with glutaraldehyde, gave the highest immobilization yield (26,60%) and the highest retained enzymatic activity (14,65%). Therefore, eupergit proved to be an efficient matrix providing the complete immobilization of the biocatalyst without inhibiting its activity.

The immobilized enzyme was then subjected to a sequential deposition of alternatively charged polyelectrolytes: it was covered by 3 ultrathin layers of PAH⁺ (poly allylamine hydrochloride, positively charged) and PSS⁻ (poly sodium 4-styrene sulfonate, negatively charged), starting with the PAH⁺ positive layer because of the negative charge retained by the eupergit-tannase system during the immobilization procedure (pH=5). The immobilization and coating procedure is clarified in Figure 5.2.

Figure 5.2: Tannase immobilization and Layer by Layer coating of supported tannase.

After the coating, the immobilized tannase (LbL-tannase) retained about 70% of its activity, evaluated once again by the rhodanine assay, carried out directly on the LbL-tannase.

5.2 ENZYME STABILITY: THE CATALYST RECYCLE

In order to evaluate the possibility of a multiple reuse of the immobilized enzyme, LbL-tannase was allowed to react with methylgallate (the standard substrate for the assay) for 7 consecutive batch reaction, one every 24 hours. Figure 5.3 shows that LbL-tannase retained about 50% of its activity after 7 cycles. The percentage was calculated with respect to the initial activity.

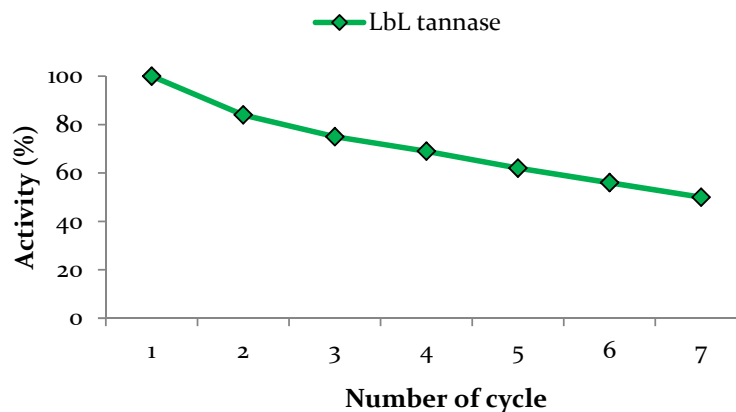


Figure 5.3: Enzymatic residual activity after 7 successive 24h catalytic cycles.

5.3 TANNIC ACID HYDROLYSIS BY MEANS OF NATIVE AND LbL-TANNASE

In order to practically evaluate the activity of LbL-tannase over time, an amount of tannic acid (30 mg) was subjected to LbL-tannase-mediated hydrolysis. The reaction was monitored using HPLC following the increase of gallic acid at the expense of galloyl substructures. In a more detailed view, tannic acid was allowed to react with the immobilized enzyme at 45°C and, every 20 minutes, an aliquot of the reaction cocktail was purified from the beads and subjected to HPLC analysis.

The HPLC profile of tannic acid obtained by a C₁₈ reverse phase column before the hydrolytic process is shown below (Figure 5.4):

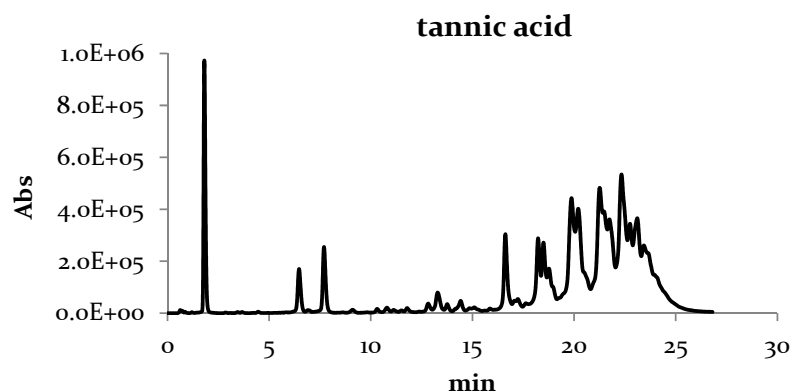


Figure 5.4: HPLC profile of tannic acid.

Commercial tannic acid is not a pure compound but it is a mixture of gallotannins, extracted usually from sumac and oak galls. Although the commercial sources provide a molecular weight of 1701,2 g/mol, the preparation is rather a heterogeneous mixture of galloyl esters. For this reason tannic acid is not a suitable standard for tannin analysis and it is commonly replaced by methylgallate.

The HPLC profile confirmed that tannic acid is a mixture of compounds, among which free gallic acid. The presence of gallic acid was highlighted by the first sharp signal of the profile, identified by comparison of its retention time with that of the pure product (Sigma-Aldrich). The multiplicity of signals among 16 and 26 min arose from galloyl esters of glucose having different degrees of esterification.

When the hydrolytic process started, a more pronounced signal of gallic acid appeared with the concomitant decrease of the galloyl esters signals (Figure 5.5).

The increasing of gallic acid was monitored over time (Figure 5.6).

After 140 min, 30 mg of tannic acid were definitively converted into gallic acid and sugars (Figure 5.7).

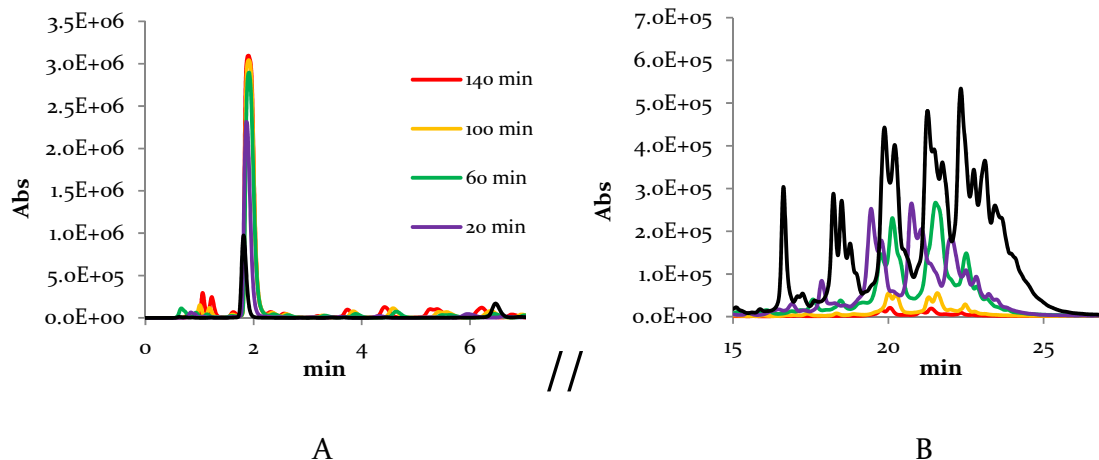


Figure 5.5: Conversion of tannic acid into gallic acid. Appearance of gallic acid (A); disappearing of galloyl esters (B).

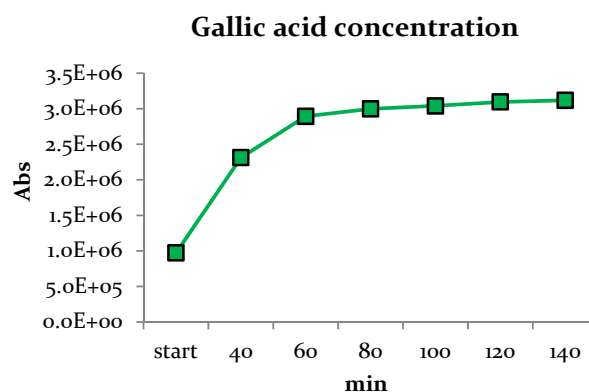


Figure 5.6: Increase of gallic acid concentration over time.

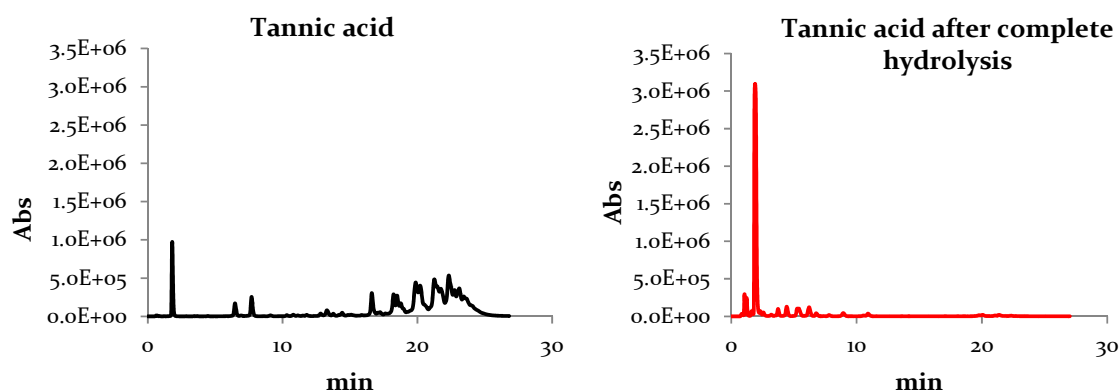


Figure 5.7: Comparison between tannic acid before and after the hydrolysis.

5.4 COMMERCIAL TANNINS HYDROLYSIS BY MEANS OF LbL-TANNASE

Two samples of commercially available hydrolysable tannins, particularly a gallotannin extracted from oak galls and an ellagitannin extracted from oak wood, were subjected to LbL-tannase-mediated hydrolysis. As well as in case of tannic acid, the reaction was followed over time using HPLC, monitoring the occurrence of gallic acid (Figure 5.8).

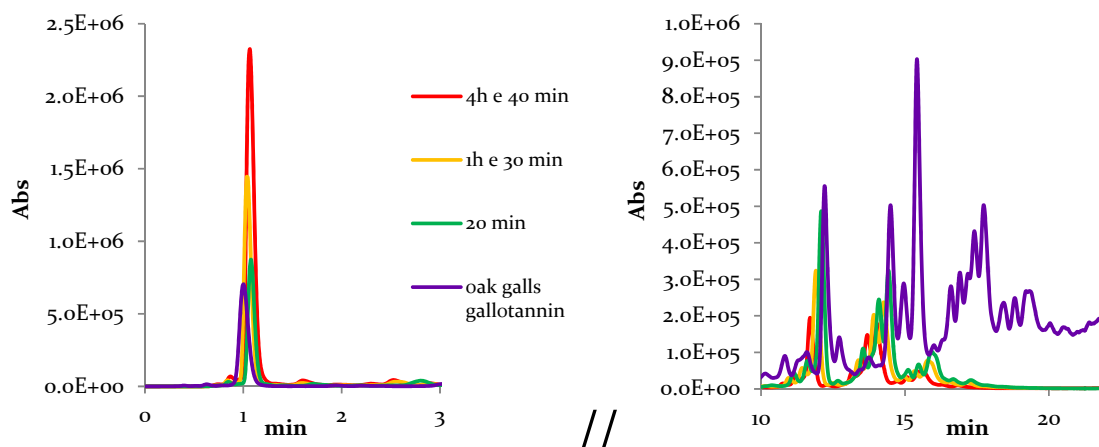


Figure 5.8: Hydrolysis of oak galls gallotannin monitored in the course of time.

The analysis of oak galls gallotannin just before the hydrolysis (purple line) showed the heterogeneity of the sample. It contained an amount of free gallic acid, highlighted by the first sharp signal, and a wide array of galloyl esters, arisen from 12 and 20 min. After about 4h and 40min the galloyl esters were completely hydrolyzed, leaving the subsequent profiles unchanged (Figure 5.9).

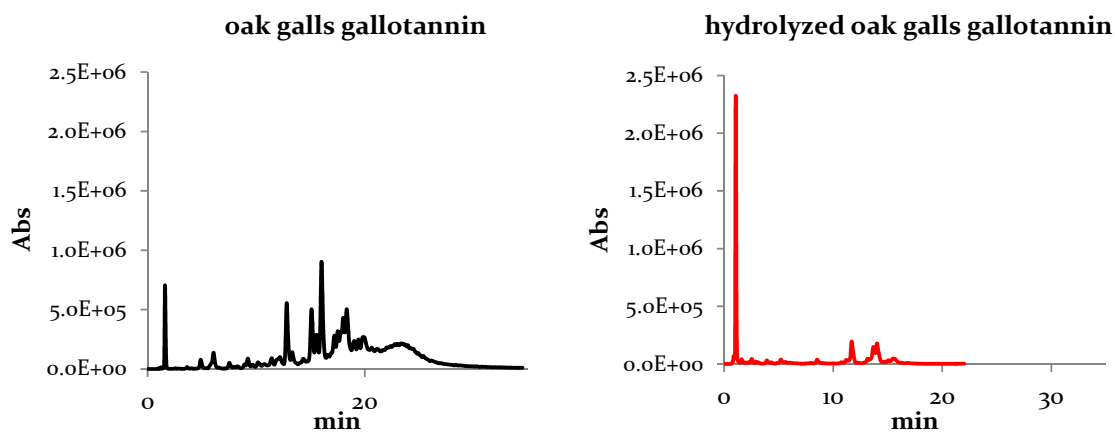


Figure 5.9: Comparison between oak galls gallotannin before and after the complete hydrolysis.

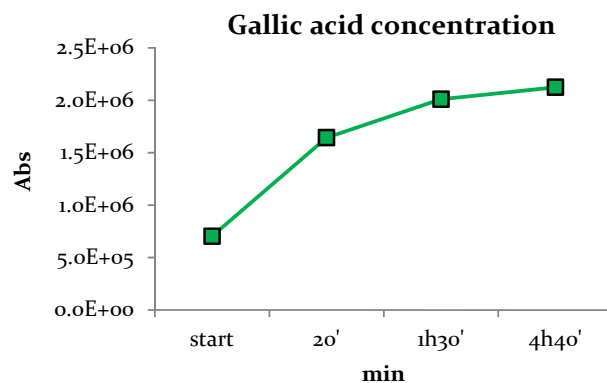


Figure 5.10: Increase of gallic acid concentration over time.

In order to clarify the nature of the compounds produced by the hydrolytic process, the hydrolyzed oak galls gallotannin was recovered from the reaction cocktail, freeze-dried and submitted to ^{31}P NMR analysis, after phosphorylation with 2-chloro-4,4',5,5'-tetramethyl-1,3,2-dioxaphospholane (Cl-TMDP), according to the procedure used to analyze tannins model compounds and commercial tannins.

The spectrum of the hydrolyzed sample (Figure 5.11 B) showed the predominance of gallic acid among the reaction products, identified by the comparison with pure gallic acid initially analyzed as tannin model compound (Chapter 3, Figure 3.2 A).

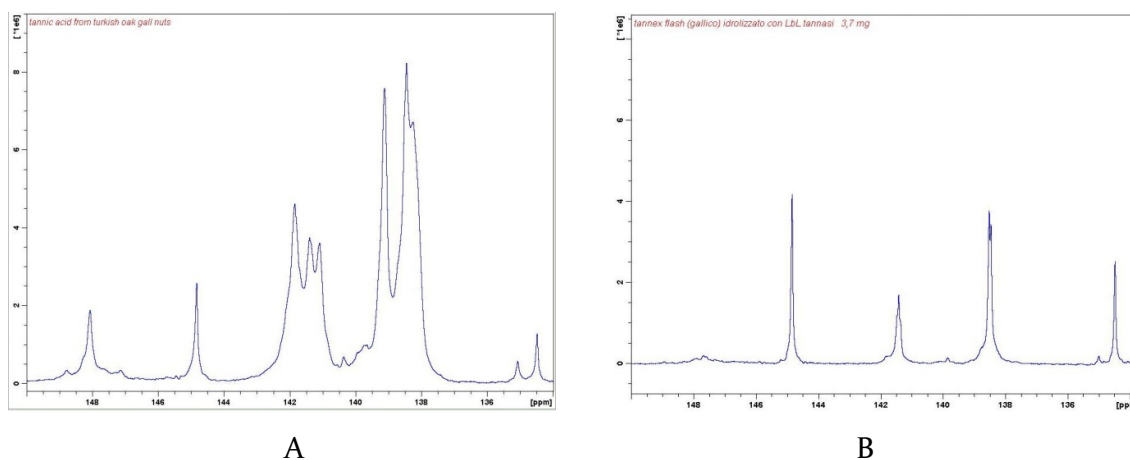


Figure 5.11: ^{31}P NMR profile of oak galls gallotannin before (A) and after (B) LbL-tannase-mediated hydrolysis.

Thanks to the quantitative ^{31}P derivatization of the sample and to the presence of a suitable internal standard, it was possible to clarify the amount of gallic acid yielded from the hydrolysis. Table 5.1 summarizes the content:

Table 5.1: Aliphatic, phenolic OH and carboxylic acid content as evaluated by ^{31}P NMR analysis.

Sample	Aliphatic OH*	<i>o</i> -disubstituted OH*	<i>o</i> -substituted OH*	<i>o</i> -unsubstituted OH*	COOH*
Hydrolyzed Gallotannin	1,19	2,34	4,62	-	2,31

*= mmol/g of sample

The results showed that oak galls gallotannin contained about 2,32 mmol of gallic acid per gram, namely about 395 mg of gallic acid per gram of sample (gallic acid MW=170,12 g/mol).

Commercially available ellagitannin extracted from oak wood could be completely hydrolysed within 15 min, as seen in the HPLC profile Figure 5.12)

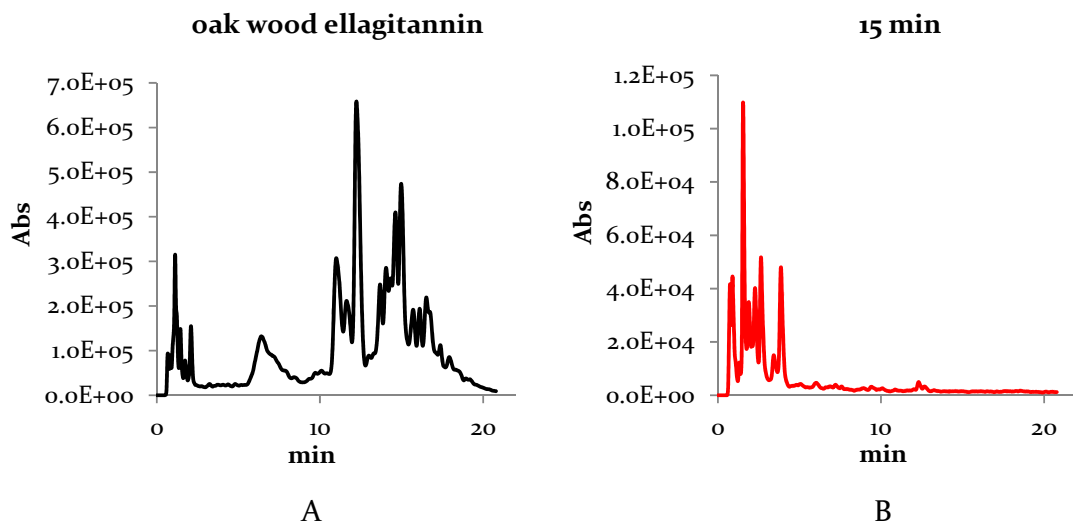


Figure 5.12: Comparison between oak wood ellagitannin before (A) and after (B) the complete hydrolysis.

Ellagic acid was not released because tannase did not hydrolyze hexahydroxydiphenyl (HHDP) moieties; despite of this, an amount of gallic acid released by tannase was detected.⁷ In any case, the substrate of tannase has to be an ester compound of gallic acid, whatever is the alcohol that forms the ester bond, while esters and carboxylic acids cannot be hydrolyzed by the enzyme unless they have phenolic hydroxyls.⁸

Although the HHDP moiety is actually an ester of gallic acid, the most part of bacteria, fungi and yeasts are not able to hydrolyze it, most likely because the C–C coupling make the structure more complex. However, some other bacteria and fungi, especially from the ellagitannin-rich soils, are able to produce a tannase that is highly active towards the hydrolysis of HHDP moieties and other residues of ellagitannins.^{9,10}

The ³¹P NMR analysis of the product recovered after the hydrolysis showed the typical signal of the HHDP moieties (basically coupled gallic acid) at 141.42 ppm (o-disubstituted OH) and 138.50 ppm (o-substituted OH). The signal in the acidic region (134.48 ppm) confirmed the presence of free gallic acid in the hydrolyzed sample (Figure 5.13). As expected, no signals related to ellagic acid (141.65 and 139.17 ppm) were reported.

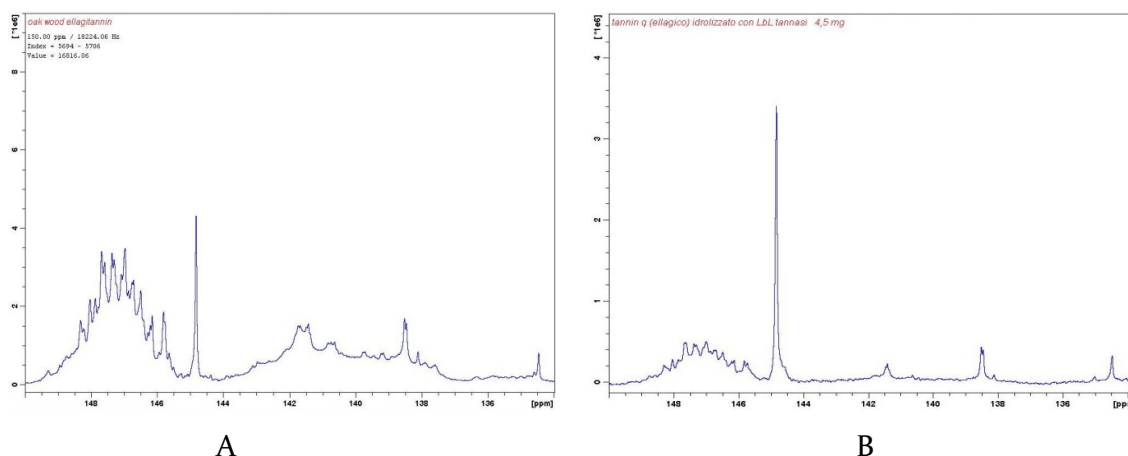


Figure 5.13: ³¹P NMR profile of oak wood ellagitannin before (A) and after (B) LbL-tannase-mediated hydrolysis.

In this case ³¹P NMR analysis could not provide absolute quantification of the gallic acid released after the treatment since its signals partially overlapped the HHDP residues adsorbancies.

5.5 CONCLUSION

The efforts of the current study have been directed to the development of a novel immobilized tannase. A wide variety of suitable supports were tested by Abdel-Naby *et al.* providing a detailed framework on the different possibilities. According to their results, the covalent binding provided the highest yield of immobilization, for this reason we chose and tested two inert matrix suitable for a covalent immobilization of the enzyme, namely functionalized alumina particle and eupergit C 250L. Functionalized alumina proven to be unsuitable for the aim since tannase lost the activity as soon as immobilized. Eupergit C 250L (10 mg each enzymatic unit) provided a complete immobilization of the enzyme ensuring the total retention of enzymatic activity, in contrast with the results obtained by Abdel-Naby.⁶

The subsequent layer-by-layer coating technique proven to be a valuable approach to assure a more enduring enzyme stability, protecting the enzyme from denaturing agents.

LbL-tannase proven to be still operating even after 7 batch reactions, retaining about 50% of its activity.

Although tannic acid is known to denature proteins, the enzyme catalyzed both the breakdown of tannic acid and of more complex gallotannins and ellagitannins, hydrolyzing only the galloyl moieties of the samples. The HPLC analysis monitored the efficiency of the enzyme over time.

³¹P NMR quantitative analysis, for which the protocol was developed before, gave the possibility to quantify the amount of gallic acid released by the LbL-tannase-mediated hydrolysis.

However, the practical use of this enzyme is at present limited due to insufficient knowledge about its properties, optimal expression, and large-scale application.

5.6 EXPERIMENTAL SECTION

5.6.1 TANNASE IMMOBILIZATION AND COATING

13,0 units of native tannase were added to a suspension of 130 mg of eupergit C250L in 20 ml of citrate buffer 0.01 M pH= 5. The reaction cocktail was allowed to stir at room

temperature for 24h. The immobilized enzyme was then washed 3 times with the same citrate buffer until no enzymatic activity was found in the washing solution.

The unreacted epoxy-groups were “inactivated” allowing the immobilized tannase to react with a solution of glycine 0.3 M for 2h at room temperature. After that, the immobilized enzyme was washed several time in order to remove the excess of glycine, then it was subjected to a sequential deposition of 3 layers of polyelectrolytes.

Polyelectrolyte solutions (polyallyl amine hydrochloride PAH^+ , 0.01 M with 0.5 M NaCl) (polystyrene sulfonate PSS^- , 0.01 M with 0.5 M NaCl) were prepared and the beads were immersed inside each solution for 20 minutes in order to deposit 3 layers according this sequence: PAH^+ , PSS^- , PAH^+ . The starting layer was positively charged since the immobilised tannase was negatively charged at the condition of the immobilization step (pH=5). After each layer, the excess of polyelectrolyte was removed by washing with 0.1 M NaCl.

5.6.2 TANNASE ACTIVITY ASSAY⁵

CALIBRATION CURVE:

Gallic acid (0.5 mM) in citrate buffer (0.05 M, pH 5.0) was used as standard and prepared fresh before use.

Aliquots of this solution containing from 0 (blank) to 100 nmol gallic acid were taken, and the volume was made to 0.5 ml with citrate buffer. Methanolic rhodanine solution (0.667%; 0.3 ml) was added to all the tubes, and they were incubated at 30°C for 5 min. Potassium hydroxide solution (0.5 M; 0.2 ml) was added to all the tubes which were again incubated at 30°C for 5 min. Then 2 mL of distilled water was added to dilute the mixture and after 5–10 min the absorbance was recorded at 520 nm. The calibration curve was obtained plotting the absorbance vs. the concentration.

NATIVE TANNASE ASSAY:

Tannase was assayed by the method based on chromogen formation between gallic acid (released by the action of tannase on methylgallate) and rhodanine. 0.25 ml of methylgallate (0.01 M in 0.05 M citrate buffer, pH 5.0) were added to the test, control and blank tubes. 0.25 ml of the buffer and 0.25 ml of the enzyme sample were added to

the blank and test respectively, and the tubes were incubated at 30°C for 5 min. 0.3 ml of methanolic rhodanine (0.667%; w/v) was added to all the tubes that were then kept at 30°C for 5 min. 0.2 ml of 0.5 M potassium hydroxide was added to each tube and these were incubated at 30°C for other 5 min. This was followed by addition of the enzyme sample (0,25 ml) to the reaction mixture in the control tube only.

The reaction mixture in the blank, test, and control tubes contained 0.25 ml of substrate solution to which 0.25 ml of the buffer and 0.25 ml of the enzyme sample were added to the blank and test, respectively. The tubes were incubated at 30°C for 5 min, and 0.3 ml of methanolic rhodanine (0.67%; w/v) was added to all the tubes that were then kept at 30°C for 5 min. After this, 0,2 ml of 0,5 M potassium hydroxide was added to each tube and these were incubated at 30°C for 5 min. This was followed by addition of the enzyme sample (0.25 ml) to the reaction mixture in the control tube only. Finally, each tube was diluted with 2,0 ml distilled water and incubated at 30°C for 10 min and the absorbance was recorded against water at 520 nm. The enzyme activity was calculated as following:

$$\Delta A_{520} = (A_{\text{test}} - A_{\text{blank}}) - (A_{\text{control}} - A_{\text{blank}}).$$

One unit of tannase activity was defined as the amount of enzyme required to release 1 μmol of gallic acid in 1 min under specific conditions.

LbL-TANNASE ASSAY:

The procedure was identical to that performed for the native enzyme. An amount of solid LbL-tannase substituted the enzyme sample added to the test tube and in the second moment to the control.. Before subjecting the sample to the spectrophotometric analysis, the tubes were centrifugated at 5000 rpm for 10 minutes, in order to separate the immobilized enzyme to the solution test.

5.6.3 TANNIC ACID AND COMMERCIAL TANNINS HYDROLYTIC TREATMENT WITH LbL-TANNASE

30.0 mg of tannic acid, 16.0 mg of oak galls gallotannin, and 14.6 mg of oak wood ellagitannin

were dissolved in 10 ml of citrate buffer 0.01 M pH=5 and the solution was heated at 30°C for 5 min. 5 U of LbL-tannase, preincubated at 45°C for 5 min, were added to the solution and the reaction cocktail was allowed to stir at 45°C.

Each 15 min, 100 µl of the reaction cocktail were collected and centrifuged at 40°C for 5 min at 5000 rpm. The supernatant was submitted to HPLC analysis.

5.6.4 HPLC ANALYSIS

The HPLC analysis was performed by Shimadzu LC 20AT liquid chromatography with a SPD M20A ultraviolet diode array (UV) detector set at 280 nm. The sample (20 µl) was injected into C₁₈ reverse phase column and the analysis was carried out using CH₃CN 0.01% TFA (pump A) and water 0.01% TFA (pump B). Compounds were separated by a linear gradient from 5% to 25% of CH₃CN in 20 min, with a flow rate of 0.80 ml min⁻¹.

5.6.5 QUANTITATIVE ³¹P NMR PROCEDURE

About 10 mg of tannin accurately weighed were dissolved in 400 ml of a solvent mixture composed of pyridine and deuterated chloroform, 1.6:1.0 (v/v) ratio. 100 ml of 2-chloro-4,4',5,5'-tetramethyl-1,3,2-dioxaphospholane were added, followed by 100 ml of the internal standard solution (cholesterol 0.1 M + 5g/L of Cr(III) acetyl acetonate as relaxation agent). The reaction cocktail was allowed to stir at room temperature for two hours. The NMR spectra were recorded on a Bruker 300 NMR spectrometer using previously published methods. To obtain a good resolution of the spectra, a total of 256 scans were acquired. The maximum standard deviation of the reported data was 2 E-2 mmol/g, while the maximum standard error was 1 E-2 mmol/g.

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6. LIGNIN BIOPROCESSING BY MEANS OF IMMOBILIZED ENZYMES

6.1 LACCASE

6.1.1 IMMOBILIZATION AND COATING

Laccase from *Trametes versicolor* was chemically immobilized onto alumina (Al_2O_3) particles, 2-3 mm diameter, initially functionalized with γ -aminopropyltriethoxysilane and glutaraldehyde. The inert alumina particles were chosen because of their known mechanical resistance at high values of pH and reaction temperature¹ and for the possibility to easily recover the catalyst from the reaction mixture.

This procedure yielded 90% of loaded enzyme respect to the soluble enzyme employed. The yield of immobilization was evaluated spectrophotometrically by the Bradford and ABTS assay, analyzing the residual enzymatic content in the waste water after the immobilization reaction and successive washing.

This result was comparable with previously reported results for the chemical immobilisation of laccase and other enzymes on functionalised alumina pellets under similar experimental conditions.**Error! Bookmark not defined.**² The immobilized enzyme was then subjected to a sequential deposition of alternatively charged polyelectrolytes: it was covered by 3 ultrathin layers of PAH^+ (poly allylamine hydrochloride, positively charged) and PSS^- (poly sodium 4-styrene sulfonate, negatively charged), starting with the PAH^+ positive layer because of the negative charge retained by the alumina-laccase system at $\text{pH}=5$.³ The immobilization and coating procedure is clarified in Figure 6.1.

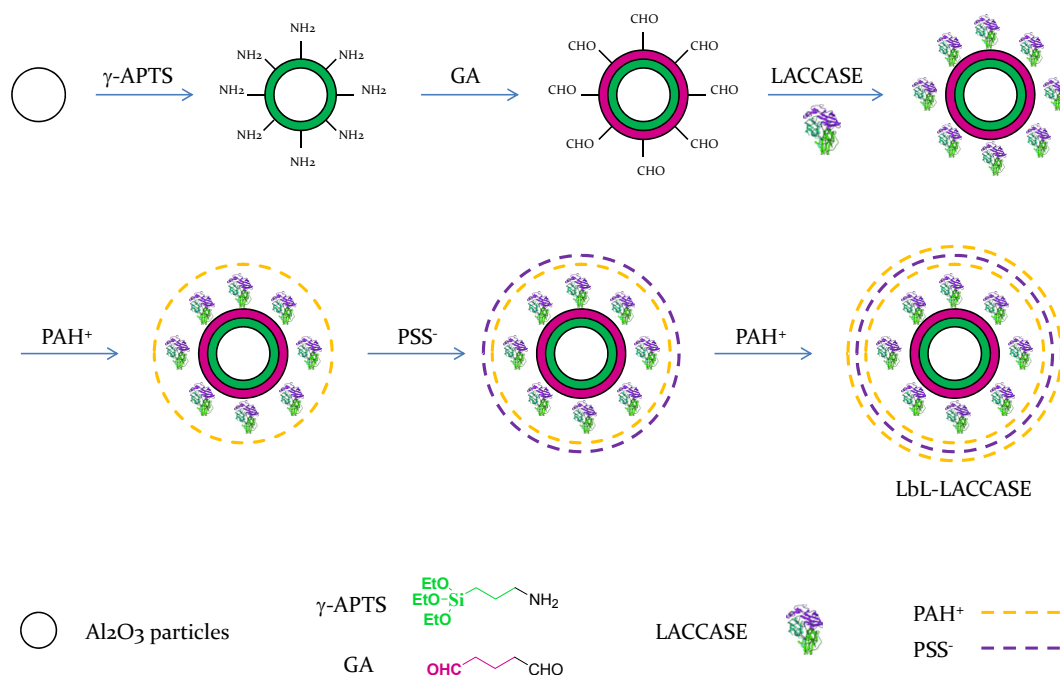


Figure 6.1: Support activation, laccase immobilization and Layer by Layer coating of supported laccase.

After the coating, the immobilized laccase (LbL-laccase) retained about 70% of its activity, evaluated once again by the ABTS assay, carried out directly on the LbL particles.

6.1.2 ENZYME STABILITY: THE CATALYST RECYCLE

In order to evaluate the possibility of a multiple reuse of the immobilized enzyme, laccase was allowed to react with ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) for 10 successive batch reaction, one every 12 hours. The same reaction was carried out on native laccase to highlight the precious advantages of the coating multi-layers. In fact, as shown in Figure 6.2, after 10 cycles, the native laccase retained only 20% of its activity while the LbL-laccase retained about 85%. The percentage was calculated with respect to the initial activity found for both the catalysts.

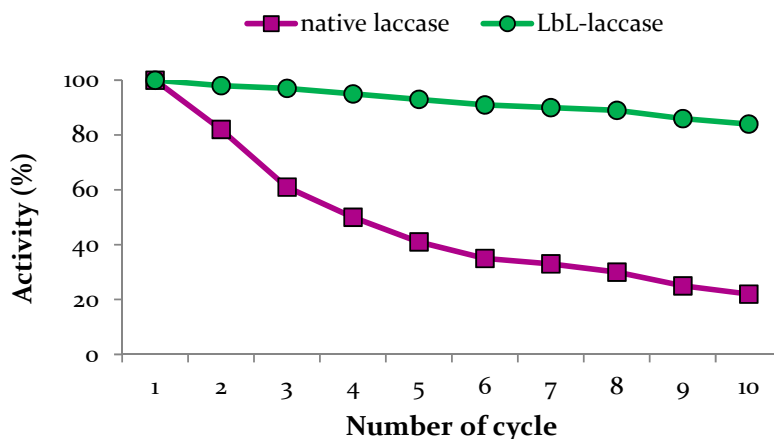


Figure 6.2: Enzymatic residual activity after 10 successive 12 h catalytic cycles.

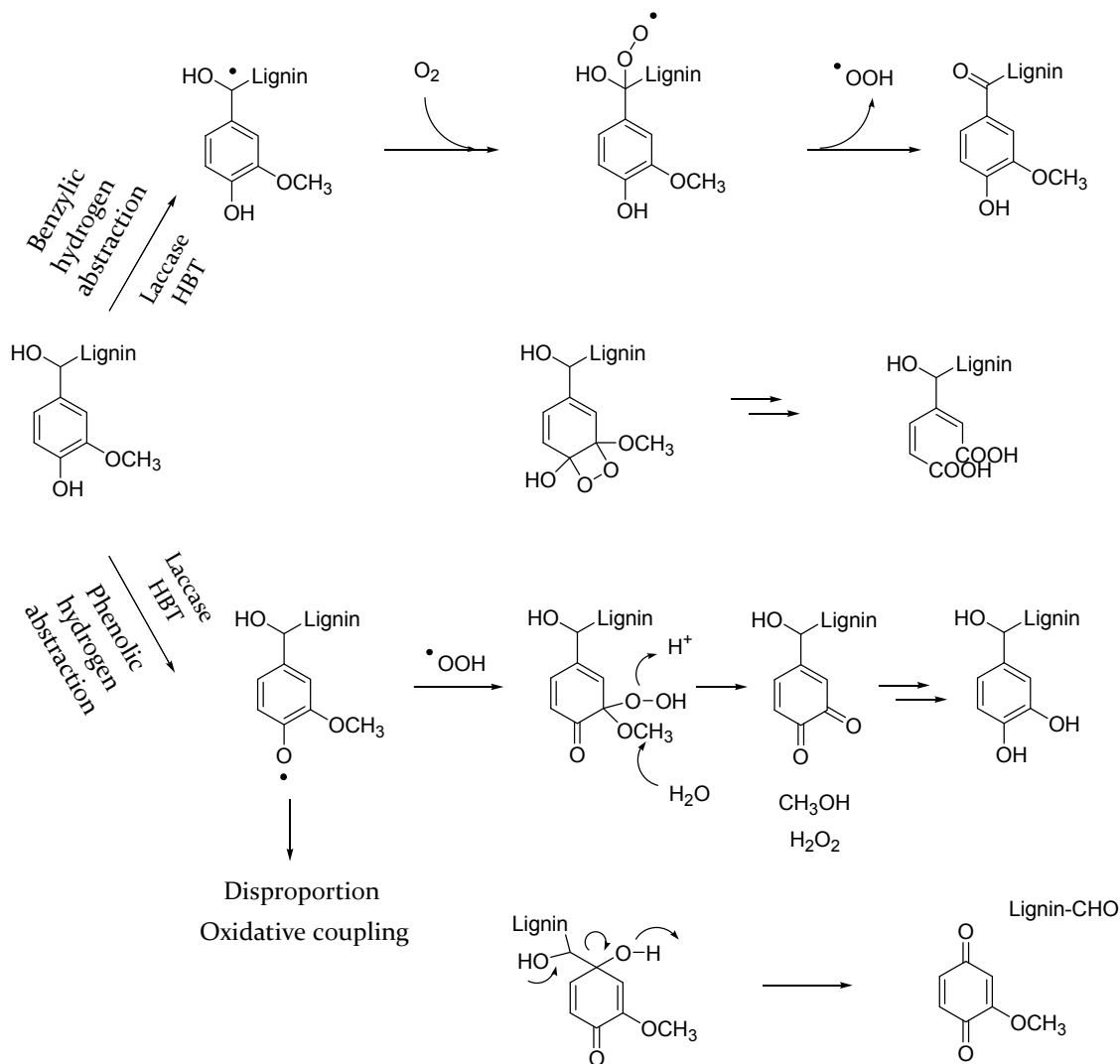
6.1.3 WHEAT STRAW LIGNIN (WL) OXIDATION BY MEANS OF NATIVE AND LbL-IMMOBILISED LACCASE

The physicochemical properties of wheat straw lignin were investigated in previous work described in literature.⁴ Wheat lignin was found to be different from softwood or hardwood lignins, characterized by high amounts of *p*-hydroxyphenyl residues that renders it having a characteristic solubility in alkaline solutions.

To investigate the efficiency and the reaction pathway of laccase-catalysed oxidations, a set of four experiments was designed. 80 mg of wheat straw lignin were suspended in acetate buffer and oxidized with 23U of soluble and LbL-laccase. Parallel reactions were carried out in presence of a chemical mediator, 1-hydroxybenzotriazole (HBT) 1 mM. For the crucial experiment that was run in order to allow both a qualitative comparison – in terms of reaction pathways – and quantitative comparison of the enzymatic activity under *these* conditions with the enzymatic activity measured in *other experimental settings* that will be discussed in the following paragraphs of this thesis, the amount of laccase used in this set of oxidations was chosen according to the activity of the enzyme as it was found in the just mentioned experiment run under alternate conditions (multi-enzyme biocatalyst experiment (see below)).

As described in literature,^{5,6} the presence of low molecular weight mediators, such as HBT, ABTS or violuric acid, gives rise to a different oxidation pathway: the enzyme first reacts with the mediator by formation of the corresponding radical species, then, the

new reactive species causes hydrogen atom abstraction on lignin, both on the phenolic and on the benzylic positions, with the subsequent oxidative functionalization or depolymerization (Scheme 6.1).



Scheme 6.1: Reaction pathway in the presence of a mediator.

The yield of conversion was calculated as the weight percentage of converted lignin. The data are summarized in Table 6.1.

Table 6.1: Conversion of wheat straw lignin after treatment with soluble and immobilized laccase, in presence or absence of HBT as oxidation mediator.

Entry	Biocatalyst	Conversion (%)*
1	Laccase	17,1
2	Laccase+HBT	14,6
3	LbL-laccase	34,7
4	LbL-laccase+HBT	58,2

*: mg of converted lignin (soluble lignin)/mg of starting material

It is known that an efficient oxidative functionalization, associated with depolymerization processes, makes lignin a more hydrophilic polymer with a high solubility in water.

After the treatment with soluble laccase, in presence or in absence of the mediator, a low amount of soluble lignin was recovered (Table 6.1, entry 1-2); on the contrary, a higher conversion was obtained after the treatment with the immobilized biocatalysts (Table 6.1, entry 3-4). On the basis of these results it is possible to gather that the multi-layer coating did not prevent lignin from reaching the catalytic site of laccase, rather it improved the enzyme stability enhancing its performance in the oxidative process.

In presence of HBT as oxidation mediator, it was noticed an increasing of the conversion (Table 6.1, entry 4 vs entry 3): it confirms the role of HBT in the enhancement of the efficiency of the oxidation.⁷ The same behavior was not observed with soluble laccase (Table 6.1, entry 2 vs entry 1).

6.1.4 ³¹P NMR STRUCTURAL CHARACTERIZATION OF WL AFTER OXIDATIVE TREATMENTS

³¹P NMR spectroscopy has proven to be a precious analytic tool for the elucidation of natural polymer structure and, to date, it has represented the most widely applied technique for highlighting lignin structural modifications. In fact, this technique allows the characterization and quantification of all OH groups present in lignins, as the aliphatics, the carboxylic acids and the phenols, distinguishing the condensed phenols (o-disubstituted) from the guaiacyl ones.

Before the analysis, the insoluble fractions of lignin recovered after the treatments, were subjected to in situ phosphitilation according to a procedure widely described in literature.^{8,9,10}

About 25 mg of the samples were suspended in a mixture of pyridine/deuterated chloroform (1.6:1.0, v/v ratio) and phosphitylated in quantitative yield with 2-chloro-4,4',5,5'-tetramethyl-1,3,2-dioxaphospholane. The presence of cholesterol as internal standard in the reaction cocktail allowed running quantitative analysis.

The assignment of the different OH signals was carried out on the basis of the comparison with the chemical shift of selected models reported in previous works.^{11,12,13}

Table 6.2 summarizes the value of the ³¹P NMR quantitative analysis for the laccase-catalysed oxidations. The amounts of the different OH groups were calculated in mmol/g of lignin sample.

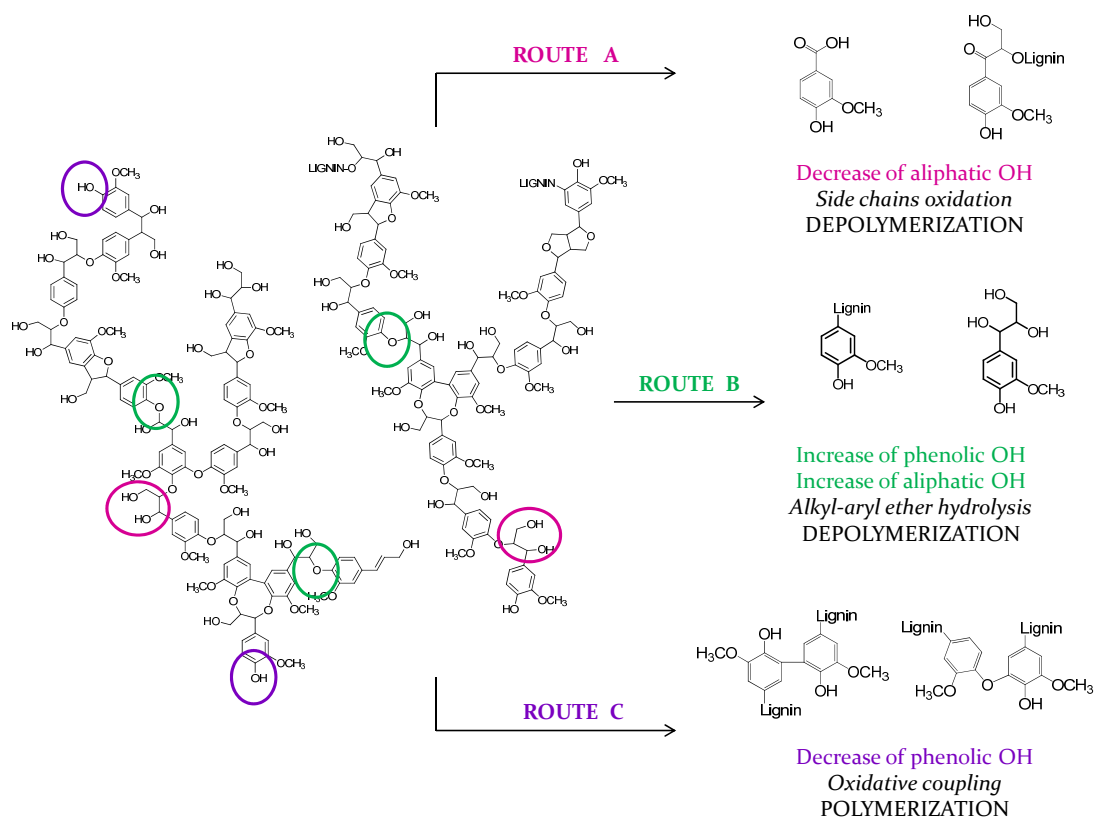
Table 6.2: Aliphatic, phenolic and carboxylic OH content evaluated by ³¹P NMR after phosphitylation.

Entry	Biocatalyst	Aliphatic OH (mmol/g)	PhenolicOH (mmol/g)	Acidic OH (mmol/g)
1	WL ^a	1,46	0,80	0,27
2	Laccase	1,46	0,71	0,25
3	Laccase+HBT	1,49	0,46	0,30
4	LbL-laccase	2,49	0,95	0,12
5	LbL-laccase+HBT	2,62	1,09	0,17

a: wheat straw lignin (WL): starting material

The content in aliphatic and acidic OH groups after treatment with soluble laccase was nearly unchanged with respect to the starting lignin (WL) while the content of phenolic OH groups decreased (Table 6.1, entry 2 vs. 1). The same results were obtained for the laccase-mediator system, with a still lower phenolic content (Table 6.1, entry 3 vs. 1, 2).

These results are consistent with an oxidation pathway characterized by oxidative coupling processes on phenolic end groups in lignin, as shown in Scheme 6.2 (route C)



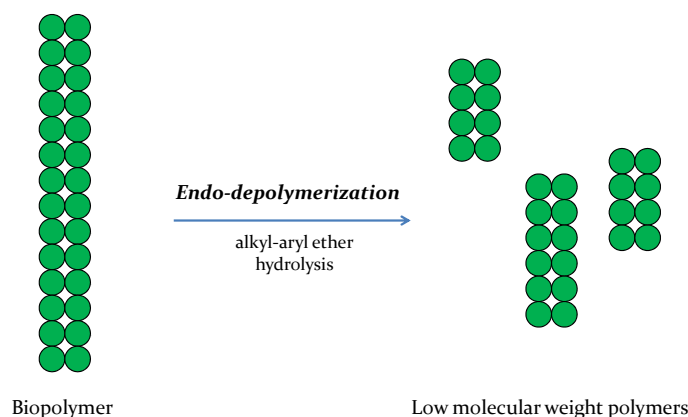
Scheme 6.2: Lignin oxidation pathways.

The low values of lignin conversion after the treatments with soluble enzymes (Table 6.1, entry 1, 2) are in agreement with the ^{31}P NMR quantitative results explained above: the occurrence of oxidative coupling gave rise to a larger and less hydrophilic polymer leaving into solution only low molecular weight fraction resulting from the depolymerization of the terminal units of the polymer. This oxidative process centered on the external units is best to be described as exo-depolymerization (Scheme 6.3).

Scheme 6.3: Exo-depolymerization process.

A different selectivity was observed with the LbL-catalysts. In fact, when WL was treated with LbL-laccase both the aliphatic and the phenolic OH groups increased while the carboxylic acid decreased. The concomitant increase of the phenolic and of the aliphatic OH were caused by alkyl-phenyl ether bond cleavage, as shown in Scheme 6.2, route **B**.

The hydrolysis of the ether bonds gave rise to a substantial increase of the polymer solubility, confirmed by a higher percentage of converted lignin respect to the soluble enzymes treatments (Table 6.1, entry 3 vs 1, 2). This kind of hydrolytic process that gives rise to alkyl-phenyl ether bond cleavage, is called endo-depolymerization, that yields a significant soluble fraction that contains oligomers (Scheme 6.4).



Scheme 6.4: Endo-depolymerization process.

The presence of the mediator enhanced the efficiency of LbL-laccase depolymerization process, as shown by the highest value of aliphatic and phenolic OH listed in Table 6.2 (entry 4 vs 3) and by the highest percentage of converted lignin in Table 6.1 (entry 4). These data indicate not only the prevalence of depolymerization reactions (Scheme 6.2, routes **B**) but also the contemporary inhibition of repolymerization processes by oxidative cross-linking coupling.

6.1.5 GPC ANALYSIS OF WL AFTER OXIDATIVE TREATMENTS

Structural modifications of wheat straw lignin were evaluated also by gel permeation chromatography (GPC).

Before running the GPC analysis, the samples of insoluble lignin recovered after the treatments were acetobrominated according to a procedure described in literature.¹⁴ Thanks to this procedure, the primary alcoholic and the phenolic hydroxyl groups are acetylated, while the benzylic α -hydroxyls are displaced by bromide, making lignin a more hydrophobic polymer.

GPC was carried out using a system of columns connected in series calibrated against

monodisperse polystyrene standards, monomeric and dimeric lignin model compounds, as 4-(1-hydroxyethyl)-2-methoxyphenol and (3-methoxy-4-ethoxy-2-phenyl)-2-oxoacetaldehyde.

The GPC profiles shown below (Figure 6.3) and the calculated M_n and M_w values (Table 6.3) confirmed the previously reported results about quantitative ^{31}P NMR: soluble laccase treatments induced an increase in M_n and M_w values respect to the untreated WL, due to oxidative coupling process (Table 6.3, entry 2, 3 vs 1).

Table 6.3: M_n , M_w and M_w/M_n of the insoluble lignin samples recovered after treatments.

Entry	Biocatalyst	M_n	M_w	M_w/M_n
1	WL ^a	2,62E + 04	1,69E + 05	6,5
2	Laccase	3,51 E + 04	1,87 E + 05	5,3
3	Laccase+HBT	2,77 E + 04	1,84 E + 05	6,6
4	LbL-laccase	1,96 E + 04	1,05 E + 05	5,4
5	LbL-laccase+HBT	1,29 E + 04	4,21 E + 04	3,3

a: wheat straw lignin (WL): starting material

The occurrence of cross-linking processes was highlighted by a broader molecular weight distribution in case of treatment with free laccase; similarly, in presence of the mediator, the distribution of molecular weight appeared shifted to higher values.

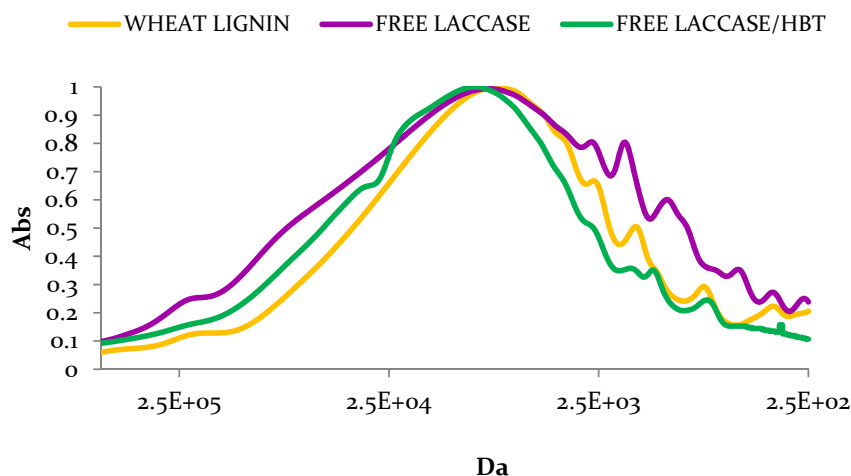


Figure 6.3: GPC analysis of the unsoluble fraction after treatments.

When the LbL-laccase was used, M_n and M_w values decreased with respect to the starting lignin (Table 6.3, entry 4 vs. 1). This confirmed that the immobilized enzyme oxidizes lignin with a different reaction pathway respect to the free enzymes, where the depolymerization processes prevail over the oxidative coupling. (Scheme 6.2, route **B** vs **C**)

A more emphasized reduction of M_n and M_w values was obtained in presence of HBT as mediator: once again, this result confirmed that the mediator enhances the efficiency of the LbL-biocatalyst in the depolymerizing capacity. (Table 6.3, entry 5 vs. 4 vs. 1)

In order to further investigate the nature of lignin modifications induced by the enzymes, a GPC analysis was carried out also on the water soluble fractions. As explained before, the native enzyme gave rise to exo-depolymerization processes that took place on the external units of lignin backbone leaving into solution only low molecular weight compounds, while the immobilized enzyme hydrolyzed the internal ether bonds, yielding oligomeric, more soluble fractions.

Figure 6.4 shows the last concept and highlights the molecular weight distribution of the soluble parts recovered after the treatment with native and LbL-laccase in comparison with the untreated lignin that did not have soluble fraction.

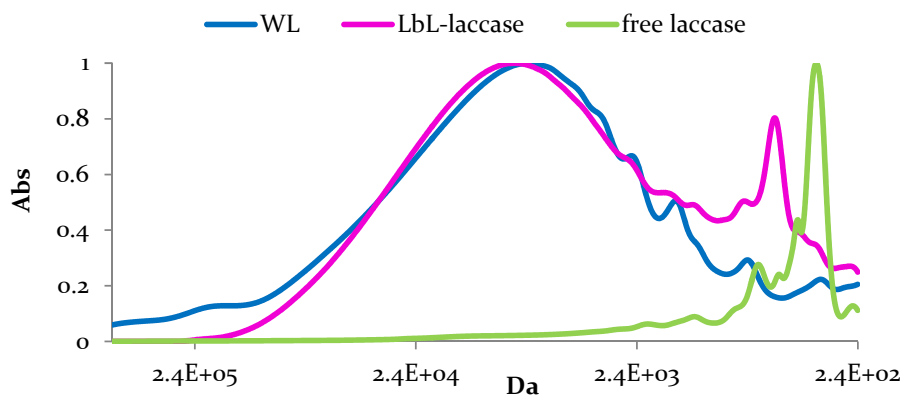


Figure 6.4: GPC analysis of water soluble fraction recovered after enzymatic treatments.

6.1.6 INTERMEDIATE SUMMARY

The results obtained in this first set of experiments showed the higher efficiency of LbL-biocatalysts in oxidative processes over the soluble ones. In a more detailed view, thanks to the immobilization strategy, it was possible to tune the selectivity of the enzyme. In fact, soluble laccase performed cross-linking reaction on lignin phenolic end groups yielding a more complex polymer and a low amount of soluble fraction, while the LbL-biocatalyst promoted a substantial and irreversible depolymerization of lignin structure. The two different oxidative reaction pathways (Scheme 6.2, route **C** vs. **B**) give rise to exo-depolymerization and endo-depolymerization processes, using a soluble and an immobilized LbL-biocatalyst, respectively.

Moreover, as demonstrated in previous works, the Layer-by-layer coating technique did not prevent lignin from reaching the biocatalytic centre but protected the enzymes from denaturant agents, conferring them a high stability and an extended efficiency.

6.2 HORSERADISH PEROXIDASE (HRP)

6.2.1 IMMOBILIZATION AND COATING

As for laccase, Horseradish peroxidase (HRP) from *Armoracia rusticana* was chemically immobilised onto alumina particles to confer high resistance at high value of pH and temperature,¹ and to ease the recovery of the biocatalyst from the reaction mixture. Before the enzyme loading, the inert alumina matrix was functionalized with γ -aminopropyl triethoxy silane, that reacts with the particles leaving free amino functions, subsequently bound by glutaraldehyde that provides the connection site to the enzyme (Figure 6.5).

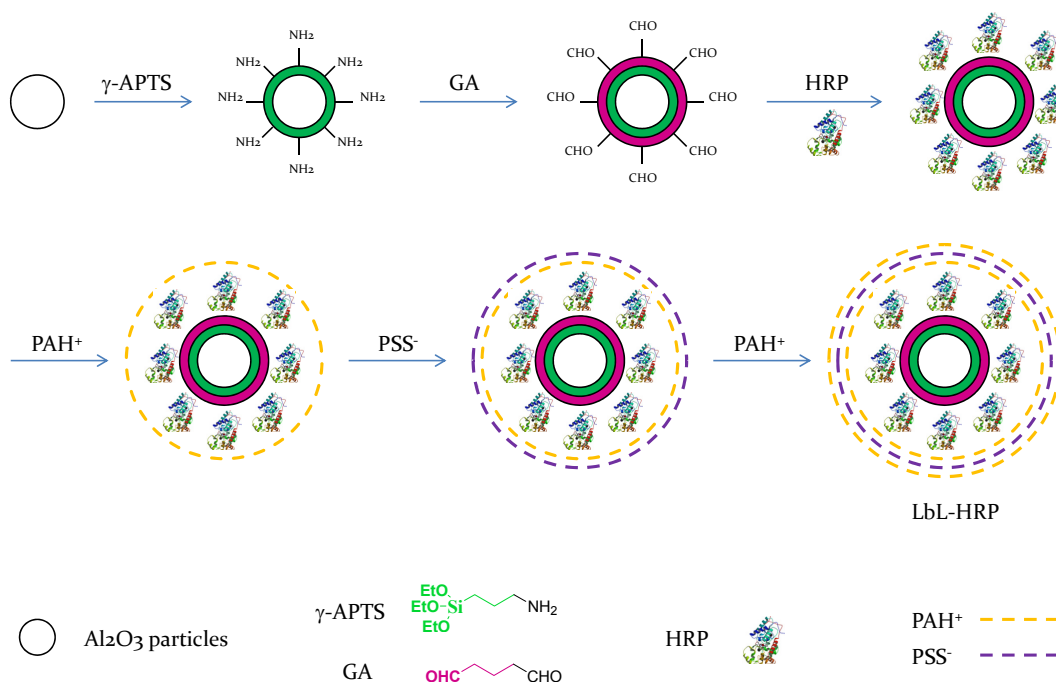


Figure 6.5: Support activation, HRP immobilization and Layer by Layer coating of supported HRP.

The Bradford assay¹⁵ showed a percentage of immobilization higher than 90%, confirming the previous results for the chemical immobilisation of HRP on functionalised alumina pellets under similar experimental conditions.¹⁶ The matrix-HRP system was then coated with a triple layer of alternatively charged polyelectrolytes, polyallylamine hydrochloride (PAH^+) and poly sodium 4-styrene sulfonate (PSS^-), starting with the deposition of the positively charged layer of PAH^+ since the system detained a negative charge at neutral pH.

After the LbL coating, the enzyme retained more than 70% of its activity with respect to native HRP, as determined by the activity assay, measured spectrophotometrically using ABTS as substrate.¹⁷

6.2.2 ENZYME STABILITY: THE CATALYST RECYCLE

Both the soluble and the immobilized enzyme were subjected to 10 successive 12 hour batch reactions in order to evaluate their activity over continuous uses. ABTS was used

as substrate for the assay, with an amount of H_2O_2 that is essential to carry out the catalytic function.

As shown in Figure 6.6, after 10 cycles, the LbL-HRP retained about 45% of its activity, while the native enzyme lost it quicker, retaining only 12% after the same amount of time. The results confirm that the LbL coating enhances enzyme stability and at the given reaction conditions, proving to be a suitable tool on the way to an industrial application of the enzyme.

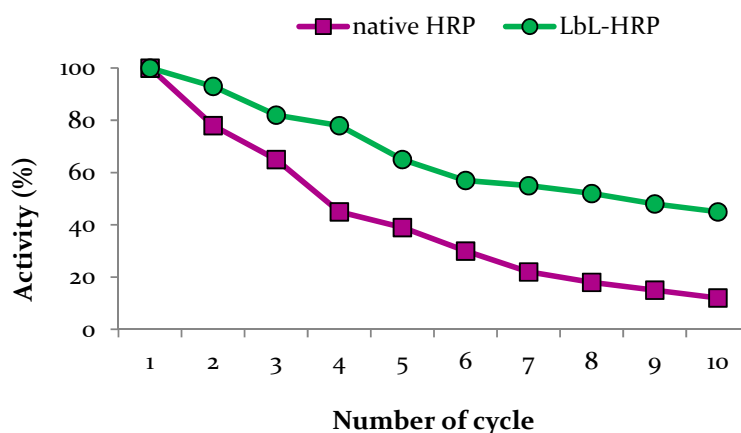


Figure 6.6: Free and LbL-HRP residual activity after 10 successive catalytic cycles. The percentage was calculated with respect to the initial activity for both the catalysts.

6.2.3 OXIDATION OF WHEAT STRAW LIGNIN (WL) BY MEANS OF NATIVE AND LbL-IMMOBILIZED HRP

Wheat straw lignin was treated both with the native and the LbL-HRP with the aim of evaluating the efficiency of the biocatalysts.

80 mg of lignin were suspended in acetate buffer pH=6 and were treated with 165 U of native HRP; a parallel reaction was carried out with an identical amount of LbL-enzyme. For the crucial experiment that was run in order to allow both a qualitative comparison – in terms of reaction pathways – and quantitative comparison of the enzymatic activity under *these* conditions with the enzymatic activity measured in *other experimental settings* that will be discussed in the following paragraphs of this thesis, the amount of laccase used in this set of oxidations was chosen according to the

activity of the enzyme as it was found in the just mentioned experiment run under alternate conditions (multi-enzyme biocatalyst experiment (see below)). In both cases, H₂O₂ was used as primary oxidant.

After the treatments, a soluble and an insoluble fraction were found and accurately separated to investigate their structure and composition.

The yield of conversion, shown in table 6.4, was calculated as the percentage of converted lignin after the treatment (mg of solubilised lignin/mg of starting material*100)

Table 6.4: Conversion of wheat straw lignin after treatment with soluble and immobilized HRP.

Entry	Biocatalyst	Conversion (%)
1	HRP	15,1
2	LbL-HRP	41,6

The results showed a higher conversion after the treatment with the LbL-enzyme with respect to the native one. It is clear that the high conversion yield is due to a more efficient oxidation that changed the chemical properties of lignin, making it more hydrophilic and soluble in water.¹⁸ It is evident also that the deposition of polyelectrolites ultrathin layers did not prevent the catalytic function of the biocatalyst but played a key role for the stability of the catalyst, improving its oxidative performance.

6.2.4 ³¹P NMR STRUCTURAL CHARACTERIZATION OF WL AFTER OXIDATIVE TREATMENTS

³¹P NMR analysis proved to be a precious analytical tool to clarify lignin structural modifications caused by the oxidative treatments. It allowed quantifying the hydroxyl groups of lignin distinguishing the different species (aliphatic, phenols and carboxylic acids). On the basis of the obtained results it was possible to make hypothesis on the oxidation pathway, investigating the peculiarities of both the native and the LbL-enzyme actions.

After the treatments, the insoluble fractions were collected, freeze dried and phosphitilated with 2-chloro-4,4',5,5'-tetramethyl-1,3,2-dioxaphospholane according to

a procedure widely described in literature.^{4,8,9} The addition of cholesterol as internal standard in the reaction cocktail allowed to run quantitative analysis, assigning the different OH signals on the basis of the comparison with the chemical shift of selected models reported in previous works.^{11,12,13} Table 6.5 shows the quantitative results referred to the native and LbL-HRP catalyzed oxidation; the amount of the different OH groups are calculated in mmol/g of lignin sample.

Table 6.5: Aliphatic, phenolic and carboxylic OH content evaluated by ³¹P NMR after phosphitylation.

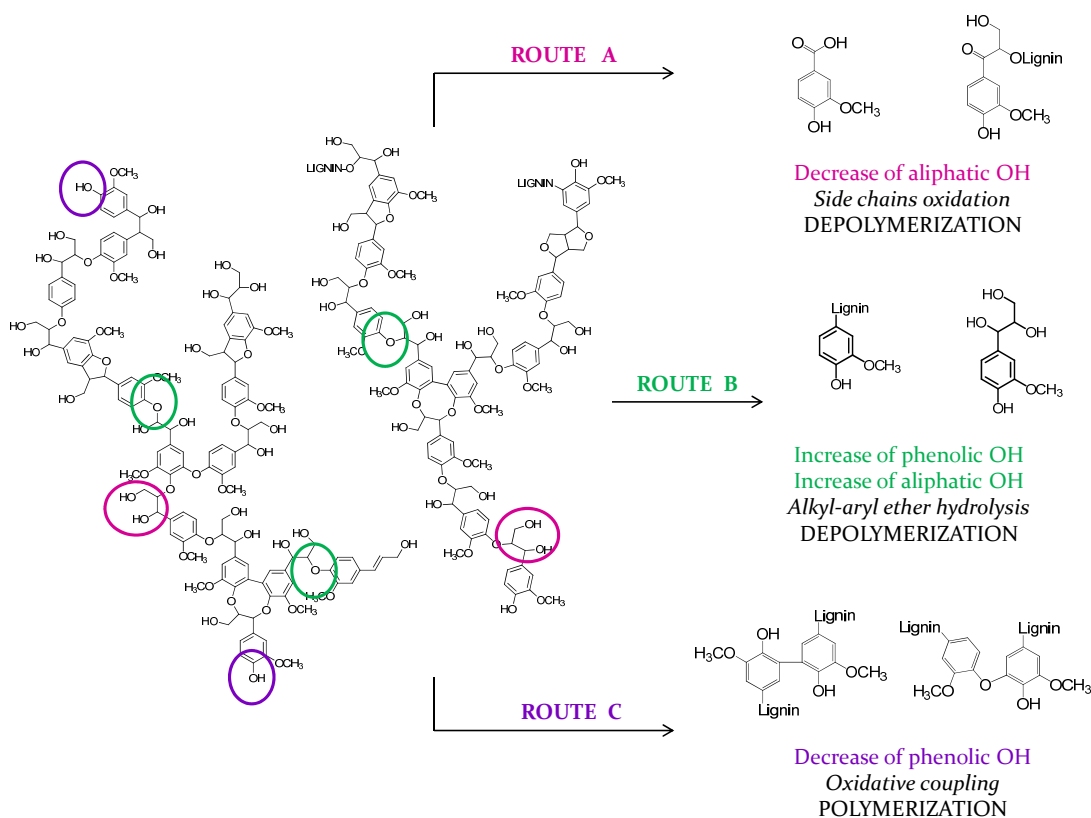
Entry	Biocatalyst	Aliphatic OH (mmol/g)	Phenolic OH (mmol/g)	Acidic OH (mmol/g)
1	WL ^a	1,46	0,80	0,27
2	HRP	1,50	0,78	0,30
3	LbL-HRP	2,84	1,20	0,18

a: wheat straw lignin (WL): starting material

The content of aliphatic, phenolic and acidic OH was found nearly unchanged after the treatment with soluble HRP. The slight decrease of the phenolic OH content could suggest the occurrence of cross-linking reactions (Scheme 6.5, route C), then confirmed by the increase of M_n and M_w values calculated after the GPC analysis (see Figure 6.7).

The lack of sensitive polymerization processes was in contrast with the results obtained in previous works, carried on with an analogously immobilized HRP toward spruce lignin.¹⁶ The different oxidative behavior can be attributed to the particular structure of wheat lignin: in fact, the high amount of syringyl units, typical of grass lignins, prevents the occurrence of oxidative coupling reactions, making the lignin less prone to polymerization processes.

Despite of this, the LbL-HRP treatment caused the increase of the aliphatic and phenolic amounts demonstrating the occurrence of alkyl and phenyl bonds cleavage (Scheme 6.5, route B). The hydrolytic process finally gave rise to a more hydrophilic polymer and a consistent soluble fraction recovered, as confirmed by the conversion yield (table 6.4, entry 2).



Scheme 6.5: Lignin oxidation pathways.

6.2.5 GPC ANALYSIS OF WL AFTER OXIDATIVE TREATMENTS

In order to obtain more information on WL structural modifications after treatment with the two different enzymatic catalysts, GPC analysis were carried out both on the insoluble and soluble fractions recovered.

Before running the analysis, the samples were acetobrominated according to a procedure described in literature and mentioned before (see above).¹⁴

The GPC profile shown in Figure 6.7 confirmed the hypothesis of the occurrence of polymerization processes after native HRP treatment. In fact, it showed a broader molecular weight distribution respect to the starting material (WL)

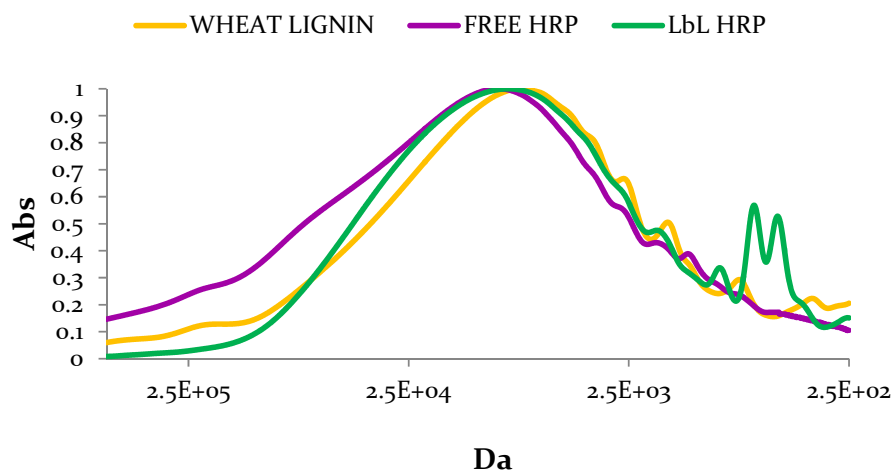


Figure 6.7: GPC analysis of the unsoluble fraction after treatments.

Moreover, the formation of a more complex polymer was underlined by higher M_n and M_w values. (Table 6.6, entry 2 vs 1)

Table 6.6: M_n , M_w and M_w/M_n of the insoluble lignin samples recovered after treatments.

Entry	Biocatalyst	M_n	M_w	M_w/M_n
1	WL ^a	2,62E + 04	1,69E + 05	6,5
2	HRP	3,58 E + 04	2,08 E + 05	6,0
3	LbL-HRP	1,81 E + 04	8,28 E + 04	4,6

a: wheat straw lignin (WL): starting material

On the other hand, the LbL-HRP treatment gave rise to a nearly unchanged molecular weight distribution (Figure 6.7) with the exception of new low molecular weight signals that show the occurrence of depolymerization processes. This was confirmed by the decrease of the M_n and the M_w value, respectively, in comparison to the untreated wheat lignin (Table 6.6, entry 3 vs. 1). Noteworthy, the immobilized enzyme oxidizes WL via a different reaction pathway than the free enzyme, where hydrolytic processes prevailed over oxidative coupling. (Scheme 6.5, route **B** vs. **C**)

The GPC analysis of the soluble fractions recovered after the treatments provided insight into the different reaction pathways triggered by the native and the LbL-enzyme (Figure 6.8).

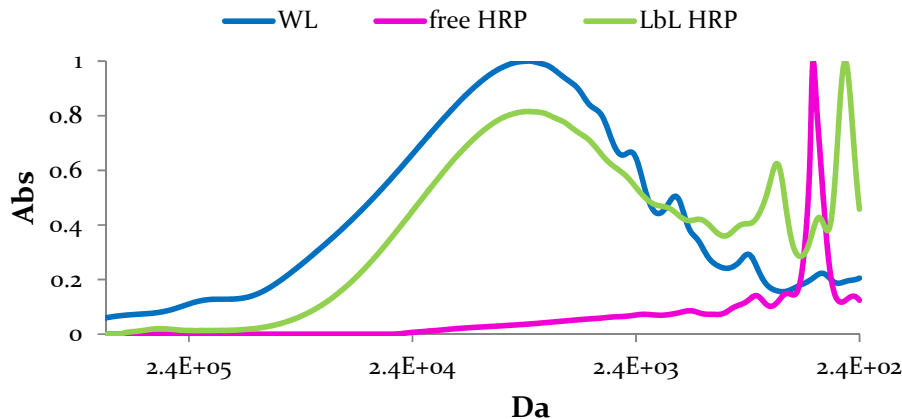


Figure 6.8: GPC analysis of water soluble fraction recovered after enzymatic treatments.

The free HRP-catalyzed oxidation was responsible for cross-linking reactions giving rise, at the same time, to oxidative processes in the external units of lignin. These oxidative processes produced a soluble fraction rich of low molecular weight compounds, as shown by the profile. On the other hand, the immobilized enzyme gave rise to hydrolytic processes yielding a predominant oligomeric soluble fraction, transposed in a broader curve in the medium molecular weight range.

6.2.6 INTERMEDIATE SUMMARY

The second set of experiments underlined that the immobilization procedure enhances the efficiency of the biocatalyst. (Table 6.4, entry 2 vs. 1) Once again, it was proven that the LbL technique does not prevent lignin from reaching the catalytic site; it rather positively influences enzymatic activity and stability. In particular, as for laccase, thanks to the immobilization strategy it was possible to tune the selectivity of the enzyme, in fact free HRP promoted an oxidation associated to polymerization processes while the LbL-enzyme carried out a prevalent hydrolytic activity. (Scheme 6.5, route **B** vs. **C**)

In a more detailed view, the GPC and the ^{31}P NMR quantitative results showed that free HRP performed its oxidative activity on the external units of lignin, giving rise to a more insoluble and complex polymer (Table 6.4, entry 1, Table 6.8, entry 2 vs. 1) and leaving into solution low molecular weight compounds (Figure 6.8). On the other hand, LbL-HRP was responsible of the hydrolysis of the internal alkyl and phenyl

bonds, converting WL in a more soluble polymer (Table 6.4, entry 2) and leaving into solution a significant oligomeric fraction (Figure 6.8).

In summary, it is possible to gather that the native and the immobilized enzymes resulted in exo- and endo-depolymerization processes, respectively, as elucidated in Figure 6.10.

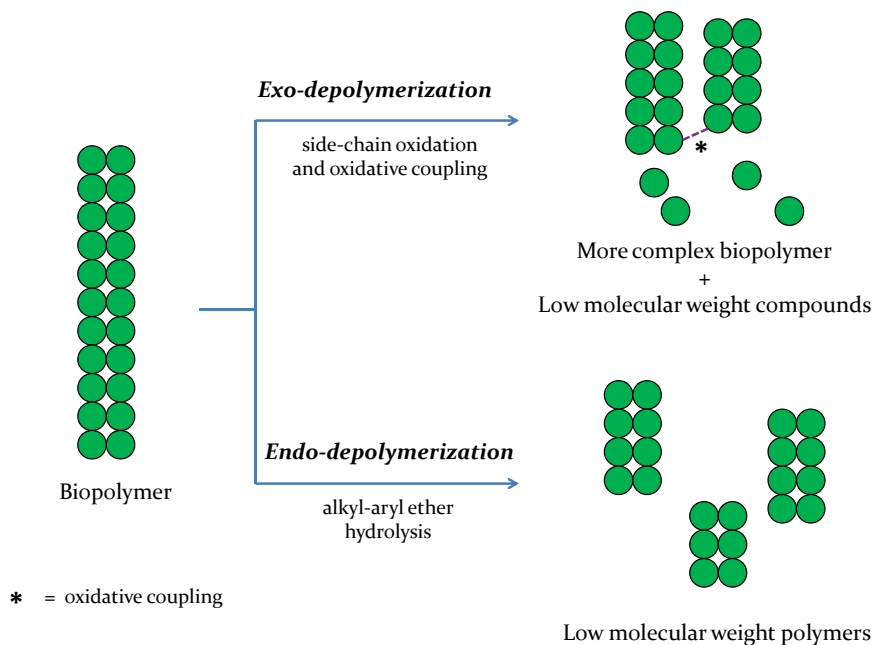


Figure 6.10: Exo- and endo-depolymerization processes.

6.3 MULTI-CATALYST

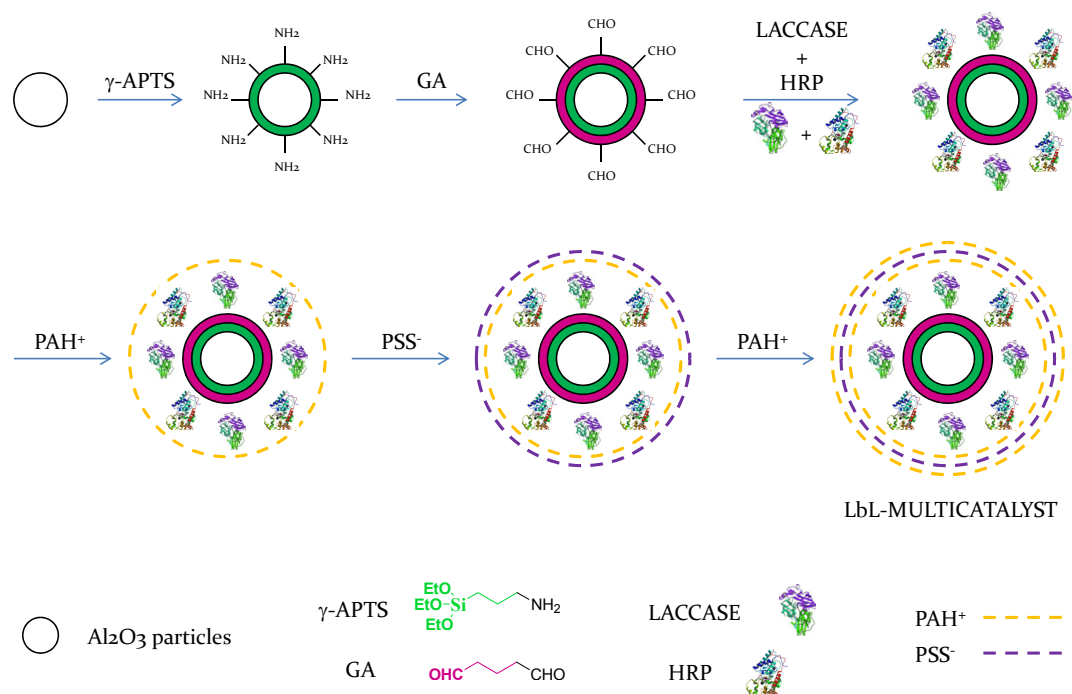
6.3.1 LACCASE+HRP CO-IMMOBILIZATION AND COATING

Laccase from *Trametes versicolor* and horseradish peroxidase (HRP) from *Armoracia rusticana* were chemically co-immobilized onto previously functionalized alumina (Al_2O_3) particles, 2-3 mm diameter. The functionalization of inert alumina particle with γ -aminopropyltriethoxysilane and subsequently with glutaraldehyde provided the opportune functionalities to allow the loading of the catalysts. (Scheme 6.6) This support proved to be convenient for our studies because of its mechanical resistance to

environmental conditions, as pH and temperature. Its properties make it appropriate also for an industrial scale-up.

This widely known procedure allowed to successfully load more than 90% of both the enzymes dissolved into solution, in agreement with previously reported results about the immobilization of the singular laccase or HRP under the same experimental conditions.^{2,16,19} For this reason, it is noteworthy that the presence of a co-immobilized enzyme, did not modify the immobilization efficiency. This data was obtained spectrophotometrically by means of the Bradford and the ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) assay, analyzing the residual enzymatic content in the waste water after the immobilization reaction.

The co-immobilized system was then coated according to the Layer by Layer (LbL) technique, based on the deposition of ultrathin layers of alternatively charged polyelectrolytes.²⁰ A triple layer composed of poly allylamine hydrochloride (PAH^+), poly sodium 4-styrene sulfonate (PSS^-) and again PAH^+ was adsorbed on the laccase/HRP co-immobilized system. The first adsorbed layer was positively charged because the alumina-catalysts system retained a negative charge at the current pH condition (pH=5) (Scheme 6.6).³



Scheme 6.6: Support activation, laccase+HRP Co-immobilization and Layer by Layer coating of supported multi-catalyst.

6.3.2 MULTI-ENZYME STABILITY: THE MULTI-CATALYST RECYCLE

The LbL-multienzyme biocatalyst was subjected to several successive batch reactions in order to investigate its activity after multiple reuses. The residual activity of both the immobilized enzymes was calculated spectrophotometrically.

The LbL-multicatalyst was allowed to react with ABTS for 10 successive batch reaction, one every 12 hours, in order to evaluate laccase activity trend. HRP did not act on the substrate, since H_2O_2 was not added in the reaction cocktail.

An identical amount of LbL-multicatalyst was allowed to react according to the same procedure in presence of H_2O_2 , in order to evaluate HRP activity. Since, in this case, the detected activity was given by both laccase and HRP performances, the HRP activity was calculated subtracting the partial laccase activity obtained from the previous experiments to the total activity detected. It was assumed that the newly

formed products, that resulted from the HRP activity, did not serve as additional substrate for the laccase.

The co-immobilized enzymes activity trends were compared to the performances of the native forms, subjected to the same 10 successive 12 hour batch reactions (Figure 6.11).

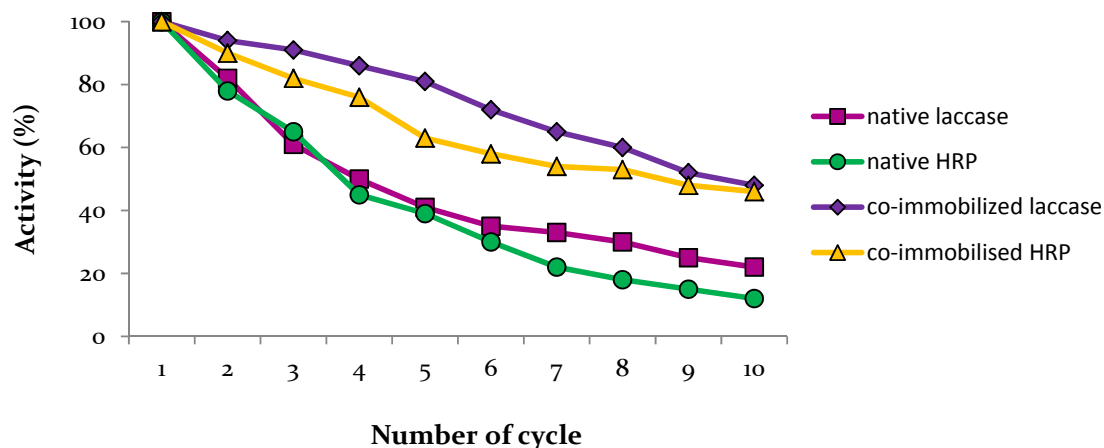


Figure 6.11: Enzymatic residual activity after 10 successive 12 h catalytic cycles.

As expected, the LbL-co-immobilized enzymes retained their activity better than the native forms, in fact LbL-co-immobilized laccase showed about 50% of its activity after 10 batch reaction while the native form retained only 22%. As well, LbL-co-immobilized HRP is still active after 10 successive batches (about 50% of the starting activity) while the native form appeared nearly inactivated (about 10%). The percentage of the retained activity was calculated with respect to the initial activity, both for the co-immobilized enzymes and for the native forms. The results confirmed that LbL technique had a key role in enzymatic activity preservation: the triple ultrathin layer of polyelectrolites protected the enzyme from denaturant agents without preventing lignin to reach its catalytic site and assuring the possibility of a multiple reuse.

6.3.3 WHEAT STRAW LIGNIN (WL) OXIDATION BY MEANS OF MULTI-CATALYST

Wheat straw lignin was tested in a similar set of consecutive experiments to cross verify the occurrence of a synergic action between the co-immobilized enzymes. More specifically, the efficiency of the LbL-co-immobilized system was compared with the performance of the native or immobilized enzymes used in mixture. Moreover, parallel reactions were carried out in presence of 1-hydroxybenzotriazole (HBT) 1mM as chemical mediator. (Scheme 6.1)

In a more detailed view, a mixture of native laccase and HRP, a mixture of LbL-laccase and LbL-HRP and the multi-catalyst system, in presence or in absence of HBT each, were employed to oxidize wheat straw lignin, taking care to use the same amount of native or LbL- enzymes in order to compare the respective efficiency and specificity.

80 g of wheat straw lignin were suspended in acetate buffer pH=6 and treated with the following oxidative systems (Figure 6.12):

- Laccase+HRP: 23 U of native laccase with 165 U of native HRP (Figure 6.12 A).
- Laccase+HRP/HBT: 23 U of native laccase with 165 U of native HRP in presence of 1-hydroxybenzotriazole (HBT) 1mM (Figure 6.12 B).
- LbL-laccase+LbL-HRP: 23 U of immobilized laccase with 165 U of immobilized HRP (Figure 6.12 C).
- LbL-laccase+LbL-HRP/HBT: 23 U of immobilized laccase with 165 U of immobilized HRP in presence of 1-hydroxybenzotriazole (HBT) 1mM (Figure 6.12 D).
- LbL-multi-enzyme system: 23 U of laccase and 165 U of HRP immobilized on the same support (Figure 6.12 E).
- LbL-multi-enzyme system/HBT: 23 U of laccase and 165 U of HRP immobilized on the same support, in presence of 1-hydroxybenzotriazole (HBT) 1mM (Figure 6.12).

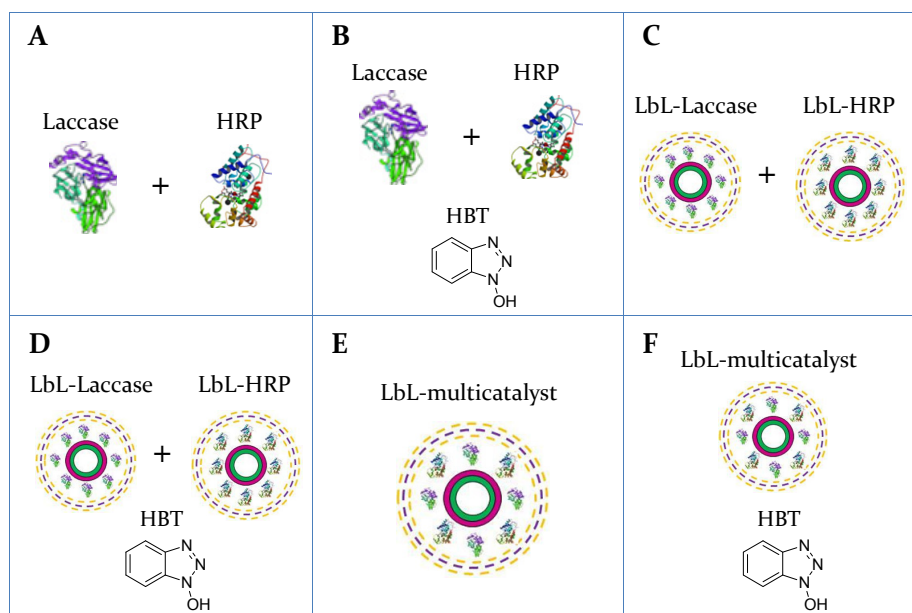


Figure 6.12: Oxidative systems.

In any case, H_2O_2 0.5mm was added to the reaction cocktail.

After the treatments, the soluble and the unsoluble fractions were accurately separated to investigate their structure and composition.

Table 6.7 shows the conversion values, calculated as the percentage of converted lignin after the treatment (mg of solubilised lignin/mg of starting material*100)

Table 6.7: Conversion of wheat straw lignin after treatment with singularly- and co-immobilized enzymes, compared with the native forms, in presence or absence of HBT as oxidation mediator.

Entry	Biocatalyst	Conversion (%)
1	Laccase+HRP ^a	23,1
2	Laccase+HRP/HBT ^a	36,4
3	LbL-laccase+LbL-HRP ^b	27,8
4	LbL-laccase+LbL-HRP/HBT ^b	35,3
5	LbL-multi-enzyme system ^c	59,8
6	LbL-multi-enzyme system/HBT ^c	61,3

^a: native enzymes used in mixture

^b: singularly immobilized enzymes used in mixture

^c: laccase+HRP co-immobilized on the same support

The conversion yield represents the measuring rod of the oxidation efficiency: a high conversion yield is caused by an efficient oxidative process, that changes the chemical properties of lignin making it more hydrophilic and soluble in water.⁵

As expected, the mixture of LbL-enzymes proven to be a more efficient oxidant with respect to the mixture of the native forms (Table 6.7, entry 3 vs. 1). The enhanced activity is due to the protective action carried out by the coating ultrathin layer of polyelectrolytes that protects the enzyme from the environmental conditions and from denaturing agents, without preventing lignin to reach the catalytic site.

It is noteworthy that the LbL-multi-enzyme system showed an additionally increased conversion with respect to the mixture of LbL-enzymes and with respect to the mixture of the native forms (Table 6.7, entry 5 vs. 3, 1). The extensive oxidative performance can be attributed to the occurrence of a synergic action between the enzymes that gave rise to domino or cascade reactions with the subsequent substantial depolymerization and solubilization of the biopolymer.

The treatments carried out in presence of the chemical mediator HBT yielded a more oxidized and soluble polymer with respect to the relative treatments in absence. (Table 6.7, entry 2 vs. 1, entry 4 vs. 3, entry 6 vs. 5) The data are in compliance with the results obtained before for the LbL-laccase and LbL-HRP treatments under the same experimental conditions (paragraph 6.1, 6.2).

6.3.4 ³¹P NMR STRUCTURAL CHARACTERIZATION OF WL AFTER OXIDATIVE TREATMENTS

With respect to ¹H NMR, that provides a pretty unclear and nonessential information about the complex lignin structure, the ³¹P NMR analysis offers the possibility to quantify and distinguish the different species of hydroxyl groups, mainly involved in the oxidation processes, after the functionalization with the opportune phosphorous label.

Samples of soluble and insoluble fractions recovered after the treatments were phosphitilated with 2-chloro-4,4',5,5'-tetramethyl-1,3,2-dioxaphospholane according to the procedure continuously used throughout the thesis.^{8,9,10}

Table 6.8 summarizes the quantitative results obtained after ^{31}P NMR analysis of the insoluble fractions recovered after the six different treatments. The amount of the different OH groups were calculated in mmol/g of lignin sample.

Table 6.8: Aliphatic, phenolic and carboxylic OH content evaluated by ^{31}P NMR after phosphorylation.

Entry	Biocatalyst	Aliphatic OH (mmol/g)	Phenolic OH (mmol/g)	Acidic OH (mmol/g)
1	WL ^a	1,46	0,80	0,27
2	Laccase+HRP ^b	1,48	0,51	0,31
3	Laccase+HRP/HBT ^b	1,48	0,53	0,29
4	LbL-laccase+LbL-HRP ^c	1,99	0,78	0,12
5	LbL-laccase+LbL-HRP/HBT ^c	2,55	1,10	0,15
6	Multi-enzyme biocatalyst ^d	1,48	0,54	0,28
7	Multi-enzyme biocatalyst/HBT ^d	1,15	0,50	0,50

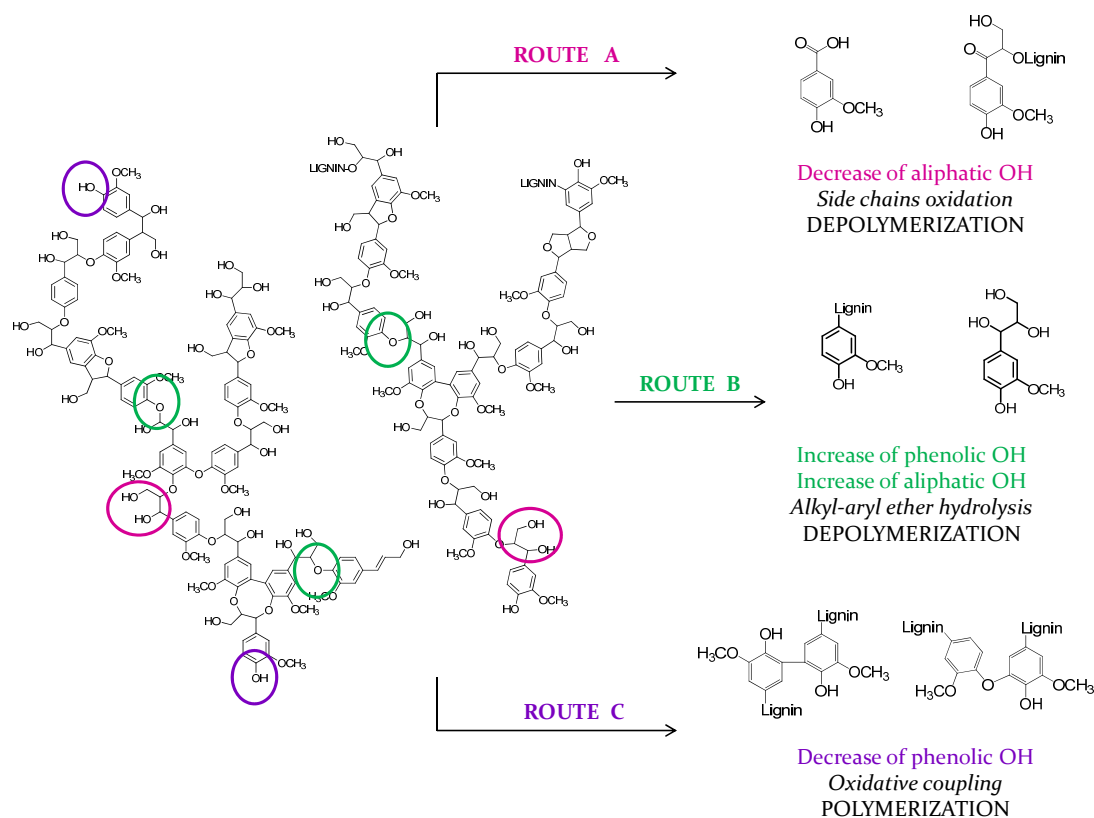
a: wheat straw lignin - starting material

b: native enzymes used in mixture

c: singularly immobilized enzymes used in mixture

d: laccase+HRP co-immobilized on the same support

The treatments with the native enzymes in mixture showed a nearly unchanged amount of aliphatic and acidic groups with respect to the starting material, while a decrease of the phenolic amount was observed (Table 6.8, entry 2,3 vs. 1). The results was consistent with an oxidation pathway characterized by oxidative coupling processes on phenolic end groups in lignin, as shown in Scheme 6.7 (route C).



Scheme 6.7: Lignin oxidation pathways.

These data were in agreement with the results obtained in the previous experiments with native laccase and native HRP separately (Table 6.2, entry 2 vs. 1; Table 6.5, entry 2 vs. 1): as expected, the mixture of the native enzymes gave rise to a synergic action in the polymerization process since the phenolic amount resulting from the treatment in mixture was lower than that obtained from the treatment with native laccase and native HRP separately.

The presence of HBT 1mM in the reaction cocktail did not show significant modifications with respect to previous experiment (Table 6.8, entry 3 vs. 2).

A different selectivity was observed after the oxidation with the LbL-enzymes used in mixture. The treatment showed an increase of the aliphatic amount (Table 6.8, entry 4 vs. 1) that suggested, at a glance, the occurrence of alkyl-phenyl ether bond cleavage, as shown in Scheme 6.8, route **B**. Comparing this result with those obtained with LbL-laccase and LbL-HRP separately, it seemed that no synergic action took place (Table 6.2 entry 4 and Table 6.5 entry 3 vs. Table 6.8 entry 4). On the contrary, and as

expected, the data resulting from the treatment with the LbL-enzymes used in mixture in presence of IMM HBT gave rise to an extensive depolymerization: the increase of the aliphatic and the phenolic amount were caused by the occurrence of hydrolytic processes on the alkyl-phenyl ether positions (Table 6.8, entry 5 vs. 1), as shown in Scheme 6.7, route B.

The treatment with the co-immobilized multi-enzymatic system, in presence or absence of HBT, yielded a particular result: the ^{31}P NMR analysis of the insoluble fractions shows a decrease of the phenolic amount that suggests the occurrence of polymerization processes (Table 6.8, entry 6, 7 vs. 1). This result was out of line with the conversion yield that proved the occurrence of an extensive oxidation with the subsequent depolymerization and solubilization of the biopolymer. The issue is resumed and discussed in the next paragraph, comparing the ^{31}P quantitative data with the GPC results.

In presence of the chemical mediator the decrease of the phenolic amount was associated with the decrease of the aliphatic OH and the increase of the acidic ones: these results suggested the occurrence of a different reaction pathway characterized by side chains oxidations (Scheme 6.8, route A). The GPC results reported in the next paragraph will highlight the association of side-chains oxidations with depolymerization processes.

6.3.5 GPC ANALYSIS OF WL AFTER OXIDATIVE TREATMENTS

The molecular weight distribution of both the soluble and insoluble fractions recovered after the treatments were investigated by means of GPC analysis. Before the analysis the samples were functionalized with acetyl bromide in acetic acid in order to decrease lignin hydrophilic properties. The acetobromination was carried out following a procedure described in literature and used throughout the entire thesis.¹⁴ On the basis of the M_n and M_w values calculated, it was possible to have additional information about lignin structural modifications.

As shown in Table 6.9, the M_n and M_w values referred to the free enzymes-catalyzed oxidations confirmed the hypothesis formulated based on the findings in the magnetic resonance investigations of the products: the, with respect to the starting material,

increased values of M_n and M_w suggest the occurrence of polymerization processes. (Table 6.9, entry 2,3 vs. 1)

Table 6.9: M_n , M_w and M_w/M_n of the insoluble lignin samples recovered after treatments.

Entry	Biocatalyst	M_n	M_w	M_w/M_n
1	WL ^a	2,62E + 04	1,69E + 05	6,5
2	Laccase+HRP ^b	3,84E + 04	2,18E + 05	6,0
3	Laccase+HRP/HBT ^b	3,33E + 04	1,69E + 05	5,9
4	LbL-laccase+LbL-HRP ^c	2,64E + 04	1,72E + 05	6,5
5	LbL-laccase+LbL-HRP/HBT ^c	2,08E + 04	9,84E + 04	4,7
6	LbL-multi-enzyme system ^d	1,37E + 04	1,14E + 05	6,5
7	LbL-multi-enzyme system/HBT ^d	8,55E + 03	3,58E + 04	4,1

^a: wheat straw lignin - starting material

^b: native enzymes used in mixture

^c: singularly immobilized enzymes used in mixture

^d: laccase+HRP co-immobilized on the same support

When LbL-laccase and LbL-HRP were used in mixture, no significant modification of M_n and M_w values was found: this data, associated to the ³¹P NMR quantitative results, appeared pretty unclear since both the LbL-enzymes, separately used, performed a depolymerizing action on wheat lignin (Table 6.3 entry 4 and Table 6.6 entry 3 vs. Table 6.9 entry 4).

In the presence of HBT, a slight decrease of the values was observed, consistent with the occurrence of depolymerization processes, as deduced from the ³¹P quantitative results.

The molecular weight distribution of the insoluble fraction recovered after the treatment with the LbL-multienzyme-system showed a substantial depolymerization (Table 6.9, entry 6 vs. 1): this data clarified the dissenting quantitative results obtained by ³¹P NMR analyses, demonstrating the high yield of conversion (about 60%) reported in Table 6.7 (entry 5). In presence of HBT the depolymerizing action carried out by the LbL-multienzyme system appeared more enhanced, as the conversion yield (more than 60%; Table 6.7, entry 6) and the M_n and M_w values (Table 6.9, entry 7 vs 6 vs 1) shown. The occurrence of side chains oxidations, highlighted by the results obtained by ³¹P

NMR analyses, proved to be associated with depolymerization precesses (Scheme 6.8, route A, B)

The oxidation pathways of the 3 different oxidative systems (free laccase+free HRP; LbL-laccase+LbL-HRP; LbL-multienzyme biocatalyst) were elucidated in detail thanks to the analysis of the soluble fractions (Figure 6.13).

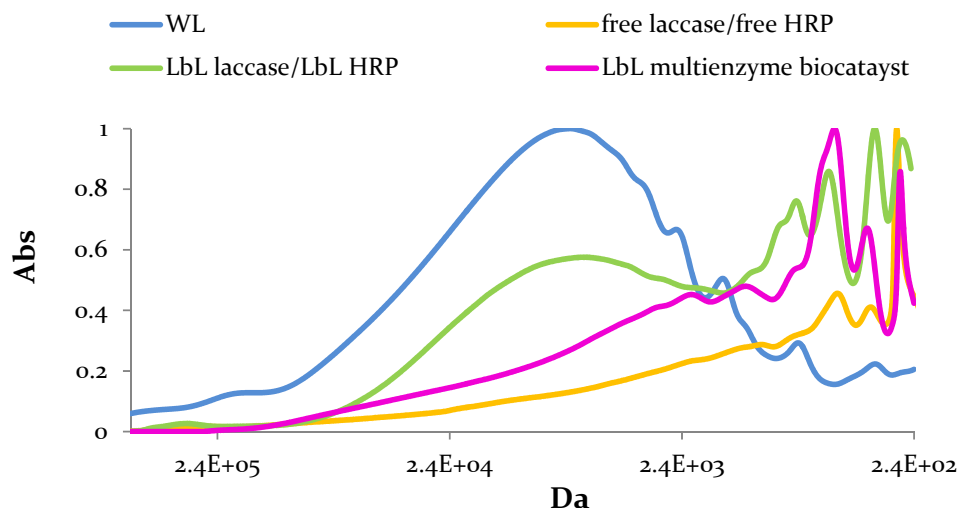


Figure 6.13: GPC analysis of water soluble fraction recovered after enzymatic treatments.

As expected, the mixture of free enzymes, responsible of polymerization processes, performed oxidation reactions on the external units of the biopolymer, leaving into solution only low molecular weight compounds (Figure 6.13, yellow vs. blue). On the other hand, the mixture of LbL-laccase and LbL-HRP yielded a soluble fraction containing oligomers and a considerable polymeric fraction (Figure 6.13, green vs. blue): this is consistent with the occurrence of alkyl-phenyl ether bonds cleavage that broke lignin backbone in the internal position.

Finally, the LbL-multienzyme system carried out a substantial depolymerizing action giving rise to a predominantly oligomeric soluble fraction (Figure 6.13, pink vs. blue): in this particular case, the hydrolytic cleavage of the internal ether bonds was associated with an oxidative activity in the external units of lignin backbone.

6.3.6 INTERMEDIATE SUMMARY

The results obtained in this set of experiments showed the high efficiency and a new different specificity of the novel LbL-multienzyme biocatalyst. The high efficiency in the oxidation process was provided by the layer-by-layer coating technique that protected the biocatalysts from the environmental conditions and from denaturing agents, conferring an enhanced and extended stability. Proof that the LbL technique increased the oxidation efficiency was given by the much higher conversion yield of the LbL-multienzyme with respect to the mixture of the native forms (Table 6.7, entries 5,6 vs. 1,2). The high performance of the novel enzymatic system was associated with a new oxidation pathway due to the occurrence of cascade reaction pattern carried out by the co-immobilized enzymes. The cascade reactions submitted wheat straw lignin to the concomitant hydrolysis of the internal alkyl-phenyl ether bonds and side chains oxidations, responsible of an endo- and exo-depolymerizing processes respectively, that originated a predominantly oligomeric soluble fraction.

The same synergic action did not occur when the LbL-laccase and LbL-HRP were used in mixture: in this case the endo-depolymerizing processes prevailed, leaving into solution both oligomers and a significant polymeric fraction.

6.4 CONCLUSIONS

The efforts of the present study were directed to the development of a novel multi-enzyme biocatalyst to combine the potentials offered by two different enzymes in lignin degrading processes. The synergic action performed by two different enzymes gives rise to domino or cascade reactions that involve several non-separable steps characterized by the presence of highly reactive intermediates. Despite the formation of unstable species, domino reactions show a remarkable synthetic advantages since the intermediates undergo subsequent conversions as soon as they are formed, thus minimizing the chance for undesired side reactions to occur..²¹

The chemical immobilization of enzymes onto the surface of functionalized alumina particles with the subsequent layer-by-layer coating technique to protect the immobilized enzymes proved to be a suitable and valuable approach for the

immobilization and protection of more than one enzyme on the same support, allowing the designing of novel LbL-multienzyme oxidative biocatalysts.

In order to investigate the efficiency and the specificity of the newly developed multi-enzyme catalyst, the pathways of its oxidative action, as well as the chemoenzymatic cascade processes that are triggered by it, a set of twelve experiments was designed: the performance of the new co-immobilized “laccase-HRP biocatalyst” was studied focusing the attention on the activity of the native enzymes first; subsequent investigation dealt with the effects that the layer-by-layer technique conferred to the chemically immobilized enzymatic catalyst; finally, a series of experiments was performed in order to highlight the peculiar reactivity stemming from the co-immobilization of laccase and HRP together on the same support. Results were generally compared to the performance of the separately immobilized enzymes in the same reactions.

The oxidative processes carried out by the twelve different enzymatic systems were studied on wheat straw lignin. The nature of the structural modification resulting from the treatments was investigated by means of GPC and ^3P NMR analyses, that allowed to elucidate the distribution of the molecular weight and the chemical peculiarity of the treated biopolymers and its degradation products, respectively.

The soluble laccase and HRP, even when used together in a mixture, gave rise to a partial depolymerization centered on the external units of lignin with a concomitant increase of the M_n and M_w values (Table 6.12, entries 2, 3, 6, 8, 9), due to the occurrence of oxidative coupling processes, as highlighted by the general decrease of the phenolic amount (Table 6.11, entries 2, 3, 6, 8, 9). The soluble fraction recovered after the treatments ranged from 14,6 to 36,4 % (Table 6.10, entries 1, 2, 5, 7, 8), and was mainly composed of low molecular weight compounds. The presence of HBT as chemical mediator enhanced the efficiency of the catalysts. The activity of the soluble enzymes can be defined as a exo-depolymerization process (Figure 6.14).

On the other hand, when wheat straw lignin was treated with the immobilized and protected enzymes, as separate, single catalysts, as mixture of separately supported catalysts, or as mixed supported catalyst, a higher conversion, ranging from 34,7 to 58,2%, is detected (Table 6.10, entries 3, 4, 6, 9, 10). It could be proven that this is due to the protective action provided by the multi-layer coating that improved enzyme

stability and enhancing enzymatic performance, while not preventing the substrate to reach the catalytic site. The decrease of the values found for M_n and M_w (Table 6.12, entries 4, 5, 7, 10, 11) with the concomitant increase of the phenolic and aliphatic amount demonstrated the prevalence of hydrolytic processes that caused the cleavage of alkyl-phenyl ether bonds with the subsequent depolymerization.

The presence of oligomers and low molecular weight polymers in the soluble fraction confirmed the occurrence of hydrolytic process also targeting internal units of lignin oligomers. The action performed by the immobilized enzymes can be defined as endo-depolymerization process (Figure 6.14).

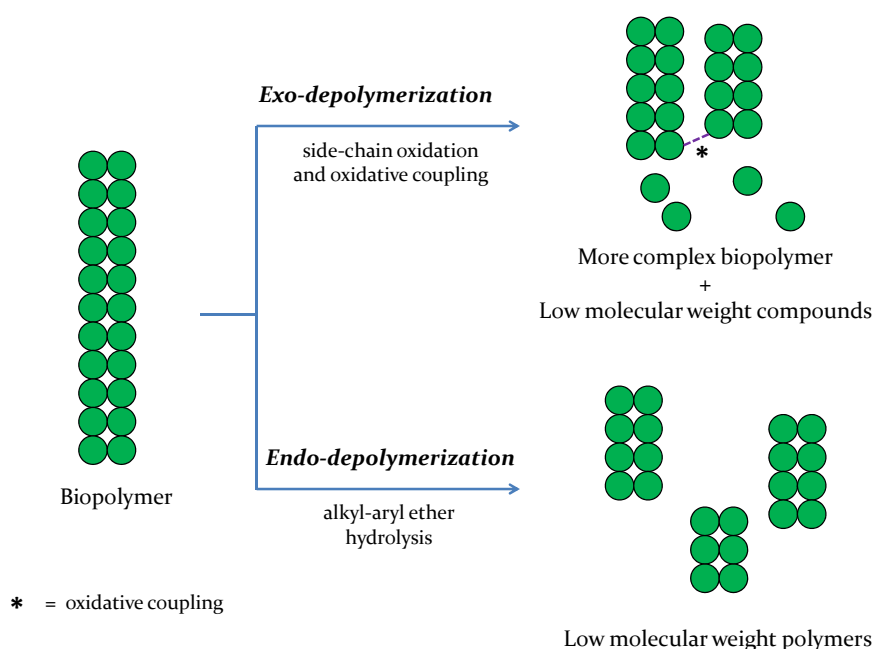


Figure 6.14: Endo- and Exo-depolymerization processes

The treatment with the new LbL-multicatalyst yielded the highest conversion with respect to both the immobilized enzymes, and the native forms, used either singularly or in mixtures, in the presence or the absence of HBT as chemical mediator (Table 6.10, entries 11, 12).

The decrease of M_n and M_w values of the recovered insoluble fraction (Table 6.12, entries 12, 13 vs 1) implied a substantial depolymerization of the structure; however, also the soluble fraction was found to be typically oligomeric. These data demonstrate

that the novel multicatalyst performs both an endo- and an exo-depolymerization, acting both as a hydrolytic and as an oxidative agent (Figure 6.15).

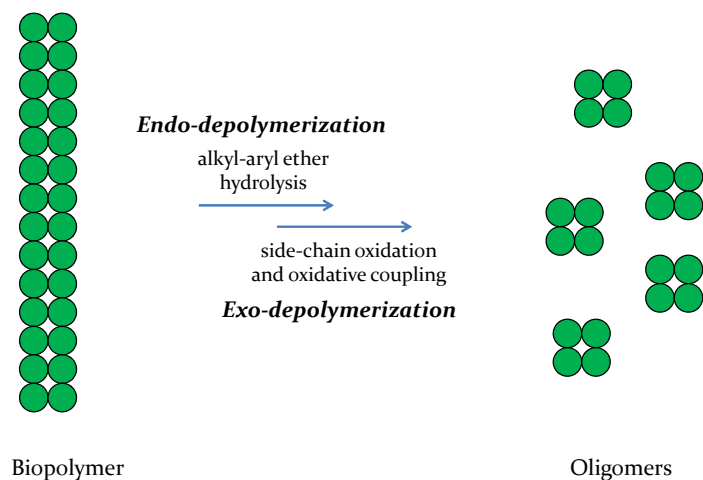


Figure 6.15: Endo-depolymerization associated with exo-depolymerization.

The different behavior of the co-immobilized multicatalyst with respect to the mixture of LbL-laccase and LbL-HRP is most probably due to the occurrence of a cascade reaction pattern arising from the co-immobilization of laccase and HRP on the same support.

Table 6.10: Conversion of wheat straw lignin after treatment with soluble, immobilized and co-immobilized enzymes, in presence or absence of HBT as oxidation mediator.

Entry	Biocatalyst	Conversion (%)
1	Laccase ^a	17,1
2	Laccase+HBT ^a	14,6
3	LbL-laccase ^b	34,7
4	LbL-laccase+HBT ^b	58,2
5	HRP ^a	15,1
6	LbL-HRP ^b	41,6
7	Laccase+HRP ^a	23,1
8	Laccase+HRP/HBT ^a	36,4
9	LbL-laccase+LbL-HRP ^b	27,8
10	LbL-laccase+LbL-HRP/HBT ^b	35,3
11	LbL-multi-enzyme system ^c	59,8
12	LbL-multi-enzyme system/HBT ^c	61,3

^a: native enzymes

^b: singularly immobilized enzymes

^c: laccase+HRP co-immobilized on the same support

Table 6.11: Aliphatic, phenolic and carboxylic OH content evaluated by ^{31}P NMR after phosphitylation.

Entry	Biocatalyst	Aliphatic OH (mmol/g)	Phenolic OH (mmol/g)	Acidic OH (mmol/g)
1	WL ^a	1,46	0,80	0,27
2	Laccase ^b	1,46	0,71	0,25
3	Laccase+HBT ^b	1,49	0,46	0,30
4	LbL-laccase ^c	2,49	0,95	0,12
5	LbL-laccase+HBT ^c	2,62	1,09	0,17
6	HRP ^b	1,50	0,78	0,30
7	LbL-HRP ^c	2,84	1,20	0,18
8	Laccase+HRP ^b	1,48	0,51	0,31
9	Laccase+HRP/HBT ^b	1,48	0,53	0,29
10	LbL-laccase+LbL-HRP ^c	1,99	0,78	0,12
11	LbL-laccase+LbL-HRP/HBT ^c	2,55	1,10	0,15
12	Multi-enzyme biocatalyst ^d	1,48	0,54	0,28
13	Multi-enzyme biocatalyst/HBT ^d	1,15	0,50	0,50

^a: wheat straw lignin - starting material

^b: native enzymes

^c: singularly immobilized enzymes

^d: laccase+HRP co-immobilized on the same support

Table 6.12: M_n , M_w and M_w/M_n of the insoluble lignin samples recovered after treatments.

Entry	Biocatalyst	M_n	M_w	M_w/M_n
1	WL ^a	2,62E + 04	1,69E + 05	6,5
2	Laccase ^b	3,51 E + 04	1,87 E + 05	5,3
3	Laccase+HBT ^b	2,77 E + 04	1,84 E + 05	6,6
4	LbL-laccase ^c	1,96 E + 04	1,05 E + 05	5,4
5	LbL-laccase+HBT ^c	1,29 E + 04	4,21 E + 04	3,3
6	HRP ^b	3,58 E + 04	2,08 E + 05	6,0
7	LbL-HRP ^c	1,81 E + 04	8,28 E + 04	4,6
8	Laccase+HRP ^b	3,84E + 04	2,18E + 05	6,0
9	Laccase+HRP/HBT ^b	3,33E + 04	1,69E + 05	5,9
10	LbL-laccase+LbL-HRP ^c	2,64E + 04	1,72E + 05	6,5
11	LbL-laccase+LbL-HRP/HBT ^c	2,08E + 04	9,84E + 04	4,7
12	LbL-multi-enzyme system ^d	1,37E + 04	1,14E + 05	6,5
13	LbL-multi-enzyme system/HBT ^d	8,55E + 03	3,58E + 04	4,1

^a: wheat straw lignin - starting material

^b: native enzymes

^c: singularly immobilized enzymes

^d: laccase+HRP co-immobilized on the same support

6.5 EXPERIMENTAL SECTION

6.5.1 WHEAT STRAW LIGNIN ISOLATION

Wheat straw lignin (WL) was prepared from ultraground extractive-free powder according to Björkman's procedure with some modifications.**Error! Bookmark not defined.** Wheat straw was milled to 40 mesh and then exhaustively extracted with a mixture of acetone:water (9:1, v/v) in a soxhlet apparatus, in order to remove the low molecular weight phenolic compounds. Then the extractive-free milled wood was ultragrounded for 3 weeks in a rotatory ball mill. The extractive-free powder was then extracted with dioxane:water (96:4, v/v), concentrated under reduced pressure and freeze-dried. The dried powder was dissolved in 90% acetic acid and the solution was then added dropwise to stirred water in order to precipitate lignin. The precipitate was recovered by centrifuging, freeze-dried and dissolved again in a mixture of 1,2-dichloroethane:ethanol (2:1, v/v). The addition of diethyl ether precipitated lignin. After centrifugation and freeze-drying lignin was recovered with a yield of 38%.

6.5.2 ENZYMES' IMMOBILIZATION

SUPPORT ACTIVATION

Alumina pellets were activated before the enzyme immobilization. They were subjected to silanisation with 2% (v/v) γ -aminopropyltriethoxysilane in acetone at 45°C for 20 hours. The silanised supports were washed with acetone and silanised again with the same procedure for 24 hours. They were then recovered and washed several times with deionised water and dried through air. In a second step, the alumina pellets were treated with 2% (v/v) aqueous glutaraldehyde (50%, v/v) during two hours at room temperature, washed again with deionised water and dried through air.^{1,22} The activated particles were subsequently subjected to the biocatalyst loading.

LACCASE IMMOBILIZATION

150 g of activated support were put in contact with the enzyme, (5,000 U/L), during 48 hours at 25°C, in 250 mL of 100mM citrate buffer pH=5 with 100 mM NaCl. The particles were then filtered off on a Buchner funnel and washed several times with 0.05 M phosphate buffer (pH 7) until no enzymatic activity was found in the filtrate of the washing process.

HRP IMMOBILIZATION

150 g of activated support were put in contact with the enzyme, (5,000 U/L), during 48 hours at 25°C, in 250 mL of 100mM citrate buffer pH=7 with 100 mM NaCl. The particles were then filtered off on a Büchner and washed several times with 0.05 M phosphate buffer (pH 7) until no enzymatic activity was found in the washing solution.

LACCASE AND HRP CO-IMMOBILIZATION

150 g of activated support were put in contact with both the enzymes, laccase and HRP (2500 +2500 U/L), during 48 hours at 25°C, in 250 mL of 100mM citrate buffer pH=6 with 100 mM NaCl. The particles were then filtered off on a Büchner and washed several times with 0,05 M phosphate buffer (pH 7) until no enzymatic activity was found in the washing solution.

LbL COATING OF IMMOBILIZED ENZYMES

The loaded particles were washed three times with 0.1 M NaCl and then subjected to a sequential deposition of polyelectrolyte (polyallyl amine hydrochloride PAH⁺, polystyrene sulfonate PSS⁻) layers. Polyelectrolyte solutions (0.01 M) with 0.5 M NaCl were prepared and the supports were immersed inside each solution for 20 minutes. Since the enzyme immobilised onto alumina particles were negatively charged at the condition of the immobilization step, the coatings procedure started with the positively charged layer of polyallyl amine hydrochloride ((PAH⁺ + PSS⁻ + PAH⁺). After each layer, the excess of polyelectrolyte was removed by washing with 0.1 M NaCl. The red particles of chemically-immobilised LbL laccase were obtained by simple filtration with Büchner from the reaction mixture.

6.5.3 ENZYMATIC ASSAYS

LACCASE ACTIVITY ASSAY

Free and LbL-laccase activity was determined spectrophotometrically using ABTS as the substrate. The laccase assay mixture contained 300 µl of 0,5 mM ABTS in 0,1 M acetate buffer (pH 5) and an amount of soluble or LbL-enzyme, brought to 3 ml volume with 0,1 M acetate buffer (pH 5). The substrate oxidation was followed at 415 nm for one and three minutes for the free and the immobilized enzyme respectively.²³ One activity unit was defined as the amount of enzyme that oxidised 1 mmol

ABTS/min. The immobilisation yield was calculated as the difference between the activity present in the starting immobilisation solution and that remaining in the supernatant at the end of the adsorption procedure.

To determine the concentration of free enzymes in a solution Bradford assay was applied.¹⁵ 400 µl of Bradford reagent were added to 2,600 ml of deionised water containing 50, 100, 150, 200 µl of the test solution. The absorbance at 595 nm was then measured against a blank made of 2,600 ml of deionised water and 400 µl of Bradford reagent.

HRP ACTIVITY ASSAY

Free and LbL-HRP activity was determined spectrophotometrically using ABTS as the substrate. The HRP assay mixture contained 300 µl of 0,5 mM ABTS and 100 µl of H₂O₂ 0,01 M in 0,1 M phosphate buffer (pH 7), and an amount of soluble or LbL-enzyme, brought to 3 ml volume with 0,1 M phosphate buffer (pH 7). The substrate oxidation was followed at 405 nm for two and six minutes for the free and the immobilized enzyme respectively.²⁴ One activity unit was defined as the amount of enzyme that oxidised 1 mmol ABTS/min. The immobilisation yield was calculated as the difference between the activity present in the starting immobilisation solution and that remaining in the supernatant at the end of the adsorption procedure.

To determine the concentration of free enzymes in a solution Bradford assay was applied.¹⁵ 400 µl of Bradford reagent were added to 2,600 ml of deionised water containing 50, 100, 150, 200 µl of the test solution. The absorbance at 595 nm was then measured against a blank made of 2,600 ml of deionised water and 400 µl of Bradford reagent.

MULTI-CATALYST ACTIVITY ASSAY

Free and LbL-enzymes activity was determined spectrophotometrically using ABTS as the substrate. The laccase assay mixture contained 300 µl of 0,5 mM ABTS in 0,1 M acetate buffer pH 5 and an amount of soluble or LbL-enzyme, brought to 3 ml volume with 0,1 M acetate buffer (pH 5). The substrate oxidation was followed at 415 nm for one and three minutes for the free and the immobilized enzyme respectively.²³ The HRP assay mixture contained 300 µl of 0,5 mM ABTS and 100 µl of H₂O₂ 0,01 M in 0,1 M phosphate buffer (pH 7), and an amount of soluble or LbL-enzyme, brought to 3 ml volume with 0,1 M phosphate buffer (pH 7). The substrate oxidation was followed at

405 nm for two and six minutes for the free and the immobilized enzyme respectively.

24

One activity unit was defined as the amount of enzyme that oxidised 1 mmol ABTS/min.

The immobilization yield was calculated as the difference between the activity present in the starting immobilization solution and that remaining in the supernatant at the end of the adsorption procedure.

To determine the activity of laccase and HRP in the co-immobilized system, the activity assay was run in two steps. In the first step an amount of LbL-multi-catalyst was submitted to laccase assay, using ABTS as substrate, as explained before. The lack of H₂O₂ in the assay cocktail did not allow HRP to express its activity, resulting in laccase activity solely. In the second step the assay was repeated with the same sample of LbL-multi-catalyst in presence of H₂O₂, according to the HRP assay procedure explained before. In this case both the enzymes expressed their activity. The activity of HRP enzymes was calculated subtracting to the value of the total activity that related to laccase one. To determine the concentration of free enzymes in a solution Bradford assay was applied.¹⁵ 400 µl of Bradford reagent were added to 2,600 ml of deionised water containing 50, 100, 150, 200 µl of the test solution. The absorbance at 595 nm was then measured against a blank made of 2,600 ml of deionised water and 400 µl of Bradford reagent.

6.5.4 WHEAT STRAW LIGNIN TREATMENTS

LACCASE-MEDIATED OXIDATION

80 mg of wheat straw lignin were suspended in 40 ml of acetate buffer (pH 6) and treated with 23 U of native or LbL-laccase. The reaction was allowed to stir at 30°C for 24h. Parallel reactions were carried out in the presence of 1-hydroxybenzotriazole (HBT) 1 mM as oxidation mediator.

HRP-MEDIATED OXIDATION

80 mg of wheat straw lignin were suspended in 40 ml of acetate buffer pH 6 with 0,5 mM H₂O₂ and treated with 165 U of native or LbL-HRP. The reaction was allowed to stir at 30°C for 24h.

MULTICATALYST-MEDIATED OXIDATION

80 mg of wheat straw lignin were suspended in 40 ml of acetate buffer (pH 6) and treated with the enzymes (23 U of laccase plus 165 U of HRP, either in solution, singularly immobilized and co-immobilized). The reaction was allowed to stir at 30°C for 24h. Parallel reactions were carried out in the presence of 1-hydroxybenzotriazole (HBT) 1 mM as oxidation mediator.

WORKUP

The mixture was then acidified at pH 3 with HCl in order to precipitate lignin. In case of immobilized enzymes, the mixture was filtered in Buchner before acidification.

The precipitate was centrifuged, washed three times with water to eliminate soluble lignin oligomers and freeze-dried.

The soluble fraction recovered after centrifugation and the waste waters were freeze-dried and put aside for GPC analysis to investigate the nature of the soluble compound yielded by the oxidative treatments.

The residual insoluble lignin after oxidation were analyzed by GPC and ^{31}P -NMR.

6.5.5 QUANTITATIVE ^{31}P -NMR PROCEDURE

The procedure of lignin derivatization is widely described in literature.^{9,11,12}

About 25 mg of lignin accurately weighed were dissolved in 400 ml of a solvent mixture composed of pyridine and deuterated chloroform, 1.6:1.0 (v/v) ratio. 100 ml of 2-chloro-4,4',5,5'-tetramethyl-1,3,2-dioxaphospholane were added, followed by 100 ml of the internal standard solution (cholesterol 0,1 M + 5g/L of Cr(III) acetyl acetonate as relaxation agent). The reaction cocktail was allowed to stir at room temperature for two hours. The NMR spectra were recorded on a Bruker 300 MHz NMR spectrometer using previously published methods.^{10,12} To obtain a good resolution of the spectra, a total of 256 scans were acquired. The maximum standard deviation of the reported data was 2 E-2 mmol/g, while the maximum standard error was 1 E-2 mmol/g.

6.5.6 GEL PERMEATION CHROMATOGRAPHY (GPC) ANALYSIS

Acetobromination of lignin samples for GPC analysis was carried out following the procedure described in literature.¹⁴

10 mg of lignin were suspended in 2,5 ml of a mixture of acetic acid glacial/acetyl bromide (92:8 v/v) and stirred at room temperature. After 2 hours the solvent is evaporated and the residue is dissolved in 5 ml THF. The GPC analyses were performed using a Shimadzu LC 20AT liquid chromatography with a SPD M20A ultraviolet diode array (UV) detector set at 280 nm. The sample (20 μ l) is injected into a system of columns connected in series (Varian PL gel MIXED-D 5 μ m, 1-40K and PL gel MIXED-D 5 μ m, MW 500-20K) and the analysis is carried out using THF as eluent at a flow rate of 0.50 ml min⁻¹. The GPC system has been calibrated against polystyrene standards (molecular weight range of 890 – 1.86 x 10⁶ g/mol⁻¹), lignin monomers and model dimers, as apocynol and (3-methoxy-4-ethoxy-2-phenyl)-2-oxo-acetaldehyde respectively.

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7. FINAL CONCLUSION

Plants have always served mankind : they function as a main source of food, as a source for energy production, as a source for many products of economic importance, such as building materials and fibers, as source for ingredients of perfumes and dyes, and last but not least as source for “biological” drugs used in medicinal applications. Latest since the first oil crisis in 1973, the drawbacks of the dependency of our economies and our lifestyles on fossil-based resources for energy and consumer products became obvious to everybody, and with the rapid increase in world population since the 1990's, the depletion of exhaustible resources is soon to have happened. Plants are naturally considered a valuable alternative source for the production of energy (biofuels) and consumer products (biomaterials), and a huge effort is currently made to manage the switch to a routine use of biomaterials instead of fossil-based resources. However, these efforts are still struggling to balance the exploitation of renewable resources with the defense of the environment and human health.

Since the early 1990's, the use of natural catalysts, namely enzymes, in the development of organic synthesis reactions has received a steadily increasing attention, especially under the slogan “green chemistry”

Within the studies towards my PhD, I focused my attention on the upgrading and valorization of plant polyphenols, thereby taking advantage of the eco-sustainable activity of natural enzymes, in order to combine the employment of renewable resources with the development of eco-friendly processes.

Among the wide variety of plant-derived polyphenols, tannins and lignins have been selected due to the myriad of possibilities that emerge based on both their structural and pharmacological features. Although tannins are used in a wide variety of industrial applications, their polyphenolic structure could be further exploited for high added value applications. Lignin represents a by-product of pulp and paper industry and of modern saccharification processes, and is still under-utilized.

In order to be able to exploit tannins to their full potential , a novel immobilized version of tannase was developed. In light of current immobilization methodologies described in literature, the covalent binding onto eupergit provided a complete

immobilization of the enzyme and ensured the total retention of enzymatic activity. The subsequent layer-by-layer coating technique (LbL technique) gave rise to an increased enzyme stability, by protecting the enzyme from denaturant agents and mechanical forces.

LbL-tannase displayed its enzymatic activity even after several consecutive batch reactions, thus proving to be suitable for use in industrial applications.

The immobilized enzyme can be potentially introduced in flow-chemistry set-ups suitable for the production of gallic acid-based biologically active compounds in preclinical scales.

In order to improve the efficiency of the common enzymatic processes used in lignin depolymerization, a novel multi-enzyme catalyst was developed combining the potential of two different enzymes that are involved in natural lignin degradation. Treatment of lignin samples with the new LbL-multicatalyst showed the highest conversion yield with respect to the immobilized enzymes and to the native forms, used singularly or in mixture.

The synergic action performed by these enzymes gives – to the best of our current knowledge – rise to domino or cascade reactions, that involve several non-separable steps characterized by the presence of highly reactive intermediates. The cascade process implies a substantial depolymerization of the structure both in the internal and terminal subunits (exo- and endo-depolymerisation activities), providing valuable building block for the potential production of bulk and fine chemicals and synthesis of new (block-) copolymers.

The lack of suitable analytical methods for tannins and lignin characterization and the need of highlighting and quantifying the structural modification after the enzymatic treatment prompted the development of new analysis protocols.

The ^{31}P NMR-based method for the quantitative analysis of tannins opened the possibility to establish the nature of a tannin sample, distinguishing gallotannins from ellagitannins and proanthocyanidines on the basis of the specific signals attributable to their related substructures, providing for each sample a specific fingerprint which can be used as quality control to evaluate the impurity. It is noteworthy that the analytical method allows to unambiguously assign the degree of esterification of gallotannins,

and to delineate the regiochemistry; furthermore, it was possible to clarify the regiochemistry in polymeric proanthocyanidines.

With respect to the quantitative analysis of lignins, the joint use of quantitative ^{31}P NMR and QQ-HSQC protocols allowed us to develop a new analytical tool for the determination of the average degree of polymerization of lignin, which is completely independent of supramolecular association phenomena.

According to the results obtained, we questioned the standard belief that lignin is a three-dimensional branched polymer: We proposed two-dimensional, hence linear and un-branched, oligomers as the only structures that are in compliance with our spectroscopic data on milled wood lignin from Norway spruces. These improved structural elucidation will greatly improve the development of strategies aiming at the valorisation of lignin samples.

The methods developed in this work are all easy to incorporated into current research activities aiming at advancing the use of biomass-deived polyphenols as valuable substitute for fossil-based polyphenols and related compounds. The analytical, but also especially the synthetic methods presented herein are easy to perform, and suitable to be adopted to scale. It is thus hoped, that the research efforts will soon spark new research activities both in academic and in industrial environments concerned with the development and industrial implementation of sustainable green chemistries.

