Agro-forest residues valorization as source of biomolecules

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Preface

The work presented in this thesis was performed at Laboratório Nacional de Energia e Geologia (LNEG) in Lisbon, Portugal, during the period of February – October 2018, under the supervision of Dr. Rafał Marcin Łukasik. The thesis was co-supervised at Instituto Superior Técnico by Prof. Maria Conceição Oliveira.

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And finally, to my parents for paying the tuition all these long years, as well as my brother, for the unconditional support throughout this journey.

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Resumo

O presente trabalho é dedicado ao estudo da implementação dos conceitos de "química verde" e biorrefinaria no pré-tratamento de biomassa lenhocelulósica (palha de trigo e resíduos extratados de *Cupressus lusitânica)*, por aplicação do solvente alcalino imidazole. As condições de pré-tratamento foram escolhidas de modo a maximizar a degradação de lenhina com produção de compostos de valor acrescentado. Temperaturas de 130, 145 e 160 °C e tempos de reação de 2, 3 e 4 horas foram estudados fixando a razão biomassa:imidazole (1:9 m/m). Obtiveram-se frações ricas em celulose e hemicelulose, enquanto a lenhina foi despolimerizada pelo imidazole. O melhor resultado para a fração rica em celulose foi obtido a 160 °C/4h, para ambas as biomassas, com 54.6 ± 0.3% e 40.7 ± 0.6% de celulose e 85.9 ± 0.3% e 65.2 ± 0.4% de remoção de lenhina, para a palha de trigo e para os resíduos extratados de *Cupressus lusitanica*, respetivamente. Tanto a recuperação de celulose como de hemicelulose foram dependentes da temperatura. No entanto, o imidazole foi mais eficaz em separar os componentes da palha de trigo nas respetivas frações do que dos resíduos extratados de *Cupressus lusitanica*. O imidazole foi recuperado e purificado. A presença de compostos fenólicos de valor acrescentado provenientes da despolimerização da lenhina no imidazole recuperado foi analisada por eletroforese capilar e pela determinação do teor em fenólicos totais e da atividade antioxidante. Os referidos compostos foram tentativamente identificados e a respetiva estrutura foi proposta por HPLC-MS.

Palavras chave: biomassa lenhocelulósica, palha de trigo, *Cupressus lusitanica*, imidazole, pré-tratamento, remoção de lenhina

Abstract

Implementation of green chemistry and biorefinery concept are needed to boost production of biomass-derived fuels, chemicals, and materials with cost-effective processing of sustainable feedstock. The use of imidazole as a novel solvent for biomass pretreatment creates an approach that helps accomplish this concept. The present work is devoted to study the pretreatment of lignocellulosic biomass, namely, wheat straw and extracted residues of *Cupressus lusitanica*, by application of the alkaline solvent imidazole. The capacity of this solvent to fractionate biomass was scrutinized. The pretreatment allowed to obtain cellulose- and hemicellulose- rich fractions, while lignin was depolymerized by imidazole. Pretreatment conditions were set to maximize delignification with production of valuable lignin derived compounds. Temperatures of 130, 145 and 160 °C and reaction times of 2, 3 and 4 hours were studied by fixing the parameter biomass:imidazole ratio (1:9 w/w). Both cellulose and hemicellulose recovery were highly dependent on reaction temperature. The best result for the cellulose-rich fraction was obtained at 160 °C for 4h for both biomasses, achieving 54.6 ± 0.3% and 40.7 ± 0.6% of cellulose content, with a delignification yield of 85.9 ± 0.3% and 65.2 ± 0.4%, for wheat straw and for the extracted residues of *Cupressus lusitanica,* respectively. The presence of added-value phenolic compounds from depolymerized lignin in recovered imidazole was analyzed by capillary electrophoresis and determination of total phenolic content and antioxidant activity. These compounds were tentatively identified, and structure proposed by HPLC-MS.

Keywords: lignocellulosic biomass, wheat straw, *Cupressus lusitanica*, imidazole, pretreatment, delignification.

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List of Abbreviations

Abs	Absorbance
C5/C6	Carbon five/six
CE	Capillary electrophoresis
CID	Collision induced dissociation
DPPH	2,2-diphenyl-2-picrylhydrazyl hydrate
ESI	Electrospray ionization
EtOH	Ethanol
FTIR	Fourier-transform infrared spectroscopy
G	Guaiacyl
GAE	Gallic acid equivalents
H	p-hydroxyphenyl
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
HPLC – MS	High performance liquid chromatography – mass spectrometry
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulfuric acid
IL	Ionic liquid
LD_{50}	Median lethal dose
MS	Mass spectrometry
MeOH	Methanol
NA_2CO_3	Sodium carbonate
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NREL	National Renewable Energy Laboratory
Pc	Critical pressure

QIT	Quadrupole ion trap
S	Syringyl
SCF	Supercritical fluid
$ScCO_2$	Supercritical carbon dioxide
SO_2	Sulfur dioxide
SPE	Solid phase extraction
Tc	Critical temperature
TE	Trolox equivalents
TIC	Total ion chromatogram
UAE	Ultrasound assisted extraction
XRD	X-ray diffraction

1. Introduction

The preservation and management of the world's diverse resources is one of the most emerging issues of the modern society. Because of the unstable supplies and diminishing reserves of fossil fuels as well as the environmental challenges that the world is facing nowadays, there is currently a growing need to explore alternative sources of energy and of chemical commodities. Therefore, in this context the green chemistry and the biorefinery concepts emerge.

The implementation of both concepts can boost a fossil-independent future with economy based on renewable feedstock like biowaste/residues and agro-industrial by-products. The development of technologies for valorization of these resources is a key role of society in the creation of a sustainable and more environmentally friendly future[1].

1.1. Green Chemistry

Although chemistry improves the quality of our daily life, in general society perspectives, chemical industry is viewed as a serious threat to the collective human health and global environment. Green Chemistry has been proposed as an alternative to how chemistry is thought out. Green Chemistry is an approach to the design, manufacture and use of chemical products to intentionally reduce or eliminate chemical hazards. The goal of green chemistry is to create better and safer chemicals while choosing the safest, most efficient way to synthetize them and to reduce wastes[2]. In 1998, Anastas and Warner outlined the Twelve Principles of Green Chemistry to demonstrate how chemical production could respect human health and the environment, while also being efficient and profitable[2]. Those are:

1) Prevent waste
2) Atom economy
3) Less hazardous chemical syntheses
4) Design safer chemicals
5) Safer solvents and auxiliaries
6) Design for energy efficiency
7) Use of renewable feedstocks
8) Reduce derivatives
9) Catalysis (vs stoichiometric)
10) Design for degradation
11) Real time analysis for pollution prevention
12) Inherently safer chemistry for accident prevention

The employment of the above-mentioned green chemistry principles into the biorefineries can make a significant contribution to sustainable development by adding value to the sustainable use of biomasses.

1.2. Biorefinery

The increase of demands for fuel consumption as well as the great number of lignocellulosic residues available for valorization led to the development of industrial units called biorefineries.

A biorefinery is a facility for the synergetic processing of biomass into several marketable bio based products (food and feed ingredients, chemicals, materials, minerals, CO_2) and bioenergy (fuels, power, heat)[3].

Biorefinery is a concept of a processing plant that covers an extensive range of combined technologies where biomass feedstocks are converted in a sustainable manner into a spectrum of valuable products. Analogous to today's petroleum refineries, which produce multiple fuels and products from petroleum, biorefineries is a "processing facility that integrates biomass conversion processes and equipment to produce fuels, energy, and chemicals from biomass"[4]. Figure 1.1 presents the biorefinery concept in a very generic manner.

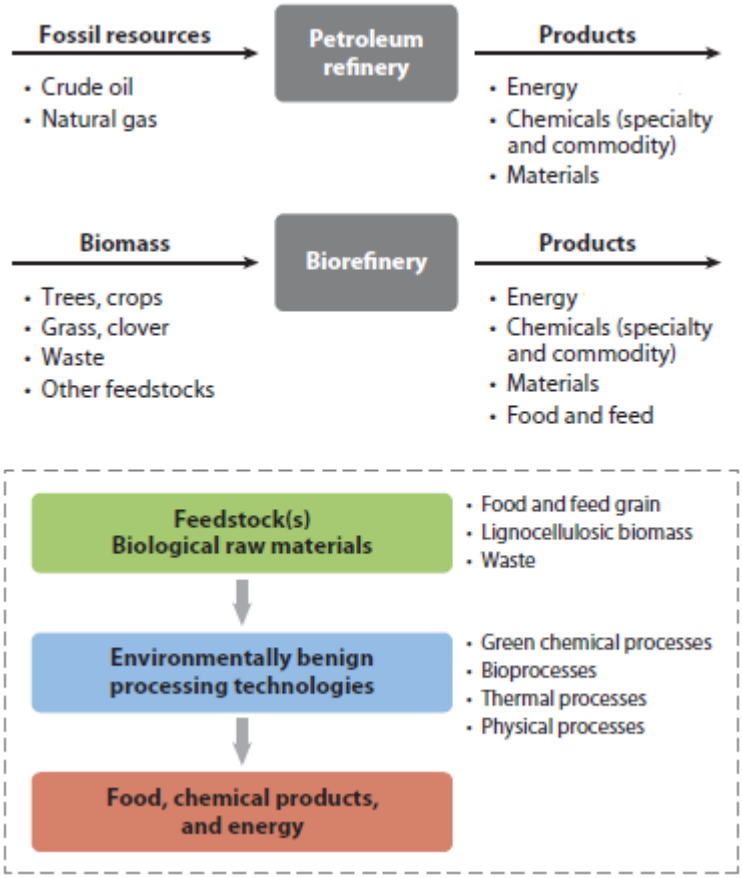

Figure 1.1 - The biorefinery concept: from biomass to valuable products via low-environmental-impact practices[5].

The objective of a biorefinery is to optimize the use of resources and minimize the wastes, integrating production of higher value bioproducts into biorefinery's fuel and power output, and thereby maximizing productivity and profitability, which are keys to sustainability and economic viability. Biorefineries can employ a variety of conversion processes – biological, chemical and thermal. The biorefinery concept has been devised that is built on different platforms, depending on the raw material, technological processes and end-products. The main two are the biochemical and thermochemical platform[6]. The first one is based on biochemical conversion processes and focuses on the fermentation of sugars extracted from biomass feedstocks, while the

latter is based on thermochemical conversion processes and aims the gasification of biomass feedstocks and resulting byproducts[7].

Biorefinery platforms, based only on main raw materials used are[6]:

1) lignocellulose feedstock biorefinery, which use chiefly cellulose containing biomass and waste;
2) the whole crop biorefinery, which uses raw material such as cereals or maize;
3) green biorefineries, which use nature-wet biomass such as green grass, alfalfa clover or immature cereal.

We are witnessing a worldwide expansion in biorefineries, in which the lignocellulosic biomass is in the spotlight. Being widely available and at moderate costs, it is expected to become a main raw material of the future. However, the industrial units need developments both at a scientific and technological level to surpass the limitations and properly answer to the challenge at hand: become generators of wealth and employment.

The use of biorefineries for the production of chemicals as well as materials and energy carrier is key to ensuring a sustainable future. Through the integration of green chemistry into biorefineries and the use of low environmental impact technologies, it is possible to establish future supply chains for green and sustainable commodities[8].

1.3. Biomass

Biomass is any organic matter that is available on a renewable or recurring basis, including dedicated energy crops, agriculture, food and feed residues, aquatic plants, wood and wood residues, animal wastes, and other waste materials usable for industrial purposes (energy, fuels, chemicals, materials) and include wastes and residues of food and feed processing technologies[4]. No other resource can compete with biomass as source of carbon for sustainably generate molecules or energy currently produced from petroleum.

Biomass is the most abundant renewable resource, with an estimated global annual production of $1.1x10^{11}$ tons[9]. Being a readily available and low-cost feedstock, it is among very few resources that can facilitate the large scale and sustainable production of the substantial volumes of energy and materials[10]. If biomass is harvested sustainably, it can also significantly reduce greenhouse gas emissions because primarily only the carbon fixed during photosynthesis can be released. Hence, biomass has the potential to solve two main current challenges of humanity: energy security and environmental concerns[11].

Many of the bio based industry products currently used are results of direct physical or chemical treatment and processing of biomass, for example cellulose, starch, oil, protein, lignin and terpenes[4].

1.3.1. Lignocellulosic Biomass

Lignocellulosic biomass, mostly existing in the form of plant materials such as agricultural residues (e.g. wheat straw, corn stover), hardwood (e.g. eucalyptus, willow), softwood (e.g. spruce, pine) forestry residues (e.g. wood chips, sawdust) and agro-industrial wastes, has been considered as one of the most promising feedstocks for biorefinery[7].

Lignocellulosic biomass is the most abundant and bio-renewable feedstock on Earth,[12-13] and is mainly composed of cellulose, hemicellulose and lignin[14]. It is a promising alternative to limited crude oil: a carbon-

neutral renewable source able to decrease CO_2 emissions and atmospheric pollution, produced quickly and at a lower cost[15].

However, the conversion of lignocellulosic biomass remains a big challenge[16]. The main reason for this is the complex chemical composition of the biomass, which makes it resistant to biological and chemical decomposition[17] and more difficult to yield target fuels and chemicals in a reasonable high yield and quality[14]. For this reason, pretreatment of biomass is essential, which increases the complexity of the process and its overall costs[18]. Figure 1.2 illustrates the lignocellulosic feedstock biorefinery concept.

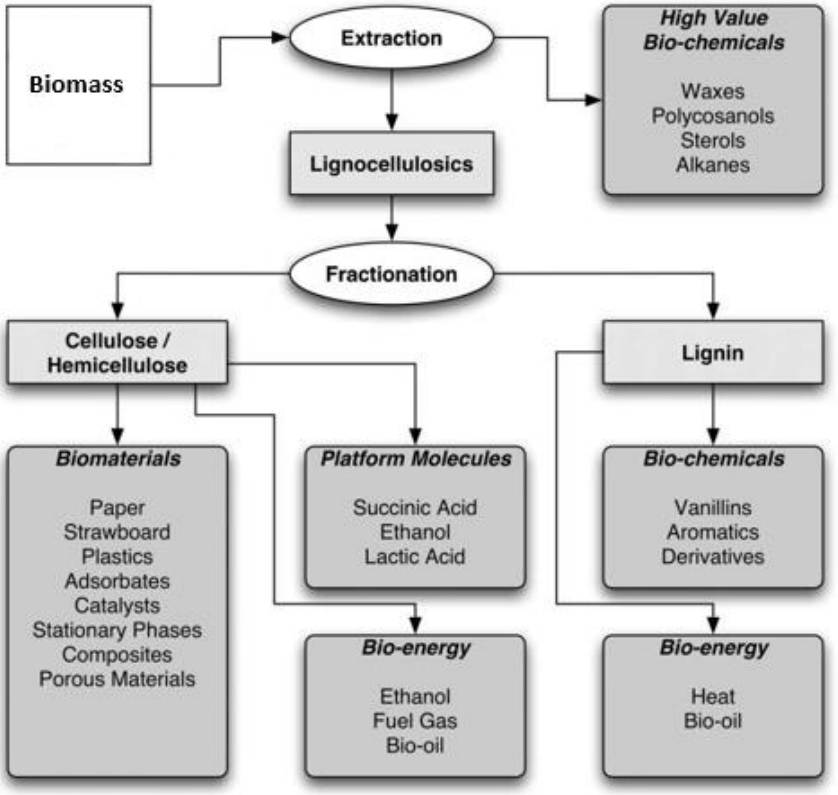

Figure 1.2 - Lignocellulosic feedstock biorefinery[19].

1.3.2. Composition and structure of lignocellulosic biomass

Lignocellulosic biomass is mainly composed by three interlinked polymers, namely: cellulose, hemicellulose and lignin (Figure 1.3 depicts a pictorial view of the structural organization of lignocellulose). It also includes minor constituents such as water and variable amounts of proteins, pectins, extractives and ash[21].

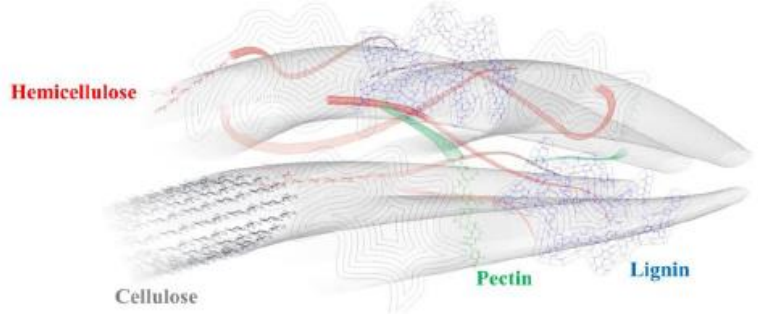

Figure 1.3 - Pictorial view of the structural organization of lignocellulose depicting the association of the three main components of biomass in the cell wall, as well as pectin[20].

Depending on the type of lignocellulosic biomass, the referred three main polymers are organized into complex non-uniform three-dimensional structures to different degrees and varying relative composition. Lignocellulose has evolved to resist degradation and this robustness or recalcitrance of lignocellulose is due to the crystallinity of cellulose, hydrophobicity of lignin, and encapsulation of cellulose by the lignin–hemicellulose matrix[15, 17, 22]. The composition of lignocellulose is highly dependent on several factors. There is a significant variation of the lignin and (hemi)cellulose content of lignocellulose depending on whether it is derived from hardwood, softwood, or grasses. The typical percentages of the three components may vary from species to species, across different parts in the same plant and can also be influenced by geography or environmental factors. Table 1.1 shows the composition of the different types of lignocellulosic biomass in their cellulose, hemicellulose and lignin content[23].

Table 1.1 – Examples of lignocellulosic biomasses and their chemical composition in cellulose, hemicellulose and lignin content.

Lignocellulosic Biomass	Type	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hardwood	Oak	40.4	35.9	24.1
	Eucalyptus	54.1	18.4	21.5
Softwood	Pine	42.0-50.0	24.0-27.0	20.0
Agricultural waste	Wheat straw	35.0-39.0	23.0-30.0	12.0-16.0
	Rice straw	29.2-34.7	23.0-25.9	17.0-19.0
	Barley straw	36.0-43.0	24.0-33.0	63.-9.8
	Sugarcane Bagasse	25.0-45.0	28.0-32.0	15.0-25.0
Grasses	Switchgrass	35.0-40.0	25.0-30.0	15.0-20.0

1.3.2.1. Cellulose

Cellulose is the major component of lignocellulosic biomass. It is a highly stable homopolysaccharide composed of β-D-glucopyranose (glucose) monomers linked by β-(1->4)-glycosidic bonds in a linear chain up to 12000 units[20, 24]. The glucose units are strongly bound by the numerous inter- and intramolecular hydrogen-bond networks, resulting in highly ordered crystalline regions that are interrupted by regions of less organized - amorphous regions of cellulose[20] (Figure 1.4 shows a simplistic visual presentation of the glucose monomer organization). This crystalline structure is obtained by the coalescence of adjacent polymers chains, which leads to the formation of microfibrils, which in turn are united to form fibres, providing rigidity and insolubility in water and in most of organic solvents[14]. This high level of molecular organization characterizes the recalcitrance of the cellulose matrix to conversion by chemical and biological means, including enzymes and microorganisms[20].

Many properties of cellulose are determined by its degree of polymerization, which represents the number of glucose units that compose a polymeric chain. This value is usually between 800-10000, but can extend to 17000, depending on the cellulose origin[14].

Since about half of the organic carbon in the biosphere is present in the form of cellulose, the conversion of cellulose into fuels and valuable chemicals has paramount importance[15].

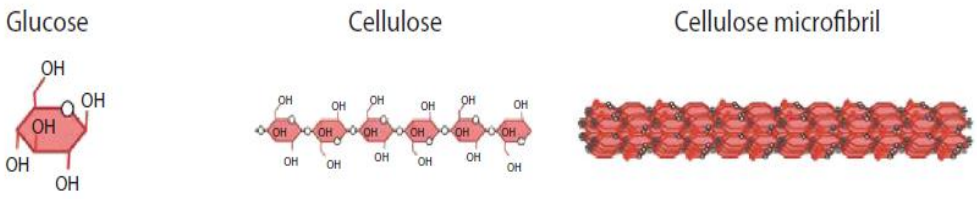

Figure 1.4 - Chemical structure of the cellulose building block. Cellulose monomer on the left, subunit of the respective polymer on the right[11].

1.3.2.2. Hemicellulose

Hemicellulose is the second most abundant polymer of lignocellulosic biomass. It is a branched-chained heteropolysaccharide composed of C5 and C6 monomers (Figure 1.5): pentoses (D-xylose and L-arabinose), hexoses (D-mannose, D-glucose, L-rhamnose, L-fucose and D-galactose), as well as acetyl groups, glucuronic and galacturonic acids. Hemicelluloses have a random and amorphous structure containing both α- and β-glyosidic bonds, lacking crystalline aggregates, which makes them less resistant to enzymatic degradation than cellulose[20]. They are also easier to be hydrolyzed under mild acid or alkaline conditions, or even hot water. Depending on their origin, hemicelluloses differ in structure and composition. An example of a hemicellulose structure is shown in figure 1.5.

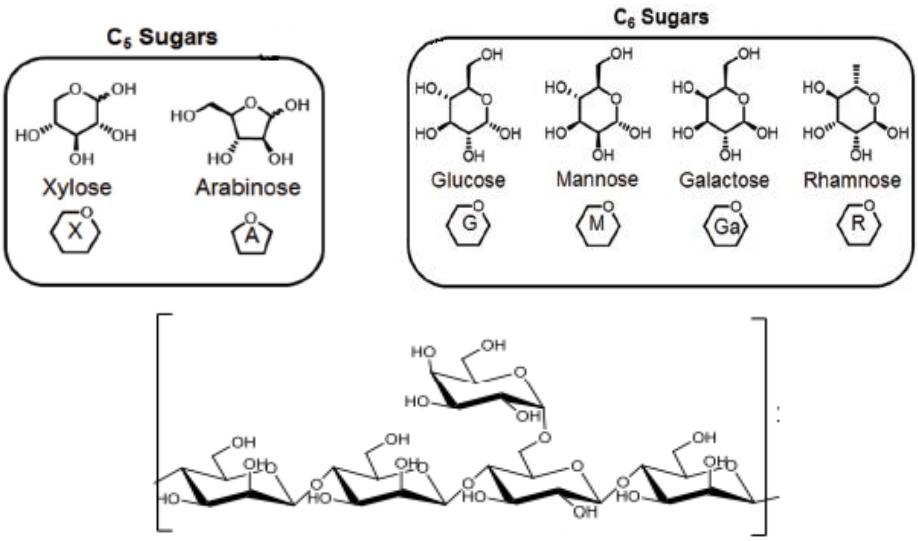

Figure 1.5 - Chemical structure of the C5 and C6 sugars that compose hemicellulose with an example of a hemicellulose structure[15].

1.3.2.3. Lignin

Lignin is the second most abundant polymer in nature[25]. It is also the most abundant and complex aromatic biopolymer in the world[14]. Lignin is an amorphous non-polysaccharidic three-dimensional heterogeneous polymer, mainly composed by methoxylated phenylpropanoid units most commonly linked by ether bonds[24]. It represents about 10 to 35% of the lignocellulosic biomass weight. It functions as a cellular glue by filling the spaces between cellulose and hemicellulose, holding the lignocellulosic matrix together, making it insoluble in water, providing compressive strength to the plant tissue and the individual fibres, stiffness and rigidity to the cell wall and resistance against insects, pathogens and oxidative stress[22].

The structure of lignin is formed by the oxidative coupling of three different phenylpropane building blocks: p-coumaryl alcohol (4-hydroxycinnamyl), coniferyl alcohol (3-methoxy-4-hydroxycinnamyl) and sinapyl alcohol (3,5-dimethoxy-4-hydroxycinnamyl) (Figure 1.6). The corresponding phenylpropanoid monomeric units in the lignin polymer are identified as p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units, respectively. These groups are monolignol precursors with different number of methoxy groups in the aromatic ring: p-coumaryl has zero methoxy groups, coniferyl has one and synapyl has two[27]. The content of each monolignol depends on the type of plant of which they are obtained. For example, softwood consists of more than 90% of guaiacyl units while hardwood has both guaiacyl and syringil units[14] and grass wood has all three[25].

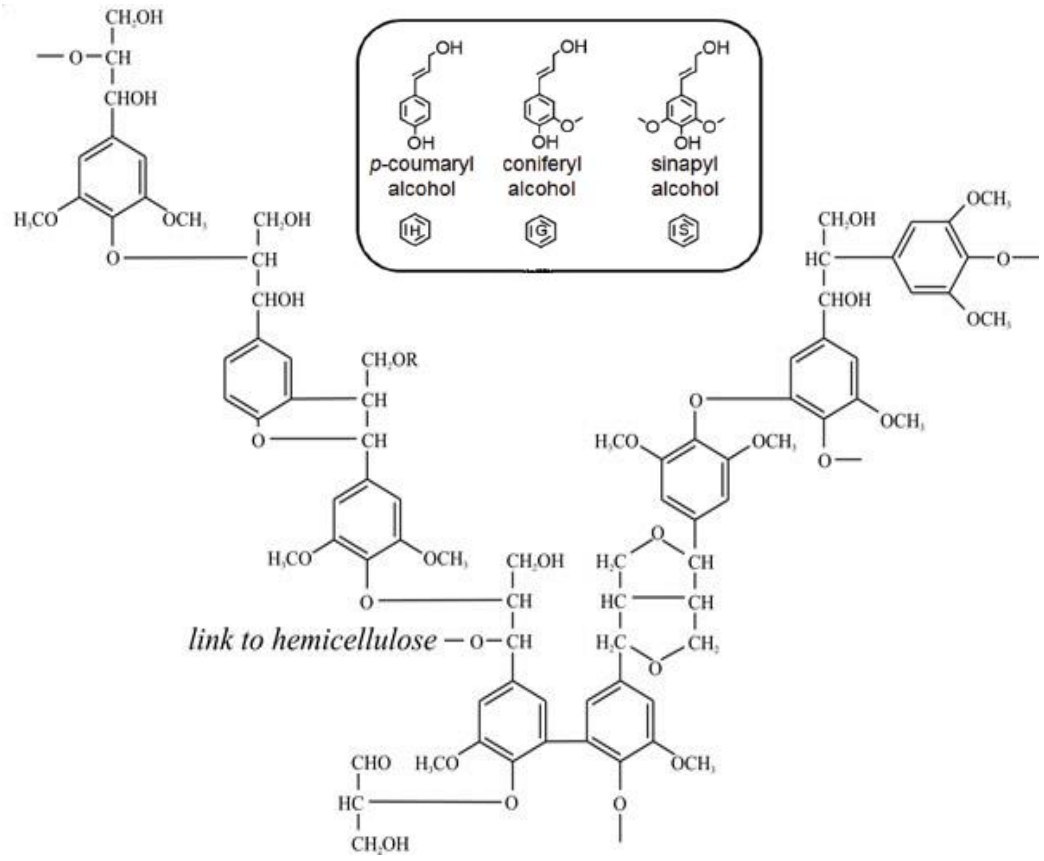

Figure 1.6 - Chemical structure of the three diferent monolignols that compose lignin with an example of lignin polymer[26].

Native lignin monolignols are linked by ether and C-C bonds. The major linkages between them are β-O-4 (β-aryl ether), β–β (resinol), and β-5 (phenylcoumaran)[25]. The close association with cellulose microfibrils makes lignin a major deterrent to enzymatic and microbial hydrolysis of lignocellulosic biomass.

1.3.3. Types of biomass used

In this work, biomass studied were wheat straw and underutilized residues of *Cupressus lusitanica* Mill. obtained during forest cleaning and management. Wheat straw is an agricultural waste while *Cupressus lusitanica* Mill. is a softwood. These were chosen to highlight the differences when subjected to pretreatment and later in the final products obtained.

1.3.3.1. Wheat Straw

Wheat straw is an agricultural by-product that results from wheat production. Wheat (*Triticum* spp.) is a cereal grain from the Family of *Poaceae* and its cultivation has been made for more than 8000 years. This cereal is a major staple food crop in many parts of the world in terms of both cultivation area and prevalence as a food source. It is widely grown throughout temperate zones and in some tropical/sub-tropical areas. The main centers where wheat is produced are Europe, North America, China and India.

As it can be seen from Figure 1.7, wheat is the second most produced cereal grain in the world with 759.75 million metric tons produced in 2017/2018. Data presented in Figure 1.8 shows that the trend of wheat production is to keep increasing, as it has been in the last 7 years. Assuming residue/crop ratio of 1.3, it can be estimated that 1000 million metric tons of wheat straw can be produced annually. Due to the relatively low cost and enormous amount available, wheat straw serves as a potentially attractive feedstock for producing biofuels (bioethanol[29]) and other high valuable chemicals.

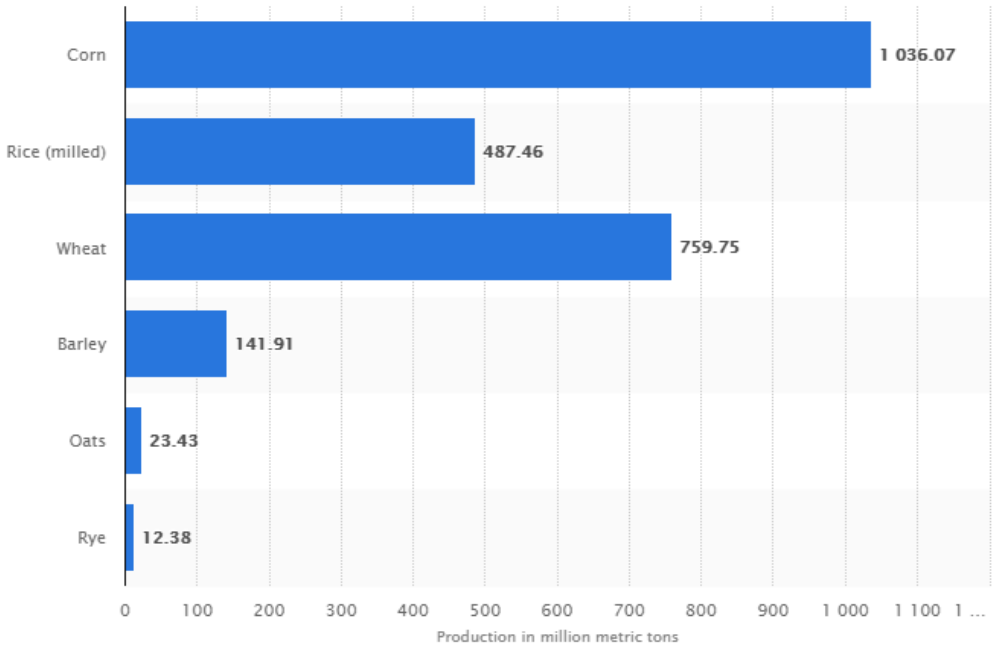

Figure 1.7 - Worldwide production of grain in 2017/2018, by type, in million metric tons[28].

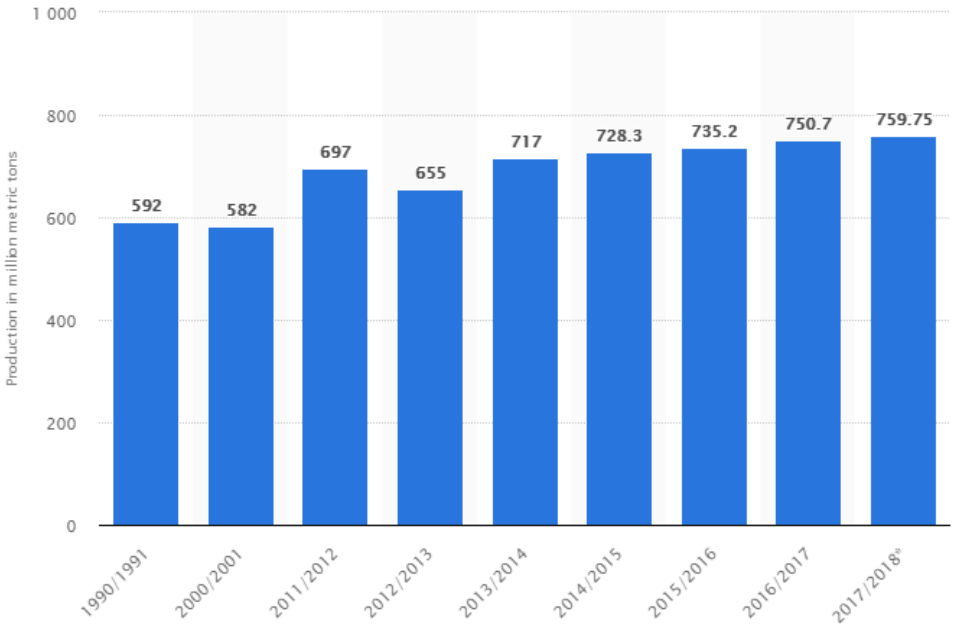

Figure 1.8 - Global wheat production from 1990/1991 to 2017/2018 in million metric tons[28].

1.3.3.2. *Cupressus lusitanica* Mill.

Cupressus lusitanica Mill., also known as Mexican Cedar, Mexican cypress and Portuguese cypress, is a conifer tree native to Mexico and Central America. The species is fast growing, produces high quality timber and tolerates a wide range of environmental conditions. As a result, it has been widely planted, both as an ornamental tree and for timber production, in many warm temperate and subtropical regions throughout the World[30]. It is the most widespread *Cupressus* species in Portugal. Its presence is mainly in the Mata do Buçaco but it is used in all country as an ornament tree in gardens and parks.

Cupressus lusitanica Mill. (Figure 1.9) grows up to 30 m height and 1.2 m breast height diameter. It is readily availability in large quantities in nature and its foliage is used in indigenous practices to treat catarrh, headache and dermatitis. The essential oil of leaves is used against rheumatism, whooping cough, and styptic problems[32].

Figure 1.9 - *Cupressus lusitanica*[31].

There are still little information available about *C. lusitanica* as a source of lignocellulosic biomass for the production of biochemicals[33], however, its high lignin content makes this species an interesting target biomass for this study .

1.4. Valorization of lignocellulosic biomass

All three structural components from lignocellulosic biomass can be valorized by the production of a wide variety of fine and bulk chemicals as well as fuel. Glucose is a monosaccharide originated mainly from cellulose hydrolysis and is the primary and most abundant sugar produced from cellulose or starch-rich biomasses. The hemicellulose hydrolysis releases C5 (xylose and arabinose) and C6 sugars (mannose, galactose and rhamnose) as well as some short alkyl chain aliphatic acids, e.g. acetic acid. Either pentoses or hexoses could be fermented to produce biofuels or other value-added products, used in a wide range of applications, such as food and feed, pharmaceuticals and other purposes[20]. The US Department of Energy (DOE), in 2004, identified the top 12 chemicals derived from lignocellulosic sugars[34] (Table 1.2). In 2007, a further review by the same author also included ethanol and lactic acid in those top chemicals.

Lignin is a desirable candidate due to its high aromaticity, making it the major aromatic resource of the bio-based economy[15]. Therefore, there have been multiple approaches for the depolymerization of lignin to collect its aromatic monomeric units for production of commercially valuable chemicals and polymers[25]. Vanillin is one of the most common type of compound obtained from lignin. Further polymerization of vanillin-based compounds can produce polymers like vinyl ester resins, cyanate ester resins, epoxyde resins, benaoxanize resins, polyester and polyurethanes. Figure 1.10 shows some more representatives of lignin derivable aromatic compounds.

Table 1.2 – Top chemicals derived from lignocellulosic biomass

Succinic, fumaric and malic acids	Glucaric acid	3-Hydroxybutyrolactone
2,5-Furan dicarboxylic acid	Glutamic acid	Glycerol
3-Hydroxypropionic acid	Itaconic acid	Sorbitol
Aspartic acid	Levulinic acid	Xylitol/arabinitol

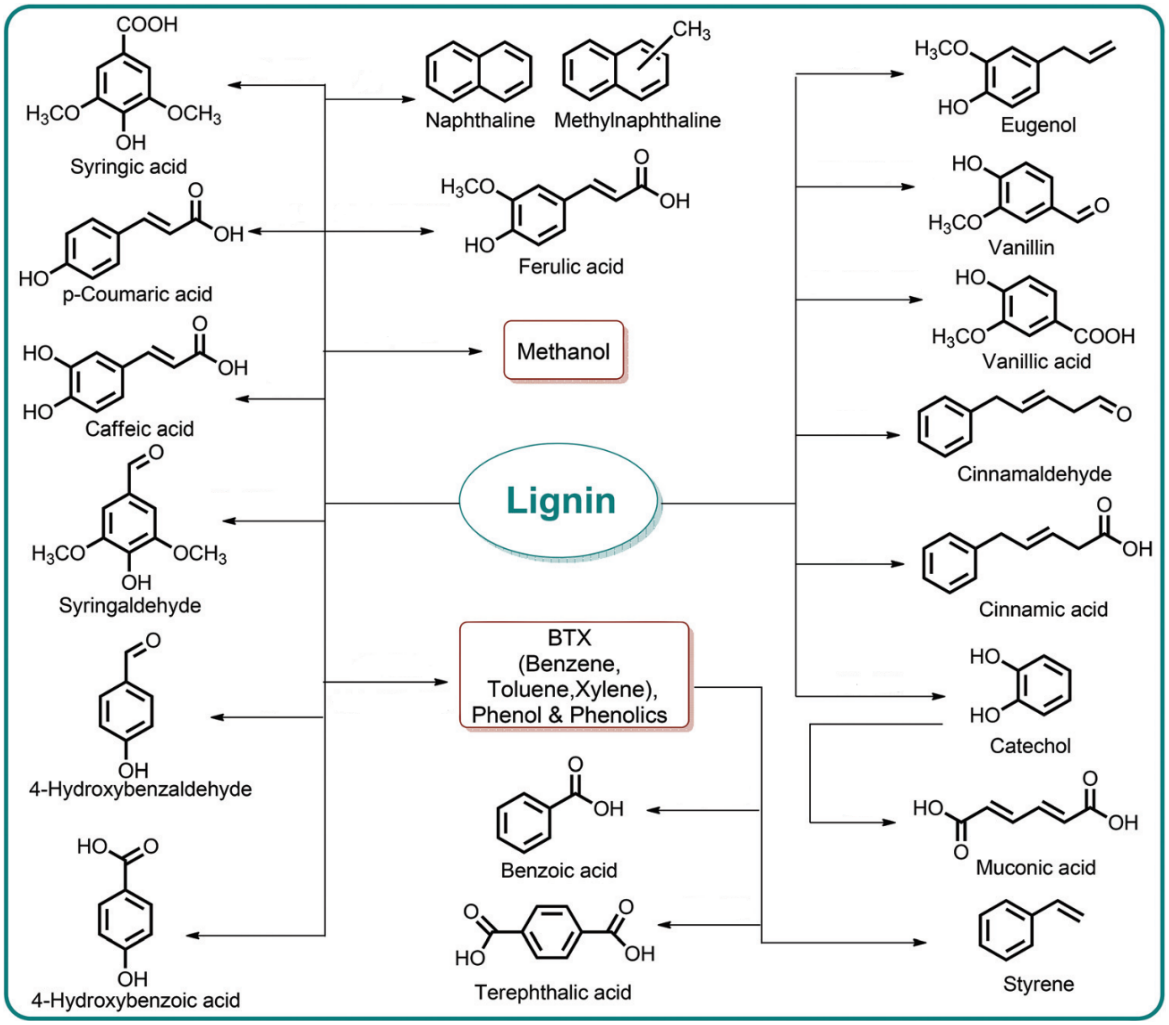

Figure 1.10 - Lignin derived chemicals[15].

Another potential application of lignin is as antioxidant. Lignin, as a highly aromatic polymer, is a free radical scavenger, and stabilizes the reaction induced by oxygen and its radical species[35]. Studies have demonstrated the antioxidant activity of lignin obtained from sugar cane[36] or carob kibbles[37], for example. The lignin and lignin derived compounds application in fields as medical, pharmaceutical (anticarcinogenic agent) or food industry has been proved[38-41]. This antioxidant property has been shown to prevent DNA and lipids oxidation by reactive oxygen species[42], and lignin derived compounds like ferulic acid and p-coumaric acid have been used in studies for the treatment of neurodegenerative diseases, such as Alzheimer's or Parkinson's disease[42-43]. Other lignin derived compounds like vanillin and 4-hydroxybenzoic acid have been studied as anticancer agents[44-46].

1.5. Biomass pretreatments

To take maximal benefits from the biomass, the lignocellulose biomass fractions must be turned available.

The main problem in using lignocellulosic biomass is its structural and compositional resilience due to the presence of covalent bonds between lignin and carbohydrates in the cell walls of plant and the crystallinity of cellulose that is embedded in a matrix of lignin and hemicelluloses. Therefore, it can be concluded that lignin is one the major barrier in e.g. enzymatic hydrolysis of cellulose, contributing to the recalcitrance of the

lignocellulosic material. Overcoming this obstacle is a key step in the production of biofuels and biochemicals. Hence, to separate the three fractions constituting lignocellulosic biomass and to make polysaccharides more accessible, not only for enzymatic hydrolysis but also for obtaining high added-value bioproducts, pretreatment is a crucial prerequisite. This is achieved by (1) degradation and/or removal of hemicellulose and lignin, (2) reduction of the crystallinity of cellulose and (3) an increase in the porosity of the pretreated lignocellulosic matrix[14]. Figure 1.11 shows the effect of pretreatment on the biomass structure. After the pretreatment, the synergetic effect of the three above-mentioned factors occur, making the transformation of biomass into fermentable sugar easier.

An effective pretreatment must meet the following requirements[48]:

1) Overcome lignocellulosic biomass recalcitrance, deconstructing the three-dimensional structure of lignocellulose, and breaking down the semi-crystalline cellulose and hemicellulose;
2) Afford high yield to sugars or chemicals and/or give highly digestible pretreated solid;
3) Avoid carbohydrates loss or degradation and preserve the utility of pentoses (hemicellulose) fraction;
4) Avoid the formation of inhibitory toxic byproducts;
5) Allow lignin recovery to generate valuable coproducts;
6) Be cost-efficient, generating energy, materials, and low wastes.

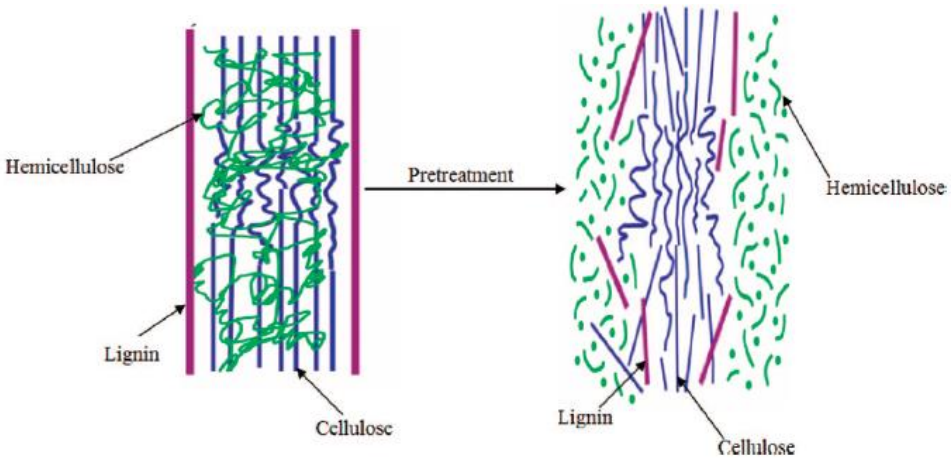

Figure 1.11 - Schematic showing the effect of pre-treatment on the lignocellulosic biomass structure[47].

All these requirements should be considered in the selection of the best pretreatment method. Many pretreatment methods have been studied and are still in development as there is a room for great improvement of efficiency, lowering the costs of the process[49]. Regardless the methods of biomass processing, pretreatment methods can be divided into conventional and alternative/novel methods.

1.5.1. Conventional methods

Table 1.3 – Overview of the conventional pretreatment methods, with description of their effect and their main disadvantages

Type	Method	Effect	Disadvantages
Physical	Mechanical comminution (milling and ultrasound)	Increase of accessible surface area by decrease of particle size, crystallinity and degree of polymerization	Need of combination with other pretreatments for complete fractionation. Higher energy consumption
	Pyrolysis	Decomposition of cellulose to produce gas and liquid products	High temperature and ash production
Physico-chemical	Steam explosion	Partial depolymerization of lignin; removal of hemicellulose; cost-effective due to low use of chemicals and energy consumption	Formation of inhibitors. Incomplete disruption of the lignin-carbohydrate matrix. Decrease of cellulose crystallinity increases
	Ammonia Fiber Explosion (AFEX)	Increase of accessible surface area; removal of lignin and hemicellulose; doesn't produce inhibitors; reduction of cellulose crystallinity	Inefficient for biomass with high lignin content. Depends on the ammonia cost.
	CO2 explosion	Increase of accessible surface area; no inhibitor production; cost effective; no toxicity and easy recovery; removal of hemicelluloses	No effect on lignin. High investment cost
	Liquid Hot Water	Removal of hemicellulose; alteration of lignin; low/moderate operational cost	Inhibitor formation
Chemical	Acid Hydrolysis	Hydrolyzes hemicellulose to sugars improving enzymatic digestibility; alteration of lignin structure	High cost, formation of fermentation toxic compounds, potential corrosion risk. Suitable for biomass with low lignin content.
	Alkaline Hydrolysis	Removes hemicellulose and lignin increasing the accessible surface area and cellulose digestibility; mild conditions	Long residence times, salts formed and incorporated in the biomass need to be removed or recycled
	Oxidative delignification (ozonolysis, wet oxidation and hydrogen peroxide)	Reduces lignin content; No/low formation of fermentation toxic residues	Formation of fermentation inhibitors. Possible degradation of lignin and hemicellulose
	Organosolv process	Removal of lignin with high quality. Hydrolysis of hemicelluloses improving enzymatic digestibility.	Solvents need to be recycled and reused; high process cost
Biological	Microorganisms	Degrades lignin and hemicellulose; low energy requirements	Rate of hydrolysis is very low

Conventional pretreatment methods are usually classified by the mechanism of action and can be considered as physical, chemical, physicochemical and biological. The selection of the feasible method for a specific process configuration considers many factors and those which can be good for one process are not necessarily optimal for another process or even biomass type. It is also important to point out that in general, none of the classical treatments is highly selective and/or material, energy and cost-efficient. The main disadvantages of these methods are: insufficient separation of cellulose and lignin (which reduces the effectiveness of subsequent enzymatic cellulose hydrolysis), formation of by-products that inhibit ethanol fermentation (e.g. acetic acid from hemicellulose, furans from sugars and phenolic compounds from lignin), high use of chemicals and/or energy, and considerable waste production. Therefore, the search for new green technologies is crucial. Table 1.3 shows a summary of the most common conventional pretreatments, describing their effects and main disadvantages[14, 20, 22].

1.5.2. Novel method: ionic liquids and supercritical fluids

In the last decade, ionic liquids (ILs) have been studied as promising solvents in numerous processes[50-51]. Ionic liquids are salts formed by large organic cations and small inorganic anions, which are liquid at low temperature (melting point below 100°C). ILs present high polarity and thermal stability, are non-flammable, have negligible vapor pressure, thus, they are inflammable. Besides that, they demonstrate a great solvent power.[50] Due to the numerous possible combinations of cations and anions, properties like viscosity, melting point, polarity and basicity can be easily tailored, allowing ILs to be a "designer" solvent[52]. The most commonly used cations are imidazolium, ammonium and phosphonium, while main anions are acetate, chloride, and phosphate.

Although ionic liquids are considered as *green solvents,* their toxicity and biodegradability are an important issue and subject of extensive study in the last years[53-54] Also, the generally claimed high cost of ILs obligates to an efficient recycling and regeneration.

Pretreatment with ILs offers several advantages: (1) alter physicochemical properties of the biomass macromolecular components, such as reducing the lignin content and cellulose crystallinity; (2) extract a specific macromolecular component, such as isolation of lignin and cellulose and (3) perform different fractionation approaches after biomass dissolution in ILs[20].

Ideally, there are two ways to fractionate lignocellulose in ILs: (1) complete dissolution of biomass followed by selective precipitation of the sought components as purified fractions by addition of an antisolvent, or (2) a selective extraction of components from the biomass.

The use of ILs as a tool for biomass fractionation have been subjected to a great number of studies and development of new methodologies[55-56], exhibiting a great potential to maximize the recovery of biomass and, simultaneously, to add value to the fractionated components within the biorefinery concept.

Another example of novel solvent is high-pressure/supercritical CO_2. Supercritical fluids (SCF) are defined as substances above their critical temperature and critical pressure. Under supercritical conditions, the fluids have unique physicochemical properties, such as liquid-like density and gas-like diffusivity and viscosity. Near the

critical point the solvation properties of the SCFs can be tuned: small variations in temperature and pressure can change solubility dramatically. The high diffusivities and extremely low surface tension of SCFs allow penetration into the inner pores of the recalcitrant structure of lignocellulosic materials and their liquid-like density permits the dissolution of some components of the lignocellulosic matrix[20].

Supercritical CO_2 has gained special importance as a solvent due to its properties. CO_2 is readily available, nontoxic, nonflammable, inexpensive, does not leave residues and is easy to bring to the supercritical conditions (Tc = 31°C and Pc = 7.38 Mpa).

Although promising studies on the employment of SCFs in the pretreatment of lignocellulosic biomass have been done, the development of this technology is still in its early phase. Further investigation is required to apply the $scCO_2$ processes in the biorefinery concept.

1.6. Imidazole

Imidazole is an organic compound with the formula $C_3N_2H_4$. It is a white/colorless powder soluble in water. The chemical structure of imidazole is depicted in figure 1.12. Classified as a diazole, it is a highly polar aromatic heterocycle with a five-membered planar ring consisting of three carbon atoms and two nitrogen atoms. Imidazole is characterized by having low toxicity (LD_{50} =970 mg kg^{-1}, oral, rat), high boiling point (256°C), stability (flame point 145°C), negligible vapor pressure (0.003 hPa at 20 °C)[58] and for being entire soluble in water and amphoteric.

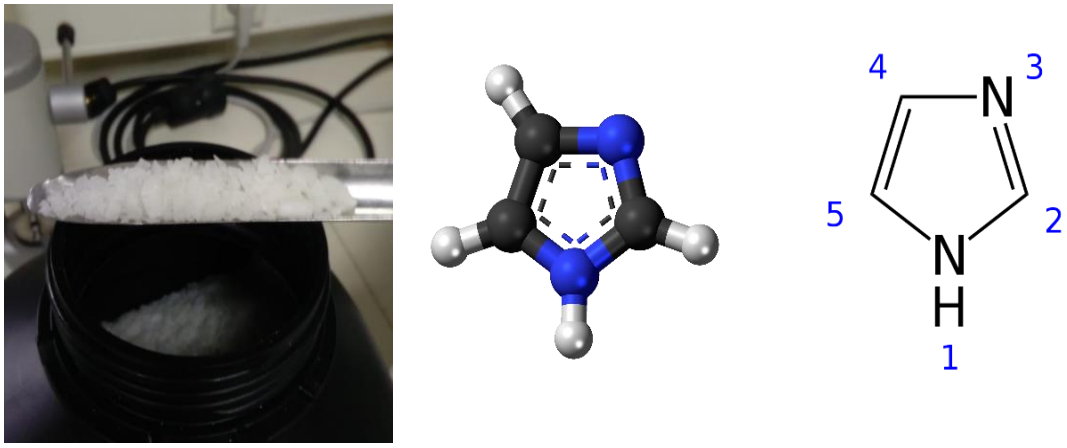

Figure 1.12 - Imidazole and its chemical structure[57].

1.7. Biomass pretreatment with Imidazole

Imidazole is an alkaline solvent and only recently explored for the pretreatment of lignocellulosic biomass[59-61]. Imidazole was only used as a solvent for starch dissolution[58] but was broadly applied as precursor of imidazolium-based ILs as described above. The imidazolium cation has a potential of π-stacking and possibility to form a hydrogen-bond-based interaction with lignin[62-63]. Despite their benefits, ILs have high costs associated with synthesis and purification of ILs. Organosolv processes, that involve the use of organic solvent, are nowadays an industrial practice. They have the advantage of efficient delignification.

Imidazole appears now as a candidate for biomass processing that can be used at industrial scale and at competitive costs. Its properties make it easy to handle and recycle, being an interesting cheap alternative to other expensive explored solvents, like ILs. While organosolvs and ILs permit to precipitate and recover lignin in an unchanged form, imidazole fractionates biomass into its respective polysaccharides and promoting the depolymerization of lignin leading to diverse range of polymers. Additionally, imidazole pretreatment can be performed at much lower temperatures than conventional pretreatments and without the need of additional catalysts[60]. One of the drawbacks of using imidazole might be the higher workup needed to recover it in comparison to other volatile solvents such as ethanol.

The pretreatment method was adapted from a previous work on ionic liquids, described elsewhere[56]. General procedure assumes and processing of the biphasic mixture (imidazole and lignocellulosic biomass at a certain w/w ratio) for a determined temperature and time with stirring. After this step, the components of the biomass will be regenerated, precipitated and filtered, one by one, in what is called of selective fractionation. The result will be solid fractions, one rich in cellulose and another rich in hemicellulose. The fractions aren't 100% pure because all compounds of the lignocellulosic biomass interact with imidazole, so, for example, the cellulose rich fraction will have hemicellulose and lignin as impurities that also regenerated and precipitated. In the end, a solution rich in imidazole and degraded lignin will go through an imidazole recovery process to recover the solid imidazole with the phenolic compounds imbued in his matrix.

The first research referring to the use of imidazole as a solvent for fractionation, delignification, and depolymerization of lignocellulosic material was reported in 2015, using wheat straw as biomass[59]. The effects of time and temperature in the pretreatment were studied: imidazole showed to be a successful solvent for biomass processing, being able to separate the wheat straw components, with noteworthy delignification. Cellulose-rich fractions up to 62.4% (w/w) of cellulose content and hemicellulose-rich fractions up to 54.5% (w/w) were achieved, reporting a maximum of 96.2% (w/w) of total lignin removal at 170 °C for 2h. Scanning electron microscopy verified that imidazole favors structural and morphological changes, while X-ray diffraction (XRD) and Fourier-transform infrared spectroscopy (FTIR) followed the crystallinity changes. Additionally, added-value phenolic compounds, like vanillin, were identified by capillary electrophoresis and HPLC-MS[2].

Imidazole was also studied in the delignification of elephant grass (*Pennisetum purpureum* Schum.)[60], as a continuation of the previous work. The authors reported on the optimization of pretreatment conditions (time and temperature) for the extraction of lignin, showing that the optimal conditions may vary with the type of biomass, while also producing polysaccharide-rich materials that can be easily converted to upgradable sugars. At temperatures up to 140 °C and reaction times up to 6h, 80% of initial lignin was removed, while producing cellulose-rich fractions up to 52.5% (w/w) of cellulose content.

There are no reports of imidazole pretreatment for *Cupressus lusitanica* or any other kind of softwood. However, pretreatments of softwood using steam explosion[64-66] and organosolv[67-68] based have been reported, with interesting results. These methods have the drawback of requiring high reaction temperatures and catalysts. Ionic liquids appear as a greener approach to the pretreatment of softwood. Some studies have been done is the past recent years[69-71] to optimize the pretreatment conditions and increase carbohydrates recovery and lignin removal.

Imidazole appears as an alternative solvent for the pretreatment of agricultural residues. Softwood has different biomass composition (higher lignin content) and requires optimization of pretreatment conditions. It is now important to study imidazole as a solvent for the pretreatment of softwood.

2. Objectives

The integration of dissolution, pretreatment, hydrolysis and conversion of lignocellulosic biomass into valuable products is one of the key issues to accomplish the economic efficiency and sustainability of lignocellulosic green biorefineries.

The main purpose of this work was to test imidazole as solvent for pretreatment of two different biomasses while also degrading lignin into valuable compounds. The biomasses used were wheat straw, one of the most common agricultural wastes in the world, and extracted solid residues of *Cupressus Lusitania*, which is a softwood. As it was referred, imidazole has previously demonstrated potential to fractionate wheat straw and to convert lignin in valuable compounds. To test its potential in the pretreatment of a softwood, a wide range of pretreatment conditions were examined to compare both biomasses in terms of composition of the solid fractions obtained in the pretreatment, efficiency of the fractionation and depolymerization of lignin. The imidazole ability to degraded lignin in phenolic compounds was tested by checking the phenolic profile, total phenolic contents and antioxidant activity of the extracts, while capillary electrophoresis and mass spectrometry were used to tentatively identify and structure proposal for some of these lignin degraded compounds. Furthermore, the recovery and purification of imidazole was tested to check for the economical sustainability of the pretreatment.

3. Materials and experimental methods

3.1. Materials

Two different lignocellulosic biomass, wheat straw and extracted residue of *Cupressus lusitanica*, were used in this work. Wheat straw was supplied by ECN (Energy Research Center of the Netherlands), from the Netherlands in the frame of AMBITION (Advanced biofuel production with energy system integration Work) project funded by the European H2020-programme under LCE-33 2016 European Common Research and Innovation Agendas (ECRIAs) in support of the implementation of the SET Action Plan., Grant Agreement 731263. Extracted residue of *Cupressus lusitanica* Mill. is the residual solid remaining after ultrasound-assisted extraction (UAE) with ethanol and subsequently with 70% (v/v) acetone aqueous solution of the hydrodestillation residues of native *Cupressus lusitanica* Mill. biomass (aerial parts, namely, leaves, cones and branches). *Cupressus lusitanica* Mill. biomass was collected by Silvapor®, Idanha-a-Nova, Portugal, and used for essential oil extraction by hydrodestillation at their premises. Hydrodestillation residues were then sent to LNEG for ethanol and 70% acetone UAE. The extracted residue was then supplied for the present work.

Both feedstocks were grounded with a knife mill using IKA® WERKE, MF 10 basic, Germany, to particles with size lower than 0.5 mm, homogenized in a defined lot and stored in plastic containers at room temperature.

For the pre-treatment experiments and post-reaction processing the following reagents were used: imidazole with a purity of 99% $w.w^{-1}$ purchased from Alfa Aesar, Karlsruhe, Germany; NaOH pellets (99% purity) supplied by Eka Chemicals/Akzonobel (Bohus/ Sweden); HCl 25% aqueous solution prepared from HCl 37% solution (VWR Chemicals/AnalaR NORMAPUR®, France) using ultrapure water (18.2 $M\Omega cm^{-1}$) produced by the PURELAB Classic of Elga system; HCl solution with pH 2 prepared from distilled water and HCl 25% solution; ethanol 96 % (v/v) and acetonitrile 99.9% purity, both from Carlo Erba Group, Aresa, Italy. For filtration, cloth (made from a cotton shirt), paper (ø = 150 mm, nº 1238, acquired from Filter-Lab®, Filtros Anoia, S.A. Barcelona) and nylon filters (0.45 µm, also from Filter-Lab®, Filtros Anoia, S.A. Barcelona) were used.

For determination of total phenolics content, Folin-Ciocalteu reagent (VWR Chemicals PROLAB®, France), Gallic acid (Sigma-Aldrich Chemie, Steinheim, Germany) and sodium carbonate anhydrous (Na_2CO_3) (Panreac, Spain) were used. In the determination of antioxidant activity, DPPH (2,2-diphenyl-2-picrylhydrazyl hydrate) and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), both from Sigma-Aldrich Chemie, Steinheim, Germany, were used.

Sodium tetraborate decahydrate (Merck – Darmstadt, Germany) and methanol (>99.9% from Honeywell/Riedel-de Haen™, Germany) were used for preparing the capillary electrophoresis (CE) electrolyte solution. Nylon syringe filters (Ø=13 mm, 0.22 µm porosity), used for sample filtration prior to analysis, were purchased from RedR analytical, Cambridgeshire, UK.

For high-performance liquid chromatography (HPLC) a solution of 5 mM sulfuric acid (H_2SO_4) by Panreac Química (Barcelona, Spain) was used as mobile phase. Standard samples of glucose, xylose and arabinose were used.

3.2. Biomass pretreatment with Imidazole

The pretreatment of biomass with imidazole was carried out on the basis of a previously developed method established in the host institution and published elsewhere[59]. Briefly, 5 g of air dried biomass and 45 g of imidazole were placed into a 100 mL Schott flask. The reaction vessel was then placed on the heating plate to guarantee continuous stirring and heating. Reaction time started to be counted from the moment when the desired temperature was reached. The reaction time and temperature are given in Table 3.1. When the process ended, the flask with the mixture was removed from the bath and cooled down to 90°C and 90 mL of distillate water was added slowly. Next, the obtained mixture was transferred to a 500 mL Erlenmeyer and stirred for 1 h. This step promotes the regeneration and precipitation of the cellulose. The resulting solution was filtered under vacuum using a cloth filter for fast filtration and the resulting solid fraction was washed in portions with 135 mL of ethanol (96% w/w) until the pH = 7 of the filtrate 1, to guarantee an efficient removal of imidazole.

Table 3.1 – Pretreatment conditions studied in this work

Time (h)	2		3	4	
Temperature (°C)	130	160	145	130	160

The solid fraction was dried for 24 h at 45°C in the oven Cassel ES.6 (Amadora, Portugal) and afterwards underwent an acid hydrolysis to quantify the sugars present, according to the methodology described elsewhere.[72]

To regenerate a second solid fraction rich in hemicellulose, water and ethanol were removed under reduced pressure at 72 mbar (Rotavapor Buchi R-210 (Switzerland)) from the filtrate 1, until a final volume of 45 mL was obtained. The pH of the solution was then adjusted to 6.8 with HCl 25% (w/w) controlled with Chemtrix Type 45AR pH controller. In the next step, 135 mL of ethanol 96% (w/w) was added to promote the hemicellulose precipitation. Analogously to cellulose regeneration, the solution was filtered under vacuum using a Nylon membrane filter (pore size 0.45 µm, slow filtration) and the resulting hemicellulose-rich fraction was washed with 135 mL of ethanol (96%) added in portions.

The hemicellulose-rich solid fraction was next dried for 24 h at 45 °C in the oven and similarly to the first solid, was subject to sugar quantification.[72]

Finally, ethanol was evaporated under reduced pressure from filtrate 2 and the pH of the solution was adjusted to 2 using HCl 25% (w/w). Such solution was next stirred for 30 min at 70 °C and filtered with filter paper to recover traces of residual lignin. The lignin-rich solid was washed with HCl solution with pH=22 and dried in oven at 45°C, while the filtrate 3 underwent the imidazole recovery.

Figure 3.1 shows a schematic representation of the experimental procedure.

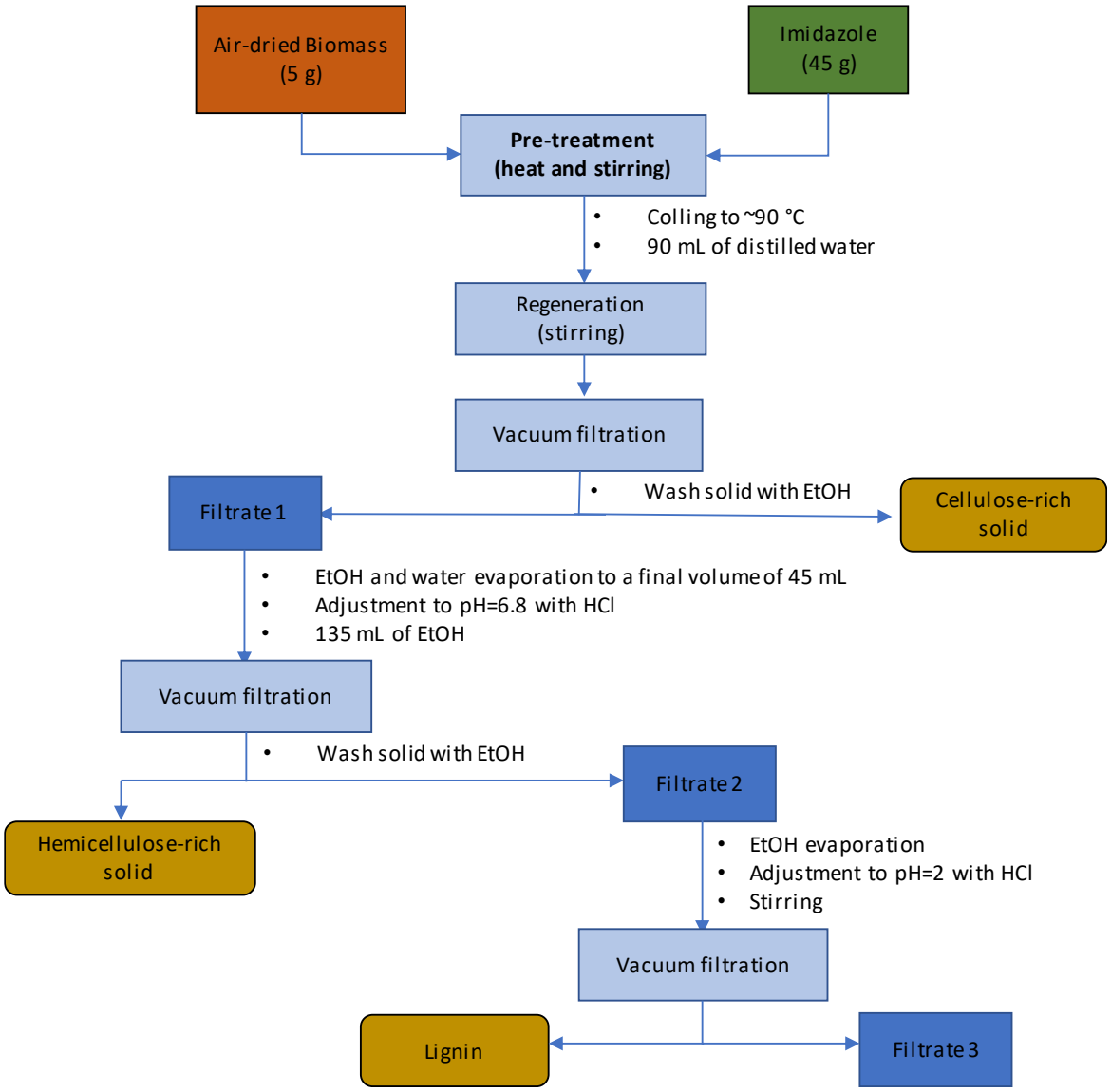

Figure 3.1 - Schematic representation of the pretreatment procedure (adopted from Morais et al.).

3.2.1. Imidazole recovery

For imidazole recovery, the method used was adapted from the one for ionic liquid recovery presented elsewhere [73]. For this purpose, filtrate 3 was neutralized with NaOH pellets to pH=10. Next, water was removed under reduced pressure and resulted in a solid formed by a mixture of NaCl and imidazole. Taking into consideration that NaCl is insoluble in acetonitrile, 130 ml of this solvent was added to dissolve the imidazole. NaCl was removed by vacuum filtration, while acetonitrile from filtrate was removed under reduced pressure (200 mbar) and the remaining solid was recovered and dried for 24h. The obtained solid was composed by imidazole and phenolic compounds imbued in his matrix. These phenolic compounds were next separated from imidazole using solid phase extraction (SPE) and analyzed by capillary electrophoresis (CE) and HPLC-Mass spectrometry (HPLC-MS), to obtain a phenolic profile and tentative identification. Figure 3.2 represents imidazole recovery.

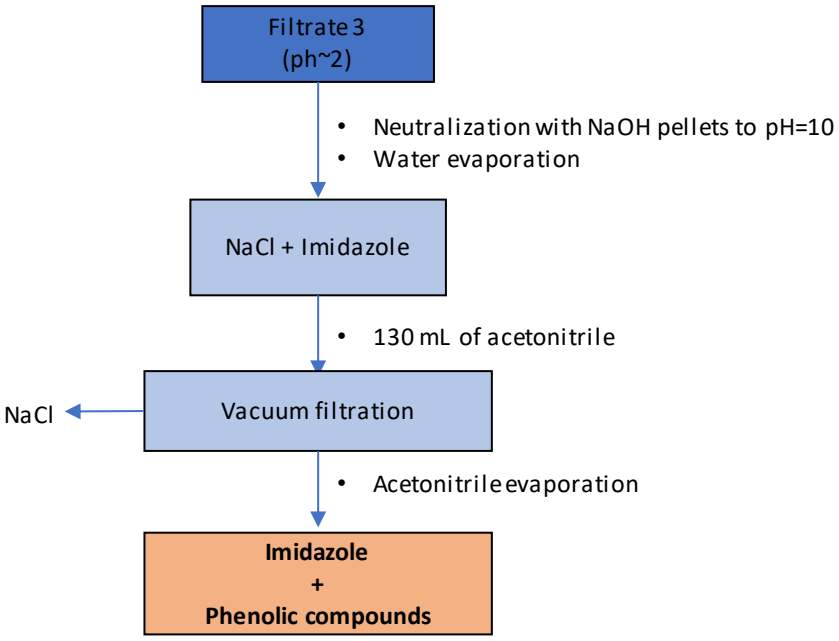

Figure 3.2 - Schematic of the imidazole recovery procedure.

Before CE and MS analysis, all the samples were subjected to SPE through a nonpolar surface as a clean-up procedure to concentrate and separate the phenolic compounds imbued in the solid imidazole matrix. Separation columns (C18 ec, 6 mL, 1000mg) from ChromabondR, (Macherey-Nagel GmbH & Co. KG, Duren, Germany) were used. The columns were preconditioned with 6 mL methanol and 6 mL water without letting the column run dry, using a high vacuum system (VacElut 20 Manifold Tall Glass Basin from Agilent Technologies, Santa Clara, CA, USA). The recovered imidazole fractions were dissolved in H_2O and resulted solutions were loaded in the columns. Ultrapure water was used to wash-out the water-soluble constituents and simultaneously remove the imidazole. The phenolic compounds were then eluted with 2 mL methanol and concentrated under vacuum prior to total phenolics, antioxidant activity, CE and MS analysis.

3.3. Solid analysis

Both biomasses and pre-treated solids were characterised to determine the moisture, total lignin and polysaccharide contents according to NREL methods.[72] The content of glucan and hemicelluloses (xylan, arabinan, and acetyl groups) was determined using high performance liquid chromatography (HPLC) . Furthermore, for native biomasses, total extractives, ash and protein contents were determined according to standard methods, namely: NREL/TP-510-42619,[74] NREL/TP-510-42622[75] and ISO 8968-1:2014,[76] respectively. All analyses were conducted in duplicate and are presented as mean values.

3.4. Analytical methods

3.4.1. HPLC for sugar and acid characterization

All liquid samples prior to HPLC analysis were filtered through Millipore filters with a pore diameter of 0.45 μm. The HPLC analyses were performed using an Agilent 1100 series HPLC system (Santa Clara, CA, USA) equipped with a Bio-Rad Aminex HPX-87H column (Hercules, CA, USA). The detection was performed using RID (refractive index detector) for monosaccharides (glucose, xylose and arabinose), and acetic acid using DAD (diode array detector) at 280nm wavelength. The quantitative analyses were performed by external calibration using standard solutions. Sulfuric acid (5 mM) at a flow rate of 0.6 mL/min (sample volume 5 μL) was used as mobile phase. Column temperature was 60 °C and detector temperature was 45 °C.

3.4.2. Capillary electrophoresis for phenolic profile

Electrophoretic analyses for the presence of phenolic compounds were carried out using an Agilent Technologies CE system (Waldbronn, Germany) equipped with a diode array detector (DAD). ChemStation data software (Rev B.04.01) and a fused-silica uncoated i.d. = 50 μm and total length of 62 cm (56 cm to the detector) extended light-path (increased sensitivity) capillary, were also from Agilent. The electrolyte was 15 mM sodium tetraborate decahydrate with 10% MeOH (used as organic modifier for improving the separation performance and selectivity of CE), adjusted to pH 9.1. The separation voltage was 30 kV with a 0.5 min ramp up and the current was at 120 μA max setting. The capillary temperature was kept at 30 °C during separations. Samples were dissolved in methanol/water (1:1) and filtered through a 0.45 μm membrane filter and were injected directly under the pressure of 50 mbar for 5 s at the anode (+) of the CE system. The capillary was preconditioned between runs by flushing with 0.1 M NaOH (3 min) followed by buffer (3 min). Electropherograms were recorded at 200, 280 and 320 nm, and phenolic compounds were identified by electrophoretic comparisons (migration times and UV spectra) with authentic phenolic standards.

3.4.3. Determination of total phenolic content

Total phenolics were determined by using the Folin-Ciocalteu colorimetric method according to an improved procedure described elsewhere[77]. In brief, the reaction mixture contained 100 μL of the extract sample dissolved in 50% (v/v) methanol/water with concentration of 1 mg/mL, 0.4 mL of ultrapure water, 0.25 mL of 1:1 (v/v) diluted Folin-Ciocalteu reagent and 1.25 mL of 20% (m/v) Na_2CO_3 in H_2O. Absorbance was measured at 725 nm in a UV/Vis spectrophotometer (Thermo Scientific™ Multiskan™ GO Microplate Spectrophotometer) after 40 min incubation in the dark at room temperature. A calibration curve of gallic acid was prepared (Appendix A). Total phenolics were converted to mg GAE (gallic acid equivalents)/g extract by means of a gallic acid standard curve. All experiments were carried out in triplicate.

3.4.4. Determination of antioxidant activity

Radical scavenging activity of extracts against stable $DPPH^{\cdot}$ (2,2-diphenyl-2-picrylhydrazyl hydrate) was determined spectrophotometrically. When $DPPH^{\cdot}$ reacts with an antioxidant compound, which can donate hydrogen, it is reduced. The changes in color (from deep-violet to light yellow) were detected at 515 nm on a

UV/Vis spectrophotometer (Thermo Scientific™ Multiskan™ GO Microplate Spectrophotometer). Radical scavenging activity of extracts was measured by a modified method presented elsewhere[78]. The solution of $DPPH^{\cdot}$ in methanol (60 µM) was prepared in the day, before UV measurements. 1950 µL of this solution were mixed with 50 µL of the same extract solution used for the determination of total phenolics (50 µL of ultrapure water for blank) and vortexed. The sample were kept in the dark for 30 min at room temperature and the absorption was measured. The decreasing of the DPPH solution absorbance indicated an increase of the DPPH radical-scavenging activity. The experiment was carried out in triplicate. A Trolox calibration curve was prepared (Appendix B). Antioxidant activity was expressed as Trolox equivalents (TE) (mM) by comparison to the Trolox standard curve and as radical scavenging-activity (%), calculated by the formula:

$$\%DPPH^{\cdot}\ inhibition = \left[\frac{Abs_b - Abs_s}{Abs_b}\right]$$

Where Abs_b is the absorption of blank sample and Abs_s is the absorption of tested extract solution.

3.4.5. HPLC-Mass spectrometry for tentative identification of compounds

Solutions of a mixture of lignin-based products dissolved in methanol were analyzed on a HPLC Dionex Ultimate 3000 composed of a binary pump HPG3200, an autosampler WPS300 and a column oven TCC3000 coupled in-line to an LCQ Fleet ion trap mass spectrometer equipped with an electrospray ion source (ESI) from Thermo Scientific. Separations were carried out with a HALO C18 (64.6 x 150 mm, 5 µm, AMT, Inc) at 35 °C constant temperature. Samples were injected into the column via a Rheodyne injector with a 100 µL loop, in the pickup injection mode. The mobile phase consisted of 1 mg/L ammonium formate in water solution (A) and 1 mg/mL ammonium formate in acetonitrile solution (B). The gradient adopted, at a flow rate of 0.2 mL/min, was as follows: 0 min B (5% v/v) isocratic, 30 min B (50% v/v) isocratic, 45 min B (70% v/v) isocratic, 70 min B (100% v/v) linear, 10 min B (equilibration time, 5% v/v). The flow rate was 0.2 mL/min and split out 0.4 mL/min. The mass spectrometer was operated in the ESI negative ion mode, with the following optimized parameters: ion spray voltage, -4.5 kV; capillary voltage, -18 V; tube lens offset, 58 V, sheath gas (N2), 80 arbitrary units; auxiliary gas, 5 arbitrary units; capillary temperature, 300 °C. Spectra typically correspond to the average of 20–35 scans and were recorded in the range between 50-1500 Da. Tandem mass spectra (MS^2) were obtained with an isolation window of 2 *m/z* units, a 20-30% relative collision energy and with an activation energy of 30 msec. Data acquisition and processing were performed using the Xcalibur software. A standard of 5% (v/v) of caffeic acid $5.56E10^{-4}$ M was used.

4. Results and discussion

4.1. Raw material composition

Wheat straw and the extracted residues of *Cupressus lusitanica* were the lignocellulosic biomass chosen to be pretreated with imidazole. The chemical composition of both oven-dried biomasses was determined and is shown in table 4.1, as weight % of the total dried biomass.

Table 4.1 – Average macromolecular composition of wheat straw and extracted residues of *Cupressus lusitanica* (in dry weight %)

	Composition (% w/w)	
	Wheat straw	**Extracted residues of *Cupressus lusitanica***
Cellulose[a]	38.8 ± 0.3	20.2 ± 0.4
Hemicellulose[b]	28.1 ± 0.2	16.2 ± 0.3
Xylan	20.5 ± 0.1	7.0 ± 0.1
Arabinosyl groups	3.1 ± 0.0	5.7 ± 0.2
Acetyl groups	4.6 ± 0.1	3.5 ± 0.0
Lignin	17.6 ± 0.1	38.5 ± 0.1
Ash	4.2 ± 0.1	6.2 ± 0.1
Moisture	8.1 ± 0.2	7.9 ± 0.0
Protein	4.5 ± 0.1	5.4 ± 0.0
Extractives	10.8 ± 0.2	27.2 ± 0.6
Ethanol extractives	1.4 ± 0.1	23.4 ± 0.6
Water extractives	9.4 ± 0.1	3.8 ± 0.1

[a]Measured as glucan; [b]Measured as sum of xylan, arabinosyl and acetyl groups

Wheat straw composition values observed in table 4.1 are within the expected range of values shown in Table 1.1 of this work. Furthermore, similar composition with a cellulose, hemicellulose and lignin content of 38.9% (w/w), 23.5% (w/w) and 18.0% (w/w), respectively, were reported in litererature[79].

The extracted residues of *Cupressus lusitanica* contain less structural carbohydrates (cellulose and hemicellulose) than wheat straw, but a lignin content of 38.5% ± 0.1 (w/w) was found. This is more than twice higher than observed in wheat straw. Also, the composition of the extracted residues of *Cupressus lusitanica* is very different from a composition of typical softwoods, given in Table 1.1. A major difference can be found in the carbohydrate content. No literature data of the composition of *Cupressus lusitanica* or other *Cupressus* trees have been found in literature. However, *Pinus radiata*, a pine from the same order (*Pinales*) as *Cupressus* and also a softwood, shows a composition of 45.3% (w/w) of cellulose, 22.2% (w/w) of hemicellulose and 26.8% (w/w) of lignin[80]. Another work by Berrocal et al. on *Pinus radiata* reports different composition dependent on the age of the tree, with cellulose varying from 31.1 to 42.5% (w/w), hemicellulose between 26.0 and 21.4% (w/w) and lignin 39.0 and 29.4% (w/w)[81]. Nevertheless, this difference in composition can expected, considering that the native biomass of the extracted residues of *Cupressus lusitanica* consisted of small

branches with sharp foliage and globular seed cones (See Figure 1.9) from the tree top, and not actual wood from the trunk of the tree. As it was stated above, there are no literature data about the macromolecular composition of native *Cupressus lusitanica*. The results obtain previously in this laboratory[82] reported a composition of 11.6 ± 0.2 % (w/w) cellulose, 9.1 ± 1.1 % (w/w) hemicellulose, 41.3 ± 0.1% (w/w) lignin and 38.0 ± 1.4 % (w/w) of others (extractives, protein and ash). It is clear that extracted residues of *Cupressus lusitanica* contains more polysaccharides that the native biomass. One of the reasons might be the fact that the extracted residues of *Cupressus lusitanica* were obtained after extraction treatments by vapor (hydrodestillation) and ultrasound with ethanol and acetone, as referred in the Materials and Experimental methods section. This resulted in the removal of some of the extractives and thus increasing the % of carbohydrates. The opposite tendency was found for lignin, which content decreased by 2.8 ± 0.2% (w/w), suggesting that employed extraction treatments are effective for some lignin removal[20].

Regardless the observed changes, one of the dominant fractions in extracted residues of *Cupressus lusitanica* is lignin. Therefore, this lignocellulosic biomass can be considered as potential feedstock to produce lignin derived compounds.

4.2. Pretreatment in Imidazole

The biomass pretreatment used in this work was carried out according to the method developed in the host institution and published elsewhere[59]. A range of temperatures between 130 and 160°C and reaction times between 2 and 4 hours with a constant imidazole:biomass mass ratio of 1:9 were examined.

Pretreatments with imidazole resulted in production of cellulose- and hemicellulose-rich fractions as well as on the delignification of biomass with the purpose to obtain phenolic compounds derived from lignin after imidazole pretreatment.

4.2.1. Wheat straw

All performed experiments resulted in two solid fractions composed mainly of polysaccharides (cellulose and hemicellulose). The macromolecular chemical composition of both fractions produced in wheat straw pretreatment as well as the % of recovered solids in both fractions is presented in Figures 4.1 and 4.2 for cellulose and hemicellulose, respectively.

Figure 4.1 shows that the mildest temperature (130 °C) studied have the highest solid recovery with a value of 73.7 ± 0.6% (w/w). An increase in the reaction temperature leads to the gradual decrease of the solid recovery achieving only 60.7 ± 0.7% (w/w) for the most severe reaction conditions, i.e. 160 °C/4 h.

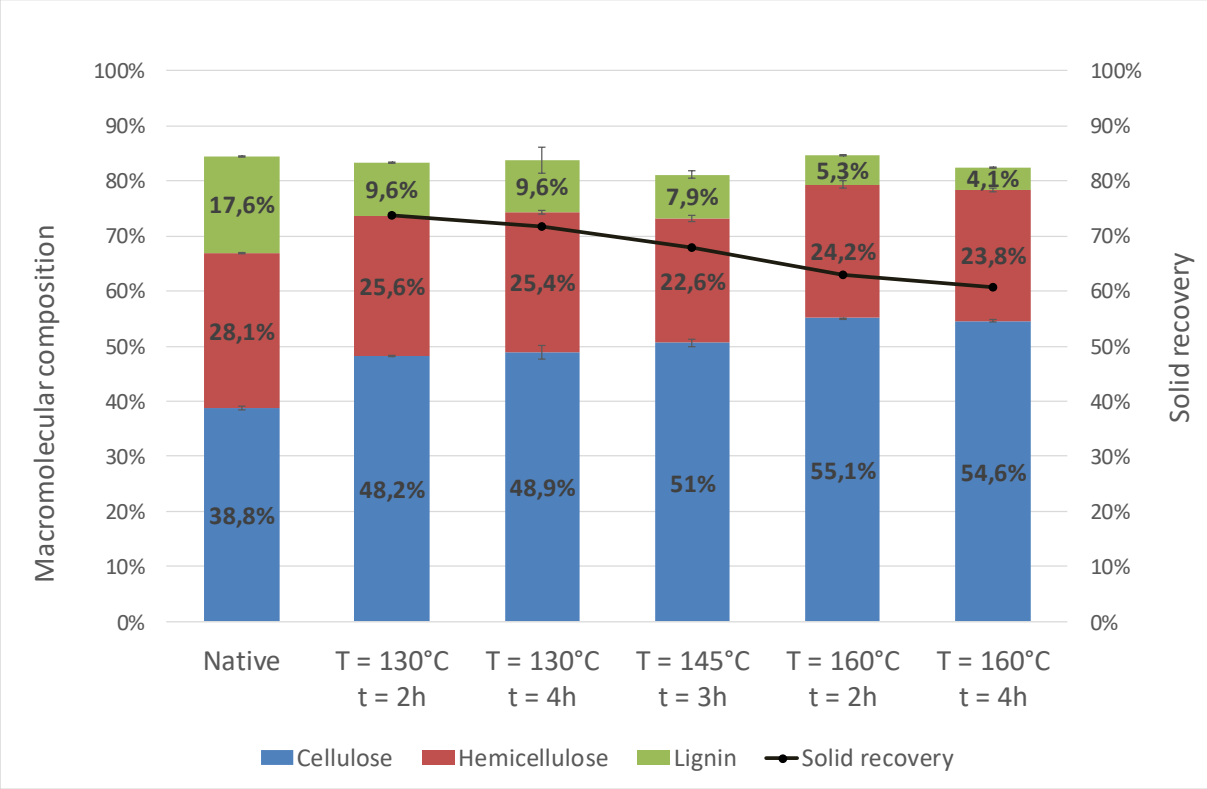

Figure 4.1 - Native wheat straw and cellulose-rich fraction compositions (% w/w) obtained from wheat straw pretreatment with imidazole at different reaction temperatures and time. The black line represents the recovered solids (% w/w). Cellulose measured as glucan content and hemicellulose measured as sum of xylan and arabinosyl group content.

Table 4.2 – Breakdown of cellulose, hemicellulose and lignin recovery in the cellulose-rich fraction in % w/w of the particular fraction in the native wheat straw used for reaction.

Cellulose-rich fraction	T = 130 °C		T = 145 °C	T = 160 °C	
	t = 2h	t = 4h	t = 3h	t = 2h	t = 4h
Cellulose recovery	91.5 ± 1.8	90.3 ± 5.3	88.5 ± 3.1	89.5 ± 1.7	85.4 ± 3.0
Hemicellulose recovery	67.0 ± 2.1	64.6 ± 2.1	54.6 ± 2.5	54.3 ± 2.9	51.4 ± 1.0
Lignin recovery	40.4 ± 0.5	39.0 ± 1.4	30.5 ± 4.0	19.1 ± 0.8	14.1 ± 0.3

Detailed analysis of the obtained results demonstrates that temperature is the dominant variable in comparison to the reaction time. As it can be observed in Figure 4.1, with an increase in temperature for the same reaction time, e.g. for 2h, the cellulose content increases from 48.2 ± 0.1% (w/w) at 130 °C to 55.1 ± 0.1% (w/w) at 160 °C. As comparison, for the same reaction temperature, an increase in the reaction time from 2 to 4 h had no influence on the cellulose content in the pretreated solids. For hemicellulose, an increase of temperature has insignificant influence, reducing the hemicellulose content only a little, e.g. for 130 °C/2h is 25.6 ± 0.5% (w/w) while for 160 °C/2h it drops to 24.2 ± 0.7% (w/w). An increase of the reaction time also has insignificant influence on the hemicellulose content, e.g. 25.6 ± 0.5% (w/w) and 25.4 ± 0.4% (w/w) for 130 °C for 2 and 4 h, respectively. Similarly to hemicellulose and cellulose contents in cellulose-rich fraction, the lignin content is significantly affected by temperature. For example, for 2h an increase of the reaction temperature from 130 °C to 160 °C guided the reduction of lignin content from 9.6 ± 0.0% (w/w) to 5.3 ± 0.1% (w/w). For 4h reaction time and the same reaction temperatures, a decrease was even more pronounced because it dropped

from 9.6 ± 2.4% (w/w) to 4.1 ± 0.0% (w/w). The results presented in Figure 4.1 are also demonstrated in Table 4.2 in the function of polysaccharide or lignin recoveries. As it can be noticed, an increase in temperature from 130 to 160 °C decreases the cellulose recovery from 91.5 ± 1.8 to 85.4 ± 3.0% (w/w) and the hemicellulose recovery from 67.0 ± 2.1 to 51.4 ± 1.0% (w/w), which is still high. A decrease in the cellulose and hemicellulose content, although notorious, is less significant than lignin recovery. An increase of the cellulose-rich fraction delignification is as high as 59.6 ± 0.5 to 85.9 ± 0.3% (w/w) when compared to lignin content in native wheat straw, respectively, for the least and the most severe reaction conditions. This result is not surprising, especially that alkaline pretreatment including imidazole[59-60] favors disruption of the ester bonds between lignin and hemicellulose, and fades the hydrogen bonds existing between lignin, cellulose, and hemicellulose[83].

Similar conclusions to those presented in this work have been reported for wheat straw processing with imidazole by Morais et al. [59]. They achieved 62.4% (w/w) of cellulose in cellulose-rich fraction with a lignin removal of 91.4% (w/w), at 170 °C/2h, which is a significant increase from the 42.2% (w/w) of cellulose content and 54.5% (w/w) lignin removal achieved at 110 °C/2h. Furthermore, they also report a reduction in solids and hemicellulose recovery % with an increase in reaction temperature. It is worth to underline that temperatures used in their work are higher than those reported in this experiment, which may explain differences in obtained results. Similar results were also reported by Toscan et al. [60]. They used imidazole for processing elephant grass and reported an increase in cellulose content from 40.3 (114.4 °C/57 min) to 52.5% (w/w) (135.6 °C/308 min), whereas lignin content dropped from 9.4% (w/w) at 125.0 °C/5.0 min to 4.6% (w/w) at 135.6 °C/308 min (increase in delignification from 50.7 to 81.8% (w/w)). Similarly to this work, they also reported a reduction in solid recovery yield with the increase in temperature, with 82.4% (w/w) yield for experiments performed at 114.4 °C/57 min, while for 140 °C/182.5 min the solid recovery yield was only 59.9% (w/w).

Comparing the obtained results to those reported in the literature for different biomass pretreatment technologies, (e.g. organosolv), it can be stated that the imidazole process is effective for delignification of biomass. Salapa et al. presented the organosolv pretreatments of wheat straw[84]. They reported a production of polysaccharide-rich fraction with 66.6% (w/w) of cellulose and 60% (w/w) lignin removal for pretreatment with 50% (v/v) ethanol at 180 °C/20 min and 23 mM H_2SO_4, while pretreatment with 50% (v/v) acetone at 180 °C/40 min and 23 mM H_2SO_4 allowed to achieve 76.4% (w/w) delignification with 67.2% (w/w) cellulose. The same authors also studied reactions at 160 °C, i.e. temperature comparable to those used herein. At this temperature, the maximum delignification achieved was 65% (w/w) with acetone as solvent, 40 min reaction and 23 mM H_2SO_4. These results are considerable worse than the delignification achieved in this work. Also, Wildschut et al. studied organosolv pretreatment of wheat straw[85] and obtained a high purity polysaccharide fraction with 75% (w/w) of cellulose and a delignification of 75% (w/w) at 190 °C, 60% aqueous ethanol, 60 min reaction time and 30 mM H_2SO_4. Although these results seem to be better than those reported herein, it is important to underline that in both above-mentioned works, temperature used was much higher and additional external catalysts were used. Wildschut et al. also studied the influence of temperature in the pretreatment without any catalyst[85]. They achieved a delignification of only 4.7 and 14.4% (w/w) for 160 and 170 °C respectively. It is also worth to mention that novel organosolv pretreatments at lower temperatures

have been studied. Park et al. pretreated corn stover and achieved 90.3% (w/w) of delignification with a flow-through process, at 150 °C/60 min reaction in a process with an aqueous solution of 30% (w/w) ethanol and 10% (w/w) H_2O_2 [86].

It is also important to compare data obtained in this work to those reported for ionic liquids (ILs). As a precursor of ILs, imidazole can also be considered as a cheaper alternative to ILs in biomass processing[59]. Ren et al.[87] studied the pretreatment of wheat straw with nine novel renewable cholinium-based ILs and reported a maximum delignification of 68.8% (w/w) with cholinium taurate under N_2 and stirred at 90 °C/6h, which is a milder temperature than those of this work. da Costa Lopes et al. reported a solid fraction with 83.4% (w/w) cellulose with only 2.8% (w/w) residual lignin obtained with 1-butyl-3-methylimidazolium thiocyanate at 120 °C for 6h with a 5% (w/w) biomass/IL ratio[73]. The same author also studied biomass fractionation with 1-ethyl-3-methylimidazolium acetate, under the above-mentioned conditions, obtaining carbohydrate-rich materials and a separated lignin fraction with 87% (w/w) purity[55]. Brandt et al. tested different 1-butyl-3-H-imidazolium hydrogen sulfate and found that this IL was able to remove up to 93% (w/w) lignin present in raw material in the process carried out at 120 °C/20 h of reaction[88]. Even though ionic liquids reactions achieve similar results at similar temperatures to those used in this work, imidazole is much cheaper reagent than most of ILs tested so far.

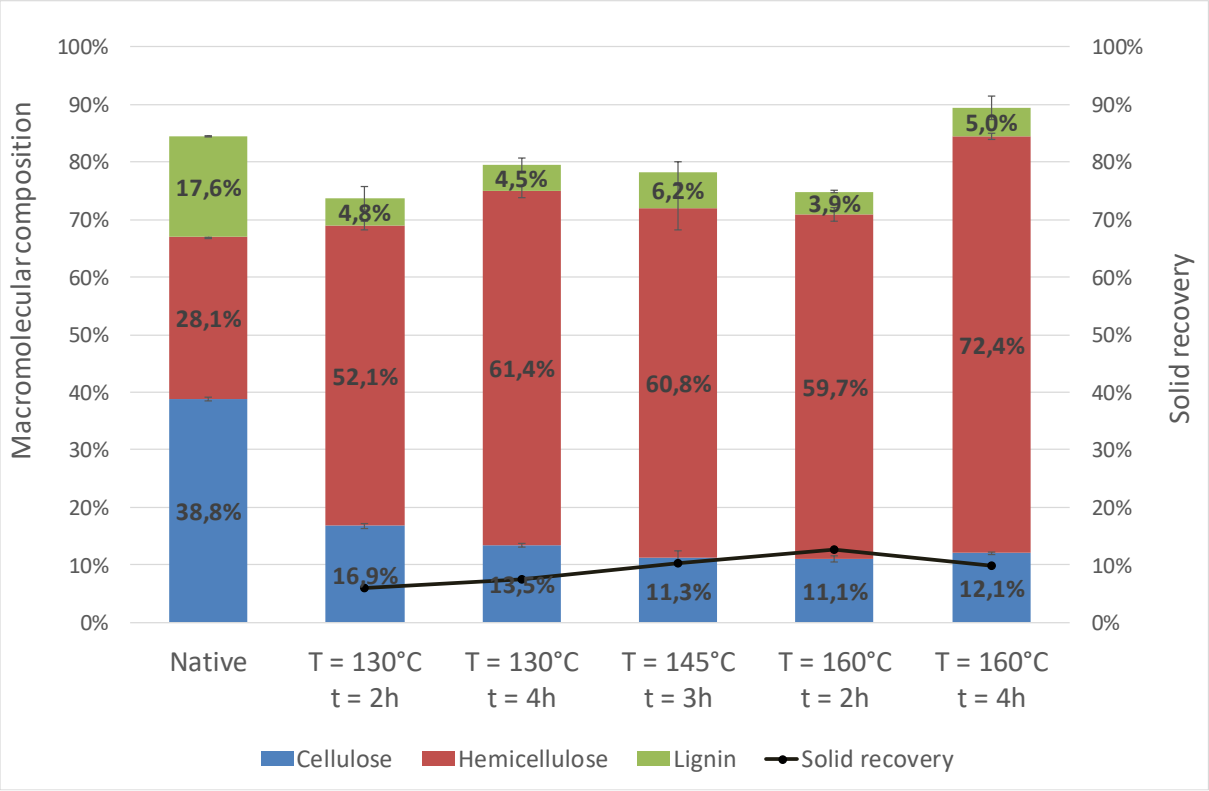

Figure 4.2 - Native wheat straw and hemicellulose-rich fraction compositions (% w/w) obtained from wheat straw pretreatment with imidazole at different reaction temperatures and time. The black line represents the recovered solids (% w/w). Cellulose measured as glucan content and hemicellulose measured as sum of xylan and arabinosyl group content.

Table 4.3 – Breakdown of cellulose, hemicellulose and lignin recovery in the hemicellulose-rich fraction in % w/w of the particular fraction in the native wheat straw used for reaction.

Hemicellulose-rich fraction	T = 130°C		T = 145°C	T = 160°C	
	t = 2h	t = 4h	t = 3h	t = 2h	t = 4h
Cellulose recovery	2.6 ± 1.8	2.6 ± 0.1	3.0 ± 0.6	3.6 ± 0.3	3.1 ± 0.1
Hemicellulose recovery	11.2 ± 0.3	16.4 ± 0.5	22.2 ± 2.1	26.9 ± 0.9	25.5 ± 0.5
Lignin recovery	1.6 ± 1.0	2.0 ± 0.7	3.6 ± 1.7	2.8 ± 0.3	2.8 ± 1.7

The second recovered fraction was the hemicellulose-rich solid. Figure 4.2 shows that an increase in temperature causes an increase in solid recovery too. For example, at 2h reaction time, an increase in temperature from 130 to 160 °C causes an increase in solid recovered from 6.0 ± 0.0 to 12.7 ± 0.1% (w/w).

Observing the obtained data in figure 4.2, it can be noticed that an increase in temperature leads to an increase in hemicellulose content in the corresponding solid fraction. For example, for 2h, the hemicellulose content increases from 52.1 ± 0.7 to 61.4 ± 1.0% (w/w) at 130 °C and 160 °C, respectively. Additionally, and in contrast to what was observed for the cellulose-rich fraction, reaction time leads to a significant increase in the hemicellulose content. At 130°C, an increase of reaction time from 2 to 4 h leads to 18% increase in hemicellulose content, while at 160 °C the same 2h increase leads to 21% increase. Therefore, it can be concluded that both temperature and reaction time influence the solid composition in a similar manner. Regarding the cellulose and lignin content in this fraction, one can observe that pretreatment conditions do not have a significant effect in their content. This result is also demonstrated in table 4.3 where cellulose recovery is between 2.6 $\pm$ 1.8% (w/w) and 3.6 $\pm$ 0.3% (w/w) while for lignin it is between 1.6 $\pm$ 1.0% (w/w) and 3.6 $\pm$ 1.7% (w/w). Similar conclusions were presented by Morais et al. [59]. They achieved an increase of hemicellulose content in the hemicellulose-rich fraction with an increase in temperature, from 37.4% (w/w) in the least severe conditions, i.e. 110 °C/2h, to 54.5% (w/w) for the most severe conditions, i.e. 170 °C/4h. However, they concluded that reaction time have negligible effect on polysaccharides composition in the solid's fractions. Even though that is the case for the cellulose-rich fraction, it is not visible for the hemicellulose-rich fraction obtained in this work, where reaction time influences the hemicellulose content. It is also important to point out that both cellulose and lignin contents as well as their recoveries barely change for different pretreatment conditions, as described above. This is also different from what was reported by Morais et al, where the cellulose content in the hemicellulose-rich fraction diminished with temperature from 35.9% (w/w) at 110 °C/2h to 17.2% (w/w) at 170 °C/4h.

Comparing the results obtained for ionic liquids use, da Costa Lopes et al. reported a hemicellulose-rich fraction with 85% (w/w) hemicellulose, obtained using 1-ethyl-3-methylimidazolium acetate[55], and 70% (w/w) using 1-butyl-3-methylimidazolium thiocyanate[73], both at 120 °C/6h with a 5% (w/w) biomass/IL ratio. Therefore, considering the results obtained in the present study, it is possible to conclude that wheat straw pretreatment with imidazole allows effective fractionation towards carbohydrate-rich fractions and high delignification yields, which are comparable to those obtained with organosolv or ionic liquid pretreatment, at milder or equally mild conditions, without any catalysts and with faster reaction times.

4.2.2. Extracted residues of *Cupressus lusitanica*

To compare the pretreatment efficiency between both biomasses, the same procedure and conditions were applied in the pretreatment of extracted residues of *Cupressus lusitanica*. All performed experiments resulted in two solid fractions composed mainly of polysaccharides (cellulose and hemicellulose). The macromolecular chemical composition of both fractions produced during pretreatment as well as the % of recovered solids from the initial biomass content in the respective fraction, are presented in Figures 4.3 and 4.4, respectively.

Figure 4.3 shows that solid recovery decreases with temperature, from 50.1 ± 0.1% (w/w) at 130 °C/2h to 44.2 ± 0.7% (w/w) at 160°C/2h, while reaction time have a negligible effect on it. It can be observed that with an increase in temperature for the same reaction time, e.g. for 2h, the cellulose content increases from 35.9 ± 0.1% (w/w) at 130 °C to 39.9 ± 0.2% (w/w) at 160 °C. For hemicellulose, to counterbalance the increase in cellulose content, an increase in temperature reduces the hemicellulose content, e.g. for 4h reaction time, 18.6 ± 0.1% (w/w) is achieved at 130 °C while for 160 °C it drops to 15.9 ± 0.3% (w/w). An increase of the reaction time also has negligible effect on the hemicellulose content, e.g. 18.9 ± 0.5% (w/w) and 18.6 ± 0.1% (w/w) at 130 °C for 2 and 4h, respectively. Additionally, a high lignin content was observed in this solid fraction for all pretreatment conditions, but without any apparent effect of the temperature and reaction time on the lignin content. For example, for 2h an increase in temperature from 130 °C to 160 °C led to an increase of lignin content from 30.4 ± 0.7 to 32.0 ± 0.7% (w/w).

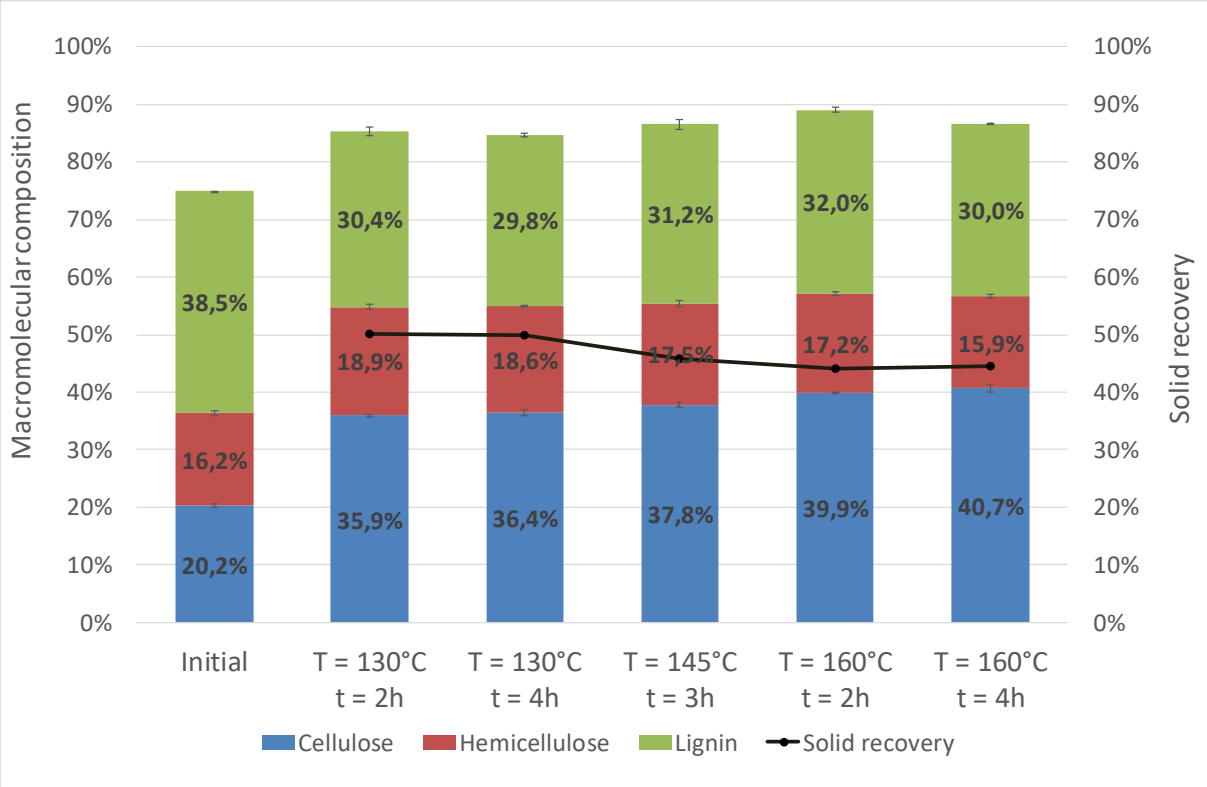

Figure 4.3 - Initial extracted residues of *Cupressus lusitanica* and cellulose-rich fraction compositions (% w/w) obtained from the extracted residues of *Cupressus lusitanica* pretreatment with imidazole at different reaction temperatures and time. The black line represents the recovered solids (% w/w). Cellulose measured as glucan content and hemicellulose measured as sum of xylan and arabinosyl group content.

Table 4.4 – Breakdown cellulose, hemicellulose and lignin recovery in the cellulose rich fraction in % w/w of the particular fraction in the initial extracted residues of *Cupressus lusitanica* used for reaction.

Cellulose-rich fraction	T = 130°C		T = 145°C	T = 160°C	
	t = 2h	t = 4h	t =3h	t = 2h	t = 4h
Cellulose recovery	89.0 ± 2.8	89.7 ± 4.6	85.8 ± 4.3	87.1 ± 3.9	89.8 ± 4.4
Hemicellulose recovery	58.5 ± 4.4	57.0 ± 3.1	49.7 ± 4.8	47.0 ± 3.8	43.8 ± 3.0
Lignin recovery	39.6 ± 1.5	38.5 ± 0.86	37.4 ± 1.8	37.1 ± 1.2	34.8 ± 0.4

The results presented in Figure 4.3 are also demonstrated in Table 4.4 in the function of polysaccharide and lignin recoveries. As it can be noticed, an increase in temperature from 130 to 160 °C for fixed reaction time, e.g. 2h, decreases the hemicellulose recovery from 58.5 ± 4.4 to 47.0 ± 3.8% (w/w) and increases the lignin removal from 61.4 ± 1.5 to 62.9 ± 1.2% (w/w). Unlike the hemicellulose and lignin recovery, the severity of the pretreatment conditions produces insignificant changes in the cellulose recovery.

There are no literature reports on the imidazole pretreatment of *Cupressus lusitanica* or any other softwood. However, one of the most studied softwood pretreatment methods was steam explosion, with and without acid catalyst[66]. One of such work deals with Lodgepole pine where a solid fraction with 52.4% (w/w) of cellulose, 1.6% (w/w) of hemicellulose and 45.9% (w/w) of lignin was achieved with a steam pretreatment performed at 200 °C and 4% SO_2 (w/w) for 5 min[65]. Although these results are very interesting, a main drawback of the use of strong acid catalysts, such as H_2SO_4 or SO_2, is formation of degradation products, such as furfural and HMF, as well as a need of high temperatures [89].

Some organosolv pretreatments have been developed and studied for softwoods too. Park et al. studied different catalysts with 1:1 ethanol:water mixture (v/v) in the pretreatment of *Pinus rigida*[67]. The use of 2% sodium hydroxide (w/v) as catalyst, at 190 °C/20 min, reduced lignin content considerably from 26 (native) to 12% (w/w), i.e. a delignification yield of 55% (w/w), which is comparable to values achieved in this work. Another example is Sannigrahi et al. who studied an organosolv pretreatment of Loblolly pine (*Pinus taeda*)[68]. With the use of an aqueous solution of 65% (v/v) ethanol containing 1.1% sulfuric acid (by weight of wood) as catalyst, at 170 °C/1h, they reached 79% (w/w) of cellulose recovery as well as a delignification yield of 61% (w/w) in the solids fraction. Again, the use of catalysts and high temperatures are drawbacks of this kind of pretreatment, and a more environmentally friendly and cheaper solvent is needed.

Also, ionic liquids were used in pretreatment of softwood biomass. Trinh et al. studied the effect of temperature and reaction time for the pretreatment of mix softwood[90] (*Pinus rigida* and *Pinus densiflora*) using 1-butyl-3-methylimidazolium and, similarly to this work, reported a decrease in solids recovery from 91% (w/w) to 73% (w/w) as pretreatment temperature increased from 70 °C to 130 °C for 18h reaction time. Moreover, the recovery of carbohydrates in the pretreated biomass decreased with an increase in temperature. On the other hand, the lignin content slightly increased (from 31 to 39% (w/w)) as the pretreatment temperature increased from 70 to 130 °C. Regarding the effects of reaction times in the composition and recovery of solids, a decrease in the recovery of solids and carbohydrates was observed with an increase of pretreatment time from 5h to 24h at temperatures of 120 °C and 130 °C, respectively. The IL pretreatment of softwood at 120 °C showed negligible changes in the carbohydrates and lignin content with reaction time when compared to the

untreated sample. However, pretreatment at slightly higher temperature (130 °C) resulted in considerable decrease in solid and carbohydrates recovery. The recovery of solids and cellulose were measured to be 53% (w/w) and 49% (w/w), respectively, in the pretreated biomass at 130 °C/24 h reaction, which are values comparable to the values present in this work, when at 5h reaction time the recovery was of 92 and 93% (w/w), respectively. It is understood that the lower recovery of carbohydrates at higher temperature and longer reaction times is related to the hydrolysis and dissolution of fermentable sugars into the liquid fraction[70, 91]. In another work[71], the same authors achieved cellulose-rich solids with cellulose contents between 41.1 and 45.5% (w/w), reporting an increase in cellulose content and decrease in hemicellulose content with an increase in temperature from 70 to 130 °C in those solids. Interestingly and similarly to the present work, they also reported that the total lignin content was kept at about 33% and did not varied for different pretreatment conditions, indicating that IL pretreatment caused insignificant biomass delignification.

It is worth to mention that none of the works reported above did any kind of extraction treatment before the pretreatment procedure and all have lower lignin content in the native biomass.

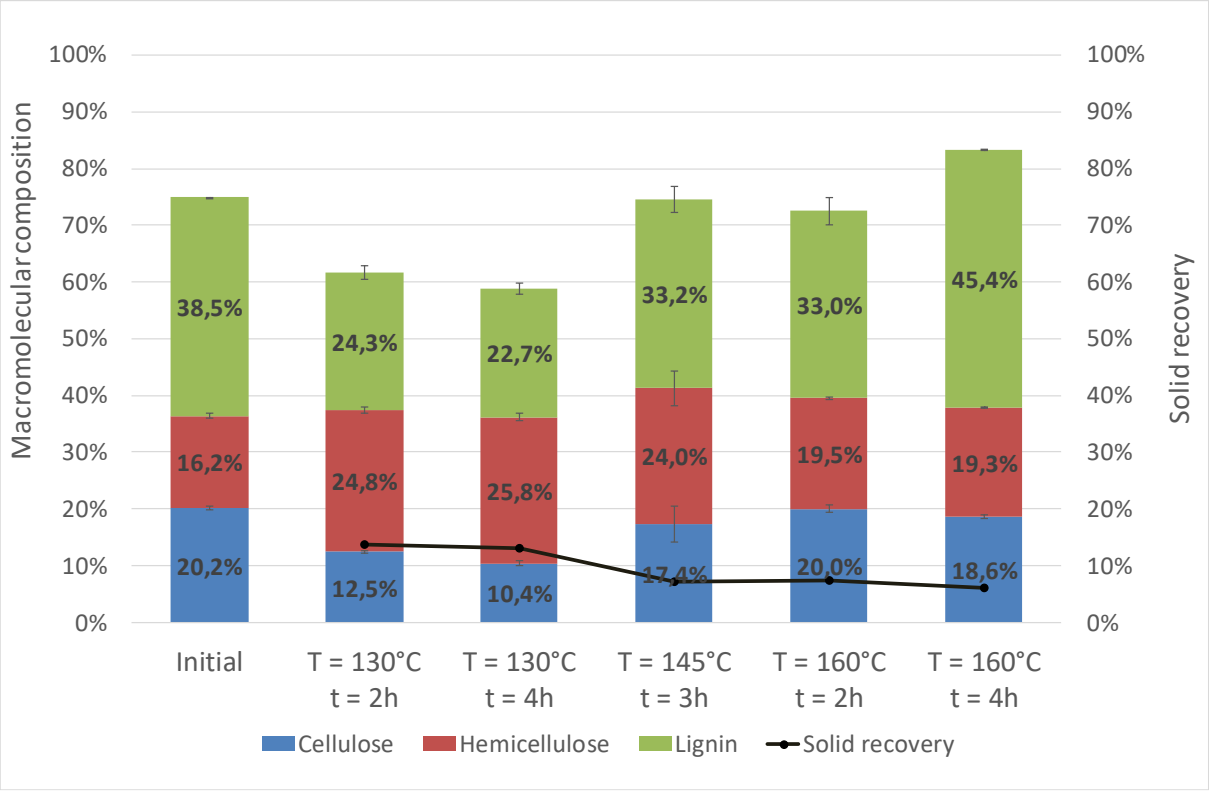

Figure 4.4 - Initial extracted residues of *Cupressus lusitanica* and hemicellulose-rich fraction compositions (% w/w) obtained from the extracted residues of *Cupressus lusitanica* pretreatment with imidazole at different reaction temperatures and time. The black line represents the recovered solids (% w/w). Cellulose measured as glucan content and hemicellulose measured as sum of xylan and arabinosyl group content.

Table 4.5 – Breakdown of cellulose, hemicellulose and lignin recovery in the hemicellulose rich fraction in % w/w from the initial content of extracted residues of *Cupressus lusitanica* used for reaction.

Hemicellulose-rich fration	T = 130°C		T = 145°C	T = 160°C	
	t = 2h	t = 4h	t =3h	t = 2h	t = 4h
Cellulose Recovery	8.5 ± 0.4	6.7 ± 0.6	6.1 ± 0.7	7.4 ± 0.6	5.5 ± 0.3
Hemicellulose recovery	21.0 ± 1.5	20.7 ± 1.5	10.5 ± 1.4	9.1 ± 0.5	7.2 ± 0.3
Lignin recovery	8.6 ± 0.6	7.7 ± 0.5	6.1 ± 0.4	6.4 ± 0.7	7.1 ± 0.0

The second recovered fraction was the hemicellulose-rich solid. Figure 4.4 shows that the percentage of solids recovered lowers with temperature, reaching as low as 6.0 ± 0.0% (w/w). In addition, figure 4.4 shows that reaction time have a negligible effect on the carbohydrate content and that the dominant variable is the reaction temperature. The hemicellulose content in this solid fraction decreases with the increase in temperature, reaching as much as 24.8 ± 0.6% (w/w) at 130 °C and decreasing to 19.5 ± 0.2% (w/w) at 160 °C, for 2h reaction time. For 4h reaction time, the same increase in temperature leads to a decrease in hemicellulose content from 25.8 ± 0.6 to 19.3 ± 0.1% (w/w). This decrease is counterbalanced by an increase in cellulose content in the hemicellulose-rich fraction associated to an increase in the pretreatment temperature. For example, for 2h, an increase of temperature from 130 to 160 °C leads to an increase by 86% in cellulose content.

Even though it is called hemicellulose-rich fraction[59], the hemicellulose content is rather low and increases only up to a maximum of 25.8 ± 0.6% (w/w). A major constituent of this fraction is lignin, whose content varies with time and temperature, increasing from 24.3 ± 1.2 to 33 ± 2.4% (w/w) at 130 °C/2h and 160 °C/2h, respectively, and from 22.7 ± 1.0% (w/w) at 130 °C/4h to 45.4 ± 0.1% (w/w) at 160 °C/4h.

Therefore, it is possible to conclude that with the pretreatment of extracted residues of *Cupressus lusitanica* in imidazole, a cellulose-rich fraction can be obtained. In addition, even though high delignification yields were achieved, a high lignin content remained in that fraction.

4.2.3. Comparison between wheat straw and the extracted residues of *Cupressus lusitanica* pretreatments

Both biomasses were subject to the same pretreatment procedure, where two variables (temperature and time) were studied to compare the efficiency of pretreatment. The efficiency was evaluated in terms of purity of both carbohydrate-rich fractions and in terms of hemicellulose and lignin removal, with alteration of the biomass structure and production of phenolic compounds derived from lignin.

Temperature and reaction time have similar effect on the solid yields because the solids recovery decreases with the increase in temperature and time for both biomasses. Regarding the cellulose-rich fraction, (Figure 4.1 and 4.3), the lowest cellulose content was achieved for the mildest conditions (130 °C), e.g. 48.2 ± 0.1 and 35.9 ± 0.1% (w/w) for wheat straw and the extracted residues of *Cupressus lusitanica*, respectively, while the highest cellulose content (55.1 ± 0.1 and 40.7 ± 0.6% (w/w), for wheat straw and the extracted residues of *Cupressus lusitanica*, respectively) was achieved for the most severe conditions used in this study (160 °C/4h). Analyzing data presented in Table 4.2 and 4.4 it can be also concluded that lignin and hemicellulose recoveries decrease predominantly with temperature, while the cellulose recovery shows negligible changes. Therefore, this indicates that a purity of cellulose in the cellulose-rich fraction increases with temperature and this is valid for both biomasses studied. Another similarity between wheat straw and the extracted residues of *Cupressus lusitanica* cellulose-fractions is that both present high hemicellulose content (wheat straw has a minimum of 51.4 ± 1.0% (w/w) of hemicellulose recovered while the extracted residues reached a minimum of 43.8 ± 0.3% (w/w)), suggesting that this step of the pretreatment procedure is not effective in separation of polysaccharides. One of the differences is the lignin content. The lignin content in the cellulose-rich fraction of wheat straw decreases with temperature, while in the extracted residues of *Cupressus lusitanica* the lignin content does not vary with pretreatment conditions. In addition, wheat straw pretreatment reaches delignification yields as high as 85.9 ± 0.3% (w/w) for 160°C/4h, indicating that imidazole is more efficient in the delignification of wheat straw than for extracted residues from *Cupressus lusitanica,* for which delignification yield was 65.2 ± 0.4% (w/w) at the same conditions.

The hemicellulose-rich fractions (Figure 4.2 and 4.4) show more pronounced differences for both biomasses. The hemicellulose content in sample produced from wheat straw demonstrate an increase with temperature from 52.1 ± 0.7 and 72.4 ± 0.6% (w/w), where the lowest hemicellulose content corresponds to the mildest conditions and the highest to the most severe reaction conditions. Hemicellulose recovery also increases with temperature while lignin and cellulose do not reveal significant changes, resulting in an increase of the purity of hemicellulose in these fractions, analogously to the cellulose fraction. The highest purity was achieved at the most severe reaction conditions, i.e. 160 °C/4h reaction time. As mentioned above, even though the hemicellulose content in the cellulose-rich solid does not vary significantly with pretreatment conditions (Figure 4.1), the hemicellulose recovery yield lowers with the increase of temperature (Table 4.2). This means that less hemicellulose is recovered in that fraction. A progressive diminishing of hemicellulose is counterbalanced by the increase of hemicellulose content in the hemicellulose-rich fraction (Figure 4.2). On the other hand, fractions obtained from extracted *Cupressus lusitanica* have a low hemicellulose content, which

decreases even more with temperature. The opposite trend has been observed for a lignin content that increases with an increase in time and temperature.

The overall mass balance for the pretreatment of each of the main components are given in table 4.6 and 4.7, for wheat straw and for the extracted residues *Cupressus lusitanica*, respectively.

Table 4.6 – Cellulose, hemicellulose and lignin recovery from all fractions, in % w/w from the initial wheat straw content used for reaction

Wheat straw	T = 130 °C		T = 145 °C	T = 160 °C	
	t = 2h	t = 4h	t = 3h	t = 2h	t = 4h
Total cellulose recovery	94.1 ± 1.9	92.9 ± 5.4	91.5 ± 3.7	93.2 ± 2.0	88.5 ± 3.1
Total hemicellulose recovery	78.1 ± 2.4	81.1 ± 2.6	76.7 ± 4.7	81.2 ± 3.8	76.9 ± 1.5
Total lignin recovery	44.4 ± 1.6	41.8 ± 1.5	40.5 ± 5.8	22.1 ± 1.1	18.1 ± 2.0

Table 4.7 – Cellulose, hemicellulose and lignin recovery from all fractions, in % w/w from the initial content of extracted residues of *Cupressus lusitanica* used for reaction.

Cupressus lusitanica	T = 130 °C		T = 145 °C	T = 160 °C	
	t = 2h	t = 4h	t = 3h	t = 2h	t = 4h
Total cellulose recovery	97.5 ± 3.2	96.3 ± 5.2	91.9 ± 5.1	94.6 ± 4.4	95.3 ± 4.7
Total hemicellulose recovery	79.5 ± 5.9	77.8 ± 4.6	60.2 ± 5.4	56.0 ± 4.3	50.9 ± 3.4
Total lignin recovery	51.9 ± 2.1	52.0 ± 1.4	48.3 ± 1.2	48.7 ± 1.9	58.9 ± 0.5

Total cellulose recovery is high in both biomasses exceeding 90 % (w/w) in almost all cases. The main difference can be observed for hemicellulose and lignin recoveries in wheat straw and extracted residues of *Cupressus lusitanica*, correspondingly. In wheat straw, pretreatment conditions show negligible effect in the total hemicellulose recovery, while in extracted residues of *Cupressus lusitanica*, the hemicellulose recovery is similar at 130 °C but drops to a minimum of 50.9 ± 3.4% with an increase in temperature to 160 °C. This suggests that the extracted residues of *Cupressus lusitanica* are more susceptible to hemicellulose degradation at higher temperatures.

Regarding lignin content, the obtained results are opposite to what was observed for hemicellulose. Pretreatment of wheat straw demonstrated that lignin recovery decreases predominantly with an increase in temperature, e.g. from 41.8 ± 1.5% (w/w) to 18.1 ± 2.0% (w/w) at 4h reaction time for 130 and 160 °C respectively. This confirms the previous statement that imidazole pretreatment is more efficient in the delignification of wheat straw than for the extracted residues of *Cupressus lusitanica*. One of the reasons might be that softwoods are more recalcitrant than hardwoods and agricultural residues, making them much more challenging for efficient pretreatment. This is attributed to their more rigid structure and higher lignin content, compared to the two other[89]. Most of the lignin in wood is bonded to hemicellulose components like a cementing agent, resulting in a complex and inaccessible structure. The differences observe in this work could also be due to the variations in the lignin-carbohydrate association, the lignin distribution, or the lignin

structure itself in agricultural residues and softwoods, which is mostly composed of guaiacyl units, while agricultural wastes contain not only guaiacyl, but also syringyl and p-hydroxyphenyl units[70]. Additionally, the UAE treatment used in the production of extracted residues of *Cupressus lusitanica* may have enhanced these effects.

One explanation for total cellulose and total hemicellulose recovery not reaching 100% in both biomasses may be due to the release of sugar by partial degradation of biomass during the dissolution and precipitation process. It was already mentioned that the lower recovery of carbohydrates at higher temperature and longer reaction times is related to the hydrolysis and dissolution of polysaccharide into the liquid fraction[70, 91]. The hydrolysis of polysaccharides, specially hemicelluloses has been well reported in literature[92-93]. This in turn leads to the formation of humins or pseudo-lignin[94], agglomeration products of furans and saccharides, which are often detected as an increase in lignin recovery as observed for extracted residues of *Cupressus lusitanica* for the most severe reaction conditions, also explaining why the hemicellulose recovery decreases for this conditions.

4.3. Imidazole Recovery

The recovery of imidazole was performed according to the method developed for ILs presented elsewhere[55]. After neutralization of the liquor with NaOH pellets and removal of NaCl formed by vacuum filtration, imidazole is obtained solid with not only degraded lignin compounds imbued in his matrix, providing a goldish color to the solid, but also remains of cellulose and hemicellulose that weren't removed in previous steps, as well as other degradation products that may exist. The yield was calculated from the initial imidazole mass used for the reaction, ~45 g of imidazole, plus the amount of lignin existing in the initial ~5 g of lignocellulosic biomass, assuming that all the lignin was degraded to phenolic compounds.

Table 4.8 – Imidazole recovered (g) with the imidazole recovery procedure with respective yield % (w/w) from initial mass in the reaction, for both biomass under all different pretreatment conditions

	T (°C)	t (h)	Solid imidazole (g)	Yield (% w/w)
Wheat straw	130	2	44,906	96%
		4	40,443	88%
	145	3	38,9305	84%
	160	2	42,487	93%
		4	38,7439	85%
Cupressus lusitanica	130	2	24,4	52%
		4	17,481	37%
	145	3	21,915	47%
	160	2	24,807	53%
		4	21,486	46%

The amount of solid imidazole recovered by this procedure is shown in Table 4.8. For wheat straw, it is noticeable that imidazole recovery yield lowers slightly with the increase of time from 2 to 4h, e.g., at 130 °C decreases from 96% (w/w) to 88% (w/w), while for the extracted *Cupressus* decreases from 52% (w/w) to 37% (w/w).

The most interesting result shown in table 4.8 was the difference in yields between both biomasses. Imidazole recovery for wheat straw samples achieved a much higher yield (between 84 and 96% (w/w)) than those of the extracted residues of *Cupressus lusitanica*, which registered yields between 37 and 53% (w/w). Due to the different composition of the liquor obtained, the water evaporation after the neutralization with NaOH was more difficult in *Cupressus lusitanica*, requiring higher temperatures in the Rotavapor (60 °C) to completely evaporate. At very low pressures (0.07 mmbar), imidazole is volatile and evaporates, explaining the lower values. It is also possible that imidazole degraded causing it to not totally dissolve in the acetonitrile and to be filtered alongside NaCl. Another possibility is that the UAE changed the lignin structure hindering the dissolution of the imidazole in acetonitrile. Comparing to ionic liquid recovery, da Silva et al. achieved recovery yields between 85.9 to 95.8% (w/w) in the pretreatment of wheat straw with 1-ethyl-3-methylimidazolium acetate[95], values comparable to those achieved in this work for wheat straw. Similarly, they report a decrease in IL recovery with the increase of reaction time from 2 to 6 hours by 5.5% (w/w) for both 120 °C and 140 °C.

One of the drawbacks of using imidazole is the higher workup, with the use of additional reagents, to recover. The whole economical sustainability of the pretreatment depends of good yields in the recovery and purification of imidazole as well on its successful reuse. da Costa Lopes et al. studied the reuse of the ionic liquid recovered in further pretreatments of wheat straw and manage to execute seven successive processes, with regeneration yields of the pre-treated biomass varying between 57.5 and 62.9% (w/w) with IL recovery always higher than 80% (w/w)[55]. Trinh et al. also reported on the IL recycling and reused it up to six times on the pretreatment of mixed softwoods without any significant change on the carbohydrate and lignin fractions[71]. The imidazole purification method and its reuse need to be studied and optimized to increase the yield and make the process economically viable.

4.4. Determination of total phenolics content and antioxidant activity

The methanolic fraction obtained from the purification of imidazole through SPE contains the phenolics derived from degraded lignin. The total phenolics content and the antioxidant capacity were evaluated in this fraction.

The methanol was evaporated, and the solid extract was re-dissolved in 50% (v/v) aqueous methanol with concentration of 1 mg/mL. Total phenolics content were determined using the Folin-Ciocalteu assay and are expressed in mg of gallic acid equivalents (GAE)/g of extract, while antioxidant activity was tested by the DPPH method and is presented in Trolox equivalents (TE) (mM) and in DPPH inhibition %. Table 4.10 shows the total phenolics and antioxidant activity for the different pretreatment conditions for both biomasses.

Table 4.9 – Total phenolics expressed as mg of Gallic acid equivalents (GAE)/g of extract, antioxidant activity expressed as mM of Trollox equivalents (TE) and % of DPPH radical inhibition for both biomasses for the different pretreatment conditions.

		Wheat straw			*Cupressus lusitania*		
t (h)	T (°C)	Total phenolics (mg GAE/g ext)	TE (mM)	DPPH inhibition (%)	Total phenolics (mg GAE/g ext)	TE (mM)	DPPH inhibition (%)
2	130	102.7 ± 7.0	0.57 ± 0.05	45.4 ± 2.7	32.0 ± 0.3	0.25 ± 0.11	27.8 ± 5.9
	160	114.2 ± 1.2	0.67 ± 0.09	50.9 ± 5.1	54.3 ± 1.9	0.52 ± 0.07	42.6 ± 3.9
3	145	107.6 ± 5.6	0.38 ± 0.06	32.2 ± 2.5	62.0 ± 8.1	0.27 ± 0.05	28.7 ± 2.5
4	130	108.4 ± 1.6	0.51 ± 0.04	41.6 ± 2.1	36.4 ± 1.2	0.25 ± 0.05	28.1 ± 2.9
	160	127.1 ± 2.5	0.48 ± 0.02	39.9 ± 0.95	77.0 ± 0.7	0.29 ± 0.06	29.7 ± 3.5

It is possible to observe for wheat straw that total phenolics increase with temperature and time. Analyzing the effect of temperature, at 2h reaction time, the increase of temperature from 130 °C to 160 °C leads to an increase of total phenolics from 102.7 ± 7.0 to 114.2 ± 1.2 mg GAE/g ext. At 4h reaction time, the same increase leads to an increase of total phenolics from 108.4 ± 1.6 to 127.1 ± 2.5 mg GAE/g ext. The effect of time can be seen at 130 °C, e.g., an increase of 2 hours leads to an increase from 102.7 ± 7.0 to 108.4 ± 1.6 mg GAE/g ext.

For the extracted residues of *Cupressus lusitanica*, the increase with reaction time is much higher at 160 °C (from 54.3 to 77.0 mg GAE/g ext, compared to the increase from 32.0 to 36.4 mg GAE/g ext at 130 °C), suggesting that, for this biomass, at higher reaction temperatures the effect of time is more significant than at lower temperatures.

The higher phenolic content was achieved in the harshest conditions in study. This is correlated with the increased delignification observed in those conditions. As shown previously in this work, the higher the reaction temperatures and reaction times, the higher is the delignification yield, meaning that more lignin is degraded by imidazole. However, the phenolic content in wheat straw is higher than in the extracted residues of *Cupressus lusitanica*, indicating that the lignin from this latter biomass is more resistant to depolymerization than wheat straw. This result may also be related with the treatment prior to this work that *Cupressus lusitanica* suffered, where ultrasound assisted extraction with ethanol and then acetone removed most of the phenolic compounds that constitutes the extractives fraction.

Regarding antioxidant activity, it can be observed that it is not very strong. Wheat straw reaches a maximum of 50.9 ± 5.1% DPPH inhibition while the extracted residues of *Cupressus lusitanica* achieved 42.6 ± 3.9% DPPH inhibition. Comparing both biomasses, at 160 °C, the antioxidant activity decreases 11.0 ± 6.1% and 12.9 ± 7.4% in wheat straw and *Cupressus lusitanica*, respectively, when the reaction time increases from 2 to 4 hours. However, at 130 °C, when reaction time increases 2h, the DPPH inhibition % decreases 3.8 ± 4.8% in wheat straw and in the extracted residues of *Cupressus* the variation is negligible. This shows that the effect of time in DPPH inhibition is bigger at higher temperatures. The decrease of antioxidant activity may result from phenolic

degradation at these experimental conditions. It is also possible to observe that the antioxidant activity is, in average, lower in *Cupressus* than in wheat straw, which might be related with lower amount phenolic content, although not all the phenolic compounds express antioxidant activity. The DPPH is a very stable organic free radical presenting the ability of accepting an electron or hydrogen radical and thus widely used in assessing free radical scavenging activity because of the ease of reaction. Phenolic compounds have redox properties, which allow them to act as antioxidants, increasing the antioxidant activity.

Imidazole is known for its potential for degrading lignin, producing several different compounds. Compounds like vanillic acid and vanillin, p-hydroxybenzoic acid or hydroxycinnamic acid are all generated off during the hydrolysis of lignin and can be classified as potent antioxidants[38]. Compared to literature[37, 96-99], the values of phenolic content and antioxidant activity in this work are lower than expected. This can be explained by the presence of imidazole lowering the purity of the methanolic fractions. Additionally, as reported by Smith et al., imidazole doesn't react with DPPH[100]. Even though the purity of each extract and its detailed chemicals were not investigated in this work, they can affect the antioxidant activity to great extent.

Due to the high content of diverse functional groups (phenolic and aliphatic hydroxyls, carbonyls, carboxyls, etc.) and its phenyl propanoic structure, lignin can act as a neutralizer or inhibitor in oxidation processes, stabilizing reactions induced by oxygen radicals and their derived species. However, this antiradical activity depends greatly on lignocellulosic material from which lignin is obtained, the method used for its extraction, and the treatments applied during its isolation and purification[97]. Heterogeneity in terms of component composition (carbohydrate admixtures), polydispersity and high molecular weight are factors which can decrease drastically the antioxidant efficiency of isolated lignins[101]. Dizhbite et al. investigations into scavenging effects of lignin related compounds on the DPPH radical have revealed the main role of the non-etherified phenolic hydroxyl groups, ortho-methoxy groups and aliphatic hydroxyl groups in the side, reporting also a negative influence on the α-carbonyl substitution in the side chain[35].

As mentioned already, both biomasses possess huge composition and structural differences and it is not clear yet the mechanism by which imidazole degrades lignin, so differences in phenolic content are expected. To have a better insight on the lignin derived compounds that exist in the extracts and to understand better the differences obtained in phenolic content and antioxidant activity, the methanolic extracts were analyzed by capillary electrophoresis.

4.5. Capillary electrophoresis and mass spectrometry

The methanolic extracts obtained through SPE, the same ones that were analyzed for total phenolic content and antioxidant activity, were analyzed by capillary electrophoresis (CE) to obtain the phenolic profile of the samples and to try to identify some phenolic compounds derived from lignin degradation by imidazole. Figures 4.5 and 4.6 show electropherograms with the phenolic profiles obtained at 280 nm for wheat straw, demonstrating the effect of the different pretreatment conditions. Figure 4.6 in particular refers to a tentative identification for some peaks according to their UV spectra and migration time, for the pretreatment conditions of 145 °C/3h, by comparison these parameters with those of authentic standards run in the same conditions and stored in library.

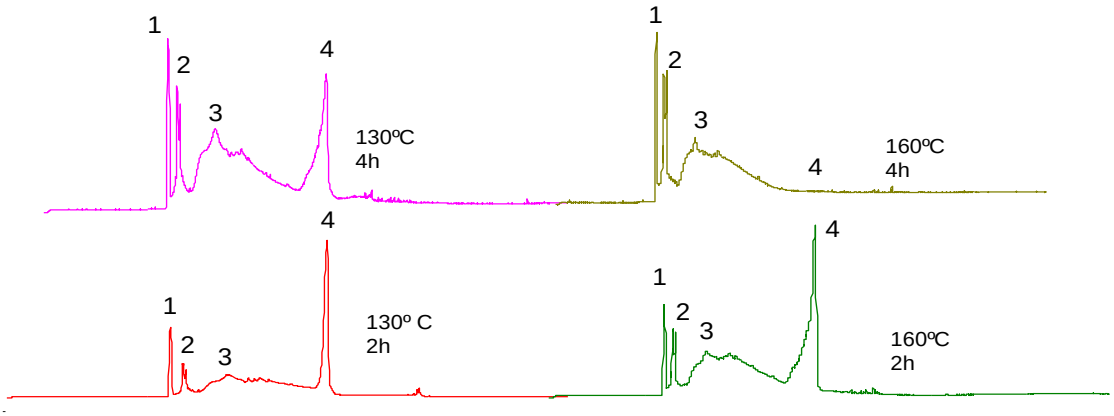

Figure 4.5 - Electropherogram with the phenolic profile of wheat straw methanolic extracts for the different pretreatment conditions. 1, 2, 3 and 4 represent the major peaks found.

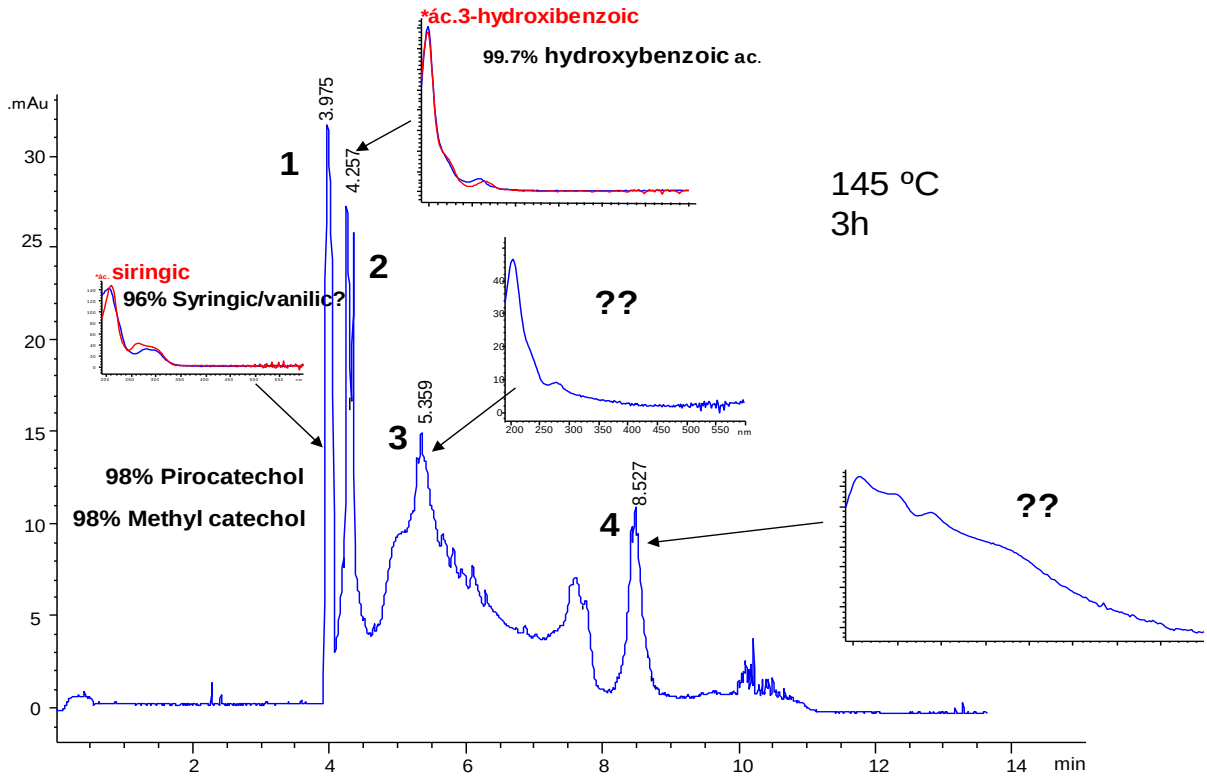

Figure 4.6 - Electropherogram at 280 nm showing the phenolic profile of wheat straw methanolic extract for pretreatment conditions of 145 °C/3h. Matching % for peaks 1, 2, 3 and 4 were obtained by comparison with authentic standards run at the same conditions as sample. See Section 3.4.2. for CE conditions.

From both Figures, one can observe that peaks 1 and 2 have very good matchings with compounds that are typically lignin monomers, often found as products of hydrothermal pretreatment of lignin. Peaks 3 and 4 show UV spectra characteristic for phenolic compounds, however, there was no relevant matching with any of the standards analyzed or present in the library. These could be non-identified fractions originating from lignin depolymerization via imidazole.

Comparing with Morais et al., the authors report the presence of rosmarinic acid with 90% similarity on the profile, and vanillin and vanillic acid with 86% and 90% similarity[59], respectively, which are not very good matchings, but still these samples, as can be depicted from Figures 4.5 and 4.6 above, are extremely complexes. Nevertheless, the pretreatment conditions used in their work were of 170 °C/2h reaction time. Rosmarinic acid isn't a lignin degradation product, therefore, this was probably a lignin degradation compound with similar structure, but vanillin is, and it was expected to be found in this work. Da Silva et al., in their work with pretreatment of wheat straw with ionic liquid[95], reported a huge peak with 99% similarity with vanillin, while also reporting peaks of coumaric acid 95% and ferulic acid 92%, both common products of lignin degradation. All these results seem to indicate that lignin depolymerization and resulting fractions are very much dependent on the temperature/time conditions and on the solvent/catalyst used for the process.

Figures 4.7 and 4.8 are the corresponding electropherograms with the phenolic profiles for different pretreatment conditions applied to the extracted residues of *Cupressus lusitanica*, according to the description stated above for wheat straw.

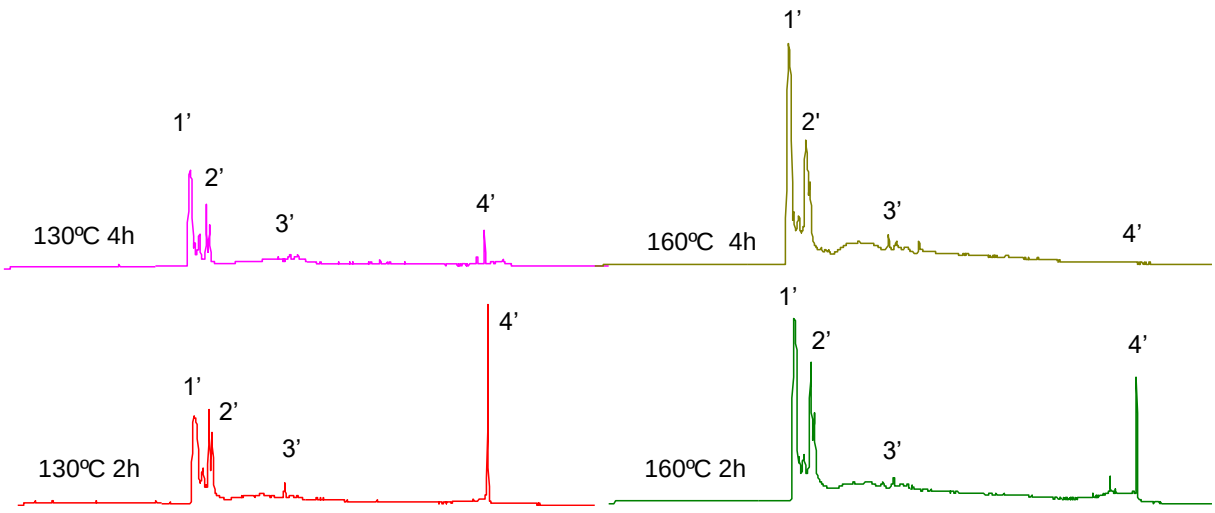

Figure 4.7 - Electropherogram with the phenolic profile of extracted residues of *Cupressus lusitanica* methanolic extracts for the different pretreatment conditions. 1', 2', 3' and 4' represent the major peaks found.

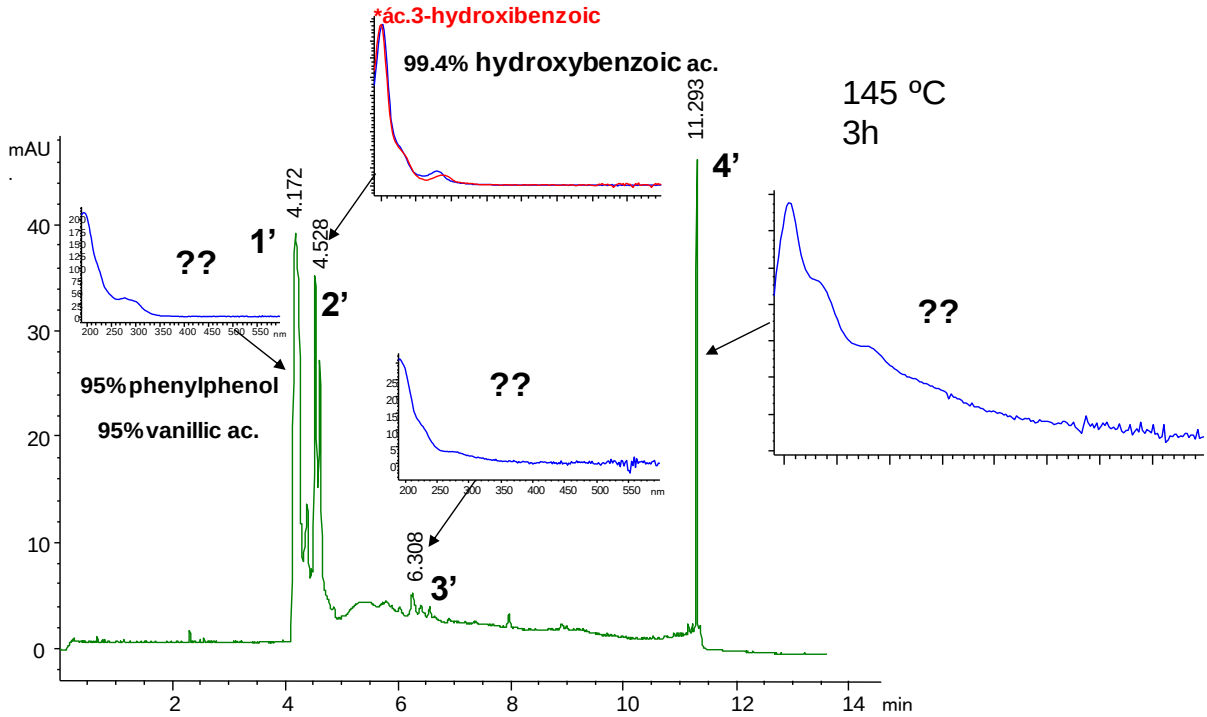

Figure 4.8 - Electropherogram recorded at 280 nm showing the phenolic profile of extracted residues of *Cupressus lusitanica* methanolic extract for pretreatment conditions of 145 °C/3h. Matching % for peaks 1', 2' 3' and 4' were obtained by comparison with authentic standards run at the same conditions as sample. See Section 3.4.2. for CE conditions.

It can be seen from the phenolic profile in Figures 4.7 and 4.8 that *Cupressus lusitanica* shows a different and apparently less complex profile than wheat straw. This is related with the differences in the biomass origin (softwood vs. herbaceous) and it suggests that lignin in extracted residues of *Cupressus lusitanica* was affected by previous UAE and is more resistant to depolymerization by imidazole than wheat straw. Nevertheless, both biomasses appear to have similar behavior at the beginning and at the end of the profiles, especially for compounds 1, 1', 2, 2' and 4, 4'. Similarly, as for wheat straw, both 1' and 2' compounds seem to be lignin monomers, while 3' and 4' might be degraded fractions from lignin, and curiously, compounds 4 and 4' seem to be totally degraded at the most severe pretreatment conditions, i.e., 160 °C/4h. To the best of our knowledge, there are no previous published results concerning lignin pretreatment to *Cupressus* species, therefore, we cannot compare our results. However, it appears from this study that imidazole has a similar degrading behavior in lignin from different biomasses. Generally, from the analysis of peak areas (data not shown), it could be observed that for both samples, there was an increase in lignin degradation with temperature, particularly for peaks 1, 1', 2 and 2', meaning that higher temperatures promotes more degradation and the formation of more monomers, and also the formation of lignin fractions (oligolignols), which, on the other hand, tend to degrade with higher temperatures and for longer treatment times (3, 3', 4 and 4').

As a proof of concept, the same samples analyzed by CE were subjected to HPLC coupled to tandem mass spectrometry (LC-MS/MS) to confirm identification and assess the structure of oligolignols contained in the depolymerized fractions of lignocellulosic biomass from the extracted residues of *Cupressus lusitanica* and wheat straw.

Figures 4.9 and 4.11 show the total ion chromatograms (TIC) of the recovered methanolic fractions obtained from the extracted residues of *Cupressus lusitanica* and wheat straw, respectively, after processing at 160 °C/4 h, which are the pretreatment conditions that exhibited higher total phenolics content. It was observed that, for all pretreatment conditions, the methanolic extracts displayed similar TIC profiles to that presented in Figures 4.9 and 4.11 (data not shown), although the intensity of the chromatographic peaks varies in function of the experimental conditions used during depolymerization. This behavior is in agreement to what was also observed in the CE.

The TIC obtained in the ESI negative ion mode showed several signals indicating the presence of deprotonated oligolignols, and the structural elucidation of the most abundant species was achieved by collision induced dissociation (CID) experiments on the selected precursor ions (CID-MS^2), using a quadrupole ion trap. The TIC for the methanolic extract of the extracted residues of *Cupressus lusitanica* (Fig. 4.9) displays a peak at retention time 23.1 min, assigned to a deprotonated molecule with *m/z* 473, whose product ion mass spectrum shows major fragments at *m/z* 427, 293 and 149. The loss of 46 u and the fragment with *m/z* 149 suggest the presence of an acid group and a coniferyl type unit in the molecular structure. Based on the fragmentation pattern, this anion is tentatively assigned to the species $[C_{28}H_{25}O_7]^-$. Peaks at retention time 24.3 and 28.0 min were attributed to deprotonated molecules with *m/z* 507 and 491, respectively. MS^2 spectra of both anions displayed similar fragmentation patterns, namely, loss of water (18 u), elimination of formaldehyde (- 30 u, CH_2O) and of formic acid (-46 u, H_2CO_2), leading to product ions at *m/z* 489 (473), 477 (461) and 461 (445), respectively. Elimination of 178 u giving *m/z* 329 (313), and a fragment with *m/z* 179 indicate the presence of a coniferyl alcohol unit[102], typical in softwoods. On the basis of these results, it was proposed that species with *m/z* 507 and 491 can be attributed, respectively, to the deprotonated cyclic trimers $[C_{28}H_{27}O_9]^-$ and $[C_{28}H_{27}O_8]^-$ containing the (8-5´)-(3´-methoxyl coumaryl) unit[103-104]. A proposed fragmentation mechanism for the precursor ion *m/z* 491 is presented in Figure 4.10. At higher retention times, it was observed species with lower deprotonated molecules, indicating the presence of dimeric oligolignol structures. The peaks at retention times of 36.2, 38.1 and 53.8 min correspond to deprotonated molecules with *m/z* 287, 285 and 271, respectively. The three precursor ions follow similar fragmentation behavior indicating similar structures, probably containing dimeric guaiacyl stilbene units. Furthermore, these low molecular weight peaks increase with the increase of temperature, analogously to CE, indicating more efficiency in lignin degradation. The peak with *m/z* 287, one of the most intense peaks in figure 4.9, presents a fragment of *m/z* 137 suggesting the presence of 3-hydroxybenzoic acid, a common product of lignin degradation also detected by CE, confirming the result shown above (Fig. 4.8).

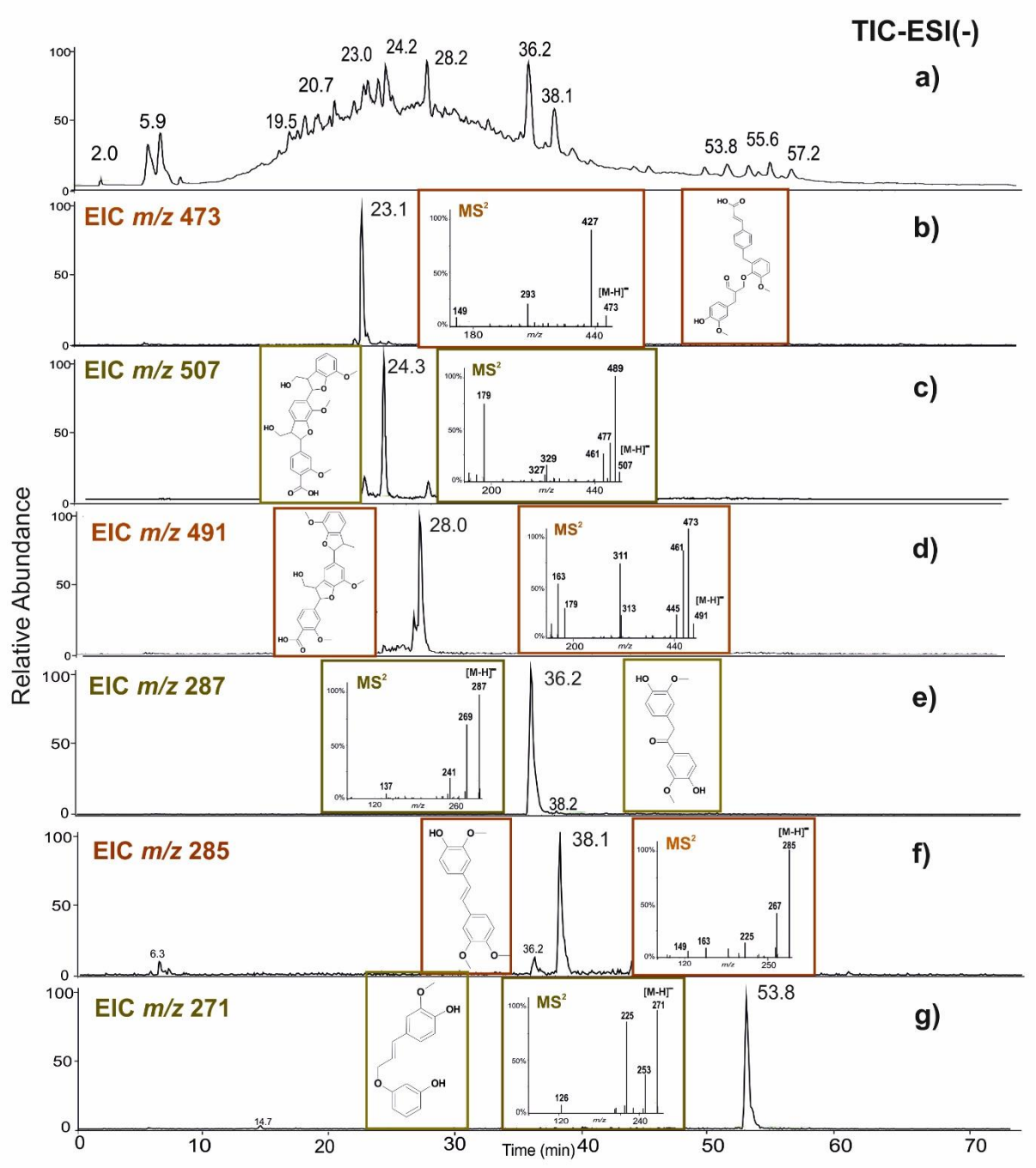

Figure 4.9 - HPLC-MS/MS analysis of the methanolic extract recovered by SPE for the pretreatment of the extracted residues of *Cupressus lusitanica* at 160 °C/4h. (a) Total ion chromatogram obtained in the ESI negative mode. Extracted ion chromatogram, MS^2 spectrum and proposed structure for the precursor ion (b) *m/z* 473, (c) *m/z* 507, (d) *m/z* 491, (e) *m/z* 287, (f) *m/z* 285 and (g) *m/z* 271.

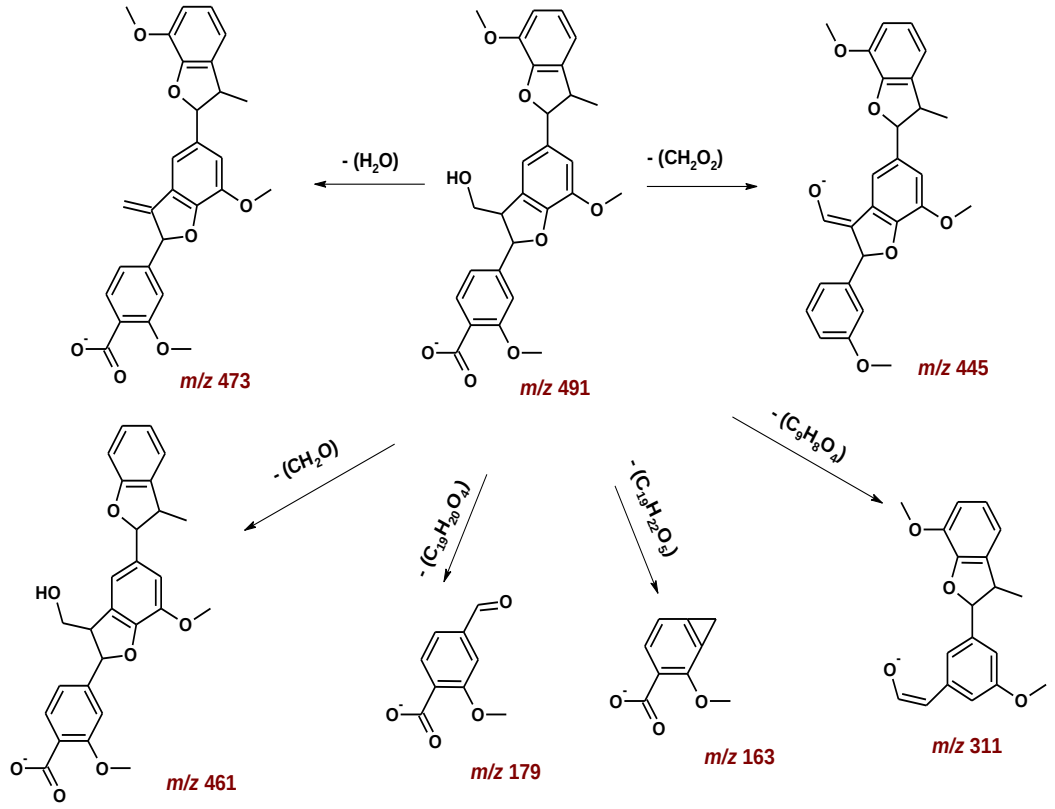

Figure 4.10 - Proposed fragmentation pattern for the deprotonated molecule with *m/z* 491 identified in LC-ESI(-)/MS extracts of *Cupressus lusitanica.*

The LC-MS profile of the methanolic fraction obtained from wheat straw (Figure 4.11) differs from the obtained from the extracted residues of *Cupressus lusitanica*, indicating that the former is constituted by different oligolignols units. At retention time 25.5 min was identified a deprotonated molecule with *m/z* 381, whose MS^2 spectrum show three main peaks at *m/z* 313, 223 and 171. Based on the fragmentation pattern, this precursor ion was attributed to the trimer structure $[C_{22}H_{21}O_6]^-$. At the retention time between 33 and 35.3 min, it was observed 3 peaks that correspond to five species. This means that the chromatographic separation was not fully achieved, occurring coelution of oliolignol fractions. The extracted ion peak at Rt = 33.9 min gave a deprotonated molecule with *m/z* 331, whose fragmentation fits well a $[C_{17}H_{15}O_7]^-$ structure. At retention time 34 min, it was detected an ion attributed to a deprotonated molecule with *m/z* 329. This precursor ion was isolated in the QIT analyzer, and CID experiments were performed. The MS^2 analysis displayed two peaks at retention time of 34.1 and 34.9 min, indicating two different precursor ions with *m/z* 329. The analysis of the product spectra confirms the presence of two distinct species, assigned to $[C_{17}H_{13}O_7]^-$ and $[C_{22}H_{21}O_6]^-$, respectively structures **A** and **B**, that displayed different fragmentation paths. The structure **A** was attributed to a tricin deprotonated molecule, an *O*-methylated flavone known to be incorporated into lignin structure of wheat straw through methoxylation of the aromatic rings present in the phenylpropanoid units[105]. Proposed fragmentation mechanisms for the precursor ions A and B were presented in figure 4.12 e 4.13, respectively.

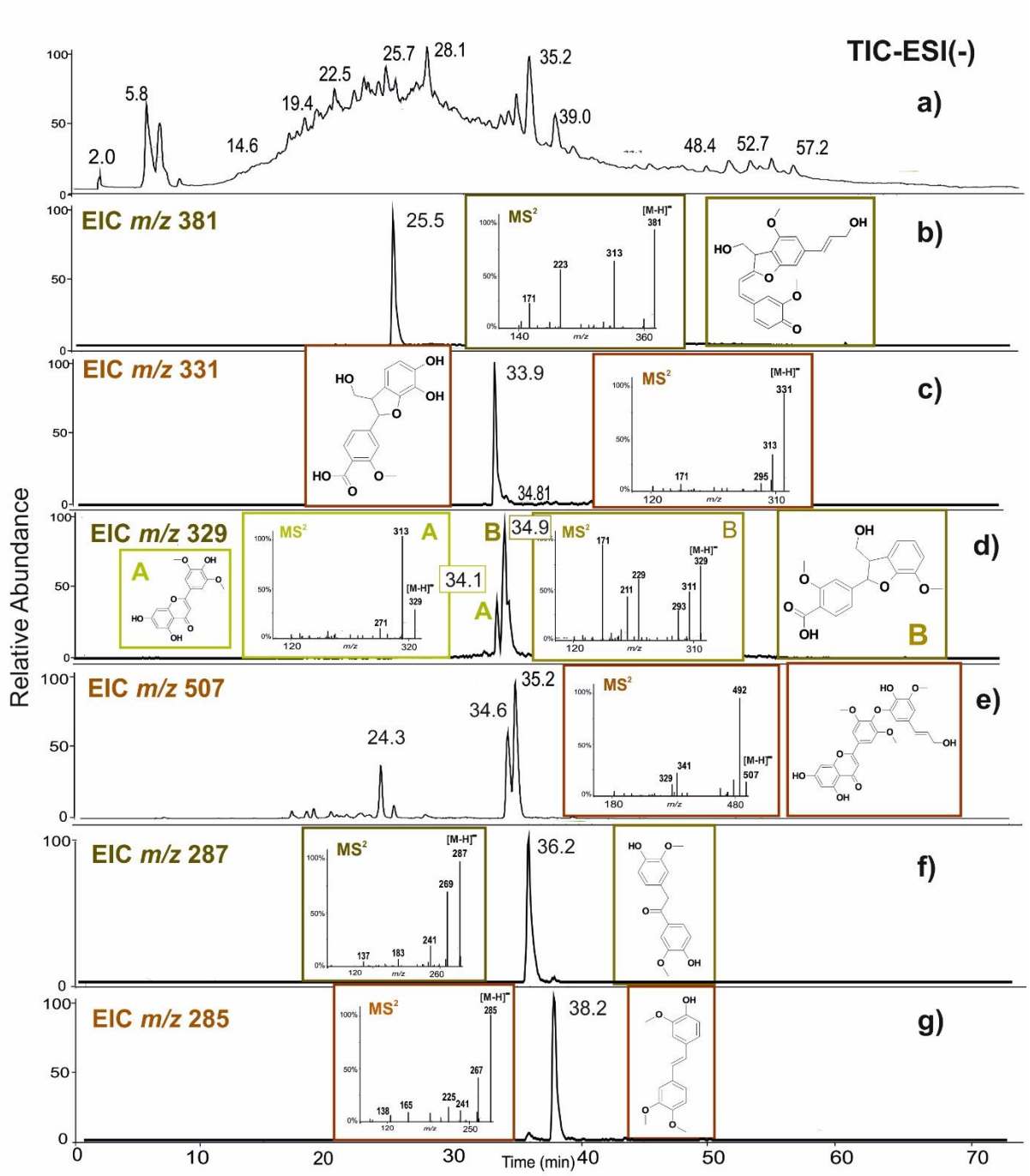

Figure 4.11 - HPLC-MS/MS analysis of the methanolic extract recovered by SPE for the pretreatment of wheat straw at 160 °C/4h. (a) Total ion chromatogram obtained in the ESI negative mode. Extracted ion chromatogram, MS^2 spectrum and proposed structure for the precursor ion (b) *m/z* 381, (c) *m/z* 331, (d) *m/z* 329 (A and B), (e) *m/z* 507, (f) *m/z* 287 and (g) *m/z* 285.

The peak at retention time 24.3 min with *m/z* 507 is the same found in the extracted residues of *Cupressus lusitanica*, attributed to the deprotonated cyclic trimer with structure $[C_{28}H_{27}O_9]^-$. The two peaks at retention time of 34.6 and 35.2 min also yield two deprotonated molecules with *m/z* 507. Considering that the methanolic fractions of both biomasses were analyzed with same LC-MS conditions, it was expected that these protonated molecules eluting at higher retention times afforded a different structure of that proposed for species $[C_{28}H_{27}O_9]^-$, identified in the sample of *Cupressus*. In fact, the product ion spectra of both precursor ions

displayed similar fragmentation patterns, showing peaks that only differ in the intensity of the signal, indicating two isomeric structures. The fragments at *m/z* 492 results from the loss of a radical methyl (- 15 u), whereas the fragments *m/z* 341 and 329 are associated with losses of 166 u and 178 u, indicating the presence of a ferulic acid unit in the structure. Based on these results, the two peaks with *m/z* 507 were assigned to a tricin-lignan unit with a $[C_{27}H_{23}O_{10}]^-$ structure. This result provides more evidence of the presence of tricin-oligolignol units in the wheat straw structure. As above mentioned, dimeric oligolignols units with *m/z* 287 and 285 were also observed, indicating the efficiency of the depolymerization process. These low molecular weight peaks only show in these pretreatment conditions (160 °C/4h), indicating more efficiency in lignin degradation, which was also verify by CE. Peak with *m/z* 287 shows a fragment with *m/z* 137, as in the extracted residues of *Cupressus lusitanica*, which indicates the presence of 3-hydroxybenzoic acid, thus confirming the identification by CE of peak 2 in figure 4.6.

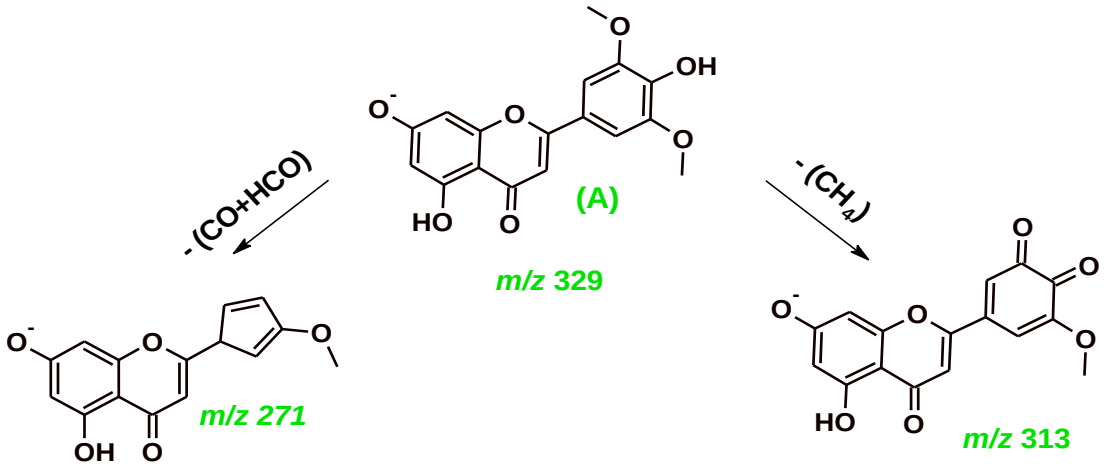

Figure 4.12 - Proposed fragmentation mechanism for the deprotonated molecule of tricin m/z 329.

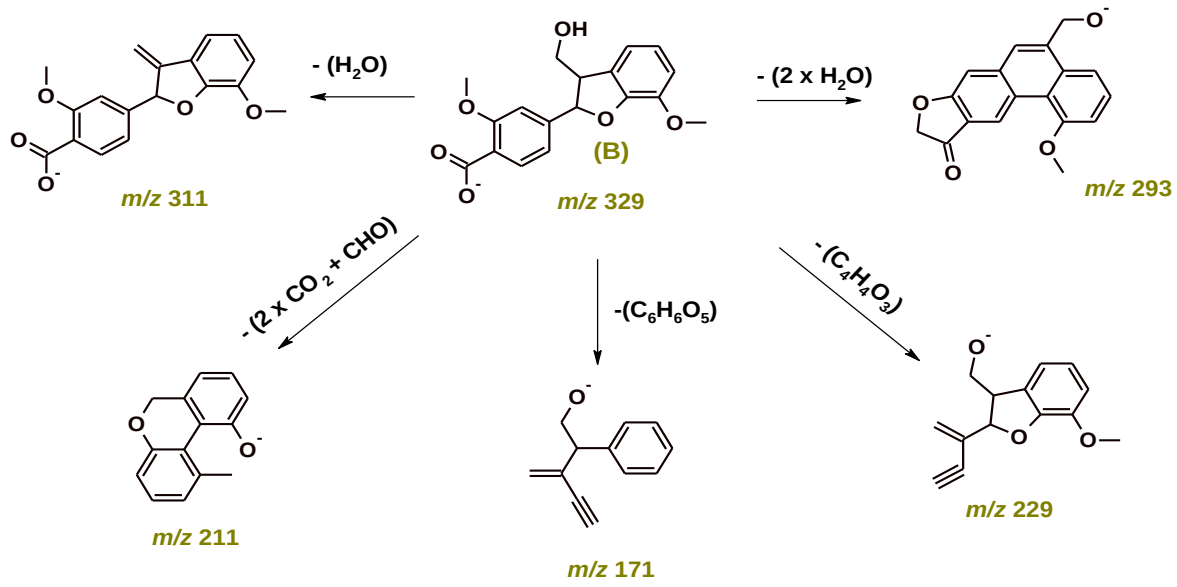

Figure 4.13 - Proposed fragmentation mechanism for the deprotonated molecule of the oligolignol structure $[C_{22}H_{21}O_6]$- *m/z* 329.

Conclusion and perspectives

The pretreatment of wheat straw and extracted residues of *Cupressus lusitanica* with imidazole revealed that the recovery of cellulose and hemicellulose, as well as the delignification of biomass, were highly dependent on temperature for both biomasses. However, imidazole was able to selectively separate wheat straw components into cellulose- and hemicellulose-rich fractions more efficiently than for the extracted residues of *Cupressus lusitanica*. Also, imidazole recovery procedure was also much more efficient for wheat straw than for the extracted residues of *Cupressus lusitanica*.

The imidazole recovery procedure was also much more efficient for wheat straw than for the extracted residues of *Cupressus lusitanica*. The major weakness of imidazole was found to be the difficulty of its recovery and of its purification. This was a drawback in the determination of the total phenolics and antioxidant activity.

Nevertheless, imidazole demonstrated to be a good and cheap alternative to ionic liquids and organosolv pretreatment for lignin depolymerization, with pretreatment performed at lower temperatures and without the need of additional catalysts. Several lignin monomers, namely 3-hydroxybenzoic acid, were found in both samples, revealing the extent of hydrolyses and consequent degradation of lignin. Other oligolignols could also be found and the structure of few were tentatively elucidated by LC-MS. Particularly for wheat straw, it was possible to identify the flavonolignan derived from tricin, which is considered one of the most potent anti-cancer agents tested most probably due to the stability of its structure.

Results showed that the difference in composition and structure between both biomasses leads to different optimum pretreatment conditions. Therefore, to get a more complete evaluation of the pretreatment, a broader range of temperatures and reaction times should be studied, also tailoring these conditions towards specific targets (tricin, vanillin, etc.). Additionally, no clear chemical mechanism of lignin extraction with imidazole was reported so far. Thus, further work into a more detailed analysis to reveal the exact mechanism of the reactions with imidazole is still necessary to develop this technology. More importantly, the recovery, purification and reuse of imidazole need to be developed to achieve economical sustainability of the pretreatment.

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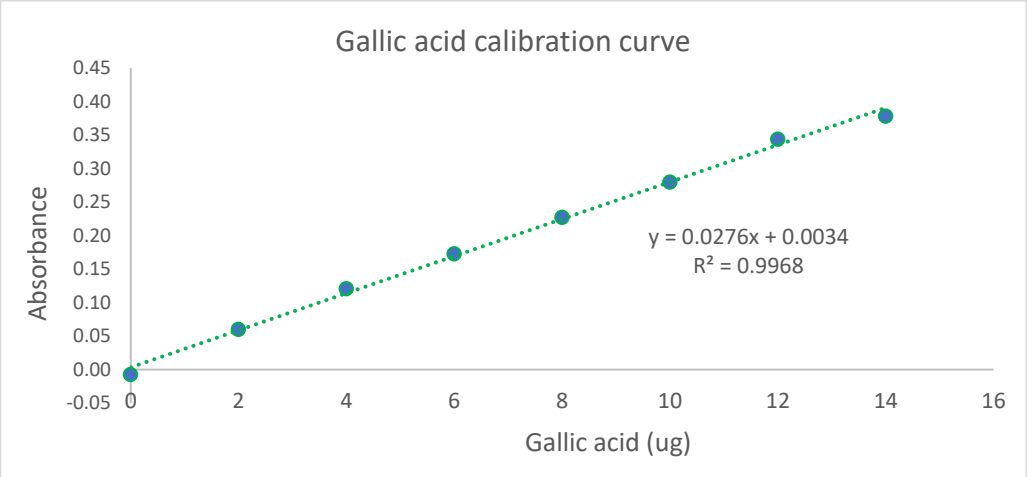
Appendix

Appendix A

Total phenolics by Folin-Ciocalteu colorimetric method

The calibration curve was plotted by mixing 0, 20, 40, 60, 80, 100, 120 and 140 ml of gallic acid solutions (diluted tenfold), where 0 is the blank, with ultrapure water up to 0.5 ml total, 0.25 ml of Folin Ciocalteu reagent (diluted 1:1) and 1.25 ml of sodium carbonate solution 20% (m/v). The absorbance was measured at 725 nm after 40 min incubation at room temperature in the dark. The procedure was done in triplicate.

Gallic acid (ug)	*1*	*2*	*3*	***Average***
0	0,0621	0,0555	0,0544	0,055
2	0,117	0,1219	0,1066	0,115
4	0,173	0,1825	0,1719	0,176
6	0,228	0,225	0,230	0,228
8	0,2939	0,272	0,2808	0,282
10	0,3284	0,3336	0,3416	0,335
12	0,4452	0,4044	0,393	0,399
14	0,4275	0,4429	0,4284	0,433



Appendix B

Antioxidant activity by DPPH free radical scavenge

The Trolox calibration curve was plotted by mixing 50 µL of Trolox solution of 0.08, 0.15, 0.3, 0.53, 0.75, 1.05 and 1.28 mM in ethanol, and 1950 µL of 0.06 mM DPPH solution. The absorbance was measured at 515 nm after 30 min.

Trolox (mM)	*1*	*2*	*3*	*Average*
0,08	0,3607	0,4212	0,4255	0,423
0,15	0,4055	0,4087	0,3965	0,404
0,3	0,3433	0,3417	0,3313	0,339
0,53	0,3244	0,2648	0,2843	0,275
0,75	0,262	0,2425	0,2428	0,243
1,05	0,221	0,1535	0,145	0,149
1,28	0,1016	0,0723	0,0797	0,076