Biomolecules extraction from forest biomass

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Biomolecules extraction from forest biomass

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"We do not need magic to change the world, we carry all the power we need inside ourselves already: we have the power to imagin better."

J.K. Rowling

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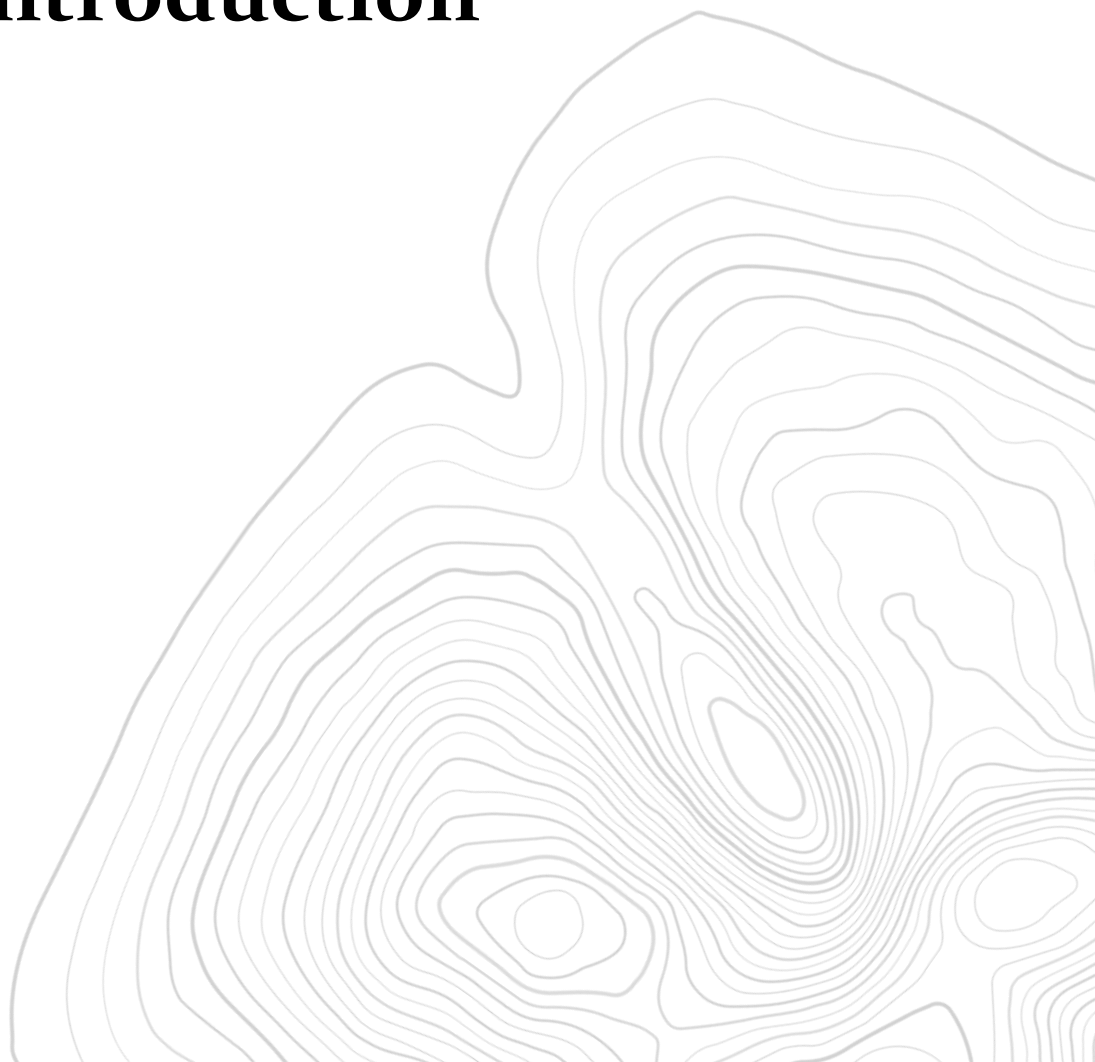
Introduction



1.1 Overview

Today, society is highly dependent on fossil resources, not only for obtaining energy but also for the production of many products derived from them, which have become indispensable. Before this situation, for centuries, humanity used other resources that were left aside with the arrival of fossil fuels. Those resources were based on the biomass, water, minerals, metals and air, where wood was one of the most used to generate energy, either heat or light. Nevertheless, in the middle of the 18th century, with the Industrial revolution, the fossil fuel use started. This change was driven largely by the problems of deforestation generated by the increased biomass demand. The term "fossil fuel" encompasses non-renewable energy sources such as coal, natural gas, crude oil and their products. They are carbon-based sources originated millions of years ago from plants and animals. Fossil fuels have satisfied most of the society energy requirements and their consumption has been rapidly increased. In addition, the society is not only very dependent on energy, but also on all the synthetic products that can be obtained from fossil fuel, such as plastics, chemicals, fertilisers etc.

Although it is true that transportation fuels represent around 70% of the total refined products [1.1], these products are not the only ones. Many of the by-products are revalorised in their great majority to obtain different products following petrochemical processes. More than 6000 items derived from the petroleum building blocks have been counted according to Ranken Energy Corporation. However, the side products of the industry, as CO_2 or sulphur contaminants among others, are the main cause of the climate change and other derived environmental problems, that affect air, water streams and soil [1.1]. An overview of recent history shows that the

unreasonable use of any of the existing resources has never been good, generating the need for a sustainable development (see **Appendix I**).

In 2009, fossil resources supply 86% of the total energy and 96% of the organic chemicals consumed by the industrially developed countries of the world [1.2]. However, due to the high environmental impact, their use in recent years has begun to shrink. While primary energy demand is expected to increase by 50-60% by 2030, mainly due to population growth and the desire for better living standard [1.3], limited fossil resources and increasing environmental problems call for a more sustainable approach. For that purpose, the Paris Agreement was signed by many countries to reduce CO_2 emissions though the use of renewable energy among other actions [1.4].

In Europe, the emissions reduction agreement has gone even further with a structured plan whence by 2050 Europe wants to become the world's first climate-neutral continent, according to the European Green Deal [1.5]. With that final objective, by 2020 there was the objective of increasing the share of renewable energy to 20% as well as reducing greenhouse gas emissions by 20%.

Petrochemical industry or oil industry, commonly known as refinery, extract fossil fuels to produce oil based products. The conversions are based on well known sequential processes. The first refinery was started 150 years ago, and its only objective was to produce fuels (gasoline, diesel, jet fuel and heating oils). However, nowadays, even if the main market is transportation fuels, in order to make them more economically and environmentally sustainable, the generated unavoidable by-products are valorised by petrochemistry processes [1.1]. These processes firstly produce "building blocks", then different chemicals intermediates and finally they are converted to final products with many applications (plastics, synthetic

rubber, fertilizers, dyes, detergents, etc.). This industry represents approximately 10%, in terms of production volume, of the total petroleum industry [1.6]. The depletion of fossil resources added to its poorer quality, has forced to optimise the technical processes in refineries in recent years [1.3]. Nonetheless, despite all the improvements made in the refineries, they are not enough to overcome all the problems.

The contribution to the global environmental crisis is one of the main drawbacks of the use of fossil resources, but it is not the only one. Thus, in the last decades, there has been a change in the use of oil resources in favour of renewable alternatives for the production of fuels and chemical products [1.7].

The International Energy Agency Bioenergy Task 42 defines biorefining as "the sustainable processing of biomass into a spectrum of marketable bio-based products (chemicals, materials) and bioenergy (biofuels, power, heat)" [1.8]. In other words, a biorefinery can be defined as a facility or a set of facilities for the transformation of many different types of biomass into building blocks associated with the production of added value products, biofuels and chemicals replacing petroleum products [1.9]. Although the principle described is adequate, it is necessary to take into account that the current situation requires taking a further step and say that it needs to be developed within the principles of sustainability. In this way, apart from replacing a resource in decline, production should be carried out in a sustainable manner and fulfilling the principles of the circular economy (see **Appendix I**) [1.9].

The biorefinery concept covers biomass conversion approach leading to a comprehensive portfolio of valuable products, drawing direct analogy to today's fossil oil refineries [1.10]. Nevertheless, biorefinery processes are

more complex compared to petroleum refinery [1.7], due principally to the heterogeneity of the biomass and its complex structure. It requires the use of higher number of separations and transformations processes, what in general makes the processes less cost effective. However, biomass-based refineries produce less greenhouse gas compared to traditional refineries, as the CO_2 generated in the conversion process is consumed in the subsequent growth stage of the biomass. Therefore, in order to make the refinery sustainable, different challenges need to be solved, such as: a) the efficient use of the biomass with minimal waste generation and energy consumption, b) flexible co-production, c) economically competitive with refineries [1.7].

The scheme proposed for biorefinery is very similar to that used in oil refineries, but instead of using fossil resources for the production of the different products, biomass and its intermediate products are used (**Figure 1.1**). With the advantage that biomass is regularly regenerated and fossil resources are on track of being depleted. In this case, that approach involves, usually, multi-step processes in which the steps and processes selected for the fractionation depend on the feedstock selection [1.11]. However, there are also biorefineries with a single step scheme.

Following the established guidelines, where achieving a circular economy as well as the idea of zero waste are priorities, biorefineries should be designed both to maximise production and to minimise waste generation by using the residues for energy production. The application of these two philosophies improves the profitability of the biorefinery and it also permits a reduction of the waste generated, solving the problems of waste management.

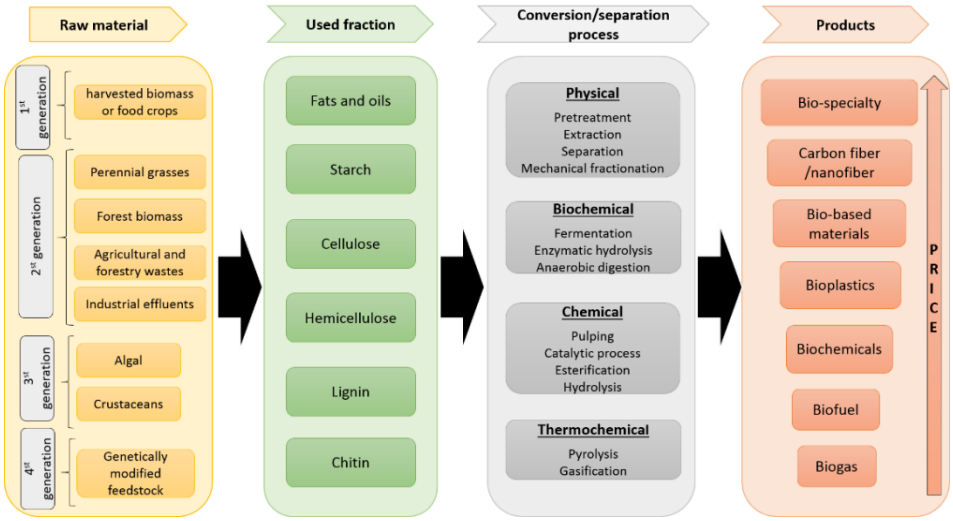


Figure 1.1 Conceptual scheme of biorefineries with the different classification criteria.

The classification of the biorefineries is not an essay task, especially due to the amount of possible feedstock and processes that could be used. The most typical classifications in the literature use different criteria such as the implementation status, raw material or conversion process (**Figure 1.1**).

Currently, although the objective is marked in the total replacement of the exploitation of fossil resources by biomass, this objective is still far from being achieved. **Table 1.1** shows some industrial scale biorefineries applied around the world [1.12], but there is still a lot to do.

New bio-based industries will appear in the near future, but there are still some challenges to overcome. Biorefineries need to be competitive producing new products or existing products at a lower cost but with similar or better properties, and for that, there are still some challenges to overcome. Some of the most important challenges are the increase in production and market generation of the obtained products, the development of more sustainable and efficient technologies and the use of non-food biomass to avoid competition, as well as preventing deforestation.

Table 1.1 Some biorefineries applied at industrial scale. Adapted from [1.12].

Company	Country	Feedstock	Product	Generation	Year
Pannonia Ethanol	Hungary	Corn	Ethanol	First	2012
Global Bioenergies	Germany	Sugar beet	Bio-isobutane	First	2017
Reverdia	Italy	Starch	Succinic acid	First	2012
BioAmber	Canada	Corn	Succinic acid	First	2015
StI	Finland	Potato flake industrial waste	Bioethanol	Second	2008
GranBio	Brazil	Straw and bagasse	Ethanol	Second	2014
StI	Sweden	Bakery food and industrial waste	Bioethanol	Second	2015
DuPont	USA	Corn stover	Ethanol	Second	2015
Fitoplancton Marino	Spain	Sea water/CO_2/ nutrients	Cosmetics	Third	2002

Due to that, society needs to take on a more sustainable and realistic model based on the sustainable used of natural resources. With the aim of zero-waste and based on the circular economy applied to biorefineries.

1.2 Lignocellulosic biomass

Our planet stores an enormous amount of available biomass in different areas, from forests to oceans, which is an advantage because it is universally available. In addition to being a renewable (non-fossil) feedstock, another important benefit is the positive contribution to the reduction of emissions of greenhouse gases, due to its theoretical zero CO_2 balance [1.13]. However, the complexity of the heterogeneous biomass structure makes its conversion a challenge.

Biomass is considered as any organic substance derived directly or indirectly from the photosynthesis process. In other words, biomass is defined as the organic material that comes from vegetables or animals, including agricultural crops and wastes, forest residues, animal wastes, municipal and industrial wastes among others (**Figure 1.2**) [1.14]. Biomass is a complex heterogeneous combination of, mainly, organic matter, and to a lesser extent, inorganic matter [1.15]. One possible classification is the one that is based on the types of biomass existing in nature. According to Vassilev et al. [1.16], all the biomass is organised in 6 different groups as it is represented in **Table 1.2**.

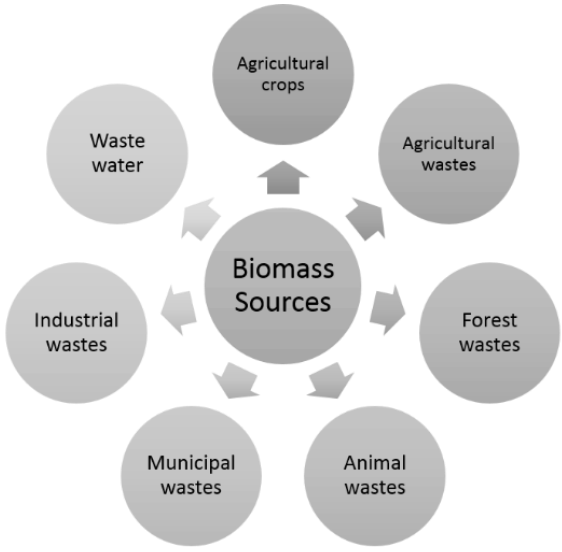


Figure 1.2 Some of the most important biomass sources. Adapted from [1.17].

Within the biorefinery context, it is important to select the type of biomass to be used, since, as it is explained in the above section, it needs to be available, abundant environmentally friendly (such as waste), low-cost and that it does not have to compete with food, so that the process is economically profitable and sustainable. For this reason, in recent decades, lignocellulosic biomass has been investigated as a viable alternative to fossil resources.

Table 1.2 Comprehensive classification of biomass varieties according to their biological diversity, source and origin. Adapted from [1.15].

Biomass group	Biomass sub-group, varieties and species
Wood and woody biomass	Coniferous or deciduous; angiospermous or gymnospermous; softwood or hardwood; stems, branches, foliage, bark, chips, lumps, pellets, briquettes, sawdust, sawmill and others from various wood species.
Herbaceous and agricultural biomass	Grasses and flowers; straws; other residues (fruits, shells, husks, hulls, seeds, cobs, bagasse, etc.).
Aquatic biomass	Marine or freshwater algae; macroalgae or microalgae; seaweed, kelp, lake weed, others.
Animal and human biomass waste	Bones, meat-bone meal, chicken litter, various manures, others.
Contaminated biomass and industrial biomass waste	Municipal solid waste, demolition wood, refuse-derived fuel, sewage sludge, hospital waste, paper-pulp sludge, waste papers, paperboard waste, chipboard, fibreboard, plywood, wood pallets and boxes, railway sleepers, tannery waste, others.
Biomass mixture	Blends from the above varieties.

Lignocellulose is a three-dimensional polymeric composite material synthesised by photosynthesis by the plant, which composes the cell walls. It consists mainly of hemicellulose, cellulose and lignin, which are considered as structural compounds, in combination with other minor non-structural compounds (pectins, inorganic compounds, proteins and extractives) [1.18]. The composition of lignocellulose varies depending on the species, plant tissue and growing conditions [1.19].

1.2.1 Cellulose

Cellulose is the most abundant polysaccharide that can be find in nature, and also the largest simple component of lignocellulose. It is a high molecular-weight and linear polymer formed by monomeric units of D-glucose linked by β-1,4-glucoside bonds, with a maximum of 15,000 monomeric units. Each glucose unit rotate 180° with respect to its neighbours in the cellulose chain, those repeat units are called cellobiose

(see **Figure 1.3**). The union of different units of cellobiose forms the structure of cellulose. Finally, the cellulose chains create a specific network between them building a structure called microfibrils. This structure is formed by hydrogen bonds and van-der-Waals forces, which generates very strong interactions between cellulose crystal chains.

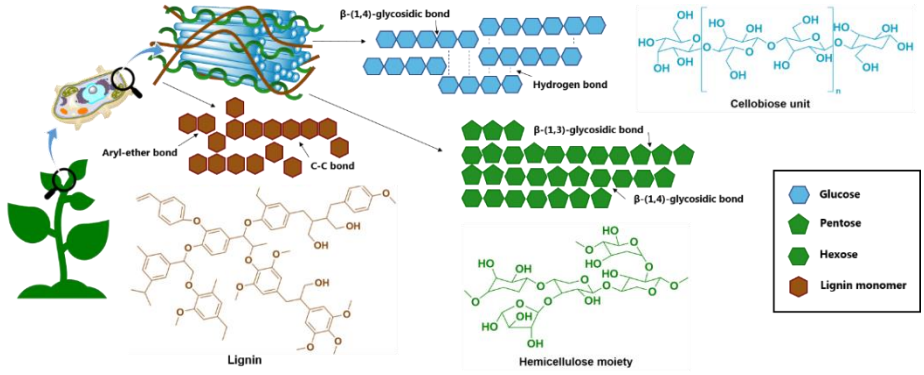


Figure 1.3 Structural representation of the plant cell wall and its main components.

The cellulose structure present in plant cells alternates between crystalline and amorphous regions. This affects to its properties because the amorphous region is more vulnerable to be degraded by any external agent. It can affect the lignocellulosic structure, since cellulose provides resistance.

Cellulose has been used mainly in paper manufacturing. However, in the last decades its applications have increased significantly. Currently, the applications of cellulose are highly varied, from different materials (nanocrystals, films, fibres, membranes, etc.) to glucose as precursors for wide variety of products such as bioethanol [1.20].

1.2.2 Hemicellulose

Hemicelluloses are an amorphous and heterogeneous group of branched polysaccharides. It is constituted mainly by pentoses (xylose and arabinose),

hexoses (mannose, glucose and galactose), uronic acids (glucuronic and galacturonic acids) and acetylated sugars [1.21] joined covalently assembling long chains. According to the way that there are joined, hemicellulose can be divided into three main groups: mannans, xylans and xyloglucans [1.22].

Hemicellulose plays an important role since it acts as a link between cellulose and lignin. Its main function consists in the stabilisation of the cell wall by the interaction with cellulose and lignin trough hydrogen and covalent bonds, respectively [1.23]. The content and structure of the hemicellulose differ considerable depending on the plant [1.17], comprising approximately between 15-35% of the lignocellulosic material [1.24]. Mannan derived sugars are the predominant monomer in hemicelluloses of softwood, while xylan derived sugars are predominant in hardwoods, annuals plants and cereals.

Hemicelluloses have good properties such as biodegradability, biocompatibility and bioactivity among others. Because of this, its application is extensive in different areas, as food, medicine, or chemical industry, with a wide variety of products. Some examples are xylooligosacharides with prebiotic applications [1.25], biopolimer production (e.g. polyhydroxybutyrate, PHB) or chemicals production as furfural or xylitol production, among others [1.21]

1.2.3 Lignin

Lignin is the third most abundant polymer in nature [1.26], and the most complex component of lignocellulose biomass. It is an aromatic biopolymer with a three-dimensional network, formed by the combination of three phenylpropane units, which differ in degree of methoxylation (p-coumaryl, coniferyl, and sinapyl alcohols), linked together. These monolignols, once

incorporated into the lignin polymer, are renamed as p-hydroxyphenyl (H), guaiacyl (4-hydroxy-3-methoxyphenyl) (G) and syringyl (4-hydroxy-3,5-dimethoxyphenyl) (S) units, respectively. These units are randomly combined by multiple ether and carbon-carbon linkages (see **Figure 1.3**), being β-O-4 ether bond the most common one [1.19].

Softwood has the highest amount of lignin, followed by hardwood and grasses, being agricultural residue those with the lowest content. Another difference between the types of wood is that softwood lignin is mainly composed by guaiacyl units, while hardwood lignin contains almost equal guaiacyl and syringyl units in its structur. The lignin provides the mechanical strength as well as the hydrophobic surface for the water transportation to the plant [1.15]. This is because it cements the cells together, binds and agglomerates the cellulose fibres and maintains the microfibrils with relatively high structural rigidity [1.15]. In addition, lignin, since it covers the structure of carbohydrates, helps in protecting tissues from external attacks [1.27].

There is a high annual generation of lignin as residue from the pulp and paper industry, around 50 million tons [1.28]. Most of the lignin is used in the same industry for energy generation to be used internally. However, since lignin is considered the most abundant renewable source of aromatic compounds, it can be valorised in multiple other ways. Therefore, at present, different applications are being sought for it, from sulphonates and additives, to more sophisticated products such as aromatic compounds (vanillin or catechol), or different materials (e.g. biopolyols) [1.28]. In addition, lignin is an antioxidant agent, which broadens its possible fields of application [1.27].

1.2.4 Inorganic matter

The inorganic matter of biomass is a non-structural part of the biomass, which content depend especially on the type of raw material as well as on environmental factors and parts of the plant. The inorganic substances have also an important role in the lignocellulosic biomass, and the common elements are calcium, sodium, potassium, magnesium, phosphorus, silicon, aluminium, nitrogen, sulphur and iron [1.17]. They are absorbed by the plant from the soil and fixed in the cell wall. For that, they are usually present as water-soluble compounds such as chlorides, sulphates, oxalates, nitrates, carbonates, silicates, phosphates etc. They are low molecular weight substances that remain as waste after incinerating the lignocellulosic material, and can cause multiple corrosion problems in the incinerators when the concentration is too high. Currently, there is no application for the inorganic matter.

1.2.5 Extractives

Extractives are non-structural components of lignocellulosic biomass. This fraction is a mixture of multiple natural chemical products that can be extracted by different solvents, such as water and/or diverse organic solvents. Although it is not an important fraction for the structure of lignocellulosic matter, its existence is necessary for plants to grow normally. They are also very important in protecting the plant from various stress situations, such as pests or extreme weather conditions. Major compounds considered as extractives are flavonoids, proanthocyanidins, lignans, fatty acids, phenols, terpenes, sterols, low molecular weight carbohydrates, resins and waxes among others [1.29].

The fraction of extractives has always been left out, mainly due to the low content in most raw materials. However, in recent years, its study and valorisation has increased considerably. The latest studies show that this fraction is rich in compounds with different capacities such as antioxidant, antifungal, antibacterial, anticarcinogenic and anti-inflammatory [1.30]. Therefore, they are a promising source for different industries such as agri-food, pharmaceutical and cosmetic among others. Obtaining added-value compounds from this fraction is a step towards the complete recovery of lignocellulosic waste from a point of view of efficient biorefinery.

Nowadays, the use of these natural resources is already being satisfactorily exploited. There are already several companies in Europe, which commercialise bio-based and natural products. The European Commission has highlighted these success stories in a report made on 2019, "Bio-based products – from idea to market "15 EU success stories" [1.31].

1.3 Forestry development

In recent years, there has been a growing awareness of the importance of forests. They are very important ecosystems that maintain biodiversity and they are key components for the biophysical and biochemical processes on earth. In addition, they provide services that are essential to human well-being. The main services apart from the supply of wood, firewood and other products (food, livestock feed, etc.) are: the capture of CO_2, the support of biodiversity, water purification, soil fixation and fertilisation, as well as other services of aesthetic/emotional pleasure [1.32]. For all these reasons, forest management is becoming very important all over the world and especially in Europe. On a proposal from the Commission [1.33], European ministries of agriculture have adopted a common strategy for

multifunctional and sustainable forest management. This strategy is based on three principles, which are: a) sustainable forest management and the multifunctional role of forests; b) resource efficiency; c) promoting sustainable production and consumption of forest products [1.34].

The world forest area is about 4,033 billion ha, and in the European Union (EU) are about 177 million ha, which correspond to the 5% of the total world forest area. However, the 37% of the total EU land area is covered by forest, mainly by boreal conifer forest, but also by many others [35]. In the case of Spain, forest area is about 28 million ha (18.5 million ha correspond to forested area), which correspond to the 55.2% of the total area of Spain. The Spanish forests are mainly formed by hardwood (57%), but the most abundant species are *Pinus sylvestris*, *Pinus pinaster* and *Pinus haleperis*, followed by Eucalyptus [1.36]. Regarding to the Basque Country, it has a forest area of 489,886 ha, which corresponds to 68% of the total area of the Basque Country. 54.6% of the surface of the Basque Country is considered as wooded forest and 35% of it corresponds to radiata pine forests.

According to EIP-AGRI, the forest-based sector is defined as "sector that covers forest resources and the production, trade and consumption of forest products and services. The woodworking and furniture industries, the pulp and paper manufacturing and converting industries and the bioenergy sector as value chains stand for the forest-based sector." [1.37]. The main product obtained from the forests is wood. It is the oldest natural resource available to humans. Traditionally, more concretely before the appearance of fossil resources, wood provided us with fuel, tools, construction material, material for furniture and utensils, as well as transport, serving for the construction of carts and boats.

The latest FAO report in 2018 on the global status of forest products shows an overall increase in the global production of wood-based products. In this study, the industries are divided according to the type of products they sell, being the categories: industrial roundwood, sawn wood, wood-based panels, fibre furnish, paper and paperboard and wood fuel, charcoal and pellets. The production of sawn wood, wood-based panels and roundwood is over 910 million m^3, while the paper industry produces more than 106 million tonnes. Only the 9% of wood in Europe is used as fuel [1.38]. This confirms the great importance of the forest-based industry in Europe.

In Spain, 18.9 million m^3 of wood are used in different sectors such as pulp and paper, firewood, sawn wood, etc. Apart from the wood-based products, in Spain other non-wood products are obtained from the forest, such as cork and resin, with 59,869 and 11,314 tonnes, respectively.

In the Basque Country, there are almost 36 million m^3 of wood stocks, but only 1.2 million m^3 are used in the wood-based industry [1.32, 1.39]. The total amount that is used is far below the available resource, which allows the territory to adapt to the future trends where the forests will take a great importance again.

There is a global trend for the multifunctional use of forests within the framework of the bioeconomy [1.34]. It encompasses the production of renewable resources and their conversion that is necessary to build zero CO_2 future according to the Paris Agreement. Thus, the forestry sector is in an exceptional situation, and it is believed that in the coming years its importance in the economy will increase due to the new forest-based development. For this reason, it is planned to cover the demand generated by the market, both for traditional uses and emerging industries and processes. The new products are based on a more efficient use of forest

resources through their exploitation in cascade decreasing the amount of waste generated, or what is the same, from the point of view of the biorefinery.

1.3.1 From waste to value: revalorisation of tree barks

The largest amount of waste generated in the wood-based industries, as well as that resulting from forest management, corresponds to the bark of the trees. Bark is the second most important tissue in a trunk, after wood, comprising approximately 10-20% of the total volume of the log [1.40, 1.41], depending on the species and growth conditions [1.42]. Moreover, considering the annual number of trees used in the different industries; it can be concluded that the amount of waste to be managed is high. This residue is generated in the debarking process, since although both bark and wood are lignocellulosic materials, their chemical composition as well as their structure are different, and so the properties provided by each of these fractions are also different.

From the point of view of sustainable development, it is necessary to eliminate and/or reduce the generation of waste. Based on this, and knowing the annual volume of generated bark, it is necessary to look for a possible valorisation route for this waste.

According to the technical definition, the bark is the most outer layer of the roots and stems of woody plants. It is a heterogeneous cellular material, which is formed by three different parts: phloem, periderm, and rhytidome [1.41]. The tree bark, as the rest of the lignocellulosic biomass, is constituted by lignin, cellulose, hemicelluloses, suberin, extractives and inorganic elements, and their chemical composition depends on different factors such as the tree species, the age and the place where grows (environmental

conditions) [1.43, 1.44]. Contrary to wood, bark is rich in extractives and suberin. These compounds allow the bark to carry out its function, which is to protect the tree against external climatic factors as well as against parasitic infections and herbivores. They are therefore constituted of different compounds, depending on the function that the cells and tissues develop. Because of that, a huge amount of organic chemicals can be isolated, such as flavonoids, alkaloids, carbohydrates, terpenoids, glycosides and lignans among others [1.45].

Up to now, the main use of bark is energy production or horticulture application [1.42, 1.46], which is not bad at all since it has an economic benefit, but other kind of valorisation could be more profitable and more environmentally friendly according to its chemical composition. Because of that, bark could be a good renewable source of many platform chemicals and bio-based products. Therefore, in the last years the research is focused in the overall use of all the bark fractions, with a particular interest in the extractive fraction, since it is the less studied and the one that has bigger potential due to the variety of compounds that form it.

As a result of the diversity of compounds that constitute the extracts, the studies carried out are varied, depending on to what will be extracted. There are many studies of bark from different trees that focus on the extraction of a mixture of compounds with specific properties, such as antioxidant, antimicrobial, antifungal, antitumor and enzyme inhibitory effects among others [1.47–1.53]. There are also several studies where more selective extractions of different compounds, such as tannins [1.54, 1.55], triterpenes [1.56, 1.57], oil [1.58] and carbohydrates [1.59] among others are carried out. There is a wide variety of value-added compounds that can be obtained from the bark, with applications in different fields, such as pharmaceutics,

cosmetics, personal care products, nutritional additives or in material production [1.40, 1.46, 1.60].

Although there are already many examples of bark extracts valorisation, it is a topic that is on the rise due to the complexity of this process, as well as the absence of homogeneity of the raw materials. The research around the different extraction and purification methods, as well as the study of the different compounds that form the extractive fraction must continue until multiple value-added compounds can be obtained, instead of just one, thus increasing the economic value of the waste in accordance with the sustainable development. To this end, there must be an evolution in both technology and knowledge so that more value-added products can be obtained from the same raw material through more environmentally friendly technologies.

1.4 Extraction methods

Separation process is one of the most crucial steps for the isolation of not only the main components from the raw material, but also for the minority compounds. The selected separation process depends on the final product, as well as on the impact generated by it on the cost of the production process and on the environment. Due to the growing concern about the current situation of the planet, as well as the scarcity of fossil resources, there is an increasing interest in the development of new biorefineries. These are based on the separation and purification of compounds from renewable materials, so separation processes are of great importance. This step corresponds up to 40-80% of the total cost of currently used most common chemical process [1.61], and one of the most exploited process is extraction.

Extraction is the main process for the separation and obtention of the interest compounds, since it converts the real matrix, in our case the lignocellulosic material, into suitable for the following procedures [1.62]. Lignocellulosic materials can be a source of a wide variety of products, from complex natural polymers to simple but high value-added organic compounds.

Extractions of natural compounds from plants are not new, as they have been carried out since ancient times. Furthermore, from a biorefinery point of view, this process is becoming very important. Currently there are many different methods, but the most widely used are solid-liquid extractions, which are considered as conventional methods. Within this group, decoction, maceration, infusion, digestion and percolation can be found. These techniques are the oldest used so far, and came to light during the 11th century. They form the basic principles applied in the new advanced extraction techniques [1.62]. During the 19th century, the "Soxhlet Extraction" technique was introduced, which is an advanced form of digestion and decoction methods. Later, in the 20th century, hydrodistillation was used to extract essential oils from plants. Maceration, infusion, Soxhlet extraction and hydrodistillation are the most used conventional techniques (**Figure 1.4**).

In general, conventional extraction (CE) methods require the use of a large amount of solvent (usually organic solvents), as well as long extraction time, even extending for days. In addition, these methods require techniques such as evaporation and concentration to achieve the final products, this being an increase in the cost of the process as well as in the environmental impact. Finally, it must also be taken into account that the use of high temperatures is counterproductive for the extraction of volatile compounds. With all this,

it can be said that these methods do not satisfy the criteria of green chemistry (see **Appendix I**), so that, in the last decades other more environmentally friendly extraction methods have been studied.

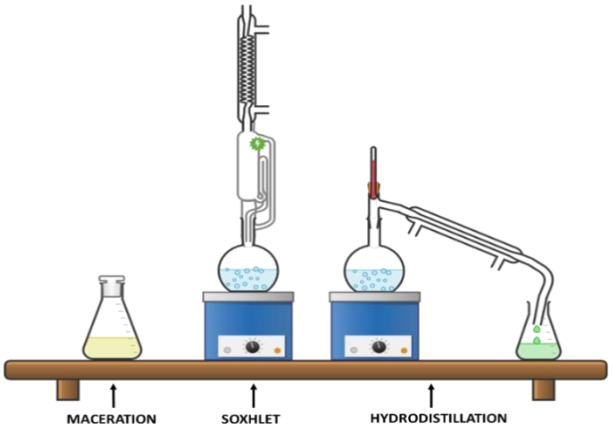


Figure 1.4 Some examples of conventional extractions.

In order to overcome specially the drawbacks related to time and solvent consumption, modern non-conventional method such as microwave assisted extraction (MAE), ultrasound assisted extraction (UAE), supercritical fluid extraction (SFE), pressurized liquid extraction (PLE) and pulse electrical field extraction (PEF) were developed. These new techniques provided a reduction of energy consumption, higher efficiency, higher yield, better temperature control and better quality extracts [1.63]. They are considered as sustainable extraction techniques [1.64].

1.4.1 Microwave assisted extraction (MAE)

MAE uses electromagnetic irradiation (microwave) with frequencies between 300 MHz (1 m of wavelength) and 300 GHz (1 mm of wavelength) (**Figure 1.5**). Typically, extractions are carried out at 2.45 GHz frequencies, where the advantage of extraction process come from the dielectric heating, which generated an efficient heating of the materials. In contrast to what

happens in CE, where heat is transferred from outside to the inside of the sample, in the MAE the heat is equally distributed inside the irradiated medium. Therefore, the heat is homogeneous throughout the mixture from the beginning, as the mass transfer and the direction of heat transfer are identical [1.65].

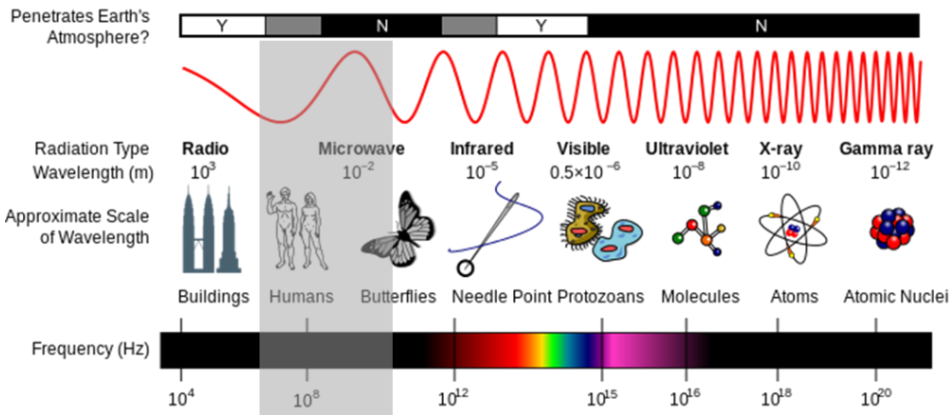


Figure 1.5 Electromagnetic spectrum. Adapted from Inductiveload, NASA / CC BY-SA (http://creativecommons.org/licenses/by-sa/3.0/).

Dielectric heating depends on the ability of the material to absorb microwave (MW) energy and transform it into heat. This heating principle is based on two different mechanisms: rotation of the dipole moment, and ionic conduction [1.66]. Only the substances that possess dipole moment are able to transform the MW radiation in heat. This occurs because they try to align with the alternating electric field in a MW medium and collide with each other creating heat [1.67]. It means that only polar substances can be heated by this kind of irradiation. Related to ionic conduction, this heating process is generated due to the collisions between particles that are generated as a result of the influence of the magnetic field. MW irradiation have a fast energy delivery, which is transformed on a fast heating of solvent and mixture in general [1.68].

The most common MAEs are carried out in a closed vessel, under controlled pressure and temperature, or in an open vessel, under atmospheric pressure conditions and the maximum temperature limited by the boiling point of the used solvent.

MAE is a good method of extraction to be used with vegetable matrices. The high pressure generated inside the raw material as a result of the direct heating of the water molecules present inside the cells causes the rupture of their walls. This effect reduces the mass transfer barrier between the solvent and the raw material, improving the penetration of the solvent, which increases extraction efficiency and reduces the extraction time [1.69, 1.70]. Currently, studies are ongoing for the use of solvent-free MAE, better known as solvent-free microwave extraction (SFME) [1.58]. This method is based on MW technology but does not require the addition of any type of solvent, since MWs directly affect the internal water of the plant and this generates the breakage of the cell wall which allows the extraction of some compounds [1.71]. This method becomes highly desirable due to the non-use of solvent, which makes it a promising green alternative. This technique is being studied especially for obtaining essential oils from vegetable matrices.

In general, MAE method has different advantages such as shorter extraction time, quicker heating, higher extraction yield, lower thermal gradient, smaller equipment size and lower solvent requirements [1.66, 1.72–1.74]. This method is gaining popularity due to the adaptability of the equipment and its low cost, as well as its easy use [1.66].

1.4.2 Ultrasound assisted extraction (UAE)

UAE works with high frequency sound waves that go beyond the human ear, but at lower frequencies than those used in MAE. UAE is usually higher than 20 KHz and below MW frequencies (up to 10 KHz) [1.75]. The driving force of the UAE is the cavitation phenomenon, which involves bubbles formation and collapse. Briefly, ultrasound (US) waves propagate through the medium by inducing a series of compression and rarefaction waves on the molecules, just as sound waves do. If these waves have enough power, gas bubbles are formed because the molecules in the medium do not re-bond. These bubbles will grow in the compression-rarefaction cycle, increasing the amount of gas inside the bubbles little by little, until they reach their maximum size and collapse (**Figure 1.6**), generating energy [1.67].

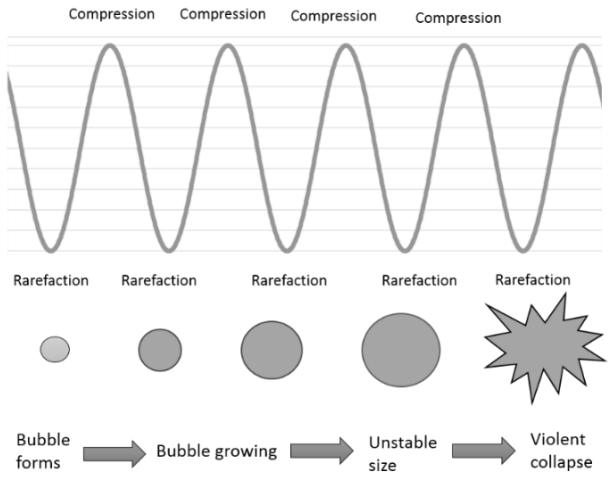


Figure 1.6 Cavitation phenomenon. Creation, development and collapse of the bubbles created by US. Adapted from [67].

The cavitation bubbles collapse violently producing extreme localized conditions (high temperatures and pressures). When the collapse is generated in a homogeneous liquid, it is symmetric. However, when the collapse occurs near a solid surface, the collapse is asymmetric, and this

forms a high velocity jet that can affect to the solid surface [1.67, 1.76]. This effect improves the extraction performance through effects such as cell wall disruption, intensive mixing, particle size reduction and hot spots. In addition, in the case of plant tissues, a modification of the tissue occurs which facilitates swelling and penetration of the solvent into the cells [1.76].

There are two different ways of carrying out the UAE. On one hand, there is the direct application of US radiation, by ultrasonic probe (horn), and on the other hand, there is the indirect application, by US bath. US baths are typically pf small volume and they can have 1 or 2 transducers (normally one) [1.67]. They have the great advantage that they are easy to use, as well as the fact that the transducers are not in direct contact with the solutions, making maintenance easier. The reason that the transducers are not in direct contact with the sample can be an advantage in avoiding sample degradation, due to the attenuation of the acoustic energy by the solvent [1.77], or it could be a disadvantage since the energy applied to the sample is lower [1.78]. Another disadvantage of the use of the US bath is that it generally operates at a fixed frequency of between 20 and 40 KHz [1.75].

On the other hand, the horn system is considered more efficient as the direct contact of the transducer with the sample and the solvent improves the extraction efficiency and minimises the loss of acoustic energy, which is very common when the US bath is used. In addition, the horns generate a higher US intensity, since it is only transmitted in the tip of the horn. This is an advantage, as the higher intensity increases the mass transfer, but can generate high temperature peaks, which can end up degrading the sample [1.77, 1.78]. Because of this the US horn is considered more difficult technique, as it has more parameters that must be controlled in the

extractions, such as amplitude, shape and size of the reaction vessel, temperature and type of horn among others [1.78].

UAE, via acoustic cavitation bubbles, improved cell disruption, milling, mass transfer and penetration [1.67]. Because of this, the main advantages of UAE are the reduction of extraction time, solvent and energy consumption, and also the increase of the extraction yield [1.79].

In the last few years, the acceleration of extraction process with a reduction of extraction time by the successive and simultaneous use of MAE and UAE is also being studied. It is a formidable technique that can reduce the use of solvent and extraction time and result in a high extraction yield in comparison not only with CE but also with MAE and UAE [1.80].

1.4.3 Supercritical fluid extraction (SFE)

SFE is based on the thermodynamic properties of fluids. Supercritical fluids (SCF) are achieved when their temperature (T_c) and pressure (P_c) are beyond the critical point established for each compound. Working with SCF, the inherent properties of each phase, liquid and gas, vanish. Under these conditions, SFC has properties of both gases and liquids. Thus, it maintains properties of high diffusivity, low viscosity and a nearly negligible surface tension of gases, and a density and solvating power similar to those of liquids [1.79, 1.81]. CO_2 is the most used SFC due to its low critical temperature (T_c= 31.1 ºC) and its moderate critical pressure (P_c = 7.38 MPa) [1.76, 1.81]. In addition to its usage conditions, the employment of CO_2 has more advantages, since it has an inert nature, is not toxic, nor is it inflammable, so it fulfils the principles of green chemistry. However, there is a problem that must be solved, because CO_2 has a low polarity, which generates problems for the extractions of polar compounds. However, this

problem can be solved with the addition of a co-solvent in low quantities [82], usually polar organic solvents [1.81].

The use of SFE has numerous advantages over conventional extractions. The most important is the improved mass transfer of this system due to the properties of SFC, which substantially reduces extraction time. Furthermore, this method is considered ideal for the extraction of thermos-labile compounds, since it is carried out at room temperature, so there is no degradation of them [1.79].

1.4.4 Pressurised liquid extraction (PLE)

PLE is based on the relationship between temperature and pressure variables, which makes the boiling point of a solvent proportional to the pressure. This means that when the pressure on the system is increased, the boiling temperature is also higher, so the solvent remains liquid beyond its normal boiling point [1.66]. The use of higher extraction temperatures promotes the extraction of the target compounds by increasing solubility and mass transfer rate. This is due to the decrease in viscosity, which favours the wetting of the plant matrix improving solubility, and also due to the break in the bonding forces, which facilitates the diffusion of the compounds into the solvent [1.83]. As with SFE, the extraction process is based on two stages, solubilisation of the analyte and then diffusion of the analyte [1.84].

One of the advantages of this method is that the system provides protection to oxygen and light to sensitive compounds and improves extraction yields, thus reducing time and solvent consumption [1.85]. Moreover, this method contributes to saving not only solvent but also energy, since less energy is required to heat a liquid than a gas.

1.4.5 Pulse electrical field extraction (PEF)

Pulse electrical field extraction (PEF) is based on the phenomenon of electroporation, which consists of increasing the permeability of the cell membrane due to the applied electrical field, allowing the recovery of intracellular compounds by diffusion [1.86, 1.87]. The pulses applied to the sample generate an electrical potential, which together with the dipole nature of the cell membranes, causes the membrane molecules to separate according to their charge, forming pores and increasing the permeability of the membrane [1.79]. This change could be temporary or permanent, depending on whether the damage to the cell is reversible or not [1.88].

One of the most important advantage of PEF technology is its non-thermal nature. This allows to reduce or eliminate the application of heat as well as the use of solvent [1.86], being very useful for the extraction of thermo-labile compounds.

All in all, it can be concluded that the methods under development generally obtain better extraction yields, are more selective, and require less or no solvent.

1.5 Extraction solvents

In order to achieve the extraction of the expected compounds from the biomass not only the selected extraction method is important, but also the solvent. With a specific solvent the efficiency of the extraction can be enhanced. At this point, the principles of green chemistry must also be taken into account with the aim of carrying out more environmentally friendly processes reducing or eliminating the use of hazardous substances.

For these purposes, the most commonly used solvents, volatile organic compounds (VOCs), should be replaced by more environmentally friendly reagents such as H_2O, EtOH or new modern solvents that are being studied, as ionic liquids (ILs) or deep eutectic solvents (DES). The main problem with VOCs is their toxicity not only to human health, but also to environment, as well as the potential explosion hazard and their high impact on the greenhouse effect [1.89], [1.90]. In order to reduce or eliminate these risks more environmentally friendly solvents need to be used.

1.5.1 Ionic liquids (ILs)

ILs are composed of organic cations that are combined with organic or inorganic anions (**Figure 1.7**), but do not package well with each other, which gives them ionic character. Nitrogen based cations are the most commonly used ones (imidazolium$^+$, pyridinium$^+$, pyrrolidinium$^+$, etc.), and the most used anions are bromide, chloride, acetate, tetrafluoroborate and hexafluorophosphate. However, more biodegradable and less toxic alternatives are being studied [1.91].

ILs are considered a new class of non-molecular liquid materials, due to their melting point below 100 °C, with unique properties. These properties are a consequence of the ionic characteristics that result from the complex interaction of hydrogen bonds, van-der-Waals and coulombic interactions of the ions that form the IL [1.92]. ILs are characterised by negligible vapour pressure, good thermal and chemical stability, low combustibility, tuneable solubility, relatively low toxicity, low nucleophilicity, good miscibility with water or organic solvents and high solvation ability (organic, inorganic and polymeric compounds) among others [1.92–1.96]. Moreover, these

properties are easily adaptable to the needs of each process thanks to the great diversity of ILs that can be synthesised by simply changing either the cation or the anion. For this reason, ILs are considered "designer solvents". The above-mentioned properties allow ILs to be used in a wide range of applications, in various fields such as analytical, electrochemical, engineering, physical chemistry, solvents and catalysis among others [1.92, 1.93]. Some of the physical properties of ILs that can be adapted include viscosity, solubility, melting point, and hydrophobicity [1.92].

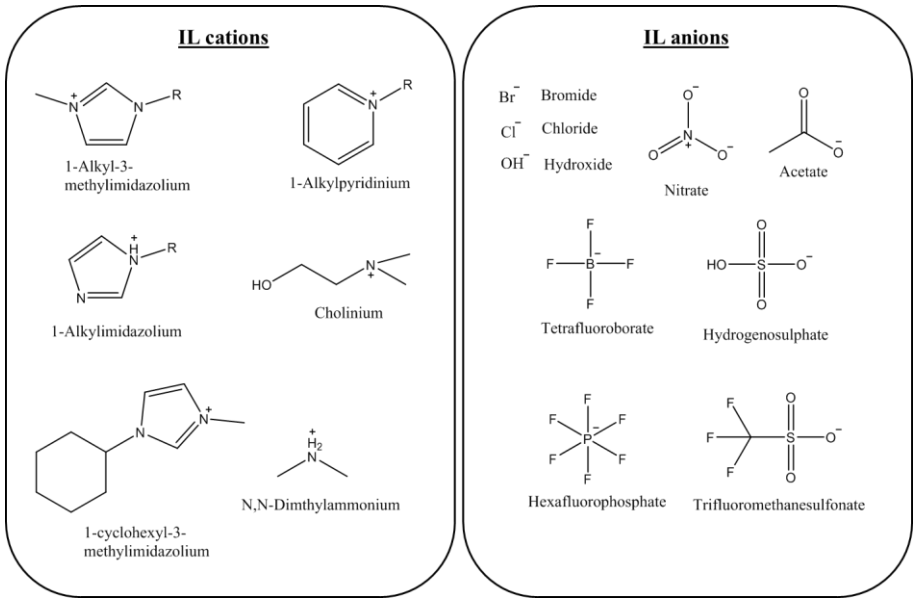


Figure 1.7 Chemical structure of some commons IL cations and anions.

Since they are easy to adapt to the needs of each process, ILs have been developed extensively in recent years. The first IL was synthesised by Paul Walden in 1914. He discovered the $[EtNH_3][NO_3]$ that had a melting point of 12 °C, and which is also the first synthesised protic IL. It was not until 1980 that John Wilkes' group introduced the most popular cation for the first time in the synthesis of the IL, dialkylimidazolium cations. At the end of the 1980s and during the 1990s the interest in ILs increased, until the end

of the XX century when the term "designer solvent" was introduced, which contributed enormously to the development of IL [1.97]. After that, the interest in the use of IL has been increased considerably in different fields, mainly because of its adaptability to each process and its low vapour pressure.

Figure 1.8 summarises the evolution of the development of ILs and the wide range of synthesised IL types [1.92, 1.93].

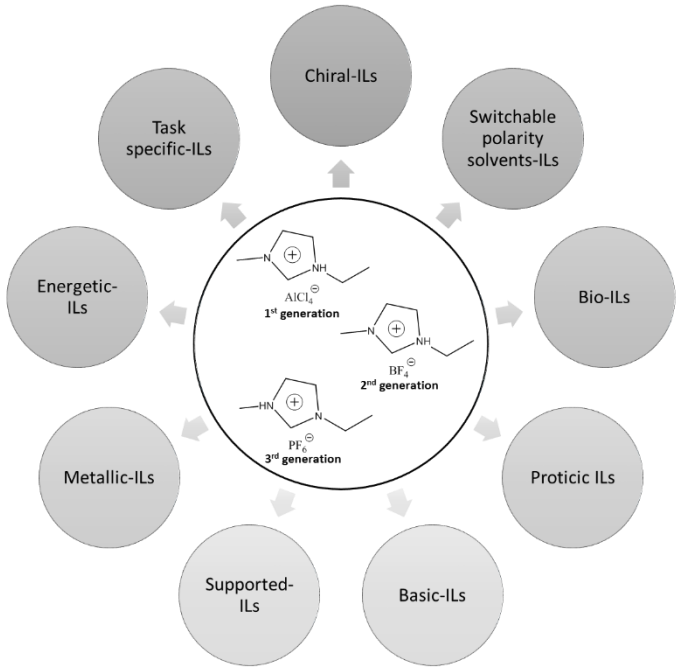


Figure 1.8 Development of ILs and their structural classification.

This work will be focused on the use of ILs as solvents for the extraction of compounds of interest from biomass. In this field, ILs are considered as green solvents mainly due to the following reasons [1.93]:

- They are non-volatile solvents due to their low vapour pressure under ambient condition.
- They remain liquid over a wide temperature range.

- They have excellent lubrication and solubility properties, as well as tuneable acidity and basicity.
- They mainly have hydrophilic nature and a wide range of solubility of biopolymers.
- They can be reused several times.

ILs may be suitable for the extraction of high added-value compounds from plants due to their high solubility [1.95]. In the last few years, ILs have been studied for the extraction of compounds such as alkaloids, lipids, flavonoids, terpenoids, aromatic compounds and phenolic acids among others [1.91]. This application is very promising as it can mitigate environmental contamination, as well as improve selectivity and extraction yield of ILs compared to those obtained by organic solvents. Moreover, this type of solvents can also be combined with the non-CE techniques explained above, thus increasing the intensification of the processes and making them more profitable.

1.5.2 Deep eutectic solvents (DES)

DES, which are considered as the 4th generation of ILs by some authors, are compounds formed by the complexation of a hydrogen bond acceptor (HBA) and a hydrogen bond donor (HBD) mainly linked by hydrogen bonds. DES differ from ILs in two aspects; on the one hand, in their chemical formation process and on the other hand, in their starting materials [1.98]. ILs are formed by ionic bonds, giving them ionic characteristics, while DES are mostly formed by non-ionic species (salts or molecular components) linked by hydrogen bonds (**Figure 1.9**). However, both share many of the characteristic properties described above for ILs, such as good chemical stability, negligible vapour pressure, tuneable solubility among others [1.81].

DES are formed by mixing two or more non-toxic compounds (cheap, renewable and biodegradable), which form an eutectic mixture [1.99, 1.100]. This makes the melting point of the resulting mixture lower than the melting points of the individual compounds, giving it specific properties, including the ability to remain liquid at temperatures below 150 °C, and many of them even between room temperature and 70 °C [1.99]. In addition, the synthesis is simple and does not require any type of purification [1.100] unlike the synthesis of ILs that are usually more complex, requiring the use of solvents and a purification step. Therefore, the use of DES has some advantages over the use of ILs, such as lower cost, inertness with water, easy to prepare and most of them are non-toxic, compatible and biodegradable [1.99].

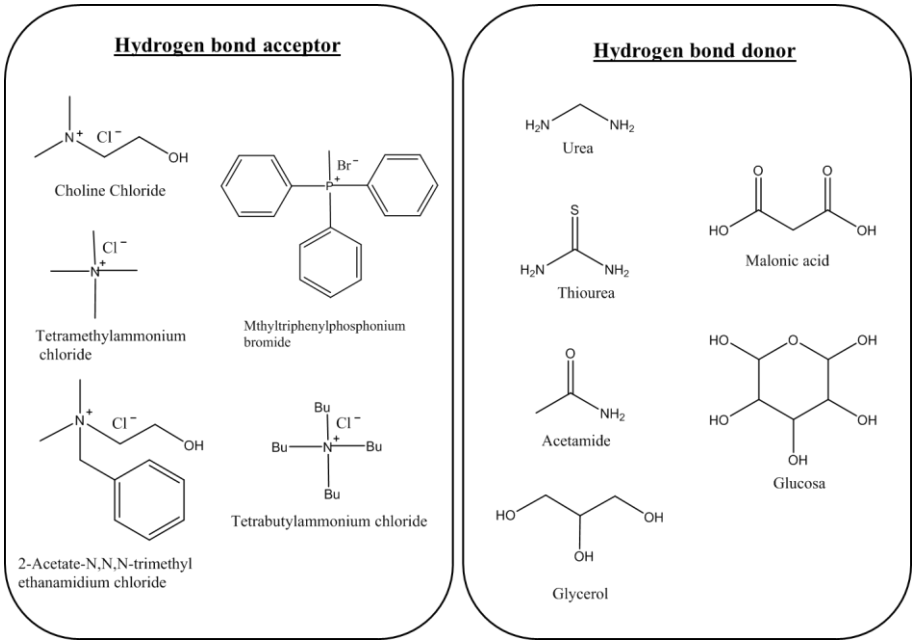


Figure 1.9 Common HBA and HBD for DES preparation.

The first DES were reported between 2003 and 2004 and since then the interest of both, the industry and the scientific community, has increased. Its production is very easy since it only requires the mixture of two or more

compounds with heat, normally at 80 °C, or freeze-drying, until the mixture is completed, without the need of purification [1.100]. However, it is important to keep the system free of humidity, since HBAs are usually very hygroscopic and this can stop the reaction [1.101]. DES are usually classified into 4 groups, type I is formed by quaternary ammonium salt (QAS) plus metal salt, type II is formed by QAS and metal salt hydrate, type III is formed by QAS and HBD (they are the most frequent ones) and type IV is formed by metal salt and HBD [1.101]. DES based on natural compounds, such as primary metabolites that are present in nature (amino acids, organic acids, sugars, or choline derivatives) are known as natural deep eutectic solvents (NADES). These types of compounds fulfil the principles of green chemistry, since they are non-toxic, renewable and have a high extraction and separation efficiency [1.90].

As well as ILs, the DES have a wide range of applications due to their tuneable properties, such as dissolution and extraction process, organic synthesis, electrochemistry, catalysis and for material chemistry [1.99]. Furthermore, they also can be combined with the non-CE techniques in order to increase the intensification of the processes and make them more profitable.

Regarding the applicability of DES in the extraction of compounds from biomass, there are already numerous studies due to the properties of these solvents. Some of them are focused on the extraction of added-value compounds such as flavonoids, polyphenolic compounds and other compounds from different biomasses [1.101].

While both, ILs and DES, are considered good solvents for the extraction of value-added compounds from biomass because of their good extraction yield and selectivity, the isolation and/or purification of compounds

obtained with such solvents remains as a challenge. This is due to one of their main advantages, which is the non-volatile nature of the compounds. This means that the separation of the solvent from the compounds of interest cannot be carried out by simple evaporation. Therefore, other isolation techniques are being studied, such as back-extraction with organic solvent, precipitation with anti-solvents, evaporation when it is applicable, or separation by the use of macroporous material or anion-exchange resins [1.91].

1.6 Thesis main objective and methodology

The general objective of this thesis was the valorisation of the extractive fraction of the lignocellulosic material in order to extract added-value compounds. The work is focused on the extraction of bioactive molecules with potential to be used as a substitute of synthetic compounds in different fields, using sustainable extraction processes. For this purpose, different extraction methods were suggested to find the most efficient and sustainable extraction method. In this context, different stages and studies were carried out.

Firstly, a study of different forest residues was performed to select the most suitable raw material for obtaining bioactive molecules. To achieve this objective, the characterisation of different raw materials has been carried out in order to study their chemical composition and their potential. In this way different parts of the same hardwood trees (bark and wood) were chemically characterised, as well as some softwood tree barks. Then, the potential of the barks was studied by characterising the different extracts.

Once the optimal raw material was selected, three different extraction methods were studied to valorise the extractive fraction for the *Larix decidua* tree bark: CE, UAE and MAE. The operation conditions of all the extraction techniques were optimised to improve the extraction yield of the processes. Afterwards, the chemical and structural characterisation was carried out, and some of the compounds present in the extracts were identified. In this way, the influence of different extraction methods on the extraction yield as well as on the properties of the obtained extracts was studied.

After considering the potential of the UAE and MAE techniques, it was decided to promote the extraction of high added-value molecules by the simultaneous use of both techniques. To this end, a study was perfomerd on the effect that the simultaneous use of both techniques could have on the improvement of the extraction yield and on the sustainability of the process. Thereafter, the extracts obtained under the optimal reaction conditions of the SMUAE were characterised, and some compounds were identified.

Finally, with the aim of increasing the sustainability of the extraction, the selective extraction of flavonoid compounds was studied using different ILs and DES. This work was done by analysing the influence of different selected solvents on the extraction yield and the composition of the extracts. For this, the previously synthesised ILs and DES were used as solvent for CE. Afterwards, solid and liquid phase's characterisations were performed to determine the selectivity of the extraction as well as the properties of the obtained extracts, discussing the enhancement of the extractions.

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Chapter 2

Bark as a potential source of biomolecules

2.1 Background

In the las decades, the use of natural products obtained from renewable sources has increased, letting aside the use of the synthetic products derived from fossil fuels. It is mainly due to the increase of the society concern about environmental problems and the impact in health care. Thus, recent research is focused on finding new sources of materials and chemicals to replace fossil fuel-based products. For that, lignocellulosic biomass is one of the most studied resources of chemicals and materials, mostly due to its universal availability, as well as its renewable nature [2.1].

Lignocellulosic biomass is mainly constituted by cellulose, hemicellulose and lignin, but it also has small amounts of inorganic compounds and other organic compounds present as extractives. The chemical composition of the biomass depends principally on its origin [2.1], as can be seen in **Table 2.1**.

Table 2.1 Typical chemical composition of different lignocellulosic materials [2.1–2.5].

Lignocellulosic biomass	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Ash (%)
Hardwood	40-55	18-40	15-25	<1
Softwood	40-50	11-35	20-35	<1
Agricultural waste	25-47	12-45	5-24	1-20
Grasses	25-40	25-50	10-30	6-8

Between all possible lignocellulosic materials, forest biomass and residues from agricultural and forestry industries are the most studied since they are available, abundant and do not compete with food production.

2.1.1 Forest as renewable resource

This thesis is focused on the valorisation of forest biomass, which is an important renewable resource. Forest are constituted by plants and trees, and they produce a wide variety of products, and also provide food for many living organisms. Trees are formed by roots, foliage, trunk and branches as it can be seen in the **Figure 2.1**. Each of the parts have their function in the tree. This way, leaves are essential for the growth of the trees, since they are in charge of performing the photosynthesis, the breathing and the transpiration. Bark is the impermeable layer that covers the tree protecting him of external atmospheric agents, cambium is the zone in charge of the growth and development of the tree, sapwood is the young wood and heartwood is the wood with hardness, formed by tissues that have reached their total development. Due to their different functions, the wood and the bark are different not only in chemical composition, but also in anatomical structure [2.6]. Although wood and bark have the main basic composition, bark is richer in extractives and suberin [2.7, 2.8], which help on the protective function that the bark has.

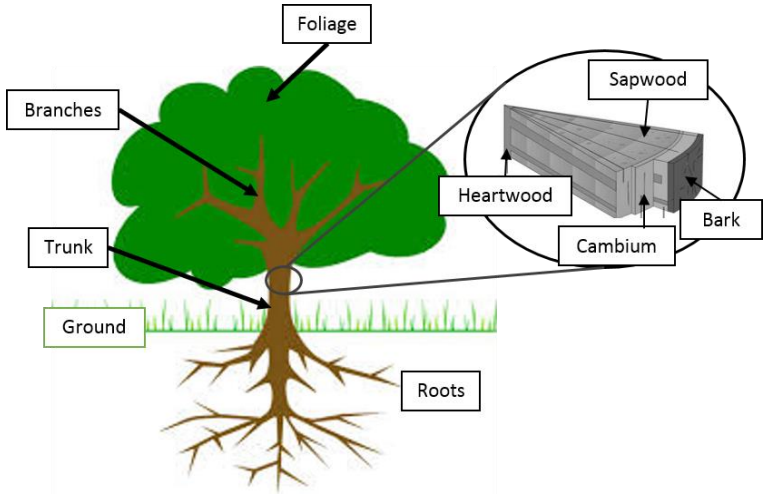

Figure 2.1 Different parts of the tree and of the internal structure of a trunk.

The wood from trees has been a resource of materials for many applications, due to its properties such as good isolation, mechanical resistance, hardness, high durability, and great flexibility. Currently there is a large market for wood, mainly in the production of materials for various uses, as well as for the generation of energy or heat. Nevertheless, in the last years, the use of the wood for the obtaining of chemical products is being studied, as well as new materials. Due to the chemical and structural difference between wood and bark, the de-barking process is used to separate both before processing the wood. Because of that, bark is considered as the most available by-product or waste stemmed by the wood-based industry.

2.1.2 Valorisation of different lignocellulosic biomass fractions

The fractionation of the biomass in its three main components is being widely studied to obtain different products or intermediates with different applications. However, the rest of the fractions are not given as much importance, mainly due to the low percentage they represent.

The three structural compounds of lignocellulosic biomass are linked by different types of bonds, which provides rigidity and stability to the structure. This is beneficial for the growth of the plants; however, it entails a problem for the separation of the fractions. Therefore, to carry out the fractionation it is necessary to use chemical, biological or mechanical processes to break these links. From the point of view of an integral valorisation, the sequential application of different procedures to obtain the fractions is being studied, following the guidelines of the biorefinery.

Hemicelluloses are the biopolymers which act as connectors between lignin and cellulose in a lignocellulosic structure. Therefore, the first step in a

biorefinery is usually the separation of this fraction, which makes the subsequential delignification stage more efficient, since cellulose and lignin are more accessible in the following steps [2.9]. To carry out this first step, some of the most used processes are alkaline treatments, organosolv treatment, ionic liquid treatment, steam explosion or autohydrolysis. The las two are the most applied ones because these treatments allow a selective solubilisation of the hemicelluloses, reducing the extraction of other fractions. Besides, they are considered green processes because they only use water.

Autohydrolysis is a treatment based on the auto-ionisation of water into H_3O^+ and OH^-. The hydronium ions act as catalysts for the hydrolysis of the glycosidic bonds of the hemicelluloses, allowing its solubilisation [2.10]. This treatment is usually carried out under subcritical conditions, at temperatures between 160 and 240 °C, with a pressure above the water saturation point (4.9-20 bars). The steam explosion process is carried out using high-pressure saturated steam (between 20 and 50 bars), temperatures between 210 and 290 °C and short periods of time [2.11]. This process produces the rupture of intermolecular and intramolecular bonds [2.11]. Unlike autohydrolysis, this process can solubilise some of the lignin. The hemicelluloses extracted during these treatments can be used to obtain different products, from xylooligosaccharides to furfural [2.12, 2.13].

The next step consists of separating the cellulose from the lignin by treatments that do not affect its structures. Among possible treatments, there are two that stand out, alkaline delignification and organosolv delignification. In alkaline delignification, alkaline agents (such as NaOH or KOH) are used to remove the lignin from the biomass by saponification of the intramolecular ester bonds. This treatment is usually used at

temperatures between 25 °C and 150 °C [2.14]. However, in organosolv delignification, lignin is dissolved using aqueous solutions of an organic solvents at temperatures between 100 and 250 °C, normally at high pressure [2.15].

The products obtained with these treatments are lignin and cellulose, which have a wide range of possible uses. Thus lignin can be used both directly, for example to obtain biopolyols [2.16], or converted to chemicals such as vanillin or catechol [2.17], with applications ranging from food industry to chemical or pharmaceutical industries [2.18]. Cellulose can also be used in many areas, from medicine to photoelectric materials and biofuel [2.19]. As in the case of lignin, its wide applicability is due to the fact that it can be used directly as a polymer, such as for cellulose nanocrystals production, or it can be converted into glucose for its subsequent transformation into bioethanol [2.20].

The extractive fraction is a mixture of different compounds, which varied depending on the raw material, so its valorisation is a challenge. Usually, tannins, waxes, lignans, fatty acids, flavonoids and extractable carbohydrates compose the extractive fraction [2.7], some of which are bioactive molecules. These types of molecules are of high interest not only because of the benefits they have on people's health, but also because of their ability to preserve food, among other things. This means that their applications can be very variable, from pharmaceuticals and chemicals to bio-based materials and green polymers [2.21–2.23].

2.2 Objective

The main objective of this chapter was to select the most suitable raw material for obtaining bioactive molecules for its possible use in different industries. For this purpose, both the composition of the wood and the bark were studied.

The second objective of this chapter, after selecting the best source of biomolecules, was the characterisation of the potential of the obtained extracts in order to select the best raw material.

2.3 Materials and methods

2.3.1 Chemical characterisation of wood

Basoekin Ltd. provided the different wood samples used in this thesis, which were collected in the local forests of the Basque Country in summer 2017. The samples collected consisted of a piece of tree from which the wood was manually separated from the bark. The samples received were northern red oak (*Quercus rubra*), common oak (*Quercus robur*), common ash (*Fraxinus excelsior*), Iberian white birch (*Betula pubescens*, var celtiberica), sweet chestnut (*Castanea sativa*) and black locust (*Robinia pseudoacacia*). All species corresponded to young stands (11-18 years old) except the older red oak (60 years old). Once debarked, the wood samples were chipped, air-dried, milled and sieved, with the objective of having a homogenised lot of each kind of tree with a particle size between 0.4 and 0.25 cm, as indicated in the standard for sample preparation (TAPPI T257 cm-85).

The different tree woods were characterised in accordance to the Technical Association of Pulp and Paper Industries (TAPPI), the National Renewable Energy Laboratory (NREL) as well as some traditional methods, which are fully described in **Appendix II**. Sample moisture, ash content and toluene-ethanol extractives content were determined by TAPPI T264 om-97, TAPPI T211 om-93 and TAPPI T204 cm-97, respectively. Lignin content, both acid-soluble lignin (ASL) and acid-insoluble lignin (AIL or Klason lignin), were measured by a quantitative acid hydrolysis (QAH) using the protocol described by the NREL (NREL/TP-510-42618). Holocellulose and α-cellulose contents were analysed using the methods proposed by Wise et al. and Rowell, respectively. Finally, the hemicellulose content was calculated by the difference between the holocellulose and α-cellulose content.

2.3.2 Chemical characterisation of bark

The barks studied in this thesis have two different origins. On the one hand, Basoekin Ltd. supplied six tree barks. These barks were manually separated from the wood in the laboratory. The species obtained were the same as those of the wood: northern red oak (*Quercus rubra*), common oak (*Quercus robur*), common ash (*Fraxinus excelsior*), Iberian white birch (*Betula pubescens, var celtiberica*), sweet chestnut (*Castanea sativa*) and black locust (*Robinia pseudoacacia*). On the other hand, another five different tree barks from the sawmill Errekondo Egur-Zerra company (Basque Country, Spain) were also studied. These barks were obtained by hand picking up directly from the dry trees existing on the company. The collection was carried out in the spring of 2017, and all the species were in adult age. These species were white spruce (*Abies alba*), Douglas fir (*Pseudotsuga menziesii*), larch pine (*Larix decidua*), cedar (Cedrus), and sequoia (*Sequoia sempervirens*). All the barks were dried, ground and sieved

to obtain a homogeneous lot with a particle size of less than 0.5 mm (NREL/TP-510-42620).

The different tree barks were characterised in accordance to the National Renewable Energy Laboratory (NREL) as well as some widely used methods for specific characterisation of some bark fractions that are completely described in **Appendix III**. Sample moisture, ash content, lignin content, hemicelluloses and cellulose content, which is measured as the glucan content, were determined by NREL/TP-510-42621, NREL/TP-510-42622, and NREL/TP-510-42618, respectively. Total extractive content was measures with sequential Soxhlet extraction with dichloromethane (CH_2Cl_2), ethanol (EtOH) and distilled water (H_2O) for 6 h, 16 h and 16 h, respectively, following the procedure determined by NREL TP-510-42619. Finally, suberin content was determined following the method described by Pereira with a slight modification [2.24].

2.3.3 Characterisation of bark extracts

In order to study the potential of the extracts obtained from the barks, each bark was extracted with a mixture of EtOH/H_2O. The method used was that previously described by Miranda et al. [2.25]. The decision of using this technique was due not only to its easy application, but also to the fact that it is a technique that has been widely used [2.26–2.28]. Thus, the comparison between the results obtained in this thesis and the values reported by other authors can be done cautiously. Briefly, 4 g of dry bark was extracted with solid-liquid ratio of 1:10 (w:v) with EtOH/H_2O (50/50 (v/v)) mixture using an ultrasound bath with temperature control (Elmasonic 570 H, Elma) at 50 °C during 1 h. After the extraction, the solid and liquid fractions were separated by vacuum filtration, and the

supernatant was stored at 4 °C until it was used. The extraction yield was calculated gravimetrically and referenced to a 100 g of dried bark, determining the non-volatile content (NVC) present in the extracts as it is described in **Appendix IV**. For the rest of the characterisations, the liquid extracts were used, since the high temperatures used to dry the extracts could degrade the compounds. The measurement was carried out three times and the results were expressed as mean ± standard deviation (SD).

The chemical compositions of the bark extracts were determined by measuring the total phenolic content (TPC) and total flavonoids content (TFC) following the procedure described in the **Appendix IV**. To analyse the potential of the obtained extracts three different antioxidant capacities were measured, using α,α-Diphenyl-β-picrylhydrazyl (DPPH) assay, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay and the ferric reducing antioxidant power (FRAP) test. The details of these procedures are fully described in **Appendix IV**. The equations of the calibration curves used are listed in **Table 2.2**.

Table 2.2 Calibration curves used for the measurement of TPC, TFC, DPPH, ABTS and FRAP.

Method	Calibration curve	R^2	Eq.
TPC	$[Galic\ acid] = 0.1596 \cdot Abs - 0.0063$	0.999	(3.2)
TFC	$[Catequin] = 0.1278 \cdot Abs - 0.0176$	0.995	(3.3)
DPPH	$[Trolox] = -0.1296 \cdot Abs + 0.0746$	0.999	(3.4)
ABTS	$[Trolox] = -1.1358 \cdot Abs + 0.7618$	0.997	(3.5)
FRAP	$[Trolox] = 0.1848 \cdot Abs - 0.0067$	0.998	(3.6)

In addition, the extracts were characterised using High Performance Size Exclusion Chromatography (HPSEC) and Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR) to provide a better understanding of their structure (see **Appendix IV**).

2.4 Results and discussion

2.4.1 Characterisation of wood and bark from the same tree

The six tree fractions supplied by Basoekin Ltd. were separated in bark and wood, and both fractions were chemically characterised, and their chemical compositions were compared. The techniques used for the chemical characterisation of the bark and wood were different, since the protocols are specific to each raw material. For this reason, the wood has been characterised by applying the TAPPI standards, which are specific for wood. While in the case of bark, the procedures used were the NREL standards. In addition, it was also necessary to determine the suberin content. Its quantification is necessary only in bark because wood does not have it. Furthermore, it is necessary to remove it from the biomass before measuring the lignin content, otherwise the results could be adulterated [2.29].

Figure 2.2 shows the characterisation results of the two different fractions (wood and bark) of the six raw materials, all of which are hardwoods. Analysing the chemical composition of the wood, it can be seen that they all have similar cellulose content (in the range of 37-41%); however, in the case of lignin content, significant differences can be observed. The highest proportion of total lignin content is found for northern red oak and black locust, while Iberian white birch and common ash have values close to 20%. However, these values correspond to the average lignin content of hardwoods (see **Table 2.1**). Regarding to the two types of lignin, there are clear differences between wood species not only for AIL but also for ASL. Northern red oak has the highest AIL value, while sweet chestnut has the highest ASL value. Black locust has one of the highest measured AIL and

total lignin content, only behind northern red oak. This high content of AIL in black locust was also found by Chow et al, which also indicates the different chemical composition for the lignin of the different species [2.30]. Hemicelluloses content ranges between 13.84 % (black locust) and 23.39 % (Iberian white birch). Being Iberian white birch, common oak and sweet chestnut those which have the highest content. These values are within the typical values for hardwoods as reported by Saidur [2.31]. EtOH-toluene extractives content was between 1.57% (Iberian white birch) and 5.34% (black locust), having the Iberian white birch the lowest extractive content and the black locust the highest. The content of inorganic compounds, measured as ash content, in all cases was less than 1%, matching the typical values for hardwood (see **Table 2.1**).

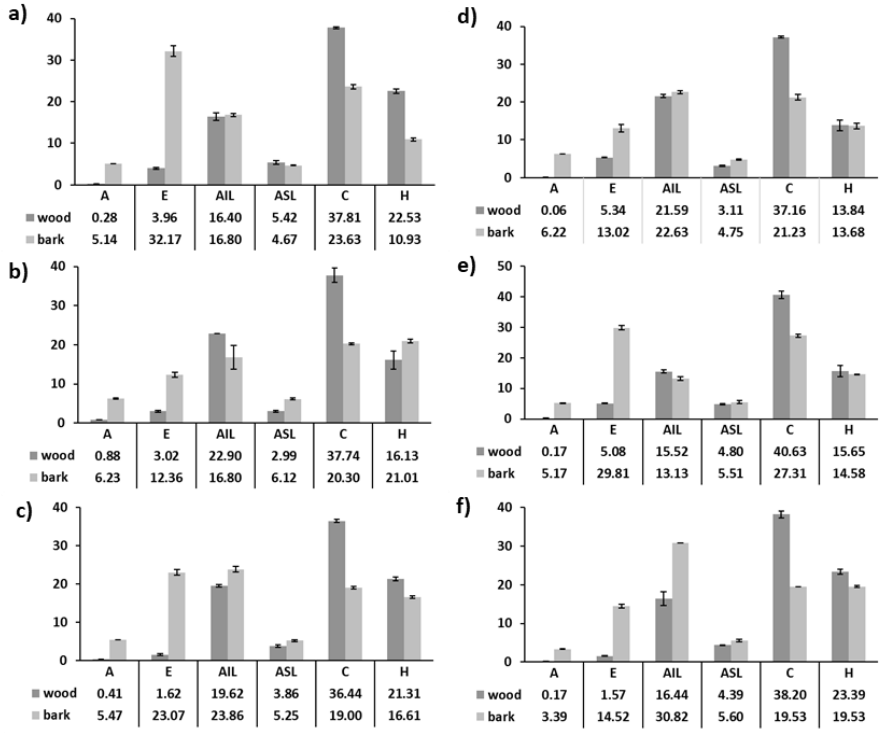

Figure 2.2 Chemical composition of bark and wood from the different raw materials. a) sweet chestnut, b) northern red oak, c) common oak, d) black locust, e) common ash, f) Iberian white birch. A: ash, E: extractives, AIL: Klason lignin, ASL: acid-soluble lignin, C: cellulose and H: hemicellulose.

According to the chemical composition calculated for the bark, which is shown in **Figure 2.2**, there are considerable differences between the different species. In terms of the total ash content, Iberian white birch has the lowest ash content with 3.39%, which is higher than the value reported by Miranda et al. [2.32]. Northern red oak and black locust have the highest ash content. The extractives content (measured by subsequential extractions with CH_2Cl_2, EtOH and H_2O) differs a lot between the different bark species with the highest concentration for sweet chestnut. This value is not in accordance with what was reported for alcohol-benzene extractives (14.55%) in other work [2.33]. The lowest extractive content was calculated for northern red oak (12.11%), close to black locust (12.72%), which is in accordance with the results reported by Putman [2.34]. The total lignin content differs also between species from 18.64 (common ash) to 36.42%, (Iberian white birch). The obtained concentrations are similar to the ones reported in the literature for other hardwoods species which are in the range of 13.1–39.7% [2.25, 2.35]. The main difference for total lignin content is due to the differences in AIL, where the concentrations vary between 13.13 and 30.82%. ASL content has not substantial differences, similar results as those reported by Lima [2.27]. There are also differences in cellulose and hemicelluloses content. The measured values for cellulose content have a difference of less than 8.5%, while in the case of hemicelluloses this increases to 10%.

When the results obtained for bark and wood are compared, the existence of differences in the composition of the two fractions of the different tree species is confirmed. Analysing the obtained results, a higher quantity of inorganic compounds (ash) is observed in the barks, reaching between 5-6% of the total dry raw material in all cases except for Iberian white birch. However, the wood does not exceed 1% in any case. For extractives content,

barks double their concentration comparing to woods. All the values obtained for the extractive content in barks are higher than 13%, while wood extractive content does not exceed 5%. Regarding to the total lignin content (measured as the sum of ASL and AIL), no overall conclusion is obtained, because it depends on each studied tree species, although the bigger difference is due to AIL and not to ASL. In the case of common oak and Iberian white birch, bark has a higher lignin content, being almost double in the case of Iberian white birch. A higher quantity of ASL is measured in the northern red oak and common ash trees wood. Sweet chestnut and black locust have similar total lignin content for both, wood and bark. Regarding to ASL, in all cases a higher content is obtained for bark, expect for sweet chestnut. It is also concluded that the cellulose content is higher in wood than in bark, as well as the hemicellulose content, except in the case of northern red oak. Finally, it is important to mention that bark also contains suberin, which if it is not represented in the graphics of the **Figure 2.2**, should not be forgotten (see **Table 2.3**). With all this, it is confirmed that the bark has a higher content of ashes and extractives, apart from the fact that it has suberin, which is in accordance with what had been reported previously in the bibliography [2.7, 2.8].

As the main objective of the thesis was to valorise the extractives and having in to account the characterisation results, the rest of the research work will be focused on barks extractives.

2.4.2 Comparison of chemical composition of different tree barks

In this section, the six barks of hardwoods already mentioned in the previous section will be studied in more detail, and in addition, the chemical characterisation of another five barks will be carried out. These new

softwood barks were collected from the sawmill Errekondo Egur-Zerra company. The chemical characterisation calculated for the different barks are shown in **Table 2.3**.

In general, it can be said that the barks of softwood trees are richer in AIL, while the barks of hardwood trees have a higher amount of ASL, polysaccharide and ash content. The ash content for hardwood is in the range of 3 to 8%, while the range for softwood is 1 to 5%. The highest AIL value is for the bark of the cedar, and the lowest for common ash. It can be seen, that the AIL content in softwood is over 28%, while in the case of hardwoods only Iberian white birch has overtaken this value. This trend is also observed for the total lignin content, being the sum of AIL and ASL, where softwoods have percentages above 30%, while most softwoods do not reach that value. Regarding to the ASL content, softwoods do not achieve the 4%, while hardwood barks reaches it very easily.

The total extractive content was determined by three consecutive extraction with CH_2Cl_2, EtOH and H_2O. The values determined for each of the different types of extracts varied depending on the specie. The aqueous extracts were in general the ones that reported the highest content, it happened in eight out of eleven of the studied barks. White spruce is the only bark that has the highest extractive content with CH_2Cl_2, which means that is the bark with more non-polar extractives. Common ash and Douglas fir have the highest total ethanolic extractive content, 18.5% and 11.7%, respectively. Larch pine and sweet chestnut are also rich in ethanolic extractives, with values around 9%. Sweet chestnut is the richest in water-soluble extractives content followed by common oak, white spruce and cedar. The bark with the highest total extractive content, calculated by the

sum of the values obtained for the three solvents, is sweet chestnut, followed by common ash, common oak, white spruce and larch pine.

The total suberin content for all the studied barks is generally between 3 and 4.5% of the total dry mass of the bark, however, this range leaves out two exceptional cases. Suberin content in black locust (16.4%) is remarkable high, close to 4 times higher than for the other barks, but is lower than the value reported by Putman et al. [2.34]. Larch pine is the other exception, which has the lowest measured content, 2.0%. All the concentrations reported in **Table 2.3** are greater than the ones reported by Miranda and Lima for different Eucalyptus species, between 0.6 and 1.9%, and in the same range that the values reported by Ruiz-Aquino et al. for *Q. faginea* (2.94%) [2.25, 2.27, 2.32, 2.35].

The polysaccharides content, determined as the sum of cellulose and hemicellulose content, reveals that there is a considerable difference between hardwood and softwood barks, where the values for hardwood barks is higher. This is mainly due to the difference in hemicellulose content. Furthermore, it can be seen that the difference in polysaccharide content for hardwoods is small. Whereas for softwoods it is higher, reaching up to the 15%. The sequoia is the specie with the highest cellulose content, while common oak, white spruce, Iberian white birch and larch pine have the lowest values (less than 20%).

In general, it can be concluded that all the studied barks have an extractive content that cannot be underestimated, since only three of them report values below 15% of the total dry mass of bark. Therefore, once the composition of the bark has been studied, it is necessary to characterise this fraction in order to know its real potential.

Table 2.3 Chemical composition (% of the total dry mass of bark) of the bark of eleven species

	Sweet chestnut	Northern red oak	Common oak	Black locust	Common ash	Iberian white birch	White spruce	Douglas fir	Larch pine	Cedar	Sequoia
Ash	5.14 ± 0.02	6.2 ± 0.2	5.47 ± 0.02	6.22 ± 0.08	5.17 ± 0.08	3.4 ± 0.1	2.73 ± 0.06	1.37 ± 0.02	3.5 ± 0.1	4.77 ± 0.05	2.28 ± 0.06
Extractive	32 ± 1	12.1 ± 0.4	23.0 ± 0.8	12.7 ± 0.7	29.4 ± 0.5	14.3 ± 0.5	24.8 ± 0.7	19 ± 1	20.4 ± 0.9	16.3 ± 0.9	8.4 ± 0.6
Dichloromethane	1.94 ± 0.04	2.7 ± 0.1	1.09 ± 0.03	3.76 ± 0.07	4.30 ± 0.01	2.65 ± 0.01	10.8 ± 0.4	1.25 ± 0.02	1.92 ± 0.09	2.6 ± 0.2	2.4 ± 0.2
Ethanol	9.5 ± 0.2	2.1 ± 0.1	7.41 ± 0,06	3.9 ± 0.2	18.5 ± 0.2	4.1 ± 0.2	4.5 ± 0.1	11.7 ± 0.2	8.7 ± 0.7	5.3 ± 0.1	2.35 ± 0.08
Water	20 ± 1	7.3 ± 0.1	14.5 ± 0.7	5.0 ± 0.4	6.6 ± 0.3	7.5 ± 0.3	9.99 ± 0.05	7.68 ± 0.08	9.9 ± 0.7	8.1 ± 0.5	3.84 ± 0.09
Suberin	4.0 ± 0.4	3.7 ± 0.2	3.9 ± 0.3	16.4 ± 0.3	3.0 ± 0.4	4.1 ± 0.2	4.16 ± 0.07	3.6 ± 0.3	2.0 ± 0.2	3.7 ± 0.8	3.4 ± 0.8
Total Lignin	21.5 ± 0.4	33 ± 3	29.1 ± 0.9	27.4 ± 0.6	19 ± 1	36.4 ± 0.3	30.8 ± 0.4	37 ± 2	36.9 ± 0.2	37.8 ± 0.9	38.0 ± 4
Klason Lignin	16.8 ± 0.3	27 ± 3	23.9 ± 0.7	22.6 ± 0.4	13.1 ± 0.6	30.82 ± 0.02	28.4 ± 0.3	33 ± 2	33.1 ± 0.2	34.9 ± 0.9	32.1 ± 4
Soluble lignin	4.67 ± 0.05	6.1 ± 0.3	5.3 ± 0.2	4.8 ± 0.1	5.5 ± 0.5	5.6 ± 0.3	2.2 ± 0.1	1.94 ± 0.09	3.71 ± 0.04	2.66 ± 0.06	2.00 ± 0.09
Polysaccharides	34.6 ± 0.9	41.3 ± 0.7	35.6 ± 0.7	34.9 ± 1.5	41.9 ± 0.6	39.7 ± 0.3	22.5 ± 0.4	27 ± 1	26 ± 2	30.1 ± 0.4	38 ± 1
Cellulose[a]	10.9 ± 0.4	21.0 ± 0.5	16.6 ± 0.3	13.7 ± 0.8	14.6 ± 0.1	20.1 ± 0.2	3.5 ± 0.3	5.05 ± 0.08	5.9 ± 0.8	5.9 ± 0.4	5.6 ± 0.6
Hemicellulose[b]	23.6 ± 0.5	20.3 ± 0.2	19.0 ± 0.3	21.2 ± 0.8	27.3 ± 0.5	19.53 ± 0.03	19.1 ± 0.1	22 ± 1	20 ± 1	24.17 ± 0.03	32.1 ± 0.7

[a] Represented as glucan content

[b] Measured as the join contribution of the rest of the sugars

2.4.3 Potential of bark extracts

With the aim of understanding the real potential for obtaining biologically active compounds from bark, the characterisation of the extractives was carried out. With that objective, the quantification of phenolic and polyphenolic compounds as well as antioxidant capacity of bark extract were performed. The fact that they show antioxidant activity makes them suitable for their use against oxidation and degradation in a variety of applications in different industries such as pharmaceutical, and food preservation among others. In addition, in order to know the structure of the extracts, they were analysed by ATR-FTIR and HPSEC.

2.4.3.1 Study of the phenolic content and the antioxidant capacities of bark extracts

The characterised extractives were all obtained using the same extraction method, so that the results are comparable. An extraction with EtOH/H_2O mixture was carried out in an ultrasonic bath to enhance the extraction of the compounds. However, the conditions of the extraction were not optimised, so the obtained extraction yields can be improved. As shown in **Table 2.4**, the measured values for extraction yield are between 2 and 16%, which, comparing with the total extractive content measured in the chemical characterisation (**Table 2.3**), are not very high. The total percentage of obtained extractives ranges from 17 to 54% of dry mas of bark, with only one of the extractions being able to extract more than the 50% (common ash). While this confirms the need to optimise the extraction parameters for each bark, in this case, as the objective was to know the potential of each raw material to choose the most suitable, the extraction is worthwhile. Moreover, different authors have used this method before, so the

comparison of the results can be done not only with the data presented in here, but also with the results reported by other authors.

Table 2.4 Bark extracts composition (TPC and TFC) and antioxidant capacity (analysed by the DPPH, ABTS and FRAP methods).

	Extraction yield (%)	TPC (mg GAE/g DBE)	TFC (mg CE/g DBE)	DPPH (mg TE/g DBE)	ABTS (mg TE/g DBE)	FRAP (mg TE/g DBE)
Sweet chestnut	9.3 ± 0.2	635 ± 24	446 ± 18	1217 ± 60	1413 ± 170	533 ± 3
Northern red oak	3.20 ± 0.07	276 ± 3	306 ± 18	400 ± 9	562 ± 98	194 ± 7
Common oak	10.0 ± 0.3	611 ± 15	480 ± 3	1521 ± 56	1557 ± 75	640 ± 22
Black locust	3.1 ± 0.2	178 ± 6	271 ± 10	167 ± 11	585 ± 17	146 ± 4
Common ash	15.8 ± 0.1	316 ± 10	206 ± 6	544 ± 14	753 ± 15	330 ± 13
Iberian white birch	5.09 ± 0.06	432 ± 3	377 ± 14	1912 ± 25	1302 ± 56	410 ± 7
White spruce	8.7 ± 0.3	244 ± 12	256 ± 12	170 ± 19	520 ± 7	178 ± 4
Douglas fir	9.3 ± 0.8	514 ± 21	443 ± 18	733 ± 49	1119 ± 20	438 ± 14
Larch pine	6.5 ± 0.4	542 ± 13	593 ± 22	617 ± 4	1040 ± 41	444 ± 6
Cedar	6.09 ±0.05	377 ± 1	586 ± 24	706 ± 20	981 ± 16	323 ± 6
Sequoia	2.6 ± 0.2	316 ± 3	330 ± 14	293 ± 7	1070 ± 30	198 ± 6

DBE: dried bark extract

Figure 2.3 provides a comparison between the obtained yield of the extractions and the total extractive content of each bark. The higher extraction yields correspond to the greater richness of the barks in extractives. The worst extraction yield was obtained for the sequoia bark, which initially was already identified as not extractive rich raw material. In contrast, the richest raw material in extractives, sweet chestnut, achieved a yield of around 9%, extracting only the 29% of total extracts.

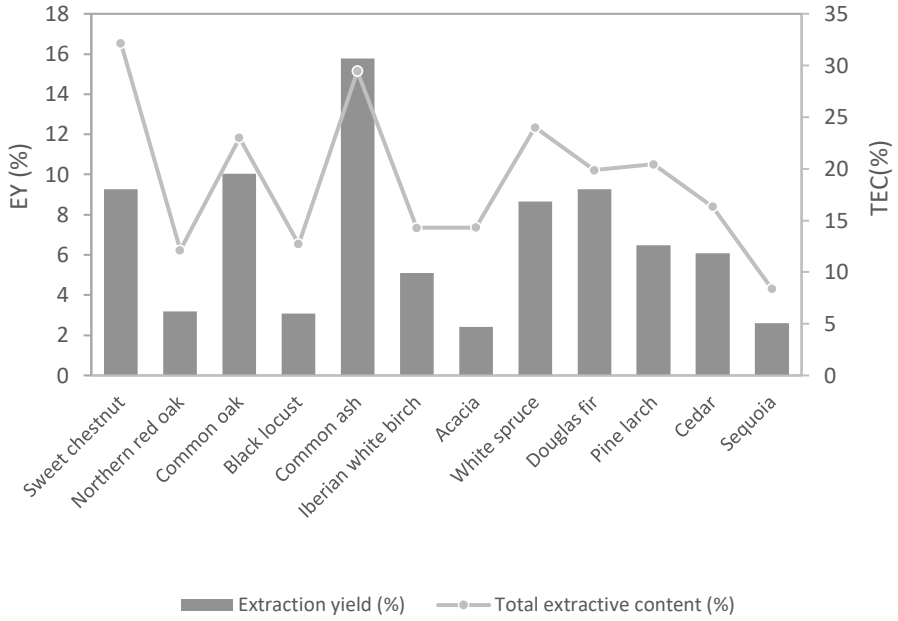

Figure 2.3 Comparative graphic of the extraction yield (EY) obtained for bark extractions against the total extractive content (TEC) of each bark.

The composition of EtOH/H_2O extract varies among the different barks. Total phenolic content (TPC) differs from 178 to 635 mg GAE/g dried bark extract (black locust and sweet chestnut, respectively). Common oak has also a high TPC, 611 mg GAE/g dried bark extract, followed by larch pine and Douglas fir. All the obtained values are in the same range that the ones reported by Lima for different Eucalyptus barks [2.27]. An analysis of the data shows that there is no correlation between the quantity of obtained extracts and the TPC. That is because the extraction method is not selective enough and there are not just phenolic compounds. In the case of total flavonoid content (TFC), there is also no correlation with the extraction yield, for the same reason as for TPC. The values for TFC are ranged from 206 to 593 mg CE/g dried bark extract (common ash and larch pine, respectively). The TFC results reported in **Table 2.4** for the different extracts have high values, being in general higher than those reported by other authors [2.23, 2.28, 2.36].

Based on the analysis of the data reported in Table 2.4, it can be found that there is a linear correlation between the TPC and the antioxidant capacities measured for the extracts. The correlation, studied by Pearson's coefficient, is a positive correlation for the three antioxidant capacities tested, obtaining 0.70, 0.88 and 0.96 coefficients for DPPH, ABTS and FRAP, respectively. The existence of strong direct linear correlations between antioxidant capacities, with values between 0.78 and 0.88, is also observed.

Concentrations obtained for scavenging capacity against the radical DPPH of EtOH/H_2O extracts of each bark are ranged between 167 and 1912 mg TE/g dried bark extract (black locust and Iberian white birch, respectively), which is a big range. Common oak and sweet chestnut have results above 1200 mg TE/g dried bark extract, while sequoia and northern red oak do not exceed 400 mg TE/g dried bark extract.

ABTS assay was carried out for EtOH/H_2O extracts of each bark and it is observed a difference between the lowest and highest results. Common oak has the greatest result, 1557 mg TE/g dried bark extract, and white spruce, northern red oak and black locust the lowest. Six of the eleven studied extracts reached values higher than 1000 mg TE/g dried bark extract, showing the great potential of the extracts.

The reducing ability of the EtOH/H_2O extracts of each bark was measured by FRAP and the obtained results differ from 146 to 640 mg TE/g dried bark extract. The lowest value corresponds to black locust extracts and the highest to common oak, followed by sweet chestnut and larch pine.

The comparison of the results with other data from literature must be done carefully because of the differences in methodology, calculations and standards. Besides, few results are reported in the literature for the

antioxidant properties of bark extracts, and usually, the only one that is measured is DPPH. The values that can be found in literature for the characterisation of the antioxidant capacity of the EtOH/H_2O extracts measured by DPPH have as much variability as those shown in **Table 2.4**. Thus, Ferreira reports a value of 1576 mg TE/g dried extract for *Quercus fagine* [2.28], whereas the lowest value (277 mg TE/g dried extract) is measured by Sartori of eucalyptus bark [2.23].

2.4.3.2 Structural characterisation of bark extracts

The molecular weight (Mw) distribution of the EtOH/H_2O extracts has been analyses by HPSEC, and the obtained results are summarised in **Table 2.5** and **Table 2.6**. All extracts consisted of a heterogeneous mixture of compounds with differentiated fractions, which may be due to a difference in the degree of polymerization of the compounds in the extract [2.37]. The global average Mw differs a lot between different bark extracts, and the global average polydispersity index (Mw/Mn) is very high. From the **Table 2.5** and **Table 2.6** can be seen that there is no a trend for hardwood or softwood, instead the values depend on the studied species.

The highest global average Mw is obtained for sweet chestnut, 57,387 g/mol, with a polydispersity index of 27.99. Analysing the different fractions, 86.69% of the total molecules have a Mw of 66,134 g/mol, with the highest polydispersity index. On the other hand, the other two fractions have a Mw of 249 and 499 g/mol. Iberian white birch bark extract has also a high global average Mw, followed by cedar and common oak, all of which have global average Mw over 20,000 g/mol. Polydispersity indexes for those extracts are also high as well as for sweet chestnut. White spruce, sequoia and common ash are the only bark extracts where more than 40% of the total compounds have a Mw inferior than 1,000 g/mol.

Table 2.5 Percentage, average molecular weight (Mw), number average (Mn) and polydispersity index (Mw/Mn) of EtOH/H_2O hardwood bark extracts.

					Global average		
	Percentage	Mw (g/mol)	Mn (g/mol)	Mw/Mn	Mw (g/mol)	Mn (g/mol)	Mw/Mn
Sweet chestnut	86.69	66,134	9,580	6.90	57,387	2050	27.99
	7.49	499	460	1.08			
	5.81	249	248	1.01			
Northern red oak	58.54	28,927	10,290	2.81	17,211	987	17.44
	13.66	1,262	1,145	1.10			
	16.97	458	428	1.07			
	9.07	243	243	1.00			
	1.76	264	262	1.01			
Common oak	76.76	26,283	6,504	4.04	20,288	1376	14.74
	6.50	843	819	1.03			
	9,86	422	399	1.06			
	6.88	245	244	1.01			
Black locust	37.22	15,661	8,430	1.86	6,334	696	9.11
	15.65	1859	1708	1.09			
	28.81	588	516	1.14			
	18.32	248	247	1.00			
Common ash	17.72	16,982	10,641	1.60	3,682	556	6.62
	35.32	1,429	1,071	1.34			
	20.94	446	433	1.03			
	14.21	268	266	1.01			
	7.17	235	235	1.00			
	4.64	438	360	1.22			
Iberian white birch	82,42	37,470	10,045	3.73	30,972	1914	16.18
	4.61	901	874	1.03			
	8.16	441	413	1.07			
	4.81	262	253	1.03			

Table 2.6 Percentage, average molecular weight (Mw), number average (Mn) and polydispersity index (Mw/Mn) of EtOH/H_2O softwood bark extracts.

					Global average		
	Percentage	Mw (g/mol)	Mn (g/mol)	Mw/Mn	Mw (g/mol)	Mn (g/mol)	Mw/Mn
White spruce	51.25	22,380	8,559	2.61	11,726	594	19.75
	7.13	1,507	1,447	1.04			
	8.34	739	710	1.04			
	15.85	344	326	1.06			
	11.46	186	184	1.01			
	3.19	161	161	1.00			
	2,79	188	186	1.01			
Douglas fir	74.57	25,436	6,331	4.02	19,073	1041	18.32
	7.08	755	724	1.04			
	7.99	386	374	1.03			
	7.07	214	209	1.02			
	3.29	172	170	1.01			
Pine larch	79.51	21,309	6,643	3.21	17,062	1540	11.08
	9.54	896	840	1.07			
	5.71	399	384	1.04			
	3.78	214	210	1.02			
	1.46	168	167	1.01			
Cedar	74.91	31,546	6,937	4.55	23,742	1116	21.27
	15.46	597	517	1.16			
	6.83	217	212	1.03			
	2.80	170	169	1.01			
Sequoia	53.19	10,808	5,244	2.06	6,009	700	8.58
	19.75	939	836	1.12			
	17.24	332	317	1.05			
	5.14	190	189	1.01			
	4.68	173	171	1.01			

This suggests that the polymerisation degree of the compounds is lower. Moreover, the extract of common ash also has the lowest global average-polydispersity index, followed by the extracts of sequoia and black locust. All the other extracts have a global average-polydispersity index higher than 11. White spruce´s EtOH/H_2O extracts have the highest percentage of the lower Mw compounds, with Mw of 186, 161 and 188 g/mol. The extracts of northern red oak, Douglas fir, larch pine and sequoia have five differentiated fractions of Mw. All of them have more than 50% of the compounds with Mw above 10,000 g/mol, even going beyond 70% in the case of larch pine and Douglas fir. This shows that, in general, the extracts are formed by a high degree of polymerisation compounds. The extracts of the barks of common oak, Iberian white birch and cedar have four differentiated fractions of Mw. Moreover, they all have more than the 76% of the total molecular content in the highest fraction, with Mw between 26,283 and 31,546 g/mol.

Figure 2.4a and **Figure 2.4b** show the HPSEC chromatograms obtained for the different EtOH/H_2O bark extracts. The differences between the samples can be seen visually, where the large size of the first peaks, which correspond to the biggest Mw, is to be highlighted. In the case of black locust common ash and sequoia, it can be seen that the percentage of the obtained different Mw fractions are more balanced. It can be concluded that the extract consisted of a heterogeneous mixture of compounds divided into different weight fractions.

Few articles have reported GPC characterisation of the extracts and the used extractions methods are not the same. In addition, the equipment used was calibrated using polystyrene standards, because of that, the comparison with the literature must be made cautiously. They all report lower Mw

values than those shown here. This may be due to the fact that the treatment used is not too severe, so that no fractionation of the molecules is achieved, resulting in the extraction of compounds with a high degree of polymerization. Different authors have reported studies of Mw for different pines bark extracts. Bocalandro et al. has studied the Mw of *Pinus radiata* bark hot-water extracts, identifying a peak assigned to some flavonoids around 300 g/mol, and other peak at 580 g/mol assigned to proanthocyanidins [2.37]. Some commercial bark extract from *Pinus pinaster* and *Pinus massoniana* analysed by Weber et al. were distinguished by having compounds with a Mw below 1180 g/mol [2.38]. In the case of hardwood bark, the average Mw of extracts of acetylated bark of *Eucalyptus globulus* extracted by using different solvents are in the range of 314 to 1,167 g/mol [2.39]. Considering all the different published results it can be concluded that the Mw of bark extracts depends on the species and the extraction conditions [2.40].

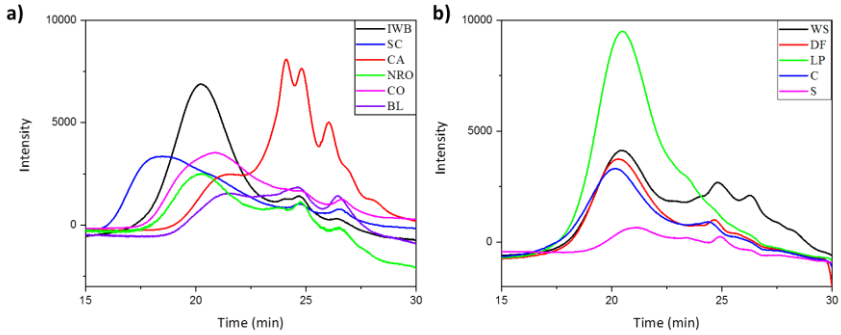

Figure 2.4 GPC chromatogram of EtOH/H_2O bark extracts, a) six different hardwood bark extracts (IWB: Iberian white birch; SC: sweet chestnut; CA: common ash; NRO: northern red oak; CO: common oak; BL: black locust) b) five different softwood bark extracts (WS: white spruce; DF: Douglas fir; LP: larch pine; C: cedar; S: sequoia).

Continuing with the determination of the chemical composition of the EtOH/H_2O bark extracts, they were subjected to ATR-FTIR analyses in order to determine the structure of the compounds. In the **Figure 2.5a** and

Figure 2.5b the spectra of the different bark extracts are presented. **Table 2.7** summarised the band assignment that is based on other authors reported results.

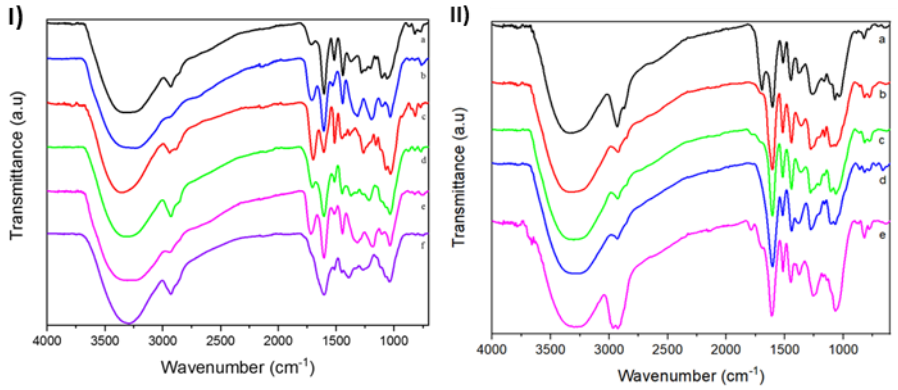

Figure 2.5 ATR-FTIR spectra of different bark extracts, I) hardwoods: a) Iberian white birch b) sweet chestnut c) common ash d) northern red oak e) common oak f) black locust. II) softwoods: a) white spruce b) Douglas fir c) larch pine d) cedar e) sequoia.

Analysing the results obtained for the EtOH/H_2O extracts of the softwood barks, in the **Figure 2.5b**, differences are observed mainly in the "fingerprint" region 1800 cm^{-1} and 700 cm^{-1}, however there are also differences in the bands around 2800-3000 cm^{-1}. Four of the five extracts have two bands, one at 2850 cm^{-1} and other at 2925-2930 cm^{-1}, being both relatively more intense in the case of white spruce. However, in the case of sequoia, there is no band at 2850 cm^{-1}, but another band is observed before, at 2957 cm^{-1}, with high relative intensity. In addition, this bark has a band at 1737 cm^{-1}, which all other extracts, both hardwood and softwood, do not have. The ATR-FTIR spectra for the Douglas fir and pine extracts are very similar, these two having the highest relative intensity band at 1515 cm^{-1}. The greatest difference between these two bark extracts is observed in the region of 1100-1300cm^{-1}, where the pine extract has the highest number of identified bands. All softwood extracts have the band corresponding to C-O stretching vibration (1040-1050 cm^{-1}), however, neither sequoia nor white

spruce have a band at the 1105-1115 cm^{-1}, that correspond to aromatic -CH bending in plane vibration. The extracts of white spruce and Douglas fir are the only ones that have an identifiable band at 1035 cm^{-1}, which in the case of Douglas fir is less intense. Finally, it is important to mention that all the extracts have different bands below 900 cm^{-1}, which are associated with the aromatic -CH stretch vibrations, where white spruce has the least relative intensity.

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Table 2.7 ATR-FTIR spectra of bark extracts

Wavenumber (cm^{-1})	Band assignment	Bark extracts	References
3300	-OH stretch vibration in phenolic and aliphatic structures	IWB; SC; CA; NRO; CO; BL; WS; DF; LP; C;S	a, b, c, d, e
2973	-CH3, CH2 stretching vibration	S	b
2925-2930	-CH stretch vibration in aromatic methoxy groups and in methyl and methylene groups of side chains	IWB; SC; CA; NRO; CO; BL; WS; DF; LP; C; S	a, b, d, e, f
2850	-CH stretch vibration in aromatic methoxy groups and in methyl and methylene groups of side chains	IWB; SC; CA; NRO; CO; BL; WS; DF; LP	a, d, e
1737	C-O stretch in unconjugated ketones	S	h
1705-1720	conjugated carbonyl-carbonyl stretching	IWB; SC; CA; NRO; CO; BL; WS; DF; C; S	a, d, f
1605	aromatic skeleton vibrations	IWB; SC; CA; NRO; CO; BL; WS; DF; LP; C; S	a, b, d, f
1515	aromatic skeleton vibrations	IWB; SC; CA; NRO; CO; BL; WS; DF; LP; C; S	a, d, e, f
1440	aromatic skeleton vibrations/ -CH deformation	IWB; SC; CA; NRO; CO; BL; WS; DF; LP; C; S	a, b, d, e, f
1412	Aromatic vibration	CA	b, d
1370-1380	phenolic stretch vibration of -OH and aliphatic -CH deformation in methyl groups	IWB; SC; CA; NRO; BL; WS; DF; LP; C; S	a, d, e
1308	C-C frame stretching (C-CHR-C)	SC; CA; NRO; CO; BL; WS	b, d
1275	C-O C asymmetric stretch vibration	IWB; NRO; CO; DF; LP; C; S	C, d, e
1260	C-O stretch vibration	CA; CO; WS	d, e, g
1245	C-O-C asymmetric stretch vibration	IWB; NRO; CA; BL; DF; LP	c, d
1200	C-O stretching vibration	IWB; SC; WS; DF; LP; C; S	a, d, e
1155	aromatic CH in-plane bending vibration	IWB; CO; CA; BL; WS; DF; LP; C; S	c, d
1105-1115	aromatic -CH bending in-plane vibration	IWB; SC; CA; NRO; CO; BL; DF; LP; C	b, d, e
1040-1050	C-O stretching vibration	IWB; NRO; CA; BL; WS; DF; LP; C; S	b, d, e
1035	C-O stretching or aromatic C-H deformation associated with the C-O, C-C stretching and C-OH bending in polysaccharides	SC; CA; NRO; CO; BL; WS; DF	a, d
921	Aromatic -CH out of plane bending vibration	CA	b, d
<900	Aromatic -CH stretch vibrations	IWB; SC; CA; NRO; CO; BL; WS; DF; LP; C; S	a, c, d, e

a: [2.41] b: [2.42] c: [2.43] d: [2.44] e: [2.45] f: [2.39] g: [2.46]; h: [2.47]

IWB: Iberian white birch; SC: sweet chestnut; NRO: northern red oak; CA: common ash; CO: common oak; BL: black locust; WS: white spruce; DF: Douglas fir; LP: larch pine; C: cedar; S: sequoia

The comparison of the ATR-FTIR spectra obtained for both hardwoods and softwoods shows some general trends. In the region 2800-2990 cm^{-1} it is noted that although there are identified bands in all extracts, these bands have a higher relative intensity in the case of hardwoods. This is due to the fact that these bands are associated with lignin [2.48], and as hardwoods have a higher amount of ASL they may have been solubilised during extraction. Regarding to the band appearing in the region 1705-1720 cm^{-1}, hardwoods have a band with a higher relative intensity. This band is associated with hemicelluloses [2.48], and as hardwoods are richer in hemicelluloses according to their chemical composition, it may be concluded that the solubilisation of these in the extraction is higher. In the region corresponding to 1000-1500 cm^{-1}, it is observed that hardwoods have a higher relative band intensity. In the 1035 cm^{-1} band there is also a notable difference between bark extracts, since in the case of hardwood bark extracts it appears in all of them, while in softwood bark extracts it is only observed for white spruce and Douglas fir.

These differences seen in the structure of the bark extracts support the reported differences in the chemical composition of the barks, and they also validate the differences in the chemical properties of the extracts.

2.5 Conclusion

The chemical characterisation of both the bark and the wood of six species of native hardwood trees of the Basque Country was carried out. From this characterisation, it is clear that the chemical composition of both fractions (bark and wood) depends mainly on the species. Summarising all the results, it can be deduced that the chemical composition of both fractions for the same species is different. This is mainly due to the high content of

extractive compounds in the barks, as well as the existence of suberin in them. Considering its high extractive content, added to the low polysaccharides content, it is concluded that bark is the best raw material for the extraction of bioactive compounds. Combining this with the fact that the bark is considered a waste in the wood-based industry, and that it is generated in large quantities, its valorisation becomes even more necessary.

The most exhaustive study of the chemical composition of hardwood barks, added to the study of other softwood barks, verifies that the barks are a source of extractive compounds. This statement is based on the high percentage of extractive content that all barks have, where only three have values below 15% of the total dry mass of bark, and all are above 12% of the total dry mass of bark. With the analysis of the extracts, it is understood that all of the studied barks can be considered as a source of polar extractives. The characterisation made to the EtOH/H_2O extracts of each bark concludes that all are rich in phenolic compounds as well as in flavonoid compounds, being the larch pine the one with the highest TFC value. However, it must be reminded that the method used for the extraction has not been optimised, so the extraction percentages are not the best they could be. Nevertheless, the objective of this study was to compare the different species in order to select the one with the highest potential, so the extractions were not optimised. The following chapters deal with the optimisation of the extractions of the most interesting raw material.

Phenols and polyphenols compounds are important free radical scavenging antioxidants with interesting bioactivities. Therefore, as the EtOH/H_2O bark extracts studied are rich in these compounds, a characterisation of their antioxidant capacities has been carried out with a view to their possible applicability. All EtOH/H_2O extracts have good antioxidant

capacities, but their values differ between species. Iberian birch bark is one with the highest antioxidant potential given by DPPH and Common oak has the higher antioxidant potential given by ABTS and FRAP. In general, all bark extracts have high antioxidant capacities. The differences observed in the chemical properties measured to the extracts as well as the difference in composition are supported by the differences observed in the structural analysis performed to the extracts by ATR-FTIR and HPSEC.

For an integrated valorisation strategy, the raw material from the wood-based industries is an interesting source of bioactive compounds or chemical intermediates due to their chemical functionalities and bioactivity. In this respect, bark could be considered as a source of bioactive compounds with a potential valorisation for cosmetic industry, drug, pharmaceuticals, additive in food, or chemicals for bio-based materials and polymers.

Once all the results presented in this chapter have been analysed, larch pine bark was select as raw material for its valorisation. Although any of the barks can provide a good yield in bioactive compounds, it has been decided to select the larch pine due mainly to its high content in flavonoid compounds. Larch pine is not the bark with the highest extractive content, but it has good potential, since apart from being the one that reports the highest TFC value, its antioxidant capacities are high, being over average. In addition, even though it was not the sample with the highest phenolic content, its content is very high, placing it third in the ranking. Apart from all the reasons derived from the study carried out in this chapter, it must also be said that this bark is a waste that is generated in large quantity in the Basque Country, so its recovery is a real need from which a benefit can be gained.

2.6 References

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Chapter 3

Bioactive molecules extraction

3.1 Background

The extraction of the different target compounds from bark is not an easy task due to its complex structure [3.1]. Therefore, its main exploitation until now has been limited to the use of them in horticulture or in energy generation as low efficiency-fuel [3.2]. The production of cork or the extraction of tannins [3.3–3.5] are other applications that have been exploited, but only in specific species.

The integral biorefineries studied so far are focused on the separation of the three main structural compounds from the lignocellulosic biomass (cellulose, hemicellulos and lignin) [3.6]. However, the extractive fraction also requires attention, especially in the case of the bark, since it is an extractive rich biomass. Thus, to propose a cost-effective biorefinery process for the bark, it is necessary to find a suitable method for the separation of extractive compounds from the rest of the main components. In addition, taking into account the evidence that confirms the potential of some of the compounds forming the extractive fraction, raises the necessity of research in this field.

3.1.1 Tree bark as a source of bioactive compounds

Bioactive compounds are typically produced as secondary metabolites. Although these compounds are not part of the cell wall structure, they are very important for plants. They provide plants with the ability to survive and overcome different challenges [3.7], being part of their defence mechanism. Among some of their properties, the antioxidant capacity of many of these compounds should be highlighted. Amorati and Valgimigli define an antioxidant as a substance that, when it is added to an oxidable

molecule in small amount is able to protect such molecules by delaying, retarding or inhibiting their autoxidation [3.8]. This helps in the elimination of free radicals and preventing the oxidation of other compounds, since they are able to capture free radicals [3.9]. That ability is desirable to protect molecules against the oxygen reactivity, so they are interesting compounds for many different applications, such as cosmetics, personal care products, and nutritional additives or in material production [3.1, 3.10, 3.11].

The interest in the extraction of natural antioxidant compounds has increased and new potential sources are being studied. Tree barks are one of the potential sources. As seen in **Chapter 2**, barks are rich in extractives, and their extracts have good potential as antioxidants.

The complex structure of the bark and its lack of homogeneity makes the recovery of this waste complex, and results difficult to select an extraction method that is applicable to every bark. Proof of this variety is the amount of work that has been published in recent years on the subject of chemical characterisation of barks [3.12–3.15] and their extracts characterisation (see **Table 3.1**).

The barks with the highest extractable content reported in literature are *Eucalyptus sideroxylon* (55.74%) [3.16], *Acacia melanoxylon* (46.4%) [3.17] and *Quercus crassifolia* (31.7%) [3.18]. In general, the extracts of the barks are usually richer in polar compounds, however, some species with high amount of cork fraction, have a higher content of non-polar extracts, such as *Quercus cerris* [3.19] and *Betula pendula* [3.20].

Table 3.1 Characterisation of the phenolic composition and antioxidant activity of the extracts following the methodology described in Chapter 2.

Raw Material	Extraction yield (%)	TPC (mg GAE/g of extract)	TFC (mg CE/g of extract)	Tannins (mg CE/g of extract)	Antioxidant capacity (mg TE/g of extract)	Ref.
Eucalyptus sideroxylon	50.0	440.7	204.4	395.0	648.8	[3.16]
Copaifera langsdorffi	12.8	589.2	441.9	54.79	720.3	[3.21]
Albizia Niopoides	11.4	247.2	59.1	118.2	839.1	[3.22]
Hybrid E. urophylla × *E. grandis*	12.5	463.4	176.3	129.7	383.7	[3.11]
Hybrid E. urophylla × *E. grandis*	10.6	550.9	234.5	153.6	494.5	
E. urophylla hybrid	11.5	287.7	98.0	183.8	308.8	
E. urophylla hybrid	14.8	266.6	92.6	157.5	286.9	
E. urophylla hybrid	12.4	215.9	119.7	76.5	277.3	
Hybrid E. urophylla × *E. camaldulensis*	14.0	210.9	128.5	128.7	279.2	
Goupia glabra	17.5	158.2	74.8	24.2	563.4	[3.23]
Eucalyptus botryoides	13.2	420.4	189.9	93.9	556.4	[3.24]
Eucalyptus camaldulensis	14.8	474.9	387.3	528.9	579.7	
Eucalyptus globulus	8.6	423.0	286.6	149.4	620.3	
Eucalyptus grandis	13.2	282.5	132.5	192.4	367.9	
Eucalyptus maculata	13.1	590.4	278.1	352.8	653.5	
Eucalyptus ovata	5.3	351.1	121.0	172.0	563.6	
Eucalyptus propinqua	13.2	543.8	361.6	544.5	667.7	
Eucalyptus resinifera	3.7	415.2	137.7	499.1	579.7	
Eucalyptus rudis	9.5	916.7	202.3	140.9	1042.2	
Eucalyptus saligna	7.3	455.2	188.4	155.2	599.8	
Eucalyptus viminalis	11.3	487.0	218.8	193.4	630.8	
Quercus faginea	6.4	630.3	204.7	220.7	1576.1	[3.9]

Bark extractives are constituted by a heterogeneous group of compounds, such as flavonoids, tannins, alkaloids, terpenoids, phenolic acids, lignans, fatty acids and extractable carbohydrates (**Figure 3.1**) [3.25, 3.26]. Most of

them are phenolic or polyphenolic compounds, and some of these biomolecules are bioactive, as can be seen in **Table 3.1**.

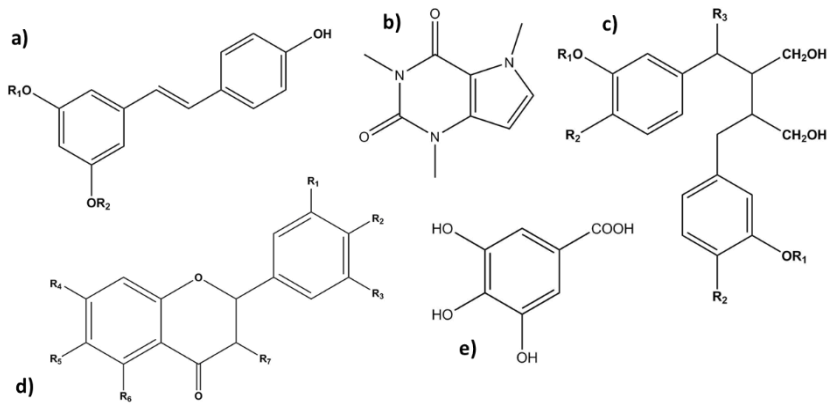

Figure 3.1 Example of structure of the main classes of compounds present in bark extracts: a) stilbenes, b) alkaloids, c) lignans, d) flavonoids, and e) phenolic acids**.**

Lima et al. analysed the bark extracts of different eucalyptus species, reporting a wide range of TPC values, 280-920 mg GAE/g of extract [3.24]. These values are higher than those reported by Sartori et al. for other eucalyptus species, since in this case none of the studied extracts reached 250 mg GAE/g of extract [3.11]. Extracts from the bark of hardwood species that has been reported in **Chapter 2** (**Table 2.4**) have even higher TPC for *Castanea sativa* and *Quercus robur*, with values over 600 mg GAE/g of dried bark extract. Similar to the value reported by Ferreria et al. for *Quercus faginea* [3.9].

Regarding the TFC, Carmo et al. reported a value of 441.9 mg CE/g of extract for *Capoifera langsdorffi* bark [3.21]. The same author measured only 59.1 mg CE/g of extract for *Albizia Niopoides* bark extracts [3.22]. This confirms that the extractive content depends on the species. Another way to characterise the extracts is by measuring their tannin content. From the studies performed on the extracts obtained from the barks of different

eucalyptus species, it can be concluded that in general they are rich in tannins, with values higher than 120 mg CE/g of extract [3.11, 3.16, 3.24].

The antioxidant capacity of the extracts can be assessed by different methods, but the most commonly used is DPPH, which measures the quality of the hydrogen donors. Looking at the values shown in **Table 3.1**, it can be seen that all the extracts have significant antioxidant capacities, which corroborates the potential of these residues as a source of bioactive molecules.

3.1.1.1 Larix decidua bark

Larix decidua (European larch) is one of the fastest growing conifers. It has an average diameter of 1.5-2.5 m and reaches 45 m long [3.27]. It is one of the most important coniferous trees species in Europe, very important for wood-based industry due to its properties, such as water-resistant and high durability, good fibre characteristics and low pest susceptibility [3.27, 3.28].

Larch pine is not a native species of the Basque Country; however, it is a Central European tree specie that has been used for years for forest recuperation. Its first introduction in the Basque Country dates back to 1849 [3.29], and since then, it has grown in size, reaching a total of 7,753 hectares [3.30]. This tree is essentially distributed in the Historical Territory of Gipuzkoa (80%), at altitudes between 600 and 1,200 m.

This specie, besides being used to recover both forests and degraded soils, is also in wide demand in the building market [3.31], mainly because of its good properties. Therefore, the amount of bark generated as waste in sawmills is not negligible.

The bark of this tree is rich in extractives, as it has been reported in **Chapter 2**. Briefly, the chemical composition of the larch pine bark was 3.5 wt.% of ash, 20.1 wt.% of extractives, 2.0 wt% of suberin, 36.8 wt.% of total lignin, 25.7 wt.% of glucan and 7.6 wt.% of hemicelluloses (measured as the joint contribution of xylan, arabinosyl substituents, mannosyl substituents and galacturonic acids). Its high extractive content is in agreement with the value reported by Piccand et al. [3.32].

3.1.2 Isolation of extractives: application of non-conventional methods for extraction in biomass

The Recovery of biomolecules from natural substrates typically involves the so-called 5-Stages Universal Recovery Process [3.33]. This process consists of 5 stages, although the second one can sometimes be omitted, and goes from the macroscopic to the macromolecular level, going then to the extraction (or elimination) of molecules, the purification of the obtained compounds and finally to their treatment for the production of the desired product (encapsulation of compounds, production of materials, etc). An example of a flowchart can be seen in **Figure 3.2**.

The steps described above are all important when valorising lignocellulosic material. However, the extraction process is the most important, since it is fundamental to obtain the desired compounds, and in general, is the most expensive step. For this reason, the choice of the optimal extraction method is particularly important.

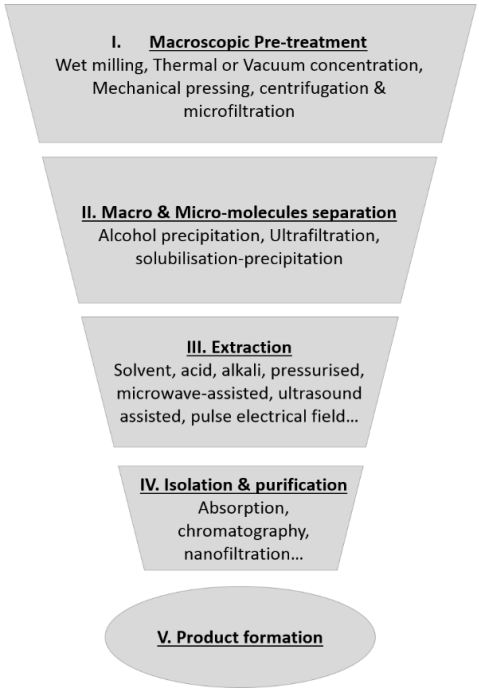

Figure 3.2 An example of 5-Stages Universal Recovery Process flowchart. Adapted from [3.33].

CE methods carried out using volatile organic solvents have been the most used techniques for the recovery of bioactive compounds from bark. The most commonly used conventional methods are maceration and Soxhlet extraction. There are many examples, but only a few of them will be collected here (**Table 3.2**). Sultana et al. reported an extraction efficiency of between 3 and 37% in the study of different barks using the maceration method with 3 different solvents, ethanol (EtOH) (80%), methanol (MeOH) (80%) and acetone (80%) [3.34]. The extraction was carried out in an orbital shaker for 8 h at room temperature. The highest extraction yield was obtained for *Terminalia arjuna* bark using EtOH (80%). The extracts with the highest TPC and TFC were the ethanolic extracts from *Acacia nilotica* bark.

Table 3.2 Different extraction methods for obtaining bark extractives.

Tree specie	Method	Extraction conditions	Yield range	Ref.
Azadirachta indica *Terminalia arjuna* *Acacia nilotica* *Eugenia jambolana*	Maceration	Solvent: EtOH (80%), MeOH (80%), acetone (80%) Time: 8 h Temperature: RT	3-37%	[3.34]
Eucalyptus grandis *Eucalyptus urograndis* *Eucalyptus maidenii*	Maceration	Solvent: MeOH/H_2O (50:50 v/v) Time: 24 h Temperature: RT	10-15%	[3.35]
Quercus Laurina *Quercus Crassifolia* *Quercus Scytophylla*	Maceration	Solvent: EtOH (90%) Time: 24 h Temperature: 22 °C	4-14%	[3.36]
	Reflux	Solvent: H_2O Time: 1 h	7-20%	
Swietenia mahagoni *Acacia mangium* *Paraserianthes falcataria*	Soxhlet	Solvent: CH_2Cl_2, acetone, toluene/ EtOH (2/1, (v/v)), H_2O (sequential)	6-19%	[3.37]
Pinus pinaster	Soxhlet	Solvent: H_2O, EtOH (50%), EtOH Time: 4 h	7-18%	[3.38]
Eucalyptus globulus *Picea abies* *Acacia melanoxylon* *Acacia dealbata*	Soxhlet	Solvent: hexane, EtOH, H_2O Time: for 16-24 h	0.5-37%	[3.39]
Pinus radiata	Soxhlet	Solvent: acetone/ H_2O (7:3, v/v) Time: 3 h	12%	[3.40]
	MAE	Solvent: acetone/ H_2O (7:3, v/v) Time: 3 min Power: 900 W	10-15%	
	UAE	Solvent: acetone/ H_2O (7:3, v/v) Time: 6 min Power: 85W	9-13%	
Fagus sylvatica	Maceration	Solvent: H_2O, EtOH (80%), MeOH (80%) Time: 2-24 h	29-46 mg/g dry bark	[3.41]
	UAE	Solvent: H_2O, EtOH (80%), MeOH (80%) Time: 10-30 min	30-50 mg/g dry bark	
	MAE	Solvent: H_2O, EtOH (80%), MeOH (80%) Time: 10 or 20 min Temperature: 60-120 °C	39-65 mg/g dry bark	
Salix eleagnos	Maceration	Solvent: EtOH (70%), EtOH (30%) Time: 48 h Temperature: 25 °C	16-22%	[3.42]
	UAE	Solvent: H_2O Time: 30 min Temperature: 25 °C		
	MAE	Solvent: H_2O Time: 5 min Power: 850 W		
Fagus sylvatica	MAE	Solvent: H_2O, EtOH (50%), EtOH (80%) Time: 2-4 min Power: 300-800 W	47-77 mg GAE/g dry plant	[3.43]
Albizia myriophylla	MAE	Solvent: EtOH (60-100%) Time: 20-40 min Power: 400-900 W	8-157 mg QE/g dry plant	[3.44]

RT: room temperature, GAE: Gallic acid equivalent, QE: Quercetin equivalent.

Santos et al. also used the maceration method to extract phenolic compounds from the barks of three different eucalyptus species [3.35]. In this case, the maceration was performed during 24 hours at room temperature, in constant agitation and with MeOH/H_2O (50/50 (v/v)) as solvents, preceded by an extraction with CH_2Cl_2 to eliminate the lipophilic compounds. The extraction yield obtained was between 10 and 15%, with the highest value being reached for the bark of the *Eucalyptus urograndis*. The three extracts from the different barks reported high TPC values, besides having potential as antioxidants, since the values measured for the antioxidant capacity of the extracts were in the range of those reported for commercial antioxidant compounds (BHT and ascorbic acid). Valencia-Avilés et al. compared the method of extraction by maceration with the method of extraction by hot water, from where it was said that the hot water extraction obtains better extraction yields than the maceration for all the studied barks [3.36]. This trend was also true for TFC, but in the case of TPC, maceration gave better results for two of the three bark extracts.

The Soxhlet extraction is one of the most widely used process mainly because of the good results it provides, as shown in the following examples. Rosdiana et al. studied the sequential extraction of bark from different tree species using CH_2Cl_2, acetone, toluene/EtOH (2/1, (V/V)), and H_2O [3.37]. The total yield of the extractions was higher than 17% for Mahoni and Acacia barks, being the extraction with acetone the one with the highest yield. In the case of Vieito et al., the exactions were performed to the pine bark using H_2O, EtOH (50%) and EtOH [3.38]. With the last two solvents, the obtained yield also overcomes the 17%, while with H_2O it does not reach the 8%. This means that besides choosing the right extraction method, it is also necessary to make a good selection of the used solvent. Neiva et al. studied the potential of different barks obtained from different wood industries, using

hexane, EtOH and H_2O [3.39]. The lowest yield in all cases was achieved with hexane, mainly due to the low content of non-polar compounds in the barks. Extractions with H_2O and EtOH reached very different yields depending on the initial composition of the studied bark, being the bark of *Acacia dealbata* the one that had the highest yields for both solvents with values above 36%. It was also confirmed the richness in TPC of the extracts obtained with EtOH and H_2O for all the barks, as well as their high antioxidant capacity.

CE methods usually have good extraction yield, but they also require long extraction times and large amounts of solvent. Furthermore, they generally involve the use of high temperatures with the risk of degradation of the target compounds. Therefore, in recent years the use of new extraction methods is being studied. The aim is to reduce the extraction time, temperature and solvent consumption, thus achieving higher efficiency and lower energy consumption [3.45] Two of the most studied modern techniques for bioactive compounds extraction from barks are microwave assisted extraction (MAE) and ultrasound assisted extraction (UAE). There are many papers related to this topic.

Aspé and Fernández did a comparison between conventional and modern techniques, from which they concluded that the best extraction yield was obtained through Soxhlet [3.40]. However, the results obtained by MAE and UAE were only 2 units smaller, but the time used in these extractions decreased by 98% compared to conventional methods. Using sequential extractions for the same substrates, it was observed that Soxhlet efficiency decreases whereas MAE and UAE increase the yield. Therefore, it was concluded that the new techniques improve the conventional ones, being MAE the one that provided better results. Hofmann et al. also conducted a

comparative study between different extractions methods with different solvents applied to beech bark [3.41]. This study shows that the best solvent for obtaining phenolic compounds is MeOH (80%), and the best method is MAE. In this paper, it can also be observed the large difference between the extraction times needed for maceration and modern techniques (MAE and UAE). Gligorić et al. studied the extraction of willow bark by different extraction methods, obtaining the best extraction yield using H_2O as solvent with MAE (21.86%), followed by UAE [3.42]. The worst values are obtained for maceration with EtOH/H_2O mixtures, although these are also high enough, above 16%.

Tanase et al. conducted the optimisation of the MAE to obtain the maximum TPC content of the beech bark, being the parameters studied the microwave (MW) power, the extraction time and the solvent [3.43]. The optimisation concluded that the mixture EtOH/H_2O (50/50) was the one that gave the best values of TPC. Furthermore, it was confirmed that the extracts obtained with the three solvents had good antioxidant and antimicrobiological capabilities. In addition, Mangang et al. performed the optimisation of *Albizia myriophylla* bark extraction by MAE to obtain the maximum amount of biflavonoid compounds [3.44]. In this case, the parameters studied were the applied power, the solid/liquid ratio, the extraction time and the EtOH concentration. The highest yield in biflavonoids was achieved with a power of 728 W and using EtOH (70%) as solvent. It was found that the use of higher power could increase the temperature too much, and could even degrade the compounds. Mangang et al. concluded that this method could save time compared to conventional methods.

Considering the results studied with conventional and non-conventional, it can be concluded that in general, modern techniques reduce significantly the extraction time, improving or equalising the extraction yield of the conventional methods. This implies an improvement in the efficiency of the processes that is promising for the integral valorisation of the bark.

3.2 Objective

The main goal of this chapter was to study the extraction capacity of different extraction methods to valorise the extractive fraction of the *Larix decidua* (from now on "pine") tree bark, which as seen in **Chapter 2** has great potential mainly due to its good antioxidant capacity. The valorisation was carried out following three different extraction techniques, conventional extraction (CE), ultrasound assisted extraction (UAE) and microwave assisted extraction (MAE). The second objective of this chapter was to evaluate the influence of the selected extraction method on the extraction yield as well as on the properties of the obtained extracts. Finally, the third aim of this chapter was to compare different extraction techniques in order to select the best technique for the extraction of bioactive molecules.

3.3 Materials and methods

3.3.1 Conventional extraction (CE)

Pine bark was subjected to an extraction in an orbital shaker with temperature control (Heidolph Unimax 1010 + Heidolph Incubator 1000) using a mixture of EtOH/H_2O (50/50 (v/v)) as solvent. 3 grams of dried bark

and 30 mL of EtOH/H_2O were disposed in 100 mL Erlenmeyer flasks, which was placed in the orbital shaker. The used experimental variables are listed in **Table 3.3**. Once the extraction was finished, the mixture was cooled down to room temperature, then it was filtered through filter paper under vacuum, and the yield of the extraction was calculated gravimetrically and referenced to a 100 g of dried pine bark, determining the non-volatile content (NVC) present in the extracts using the methodology described in **Appendix IV**. The measurement was carried out three times and the results were expressed as mean ± SD.

The studied variables in this extraction method were temperature and extraction time. The selection of the values of the variables was based on the literature as well as on the limitations of the used equipment, which in this case has a temperature limit of 65 °C.

Table 3.3 Experimental variables used for the optimisation of CE.

Variable	Definition	Unit	Value or range
Fixed	solid/liquid ratio	w/v	1:10
	Solvent: EtOH/H_2O	v/v	50/50
	Shaking speed	rpm	120
Independent	Temperature	°C	40-65
	Extraction time	min	30-180
Dependent	Extraction yield	%	

3.3.2 Ultrasound assisted extraction (UAE)

The extraction of the pine bark was performed in a temperature-controlled ultrasound (US) bath (Elmasonic S 70 H, Elma) using EtOH/H_2O (50/50 (v/v)) mixture as solvent. 3 grams of dried bark were mixed with 30 mL of EtOH/H_2O mixture in a 100 mL Pyrex™ Borosilicate Glass with a fixed

solid/liquid ratio of 1:10 (w/v) (see **Table 3.4**). Once the extraction was finished, the mixture was cooled down to room temperature and the extracts were separated from the solids by filtration. It was done under vacuum with a filter paper, and the yield of the extraction was calculated gravimetrically and referenced to a 100 g of dried pine bark, determining the non-volatile content (NVC) present in the extracts following the methodology described in **Appendix IV**. The results were expressed as mean ± SD of the three carried out measurements.

The studied variables in this extraction method were temperature and extraction time. The selection of the variables values was based on the limitations of the used equipment as well as on the literature. The Elmasonic S 70 H equipment has a fixed US frequency of 37 kHz, and it can work at temperature range of 30-80 °C. Although it is true that the maximum temperature at which it could work is 80 °C in theory, due to the fact that the US bath is an open system, it is very difficult to keep that temperature stable, so the real temperature limit was 65 °C.

Table 3.4 Experimental variables used for the optimisation of UAE.

Variable	Definition	Unit	Value or range
Fixed	solid/liquid ratio	w/v	1:10
	Solvent: EtOH/H_2O	v/v	50/50
Independent	Temperature	°C	40-65
	Extraction time	min	10-120
Dependent	Extraction yield	%	

3.3.3 Microwave assisted extraction (MAE)

MAE of the pine bark was performed in an open vessel MW (CEM Discover) under reflux, using EtO

H/H_2O (50/50 (v/v)) mixture as solvent. 3 grams of dried bark were mixed up with 30 mL of EtOH/H_2O mixture were placed in a 100 mL round bottomed flasks (see **Table 3.5**). Once the extraction was ended, the mixture was cooled down to room temperature, the extracts were filtered through a filter paper under vacuum and the yield of the extraction was calculated gravimetrically and referenced to a 100 g of dried pine bark, determining the non-volatile content (NVC) present in the extracts following the methodology described in **Appendix IV**. The measurement was carried out three times and the results were indicated as mean ± SD.

Table 3.5 Experimental variables used for the optimisation of MAE.

Variable	Definition	Unit	Value or range
Fixed	solid/liquid ratio	w/v	1:10
	Solvent: EtOH/H2O	v/v	50/50
	Shaking speed		max
Independent	Extraction time	min	10-120
	Power	W	100-300
Dependent	Extraction yield	%	

Extraction time and MW power were the studied variables in this extraction method. The choice of the variables values was based on the literature as well as on the limitations of the used equipment, which in this case has a maximum MW power of 300 W.

3.3.4 Optimisation method

A study of the effect of two variables on extraction yield (%) was carried out for the three extraction methods. The independent variables studied are shown in **Table 3.3**, **Table 3.4** and **Table 3.5**, for each of the methods. Briefly, for the CE and UAE methods, the analysed variables were extraction

time (min) and temperature (°C); while in the case of the MAE, the studied parameters were extraction time (min) and MW power (W).

An analysis of the influence of the different operational conditions for the different extraction methods were performed using three-level two factor experimental design with 10 experiments and 1 replicate of the central point. The optimisations were done by a response surface methodology (RSM), where the selected response variable was maximised, extraction yield (%). The Statgraphics Centurion XV.II software was used to perform the experimental design as well as the optimisation. The data were fitted using a secondary-order polynomial described by the **Equation 3.1**.

$$y = \beta_0 + \sum_{i=1}^{2} \beta_i x_i + \sum \sum_{i<j=2}^{2} \beta_{ij} x_i x_j + \sum_{i=1}^{2} \beta_{ii} x_i^2 + \varepsilon \quad (3.1)$$

where y is the predicted response, β_0 is the constant coefficient, β_i, β_{ij}, and β_{ii} are the coefficient of interaction, linear and quadratic, respectively, and x_i and x_j are the independent variables. The suitability of the model was measured by the coefficient of determination (R^2). The adequacy of statistical significance of the regression coefficients and analysis of variance (ANOVA) was used with a confidence level of 90%. Models validation were implemented comparing the extraction yield (%) values obtained experimentally at the optimal point and the ones predicted by the model.

Once the different extraction methods were optimised, the results obtained at the optimum point of each technique were compared. For this purpose, a statistical analysis was performed using one-way analysis of variance (ANOVA) by IBM SPSS Statistic 24 software. The values of the significant differences were determined by Tukey's range test. The experiments were

replicated three times, and the results were expresses as mean ± SD. The values of $p < 0.05$ were considered to be statistically significant.

3.3.5 Characterisation of the extracts of the optimal point

The characterisations of the extracts were carried out to the liquid extracts instead of to the dried extracts. It was done this way, to avoid sample degradation and the loss of volatile compounds. If the extracts dried at 105 °C had been used, the extracts could be degraded due to the high temperatures, and some of the most volatile compounds would be lost, thus losing information. Therefore, to avoid this, the liquid extracts were used.

The chemical compositions of the bark extracts at the estimated optimal conditions were determined by measuring the total phenolic content (TPC) and total flavonoids content (TFC) following the procedure described in the **Appendix IV**. The analysis of the potential of the obtained extracts was carried out by measuring three different antioxidant capacities, DPPH, ABTS and FRAP following the methodology described in the **Appendix IV**. The equations of the calibration curves used are given in **Table 3.6**.

Table 3.6 Calibration curves used for the measurement of TPC, TFC, DPPH, ABTS and FRAP.

Method	Calibration curve	R^2	Eq.
TPC	$[Galic\ acid] = 0.1373 \cdot Abs - 0.0037$	0.998	(3.2)
TFC	$[Catequin] = 0.1278 \cdot Abs - 0.0176$	0.995	(3.3)
DPPH	$[Trolox] = -0.1394 \cdot Abs + 0.0724$	0.988	(3.4)
ABTS	$[Trolox] = -0.9827 \cdot Abs + 0.7467$	0.997	(3.5)
FRAP	$[Trolox] = 0.1706 \cdot Abs - 0.0141$	0.999	(3.6)

In addition, the extracts were characterised using Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR) and High

Performance Size Exclusion Chromatography (HPSEC) to provide a better understanding of their structure, following the methodology explained in the **Appendix IV**. Finally, the identification of some compounds that constitute the extracts of the pine bark was carried out by using Ultraperformance Liquid Chromatography-Diode Array Detector-Electrospray Ionisation-Mass Spectrometry (UPLC-DAD-ESI-MS) (see **Appendix IV**).

3.4 Results and discussion

All the extractions were carried out using EtOH/H_2O mixture. The selection of this solvent was based on the literature. The literature study was done to look for methods that comply with green chemistry. In this way, the most used volatile organic compounds (VOCs), such as MeOH and hexane, are discarded as solvents [3.46–3.48]. EtOH, although it is a VOC, is generally accepted as environmentally friendly, but it is a flammable compound, so caution should be taken when handling it. The use of a binary mixture of EtOH/H_2O improves the efficiency of phenolic compound extraction [3.49]. In addition, H_2O is considered the green solvent by excellence.

It has been found that the amount of EtOH/H_2O in the binary mixture affects the extraction yield. In this thesis, the ratio has been set at 50/50 (v/v) based on the studied literature. In the study conducted by Cho et al. an analysis of how the EtOH concentration affects the extraction yield of the *Ulmus pumila* bark was carried out [3.50]. After studying concentrations of EtOH from 30 to 99%, it was observed that the best extraction yields were obtained using a 50% EtOH mixture. Other authors who have also carried out studies for the selection of the best binary mixture for the extraction of

bioactive compounds also agree with this [3.43, 3.51, 3.52]. Therefore, EtOH/H_2O (50/50 (v/v)) was selected as a solvent for this research.

3.4.1 Optimisation of conventional extraction (CE)

CE was the first optimised method. **Table 3.7** present the 10 experiments performed for the three-level two factor experimental design. It includes the variables studied with the experimental results obtained for each of the experiments. The value determined by R^2 was used to measure the correlation and significance of the models. This value is shown in **Table 3.8**, where the regression coefficients are also listed.

Using the significant regression coefficients given by the software, and summarised in the **Table 3.8**, a quadratic regression equation for the extraction yield (%) was calculated (**Equation 3.7**).

$$\%\,\mathrm{CE} = -16.557 + 0.816x_1 + 0.030x_2 - 0.0068x_1^2 - 0.00026x_1x_2 - 0.00008x_2^2 \qquad (3.7)$$

Table 3.7 Tested operational conditions for CE expressed in terms of dimensionless and dimensional independent variables (X_1 (temperature, °C), X_2 (time, min) and their response.

Nº Exp	X_1	X_2	Extraction yield (%)
1	1 (65)	1 (180)	7.78
2	0 (52.5)	-1 (30)	8.23
3	0 (52.5)	0 (105)	8.01
4	1 (65)	0 (105)	8.12
5	0 (52.5)	1 (180)	7.83
6	-1 (40)	1 (180)	6.15
7	0 (52.5)	0 (105)	8.71
8	-1 (40)	0 (105)	6.70
9	-1 (40)	-1 (30)	5.50
10	1 (65)	-1 (30)	8.11

Table 3.8 Regression coefficients and R^2 measured for CE model.

Coefficient	Value
b_0	-16.56
b_1	0.816[a]
b_2	0.030
$b_{1.1}$	-0.007[b]
$b_{1.2}$	-0.0003
$b_{2.2}$	-0.00008
R^2	0.945

[a] Significant coefficients at the 99% confidence level.
[b] Significant coefficients at the 95% confidence level.

The results of the experiments show that the variability of the extraction yield reaches up to 50% compared to the measured lowest yield value, 5.50% (experiment 9). This indicates that the selected conditions for this method have a considerable impact on the extraction yield, with a variability of 2.71%.

According to the regression coefficients (**Table 3.8**), the temperature is, from the two studied variables, the one that has a significant influence on the extraction yield. Since both, its linear and quadratic effects have a $p < 0.05$. In **Figure 3.3a** and **Figure 3.3b**, the response surface and the contour of the response surface of the optimisation performed can be observed. It is clear that small temperature increments increase the extraction yield considerably. However, focusing on how time affects, it can be observed that setting the temperature and increasing the time, the extraction yield increases but very few. In addition, it is noted that once the extraction time exceeds 120 min, the extraction yield gradually decreases reducing the extraction yield. This may be due to the compounds degradation because of long reaction time [3.53].

The optimisation of CE was carried out using the Statgraphic Centurion XV.II software, which predicted a model to achieve the maximum extraction yield, estimated at 8.63%. According to the model, the estimated optimal conditions to reach the maximum yield correspond to 58.27 °C and 94.27 min, with a R^2 of 0.945. The model was validated making a comparison of the predicted value with the experimental one, obtained by performing three experiments at the adjusted optimal point conditions (58 °C and 94 min). The experimental mean value of extraction yield was 8.24%, which was close to the predicted value. This fact confirms the suitability of the optimisation, so the optimisation of the CE is confirmed.

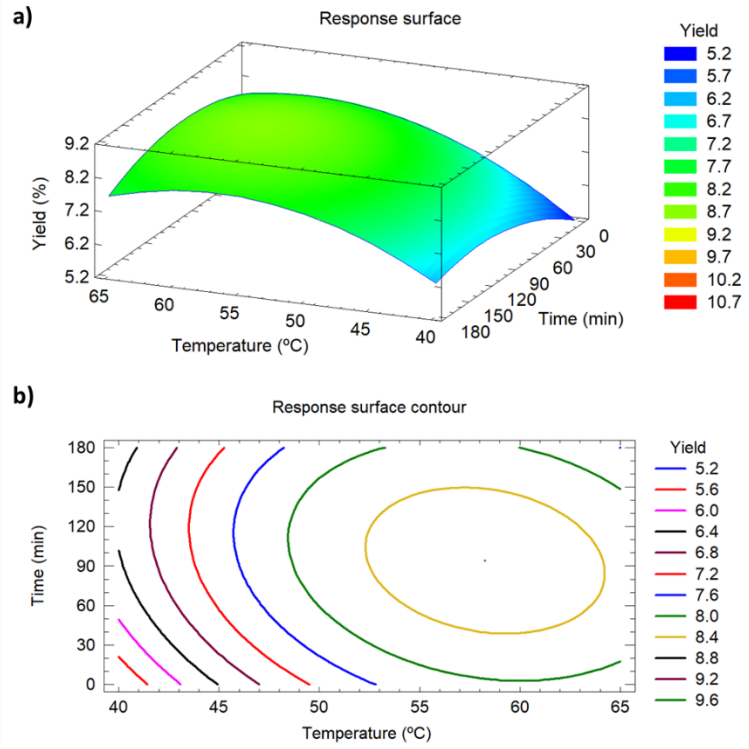

Figure 3.3 a) RSM plots for CE extraction yield. b) Response surface contour for CE extraction yield.

3.4.2 Optimisation of ultrasound assisted extraction (UAE)

UAE was also optimised using experimental design. The variables studied for each of the 10 experiments of the three-level two factor experimental design are presented in **Table 3.9**, together with the experimental results obtained for each of the experiments. The correlation and the importance of the models were determined by the R^2 value, which is shown in **Table 3.10**, where the regression coefficients are also displayed.

A quadratic regression equation for UAE extraction yield (**Equation 3.8**) was determined using these coefficients.

$$\%\ \text{UAE} = 3.894 + 0.119x_1 - 0.069x_2 - 0.0015x_1^2 + 0.0014x_1x_2 - 0.00013x_2^2 \qquad (3.8)$$

Table 3.9 Tested operational conditions for UAE expressed in terms of dimensionless and dimensional independent variables (X_1 (temperature, °C), X_2 (time, min) and their response.

Nº Exp	X_1	X_2	Extraction yield (%)
1	0 (52.5)	0 (65)	6.48
2	0 (52.5)	-1 (10)	5.71
3	-1 (40)	0 (65)	4.57
4	-1 (40)	1 (120)	3.47
5	1 (65)	0 (65)	5.18
6	1 (65)	1 (120)	7.33
7	1 (65)	-1 (10)	6.04
8	0 (52.5)	1 (120)	3.71
9	0 (52.5)	0 (65)	7.12
10	-1 (40)	-1 (10)	6.16

Table 3.10 Regression coefficients and R^2 measured for UAE model.

Coefficient	Value
b_0	3.89388
b_1	0.119
b_2	-0.069
$b_{1.1}$	-0.0014
$b_{1.2}$	0.0014
$b_{2.2}$	-0.00013
R^2	0.603

Looking at the results obtained for the different experiments of the design, it can be seen that the difference between the lowest extraction yield, 3.71% (experiment 8), and the highest, 7.12% (experiment 9) is equivalent to 3.41%. This value is almost equal to the lowest extraction yield obtained, so the variability of the reported results is significant.

In accordance with the data collected in the **Table 3.10**, it can be concluded that neither of the two studied variables have a significant influence on the extraction yield, since neither of the regression coefficients have a $p < 0.1$. This effect is also visible in **Figure 3.4a** and **Figure 3.4b**. From the response surface graph, it can be deduced that the best yield is obtained with the highest studied temperature. By setting the time at 120 min, it can be seen that the increase in extraction yield is linear with the increase in temperature. Looking at the response surface contour graph, it can be deduced that the increase in reaction time causes the extraction yield to decrease, which could be due to an over-exposure of the sample, which causes the degradation of the compounds [3.54].

The Statgraphic Centurion XV.II software was used to carry out the optimisation of the UAE. The model obtained maximises the extraction yield. However, the R^2 provided by the software for this optimisation was

low, 0.603, which is far from the minimum value required to ensure that the model fits correctly. In order to confirm the adequacy of the predicted model, a comparison was made between the software's predicted value and the one measured experimentally. The experimental value was measured in triplicate under the optimal conditions estimated by the designed model. This establishes that the optimal conditions are 65 °C and 94.76 min (rounded up to 95 min). The measured experimental value for the extraction yield was 6.13%, while the predicted value is 6.56%, which is a difference of the 7%. The similarity of both values confirms the suitability of the model, so the low R^2 may be due to the low influence of the tested variables. As the optimal temperature value is assigned to the maximum established for that variable, the doubt remains of whether the extraction yield will continue to rise with the increase in temperature or if the optimal temperature has already been reached.

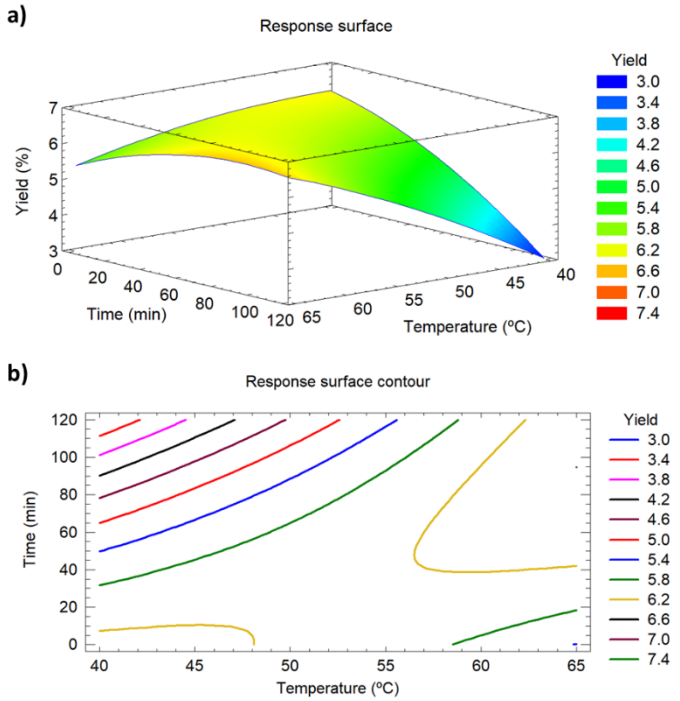

Figure 3.4 a) RSM plots for UAE extraction yield. b) Response surface contour for UAE extraction yield.

US is a method that relies on cavitation, and it requires the right conditions for this to happen. Although it has been seen that temperature can be an important variable, it may be necessary to study others, such as the solid/liquid ratio, to make cavitation easier. Other authors who worked with UAE obtained better results with higher solid/liquid ratios, so that cavitation is enhanced by the presence of a larger amount of solvent. Wei et al. conducted a study on the optimisation of UAE extraction from the *Abies nephrolepis* bark and concluded that the solid/liquid ratio of 10 mg/L to 25 mg/L considerably increased the extraction yield [3.55]. Therefore, it is concluded that the increase of the amount of solvent favours the extraction.

3.4.3 Optimisation of microwave assisted extraction (MAE)

Finally, the optimisation of MAE was conducted. The variables of the three-level two factor experimental design studied for each of the 10 experiments are presented in **Table 3.11**, together with the experimental results for each experiment. In **Table 3.12** the regression coefficients of the model are shown together with the R^2, which was used to measure the significance of the model.

In addition, with these coefficients, a quadratic regression equation was determined for the MAE extraction yield (**Equation 3.9**).

$$\%\,\mathrm{MAE} = 9.821 + 0.0505x_1 - 0.024x_2 - 0.00034x_1^2 - 0.00008x_1x_2 + 0.000051x_2^2 \qquad (3.9)$$

Table 3.11 shows that the difference between the lowest extraction yield (experiment 7) and the highest yield (experiment 8) is about 4%, which is almost double of the lowest obtained value. As a result, it is clear that the variability of the results measured is significant. Looking at the regression coefficients presented for each of the variables (**Table 3.12**), it is concluded that

the MW power applied to the sample has a significant influence on the extraction yield.

Table 3.11 Tested operational conditions for MAE expressed in terms of dimensionless and dimensional independent variables (X1 (time, min), X2 (power, W) and their response.

Nº Exp	X_1	X_2	Extraction yield (%)
1	0 (65)	0 (200)	7.83
2	0 (65)	0 (200)	7.91
3	-1 (10)	1 (300)	7.81
4	1 (120)	-1 (100)	7.87
5	-1 (10)	-1 (100)	8.93
6	1 (120)	0 (200)	7.27
7	1 (120)	1 (300)	4.95
8	0 (65)	-1 (100)	8.96
9	-1 (10)	0 (200)	6.57
10	0 (65)	1 (300)	7.97

Table 3.12 Regression coefficients and R^2 measured for MAE model.

Coefficient	Value
b_0	9.82065
b_1	0.051
b_2	-0.024[a]
$b_{1.1}$	-0.0003
$b_{1.2}$	-0.00008
$b_{2.2}$	0.00005
R^2	0.777

[a] Significant coefficients at the 90% confidence level.

The influence of the MW power is confirmed both in the response surface graph and in the response surface contour (**Figure 3.5a** and **Figure 3.5b**). It can be seen that, in general, an increase in the MW power decreases the extraction yield, with the best results being obtained using the lowest MW power. Regarding to the effect of the time, it is observed that the medium

extraction times are the most suitable for the highest extraction of compounds from the bark. This, just as with UAE, may be due to the fact that too long exposure may lead to the degradation of the compounds, as well as the application of high power [3.44, 3.56]. This effect can also be the reason why when the power is set at 300 W, the extraction yield, at low extraction times, increases with the increase of the time. However, once the maximum is reached, around 1 h, the yield decreases drastically with the increase of the extraction time.

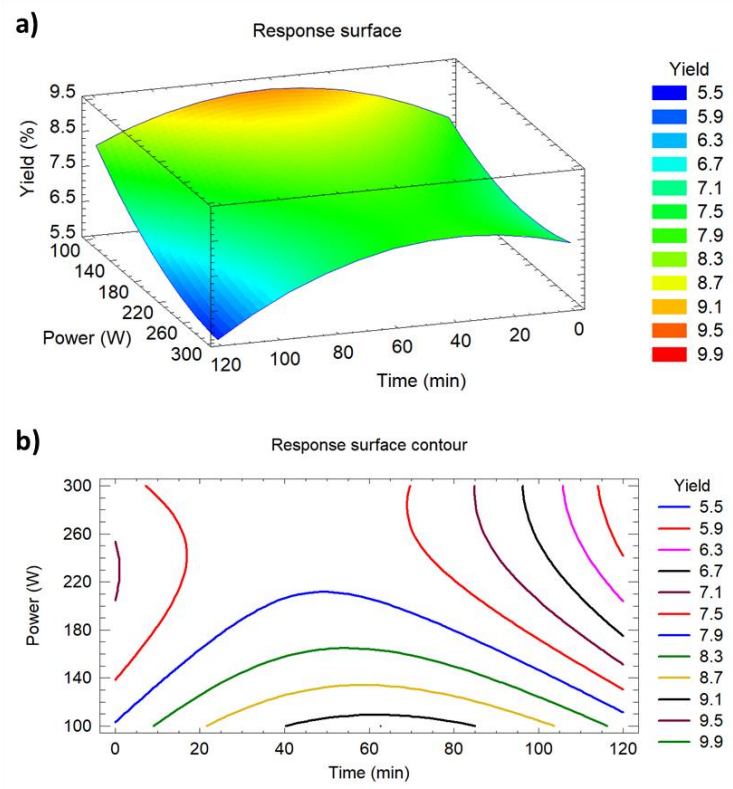

Figure 3.5 a) RSM plots for MAE extraction yield. b) Response surface contour for MAE extraction yield.

The optimisation of the MAE was carried out using the Statagraphic Centurion XV.II software, maximising the extraction yield. The R^2 obtained here was 0.777, which even though it is less than 0.85, which would be the

minimum desired to confirm the correct fit of the model, it can be considered a good fit. However, comparing the value predicted by the software (9.27%) with the experimental value (8.25%), it can be seen that the difference is big, so it can be said that the model does not fit correctly. The experimental value was obtained by performing the extraction in triplicate under the optimal conditions estimated by the designed model, which are 100 W and 62.66 min (rounded up to 63 min).

The lack of suitability of the model could be due to a poor selection of the limits of the studied variables, or the variables themselves. For this extraction method, other variables could have been studied, such as the solid/liquid ratio or the solvent. Li et al. found that the solid/liquid ratio also influences the MAE during the extraction of *Eucommia ulmoides* bark, although power remains the most important variable [3.57]. Another factor to have into a count is the power/time ratio. Tanase et al. studied times of less than 10 min for MW powers equal or greater than 300 W in their optimisation of MAE extraction from beech bark [3.43]. On the other hand, Bouras et al. performed the optimisation of *Quercus* bark extraction using MAE, and operated with MW powers lower than 100 W using reaction times of up to 1 h, achieving the highest yield with 45 W and 1 h [3.58]. From the analysis of these two works, it can be deduced that at MW powers higher than 100 W the reaction times must be short to avoid the compounds degradation. Therefore, for lower MW powers, the times must be longer than 10 min to ensure that there is enough time to achieve the total extraction of the compounds. Thus, it is concluded that the variables of this work should have been better fitted to make a good model.

3.4.4 Comparison of the extraction yield of the different extraction method.

The extraction yields obtained for the different experiments of each design demonstrated the existing differences between the methods. Although some similarity is observed between CE and MAE, the MAE yields are slightly higher. Therefore, it is expected that the yield at optimal conditions will be higher than that of the CE.

The extraction yields in the optimal conditions for the three studied extraction methods are summarised in **Table 3.13**. This table shows that there are no significant differences between CE and MAE ($p > 0.05$). However, the results reported for UAE are significantly different from both, CE and MAE.

Table 3.13 Comparison of extraction yield obtained at the optimum point for the different extraction methods. (The values were average ± SD (n = 3). Superscript letters depict significant differences (Tukey test, $p < 0.05$)).

Extraction method	Yield (%)
CE	8.24 ± 0.52^{a}
UAE	6.13 ± 0.41^{b}
MAE	8.25 ± 0.20^{a}

Comparing the extraction yields obtained at the optimal conditions of each of the models, it can be seen that MAE and CE obtained almost the same results. However, considering the trend of the experiments of the model, the yield of MAE was expected to be higher. The yield of the optimal value obtained for MAE was low because the designed model was not good predictor. However, it is clear that the worst yield is obtained using the UAE. These results are in accordance with the conclusions reported by other authors [3.40, 3.42, 3.59].

For the CE the obtained values were for medium temperature and not too long times. This could be because with too much time the extracted compounds can be degraded, and the same with high temperatures. In the case of UAE, the best results were obtained with the highest temperature and long time. But still with the fixed variables it seems that the system needs something more to increase the extraction yield to obtain at least the same as CE. Therefore, other variables should be studied, such as the solid/liquid ratio, the sonication frequency or the direct use of the US horn. In the case of MAE, the least power and long extraction time were needed to obtain the best results. However, the yield obtained under optimal conditions was very similar to the CE. The fact that lower MW power gives better results could be because with a higher MW power the extracted compound could be broken down [3.56] reducing the number of extracted compounds and their antioxidant capacity. In addition, the degradation of the compounds could also result from the higher temperature reached, which could lead to the degradation of the most volatile compounds

3.4.5 Characterisation of pine bark extract for each optimum point

The extraction yield is an easy indicator to know the extraction capacity of each method. Nevertheless, as the aim of this thesis is to obtain biomolecules, it is necessary to characterise more deeply the obtained extracts from the pine bark to be able to select the most suitable technique.

3.4.5.1 Total phenolic content (TPC) and total flavonoid content (TFC)

According to **Table 3.14**, UAE was the extraction method that provided the highest TPC, followed by CE and MAE. Furthermore, this value is higher

than the one estimated in the characterisation (**Table 2.3, Chapter 2**), which indicates that UAE is a good technique for the extraction of phenolic compounds. The lowest value measured for MAE can confirm the degradation of the sample under the optimised conditions, thus leading to a decrease in the extraction efficiency. In the comparative study done by Asé and Fernández, an opposite trend between the extraction techniques is concluded [3.40]. The best TPC value is obtained with Soxhlet, followed by MAE, with the worst value for UAE.

Table 3.14 Extraction yield and characterisation of the optimised point of pine bark extract for the different extractions.

	CE	UAE	MAE
TPC (mg GAE/g dried bark extract)	562 ± 1	605 ± 32	529 ± 20
TFC (mg CE/g dried bark extract)	417± 16	412 ± 14	430 ± 10
DPPH (mg TE/g dried bark extract)	742 ± 7	763 ± 14	722 ± 9
ABTS (mg TE/g dried bark extract)	807 ± 44	713 ± 54	853 ± 35
FRAP (mg TE/g dried bark extract)	345 ± 36	385 ± 37	370 ± 4

In general, the values measured in this work for TPC are higher than those reported by Santos et al. in the extraction of different Eucalyptus barks with MeOH/H_2O (50/50), since the maximum TPC value reported was 385.63 mg GAE/g extract for *Eucalyptus grandis* bark extract [3.35]. Furthermore, our values are in the range of those reported by Valencia-Avilés et al., 329-860 mg GAE/g extract for aqueous or 90% ethanolic extracts of different *Quercus* barks [3.36]. Comparing the results shown here with those obtained by Rhazi et al. for the optimisation of the extraction of the bark of *Acacia mollissima*, it can be concluded that the TPC are higher, since in that work the maximum value was 444.3 mg GAE/g extract [3.60].

Regarding the values reported for TFC, it is observed that the best method for the extraction of flavonoids is MAE. UAE is the one with the worst result,

very close to the values of CE. But in this case, the differences between the measurements for all techniques are low.

None of the obtained extracts with the different methods gives a better TFC than the one reported in the pine characterisation (**Table 2.3, Chapter 2**). However, the values measured here are better than those reported by other authors. The values presented in

Table 3.14 for TFC are higher than the maximum value provided by Soto-García and Rosales-Castro for hydroalcoholic extracts from the bark of *Quercus sideroxyla* (385.95 mg CE/g extract) [3.51]. However, the maximum value that they reported for the *Pinus durangensis* bark extract was higher (614.68 mg CE/g extract). Nevertheless, the value calculated for the extracts obtained with an acetone/H_2O mixture for the same raw material showed a lower TFC, 379.3 mg CE/g extract [3.61]. The results of the

Table 3.14 are also better than those obtained by Chupin et al. for maritime pine bark, although the difference is not big, since the MAE value obtained from maritime pine bark is 403 mg GAE/g extract [3.62]. These differences are mainly due to the tree species, although the used particle size can also be an important factor. The smaller the particle size, the bigger the contact surface with the solvent, so the extraction of the compounds is enhanced.

3.4.5.2 Antioxidant activity of the different bark extracts

All the extracts under study were found to be rich in phenolic and flavonoid compounds and their antioxidant capacity was measured in order to know the opportunities for their application.

Looking at the values obtained for the three antioxidant capacities of the extracts (**Table 3.14**), there is no clear trend in the three cases. Therefore,

each case will be studied separately. Regarding the DPPH, the extract with the highest activity is the one obtained with UAE, and the one with the lowest activity is the one obtained by MAE. However, all of them report a higher antioxidant activity than the one measured in the characterisation of the raw material (**Table 2.3, Chapter 2**). In the case of the activity measured by ABTS, it can be seen that all the values measured here are lower than the value reported for the bark characterisation. Unlike what happens for DPPH, in this case, the extracts obtained by MAE are those with the highest antioxidant activity, followed by the CE and far from those obtained by UAE. Finally, concerning the antioxidant capacity measured using FRAP, it is observed that the three values obtained are not very different. The worst value reported is for CE and the best for UAE. In this case, the value measured at the characterisation of the extracts is also higher (**Table 2.3, Chapter 2**).

The antioxidant capacities of the extracts, as discussed in **Chapter 2**, vary depending on the raw material used as a starting material. This makes the comparison of the obtained values with other works difficult, since as far as it is known, there are no published works with the characterisation of the extracts obtained from *Larix decidua* bark. Therefore, a comparison with extracts from different barks will be made cautiously.

The comparison of *Chrysophyllum perpulchrum* bark extracts obtained with different solvents by Baloglu et al. indicates that the highest DPPH value obtained was for the MeOH extracts (73.23 mg TE/g extract) [3.63]. This value is far below to those obtained here for the pine bark extracts. The same happened for the aqueous extracts for the ABTS antioxidant capacity. Bibi Sadeer et al. conducted a research on the antioxidant capacities of MeOH extracts from different barks [3.64]. Comparing our results with those ones,

it is observed that the antioxidant capacity of DPPH is lower than that measured here in all cases. However, the values obtained for ABTS indicate that the methanolic extracts from *Macaranga hurifolia* bark (784.21 mg TE/g extract) are in the same range as those obtained for CE pine extracts, while the methanolic extracts from *Sterculia tragacantha* are higher (943.26 mg TE/g extract). Regarding the reduction ability measured by FRAP, it is observed that for methanolic extracts of both, *Macaranga hurifolia* and *Sterculia tragacantha*, the results reported are higher than those presented here for the different pine extracts. Neiva et al. also characterises the potential of different barks using the FRAP assay [3.39]. From their results, it is concluded that the ethanolic extracts have higher antioxidant capacity than the others, being the extracts of the bark of *Acacia dealbata* the best antioxidant (1295 mg TE/g extract). This result is superior to the one obtained for the pine extracts here. However, the EtOH extracts of *Acacia melanoxylon* bark have a slightly lower antioxidant capacity (323 mg TE/g extract).

The results of the characterisation of the extracts obtained by the different techniques demonstrate that all of them have a great potential. However, a comparison of the results of this section with those obtained for the characterisation of the raw material (**Chapter 2**) indicates that there is still space for the improvement of the selected methods.

3.4.5.3 Structural characterization of the different bark extracts

For a better understanding of the compounds that compose the pine bark extracts, they were characterised with different techniques. The molecular weight (Mw) distribution of the different extracts was analysed by HPSEC, and the obtained results are summarised in **Table 3.15**. These extracts consisted on a heterogeneous mixture of compounds with separated

fractions, probably caused by a difference in the degree of polymerisation of the compounds [3.65].

Table 3.15 Percentage, average Mw, number average (Mn) and polydispersity index (Mw/Mn) of EtOH/H_2O bark extracts of each method at the optimal point.

					Global average		
	Percentage	Mw (g/mol)	Mn (g/mol)	Mw/Mn	Mw (g/mol)	Mn (g/mol)	Mw/Mn
CE	78.83	19,308	6,207	3.11	15,332	1,376	11.15
	8.97	836	791	1.06			
	7.66	357	335	1.07			
	4.53	177	176	1.01			
UAE	81.36	25,591	7,402	3.46	20,956	2,191	9.56
	10.92	968	895	1.08			
	7.72	370	344	1.08			
MAE	81.78	31,346	7,829	4.00	25,744	1,750	14.71
	9.43	900	828	1.09			
	5.70	346	325	1.07			
	3.08	175	174	1.01			

Figure 3.6a depicts the chromatogram, where it can be observed that there are not many differences between the extracts obtained by the different methods. Nevertheless, analysing the data in **Table 3.15**, it can be seen that the average Mw is different according to the extraction method. The extracts obtained using MAE are those with the highest global average Mw, and also those with the highest global average polydispersity index. Extracts obtained using CE are those with lower global average Mw, but also have a very high polydispersity index. The extracts obtained using UAE have the lowest global average polydispersity index, since they are only separated into three groups with different Mw. Both MAE and UAE extracts consist of compounds with Mw over 1,000 g/mol, while the CE extract has more than 11% of its compounds with lower molecular weights. The values reported for CE extracts are similar to those described in the characterisation of the raw material (**Table 2.5, Chapter 2**). The fact that more than 78% of the

compounds present in all the studied extracts have Mw higher than 1,000 g/mol is because the degree of polymerisation of the compounds is high.

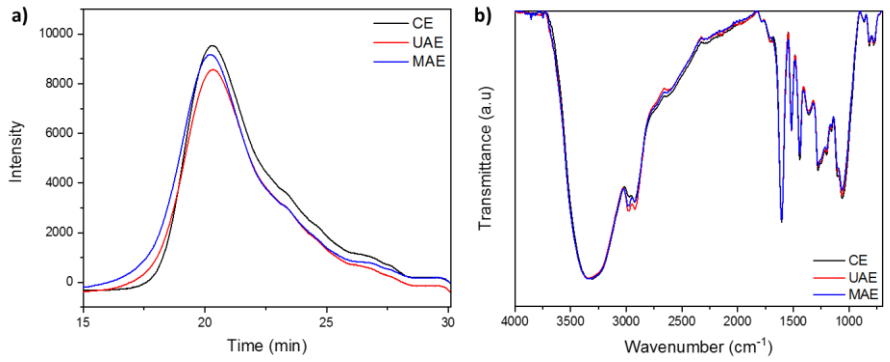

Figure 3.6 a) GPC chromatogram of EtOH/H_2O bark extract under optimal conditions. b) ATR-FTIR spectra of EtOH/H_2O bark extract obtained under optimal conditions

Following the analysis of the structure of the extract compounds, a study of the bond types was performed using ATR-FTIR. **Figure 3.6b** shows the spectra of the three bark extracts, confirming the similarity of their structures. The spectra of the pine bark extracts are practically the same, except for the difference in the intensity of the peaks between 2900 and 3000 cm^{-1}, and between 1040 and 1105 cm^{-1}. The band assignment is based on the assignments found in the literature given by Chupin et al. and Boeriu et al. [3.62, 3.66].

According to the band assignment, the peak at 3300 cm^{-1} is attributed to –OH stretch vibration in phenolic and aliphatic structures. The band at 2973 cm^{-1} is identified as -CH_3, CH_2 stretching vibration. The peak at 2925 cm^{-1} is detected to –CH stretch vibration in aromatic methoxy groups and in methyl and methylene groups of side chains. The conjugated carbonyl-carbonyl stretching is identified at 1705 cm^{-1}. The peaks at 1605 cm^{-1}, 1515 cm^{-1} and 1440 cm^{-1} are originated by aromatic skeleton vibration, and 1440 cm^{-1} also correspond to –CH deformation. The phenolic stretch vibration of -OH and aliphatic -CH deformation in methyl groups is detected at 1370

cm^{-1}. The peak 1275 cm^{-1} and the small peak at 1245 cm^{-1} are attributed to C-O-C asymmetric stretch vibration [3.67]. The bands at 1200 cm^{-1} and 1050 cm^{-1} can be assigned to C-O stretching vibration. The peak at 1150 cm^{-1} is identified as aromatic CH in-plane bending vibration. Aromatic –CH bending in-plane vibration is detected at 1105 cm^{-1}. Finally, al the bands with wavelength smaller than 900 cm^{-1} are attributed to an aromatic –CH stretch vibration.

The peak at 2973 cm^{-1} has low intensity for CE extracts; however, it is more intense for the other two extracts. This peak, together with the peak at 2925 cm^{-1}, exhibits major intensity for UAE extracts. The peak 1705 cm^{-1} appears in all extracts, but in the case of the CE extract it is better defined. The peaks at 1150 cm^{-1} and 1105 cm^{-1} have small differences, since in the case of MAE extracts, they are less intense than in the others. In addition, the 1150 cm^{-1} band has a higher intensity for CE extracts.

The vibration peaks assigned to different aromatic structures confirmed the high content of phenolic compounds in the extracts. Furthermore, the band corresponding to -OH in phenolic and aliphatic structure is very big, which evidences the good measured antioxidant capacities.

3.4.6 Identification of the extracted compounds by UPLC-DAD-ESI-MS

From the data obtained about the characterisation of bark extracts at optimal point and from the HPSEC analysis, it is expected that the obtained extracts consist on a mixture of compounds with different Mw and polymerisation rate, with a high content of phenolic compounds. In order to know better the compounds that form the extracts, these were analysed by UPLC-DAD-ESI-MS.

The UV chromatograms of these extractives permitted the identification of six peaks corresponding to different co-elutions of some compounds. **Figure 3.7** shows only the chromatogram measured for MAE extracts because the other two are identical. These peaks have been identified in the three extracts (CE, UAE and MAE) by analysing the results obtained for three different wavelength (254, 320 and 350). The compounds from the different peaks were tentatively identified (**Table 3.16**).

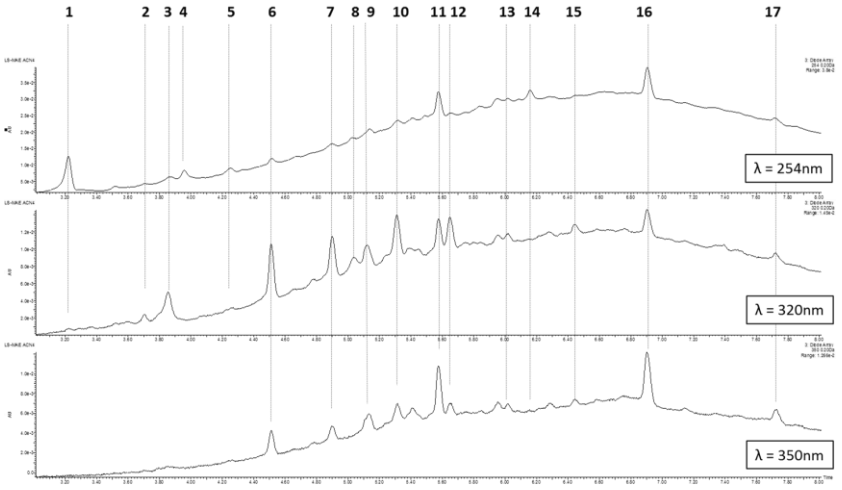

Figure 3.7 UV and MS chromatograms of the EtOH/H_2O bark extract obtained under MAE optimal conditions analysed by UPLC-DAD-ESI-MS (From minute 3 to 8).

The identification of the compounds present in the pine bark extracts was performed based on the literature. In general, each peak are a mixture of different compounds, which co-eluted, making the identification difficult. **Table 3.16** shows the compounds that have been tentatively identified in each peak and for each extract. However, the non-identification of a compound in one of the extracts does not mean that this compound is not present in that extract.

This identification confirms the existence of phenolic and polyphenolic compounds in EtOH/H_2O extracts from pine bark. Furthermore, the

existence of flavonoid compounds is also confirmed by the presence of its derivatives (derived from kaempferol, catechin and quercetin). The presence of dimers and trimers is also confirmed, proving the degree of polymerisation discussed in the molecular weight analysis of the extracts.

Among the identified compounds, the flavonoids Catechin, Quercetin, Kaempferol and Luteolin are of high interest. This is because they all have antioxidant, anti-inflammatory, and anti-carcinogenic properties among others. These properties convert them into perfect compounds for applications related to human health. Luteolin has been effectively tested as cardio-protective agent and as cancer preventer [3.68, 3.69]. Kaempferol has been tested as an agent to prevent various diseases, with good results [3.70]. However, its absorption in the body is not good, so it is necessary to continue studying mechanisms to promote that.

n the case of Quercetin, this has also been tested as a nutritional supplement with clear benefits for human health in different diseases such as cancer, cardiovascular problems and osteoporosis among others [3.71]. Furthermore, due to its poor absorption in the organism, some studies have also been carried out to improve this area, mainly through the study of nanoparticles [3.72]. Finally, Catechin is one of the most studied flavonoids, especially in the field of human health, in different industries (pharmaceutical industry, cosmetic industry, food industry, etc.) [3.73]. In addition to its benefits in human health, there are also known benefits from its use in different materials, mainly focused on packaging, where the good properties of this flavonoid allow to improve the oxidation resistance, it can act as an age indicator agent and works as a stabiliser [3.73, 3.74].

Table 3.16 Tentative identification of the compounds of the extracts in the UPLC-DAD-ESI-MS spectra of the eluted peaks during the UPLC analysis of the EtOH/H2O bark extracts.

$[M-H]^-$	CE	UAE	MAE	Identification	Peaks	Reference
153	√	√	√	Protocatechuic acid	1	[3.75]
169		√	√	Gallic acid	10, 14, 16, 17	[3.76]
179	√	√		Caffeic acid	12, 13, 16	[3.77]
279	√	√		(epi)afzelechin	11	[3.78]
285	√	√	√	Luteolin	17	[3.79]
289	√	√	√	Catechin	2,3	[3.79]
301	√	√	√	Quercetin	15, 16	[3.75]
345	√	√		Methyl gallic acid hexoside	5	[3.76]
354	√	√		5-O-caffeolyquinic acid or chlorogenic acid	14	[3.80]
359	√	√	√	Rosmarinic acid	1	[3.77]
405	√	√	√	Junipediol A 8-glucoside or nikoenoside	6,7	[3.80]
426		√	√	Catechin derivative	7, 8, 16	[3.80]
446	√	√	√	Quercetin-hexoside	11, 17	[3.79]
505	√			Quercetin hexoside derivative	5, 11, 12	[3.81]
541		√	√	Larixinol	16	[3.79]
573	√	√	√	Kaempferol Derivative 1	8,9, 12, 13, 15	[3.79]
577	√	√		Procyanidin dimer	3,4,7	[3.82]
609	√	√	√	(Epi)gallocatechin dimer	1	[3.64]
687	√	√	√	Gallic acid quinate	8, 12, 14	[3.76]
787	√	√	√	Tetragalloylglucose	10,13	[3.75]
831		√		4´-methyl-(epi)afzelechin trimer	2	[3.78]
865	√	√		Procyanidin trimer	2,3,4	[3.79]

3.5 Conclusion

The optimisation of three different extraction methods was performed. From which it is concluded that there is a direct influence of the extraction technique not only on the extraction yield, but also on TPC, TFC and antioxidant capacity of pine bark extracts. Thus, the model obtained for the CE fits perfectly, while in the case of UAE and MAE should be improved. Although it is true that the UAE model fits well, it has been seen that the studied variables do not have a significant influence on the extraction yield. Therefore, other variables should be studied in order to improve the extraction yields obtained. The adjustment of the MAE model is not good, so it is necessary to adjust the ranges of the studied variables in order to improve them.

The best extraction yield obtained corresponds to the MAE, closely followed by the CE. Even if values for CE and MAE are not so different, the main advantage of MAE is the lower processing time and therefore the potential of energy saving. However, the difference should be higher with the optimised operating parameter of the MAE. The extraction yield obtained for UAE was lower than the CE, which once again highlights the importance of the investigation of other variables that have a significant influence on this extraction.

All extraction methods showed good capacity for the extraction of biomolecules, more specifically phenolic and polyphenolic compounds. The extracts obtained by UAE were the richest in phenolic compounds, while the MAE extracts had the lowest TFC. However, all three cases had high TPC results, which confirms that the selected methods are suitable for the extraction of this type of compounds. As well as with phenolic compounds,

these methods have also been confirmed as suitable techniques for the extraction of flavonoid compounds, since all the studied extracts have a high TFC, with similar values in the three cases. Furthermore, the presence of phenolic and flavonoid compounds is confirmed by the analysis of UPLC-DAD-ESI-MS. The presence of flavonoids such as Catechin and Quercetin not only confirms pine bark as a source of flavonoid compounds, but also increases the potential industrial interest of these extracts.

Pine bark extracts, in addition to their richness in phenolic compounds, also show good qualities as antioxidants. In the three studied antioxidant capacity approaches, all the extracts are demonstrated to have high antioxidant capacities. However, the comparison of the values of each extract with those measured for pine characterization shows that there is still room for improvement. The structural analysis carried out by HPSEC and ATR-FTIR shows no significant differences between the pine bark extracts obtained by the different methods of extraction.

In conclusion, there are not many differences between all the obtained extracts. Therefore, it is clear that the US and MW intensification methods can provide at least similar extracts to those obtained with conventional methods. For this reason, and considering the evolution towards a sustainable development, it is confirmed that MAE and UAE can be promising techniques for the extraction of bioactive molecules from pine bark.

3.6 References

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Chapter 4

Bioactive molecules extraction by process intensification

4.1 Background

Lignocellulosic biomass is considered an attractive raw material for biorefineries. However, there are still limitations, especially economic ones, to the extensive application of biorefineries. To overcome these limitations different intensification processes are being developed.

The objective of the processes intensification is to obtain a higher extraction yield and high-quality products by reducing the number of operating units, extraction time, raw material consumption, environmental impact, global energy consumption, cost and waste generation [4.1], as well as improving the quality and selectivity of the method (see **Figure 4.1**). Currently there are many innovative processes available to carry out extractions using green processes, such as ultrasound, microwave, pulse electrical field, supercritical fluids, pressurised liquids, supercritical water, and thermal magnetic induction among others [4.1]. Microwave assisted extraction (MAE) and ultrasound assisted extraction (UAE) are two of the most studied and promising non-conventional techniques.

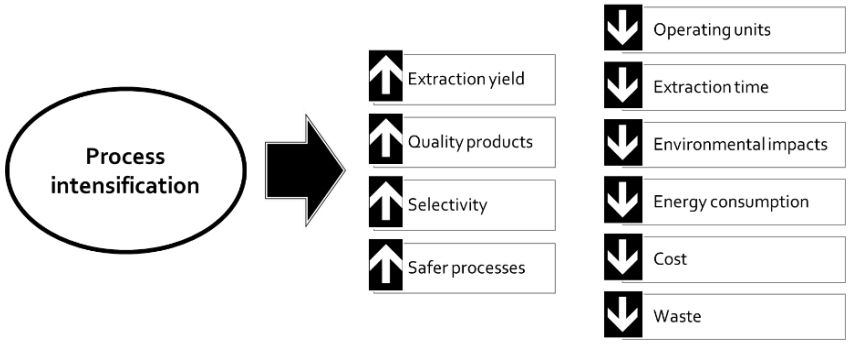


Figure 4.1 Process intensification.

MAE heats by two different mechanisms: dielectric heating, generated by the rotation of the dipole moment, and ionic conduction [4.2], which are connected with the interaction between the electromagnetic waves with the

solvent [4.3]. In the case of biomass, the heating creates a rapid evaporation of the moisture, generating high pressure inside the plant cells which break these up facilitating the extraction of intracellular compounds [4.4].

UAE, defined as inaudible sound waves at frequency over 20 kHz, is based on the cavitation phenomenon, which enhances cellular disruption and penetration of the solvent in the solid matrix due to compression and expansion effects on the plant cells [4.4].

The use of both techniques individually improves the extraction of intracellular compounds. However, each technique has its own limitations. Generally, the MAE obtains better results for the extraction from lignocellulosic materials, but even so, as seen in **Chapter 3**, there is still room for improvement. These limitations can be compensated by the characteristics of the UAE. The combination of these two techniques offers a synergistic effect induced by the improvement of mass (ultrasound) and heat (microwave) transfer [4.5], which influences the efficiency of the extraction [4.6]. Thus, the simultaneous use of both techniques, not only combines the advantages of both technologies, but also compensates for their shortcomings [4.7].

4.1.1 Combined microwave-ultrasound irradiation

The use of combined microwave (MW) and ultrasound (US) as a hybrid technology was first introduced in the mid-1990s. In 1995, the synergistic effects occurring under simultaneous irradiation of MW and US were described for the first time. Japanese researchers first described these effects seen in a sono- and chemi-luminescence experiment [4.8]. Although popular wisdom simply associates MW with superior heating and US with efficient agitation, these techniques are capable of doing much more, which

has resulted in its increased use in chemical processes [4.9]. Combined irradiation can be performed simultaneously or otherwise sequentially [4.10].

Different equipment needs to be used depending on whether the process is simultaneous or sequential. The sequential use of MW and US allows the use of two separate equipment's, which permits to use the same equipment that have been used up to now. However, to use MW and US simultaneously, the equipment must be adapted. To do this, MW oven is modified by drilling a hole in the wall, where the US horn is inserted. This must be of a non-metallic material, so typically they are ceramic, Pyrex®, quartz or PEEK® [4.11]. In addition, this equipment usually needs a cooling system of the vessel to avoid the increase of temperature to the boiling point, which results in a negligible cavitation. The first prototype was designed at the university of Turin in 2004 [4.11], and it was built by customising a domestic oven.

This technique was first developed in the field of organic chemistry, and nowadays it is still one of the most used fields. However, at present, it is also used in inorganic synthesis, degradation of contaminants, chemical digestion and natural compounds extraction. This advance is mainly due to the fact that this technique is considered sustainable. The combination of improved energy and material transport effects enhances the reaction, reducing the extraction time and energy consumption [4.12].

In the field of organic chemistry, the applications of the combination of MW and US are many; from the improvement of the transesterification to obtain biodiesel [4.13, 4.14], to the digestion of different samples to calculate their nitrogen content (improvement of the Kjeldahl method) [4.15], as well as

different reactions for the synthesis of heterocyclic compounds, C-C couplings or C-heteroatom bond formations [4.11].

In recent years, nanoparticles are becoming key components in a wide range of applications (nanotechnology, chemistry, physics, and polymer science among others). Size, morphology and dimensionality strongly affect the properties of nanostructured materials, so the control of these parameters is essential. Due to this, in the last years, the use of the combination of MW and US for the synthesis of nanoparticles is being studied, reducing the processing time and controlling the properties of the nanoparticles [4.16].

Recently, the study of combining MW and US for natural compounds extraction from plants is becoming popular. Due to the good results obtained comparing to when the techniques are used separately.

4.1.2 Microwave-ultrasound assisted extraction (MUAE) applied in biomass

The combined use of MW and US, due to the effects of each technique on the plant's cell structure, enhances the extraction of the compounds, resulting in a greater solubilisation of compounds, shortening the extraction time and reducing the loss of solvent. This technique is being studied not only to obtain extracts, but also to isolate other compounds such as essential oils, dyes or oligosaccharides.

Table 4.1 lists some examples of works that have been carried out where MW and US are used in combination to obtain different compounds from different biomasses. In all the studied cases, reported extraction yields were equal or higher than those obtained by conventional techniques, and in all cases a considerable reduction in extraction time was reached.

Table 4.1 Example of extraction of different products from different biomass using MUAE.

Raw material	Type of MUAE	Products	Reference
Soybean germ Seaweed	Simultaneous	Vegetable oil	[4.17]
Leaves	Simultaneous	Flavonoids	[4.18]
Black rice husk	Sequential	Polyphenols	[4.19]
Lotus seeds	Simultaneous	Oligosaccharides	[4.20]
Cumin seed	Simultaneous	Essential oil	[4.21]
Sorghum husk	Simultaneous	Natural colorants	[4.22]
Sea buckthorn leaves	Simultaneous	Flavonoids and essential oil	[4.23]
Wet oleaginous yeast biomass	Sequential	Lipids	[4.24]
Fruit	Sequential	Essential oil and polysaccharides	[4.25]
Chicory leftovers	Simultaneous	Phenolic compounds	[4.12]
Coffee silverskin	Simultaneous	Dietary fibre	[4.26]
Brown Macroalgae	Simultaneous	Carbohydrates and phenolic compounds	[4.27]

Cravotto et al. reported a yield increase between 50 and 500% of vegetable oil extraction from soybean germ and seaweed by simultaneous microwave-ultrasound assisted extraction (SMUAE) [4.17]. The values obtained for SMUAE were also better than those obtained by the independent use of US and MW. Furthermore, in this work the extraction time was reduced up to 10 times compared to the conventional method.

Regarding the oligosaccharides, Lu et al. carried out the optimisation of the SMUAE improving the yields between 17 and 76%, depending on the type of oligosaccharides [4.20]. The optimal conditions reported were 325 sec, microwave power 250 W, ultrasonic power 300 W, and solid/liquid ratio 10 mL/g, highlighting the reduction of the extraction time.

As shown in **Table 4.1**, this technique is also studied for the extraction of essential oils. An example of this is the study carried out by Ascrizzi et al. to obtain essential oils from cumin seeds [4.21]. In this work, SMUAE is combined with the use of ILs as a solvent. Additionally, Li et al. have optimised the use of SMUAE in combination with ILs for the extraction of essential oils from leaves [4.23]. In addition, the sequential use of MW and US has also been studied. Li et al. apply this technique combined with the use of DES to improve the extraction yield of essential oils from fruit [4.25]. In all these cases, the extraction yields were improved compared to conventional methods and the extraction time was reduced.

The use of both natural dyes and natural dietary fibres is increasing, which requires finding cost-effective process. Therefore, the use of SMUAE is also being studied in this field. In the works conducted by Wizi et al. [4.22] and Wen et al. [4.26], an increase in the recovery of the target compounds is detected, highlighting the potential of this technique.

The extraction of phenolic and polyphenolic compounds is one of the fields that is attracting more interest for the application of the combination of MW and US. So far, this technique has been applied, either simultaneously or sequentially, to different raw materials (see **Table 4.1**), always having a positive effect on the extraction process. This implies an efficiency improvement in the processes that are promising for the integral valorisation of the bark. To the best of our knowledge, there are no works where SMUAE is applied to tree bark, so it seems interesting to perform it due to its great potential.

4.2 Objective

The main objective of this chapter was to go one step further in the intensification of the pine bark extraction process by using SMUAE. After studying the potential of both MAE and UAE methods (**Chapter 3**), it was decided to apply them simultaneously to improve the extraction. With this aim, an evaluation of the influence of different variables of the SMUAE on the extraction yield and the total phenol content was carried out. The secondary aim of this chapter was the analysis of the influence of the selected extraction technique on the properties of the obtained extracts.

4.3 Materials and methods

4.3.1 Simultaneous microwave-ultrasound assisted extraction (SMUAE)

SMUAE was performed in an open vessel microwave reactor (MILESTONE flexiWAVE) under reflux with an added ultrasonic unit (HIELSCHER UIP500hdT). To avoid possible solvent losses by evaporation, the condenser operates with a constant air flow. The assembly is illustrated in **Figure 4.2**.

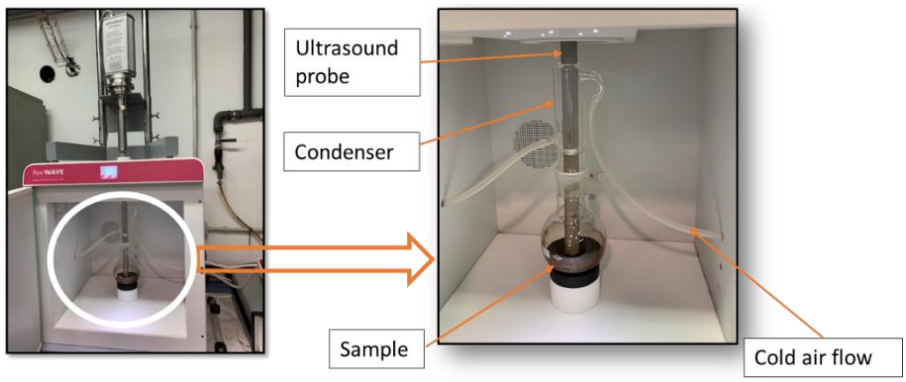


Figure 4.2 Assembly for SMUAE.

The extractions were carried out using pine bark with a particle size below 0.5 x 0.5 mm, EtOH/H_2O (50/50 (v/v)) mixture as solvent and fixed solid/liquid ratio of 1:10 (w/v). 10 g of dried bark were placed in a 500 mL borosilicate round bottomed flask with 100 mL of solvent and medium stirring level. After the extraction, the extracts were filtered through filter paper under vacuum and then centrifuged. The yield of the extraction was determined gravimetrically and referenced to a 100 g of dried pine bark determining the non-volatile content (NVC) present in the extracts as it is described in **Appendix IV**. The measurement was carried out three times and the results were expressed as mean ± SD.

The solid/liquid ratio (1:10) as well as the used solvent mixture (EtOH/H_2O (50/50)) were selected and fixed according to the parameters of **Chapter 3**. It is in accordance with the solvent mixture estimated by Yu et al. in the optimisation done to the leaves extraction using sequentially MAE and UAE [4.4]. These variables are fixed in order to compare the effect generated by the simultaneous use of MW and US, with the effect they generate separately.

4.3.2 Experimental design and statistical analysis

The variation on extraction yield and total phenolic content (TPC) were studied changing the values of the microwave power (W), extraction time (sec) and the ultrasound amplitude (%). In this work, the extraction time represents the time from the start to the cessation of the MW and US simultaneous application on the mixture. The experimental design and the optimisation were carried out using response surface methodology with a Box-Behnken design including three replicates in the central point. The variables are reported in **Table 4.2**.

Table 4.2 Experimental variables used for the optimisation.

Variable	Definition	Unit	Value or range
Fixed	solid/liquid ratio	w/v	1:10
	Solvent: ethanol/water	v/v	50/50
	Shaking speed	%	40
Independent	Extraction time	sec	30-120
	Microwave power	W	100-300
	Ultrasound amplitude	%	0-100
Dependent	Extraction yield	%	
	Total phenolic content	mg GAE/g dried bark extract	

The independent variables selected were microwave power, extraction time and percentage of ultrasound amplitude. The most commonly used parameter for the study of the ultrasound influence on the extraction is usually the power. In this case, the used equipment had a fixed power, so it was decided to vary its percentage of amplitude. This equipment limitation is related to the fact that the chosen US horn must fulfil specific conditions in order to work inside the microwave cavity without being affected by microwaves. Therefore, the equipment selected was a ceramic horn, with a fixed operating frequency of 19-20 KHz and a fixed amplitude of 25 μm (HIELSCHER UIP500hdT). The microwave power range tested was determined according to the previous work done and in line with the results obtained by Luo et al. [4.5]. Finally, for the selection of the time range, the conclusions obtained in **Chapter 3** were taken into account. Thus, since the powers tested were greater than 100 W, the studied extraction times were very short, less than 2 minutes. Another factor that was taken into account to select the extraction time range was to avoid the degradation of the sample caused by exposure to high temperatures. The application of both MW and US causes an increase in the temperature of the solvent/raw

material mixture. Therefore, very short reaction times were chosen, since it was assumed that the combination of both would generate a bigger temperature increase [4.28].

Statgraphics Centurion XV.II software was used to generate the experimental design and the optimisation. A second-order polynomial equation was used to fit the data (**Equation 4.1**).

$$y_n = \beta_0 + \sum_{i=1}^{3} \beta_i x_i + \sum \sum_{i<j=2}^{3} \beta_{ij} x_i \, x_j + \sum_{i=1}^{3} \beta_{ii} x_i^2 + \varepsilon \qquad (4.1)$$

where *y* is the predicted response, x_i and x_j are the independent variables, β_i, β_{ij}, and β_{ii} are the coefficient of interaction, linear and quadratic, respectively, and β_0 is the constant coefficient. The suitability of the model was determined by the evaluation of the coefficient of determination (R^2), the significance of the regression coefficients, and the F-test value obtained from the analysis of variance.

With the aim of optimising the selected response variables simultaneously, a multiple response surface optimisation was conducted. The selection criteria were relied on obtaining the highest extraction yield in addition to a high TPC in the defined range of conditions. A comparison between the experimental values obtained at the optimal point and the ones predicted by the model was done for the validation of the model.

The results measured in this work at optimal conditions were compared with those reported in **Chapter 3** under the optimal conditions of each of the methods. Statistical analysis was carried out by the analysis of unidirectional variance (ANOVA) using the IBM SPSS Statistic 24 software. The significance study was carried out by Tukey's range test, and the values

of $p < 0.05$ were considered statistically significant. The experiments were repeated three times and the results were reported as mean ± SD.

4.3.3 Characterisation of bark extracts in optimal conditions

The characterisations of the extracts were carried out on the liquid extracts. Chemical composition of bark extracts obtained at optimal conditions were determined by measuring the TPC and the total flavonoid content (TFC) following the procedures described in **Appendix IV**. The potential of the bark extracts was studied by measuring three different antioxidant capacities. The assays used were DPPH, ABTS and FRAP, which were conducted following the methodology described in **Appendix IV**. The equations of the used calibration curves are listed in **Table 4.3**.

Table 4.3 Calibration curves used for the measurement of TPC, TFC, DPPH, ABTS and FRAP.

Method	Calibration curve	R^2	Eq.
TPC	$[Galic\ acid] = 0.166 \cdot Abs - 0.0147$	0.998	(4.2)
TFC	$[Catequin] = 0.1278 \cdot Abs - 0.0176$	0.995	(4.3)
DPPH	$[Trolox] = -0.1222 \cdot Abs + 0.0778$	0.988	(4.4)
ABTS	$[Trolox] = -1.0202 \cdot Abs + 0.6899$	0.997	(4.5)
FRAP	$[Trolox] = 0.189 \cdot Abs - 0.0125$	0.999	(4.6)

In order to have a better comprehension of the structure of the extracts, they were characterised by Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR) and High Performance Size Exclusion Chromatography (HPSEC) following the methodology described in **Appendix IV**. Finally, Ultraperformance Liquid Chromatography-Diode Array Detector-Electrospray Ionisation-Mass Spectrometry (UPLC-DAD-ESI-MS) was used for the structural analysis of the extracts (described in **Appendix V**).

4.4 Results and discussion

The simultaneous use of MW and US for the extraction of natural compounds is neither well studied nor well developed, perhaps due to technical difficulties. For this reason, no literature evidence has been found on the combination of both extraction techniques for tree bark. However, it is considered that the synergy generated by the simultaneous use of both techniques could be beneficial for the extractions. Therefore, in this work, the study of SMUAE extraction from pine bark has been conducted and the obtained results are discussed below.

4.4.1 Modelling and optimisation of SMUAE conditions

Table 4.4 presents the 15 experiments performed for the Box-Behnken experimental design along with the obtained experimental results. The fit of the model was evaluated by analysis of variance (ANOVA). **Table 4.5** shows the regression coefficient and the statistical parameters obtained for the model. The measured statistical are determination coefficient (R^2), Student's t-test for statistical significance and Fisher's F test for the models' statistical significance. Using the multiple regression analysis of the experimental data a second-order polynomial equations were calculated for the extraction yield (**Equation 4.7**) and the TPC (**Equation 4.8**).

$$Yield=13.37+1.05x_1+0.68x_2+0.68x_3-0.50x_1^2-0.12x_1x_2+1.03x_1x_3+0.35x_2^2-0.32x_2x_3+0.05x_3^2 \quad (4.7)$$

$$TPC=594.96-0.06x_1+0.82x_2-6.39x_3+0.35x_1^2+2.53x_1x_2+9.39x_1x_3+6.59x_2^2+5.35x_2x_3-2.99x_3^2 \quad (4.8)$$

Table 4.4 Tested operational conditions expressed in terms of dimensionless and dimensional independent variables (X_1 (extraction time, sec), X_2 (microwave power, W) and X_3 (Ultrasound amplitude, %)) and their responses.

Nº Exp	X_1	X_2	X_3	Yield (%)	TPC (mg GAE/g DBE)
1	0 (75)	0 (200)	0 (50)	12.94	597.08
2	-1 (30)	0 (200)	-1 (0)	12.25	613.36
3	0 (75)	-1 (100)	1 (100)	14.57	571.95
4	1 (120)	0 (200)	1 (100)	15.64	590.06
5	-1 (30)	1 (300)	0 (50)	13.42	581.01
6	0 (75)	-1 (100)	-1 (0)	11.68	610.71
7	1 (120)	0 (200)	-1 (0)	13.13	568.79
8	0 (75)	0 (200)	0 (50)	13.07	573.11
9	1 (120)	-1 (100)	0 (50)	13.26	617.72
10	-1 (30)	0 (200)	1 (100)	10.66	597.06
11	0 (75)	0 (200)	0 (50)	14.11	614.70
12	1 (120)	1 (300)	0 (50)	14.44	611.61
13	-1 (30)	-1 (100)	0 (50)	11.76	597.26
14	0 (75)	1 (300)	-1 (0)	13.62	614.48
15	0 (75)	1 (300)	1 (100)	15.22	597.12

DBE: dried bark extract

Table 4.5 Regression coefficients and statistical parameters measuring the correlation and significance of the models.

Coefficient	Value for extraction yield	Value for TPC
b_o	13.37[a]	594.96[a]
b_1	1.05[b]	-0.06
b_2	0.68[c]	0.82
b_3	0.68[c]	-6.39
b_{12}	-0.12	2.53
b_{13}	1.03[c]	9.39
b_{23}	-0.32	5.35
b_{11}	-0.50	0.35
b_{22}	0.35	6.59
b_{33}	0.05	-2.99
R^2	0.854	0.507
F-exp	3.24	0.19
Significance level (%)	89.57	1.60

[a] Significant coefficients at the 99% confidence level.
[b] Significant coefficients at the 95% confidence level.
[c] Significant coefficients at the 90% confidence level.

4.4.1.1 Analysis of the model generated for the maximisation of the extraction yield

The results showed that the extraction yield varied between 10.66% and 15.64%, experiments 10 and 4, respectively (**Table 4.4**). This indicates that the treatment conditions greatly influenced the extraction yield, with a variability higher than 30%.

The variables that had a significant influence on extraction yield were the linear effect of the three independent variables and the effect of interaction between extraction time and ultrasound amplitude (**Table 4.5**). The interaction between extraction time and microwave power, and also microwave power and ultrasound amplitude did not have a significant effect on the response, as it can be seen in **Table 4.5** and **Equation 4.7**. Our results cannot be properly compared with other studies because as far as we know, there are no SMUAE for tree bark extractions. But comparing them with the results obtained by Jha et al. for black rice husk at a sequential US and MW extraction, it can be said that our results are in agreement with theirs results in regards to the importance of the extraction time [4.19]. Contrasting the results with the SMUAE studied by Luo et al. for walnut flour, there is a coincidence in the importance that microwave power and extraction time have, and also in the significance of the interaction of US and extraction time [4.5].

The interaction effects of MW power and US amplitude in the extraction yield for a fixed middle point value of extraction time 75 sec ($X_1 = 0$) is showed in **Figure 4.3a**. In this plot, it can be noticed that the maximum extraction yield was achieved for the maximum MW power and US amplitude. Nevertheless, for the maximum value of US amplitude and with 100 W of MW power the obtained extraction yield was high. In addition, it

was observed that for low MW power the effect of the US amplitude is large, while when the maximum power of MW was used the effect of the US is reduced. In the graph, it can also be seen the influence of the MW power looking at the part where the US amplitude is set in 0%. It is bserved that the increase in the extraction yield has an almost linear growth of up to 2% of extraction yield, but without reaching the maximum.

Figure 4.3b allows to visualise the interaction between extraction time and US amplitude keeping the microwave power constant at 200 W ($X_2 = 0$). This relation has the highest significance level, so is the relation with the greatest influence on the optimisation. As it can be seen, with the shortest extraction time the influence of the US is low. This could be due to the lack of time for cell disruption that should be generated as a result of the application of US. Moreover, when the amplitude of the US is the maximum, the extraction yield is proportional with the extraction time and US amplitude. This increase is close to 4% of extraction yield and is generated by increasing the extraction time at the maximum US amplitude. It confirms the hypothesis that it takes a minimum time to have an efficient cell disruption.

In **Figure 4.3c** can be seen the response surface in function of extraction time and MW power for a constant value of US amplitude ($X_3 = 0$). It can be noted, that for the minimum MW power (100 W) and maximum extraction time (120 sec) the obtained extraction yield is high, and it is improved raising the MW power. It can be also seen how there is a large increase, greater than 1% of extraction yield, when the minimum time was used and the MW power is increased from 100 to 300 W. Finally, on the plot it can be seen how, unlike US amplitude, the MW power does affect the extraction yield at lower times.

The R^2 value for the extraction yield response was 0.854, which illustrates the competence of the model. Taking into account all the analysed results, it can be concluded that the bark extraction yield is enhanced by the use of SMUAE.

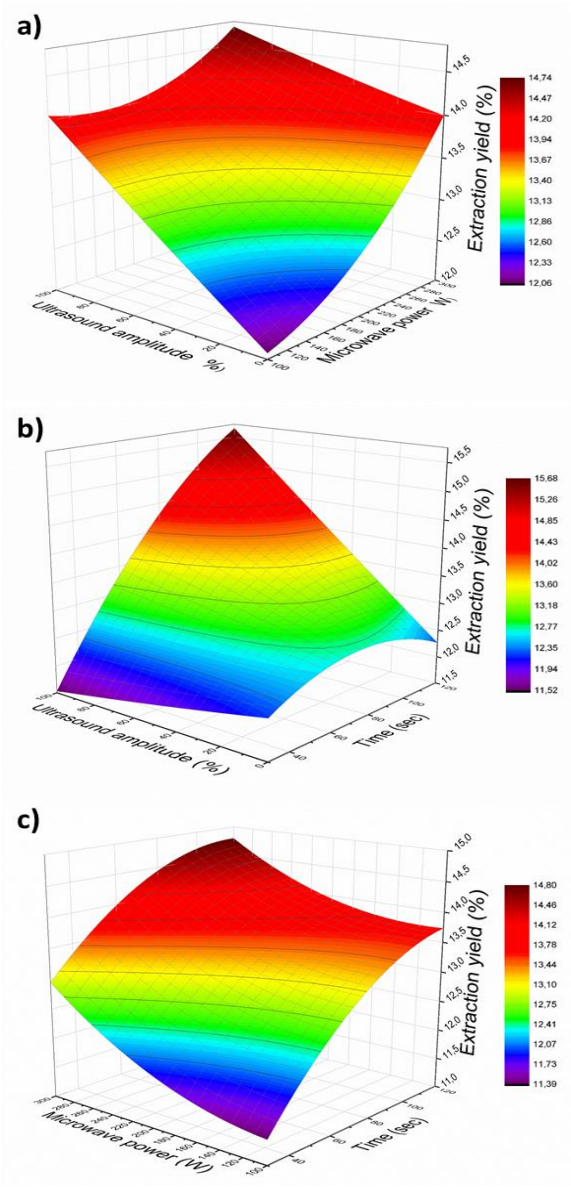


Figure 4.3 Response surface plots for extraction yield. (a) Microwave power and Ultrasound amplitude at a fixed extraction time (X_1 = 0); (b) Extraction time and Ultrasound amplitude at a fixed microwave power (X_2 = 0); (c) Microwave power and extraction time at a fixed ultrasound amplitude (X_3 = 0).

4.4.1.2 Analysis of the model generated for the maximisation of the TPC

The values measured for TPC varied between 571.95 and 617.72 mg GAE/g dried bark extract, experiments 3 and 9, respectively (see **Table 4.4**). This corresponds to less than 8% of variability, which indicates that the treatment conditions do not have a significant influence. In addition, the obtained results are in the range of the one measured in the characterisation (**Table 2.3, Chapter 2**). A more in-depth study of the model shows that none of the variables studied had a significant influence on the TPC. It can be observed in **Table 4.5**, where none of the regression coefficients are labelled as significant according to the statistical analysis. In addition, the determination coefficient obtained was 0.507, which indicates the lack of suitability of the model.

Figure 4.4 presents the interactions between the different variables in TPC. The **Figure 4.4a** and **Figure 4.4b**, show that the best TPC values are reached in the absence of US. However, it can also be seen that high TPC values are obtained when maximum US amplitude is applied. This suggests that there is no direct relation between the application of the US and the TPC. In the **Figure 4.4c**, where the US amplitude value is kept fixed, it can be seen that neither the MW power nor the extraction time affect the TPC in a proportional way, since both extremes (the maximum and the minimum) reported the best TPC values. These trends are not consistent, since in none of the studied cases does the variability between the maximum and minimum value exceed 6%. This variability value is close to the value accepted as an experimental error, 4%. Thus, the lack of influence of the variables is once again demonstrated.

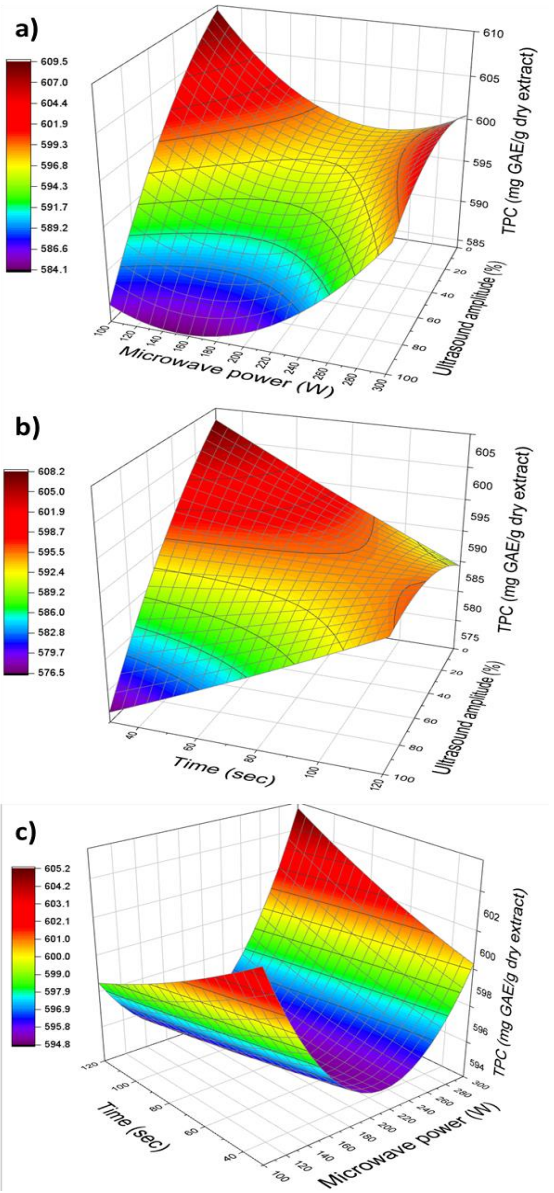


Figure 4.4 Response surface plots for TPC. (a) Microwave power and Ultrasound amplitude at a fixed extraction time ($X_1 = 0$); (b) Extraction time and Ultrasound amplitude at a fixed microwave power ($X_2 = 0$); (c) Microwave power and extractin time at a fixed ultrasound amplitude ($X_3 = 0$).

Considering these facts, it can be concluded that the parameters studied in this work do not have a direct effect on TPC of the extracts. Even if it is true that the TPC is not influenced by the studied variables, the yield does.

Therefore, if the yield increases and the TPC remains unaltered, this results in an increase in the amount of extracted phenolic compounds. Thus, with yield optimisation, both objectives are reached. Therefore, the discussion of the results of the TPC optimisation was finally not taken into account.

4.4.1.3 Optimisation of extraction conditions and validation of the model

The optimisation of SMUAE to achieve the maximum extraction yield was carried out using Statgraphics Centurion XV.II software. TPC was not considered in that optimisation due to the reasons explained above. The model predicted the maximum extraction yield (16.25%), which correspond nearly to the highest extraction time (119.95 sec), MW power (300 W) and US amplitude (99.68%).

To validate the model, three experiments were performed under the optimum conditions. The adequacy of the model for quantitative predictions was validated by the successful agreement between the measure and the predicted value. The experimental mean value of extraction yield was 15.72 ± 0.08%, which was close to the predicted value of 16.25% from the model. This fact confirms the suitability of the response surface methodology.

4.4.2 Study of the improvement of extraction yield using SMUAE

Looking at the extraction yield obtained for the optimum point it can be concluded that the obtained yield was close to the total extractive content determined by sequential extraction for the characterisation of the raw material (**Table 2.2, Chapter 2**). Thus, the suitability of the extraction method as well as the high content of extracts of the bark is confirmed.

Comparing the value obtained for SMUAE with that obtained using conventional extraction (CE) method (see **Table 4.6**); it is observed that the extraction yield value is almost the double, indicating a considerable improvement. This increase is very good, extracting around 75% of the total extractive content of the raw material.

Table 4.6 Comparison of extraction yield obtained at the optimum point for the different extraction methods.

Extraction method	Yield (%)
CE	8.24 ± 0.52^{a}
UAE	6.13 ± 0.41^{b}
MAE	8.25 ± 0.20^{a}
SMUAE	15.72 ± 0.08^{c}

The values were average ± SD (n = 3). Superscript letters depict significant differences (Tukery test, $p < 0.05$).

Table 4.6 presents a comparison between the extractions yields obtained in **Chapter 3**, with those obtained by SMUAE. It is evident that there are significant differences ($p > 0.05$) between SMUAE and the rest of the used extraction methods, being the SMUAE extraction yield the highest of all cases. It also improves the values of other studies carried out with MAE for maritime pine bark and spruce bark [4.29, 4.30]. Therefore, it can be concluded that the use of both techniques simultaneously improves the extraction yield. This is due to the synergetic effect induced by the simultaneous use of MW and US irradiation. As a result of this effect, the reaction time is considerably reduced by the use of a single operation unit, thus complying with one of the principles of green extraction [4.31].

4.4.3 Characterisation of pine bark extract at optimal conditions

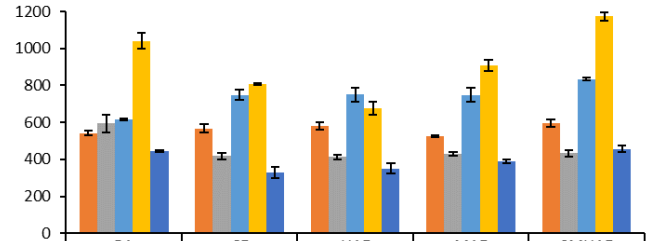
Once the positive effect of the use of SMUAE as an extraction method has been demonstrated, it is necessary to determine if it has any effect on the characteristics of the extracts. For this purpose, and keeping in mind that the aim is to obtain biomolecules selectively, more detailed characterisation of the obtained pine bark extracts was performed.

4.4.3.1 Total phenolic content (TPC) and Total flavonoid content (TFC)

TPC and TFC for SMUAE extracts are presented in **Figure 4.5**, along with the values reported for CE, UAE and MAE extract in **Chapter 3**, and the values reported for bark characterisation in **Chapter 2**. The TPC value is higher than that obtained for the characterisation of pine bark extracts, as well as for the rest of the pine bark extracts obtained in **Chapter 3**. The biggest difference is with MAE extract. Nevertheless, the differences are not significant for all the values measured for this raw material. The obtained value for SMUAE is higher than that reported for maritime pine bark by Chupin et al. [4.29]. The maximum value obtained in this work using MAE is 306 ± 33 mg GAE/g extract, far below the value that have been determined here by combining MW and US.

As in the case of the TPC, there is no improvement for the TFC with respect to conventional extraction, as seen in **Figure 4.5**. Furthermore, the value obtained is below the total potential of the bark, which was measured in the characterisation of the raw material. However, this is better than those reported in other works. Comparing these results with those obtained by Chupin et al. for the bark of maritime pine [4.29], it can be concluded that

a better TFC is achieved, being the value obtained of MAE of the maritime pine bark 403 ± 42 mg GAE/ dry plant.



	RA	CE	UAE	MAE	SMUAE
TPC	542.41	567.42	578.70	524.94	593.40
TFC	593.00	417.10	411.84	429.78	433.07
DPPH	616.95	749.08	749.58	747.82	833.94
ABT	1040.32	807.13	676.65	906.24	1172.59
FRAP	444.18	329.52	350.74	389.55	456.60

Figure 4.5 Comparison of the characterisation of different pine bark extracts; RA: raw material characterisation, CE: Extract obtained by CE; UAE: Extract obtained by UAE; MAE: Extract obtained by MAE; SMUAE: Extract obtained by SMUAE.

Taking into account all the above, it can be concluded that the TPC and the TFC seem not to be greatly influenced by the extraction method used for this pine bark. This can indicate that the SMUAE, at the optimised conditions, does not degrade the extracts.

4.4.3.2 Antioxidant capacity

Figure 4.5 presents a comparative summary of the results obtained for the analysis of the antioxidant capacities of the extracts. The values obtained with SMUAE for the three antioxidant capacities under study (DPPH, ABTS and FRAP) are higher than those obtained by CE method as well as for the characterisation of the extracts of raw material, which confirms the technique's potential. The obtained scavenging capacity against the radical DPPH was close to 100 mg TE/g dried bark extract higher than the value obtained for CE. In the case of ABTS, the results obtained were better, since the value obtained for SMUAE increased respect to CE by more than a third of the total value obtained for CE. Finally, in the case of FRAP, the increase

in the value obtained for SMUAE is of the same magnitude as that given for ABTS.

Comparing the results reported for DPPH by MAE and UAE with the one measured by SMUAE, higher antioxidant capacity is observed. Both results obtained previously for MAE and UAE have quite similar results to each other (≈ 748 and 750 mg TE/g dried bark extract, respectively). The value obtained in this study exceeds it by more than 100 mg TE/g dried bark extract. The values for ABTS and FRAP of the extracts obtained through MAE and UAE are also lower than the values calculated for SMUAE. In the case of ABTS, the results reported for MAE and UAE are considerably lower than those obtained for SMUAE. The values reported for FRAP (MAE: ≈ 390 mg TE/g dried bark extract: UAE: ≈ 351 mg TE/g dried bark extract) are not so different from that obtained by SMUAE, although they also remain lower.

There are few results in the literature regarding the antioxidant capacity of these types of extractions performed to tree barks, and in general, the only one used is DPPH. Due to that, the comparison of the results with other literature data is not easy, and it should be done carefully. Comparing the results with those of another raw material, in this case *Morus nigra* leaves, it can be seen that the one obtained by SMUAE is considerably higher. Radojković et al. reported a range of values for DPPH between 11 and 18 mg TE/g dried plant [4.32], while the value obtained for SMUAE (123 ± 2 mg TE/g dried bark) can be up to 10 times greater. Zoumpoulakis et al. reported 22.83 mg TE/g dried extract for the ABTS antioxidant capacity of the commercial antioxidant BHT, which is 18 times lower than that obtained for the SMUAE extracts [4.33].

The evidence of antioxidant activity makes the obtained products suitable for use against oxidation and degradation in a variety of applications. It can

be concluded that using SMUAE the antioxidant capacity of the extracts is improved, showing the potential use of pine bark as a promising antioxidant source in different industries such as agri-food, pharmaceutical and cosmetic among others. The use of antioxidant compounds in sunscreens improves their properties [4.34], and they can also be used in the food industry to protect against food degradation [4.35, 4.36]. However, the use of the compounds obtained in this work in food must be studied in more detail prior to their use, particularly concerning their toxic effects, interaction with the food and their effect on organoleptic properties of food.

4.4.3.3 Structural characterisation of the SMUAE pine bark extract

Figure 4.6a illustrates the molecular weight (Mw) distribution of the extract obtained from pine bark under the optimal condition. As can be seen in that figure, the extract consisted of a heterogeneous mixture of compounds divided into different Mw fractions. The difference in Mw may be due to a difference in the degree of polymerisation of the compounds in the extract [4.37].

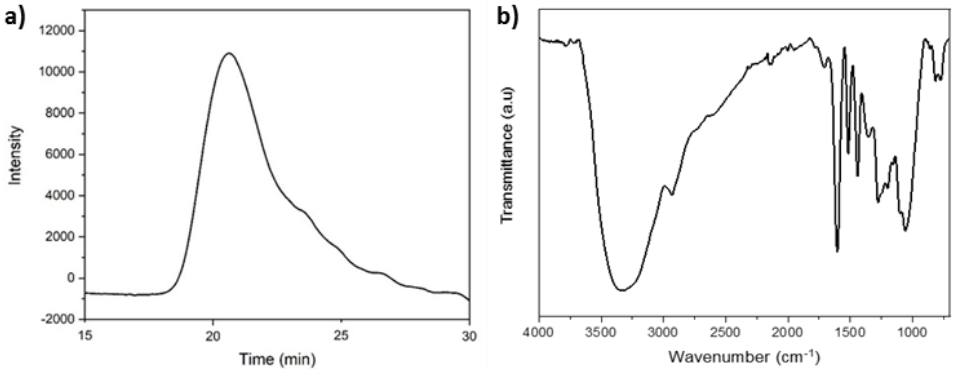


Figure 4.6 a) GPC chromatogram of EtOH/H_2O bark extract under optimal conditions. b) ATR-FTIR spectra of EtOH/H_2O bark extract obtained under optimal conditions.

As it can be seen in **Table 4.7**, the global polydispersity index of the extract is far from 1. This is because there are considerable differences in the Mw of the compounds present in the extracts. Since the extractions are carried out at low temperature, in the extract it can be found from monomers and dimers of low Mw, to oligomers and high Mw flavonoids. More than the 82% of the compounds have a Mw of 20,446 g/mol, which it means that the degree of polymerisation is high. However, the rest of the compounds have a much smaller Mw, below 1000 g/mol. This distribution is in the same range as the ones reported in **Chapter 2** for the characterisation of extracts from different tree barks. In **Chapter 2**, extracts of up to 6 different barks are characterised, of which 4 have a global Mw similar or higher than that obtained in this study. The sweet chestnut has the highest global Mw (57,387 g/mol), while the northern red oak, the common oak and the Iberian withe birch have it lower (17,211 g/mol; 20,288 g/mol and 30,972 g/mol, respectively). In the case of the sweet chestnut and the Iberian white birch, they also have less than 20% of the compounds with Mw below 1000 g/mol.

Table 4.7 Percentage, average Mw, number average (Mn) and polydispersity index (Mw/Mn) of EtOH/H_2O bark extract under optimal conditions.

					Global average		
	Percentage	**Mw (g/mol)**	**Mn (g/mol)**	**Mw/Mn**	**Mw (g/mol)**	**Mn (g/mol)**	**Mw/Mn**
Pine	82.21	20,446	8,221	2.49	16,939	2,287	7.41
	10.26	1,016	933	1.09			
	5.97	392	370	1.06			
	1.57	237	237	1.00			

The comparison of the data in **Table 4.7** with those reported for the extracts obtained using CE, UAE, MAE (**Table 3.15, Chapter 3**) indicates that the average Mw is different according to the method of extraction. The extracts

obtained by SMUAE have lower average Mw than those obtained using UAE and MAE. This may be due to the fact that the application of both techniques simultaneously facilitates the disruption of bonds decreasing the polymerisation degree of the compounds. These extracts also have the lowest polydispersity index. However, more than 78% of the compounds present in all the extracts studied have a Mw greater than 1,000 g/mol, which confirms the high degree of polymerisation of the compounds.

The analysis of the structure of the extract compounds continues with the study of the types of bonds using ATR-FTIR. The spectra of the extract are presented in the **Figure 4.6b**. The band assignation is relying on the assignments given by Boeriu, Ping, Soto and Chupin [4.29, 4.38–4.40].

According to the band assignment, the bands with wavelengths smaller than 900 cm^{-1} are assigned to –CH stretch vibration. Aromatic –CH bending in-plane vibration is detected at 1105 cm^{-1}. The bands 1200 cm^{-1} and 1050 cm^{-1} can be attributed to C-O stretching vibration. The peak 1275 cm^{-1} is assigned to a C-O-C asymmetric stretch vibration [4.43]. The bands at 1605 cm^{-1}, 1515 cm^{-1} and 1440 cm^{-1} are originated from aromatic skeleton vibration. 1440 cm^{-1} also correspond to –CH deformation. The conjugated carbonyl-carbonyl stretching is detected at 1705 cm^{-1}. The peak at 2925 cm^{-1} is identified as –CH stretch vibration in aromatic methoxy groups and in methyl and methylene groups of side chains. Finally, the band at 3300 cm^{-1} is attributed to –OH stretch vibration in phenolic and aliphatic structure.

There are no major differences compared to the ATR-FTIRs of the extracts obtained using CE, UAE, MAE (**Chapter 3**). Nevertheless, it should be noted that the extracts obtained using SMUAE do not have the band identified as $-CH_3$, CH_2 stretching vibration, at 2973 cm^{-1}. In addition, the peak at 1705 cm^{-1} is well defined, as it is in the case of CE extracts. Finally, the band at

1105 cm^{-1} is similar to the one reported for UAE, with a higher intensity than for the CE and MAE extracts.

All in all, the band corresponding to –OH in phenolic and aliphatic structure is very big, as happens for the rest of the extracts, which confirmed the high activity of the sample measured by antioxidant capacities. The peaks assigned to different aromatic structure vibration ratified the high content on phenolic compounds of the extract.

4.4.3.4 Compounds identification by UPLC-DAD-ESI-MS

The analysis carried out by UPLC-DAD-ESI-MS confirmed the content of phenolic and polyphenolic compounds in the EtOH/H_2O pine bark extract. The UV chromatograms of these extractives permitted the identification of 14 peaks corresponding to different co-elutions of some compounds (**Figure 4.7**). The tentative identification of the compounds present in the different peaks was done based on the literature (**Table 4.8**). In general, each of the peaks are a mixture of different compounds, which are co-eluting, because they are very close in their structure and polarity, so its separation is difficult. It makes difficult the compounds identification, which is interesting for a future study.

All the compounds identified in **Table 4.8** are phenolics and polyphenolics. A high presence of flavonoid compounds is found, as suggested by the high TFC measured in the characterisation (**Figure 4.5**). The presence of dimers and trimers is also confirmed, demonstrating the degree of polymerisation discussed in the HPSEC analysis of the extracts.

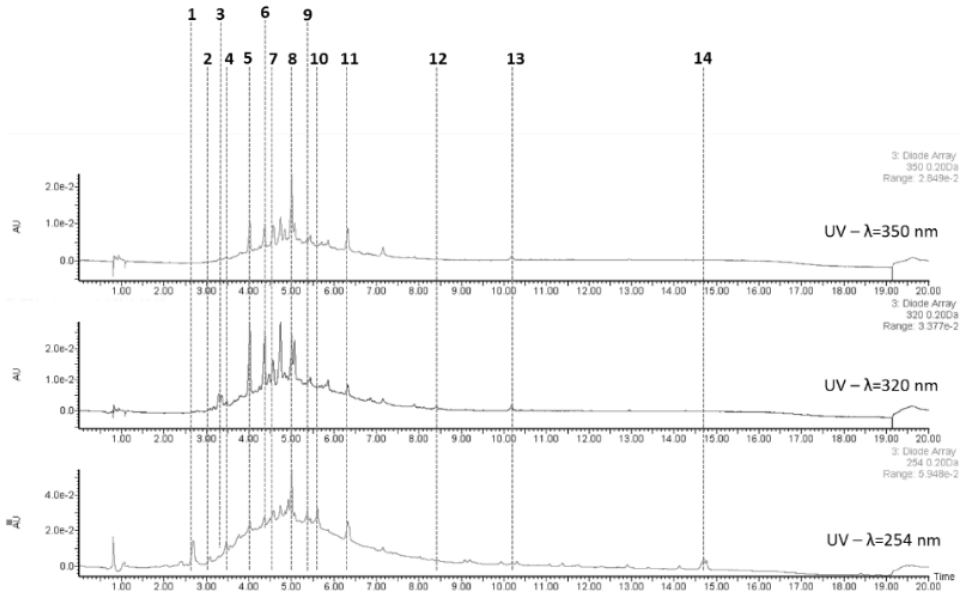


Figure 4.7 UV and MS chromatograms of the EtOH/H_2O bark extract obtained under optimal conditions analysed by UPLC-DAD-ESI-MS.

Comparing the results reported here for the SMUAE extracts with those reported in **Chapter 3**, it can be seen that the compounds identified in all cases are similar. However, the SMUAE extracts have fewer compounds with m/z less than 400. This suggests a higher degree of polymerisation, which may be due to the short extraction time. The extraction yield is higher than those obtained with CE, UAE and MAE, but there is a lack of small molecules (precursors), which suggests that the applied method facilitates the extraction without breaking the intermolecular bonds (without depolymerisation). Thus, the synergic effect of the simultaneous use of both techniques on the cell wall of the lignocellulosic biomass is confirmed.

Table 4.8 Tentative identification of the compounds of the extracts in the UPLC-DAD-ESI-MS spectra of the eluted peaks during the UPLC analysis of the EtOH/H_2O bark extracts obtained by SMUAE.

Peak	t_R (min)	$[M-H]^-$	Identification	Ref.
1	2.7	169	Gallic acid	[4.42]
		609	(Epi)gallocatechin dimer	[4.43]
2	3.1	577	Procyanidin dimer isomer 1	[4.44]
3	3.25	577	Procyanidin dimer isomer 2	[4.44]
4	3.4	289	Catechin	[4.45]
		865	Procyanidin trimer	[4.45]
5	4	405	Junipediol A 8-glucoside or nikoenoside	[4.46]
		577	Procyanidin dimer isomer 3	[4.44]
6	4.55	463	Quercetin derivative 1	[4.44]
		573	Kaempferol derivative 1	[4.45]
7	4.36	863	(epi)afzelechin derivative	[4.47]
8	5	446	Quercetin-hexoside	[4.45]
		575	Catechin derivative 1	[4.47]
9	5.35	573	Kaempferol derivative 2	[4.45]
10	5.55	505	Quercetin derivative 2	[4.48]
11	6.3	541	Larixinol	[4.45]
12	8.4	505	Quercetin derivative 3	[4.45]
		426	Catechin derivative 2	[4.46]
13	10.2	293	Oxo-octadecadienoic acid	[4.46]
14	14.7	301	Quercetin	[4.46]

4.5 Conclusion

The optimisation of the SMUAE with a Box-Behnken design was successfully carried out. The results of this optimisation proved that the interaction between the extraction time and US amplitude had the greatest impact on the extraction yield. Although it has not been possible to optimise the total phenolic content due to its low variability, the optimisation of the extraction yield has been carried out correctly. The value predicted by the

model was consequent with the experimental value, and it is considerably larger than the one obtained by CE, UAE and MAE. Although the optimisation of the TPC was not realised, by optimising the extraction yield a greater quantity of extracts is obtained, which they have an unaffected TPC, so the quantity of extracted phenolic compounds is also higher.

The characterisation of the extract obtained under the optimal conditions showed that the extract has a high content not only in phenolic compounds, but also in flavonoids, which was confirmed by the analysis of the UPLC-DAD-ESI-MS. The content of high Mw compounds is also confirmed by HPSEC, since it is observed that the average Mw is large. The antioxidant capacities were improved significantly compared to the previously obtained pine bark extracts (CE, UAE, MAE). In conclusion, these extracts are more biologically active, which is very interesting for different applications in fields as varied as food industry, cosmetic or bio-based materials.

In addition, the comparison of the extract antioxidant capacities obtained using SMUAE with those measured for pine characterisation demonstrates an improvement in the results. Therefore, it is concluded that this method is an effective technique to get the maximum benefit from this raw material.

Taking all the above into account, it can be concluded that SMUAE is a very good extraction method not only for the extraction of good quality extracts, but also for the reduction of extraction time, which is reduced by 47 times. The results obtained in this research confirm the improvement of the competitiveness of the wood industry due to the possibility of using the bark as a natural source of bioactive compounds and the generation of economic value from a waste through the use of sustainable innovative extraction technique.

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Chapter 5

Selective extraction of flavonoid compounds

5.1 Introduction

Tree barks are an important source of phenolic compounds, as has been demonstrated throughout this study. Nevertheless, the extraction and separation of the different compounds is not an easy task, mainly because of the complexity of the lignocellulosic material's structure [5.1]. As a result, the selection of a sustainable extraction process becomes very important.

Although it is true that tree bark is rich in extractives, especially in comparison with wood, this value normally does not overcome the 30% in weight of the bark [5.2]. Moreover, this fraction is composed of a large variety of different compounds [5.3], which means that the concentration of interesting compounds is small. Therefore, in order to make the extraction process efficient, it is necessary to choose not only the proper extraction technique, but also the most selective solvent. The use of selective solvents allows the exclusive extraction of some types of compounds, reducing the subsequent purification stages.

Ionic liquids (ILs) and deep eutectic solvents (DES) are becoming one of the most popular solvents based on their specific properties. Which make them both to be considered as green and designer solvents [5.4]. Both families of compounds are very large since there are many combination possibilities. This allows the adaptation of properties such as viscosity, polarity, melting point and solubility [5.5] in order to facilitate and optimise the extraction of the target compounds [5.4].

5.1.1 Flavonoid compounds

Flavonoids are becoming popular as part of bark extractives because they are bioactive compounds, which means that they are capable to modulate

different biological activities [5.6]. As a result of this property, flavonoids have a great amount of benefits, such as antioxidant, anti-allergic, anti-inflammatory and vasoprotective properties, among others [5.7]. For that reason, their application in different fields is increasing, from the food industry to personal care industry and even in the creation of new bio-based materials [5.8–5.10].

Flavonoids are phenolic compounds constituted by two aromatic rings joined by a three atoms carbon unit, C6-C3-C6 [5.11]. Due to their skeleton, the flavonoid family has a great chemical diversity, so this family is divided into 6 sub-groups that mostly are present as glycosides in plants: flavonols, flavones, isoflavones, flavanones, chalcones and anthocyanins (see **Figure 5.1**) [5.12]. Moreover, these compounds are also rich in phenolic hydroxyl groups.

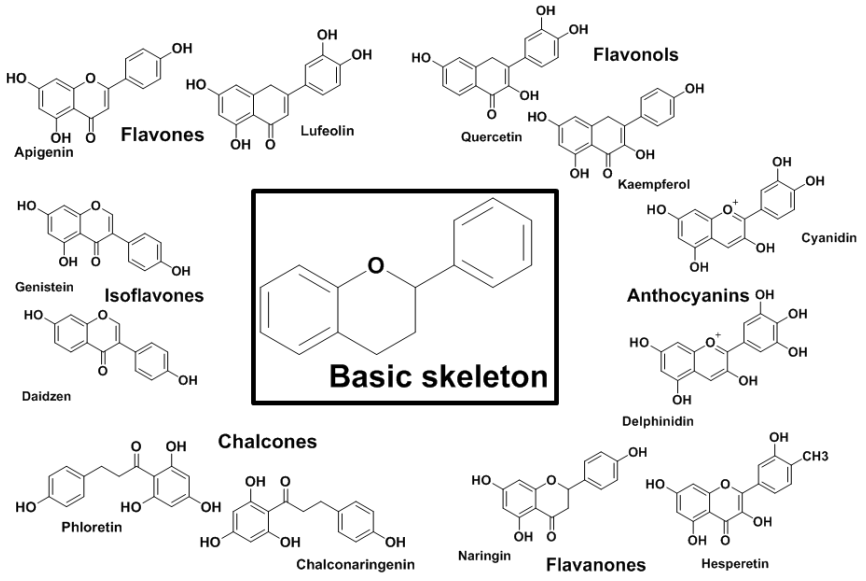

Figure 5.1 Flavonoid sub-groups and its basic structure.

One of the most important properties of flavonoid compounds is their antioxidant capacity. The oxidation process of the biological systems is

based on an excess of free radicals with at least one electron missing in the outer orbit, usually called reactive oxygen species (ROS). These species are very reactive and produce degradation by oxidation. The oxidative stress caused by ROS plays an important role in the development of different diseases, including cardiovascular and neurodegenerative diseases, cancer and diabetes [5.13]. This property is mainly a result of the presence of phenolic hydroxyl groups as well as the conjugated aromatic system [5.14]. Thus, antioxidant capacity prevents the oxidation process, which allows the protection of other compounds, such as lipids, DNA and proteins of the biological systems [5.13].

Conventional or traditional methods are the most exploited technologies for the extraction of polyphenolic compounds from plants, but they required large amount of solvent and energy [5.15, 5.16]. Therefore, with the aim of improving the extraction, in recent years both the industry and the academic community are studying the intensification of the processes through the use of new techniques [5.17]. However, although these new techniques permit the reduction in extraction time, solvent and energy consumption, as well as the improvement of extraction yield [5.18], it is true that there is still a lot to do in order to fulfil the principles of green chemistry. **Table 5.1** lists some examples of the different techniques used for the extraction of phenolic compounds from bark.

The most commonly used solvents are volatile organic compounds (VOCs), which generate a large impact on the climate change and they can also put people's health at risk [5.19]. Due to their environmental impact and low selectivity, the use of new modern solvents for the extraction of polyphenolic compounds is being studied.

Table 5.1 Different extraction methods for bark extractive and their possible applications.

Tree specie	Extraction method	Application	Reference
Different Canadian forest species	CE (EtOH (95%))	Natural anti-agent	[5.20]
Prunus padus	CE (H_2O)	Natural antioxidative cosmetic agent	[5.21]
Eucalyptus nitens *Eucalyptus globulus*	CE (MeOH/H_2O)	Natural antifungal	[5.22]
Birch	Supercritical fluid extraction	Oleogel stabilizing agent	[5.23]
Alnus incana *Alnus glutinosa* *Salix caprea*	Pressurized liquid extraction (EtOH (40%))	Lipid oxidation stabiliser	[5.24]
Acacia mearnsii	CE (EtOH (80%))	Natural antidiabetic agent	[5.25]
Acacia mearnsii	CE (EtOH (50%))	Functional food additive	[5.26]
Catalpa speciosa *Taxus cuspidate* *Magnolia acuminata*	CE (MeOH)	Anticancer agent	[5.27]
Salix eleagnos	MAE (H_2O) UAE (H_2O)	Anti-inflammatory agent	[5.28]

CE: conventional extraction; MAE: Microwave assisted extraction; UAE: Ultrasound assisted extraction.

5.1.2 Use of ILs and DES for flavonoid extraction in biomass

ILs are solvents with unique properties, mainly due to their dislocated charge, they are considered salts with low melting points, most of them below 100 °C. These properties are adaptable thanks to the great variety of ILs that can be synthesised with only a small change in the cation or anion. DES share many of the same properties with ILs, but they differ mainly in

their chemical formation and starting compounds [5.29]. The possibility of synthesising this type of compounds with the required characteristics for each type of extraction makes them very attractive for their use as selective solvents.

These compounds were first used in organic chemistry and material synthesis [5.30]. Another possible use of these solvents that has been successfully studied in the las years is chromatography [5.31, 5.32]. This is due to their good separation characteristics, mainly because of their good physicochemical properties. Furthermore, the fact that their miscibility may be adjusted for different solvents, results in a wide range of possible applications of these compounds, both as part of the stationary and the mobile phase [5.32–5.34]. In the case of IL, thanks to their good thermal stability for working at high temperatures, there are already IL-coated capillary GC columns on the market [5.31].

However, in recent years the interest of their application for the treatment of biomass has increased. The fractionation of the lignocellulosic material is a challenge due to its complex structure. Therefore, with the aim of achieving a more sustainable fractionation, the use of more selective solvents is being researched. Several studies have been carried out for the solubilisation of the two main fractions of the lignocellulosic material, cellulose and lignin [5.35].

The increasing interest in the use of natural compounds in replacement of fossil fuel derivatives, leads to a considerable increase in the interest of the application of ILs and DES for the extraction of bioactive compounds from lignocellulosic biomass. This is demonstrated by the increase in related studies [5.4, 5.36, 5.37]. Among the different bioactive compounds belonging to the biomass extractive fraction, flavonoids are becoming very

relevant also in IL and DES fields. **Table 5.2** lists some of the latest works carried out for the selective extraction of flavonoid compounds from different raw materials using ILs and DES.

Table 5.2 Example of extraction of flavonoids from different biomass using ILs and DES.

Raw material	Extraction method	Solvent	Product	Reference
Leaves	UAE	Imidazole-based ILs	Flavonoids	[5.38]
Leaves and flowers	UAE	Imidazole-based ILs	Flavonols	[5.39]
Leaves	SMUAE	Imidazole-based ILs	Flavonols	[5.40]
Leaves	UAE	Imidazole-based ILs	Flavones	[5.41]
Sprouts	CE	ChCl based DES	Rutin	[5.42]
Flowers	UAE	ChCl based DES	Myricetin	[5.43]
Root	UAE	ChCl, proline and citric acid based DES	Flavones	[5.44]
Sprouts	UAE	ChCl based DES	Flavonoids	[5.45]

Wei et al. carried out a study of the selective extraction of flavonoids from both the leaves and the flowers of *Lysimachia clethroides* [5.39]. They proved the efficacy of four imidazole derivatives ILs using UAE, of which $[C_4C_1im][BF_4]$ was the one with the best extraction yield. Among the flavonoids extracted from the leaves, two were identified, isoquercetin and astragalin. Wang et al. conducted the optimisation of the extraction of flavonoid compounds from bamboo leaves, studying 15 imidazole-based ILs [5.41]. The optimisation of UAE was carried out with $[C_4C_1im][Br]$ because it was the IL with the highest yield of flavonoids. The total amount of flavonoids at the optimum point was higher than that obtained by CE with

EtOH (80%), 4.5 mg/g and 2.5 mg/g, respectively. Li et al. also determined [C_4C_1im][Br] as the best IL, in this case for the extraction of flavonoids from Seabuckthorn leaves [5.40]. In this work, apart from flavonoids, essential oil was also obtained thanks to the SMUAE process designed by the authors. This demonstrates the great potential of IL for the selective extraction of different compounds.

Besides ILs, DES are also being studied as possible solvents for the extraction of flavonoid compounds. Zhao et al. conducted a study with 20 different DES for the extraction of rutin from *Sophora japonica* buds [5.42]. All the studied DES were based on choline chloride (ChCl) and the one that obtained the best yield was ChCl:levulinic acid (1:2), with about 200 mg/g. This represents an improvement of between 50 and 75 mg/g in comparison with ethanolic and methanolic extracts, respectively. Mansur et al. also studied the extraction of rutin, but this time it was from buckwheat sprouts [5.45]. The optimisation of the extraction was carried out using UAE and nine different ChCl-based DES. ChCl:Triethylene glycol (1:4) with 20% of water was the solvent that obtained the best results with between 2 and 7 mg/g for each studied flavonoid at the optimal point.

Finally, it is interesting to mention the work done by Xiong et al. where, in addition to studying ChCl-based DES, they also studied other DES based on L-Proline and citric acid [5.44]. The study was carried out for the optimisation of the extraction of different flavonoids from *Radix scutellariae* using UAE. Among all the used solvents, L-Proline:glycerol (1:4) was in general the one with the highest flavonoids concentration. Baicalin in particular had very good yield, with a concentration of around 170 mg/g. A comparison of the results at the optimal point with the results of the extractions carried out with ethanol (70%), methanol or H_2O showed a

considerable improvement. With conventional solvents, the Baicalin concentrations were around 100 mg/g, while the extraction at the optimum point with DES was in the range of 151-176 mg/g, depending on the origin of the plant. Hence, the potential of these solvents for the selective fractionation of biomass is once again demonstrated.

The growing interest in flavonoids extraction from natural sources coupled with the enormous potential that ILs and DES have for the selective extraction of these compounds, results in a growing interest in this area. Therefore, knowing the excellent potential of tree bark as a natural source of flavonoids, the combination of both becomes necessary with the aim of a more sustainable extraction processes. It is important not to ignore the fact that the ILs and DES properties allow operating at lower temperatures with the consequent advantages that it can generate. From the protection of volatile compounds to the reduction of energy consumption.

5.2 Objective

The main objective of this chapter was to study the selective extraction of flavonoid compounds from pine bark using different ILs and DES, leaving aside the use of alcohols as solvent. The goal was to evaluate the influence of the selected solvent on the extraction yield, as well as on the composition of the extracts.

The second goal of this chapter was the comparison between the selected ILs and DES in order to select the best solvent for the selective extraction of flavonoids.

5.3 Materials and methods

For this purpose, extractions were carried out with the different solvent mixtures by conventional extraction (CE). All the used ILs and DES were previously synthesised in the laboratory. Then, the characterisations of both the solid and the liquid phases were performed in order to determine the selectivity of the extraction.

5.3.1 Synthesis of the ionic liquids (IL) and deep eutectic solvents (DES)

For the selective extraction of flavonoids, three ILs and two DES were selected based on previous literature study. The chosen solvents were $[C_4C_1im][Br]$, $[C_4C_1im][OAc]$ and $[C_4C_1im][BF_4]$ as ILs, and choline chloride:urea (1:2) and choline chloride: 1,4-butanediol (1:2) as DES. All the selected ILs have the same cation, so the influence of the anion was studied. In the case of DES, the effect of the hydrogen bond donor (HBD) was studied since the hydrogen bond acceptor (HBA) was the same in both solvents. All the selected solvents were specifically synthesised in the laboratory before their use in the extraction.

5.3.1.1 Synthesis of ILs

The synthesis of $[C_4C_1im][Br]$ (IL 1) was done following the method described by Brandt et al. with a slight modification [5.46]. Briefly, 90.00 g of 1-methylimidazole previously distilled was transferred into a 1 L two-neck round-bottomed flask. The reagent was then stirred and 70.00 g of acetonitrile was added. Later, previously distilled 1-bromobutane in excess (217.33 g) was added dropwise. Once the addition was finished, the mixture was heated to 75 °C and it was left at these conditions for 24 h. Then IL was

crystallised cooling down to -20 °C overnight. Finally, the IL was recrystallised with acetonitrile under nitrogen atmosphere, and it was dried and stored until it was used (96.15% yield).

The synthesis of the $[C_4C_1im][OAc]$ (IL 2) was carried out using a two steps method. The first step was performed in a 600 mL stainless steel 4545 Parr reactor with a 4848 Parr controller. 121.00 g of previously distilled N-butylimidazole was introduced into the reactor with 194 mL of MeOH. After that, dimethyl carbonate ($C_3H_6O_3$) in excess (263 g) was added and the mixture was heated to 140 °C, under mechanical stirring for 24 h. Once verified that the reaction was complete, the next stage of the synthesis was carried out. The entire mixture was transferred into a 2 L two-neck round-bottomed flask, and it was placed in an ice bath. Later, 58.86 g of acetic acid (CH_3COOH) was added dropwise, and then it was left stirring overnight. Finally, the solvent was removed with a rotary evaporator, and it was dried under vacuum overnight obtaining a pale yellow liquid with a yield of 94.59%.

$[C_4C_1im][BF_4]$ (IL 3) was synthesised by a metathesis following the method reported by Ab Rani et al. with the difference of the used starting reactive [5.47]. Briefly, sodium tetrafluoroborate ($NaBF_4$) (115.46 g) was added to a flask which contains $[C_4C_1im][Br]$ (222.31 g) in CH_2Cl_2 (250 mL) under N_2. The mixture was stirred under N_2, at room temperature for 24 h. Once the synthesis was completed, it was left to sediment, where a white precipitate (NaBr) appeared, which was separated from the IL by cannula filtration. In order to carry out the complete removal of the NaBr, the IL was washed twice with CH_2Cl_2 (2 × 50 ml). Once cleaned, the IL was filtered by acid and basic alumina, and finally it was dried in vacuum at 45 °C overnight, obtaining a colourless liquid with a yield of 71.63%.

5.3.1.2 Synthesis of DES

The same method was used for the synthesis of both DES. First of all, the reagents were dried under vacuum overnight. Then choline chloride (ChCl) was mixed up with HBD in the desired proportion. In this work, urea and 1,4-butanediol were used as HBD, both in a ratio of 1:2 (ChCl:HBD). Then, the mixture was heated at 80 °C under constant stirring for 2 h, where a clear and homogeneous liquid was obtained. The results were choline chloride:urea (DES 1) and choline chloride: 1,4-butanediol (DES 2), with a reaction yield close to 100% in both cases.

5.3.1.3 Characterisation of the ILs and DES

Before being used, the ILs and DES were characterised to verify their correct synthesis. For this purpose, ILs and DES were characterised by Attenuated Total reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR) and Nuclear Magnetic Resonance (NMR). All the solvents were characterised by ^{1}H-NMR and ^{13}C-NMR. The IL 3 was also subjected to ^{19}F-NMR to verify its structure. These techniques were performed following the methodology described in **Appendix V**.

5.3.1.4 Characterisation of the solvents

For the extractions, ILs and DES mixed with water in different proportions were used. The concentrations of IL and DES selected for all cases were 0%, 25%, 50% and 75% in weight. Once they were prepared, these solvents were characterised by measuring their pH, by a pH-meter (pH-2005 SELECTA). The measure was repeated twice and the results were expressed as mean ± SD.

The study of the polarity and the toxicity of the solvents was carried out based on the data found in the literature. The toxicity of water and ethanol is well known for all the scientific community, but in the case of the IL and DES, it is not so clear. For the analysis of water and ethanol as solvents, already published solvent guides were consulted (GSK and Pfizer solvent guides) as well as the guide published by Prat [5.48]. However, to understand the toxicity of the ILs and DES exhaustive search was carried out in literature to understand their properties. In this work, not only the final properties of the ILs and DES have been taken into account, but also the characteristics of the synthesis of these solvents. ILs and DES are often considered as "green-solvents", but there is a need to analyse the risk associated with their productions.

The polarity study was conducted using the most commonly used polarity scale, which is based on solvatochromism. In this work the multiparameter polarity scale developed by Kamlet and Taft was investigated, which is based on three solute-solvent interactions. The studied parameters are: polarisability, π^*; hydrogen bond accepting ability (basicity), β; and hydrogen bond donating ability (acidity), α. The three parameters are determined by a UV-Vis spectrophotometer using a different dye for each parameter, whose maximum absorbance is shifted in response to the surrounding molecule. The chosen reference studies employed the Reichardt´s dye, the N,N-diethyl-4-nitroaniline dye and the 4-nitroaniline dye for the determination of all the parameters [5.47, 5.49].

5.3.2 ILs and DES extractions

The extractions were carried out with several aqueous mixture of ILs and DES at different concentrations (0%, 25%, 50% and 75%) calculated by

weight. In order to study the influence of the used solvents in the extraction of flavonoid compounds, the selected extraction method was CE. The conditions used were the same as those previously optimised for CE with EtOH/H_2O (50/50 (v/v)) in **Chapter 3**. Briefly, 1 g of pine bark was weighted in 100 mL Erlenmeyer flask and 10 mL of the previously prepared solvent (SLR of 1/10 (w/v)) were added. The extraction was carried out under controlled temperature (65 °C) and constant shaking (120 rpm) in an orbital shaker with a heating module (Heidolph Unimax 1010 + Heidolph Incubator 1000) for 94 min. After the extraction time was over, the solid was separated from the liquid phase by vacuum filtration. Then, the solid phase was washed with distilled water to remove the solubilised extracts that could remain attached to the solid, as well as the IL or DES that could also be attached to it. Finally it was air dried. The extraction yield was gravimetrically calculated using the following equation (**Equation 5.1**):

$$Extraction\ yield\ (\%) = 100 - \left[\left(\frac{W_{dry\ solid\ without\ extracts}\ (g)\frac{(100-moisture(\%))}{100}}{W_{dry\ bark}(g)}\right) x\ 100\right] \qquad (5.1)$$

Once all the extractions were carried out, the results were compared. For this purpose, a statistical analysis was performed by one-way analysis of variance (ANOVA) with IBM SPSS Statistic 24 software. The study of the significance was done using Tukey's range test. The experiments were replicated three times, and the results were expresses as mean ± SD. The values of $p < 0.05$ were considered to be statistically significant.

5.3.3 Chemical characterisation of bark after extractions

The cleaned and air-dried solids were subjected to a quantitative acid hydrolysis (QAH) (NREL/TP-510-42618) to determine their lignin,

hemicelluloses and glucan content following the methodology described in the **Appendix III**.

5.3.4 Characterisation of bark extracts

The characterisation of the extracts was carried out directly on the liquid phase obtained after separation by filtration. This means that the characterised extracts were a mixture of water, IL or DES and extracted compounds. Since no separation of the extracts from IL or DES was carried out, the use of characterisation techniques that could be affected by the presence of these reagents was avoided.

The total flavonoid content (TFC) of the bark extracts obtained after the extractions were determined by aluminium chloride colorimetric assay following the procedures described in **Appendix IV**. The potential of the bark extracts was studied by measuring their antioxidant capacity using DPPH, ABTS and FRAP assays, which were conducted following the methodology described in **Appendix IV**. The equations of the calibration curves used for each characterisation are listed in **Table 5.3**.

Table 5.3 Calibration curves used for the measurement of TFC, DPPH, ABTS and FRAP.

Method	Calibration curve	R^2	Eq.
TFC	$[Catequin] = 0.1316 \cdot Abs - 0.0033$	0.999	(5.2)
DPPH	$[Trolox] = -0.1144 \cdot Abs + 0.0685$	0.999	(5.3)
ABTS	$[Trolox] = -0.9987 \cdot Abs + 0.6844$	0.991	(5.4)
FRAP	$[Trolox] = 0.1794 \cdot Abs - 0.0133$	0.999	(5.5)

Finally, in order to have a better comprehension of the structure of the extracts, the dried extracts were characterised by Attenuated Total

Reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) following the methodology described in **Appendix IV**.

5.4 Results and discussion

5.4.1 Characterisation of the synthesised ILs and DES

The syntheses were monitored by ^{1}H-NMR measurements. The analysis of the NMRs allowed to check the end of each stage of the synthesis process, as well as to identify the moment when the synthesis was completed for each of the ILs and DES. The characteristics of the ILs and DES synthesised in this work for the extraction of flavonoid compounds from the bark are detailed below.

Figure 5.2 shows the proton and carbon NMR of the IL 1. The structure band assignment was carried out according to the structural data of other authors [5.47, 5.50].

The bands of the ^{1}H-NMR spectra of IL 1 are assigned as follow; δ_H (270 MHz, DMSO-d^6)/ppm: 9.18 (1H, s, NCHN), 7.80 (2H, t, NCHCHN), 7.73 (2H, t, NCHCHN), 4.17 (2H, t, NCH_2CH_2), 3.86 (3H, s, NCH_3), 1.76 (2H, m, $CH_2CH_2CH_2$), 1.25 (2H, m, $CH_2CH_2CH_3$), 0.90 (3H, t, CH_2CH_3)

The bands of the ^{13}C-NMR spectra of IL 1 are assigned as follow; δ_C (68 MHz, DMSO-d^6)/PPM: 136.98 (s, NCHN), 124.07 (s, NCHCHN), 122.73 (s, NCHCHN), 48.94 (s, NCH_2CH_2), 36.23 (s, NCH_3), 31.83 (s, $CH_2CH_2CH_2$), 19.24 (s, $CH_2CH_2CH_3$), 13.76 (s, CH_2CH_3).

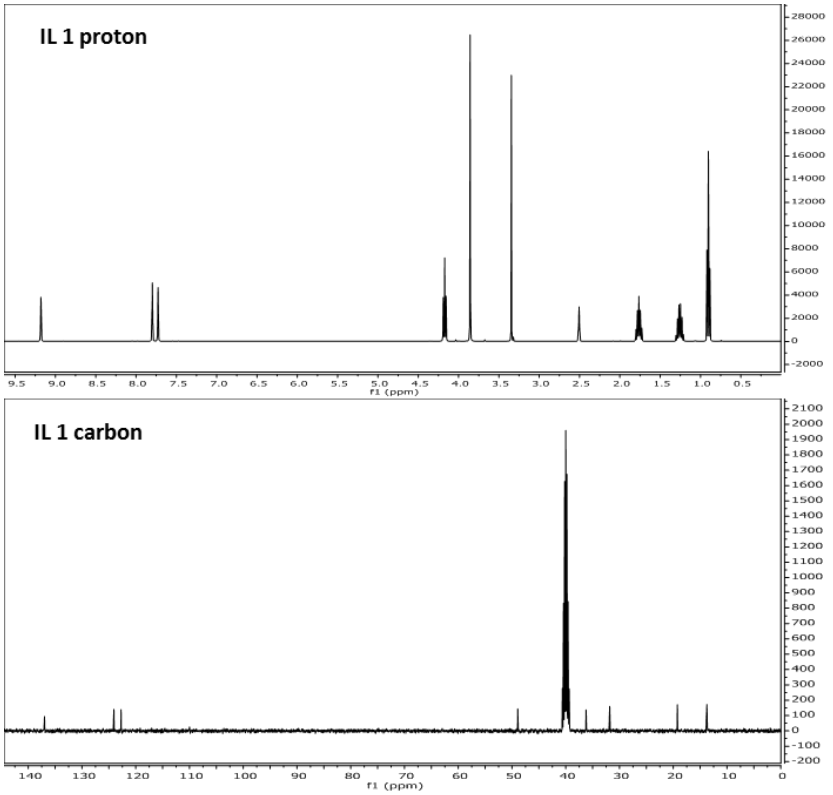

Figure 5.2 ^{1}H-NMR and ^{13}C-NMR of [C_4C_1im][Br] (IL 1).

The ^{1}H-NMR and ^{13}C-NMR spectra of the IL 2 are shown in **Figure 5.3**. The structure band assignment was performed according to the structural data of another author [5.50].

The bands of the ^{1}H-NMR spectra of IL 2 are assigned as follow; δ_H (270 MHz, DMSO-d^6)/ppm: 10.06 (1H, s, NCHN), 7.87 (2H, t, NCHCHN), 7.80 (2H, t, NCHCHN), 4.19 (2H, t, NCH_2CH_2), 3.88 (3H, s, NCH_3), 1.76 (2H, m, $CH_2CH_2CH_2$), 1.58 (3H,s, OCH_3),1.23 (2H, m, $CH_2CH_2CH_3$), 0.88 (3H, t, CH_2CH_3). In addition to this band, a small peak at 3.14 is also observed, indicating that there is a small impurity of N-butylimidazole because initially not all of the reagent had reacted.

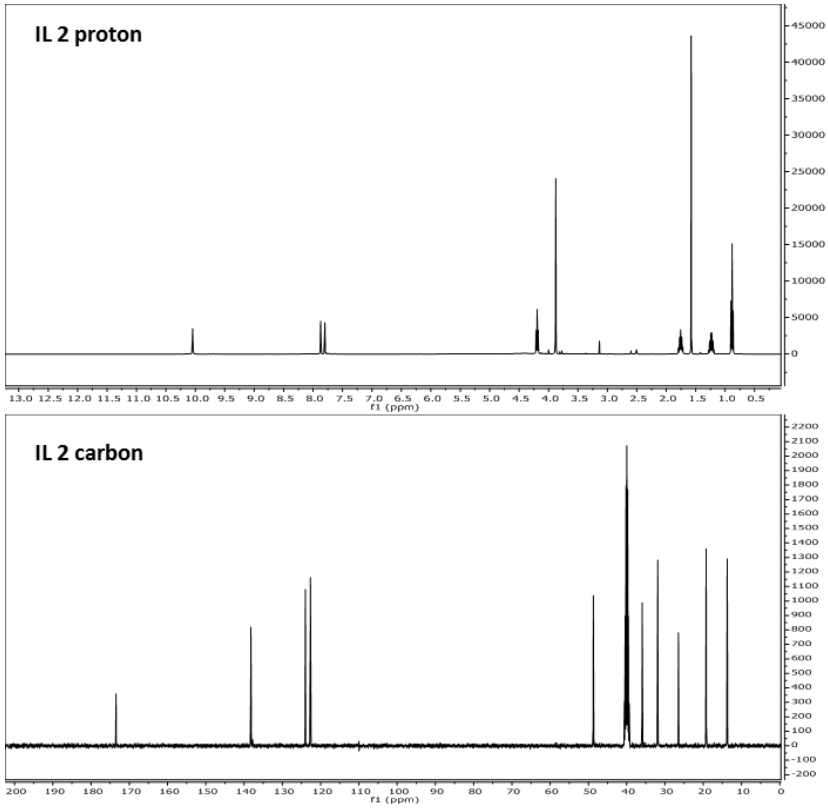

Figure 5.3 ^{1}H-NMR and ^{13}C-NMR of [C_4C_1im][OAc] (IL 2).

The bands of the ^{13}C-NMR spectra of IL 2 are assigned as follow; δ_C (68 MHz, DMSO-d^6)/ppm: 173.43 (s, OCH_3), 138.24 (s, NCHN), 123.98 (s, NCHCHN), 123.78 (s, NCHCHN), 48.73 (s, NCH_2CH_2), 35.96 (s, NCH_3), 31.88 (s, $CH_2CH_2CH_2$), 19.23 (s, $CH_2CH_2CH_3$), 13.72 (s, CH_2CH_3). In this spectrum there is also a small band at 26.47, which is also associated with N-butylimidazole, so it is confirmed that IL 2 has a small impurity.

Figure 5.4 presents the ^{1}H-NMR and ^{13}C-NMR spectra of the IL 3. The structure band assignment was performed according to the structural data of another author [5.47].

The bands of the ^{1}H-NMR spectra of IL 3 are assigned as follow; δ_H (270 MHz, DMSO-d^6)/ppm: 9.15 (1H, s, NCHN), 7.78 (2H, t, NCHCHN), 7.71 (2H,

t, NCHCHN), 4.17 (2H, t, NCH_2CH_2), 3.86 (3H, s, NCH_3), 1.76 (2H, m, $CH_2CH_2CH_2$), 1.26 (2H, m, $CH_2CH_2CH_3$), 0.90 (3H, t, CH_2CH_3).

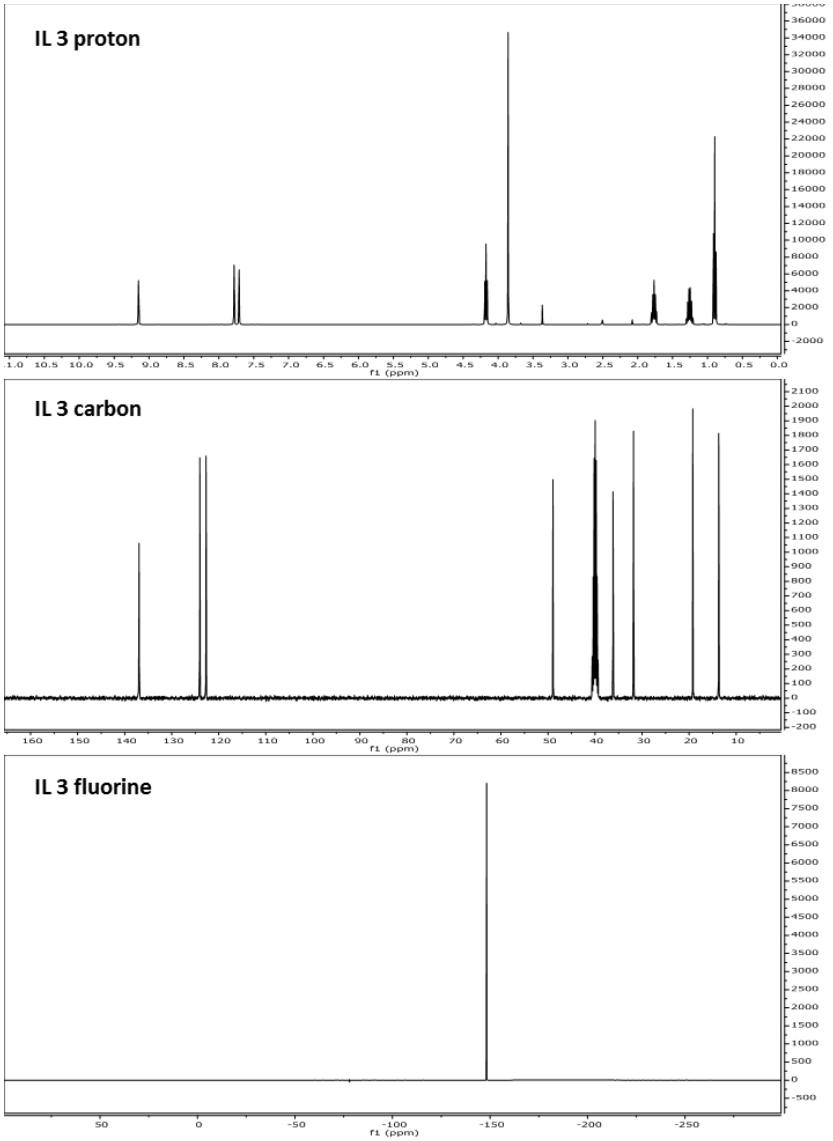

Figure 5.4 ^{1}H-NMR, ^{13}C-NMR and ^{19}F-NMR of $[C_4C_1im][BF_4]$ (IL 3).

The bands of the ^{13}C-NMR spectra of IL 3 are assigned as follow; δ_C (68 MHz, DMSO-d^6)/PPM: 136.94 (s, NCHN), 124.02 (s, NCHCHN), 122.70 (s,

NCHCHN), 48.94 (s, NCH_2CH_2), 36.17 (s, NCH_3), 31.81 (s, $CH_2CH_2CH_2$), 19.22 (s, $CH_2CH_2CH_3$), 13.70 (s, CH_2CH_3).

^{19}F-NMR spectra of IL 3 was carried out in order to confirm that the reaction was finished. There was only one band, which confirms that the reaction has finished, since all the Br has been replaced by BF_4. The band is assigned as follow; δ_F (DMSO-d^6)/ppm: -148.3 (s)

The ^{1}H-NMR and ^{13}C-NMR spectra of the DES 1 are shown in **Figure 5.5**. The structure band assignment was performed according to the structural data of other authors [5.51, 5.52].

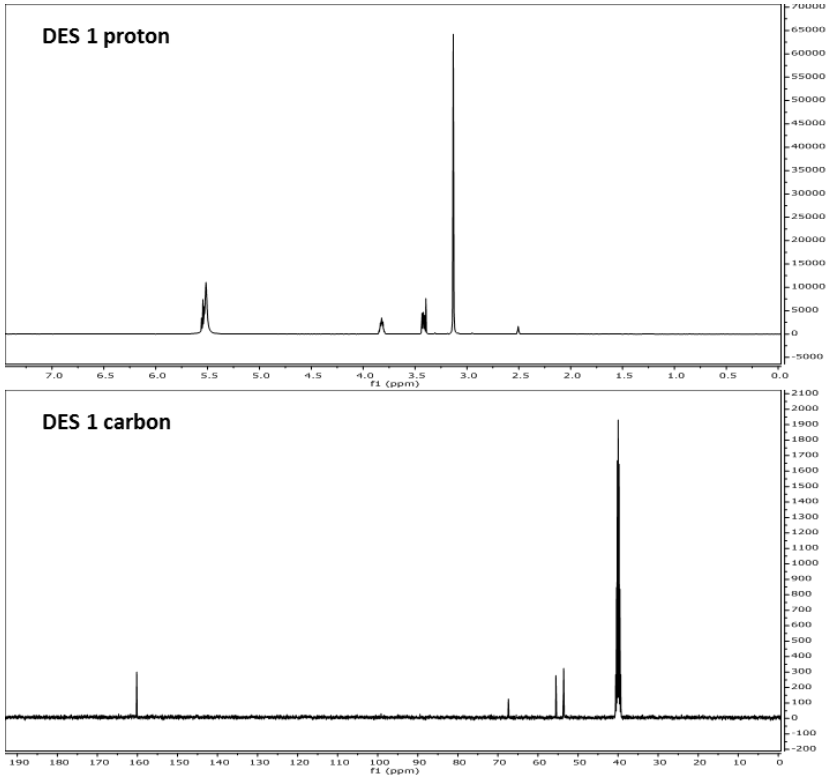

Figure 5.5 ^{1}H-NMR and ^{13}C-NMR of ChCl:Urea (1:2) (DES 1).

The bands of the ^{1}H-NMR spectra of DES 1 are assigned as follow; δ_H (270 MHz, DMSO-d^6)/ppm: 3.25 (s, 9H, NCH_3), 3.39 (s, 2H, NCH_2CH_2) 3.42 (t, 2H, CH_2 CH_2O), 3.83 (m, 2H, CH_2O), 5.51 (s, 8H, CNH_2).

The bands of the ^{13}C-NMR spectra of DES 1 are assigned as follow; δ_C (68 MHz, DMSO-d^6)/ppm: 54,0 (t, 3C, NCH_3), 55,3 (s, CH_2CH_2O), 67,7 (s, N CH_2CH_2), 160.1 (s, 2C,NH_2CONH_2).

Figure 5.6 presents the ^{1}H-NMR and ^{13}C-NMR spectra of the DES 2. The structure band assignment was performed according to the structural data of other authors [5.51].

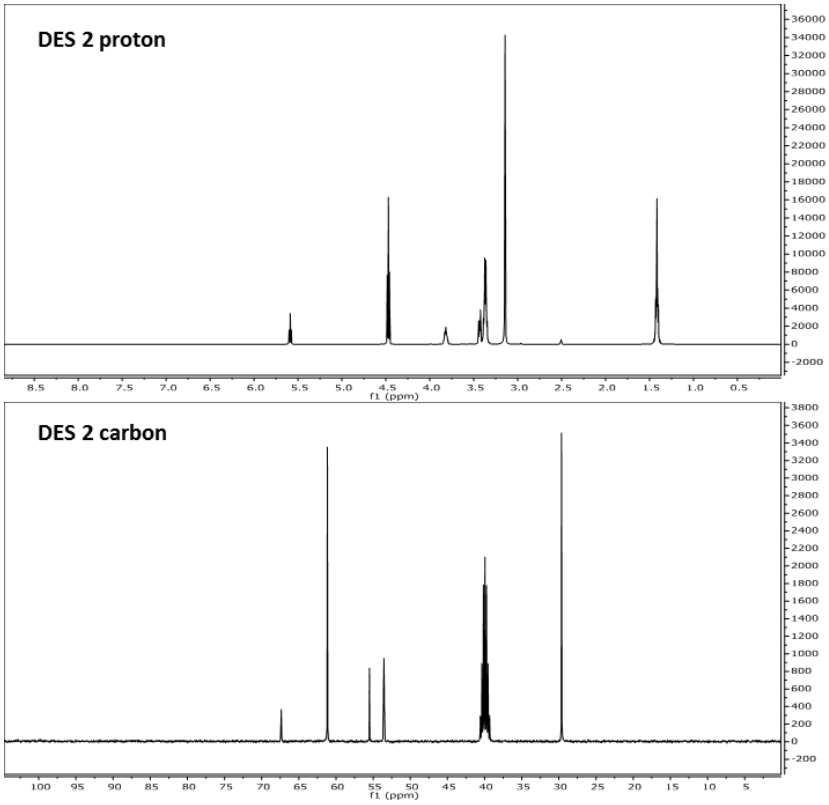

Figure 5.6 ^{1}H-NMR and ^{13}C-NMR of ChCl:1.4-Butanediol (1:2) (DES 2).

The bands of the ^{1}H-NMR spectra of DES 2 are assigned as follow; δ_H (270 MHz, DMSO-d^6)/ppm: 1.42 (t,4H, $CH_2CH_2CH_2$) 3.14 (s, 9H, NCH_3), 3.35 (t, 8H, CH_2CH_2O) 3.42 (t, 2H, NCH_2 CH_2), 3.83 (m, 2H, CH_2CH_2O), 4.47 (s, 4H, CH_2OH), 5.59 (t, IH, CH_2OH).

The bands of the ^{13}C-NMR spectra of DES 2 are assigned as follow; δ_C (68 MHz, DMSO-d^6)/ppm: 29.5 (t, $CH_2CH_2CH_2$) 53,5 (t, 3C, NCH_3), 55,3 (s, CH_2CH_2O), 61.2 (s, 2C, OCH_2CH^2) 67,7 (s, NCH_2CH_2).

Following the analysis of the structure of synthesised ILs and DES an ATR-FTIR analysis was performed. **Figure 5.7a** shows the spectra of the three ILs, where the structural differences can be seen. All the spectra had more or less the same main bands with some differences. The band assignment is based on the assignments given by other authors [5.53–5.56].

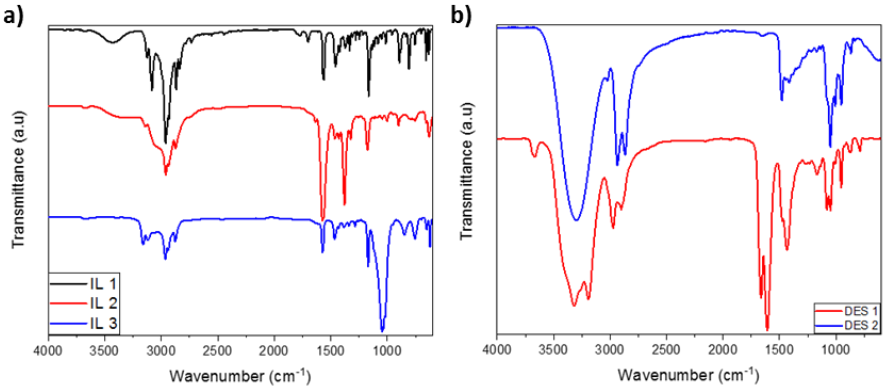

Figure 5.7 a) ATR-FTIR spectra of the 3 ILs. b) ATR-FTIR spectra of the 2 DES.

In these spectra, the following typical 1-Butyl-3-methylimidazolium$^+$ structures can be identified. Bands at 3159 and 3082 cm^{-1} are assigned to the –CH stretching vibration of the imidazole ring. The peaks at 2962, 2932 and 2868 cm^{-1} are identified as stretching of the -CH_3 of the butyl chain attached to the imidazole ring. The stretching of the imidazole ring is detected at

1569 cm^{-1}. The peaks at wavenumber 1457 cm^{-1} correspond to C=C and C=N stretching. The 1164 and 1114 cm^{-1} bands correspond to the in-plane bending vibrations of the methyl group. The peak at wavenumber 848 cm^{-1} is assigned to the in-plane bending of the imidazole ring, and the band 755 cm^{-1} is identified as the out-of-plane C-N bending of the imidazole ring. In the spectrum of IL 3 a very intense peak is observed in the 1012 cm^{-1} band, which is assigned to the stretching of BF. On the other hand, looking at the spectrum of the IL 2, it can be seen that the peaks for the wavenumbers 1574 and 1378 cm^{-1} are more intense. These are identified as the stretching of C=O and C-O at the acetate ion, respectively.

Figure 5.7b shows the spectra of the two DES, where the structural differences can be seen. The band assignment is based on the assignments given by other authors [5.57–5.59].

The main differences between the spectra of the two DES are in the ranges 3500-2500 cm^{-1} and 1750-1250 cm^{-1}. The DES 1 has, apart from the peak at 3300 cm^{-1} associated with -OH vibration of the pure choline chloride, another at 3189 cm^{-1} which is identified as -NH stretching, coming from urea. Furthermore, it also has two high-intensity peaks in the range of 1600-1700 cm^{-1} that correspond to the stretching of the -CN of urea. DES 2, however, has the bands associated with -CH stretching with higher intensity (2900-2700 cm^{-1}), due to the presence of 1,4-butanediol. The rest of the bands are characteristic of choline chloride, so they are similar, although they vary in intensity.

All these facts confirm that the syntheses were completed successfully, and that the obtained compounds were the desired ones.

5.4.2 Characterisation of the mixtures

The ILs and DES in this work were used as additives. In other words, they were used in combination with water. The use of water as solvent for the extraction of different compounds from the lignocellulosic biomass in general is the best option. However, due to the properties of water itself as well as those of the target compounds, its use is not always suitable. Unfortunately, many bioactive compounds, including flavonoids, have limited water solubility due to their properties [5.60]. The aim of this work is the selective extraction of flavonoid compounds, which has been demonstrated to be facilitated by the use of organic solvents, especially EtOH, as it has been shown in the previous chapters. Moreover, other authors have studied the benefits of using the H_2O/organic solvent mixture to obtain bark extracts [5.61, 5.62]. Therefore, in this work, EtOH is going to be replaced by different ILs and DES, in order to improve the extraction.

The use of water in mixtures with ILs and DES decreases the viscosity of the solvent, which facilitates the extraction of the target compounds [5.63]. This mixture also modifies their polarity, which has a direct effect on the extraction, as discussed in the following sections. Moreover, water is considered as the most recommended green solvent, so its use is fully justified.

5.4.2.1 Polarity

The polarity of the solvent is defined as the sum of all possible intermolecular interactions that occur between the solute and the molecules of the solvent. Therefore, it should be expected that the polarities of the ILs and DES studied are not equal due to the diverse degrees of intermolecular interactions experienced resulting from structural

differences. Proof of this can be found in the solvatochromic parameters measured for each of the solvents listed in **Table 5.4**.

Table 5.4 Kamlet–Taft parameters, using the dye set Reichardt's Dye, N,N-diethyl-4-nitroaniline and 4-nitroaniline.

Abbreviation	Solvent	α	β	π*	Reference
H_2O	H_2O	1.23	0.47	1.14	[5.64]
IL 1	$[C_4C_1im][Br]$	0.36	0.87	-	[5.65]
IL 2	$[C_4C_1im][OAc]$	0.48	1.20	0.96	[5.47]
IL 3	$[C_4C_1im][BF_4]$	0.63	0.37	1.05	[5.47]
DES 1	ChCl:Urea	1.42	0.50	1.14	[5.49]
DES 2	ChCl:1,4-butadienol	0.65	0.79	1.74	[5.66]

The values on π* are affected by both cation and anion in the case of ILs and by HBD and HBA in the case of DES. For these types of solvents, the values of π* trend to be higher than for most organic solvents due to the degree of delocalisation of the charge. Taking water as a reference, it can be seen that DES 2 had the highest π* value, while the lowest value reported was for IL 2. This may be due to the fact that in the case of the ILs, when more atoms are introduced into the anion there is a decrease in the strength of the Coulombic interactions between the solute and the ion due to the increased dispersion in the delocalised charge [5.66].

The parameter α is mainly influenced by the cationic component of the IL or HBD of the DES. In the chosen ILs and DES, these compounds remain constant, so it is expected that this value would be similar. However, as can be seen in **Table 5.4** it is not the case. The reason for this is that the α values are controlled by the ability of the compounds to act as a cation or HBD which in turn are moderated by what they have around them (ion and HBA). In the case of DES this difference is much higher, more than double.

The β parameter, which describes the ability of the solvent to donate electron density to form a hydrogen bond with the protons of a solute, is more dependent on the anion or HBA for IL and DES, respectively. In this case, all anions and HBAs are different, so the values are different in all cases. Considering the case of DES, the lowest value is for DES 1, because urea is a very basic compound.

The solvents used in this work were a mixture of ILs and DES with water, so the polarities were not the same as those reported in **Table 5.4**. Therefore, a theoretical estimation of these polarities was done. In general, as the mixtures were made in weight percentage, the total volume of IL or DES was not very large, so the parameters reported for water will not change too much. However, as the concentration increases, the polarity parameters of the water will be more and more affected, varying according to the parameters of the IL or DES used for the mixture. This discussion is continued in the following sections.

5.4.2.2 Solvent toxicity

To analyse the toxicity of the different solvents, each constituent of the mixture has to be studied separately. Starting with water, it is considered the most recommended and greenest solvent in all the consulted solvents guidelines, in addition to the fact that it facilitates the extraction. Regarding the ILs, these solvents are usually considered as "green solvents", mainly due to their possibility of reutilisation and low volatility, which is summarised in almost no risk of flammability and atmospheric contamination [5.67]. However, it is a mistake to consider only with these characteristic that ILs are environmentally friendly. To be able to affirm this, it is necessary to take into account other factors such as their toxicity in water, humans or soil, as well as their biodegradability and their full life cycle analysis (LCA).

Most of the ILs are not biodegradable; therefore, they can be accumulated in water or soil. The toxicity of imidazolium based ILs in aquatic environments is confirmed [5.68], with a higher toxicity for IL 3 than for IL 1 [5.69]. In the case of terrestrial toxicity, it is also verified that not all the ILs are toxic, but according to Frade, IL 3 has an effect on the growth of some bacteria presented in the soil [5.69]. For the other two ILs no results were found.

Focusing on the LCA, first it must be said that it is difficult to carry out the LCA for ILs or DES, since there is a lack of data. At this point, the synthesis, use and degradation of ILs in ecosystems are taken into account to understand their real impact. Making a theoretical analysis of the synthesis process, it can be said that this may be a very limiting factor to consider these as a "green solvent", since it must comply with the principles of green chemistry. It can be said that IL 2 is a high energy consuming product, so its impact is considered to be high, the same for IL 3. The synthesis can also be a high solvent consumption step, more if high purity ILs are needed. The reactions done at this work are not very high time and energy consuming, so there will not be a high impact at this step. Finally, the effect of the ILs in the ecosystems should be measured. It is closely related with the toxicity explained above, and with the biodegradability. The two best properties for the use of the ILs in the industry, thermal stability and non-volatility, are potential problems with degradation or persistence in the environment as has been reported by Thuy Pham et al. [5.19]. In the same work, the effect of both the anion and the cation on biodegradability is discussed. The conclusion is that the use of oxygen containing functional groups, such as acetate, makes it easier to degrade, and the case of the halides, they are more stable, so less biodegradable, with $[BF_4]^-$ being worse than $[Br]^-$. They also conclude that increasing the alkyl chain leads to an increase of the

biodegradability. Taking into account all the above, it can be said that processes using ILs could have a higher environmental impact than other conventional methods where the life-cycle is concluded [5.70]. Therefore, it is always advisable to analyse each case separately.

In the case of DES, there are limited studies, so their classification becomes more difficult. In our case of study, all the reactants used for the generation of the DES are natural, so they can be from renewable resources, having a lower environmental impact [5.71]. They are also considered non-toxic, biodegradable and with good bio-compatibility [5.68].

5.4.3 ILs and DES extractions

In this work, fifteen different solutions, based on three ILs and two DES, were prepared and tested for the extraction of pine bark. **Table 5.5** presents the average extraction yield obtained for each of the experiments.

The extraction yield obtained varies from 9% to almost 22% of the dry weight of the bark. The lowest value measured was recorded for water as solvent, while the highest yield was obtained for DES 1 (75%). In general, the extractions carried out with DES at different concentrations resulted in better extraction yields in all cases except for IL 2 (75%). All the extractions carried out are significantly different from the one with water as shown in **Table 5.5**. It is also observed that among all the extractions carried out with the different mixtures of ILs the results obtained do not show significant differences, except in the case of IL 2 (75%), which is significantly different. In general, there are also significant differences between the results obtained with the mixtures of ILs and the mixtures of DES, except in the case of the lowest concentration of DES. The result of IL 2 (75%) does not show significant differences with DES 1 (25%) and DES 2 (50 and 75%).

Table 5.5 Extraction yield obtained for the different solvents. (The values were average ± SD (n = 3). Superscript letters depict significant differences (Tukery test, $p < 0.05$)).

Solvent	[IL or DES] (%)	pH	Yield (%)
H_2O	0	5.80	9.3 ± 0.2[a]
IL 1	25	3.95	15.2 ± 0.6[b,c]
	50	4.51	15.3 ± 0.4[b,c]
	75	4.91	15.6 ± 0.2[b,c]
IL 2	25	6.53	14.7 ± 0.7[b,c]
	50	7.57	14.8 ± 0.1[b,c]
	75	9.55	19.3 ± 0.4[d]
IL 3	25	1.93	14.6 ± 0.5[b,c]
	50	0.90	14.3 ± 1.8[b]
	75	1.19	16.0 ± 0.2[b,c]
DES 1	25	9.49	16.2 ± 0.7[b,c]
	50	9.77	19.4 ±0.1[d]
	75	10.16	21.9 ± 0.8[e]
DES 2	25	4.63	16.5 ± 0.7[c]
	50	5.70	20.0 ± 0.5[d,e]
	75	5.29	20.4 ± 0.1[d,e]

The lower extraction yield when using DES was measured for the lowest DES concentration. According to Wan et al. a water proportion of more than 70% in the mixture decreases the extraction yield due to the destruction of the DES structure [5.43].

Regarding to the pH of the different solvents (**Table 5.5**), no general trend was observed in the influence on the extraction yield. Mixtures of ILs with water showed pH values from very acidic to basic; however, the extraction yield does not seem to be directly affected by this factor in the selected working conditions. Among these experiments, the highest yield was obtained with the most basic pH, being this value the only one significantly different and comparable with the obtained results and the ones obtained by DES mixtures. In the case of the DES mixtures, the studied pH variation

is lower, however no clear influence in the extraction yield was seen. It is observed that with pH between 5 and 6, the extraction yields are similar to those at pH 9.77. Therefore, it can be concluded that the pH does not have a direct influence on the extraction of the pine bark under the studied extraction conditions.

In the case of IL 2, the extraction yield remains stable at concentrations 25 and 50, but when it increases to 75% of IL, the yield rises. This may be due to a greater variation in the α and β polarity parameters, which are decreased and increased, respectively. This means that a higher basicity enhances the extraction. In the case of the extraction yield with different concentration of IL 3, it is similar to the previous case, with the difference that the yield with the highest concentration of IL is lower than the one measured for IL 2 (75%). However, in this case the polarity parameters studied, they vary, mainly because of the β parameter, which decreased instead of increasing. Perhaps that is why there is less increase in the extraction yield.

Looking at the measured data for the different DES mixtures and analysing them from the point of view of the different polarity parameters, no clear trend can be observed. In the case of DES 1 only the α parameter can change (it will increase in this case) since both β and π* are similar to those of water, so they will not change much. But in the case of DES 2 all parameters change, β and π* increased while α, contrary to what happens for DES 1, decreases. The trend of the DES 1 (75%) is contrary to the one observed for the highest yield IL mixture (IL 2 (75%)). Thus, there is no clear trend on how the different polarity parameters can affect the extraction yield of the pine bark in the studied conditions.

Comparing the values obtained in this work for the extraction of pine bark with to those obtained with conventional solvents (EtOH/H_2O), a considerable increase in the extraction yield is noted compared to the extracts measured in **Chapter 3**. None of the extracts obtained by the different techniques (CE, UAE and MAE) overcome the 10% extraction yield, while all the extractions carried out with the different mixtures of IL and DES enhance it. The extraction yield obtained for SMUAE extracts was 15.72% (**Table 4.6, Chapter 4**). This is similar and even higher than some of the yields obtained here (**Table 5.5**). The extraction yields obtained for the IL 2 and IL 3 mixtures with the concentrations of 25 and 50% are lower. Furthermore, all the extractions carried out with the different concentrations of IL 1 are also lower, although they all obtain values above 15%. Regarding the different mixtures of DES, all obtain values over 16% of the yield, so their extraction yield is better than that of SMUAE.

Škulcová et al. carried out the extraction of Spruce bark using nine different DES [5.72]. The extraction yield reported in this study ranged from 11.4% to 27.7%, so it can be said that all our results are within the range. The highest yield was obtained by DES ChCl:tartaric acid (1:1). In the case of the results reported by Haz et al. for the same raw material using three different DES, the reported extraction yield was lower [5.73]. The results varied between 11.40% and 14.68%, being the results obtained here higher. In this case, the best yield was obtained with the DES ChCl:malic acid (1:1).

Studies conducted by Yang et al. and Sun et al. for the extraction of Larix bark have shown that [C_4C_1im][Br] is the best IL for extraction compared to other IL or conventional solvents [5.74, 5.75]. Furthermore, in both cases the best extraction yield was obtained for a 1.25 M concentration of IL. **Table 5.5** illustrates that among the ILs, IL 1 is the one that provides the

best extraction yield for concentrations of 25% and 50%, while for the concentration of 75%, the best value is obtained by IL 2.

In order to confirm that only extractive compounds have been extracted, the characterisation of the solids was carried out once the extraction was completed. In this characterisation, the lignin (acid-soluble lignin (ASL) and acid-insoluble lignin (AIL)), cellulose (represented as glucan content) and hemicellulose content were measured directly by QAH (**Figure 5.8**).

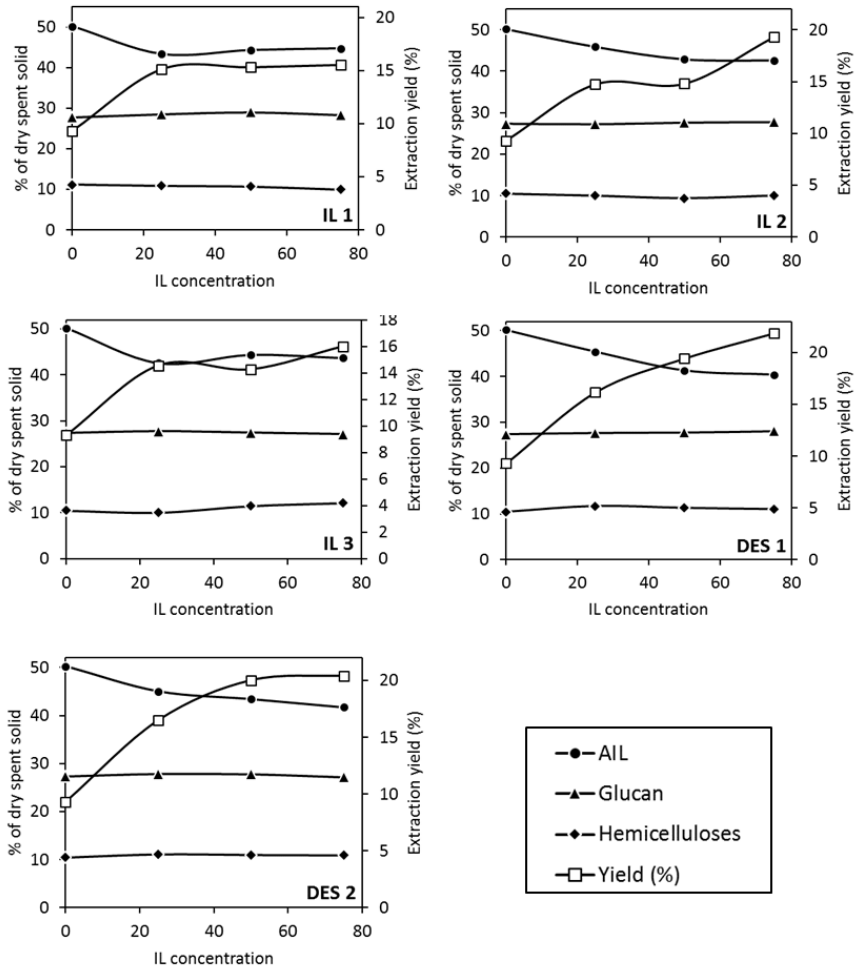

Figure 5.8 Chemical composition of the pine bark after the extraction vs extraction yield.

In general, it can be said that there is no solubilisation of glucan in any of the extractions carried out. The evolution of the solubility of each IL and DES mixes revealed that there was practically no variation between them, neither in comparison with the extraction carried out only with water (values between 27% and 28%). It confirms that in the studied conditions there is no glucan solubilisation.

In the case of the total hemicellulose content, it is observed that the variation is not very high either, although some trend can be seen. For example, in the case of mixtures with IL 1 and DES 1, a greater solubilisation of this fraction is observed when the concentration of IL or DES increases. In the case of DES 1 the solubilisation of hemicellulose is lower than that obtained with water (11.1% of hemicellulose content). DES 2 mixtures also shown a slight increase in solubilisation of hemicelluloses with higher DES concentrations. In the case of IL 2 and IL 3 the trend is inverted. These mixtures decrease the solubilisation of hemicelluloses by increasing the concentration of IL. In the case of IL 2 the variation is small and the solubilisation is higher than that obtained with water. However, in the case of IL 3, it can be seen that when the concentration of IL increases, the solubilisation of the hemicellulose decreases considerably, with even less solubilisation than when only water is used (hemicellulose content varies from 10% to 12%). This may be due to the fact that the solubilisation of the hemicellulose fraction is diminished due to the low pH of the solvent.

AIL appears as the main component and the one with the greatest variation. The solid that was extracted with water shows a 50.2% content in AIL. This value may be somewhat overestimated due to the presence of extracts and suberin in the sample. In general, for the extractions carried out with mixtures of IL 2, DES 1 and DES 2, it can be seen that the solubilisation of

the AIL increases with the increase of the IL or DES concentration. However, in the case of the IL 1 mixture, the opposite happened, the solubilisation decreased with the increase of IL. In the case of IL 3, when IL concentration was increased from 25% to 50% there was a decrease in solubility, while with the next concentration, the solubility decreased again. However, since the measurement of AIL can be affected by the presence of extracts [5.76], it cannot be confirmed that in the extractions carried out under these conditions there is a real solubilisation of lignin. The variations observed could be due to solubilisation of the extractable fraction. Finally, considering the ASL, which is not present in **Figure 5.8**, it is concluded that mixtures of ILs decrease the solubility of these compounds (range of ASL 1.6-2.6%). While the mixtures with DES are in the same range as the values obtained for water (about 1% of ASL).

In conclusion, it can be said that there was no solubilisation of glucan, but in some cases, there was solubilisation of hemicellulose and ASL. However, lignin solubilisation could not be confirmed.

5.4.4 Total flavonoids content (TFC)

Seeing that the extraction yield is considerably higher than that reported in previous chapters, the next step was to verify that the extraction was selective. For this purpose, the TFC of the liquid phase was measured, as shown in **Table 5.6**. It can be noticed that all the studied solvents extracted higher flavonoids concentrations than those obtained with water. This confirms the low affinity of this type of compounds with water [5.57].

The TFC varies from 96 to 779 mg CE/g dried bark extract. In general, the best values were obtained with the IL 1 mixtures, while the lowest value was determined for IL 2 (25%), not even reaching 100 mg CE/g dried bark

extract. IL 2 was the worst IL, and none of the DES were able to reach the lowest TFC value obtained for IL 1 (431 mg CE/g dried bark extract).

Table 5.6 Characterisation of the pine bark extracts of the different extractions.

Solvent	[IL or DES] (%)	TFC (mg CE/g DBE)	DPPH (mg TE/g DBE)	ABTS (mg TE/g DBE)	FRAP (mg TE/g DBE)
H_2O	0	34 ± 2	22.3 ± 0.3	106 ± 2	31 ± 4
IL 1	25	779 ± 32	1075 ± 21	1933 ± 89	608 ± 6
	50	540 ± 12	728 ± 20	1095 ± 27	403 ± 3
	75	431 ± 34	650 ± 28	903 ± 61	384 ± 12
IL 2	25	96 ± 4	102.5 ± 0.6	599 ± 58	57 ± 18
	50	371 ± 18	368 ± 16	711 ± 15	202 ± 5
	75	369 ± 11	306 ± 9	610 ± 11	155 ± 2
IL 3	25	435 ± 20	279 ± 19	1053 ± 43	432 ± 8
	50	532 ± 93	316 ± 56	1136 ± 5	493 ± 57
	75	431 ± 9	251 ± 8	1034 ± 23	368 ± 12
DES 1	25	159 ± 7	215 ± 20	391 ± 17	98 ±2
	50	305 ± 9	297 ± 17	593 ± 16	155 ± 8
	75	275 ± 1	215 ± 4	637 ± 45	120 ± 2
DES 2	25	376 ± 3	460 ± 17	850 ± 54	309 ± 7
	50	376 ± 12	453 ± 8	736 ± 22	314 ± 6
	75	383 ± 7	452 ± 19	799 ± 19	289 ± 11

DBE: dried bark extract

The TFC values obtained for the IL 1 and IL 3 mixtures were the highest, coinciding with the lowest pH values. In the case of IL 1, it is observed that the increase of pH above 4 led to a decrease of TFC. In the case of IL 3 the opposite is observed, since the highest TFC was determined with the lowest pH. Therefore, it can be confirmed that the pH of the mixture affects the

extraction of flavonoids, although other factors such as viscosity or polarity certainly have an influence. It can be seen that the viscosity has an influence on the extraction, as in no case the best values are obtained with the highest viscosities. In addition, the influence of the anion on the extraction is evident. This suggests that the bigger the β, the more favoured the flavonoids extraction is. However, the biggest β value is found in IL 2, but their TFC are lower. This is because when the anion has more atoms, the dispersion of the charges is increased reducing the force of solute-IL interaction [5.66]. In summary, the flavonoid extraction is reduced.

According to the TFC values measured for the different mixtures of DES, it is observed that all the results obtained are below those reported in **Chapter 4** (**Figure 4.5**) for the different extraction methods (about 400 mg CE/g dried bark extract). Furthermore, the results reported for IL 1 (75%), which is the one that has the worst results in this family, is far from being achieved even in the case of DES 2. This may be due, on one hand, to the difference in pH, and, on the other hand, to the difference in viscosity. Since DES mixtures had a higher viscosity. Finally, analysing the polarity, the β parameter indicates that DES 2 and IL 1 are similar, so their mixtures should also have similar β. However, the TFC differs a lot. This may be due to a possible steric hindrance that prevents the solvent from getting close to the target compounds. DES are bigger than IL 1, so their accessibility to extract flavonoids may be limited, since these compounds are inside the lignocellulosic matrix.

In contrast to what happened with the intensification of processes, in the extractions carried out with different ILs and DES, at least 3 of the extractions have a TFC value similar or higher than that reported in the characterisation of the pine (**Table 2.3, Chapter 2**). Moreover, they are

higher than the values obtained by using EtOH/H_2O in combination with other techniques (UAE, MAE or SMUAE).

Considering the values obtained in this work with the values reported by other authors, in general it can be said that good results have been obtained. The value obtained by IL 1 (25%) is higher than the value provided by Soto-García and Rosales-Castro for hydroalcoholic extracts from the bark of *Pinus durangensis* (615 mg CE/g extract) [5.77]. Furthermore, the values measured with IL 2 (50 and 75%) and all those determined with DES 2 are in the range of the ones reported for acetone/H_2O extracts from *Pinus durangensis* (379 mg CE/g extract) [5.78], and the ones reported for hydroalcoholic extracts from *Qercus sideroxyla* (386 mg CE/g extract) [5.77]. Finally, all the TFCs of the different mixtures of IL 1 and IL 3 reported in **Table 5.6** were better than the TFC determined by Chupin et al. for the ethanolic extracts from maritime pine, 403 mg GAE/g extract [5.79].

5.4.5 Characterisation of the extracts: Antioxidant capacity.

A further point to consider for the possible application of the extracts is their antioxidant capacity. Three antioxidant capacity measurements have been performed in this work, providing a more accurate idea of the capacity of these extracts. All the methods are based on the reaction of a specific radical with the extracts, which are measured by UV-vis spectroscopy. DPPH provides information about the amount of hydrogen donors, ABTS is based on the lost electron of the ABTS radical, and FRAP indicates the capacity of the sample to reduce the complex ferric ion-TPTZ.

The values measured for different antioxidant capacities are different for the same extract, which is normal because they measure different aspects. The analysis of the data in **Table 5.6** shows that there is a positive linear

correlation between TFC and antioxidant capacities for the pine bark extracts. Mainly for ABTS and FRAP, which both obtain a Pearson's correlation coefficients of 0.93, being DPPH the one with the lowest coefficient, 0.85. A strong direct linear correlation was also found between the antioxidant capacities of ABTS and FRAP, 0.90.

From the data in **Table 5.6**, the first thing to remark is the low antioxidant capacity reported for the pine bark extracts obtained with water. This is consistent with the low TFC, which suggests that the use of ILs and DES as additives for the extraction has a strong effect on the extraction of bioactive molecules. DPPH has a wide range of values for the different tested extracts, from 102 to 1075 mg TE/g dried bark extract. The highest (IL 1 (25%) extract) and lowest (IL 2 (25%) extract) calculated values correspond to the highest and the lowest TFC, respectively. In general, there is a tendency: the higher the TFC, the higher the DPPH value. This suggests that the solvent properties affecting this parameter are the same that for TFC.

Comparing these values with those reported for the extracts obtained by EtOH/H_2O from pine bark, it could be seen that in most cases, the reported capacities are lower. The values determined for the extractions carried out using CE, UAE MAE and SMUAE are in the range of 740-840 mg TE/g dried bark extract. Only the DPPH of the IL 1 (25%) extract was higher, and the IL 1 (50%) extract was similar. Since the antioxidant capacities are not only due to flavonoid compounds, the number of other compounds that also provide antioxidant capacity may have decreased (e.g. other phenolic compounds). Hence, the reported values are lower.

The ABTS antioxidant capacity in general were higher than those reported for scavenging capacity against the radical DPPH. The best values were determined for the extracts obtained by the mixtures of IL 1 and IL 3

followed by those calculated for the extracts of the mixtures of DES 2. The best value was determined for IL 1 (25%) extracts, 1933 mg TE/g dried bark extract. This result was better than those reported for CE, UAE, MAE and SMUAE extracts (807, 677, 906 and 1173 mg TE/g dried bark extract, respectively). All the values measured for the extracts of the different mixtures of DES 1 and the IL 2 mixtures of 25 and 75% were lower than those previously reported for the different EtOH/H_2O extracts.

Finally, regarding FRAP assay, the extracts obtained by IL 2 and DES 1 mixtures have the lowest antioxidant capacities. These values are far below the ones reported for the EtOH/H_2O extracts obtained by different extraction techniques, which are in the range of 330-460 mg TE/g dried bark extract. The worst results, as it has happened for the other antioxidant capacities, were obtained with the lowest IL 2 and DES 1 concentrations. The values in this case are especially low, since they did not even reach 100 mg TE/g dried bark extract. This is consistent with the fact that they are the mixtures with the lowest flavonoid compounds extraction. This suggests that these solvents are not good for the selective extraction of flavonoids. The extracts of IL 1 (25%) were the ones with the best measured antioxidant capacity (608 mg TE/g dried bark extract).

The comparison of the results obtained in the antioxidant capacity measurements should always be done with caution, as it is not usual to be able to compare with the values of the extracts of the same raw materials. In the case of pine bark, no other work has been carried out apart from those described in this thesis. Therefore, a comparison with the values reported for other raw materials has been made cautiously.

In the work conducted by Bibi Sadeer et al. to obtain methanolic extracts from three different tree stem barks, it was observed that the lowest values

of ABTS and DPPH were measured for *Zanthoxylum gilletii* (178 and 82 mg TE/g extract, respectively) [5.80]. These values are lower than those reported in **Table 5.6**. The methanolic extracts of *Sterculia tragacantha* had the highest ABTS value (943 mg TE/g extract). This value is exceeded by the pine bark extracts obtained with the mixtures of IL 1 and IL 3. In the case of DPPH, *Macaranga hurifolia* as *Sterculia tragacantha* reported values close to 495 mg TE/g dried bark extract, far below those calculated for the extracts obtained with IL 1 mixtures (650-1075 mg TE/g dried bark extract). Analysing the values measured by Bibi Sadeer et al. for FRAP, the value reported for the methanolic extract of *Macaranga hurifolia* (622 mg TE/g extract) was higher than the highest value obtained in this work.

Tanase et al. characterised the extracts obtained with different solvents using MAE from the bark of *Fagus sylvatica* [5.62]. The FRAP values reported were in the range of 592-784 mg TE/g extract, being higher than those reported in this work. Only the IL 1 (25%) extracts surpassed the value reproduced for the 80% ethanol bark extract, which has the lowest value. The DPPH values measured by Tanase et al. (505-620 mg TE/g extract) are higher than those reported in **Table 5.6**, except for all the extracts obtained by IL 1 mixtures, which were higher. Regarding ABTS and the antioxidant capacity reported for 80% ethanol extracts of *Fagus sylvatica* bark, was 472 mg TE/g extract. These values were exceeded in all the experiments of this work except in the case of DES 1 (25%) extracts.

Neiva et al. also characterised the potential of different barks using FRAP test [5.81]. From their results, it is concluded that the range of values for ethanolic and aqueous extracts from different barks is large, from 323 to 1295 mg TE/g extract. Comparing these results with those measured in this work, only the extracts obtained with the IL 1 and IL 3 mixtures are in the

range, the rest of the extracts showed lower values. The best antioxidant capacities were far from the best determined in this work: 608 mg TE/g dried bark extract measured for IL 1 extracts (25%) from pine bark.

A comparison of the antioxidant capacities of *Chrysophyllum perpulchrum* extracts obtained using different solvents by Baloglu et al. shows that the highest DPPH value obtained was for MeOH extracts (73.23 mg TE/g extract) [5.82]. This value is significantly lower than those obtained here for pine bark extracts (**Table 5.6**). Something similar occurs with the aqueous extracts for ABTS antioxidant capacity (491 mg TE/g extract).

5.4.6 Best solvent selection

Considering all the aforementioned, it can be seen that the extraction yield is not linked to the number of flavonoid compounds that are extracted. This may be because the solvents used are not very selective. All the solvents studied are mixtures of IL or DES with water, and as shown in **Table 5.5**, water also extracts compounds from pine bark by itself. Nevertheless, these compounds are generally not flavonoids, since the TFC value reported for aqueous extracts is only 34 mg CE/g dried bark extract. Therefore, although IL and DES enhance the extraction of flavonoids as well as other phenolic compounds, the presence of water in the mixture will allow the solubilisation of other compounds. This finally makes the extraction not completely selective.

The extracts obtained with the DES mixtures showed better extraction yields, but the TFC is not the highest. Even though the obtained values are good, it is observed that the use of IL in general provides better flavonoid extractions, especially IL 1. This result is in agreement with that obtained by Ma and Row [5.59]. In that work, they studied the extraction of three

flavonoid compounds from *Herba Artemisiae Scopariae* using different IL and DES, including IL 1, IL 3, DES 1 and DES 2. The IL 1 was the one that extracted the greatest amount of flavonoids, 10275.92 μg/g rutin, 899.73 μg/g quercetin, and 554.32 μg/g scoparon.

Studying different works performed for the extraction of flavonoid compounds from different lignocellulosic materials with DES, both Wang et al. and Cui et al. conclude that the ChCl:1,4-butanediol was the best [5.83, 5.84]. Although in the case of Ciu et al. the best ratio was 1:3, while in the case of Wang et al. the best ratio was 1:2. This is in accordance with the results obtained here, where among all the extractions done with DES, the best TFC was obtained for DES 2 (75%) extract. The use of 25% of water to facilitate the extraction of flavonoid compounds is in accordance with that reported by Wang et al [5.84].

According to **Table 5.5**, the best extraction yield for IL mixtures was obtained for IL 2 (75%), while in the case of DES it was obtained for DES 1 (75%). However, this yield is not consistent with a higher TFC. This may result from a lower selectivity of these solvents, since they have also been studied for the delignification of different lignocellulosic materials [5.35, 5.85, 5.86]. This indicates that the use of the mixture of IL 1 and DES 1, together for the extraction of flavonoids, can also solubilise part of the lignin. Thus, reporting a high extraction yield whereas TFC would not be consistent. This is confirmed by the decrease in the measured AIL content of the solids after extraction (**Figure 5.8**).

In conclusion, it could be said that the best flavonoids extractions from pine bark were those carried out with different concentrations of IL 1. Not only because they had higher TFC but also because they showed very high values for antioxidant capacities. All IL 1 mixtures had similar extraction yields

(15.15-15.55%), but IL 1 (25%) extracted more flavonoid compounds and reported the best antioxidant capacity. Therefore, it is selected as the best choice. This is in line with the optimisation carried out by Zhang et al. for the extraction of isoflavones compounds from *Radix puerariae*, where the solvent with the best yield was IL 1, with a concentration of 1.2 mol/L [5.87]. Yang et al. also established IL 1 as the best solvent for proanthocyanidins extraction from *Larix gmelini bark* [5.74]. The optimal concentration in this case was a little bit higher, 1.25 mol/L.

5.4.7 Structural characterisation of extracts

The presence of flavonoid compounds in the different extracts was confirmed by ATR-FTIR analysis. The following figures show the extracts with different concentrations of solvents compared to the spectrum of the pure IL or DES used as a solvent. In the **Figure 5.9** and **Figure 5.10**, it can be seen how the intensities of the different bands change depending on the concentration of solvent used, as well as the appearance of new bands.

In the 5 study cases, a significant increase in intensity is observed in the band attributed to -OH stretch vibration in phenolic and aliphatic structure (between 3400-3300 cm^{-1}). The **Figure 5.9a** and **Figure 5.9b** of the IL 1 and IL 2 extracts show that the band belonging to wavenumber 1630 cm^{-1} undergoes a considerable increase in intensity. This band also appears in the spectra of the IL 3 and DES 2 extracts (**Figure 5.9c** and **Figure 5.10b**), which is assigned to the valence vibrations C=O, typical of the flavonoid compounds [5.88]. Thus, the extraction of these compounds is confirmed. In the case of DES 1, as shown in **Figure 5.10a**, instead of the typical C=O band of the flavonoids, another band appears at a wavenumber of 1705 cm^{-1}. This band is related to the presence of lignin, since it corresponds to the stretching vibration of non-conjugated carbonyl groups from the aromatic

lignin skeleton [5.89]. It confirms the capacity of this solvent to solubilise lignin, as it was mentioned in **section 5.4.5**.

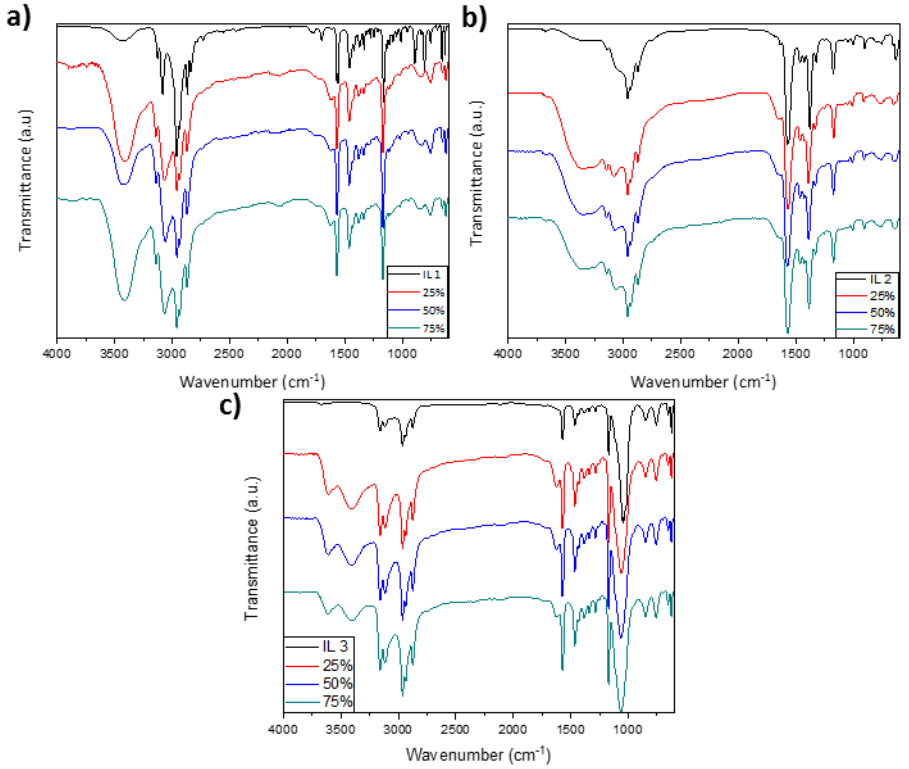

Figure 5.9 ATR-FTIR spectra of different bark extract. a) Extracts obtained with different IL 1 concentrations. b) Extracts obtained with different IL 2 concentrations. c) Extracts obtained with different IL 3 concentrations.

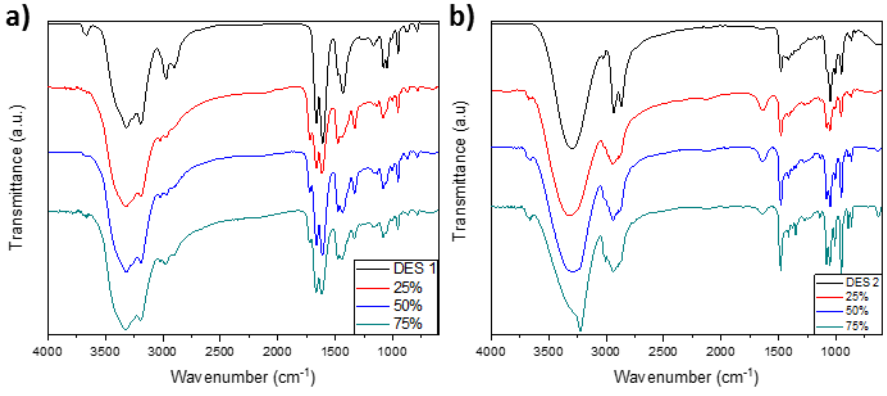

Figure 5.10 ATR-FTIR spectra of different bark extract. a) Extracts obtained with different DES 1 concentrations. b) Extracts obtained with different DES 2 concentrations.

5.5 Conclusion

A total of three IL and two DES have been successfully synthesised in this work. Moreover, these compounds have been used as additives in aqueous mixtures for the extraction of bioactive compounds from pine bark.

It has been proved that the use of aqueous mixtures of ILs and DES can be used as an alternative solvent for the extraction of flavonoid compounds from pine bark. All the studied cases presented an improvement in the extraction yield compared to the aqueous extraction. Furthermore, these solvents obtained higher extraction yields than those obtained with EtOH/H_2O using intensification techniques such as UAE and MAE. Thus, the potential of these alternative solvents is demonstrated. However, as far as TFC is concerned, only mixtures of IL 1 and IL 3 showed an improvement compared to the results reached with the conventional solvent. Throughout this work, the influence of polarity and pH on the extraction of flavonoid compounds was also confirmed.

IL 1 (25%) was chosen as the optimal solvent not only because of its good flavonoid extraction ability, but also because of its good antioxidant properties. The characterisation of these extracts showed that the extract had a high flavonoid content, considerably higher than that measured for extracts obtained using the different intensification methods (MAE, UAE and SMUAE). It was confirmed by the FTIR analysis of the extracts, which showed a large increase in the band typically assigned to the flavonoids at 1630 cm^{-1}. Regarding antioxidant capacities, the previously measured values for the pine bark extracts were far exceeded. In conclusion, these extracts are biologically more active, which is very appropiate for different applications in fields as varied as cosmetics, food industry or bio-based

materials among others. However, a more in-depth characterisation of the compounds obtained should be carried out, as well as their purification before their application.

In conclusion, it can be noted that the use of these new solvents, in particular IL 1, is promising. However, as happened when CE with EtOH/H2O was used as a solvent, the energy and time consumption was elevated. Therefore, it will be useful to try to use these solvents with some of the intensification methods previously studied to see how they affect the extraction. The purpose will be to reduce the extraction time and the required energy for the extraction, developing a more sustainable extraction process from the pine bark. Furthermore, a comprehensive study should be carried out in order to achieve a complete recovery of the IL, in order to avoid its effect on the environment and/or on humans.

5.6 References

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Chapter 6

Conclusions, future work and published research

6.1 Conclusions

In this work, a valorisation of the bark was carried out to obtain added-value products from the extractive fraction. The objective was to convert a waste into an added-value raw material suitable for extracting different compounds, instead of burning or discarding it.

The study of the selection of the raw material demonstrated that the barks of the trees, in general, are richer in extractive materials than the wood, with a percentage higher than 12% in all the studied cases. More exhaustive study of bark extracts revealed that they are potential sources of phenolic and flavonoid compounds that also have high antioxidant capacities. This property is very interesting for its possible application. Therefore, barks can be considered as an interesting source of bioactive compounds due to their chemical functionalities and bioactivity.

Conventional extraction (CE), ultrasound assisted extraction (UAE) and microwave assisted extraction (MAE) techniques were successfully used for the extraction

of bioactive compounds from the pine bark. All extraction methods showed good capacity for the extraction of biomolecules, more specifically phenolic and polyphenolic compounds. It was confirmed by the identification of 22 different compounds by UPLC-DAD-ESI-MS. Pine bark extracts also exhibited good qualities as antioxidants, as can be seen from the good results obtained for the different antioxidant capacities. This confirms the effectiveness of the studied techniques. MAE was the intensification process that achieved the highest extraction yield (8.25%) at the optimum point in addition to having the highest flavonoids content (430 mg CE/g dried bark extract). Another advantage of this technique was the reduction of the

extraction time with the consequent economic benefit that this can imply. Although MAE was the most successful intensification technique, the potential of UAE for the bioactive molecules' extraction was also proved.

Simultaneous microwave-ultrasound assisted extraction (SMUAE) was a further step in the development of a sustainable extraction process using process intensification. The simultaneous use of microwaves and ultrasound improved the extraction yield (15.72%) by up to double of that obtained by both extraction techniques separately. The optimisation of the process was successfully completed, and the analysis of the extracts obtained at the optimum point reveals their great capacity to extract bioactive compounds. The reason for this was that, apart from improving the extraction yield, a bigger quantity of phenolic compounds was extracted, resulting in a higher antioxidant capacity of the extracts. It was confirmed with the identification of 14 compounds by UPLC-DAD-ESI-MS. This intensification process, besides to bringing significant benefits to the amount of the extracted bioactive compounds, also has the advantage of reducing extraction time. This technique improves considerably the amount of extracted compounds and their properties with only 2 minutes of extraction, reducing the extraction time up to 47 times. Furthermore, this can lead to a reduction in energy consumption, making the process more efficient and sustainable, in line with the sustainable development.

Finally, in the study conducted for the identification of selective solvents, it was concluded that aqueous mixtures of ILs and DES could be used as an alternative solvent for the extraction of flavonoid compounds from pine bark. The extraction yield (between 14% and 22%) was improved in all the studied cases compared to those measured for UAE and MAE. However, only the IL 1 and IL 3 mixtures showed an improvement in the extraction of

flavonoid compounds (between 430 and 780 mg CE/g dried bark extract). Therefore, it was concluded that these extractions were influenced by the polarity of the solvents and the pH of the mixtures. IL 1 was the solvent that generally extracted the highest amount of flavonoids. The IL 1 (25%) mixture was selected as the best solvent, with a TFC of 779 mg CE/g dried bark extract, with high antioxidant capacities.

Once the great potential of this new solvent is known, it is necessary to go further to develop a sustainable extraction method. In this study, in order to understand the capacities of these solvents, the conventional method was used. However, knowing all the limitations and disadvantages of this method, it becomes necessary to study how this solvent works in the intensification process. The combination of selective extraction and the benefits of intensification methods could be the key to achieve a more sustainable process. Therefore, further research is still needed in this area.

6.2 Future work

To continue the work in this field, the following lines of research could be tackled.

- Study the environmental impact of the studied extraction methods.
- Perform an economic and energetic analysis of the different intensification process.
- Separation and purification of the different extracted compounds by different techniques. Especially the ones extracted with ILs and DES.

- Intensification of the extractions with the ILs and DES by the employment of microwave, ultrasound, or both.
- Study the extraction of bioactive compounds by a continuous SMUAE.
- Evaluate the influence of the extraction on the subsequent stages of lignocellulosic material fractionation to achieve a complete valorisation of the raw material from a biorefinery approach.

6.3 Published research

The research carried out during this thesis led to various papers published in different scientific journals. The results obtained have also been presented at various conferences

Papers in Scientific Journals

(JC: Journal Category, JR: Journal Ranking)

Sillero, L., Prado, R., Welton, T., Labidi, J. 2020.

Energetic and Environmental Analysis of Different Techniques for Biomolecules Extractions. Chemical Engineering Transaction 81, 631-636.

Impact factor: 0.76 (2019)
JC: Chemical Engineering; JR: 176/281

Sillero, L., Prado, R., Labidi, J. 2020.

Simultaneous Microwave-Ultrasound Assisted Extraction of Bioactive Compounds from Bark. Chemical Engineering and Processing - Process Intensification 156, 108100.

Impact factor: 3.731 (2019)

JC: Chemical Engineering; JR: 41/143

Sillero, L., Barriga, S., Izaguirre, N., Labidi, J., Robles, E. 2020.

Fractionation of non-timber wood from Atlantic mixed forest into high-value lignocellulosic materials. Journal of Wood Chemistry and Technology 40, 200-212.

Impact factor: 1.899 (2019)

JC: Materials science, paper & wood; JR: 5/21

Sillero, L., Prado, R., Andrés, M.A., Labidi, J. 2019.

Characterisation of bark of six species from mixed Atlantic forest. Industrial Crops and Products 137, 276-284.

Impact factor: 4.244 (2019)

JC: Agricultural Engineering; JR: 2/13

Sillero, L., Prado, R., Labidi, J. 2018.

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Impact factor: 0.76 (2019)

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Appendix I. Concept clarification

Circular economy

The circular economy concept was described as "an industrial system that is restorative or regenerative by intention and design. It replaces the end-of-life concept with restoration shifts towards the use of renewable energy, eliminates the use of toxic chemicals, which impair reuse and return to the biosphere and aims for the elimination of waste through the superior design of materials, products, systems and business models" [A.1]. In other words, it consists of changing the linear flows of materials, on which everything is currently based, to circular flows (see **Figure A.9.1**).

Figure A.9.1 Simplified scheme of circular economy.

Sustainable development

The sustainable development goals are the roadmap for a better and more sustainable future for all addressing different global challenges [A.2]. To this end, 17 goals have been set, which are interconnected, and it is important that the goals are achieved by 2030. The goals are:

1. No poverty
2. Zero hunger
3. Good health and well-being
4. Quality education
5. Gender equality
6. Clean water and sanitation
7. Affordable and clean energy
8. Decent work and economic growth
9. Industry, innovation and infrastructure
10. Reduced inequalities
11. Sustainable cities and communities
12. Responsible consumption and production
13. Climate action
14. Life below water
15. Live on land
16. Peace, justice and strong institutions
17. Partnerships for the goals

Principles of green chemistry

Green chemistry is an integrated approach to chemistry that focuses in maximising efficiency and minimising hazardous effects on human health and the environment. To achieve these goals, 12 Principles of Green Chemistry [A.3] have been defined:

- Waste/by-products prevention.
- Atom economy by reducing the waste at the molecular level maximising the incorporation of the reactants into the final product.
- Prevention or minimization of hazardous chemical synthesis by designing safer processes. Considering the hazards of all the substances handled.
- Designing safer chemicals.
- Designing energy efficient processes, minimising the energy requirement.
- Selecting the appropriate starting materials, use of renewable feedstocks.
- Reduce derivatives. Avoid the use of the protecting group whenever possible.
- Use of catalysts should be preferred instead of stoichiometric agents.
- Design for degradation. Products obtained should be biodegradable.
- Eliminate the possible accidents during operations at manufacturing plants by good processes design.
- Real-time pollution prevention strengthening of analytical techniques to control hazardous compounds.

Appendix II. Procedure for wood characterisation

In this appendix, the experimental procedure for the chemical characterisation of wood is described. It was characterised following the procedures described by the Technical Association of Pulp and Paper Industries (TAPPI), the National Renewable Energy Laboratory (NREL) as well as some traditional methods.

The TAPPI protocols [A.4] were used to prepare the raw material and for the determination of moisture content, ash content and extractive content. NREL technical report [A.5] was used for the determination of lignin, and cellulose and holocellulose content were measured following the methods proposed by Rowell (1983) and Wise et al. (1946) respectively.

All measurements were performed in triplicate, giving the results as the mean ± standard deviation on an oven-dried basis.

Sample preparation

The aim of this standard is to prepare a homogeneous lot of raw material with a particle size suitable for the chemical treatment to determine its chemical composition.

The homogeneity of the biomass is very important for its uses, because there are many factor that can affect the composition of the biomass, and one of the most important is the particle size. This can affect the rate of solvent impregnation on the raw material, resulting in a change in the measured composition depending on the particle size. For that reason, the sample

preparation is a very important step in the chemical characterisation of the raw material.

The conditioning of the sample consists of drying and grinding the sample until a specific particle size. In this thesis, all the analysed woods were air-died, milled (Restch SM 100) and sieved to a size between 0.4 and 0.25 cm.

Determination of moisture content (TAPPI T264 cm-97)

The moisture contained in the biomass is that which is in equilibrium with that in the environment. It is therefore necessary to know it, since it will be taken into account in the subsequent analyses, since the results are typically reported on an oven-dried basis. The procedure used to determine the moisture content consists of:

- Prepare the recipient that will be used for the measurement. It need to be clean and dry, so clean and dry placing it in the oven at 105 ± 3 °C for 6 h. Then after that, cool it down until room temperature in a desiccator, and weigh it on the analytical balance to the nearest 0.1 mg (m_0).
- Weigh accurately 2.00 ± 0.01 g of sample in the previously tared recipient with the same analytical balance (m_1).
- Place the recipient with the sample in the oven at 105 ± 3 °C for 24 h.
- Then, placed the recipient with the sample in a desiccator until it is cooled down to room temperature. Finally, weigh the recipient with the sample (m_2) until the weight of the sample is constant to ± 0.2 mg.

The moisture content is determined as follows:

$$Moisture\ content\ (H)\ (\%) = \frac{(m_2 - m_0)}{m_1} \cdot 100$$

Determination of ash content at 525 °C (TAPPI T211 om-93)

The ash content of the sample measures the amount of inorganic matter present in the sample, whether it belongs to the structure of the sample or has been transferred to it externally.

The protocol to determine the ash content of the wood consists in the measurement of the remaining solid material after the ignition at 525 °C. The used procedure is:

- Weigh accurately 1.00 ± 0.01 g of sample (m_1) in a previously tared crucible. The crucible needs to be clean and previously ignited in a muffle furnace at 525 ± 25 °C for 30-60 min, after that, place it in a desiccator until it is cooled down to room temperature. Then, weigh it on the analytical balance to the nearest 0.1 mg (m_0).
- Place the crucible with the sample in a muffle furnace at 525 ± 25 °C for 3 h.
- After this time, remove the crucible with the sample from the muffle furnace and kept in a desiccator until it is cooled to room temperature.
- Finally, weigh the crucible with the remaining solid (m_2) until the weight of the sample is constant to ± 0.2 mg.

The ash content at 525 °C is determined as follows:

$$Ash\ content\ (AC)\ (\%) = \frac{(m_2 - m_0)}{m_1 \frac{(100 - H(\%))}{100}} \cdot 100$$

Determination of solvent extractives content (TAPPI T204 cm-97)

This method describes a procedure for determining the amount of solvent-soluble material present in wood and pulp, such as resin, fatty acids and their esters or waxes. The solvent-soluble compounds can be extracted with a simple extraction, since these compounds are not part of the cell wall of the biomass. Although this compound is not present in much quantity in the wood, it is necessary to make this determination prior to the following analysis since these compounds could generate interferences in the following measurements.

The used procedure to determine the solvent extractives consists of:

- Clean and dry a 250 mL extraction flask. After 6 h in the oven at 105 ± 3 °C, cooled it down in a desiccator and weigh on the analytical balance to the nearest 0.1 mg (m_0).
- Weigh accurately 4.0 ± 0.1 g (m_1) on an extraction thimble (cellulose cartridge), and then put another thimble on the top to avoid the losing of any raw material.
- Place the thimble with the sample in position in the Soxhlet apparatus. Fill the previously tared extraction flask with 150 mL of toluene-EtOH mixture (2:1 v/v).
- Connect the flask to the extraction apparatus, and start water flow to the condenser section. Adjust the heaters to provide a boiling rate which will cycle the samples for not less than 24 extractions over a 4-5-h period.

- When the time is over, remove the flask from the apparatus and partially evaporate the solvent in the extraction flask to a volume of 20-25 mL.
- Place the extraction flask with the extracts in the oven at 105 ± 3 °C for 24 h.
- Finally, cool down the flask to room temperature in a desiccator, and then weigh the extraction flask with dried extracts (m_2) until the weight of the sample is constant to ± 0.2 mg.

The percentage of extractives is determined as follows:

$$Solvent\ extractives\ content\ (\mathrm{SEC})(\%) = \frac{(m_2 - m_0)}{m_1 \frac{(100 - H(\%))}{100}} \cdot 100$$

Determination of lignin content (NREL/TP-510-42618)

This protocol described a procedure to determine the structural carbohydrates the acid-insoluble lignin (AIL) and the acid-soluble lignin (ASL) present in extractives-free lignocellulosic biomass. The raw material must be free of extractives because otherwise the lignin measurement could be overestimated. This procedure was only used in the wood characterisation to measure the ASL and AIL content, leaving aside the quantification of carbohydrates. The quantitative acid hydrolysis (QAH) of biomass samples was carried out as follows:

- Weigh accurately 0.25 ± 0.001 g of the extract-free sample (m_0) in a test tube. The particle size of the sample should be less than 0.5 mm and the moisture of the sample (H) needs be previously determined.

- Add 2.5 mL of 72.0 wt.% H_2SO_4 to the test tube and stir the sample + H_2SO_4 mixture to have an homogeneous mixture. Then, place the test tube in a water bath at 30 °C for 1 h, stirring it periodically.
- After the hour, add distilled H_2O to the mixture in order to stop the reaction. Then, transfer the content of the test tube to a previously tared pressure flask and add distilled H_2O is until the weight of the whole mixture is 74.33 g (m_1), which corresponds to a H_2SO_4 concentration of 4.0 wt.%.
- Weigh the pressure flask with the mixture (m_2) and autoclave it for 1 h at 121 °C.
- After this hour, cool down the pressure flask and note down the weight of the pressure flask (m_3).
- Separate the mixture by filtration using a previously tared Gooch crucible № 3. The Gooch crucible should be previously dried in an oven for 6h at 105 ± 3 °C, cooled down in a desiccator and weighed (m_4).
- Dry the solid residue contained in the Gooch crucible in an oven at 105 ± 3 °C for 24 h.
- After this time, cool down to room temperature the Gooch crucible with the solid phase in a desiccator and weigh it (m_5) until the weight of the sample is constant to ± 0.2 mg.
- Analyse the liquid phase for ASL by spectrophotometry (UV absorption at an appropriate wavelength). Dilute the sample with 4% H_2SO_4 to bring the absorbance into the range 0.7-1.0, and measure it. The ASL is determined as follows:

$$Acid - soluble\ lignin\ (ASL)(\%) = \frac{UV_{abs} \cdot V \cdot D}{\varepsilon \cdot W \cdot Pathlength} \cdot 100$$

Where: UV_{abs} is the average UV-vis absorbance at 205 nm, V is volume of filtrate (L), D is the dilution, ε is the absorption coefficient (110 $L \cdot g^{-1} \cdot cm^{-1}$),

W is the weight of sample (g) and Pathlength is the width of the cuvette (1 cm).

Throughout this thesis, the UV-vis spectrometry analyses were carried out using Jasco V-630 UV-VIS spectrophotometer.

The acid-insoluble lignin or Klason lignin content of the sample is estimated as follows:

$$Acid-insoluble\ lignin\ (AIL)(\%) = \left(\frac{m_5 - m_4}{m_0 \cdot \frac{(100 - H(\%))}{100}} \cdot (100 - SEC) \right)$$

Determination of holocellulosic content (Wise et al. 1946)

The holocellulose is described by the fraction of water-insoluble carbohydrate present in plant raw materials, being it formed by the sum of cellulosic and hemicellulosic fractions. The total hollocellulose content was determined following the procedure previously described by Wise et al. [A.6], which is based on a delignification process with sodium chloride in an acid medium. This process achieves a total solubilisation of the lignin while carbohydrates remain unchanged.

The procedure to determine holocellulosic content consists of:

- Weigh accurately 2.5 ± 0.1 g of sample (m_0) in a 250 mL beaker.
- Add 80 mL of hot distilled H_2O (70-80 °C), and introduce the beaker with the mixture in a bath at 70 °C and stirred periodically to homogenize.

- Every hour, add 2.6 mL of 25% sodium chlorite and 0.5 mL of glacial acetic acid to the beaker. Repeat this step every hour until cover a total period of 6-8 h.
- After that period of time, keep the beaker with the mixture in the bath for 12 h without further additions.
- After 12 h, separate the solid and liquid fractions by vacuum filtration using a previously tared grade 2 pore size Gooch crucible. The Gooch crucible should be previously dried in an oven at 105 ± 3 °C for 6 h, cooled down until room temperature in a desiccator and weighed (m_1).
- Then, wash the solid phase that remain in the crucible with distilled hot H_2O until neutral pH and dry it in an oven at 105 ± 3 °C for 24 h.
- Place the Gooch crucible with the solid phase in a desiccator to cool it down, and then weigh it (m_2) until the weight of the sample is constant to ± 0.2 mg.

The percentage of holocellulose is determined as follows:

$$\text{Holocellulosic content}(\%) = \frac{(m_2 - m_1)}{m_1 \frac{(100 - H(\%))}{100}} \cdot 100$$

Determination of α-cellulosic content

The decision not to use TAPPI T203-om93 ("Determination of a, β and γ cellulose pulp") for the determination of the α-cellulose content is based on the fact that this procedure is only defined for paper pulp, and places special emphasis on it, so it is understood that it is not applicable to wood. Therefore, in this thesis the protocol followed to determine α-cellulose and hemicellulose content is the one described by Roswel [A.7]. According to

this method, the α-cellulose corresponds to the fraction remaining insoluble from the holocellulose after a treatment with sodium hydroxide and acetic acid, considering the fraction that is solubilised as hemicellulose. This is not totally true, since only α-cellulose is considered in the method, so β and γ-celluloses are considered as hemicellulose, which would result in some error in the characterisation.

The procedure to determine α-cellulosic content consists of:

- Weigh accurately 2.0 ± 0.1 g of sample of dry holocellulose (m_0) in a 100 mL beaker. Holocellulose has to be extracted from the fibres by the method described in the previous section (Determination of holocellulose content).
- Add 10 mL of 17.5% NaOH solution to the beaker. After 5 min, add 5 mL of the same solution and repeat this step 2 times more.
- After the last addition, let the alkali solution react with the sample for 30 min at room temperature.
- Then, stop the reaction adding 33 mL of distilled H_2O to the beaker, and keep the solution at room temperature for 1 h.
- Separate the solid and liquid fractions by vacuum filtration using a previously tared grade 2 pore size Gooch crucible. The Gooch crucible should be previously dried in an oven at 105 ± 3 °C for 6 h, cooled down until room temperature in a desiccator and weighed (m_1).
- Wash the solid residue (α-cellulose) with 100 mL of NaOH solution (8.3%), and then two times more with the same volume of distilled H_2O.
- Then, add 15 ml of acetic acid solution (10%) to the crucible where it remains the solid fraction and let it reacts 3 min.

- After that, remove the acid by vacuum filtration and wash the solid with hot distilled H_2O until the filtrate is neutralised.
- Dry the Gooch crucible with the solid in an oven at 105 ± 3 °C for 24 h.
- Finally, place the crucible in a desiccator until it is cooled down to room temperature and weigh it (m_2).

The α-cellulosic content is determined as follows:

$$\alpha\ cellulosic\ content\ (\%) = \frac{(m_2 - m_1)}{m_1 \frac{(100 - H(\%))}{100}} \cdot 100$$

The hemicellulosic content is estimated by the difference between the initial holocellulosic content and the α-cellulose content of the sample.

$$Hemicellulosic\ content\ (\%) = \text{Holocellulosic content (\%)} - \text{ α cellulosic content (\%)}$$

Appendix III. Procedure for bark characterisation

This appendix collects the procedure used for the chemical characterisation of bark. It was characterised following the procedures described by the National Renewable Energy Laboratory (NREL) as well as other widely used methods.

The NREL technical report [A.5] were used to prepare the raw material and for the determination of moisture, ash, lignin, cellulose and hemicellulose content. The extractive content was measured following the method proposed by Miranda et al. 2016 [A.8] and the suberin content was determined using the method described by Pereira [A.9].

All measurements were performed in triplicate, giving the results as the mean ± standard deviation on an oven-dried basis.

Preparation of sample (NREL/TP-510-42620)

The aim of this procedure is to prepare a uniform lot of raw material suitable for compositional analysis. The homogeneity of the biomass is very important for its uses, because there are many factor that can affect the composition of the biomass, and one of the most important is the particle size. Because of that, the sample preparation is a very important step in the chemical characterisation of the raw material.

The conditioning of the sample consists of drying and grinding the sample until a specific particle size. In this thesis, all the analysed barks were air-died, milled (Restch SM 100) and sieved to a particle size less than 0.5 mm.

Determination of moisture content (NREL/TP-510-42621)

The moisture contained in the biomass is that which is in equilibrium with that in the environment. It is therefore necessary to know it, since it will be taken into account in the subsequent analyses, since the results are typically reported on an oven-dried basis. The procedure used to determine the moisture content consists of:

- Prepare the recipient that will be used for the measurement. It need to be clean and dry, so clean and dry placing it in the oven at 105 ± 3 °C for 6 h. Then after that, cool it down until room temperature in a desiccator, and weigh it on the analytical balance to the nearest 0.1 mg (m_0).
- Weigh 2.00 g of sample in the previously tared recipient with the same analytical balance (m_1).
- Place the recipient with the sample in the oven at 105 ± 3 °C for 24 h.
- Then, placed the recipient with the sample in a desiccator until it is cooled down to room temperature. Finally, weigh the recipient with the sample (m_2) until the weight of the sample is constant to ± 0.1 mg.

The moisture content is determined as follows:

$$Moisture\ content\ (H)\ (\%) = \frac{(m_2 - m_0)}{m_1} \cdot 100$$

Determination of ash content (NREL/TP-510-42622)

The ash content of the sample measures the amount of inorganic matter present in the sample either structural or extractable. Structural ash belongs to the sample and extractable ash is inorganic material that has been transferred to it externally.

This protocol determine the ash content of the bark by measuringof the remaining solid material after the ignition at 575 °C. The used procedure is:

- Weigh accurately 1.00 ± 0.01 g of sample (m_1) in a previously tared crucible. The crucible needs to be clean and previously ignited in a muffle furnace at 525 ± 25 °C for 4 h, after that, place it in a desiccator until it is cooled down to room temperature. Then, weigh it on the analytical balance to the nearest 0.1 mg (m_0).
- Place the crucible with the sample in a muffle furnace at 575 ± 25 °C for 24 ± 6 h.
- After this time, remove the crucible with the sample from the muffle furnace and kept in a desiccator until it is cooled to room temperature.
- Finally, weigh the crucible with the remaining solid (m_2) until the weight of the sample is constant to ± 0.1 mg.

The ash content at 575 °C is determined as follows:

$$Ash\ content\ (AC)\ (\%) = \frac{(m_2 - m_0)}{m_1 \frac{(100 - H(\%))}{100}} \cdot 100$$

Determination of extractives content (NREL TP-510-42619 and Miranda et al. 2016)

This method describes a procedure for determining the amount of non-structural material from biomass, such as resin, fatty acids and their esters or waxes. They are solvent-soluble compounds, and can be extracted with a simple extraction, since these compounds are not part of the cell wall of the biomass. This fraction is important in the bark, because of that three different solvents (CH_2Cl_2, EtOH and distilled H_2O [A.8]) are used for the sequential extraction in order to complete the extraction. Additionally, the present of extractives in biomass could generate interferences in the following measurements, so prior to the following analysis its removal is necessary.

The used procedure to determine the extractive content for each of the selected solvents is based on NREL TP-510-42619, and consists of:

- Clean and dry a 250 mL extraction flask. After 12 h in the oven at 105 ± 3 °C, cooled it down in a desiccator and weigh on the analytical balance to the nearest 0.1 mg (m_0).
- Weigh accurately 5.0 ± 0.1 g (m_1) on an extraction thimble (cellulose cartridge), and then put another thimble on the top to avoid the losing of any raw material.
- Place the thimble with the sample in position in the Soxhlet apparatus. Fill the previously tared extraction flask with 190 mL of the selected solvent (CH_2Cl_2, EtOH or distilled H_2O).
- Connect the flask to the extraction apparatus, and start water flow to the condenser section. Adjust the heaters to provide a boiling rate which will cycle the samples between 6 h and 16 h depending on the solvent. Extraction is carried out sequentially with each of

the solvents listed, and the extraction times are: 6 h for CH_2Cl_2, 16 h for EtOH and 16 h for distilled H_2O.

- When the time is over, remove the flask from the apparatus and partially evaporate the solvent in the extraction.
- Place the extraction flask with the extracts in the oven at 105 ± 3 °C for 24 h.
- Finally, cool down the flask to room temperature in a desiccator, and then weigh the extraction flask with dried extracts (m_2) until the weight of the sample is constant to ± 0.1 mg.

The percentage of extractives for each solvent is determined as follows:

$$Total\ extractive\ content\ (\mathrm{TEC})(\%) = \frac{(m_2 - m_0)}{m_1 \frac{(100 - H(\%))}{100}} \cdot 100$$

Determination of suberin content (Pereira 1988)

This method described a procedure to determine the suberin content. Suberin is a complex aromatic–aliphatic cross-linked biopolyester, which is particularly abundant in tree barks. The procedure for the determination of suberin consist on a methanolysis depolymerisation. The present of suberin in biomass could generate interferences in the following measurements, so prior to the following analysis its removal is necessary.

The procedure to determine the suberin content consists of:

- Clean and dry a 250 mL extraction flask. After 12 h in the oven at 105 ± 3 °C, cooled it down in a desiccator and weigh on the analytical balance to the nearest 0.1 mg (m_0).

- Weigh 1.0 ± 0.1 g (m_1) on previously tared flask and fill it with 167 mL of $NaOCH_3$ (3%, in MeOH).
- Connect the flask to the refrigeration apparatus, and start water flow to the condenser section. Adjust the heaters to provide a boiling rate and let it under reflux for 3 h.
- When the time is over, let the system cool down, remove the bottle and separate the solid from the liquid by filtration.
- Place the filtrated solid again in the flask again, filled with 70 mL of MeOH and let it under reflux for another 15 min.
- When the time is over, let the system cool down, remove the bottle and separate the solid from the liquid by filtration, and mix the liquid phases obtained in both stages.
- Acidify the mixture of the liquid phases up to pH 6 adding H_2SO_4 2M and remove the solvent with a vacuum rotary evaporator.
- Suspend the residue with 70 mL H_2O.
- Performs a liquid-liquid extraction with 150 mL of Cl_3CH. Let the phases separate well and pick up the organic phase. Repeat this process two more times and mix all the organic phases.
- Add Na_2SO_4 to verify that no water is present in the organic phase and then filter it.
- Place the extraction flask with the suberin in the oven at 105 ± 3 °C for 24 h.
- Finally, cool down the flask to room temperature in a desiccator, and then weigh the extraction flask with dried extracts (m_2) until the weight of the sample is constant to ± 0.1 mg.

The percentage of suberin is determined as follows:

$$Total\ suberin\ content\ (\mathrm{TSC})(\%) = \frac{(m_2 - m_0)}{m_1 \frac{(100 - H(\%))}{100}} \cdot 100$$

Quantitative acid hydrolysis (NREL/TP-510-42618)

This protocol described a procedure to determine the structural carbohydrates the acid-insoluble lignin (AIL) and the acid-soluble lignin (ASL) present in extractives-free lignocellulosic biomass. The raw material must be free of extractives because otherwise the lignin measurement could be overestimated. This procedure consists in two consecutive acid hydrolyses, which permits the estimation of the hemicellulosic content and the glucan content of the lignocellulosic biomass by determination of the concentration of the monosaccharides. After the hydrolysis, the solid residue obtained corresponds to the AIL, and the ASL is solubilised in the liquid phase. The main disadvantage of this procedure is that it does not provide an estimation of the cellulose itself, since the glucan content determined in the biomass could correspond to both the cellulosic and hemicellulosic fractions. The quantitative acid hydrolysis (QAH) of biomass samples was carried out as follows:

- Weigh accurately 0.25 ± 0.001 g of the extract-free sample (m_0) in a test tube. The particle size of the sample should be less than 0.5 mm and the moisture of the sample (H) needs be previously determined.
- Add 2.5 mL of 72.0 wt.% H_2SO_4 to the test tube and stir the sample + H_2SO_4 mixture to have an homogeneous mixture. Then, place the test tube in a water bath at 30 °C for 1 h, stirring it periodically.
- After the hour, add distilled H_2O to the mixture in order to stop the reaction. Then, transfer the content of the test tube to a

previously tared pressure flask and add distilled H_2O is until the weight of the whole mixture is 74.33 g (m_1), which corresponds to a H_2SO_4 concentration of 4.0 wt.%.

- Weigh the pressure flask with the mixture (m_2) and autoclave it for 1 h at 121 °C.
- After this hour, cool down the pressure flask and note down the weight of the pressure flask (m_3).
- Separate the mixture by filtration using a previously tared Gooch crucible N° 3. The Gooch crucible should be previously dried in an oven for 6h at 105 ± 3 °C, cooled down in a desiccator and weighed (m_4).
- Dry the solid residue contained in the Gooch crucible in an oven at 105 ± 3 °C for 24 h.
- After this time, cool down to room temperature the Gooch crucible with the solid phase in a desiccator and weigh it (m_5) until the weight of the sample is constant to ± 0.2 mg.
- Analyse the liquid phase for ASL by spectrophotometry (UV absorption at an appropriate wavelength). Dilute the sample with 4% H_2SO_4 to bring the absorbance into the range 0.7-1.0, and measure it.
- Analyse the liquid phase obtained after the filtration by High Performance Liquid Chromatography (HPLC) to measure the concentration of monosaccharides, acetic and galacturonic acid and degradation products (furfural and hydroxymethylfurfural (HMF)).

Throughout this thesis, the HPLC analyses were carried out using a Jasco LC Net II/ADC chromatograph equipped with a refractive index detector and a photodiode array detector. For the determination of the

monosaccharides (glucose, xylose, arabinose, galactose and mannose) Transgenomic 2l1 CARBOSep CHO-682 column was used, working with a flow rate of 0.4 mL water/min at 80 °C and eluting 40 µL of the sample, after neutralizing it with $BaCO_3$. For the determination of the acetic acid, galacturonic acids, furfural and HMF an 300 x 7.8 mm Aminex HPX-87H column (Bio-Rad Laboratories, USA) was used, working with a flow rate of 0.6 mL/min at 50 °C and eluting 20 µL of the sample with a mobile phase of 0.005 M H_2SO_4. The UV-vis spectrometry analyses were carried out using Jasco V-630 UV-VIS spectrophotometer.

The AIL or Klason lignin content of the sample is estimated as follows:

$$Acid-insoluble\ lignin\ (AIL)(\%) = \left(\frac{m_5 - m_4}{m_0 \cdot \frac{(100 - H(\%))}{100}} \cdot (100 - TEC(\%) - TSC(\%)) \right)$$

The ASL is determined as follows:

$$Acid-soluble\ lignin\ (ASL)(\%) = \frac{UV_{abs} \cdot V \cdot D}{\varepsilon \cdot W \cdot Pathlength} \cdot 100$$

Where UV_{abs} is the average UV-vis absorbance at 240 nm, V is the volume of filtrate (L), D is the dilution, ε is the absorption coefficient (l10 $L \cdot g^{-1} \cdot cm^{-1}$), W is the weight of sample (g) and Pathlength is the width of the cuvette (1 cm)

The structural carbohydrates content, measured as glucan, xylan, arabinosyl (ArOS), mannosyl (MaOS), galactosyl (GalactOS), acetyl groups (AcOS) and galacturonic acids (GaAc), is estimated as follows:

$$\text{Glucan/xylan/ArOS/MaOS/GalactOS/AcOS/GaAc (\%)} = F \cdot C_{est} \cdot \frac{[X]}{\rho} \cdot \frac{P}{m_0 \cdot \left(\frac{100 - H(\%)}{100}\right)} \cdot (100 - TEC(\%) - TSC(\%))$$

$$P = \left(m_1 - \left(m_0 \cdot \left(\frac{100 - H(\%)}{100} \right) \cdot \frac{AIL(\%)}{100} \right) \right) \cdot \frac{m_1 - (m_2 - m_3)}{m_1}$$

Where F is degradation of the carbohydrates (see **Table A.I**); C_{est} is a parameter that takes into account the increase of the molecular weight of the monosaccharide during the hydrolysis (see Table A.I); [X] is the concentration (g/L) of the monosaccharide or acids; ρ is the density of the liquid phase obtained in the QAH (1.022 g/L); P is the weight of the liquid phase at the end of the QAH taking into account the losses that could have taken place during the second stage of the QAH.

The hemicellulose content was determined as follows:

$$Hemicellulose\ content(\%) = xylan(\%) + ArOS(\%)) + MaOS(\%) + GalactOS(\%) + AcOS(\%) + GaAc(\%)$$

Table A.I Standarised values of the the F and C_{est} parameters for the different monosaccharides, the acetic acid and the galacturonic acid.

	Glucan/Manosyl/ Galactosyl substituents	**Xylan/Arabynosyl substituents**	**Acetyl substituents**	**Galaturonic acid substituents**
	$GC_n/CMa_n/CGa_n$	CX_n/CAr_n	CG_A	CG_{GaAc}
	Flucose/Mannose/galactose	Xylose/Arabinose	Acetic acid	Galacturonic acid
F	1.04	1.088	1.00	1.00
C_{est}	162/180	132/150	43/60	212/230

Appendix IV. Procedure for the characterisation of the obtained extracts

In this appendix, the experimental procedure for the chemical and structural characterisation of the extracts obtained at the characterisation of the raw material as well as in the studied extraction is described.

The chemical characterisation of the extracts was carried by determining the extraction yield measured as their non-volatile content. The total phenolic content (TFC) and total flavonoid content (TFC) of the extracts were also determined using the Folin-Ciocalteau (FC) method [A.10] and the $AlCl_3$ colorimetric assay procedure described by Lima et al. [A.11], respectively. These analyses were carried out in triplicates. In addition, three methods were used to evaluate the antioxidant capacity of the extracts, DPPH, ABTS and FRAP. Methodology of Gullón et al. [A.12] was used for the DPPH measurement. FRAP assay was performed according to the methodology described by Benzie and Strain [A.13]. Finally, the methodology described by Re et al. was used to measure ABTS assay [A.14].

The structural characteristics of the compounds present in the extracts were determined by subjecting them to instrumental analytical techniques such as HPSEC, ATR-FTIR and UPLC-DAD-ESI-MS. Prior to the HPSEC and ATR-FTIR analyses the extracts were over dried to facilitate their analysis.

Determination of extraction yield by determining the non-volatile content (NVC)

The non-volatile content (NVC) of the extracts corresponds to its solid content, which can be constituted by waxes, fatty acids, terpenes,

flavonoids, lignans, tannins and extractable carbohydrate. The NVC value corresponds to the extraction yield because the aim is to measure the amount of all the extracted compounds. The procedure used for the determination of the NVC consists of:

- Clean and dry a glass vessel. After 12 h in the oven at 105 ± 3 °C, cooled it down in a desiccator and weigh on the analytical balance to the nearest 0.1 mg (m_0).
- Weigh accurately 2.00 ± 0.01 g of the liquor (m_1) in a previously tared recipient.
- Keep the recipient with the sample in the oven at 105 ± 3 °C for 24 h.
- After this time, introduce the recipient with the sample in a desiccator until it cools down to room temperature and weigh it (m_2) until the weight of the sample is constant to ± 0.1 mg.

The extraction yield of the extracts is determined as follows:

$$Extraction\ yield\ (\%) = \left(\frac{m_2 - m_0}{m_1 \cdot \left(\frac{100 - H(\%)}{100} \right)} \right)$$

Determination of the total phenolic content (TPC)

The tree bark extracts are rich in phenolic compounds; this is why the total phenolic content (TPC) was determined. The measurement of the TPC of the extracts was carried out according to the Folin-Ciocalteau (FC) method, which is based on the measurement of the colour change of the FC reagent in contact with a reducing agent.

Prior to the determination of the TPC, a calibration curve has to be constructed to interpret the absorbance measurements, and to transform

them in TPC, expressed as gallic acid equivalent (GAE). The calibration curve was constructed using 10 MeOH solutions of gallic acid with concentrations between 0 and 0.34 g/L.

The determination of the gallic acid present in the calibration solutions and the phenolic content of the extracts was performed as follows:

- Place 300 µL of the extract diluted with MeOH or the calibration solution in a test tube.
- Add 2.5 mL of a 1/10 (v/v) aqueous solution of the FC reagent to the test tube and stir it for 1 min by a vortex.
- Add 2 mL of 7.5 % (w/v) Na_2CO_3 solution and stir it for another minute by the vortex.
- Fill the test tube with parafilm, cover it with aluminium foil and keep it in a bath for 5 min at 50 °C.
- After this time, cool down the test tube at room temperature and then measure the absorbance of the sample at 760 nm. In this thesis, the measurements were carried out in a Jasco V-630 UV-vis spectrophotometer.

Determination of the total flavonoid content (TFC)

The tree bark extracts are rich in flavonoid compounds; this is why the total flavonoid content (TFC) was determined. The measurement of the TFC of the extracts was carried out according to the method described by Lima et al. [A.11], which is based on a spectrometric analysis using $AlCl_3$. This method is widely used for the determination of flavonoids as it does not present interferences of other phenolic compounds.

Prior to the determination of the TFC, a calibration curve was constructed using catechin as reference. This curve permits the interpretation of the absorbance measurements, and their transformation in TFC, expressed as catechin equivalent (CE). The calibration curve was constructed using 6 MeOH solutions of catechin with concentrations between 0 and 0.4 g/L.

The determination of the catechin present in the calibration solutions and the flavonoid content of the extracts was carried out as follows:

- Place 1 mL of the extract diluted with MeOH or the calibration solution in a test tube.
- Add 0.3 mL of $NaNO_2$ (5.0 % (w/v)) and stir it for 1 min by a vortex.
- Wait 5 min, add 0.3 mL of $AlCl_3$ (10.0 % (w/v)) and stir it for another min by a vortex.
- Wait 6 min and add 2 mL of NaOH (1 N) and stir it for another minute by a vortex.
- Wait 5 min and measure the absorbance of the sample at 510 nm. In this thesis, the absorbance measurements were carried out in a Jasco V-630 UV-vis spectrophotometer.

Determination of the antioxidant capacity by DPPH

One of the procedures used for the determination of the antioxidant activity of the extracts was the DPPH [A.12], which is a method that measures the quality of hydrogen donors. In this assay, the capacity of the extract to reduce the DPPH radical is measured spectrophotometrically.

Prior to the determination of the capacity of the extracts to reduce the DPPH radical a calibration curve was constructed using Trolox as reference. The calibration curve permits the interpretation of the absorbance

measurements to transform them in Trolox equivalente antioxidant capacity, expressed as Trolox equivalent (TE). The calibration curve was constructed using 10 MeOH solutions of Trolox with concentrations between 0 and 0.05 g/L.

- Place 300 µL of an extracts diluted with MeOH or of the calibration solutions in a test tube.
- Add 3 mL of DPPH solution in MeOH (0.06 mM) and stir it for 1 min using a vortex.
- Fill the test tube with parafilm, cover it with aluminium foil and keep it in the dark at room temperature for 15 min.
- After that time, measure the absorbance of the sample at 515 nm. The measurements were carried out in a Jasco V-630 UV-vis spectrophotometer.

Determination of the antioxidant capacity by ABTS

Another procedure used for the determination of the antioxidant activity of the extracts was the ABTS [14]. In this assay, as it happens with the DPPH assay, the capacity of the extract to reduce the ABTS radical is measured spectrophotometrically.

Prior to subjecting the extracts to the ABTS assay, a calibration curve was constructed using Trolox as reference. The calibration curve permits the interpretation of the absorbance measurements in order to transform them in Trolox equivalent antioxidant capacity, expressed as Trolox equivalent (TE). The calibration curve was constructed using 10 MeOH solutions of Trolox with concentrations between 0 and 0.6 g/L.

- Prepare the ABTS radical solution by mixing 7 mM ABTS stock solution with 2.45 mM potassium persulfate add PBS buffer up to 25 mL. Then, left the mixture for 12-16 h under stirring in the darkness at room temperature to ensure the radical full formation.
- Afterwards, dilute the ABTS radical solution with PBS buffer to have an absorbance of 0.70 at 734 nm. In this thesis, the absorbance measurements were carried out in Jasco V-630 UV-vis spectrophotometer.
- Place 30 µL of extracts diluted with MeOH or of the calibration solutions in a test tube.
- Add 3 mL of the diluted ABTS radical solution.and wait 6 min.
- Wait 6 min and measure the absorbance of the samples at 734 nm.

Determination of the antioxidant capacity by FRAP

The las procedure used for the estimation of the antioxidant capacity of the extracts was FRAP assay [A.13], which is based a reduction of the complex ferric ion-TPTZ. This reaction causes a change in the colour of the solution, and is measured in a spectrophotometrically.

As it was done previously in antioxidant trials before subjecting the extracts to the FRAP assay, a calibration curve was constructed using MeOH solutions containing between 0 and 0.5 g/L of Trolox. The results were expressed as Trolox equivalent (TE).

- Mix 25 mL of a 300 mM acetate buffer (pH 3.6), 2.5 mL of a 10 mM solution of TPTZ and 2.5 mL of 20 mM $FeCl_3 \cdot 6\ H_2O$ to prepare the FRAP reagent solution.

- Place 100 µL of the calibration solutions or of extracts diluted with MeOH.
- Add 3 mL of the FRAP reagent and stir it for 1 min by a vortex.
- Wait 6 min and measure the absorbance of the sample at 593 nm. In this work, the measurements were carried out in a Jasco V-630 UV-vis spectrophotometer.

Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR) spectroscopy analysis

Attenuated Total Reflectance Fourier transform infrared spectroscopy (ATR-FTIR) was used to analyse the main chemical functionalities of the extracts. It was determined on a PerkingElmer Spectrum Two spectrometer fitted with a Universal Attenuated Total Reflectance accessory. The defined working range was from 700 to 4000 cm^{-1} with 4 cm^{-1} resolution with 12 registered scans.

High Performance Size Exclusion Chromatography (HPSEC) analysis

The molecular weight distribution of the extracts was determined by HPSEC. The measured parameters were average molecular weight (Mw), number-average (Mn) and polydispersity index (Mw/Mn). Prior to the HPSE analysis, a solution of dried solid in dimethylformamide (DMF) with 0.1% of lithium bromide was prepared (5 g/L). The analyses was carried in a Jasco LC Net II/ADC chromatograph equipped with a RI 2031Plus reflex index detector and two PolarGel-M columns in series (Varian Polymer Laboratories) and PolarGel-M guard (Varian Polymer Laboratories). The

used conditions were 0.7 mL per min flow, 20 µL of injection volume and temperature of 40 °C using DMF with 0.1% of lithium bromide as eluent.

The calibration of the HPSEC was carried out using polystyrene standards ranging from 266 to 62,500 g/mol (Sigma Aldrich). The results obtained by the HPSEC analysis are indicative so their comparison with other works should be done cautiously.

Ultraperformance Liquid Chromatography-Diode Array Detector-Electrospray Ionisation-Mass Spectrometry (UPLC-DAD-ESI-MS) analysis

The components in the extracts were identified by UPLC-DAD-ESI-MS dissolving them in CH_3CN at a concentration of 0.5 mg/mL. The UPLC-DAD-ESI-MS analysis was performed on a UPLC instrument (Acquity, Waters) fitted with a diode array detector. The compounds separation was carried out at 30 °C using C18 analytical column (Acquity (Waters), 100 mm × 2.1 mm, 1.7 mm particle size). The mobile phase consisted of 0.1% formic acid, v/v (phase A) and MeOH (phase B). A 0.3 mL/min constant flow rate was applied with the following elution gradient: 0 min 95% A up to 0.5 min, 16 min 1% A up to 18 min, and 18.5 min 95% A up to 20 min. 5 µL were used as injection volume in the UPLC system. The UV spectra were recorded from 190 to 500 nm, but only cromatogramas at wavelengths 254, 320 and 350 nm have been studied. For the mass spectrometry analysis a LCT Premier ESI-TOF (Waters) was used. The analyses were performed using scans from m/z 50 to 2000. The capillary and cone voltages were set at 2000 and 50 V, respectively, in positive and negative ionization mode.

Appendix V. Procedure for the characterisation of ionic liquids and deep eutectic solvents

This appendix collects the spectroscopy techniques used for the structural characterisation of the synthesised ionic liquids (ILs) and deep eutectic solvents (DES). Their structural characteristics were determined by subjecting them to instrumental analytical techniques such as ATR-FTIR and NMR.

Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR) spectroscopy analysis

The chemical structure of the ILs and DES was evaluated by Attenuated Total Reflectance Fourier transform infrared spectroscopy (ATR-FTIR). It was determined on a PerkingElmer Spectrum Two spectrometer fitted with a Universal Attenuated Total Reflectance accessory. The defined working range was from 700 to 4000 cm^{-1} with 4 cm^{-1} resolution with 12 registered scans.

Nuclear magnetic resonance (NMR)

Nuclear magnetic resonance (NMR) spectra were recorded at 30 °C on a Bruker Ultrashield 400 MHz equipped with a z gradient BBI probe. Typically, 40 mg of sample were dissolved in DMSO-d6. 2D-NMR (HSQC) spectra were recorded with a relaxation delay of 1.43 over 32 scans. The spectral widths were 5000, 25000 and 55000 Hz for the ^{1}H, ^{13}C and ^{19}F dimensions, respectively.

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The valorisation of biomass for fuel and chemicals appears to be an alternative to the current situation of fossil fuel depletion and environmental awareness. The employment of wastes coming from forest biomass to obtain added-value compounds could also involve an economic benefits in favour of the sustainable development. In this context, different methods are proposed for obtaining bioactive molecules as added-value products from the extractive fraction of the tree bark. The valorisation of this fraction could be a key step in the development of a multiproduct biorrefinery of extractive rich lignocellulosic materials.

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