HIGH ADDED-VALUE COMPOUNDS FROM THE INTEGRAL REVALORISATION OF WINERY RESIDUES

Izaskun Dávila Rodríguez

Donostia-San Sebastián 2019

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HIGH ADDED-VALUE COMPOUNDS FROM THE INTEGRAL REVALORISATION OF WINERY RESIDUES

A dissertation presented by

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In fulfilment of the Requirements for the Degree Doctor of Phylosophy by the University of the Basque Country

In the program Renewable Materials Engineering

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Donostia-San Sebastián 2019

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AGRADECIMIENTOS

Hasta el momento en el que uno empieza a escribir la tesis no se da cuenta de todo lo que ha sucedido, ni de toda la gente que de manera más o menos directa ha formado parte de esta etapa de tu vida. Y aunque no sea una experta en verbalizar sentimientos, no podían faltar unas líneas para darles las gracias.

Primeramente, querría agradecer al Dr. Jalel Labidi por el apoyo, confianza y por haber visto algo en mí que le hizo darme la oportunidad de llevar a cabo esta tesis. Al Dr. Vitaliy Budarin por darme la opción de realizar la estancia en el Green Chemistry Centre of Excellence y por la ayuda suministrada. A María, Almudena y Javi por hacer que el estar fuera de casa fuese llevadero y por mantener rutinas tan cotidianas como el comer fuera del lugar de trabajo, que por lo general no valoramos tanto. También, quisiera agradecerle a Almudena las excursiones, los cafés y los momentos de confidencias, vamos en definitiva por hacer de York una estancia agradable.

Al grupo de investigación BioGroup de la Universidad de Santiago de Compostela y en particular a la Dra. Beatriz Gullón por darme la oportunidad de colaborar con ellos y por ese trabajo en equipo.

Tampoco podrían faltar agradecimientos para mis aitas, mi hermano y mi chico por aguantarme cuando he estado más tensa y por los consejos, ánimo, confianza y apoyo suministrados a lo largo de esta etapa y que sigáis igual en las siguientes etapas que puedan venir. Además, agradecer a este último su habilidad de distraerme y hacerme desconectar. También agradecer a mis amigas el estar ahí cuando me ha hecho falta proporcionando el apoyo o la fuerza necesarios.

A mis chic@s les tengo que agradecer su capacidad por hacer que el ir a trabajar no sea solo eso, sino que sean momentos de risas, desconexión o desahogos. Resaltar ese trabajo de equipo tanto dentro como fuera del trabajo. ¡Seguid así!

Agradecer al grupo de investigación BioRP tanto a profesores como compañeros por el apoyo y ayuda proporcionados a lo largo de este trabajo.

Y por último y no por ser menos importante, a la Dra. Patricia Gullón sin la cual esta tesis no hubiese sido la misma. Gracias por estar ahí tanto cuando hemos tenido buenas rachas como cuando nos ha tocado sortear obstáculos y además por ser una amiga o compañera más que una codirectora.

Muchas gracias, eskerrik asko a todos.

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La crisis medioambiental es un problema global y solo la acción global lo resolverá.
-Barry Commoner

Chapter 1

Introduction

1.1 From fossil to renewable resources

The actual society presents a huge dependence on fossil resources, being their main application the production of energy, and to a lesser extent the production of chemicals and materials. However, the natural reserves of coal, crude oil/petroleum, natural gas etc. are limited due to their high exploitation and their lack of renewability. The depletion of these resources could be associated with their use under a linear economy, according to which goods are produced from virgin resources and once they are employed, they are thrown away as no more usable wastes. It has been estimated that the crude oil and the natural gas would last for 22 and 24 years more, respectively, being the coal the only remaining fossil resource after 2042, which will last until 2112 [1.1]. However, although the fossil resources would tend to disappear due to their usage under a linear economy, the energy consumption is not expected to go decreasing with the time, as its trend is opposite to the one observed for the fossil resources. According to the International Energy Agency (IEA) the world energy demand seem to be growing with a rate of 1.6 % per year [1.2], being 1400 million tonnes of oil equivalent (mtoe) of primary energy consumed worldwide in 2017 according to the British Petroleum Company (BP) [1.3]. 79.7 % of the energy consumed nowadays is provided by non-renewable fossil energy sources or by nuclear energy, while only a 20.3 % is obtained from renewable energy sources, being the 15.3 % provided by hydropower and the remaining 5.0 % by other energy sources [1.4].

Several actions could be taken to delay the depletion time of the fossil resources; as it could be the improvement of the energy efficiency, the exploration of new fossil reserves or the promotion of the development of renewable energies and their market introduction [1.5].

The European Commission, for instance, has developed the Horizon 2020 funding programme with the objective of accelerating the conversion of fossil-based European industries, to low carbon, resource efficient and sustainable industries for the production of energy and chemicals [1.6]. They estimate that the shift from fossil-based industries to bio-based ones, in which biological raw materials and processing methods are used, could save up to 2.5 billion tons of CO_2 equivalent per year by 2030. This substitution could reduce the environmental problems

associated with the fossil-based industries, such as the acid rain, the climate change, the global warming or the harmful effects on the human health and on the aquatic life [1.4].

The use of biological raw materials such as lignocellulosic biomass instead of fossil resources for the production of chemicals, fuels and materials has gained attention in the last decades [1.7]. The employment of this biomass in bio-based industries is favoured not only by their quick production, high availability and limited price [1.8] but also because they constitute the non-edible portion of the plants and they do not interfere with the food supplies. This usage of the lignocellulosic biomass could reduce the serious environmental problems associated with the disposal of high volumes of these wastes on the soils and landfills.

A new approach of the actual energy and chemical production systems is necessary due to the depletion of the fossil resources. The biorefineries are potential candidates to be considered as feasible alternatives to the fossil-based industries due to the employment of renewable feedstock and environmentally friendly technologies.

1.2 Biorefineries

The biorefinery concept was defined by the International Energy Agency (IEA) Bioenergy Task 42 as "the sustainable processing of biomass into a spectrum of marketable bio-based products and bio-energy" [1.9]. In other words, a biorefinery is an industrial facility, or network of facilities, that covers an extensive range of combined technologies aimed at full sustainable transformation of biomass into building blocks with the concomitant production of biofuels, energy, chemicals and materials, preferably of added-value [1.10].

The biorefinery approach presents many similarities with the fossil oils refinery as instead of using fossil oils to produce multiple fuels, basic chemicals, intermediate products and sophisticated products, the components of the biomass and their intermediates are used. However, the heterogeneity of the biomass and its more complex structure compared with the fossil oils makes the biorefinery more challenging, as a higher number of transformations and separations are needed. Nevertheless, the transformation and the separation processes applied in the

biorefineries have a relatively lower impact on the greenhouse gas emissions than the ones used in the fossil-based industries [1.11].

Independently of the feedstock used, the biorefinery approach commonly involves a multiple-step process (Figure 1.1) for the fractionation and further up-grading of the components of the biomass. Commonly, the biomass is firstly treated to make it more susceptible for a further process, being this first procedure referred as the pre-treatment. In some cases, depending on the process, the isolation of a single fraction takes place, being possible to subject the obtained residue to an additional treatment to fractionate the biomass to a greater extend. Then, each component of the biomass are subjected to a subsequent process in which the isolated fractions could be converted into added-value products for further applications [1.12].

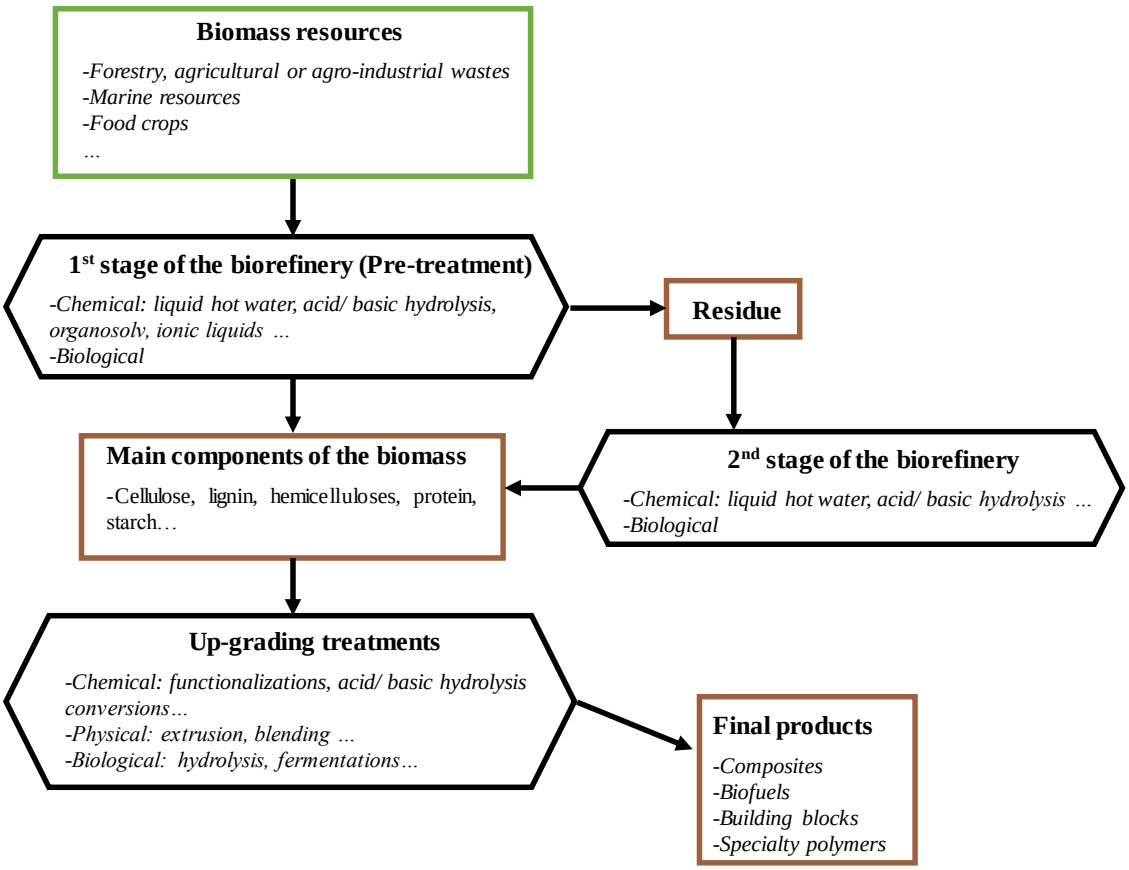


Figure 1.1. Common stages of a multiple-step biorefinery.

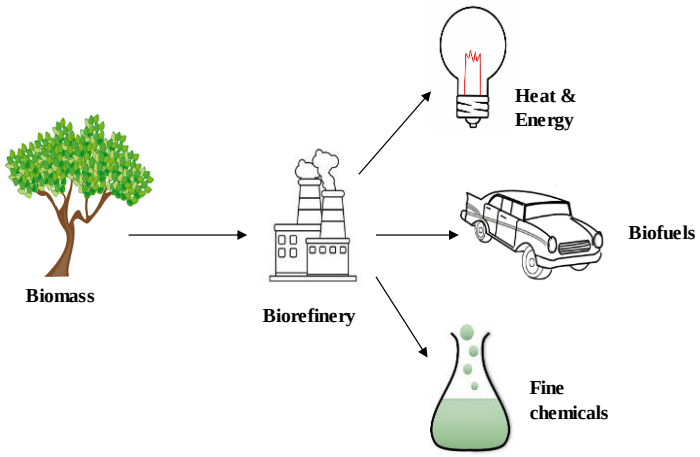
Nevertheless, not all the biorefineries follow the scheme shown in Figure 1.1, as there are treatments that could separate the main fractions of the lignocellulosic biomass in a single step. The thermochemical treatments, such as the steam explosion or the ammonium recycle

percolation (ARP) process, for instance, could disrupt the lignocellulosic biomass in its different fractions [1.13], being possible to upgrade each one separately for the obtaining different added-value products. The separation of the main fractions of the lignocellulosic biomass in a single step could also be carried out using concentrated acids , as its cellulosic and hemicellulosic fractions are hydrolysed leaving the lignin unaltered [1.13].

The different stages of the biorefinery are designed to maximise the production of added-value compounds while the waste streams are minimised by converting the residues into energy. This makes the biorefineries work as a zero waste systems in which the generation of the high-value products increases the profitability of the biorefinery while the use of the residues could help decreasing its global energy demand. This philosophy works in parallel with the circular economy concept, which 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" [1.14]. Thus, the application of the circular economy and the zero waste philosophies in the biorefineries permit the prevention, minimisation and valorisation of the wastes generated once the natural goods reach their end-of-life, solving the problems of the landfills and the fossil-source depletion [1.15].

1.2.1 Types of biorefineries

The great variety of feedstock and the number of processes employed to generate multiple added-value products make it necessary to somehow classify the different biorefineries that could be settled down. Up to now, the bio-based industries have been characterised using different criteria such as the technological implementation status, the type of raw material or the main type of conversion process used. Depending of the criterion applied the biorefineries are classified in different groups as it can be seen in Figure 1.2.



Types of Biorefineries

Technological implementation status	Raw material	Conversion process
1st generation *Harvested biomass or food crops* 2nd generation *Agricultural and forestry wastes and industrial effluents* 3rd generation *Sea resources* 4th generation *Genetically modified feedstock*	Whole-crop biorefinery *Entire crops, such as wheat, rye or maize* Green biorefinery *Wet feedstock derived from untreated products as grass or green plants* Lignocellulosic biorefinery *Nature-dry feedstock, such as cellulose containing biomass and wastes* Two-platform concept biorefinery *Sugar and syngas platforms*	Physical *The chemical structure of the biomass components is not changed* Biochemical *Microorganisms or enzymes are used* Chemical *Chemical changes occur on the biomass* Thermochemical *Feedstock undergoes extreme conditions of temperature and/or pressure*

Figure 1.2. Classification of the biorefineries according to different criteria.

However, as the same bio-based industry could be classified in more than one group depending on the criterion used, in 2008 the IEA Bioenergy Task 42 developed a biorefinery classification system based on a schematic representation of full biomass to end-products chains [1.16]. The classification of the biorefineries according to this system consists on describing them using the following four main features, which are further categorised in the groups shown in Table 1.1 [1.17]:

- **Platforms**. It considers the intermediates generated by the fractionation of the biomass. The further processing of the platform converts it into energy and/or chemical products. This feature is the main pillar of this classification as platforms could be obtained by different conversion processes from different raw materials.

- **Products**. It considers the final products generated in the biorefineries and they are subcategorised as energetic (energy-products) and non-energetic products (material products).
- **Feedstock**. It considers the renewable raw material used to obtain the final products and it is subcategorised as dedicated crops and residues.
- **Processes**. It considers the type of the main process used to convert the feedstock into the main marketable product. The processes are subcategorised as thermochemical, biochemical, chemical and mechanical/physical.

By classifying the biorefineries using these four features a complete description of their activity is given, leaving no point to ambiguity.

Table 1.1. The features and the subcategories used to classify the biorefineries.

Platforms	Products	Feedstock	Processes
C5 sugars	**Energy products**	**Dedicated crops**	**Thermochemical**
C6 sugars	Biodiesel	Oil crops	Combustion
Oils	Bioethanol	Sugar crops	Gasification
Biogas	Biomethane	Starch crops	Hydrothermal up-grading
Syngas	Synthetic biofuels	Lignocellulosic crops	Pyrolysis
Hydrogen	Electricity and Heat	Grasses	Supercritical
Organic juice		Marine biomass	
Pyrolytic liquid	**Material products**		**Biochemical**
Lignin	Food	**Residues**	Fermentation
Electricity and Heat	Animal feed	Lignocelullosic residues	Anaerobic digestion
	Fertilizer	Oil-based residues	Aerobic conversion
	Glycerin	Organic residues and others	Enzymatic processes
	Biomaterials		
	Chemicals and Building Blocks		**Chemical processes**
	Polymers and Resins		Catalytic processes
	Biohydrogen		Pulping
			Esterification
			Hydrogenation
			Hydrolysis
			Methanisation
			Steam reforming
			Water electrolysis
			Water gas shift
			Mechanical/Physical
			Extraction
			Fiber separation
			Mechanical fractionation
			Pressing/Disruption
			Separation

1.2.2 Future perspectives of the biorefineries

The fact that drives the implementation of the biorefinery concept in the industrial sector is its capacity to revalorise biomass by obtaining products of added-value by less energy requiring and waste generation technologies than the ones employed in the fossil oil refineries [1.10]. Nevertheless, although the implementation of the biorefineries is promoted in some cases by funding programs such as the Horizon 2020, their development is still slow. Nowadays, there are examples of bio-based industries in countries such as USA, Brazil or Europe [1.10], being the different European biorefineries shown in Figure 1.3.

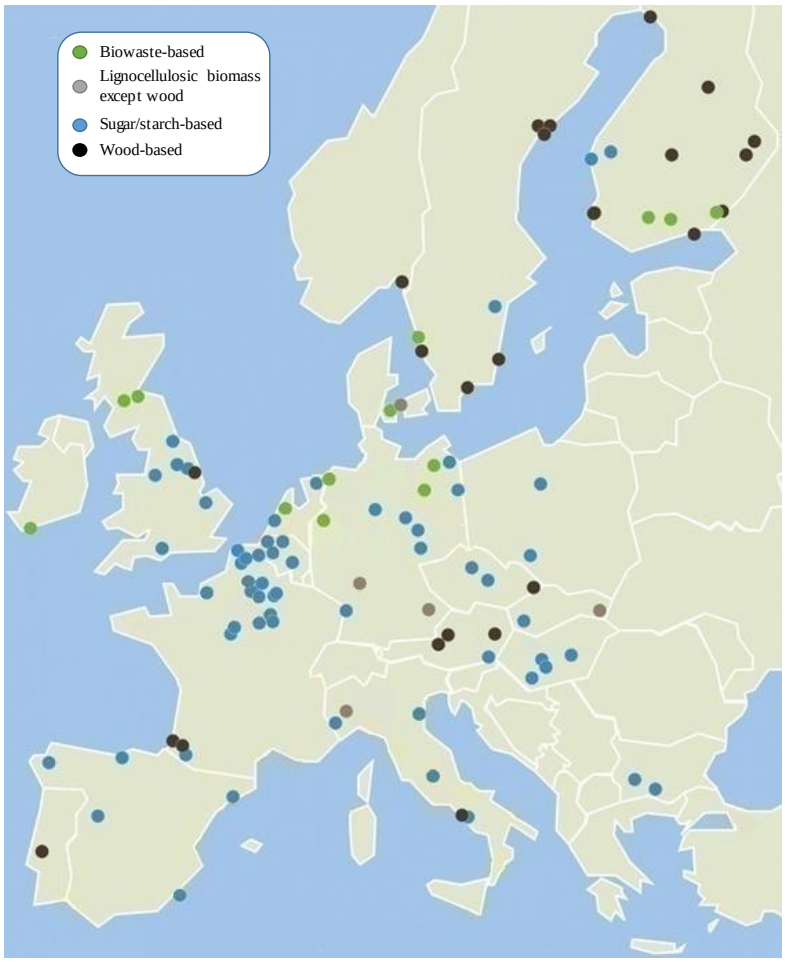


Figure 1.3. Different types of biorefineries based on the employed feedstock, that were settled in Europe in 2017 [1.18].

Examples of the implementation of biorefineries in Europe are the pulp industries Lenzing AG and Borregaard´s, which have gradually evolved into biorefineries. The first one is located in

Austria and it employs wood components to produce pulp (40.0 % of the wood), bioenergy (50.0 % of the wood) and acetic acid, xylitol, furfural or soda from the remaining 10.0 % of the wood. Borregaard`s is a Norwegian biorefinery which manufactures cellulose, lignin, vanillin and bioethanol from wood.

It is foreseen that in the near future the appearance of new bio-based industries can take place [1.10]. Nevertheless, some challenges need to be achieved in order to accelerate their implementation and their spread worldwide. It is necessary to increase the production of added-value compounds from basic raw materials, to promote the introduction and the establishment of biorefinery demonstration plants and to boost the development of the ecological transport of biomass [1.19].

In order to facilitate the implementation of the biorefinery products in the market, the research, the development and the production methods should be oriented to carry out an efficient revalorisation of undervalued non-food biomass into carbohydrates, lignin or proteins and to perform efficient pulping and fractionation processes, between other aspects [1.19]. This could lead to the development of more sustainable technologies that could make the use of the resources in each step of the biorefinery process more efficient, by reducing its operation time [1.10].

Another aspect that could make the bio-based industries economically more competitive against the fossil oil refineries is the obtaining at lower cost new products with similar or enhanced properties to the ones existing in the market. They could also be competitive by producing compounds with unique properties, instead of trying to develop new processes to generate existing products [1.12].

1.3 From wineries towards the future biorefineries

The biorefineries can be used for the exploitation of agricultural, forestry and agro-industrial residues increasing the sustainability and the economic viability of the corresponding activities, since added-value products could be obtained from the generated residues. In particular, in this thesis the attention was focused on agricultural residues, whose availability is estimated to be 10^{10}

Mt worldwide, being the wheat and rice straw the most employed raw materials in biorefineries [1.2]. The corn stover, cotton stalk, barley stalk, sugarcane bagasse and empty oil palm fruit bunch, between others, are also starting to be considered as potential biorefinery feedstocks [1.2]. Apart from the mentioned agricultural residues, the ones generated from fruit crops also have their importance, being the grapes the largest fruit crops after the watermelon, bananas and apples [1.20]. According to the Food and Agriculture Organisation of the United Nations Statistics (FAOSTAT) 74.08 Mton of grapes were produced worldwide in 2014, being China (12.63 Mton), USA (7.15 Mton), Italy (6.93 Mton), Spain (6.22 Mton) and France (6.20 Mton) the main producers [1.21]. From the grapes produced annually, a 15.0 % is destined to the production of wine, being 29.10 Mton of wine produced in 2014 [1.21]. Italy (4.79 Mton), Spain (4.61 Mton), France (4.29 Mton), USA (3.30 Mton) and China (1.70 Mton) were the most important wine producers worldwide that year [1.21]. In Figure 1.4, the amount of wine produced worldwide is shown, using the data provided by FAOSTAT [1.21].

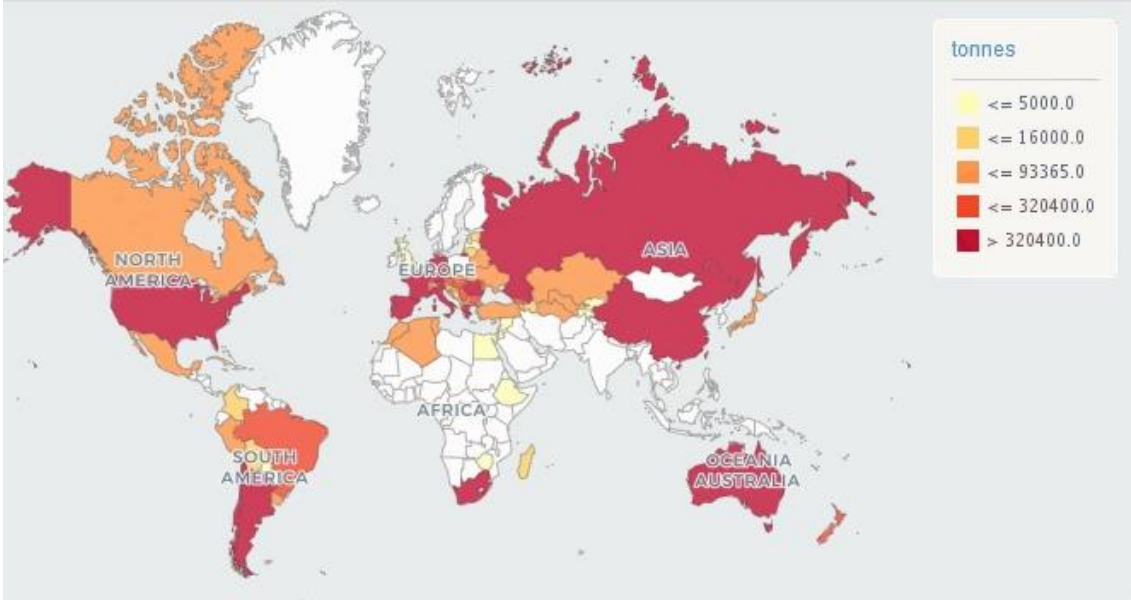


Figure 1.4. Quantity of wine produced worldwide in 2014 by the FAOSTAT [1.21].

Nevertheless, not the whole grape is employed during the wine making process, as up to a 30.0 wt.% of it remains as a solid waste [1.22]. Only in Europe 14.5 million tons of grape´s residues

are produced annually [1.23], thus the high quantity of generated by-products makes the viticulture an agricultural activity in which the biorefinery concept could be implemented.

The by-products are generated throughout the entire wine production process, starting from the removal of the grapes from the vines to the bottling of the wine, as it can be seen in Figure 1.5.

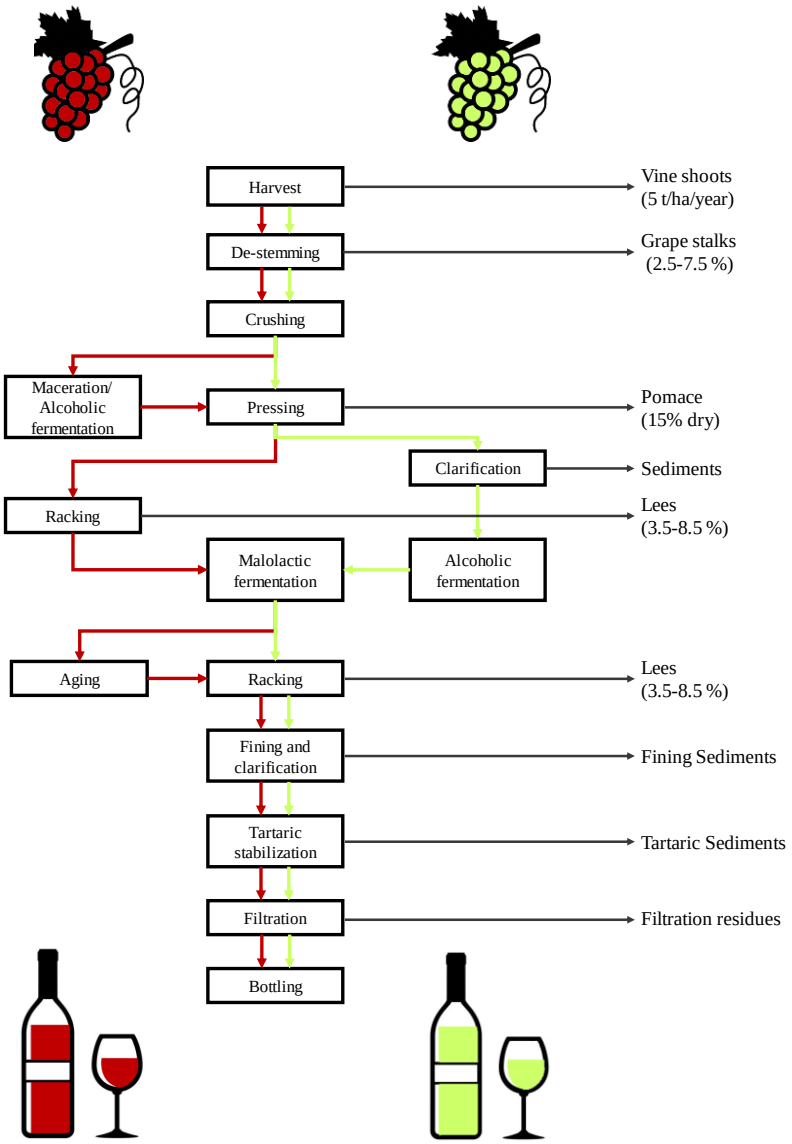


Figure 1.5. Stages of the white and red wine production and the residues generated in each stage, adapted from Dávila et al. [1.24]. The amount of wine residues is represented as its percentage in the grape, being the data adapted from Nerantzis et al. [1.25].

The main residues generated during the wine production, are represented by: organic wastes (vine shoots (also denoted as vine pruning or vine cane), grape pomace (containing seeds, skins, and pulp), grape stalks, grape leaves…) wastewater, emission of greenhouse gases (CO_2, volatile

organic compounds, etc.) and inorganic wastes (diatomaceous earth, bentonite clay and perlite) [1.26].

The disposal of the winery residues could be a problem with ecologic and economic impacts, as not all the wineries always follow the legislation and the winery residues increase constantly. The environmental problems associated with the wineries include the water pollution, the soil degradation, the damage of vegetation, the odours and air emissions or the noise from vehicles and equipments [1.24]. The environmental problems of the wineries could be minimised by using technology adapted to the environmental restrictions, reducing the water consumption, recovering the residues or reducing the produced wastes according to the ISO 14000 [1.26]. The economic viability of the wineries could be increased by using the winery residues under a biorefinery approach in order to recover valuable compounds.

1.3.1 Revalorisation of the winery residues using a biorefinery approach

Up to now, the winery residues are mainly sold to biogas companies to produce energy, they are also used to elaborate soil fertilizers [1.24] and they are employed as fermentation substrates for biomass production and livestock feeds [1.27]. However, there are several constraints for the current revalorisation routes of these residues. For example, the polyphenolic compounds present in some winery residues could present antimicrobial activities making these residues not suitable for their employment as livestock feeds [1.22].

Thus, in the recent years the revalorisation of the different winery by-products under a biorefinery approach for the obtaining of added-value compounds has gained attention. The type of residues and their physicochemical characteristics determine their use and their specific valorisation route. The research on the revalorisation of the winery residues has been focused on the obtaining of a single or multiple added-value compounds, using in some cases a pre-treatment of the biomass to facilitate the subsequent fractionation processes. The variety of added-value compounds obtained from winery residues is very diverse with application in many fields, such as medicine or the

cosmetic, material or food industries, between others. In addition, they have also been used as fuels and energy sources. In Table 1.2 examples of single added-value products obtained from different winery residues are shown.

Table 1.2. Added-value products obtained from different winery by-products.

Winery by-product	Composition	Added-value products	Extraction procedure	References
Grape pomace or grape marc	Polysaccharides, lignin, acid pectic substances, phenols, proteins and insoluble proanthocyanidins	Ethanol	Solid state fermentation	[1.29]
		Methane	Anaerobic digestion	[1.30]
Grape stalks	Cellulose, lignin, hemicelluloses and tannins	Ethanol	Autohydrolysis or acid hydrolysis + fermentation	[1.31]
		Polysaccharides	Delignification with 14.0 % peracetic acids + extraction with DMSO + precipitation with EtOH	[1.32]
Grape seeds	Fiber, proteins, lipids (fat and oils), minerals, carbohydrates and tannins	Polyphenols	Extraction with Acetic acid/MeOH (5/100 mL)	[1.33]
			Ultrasound assisted extraction with 44.0 % EtOH	[1.34]
		Oil	Extraction with supercritical CO_2	[1.35]
Vine shoots	Cellulose, lignin, hemicelluloses and proteins	Polyphenols	Extraction with 1000mM NaOH	[1.36]
Wine lees	Yeast, bacterias, insoluble carbohydrates, phenolics, lignin, proteins, metals and organic salts	Methane	Thermophilic anaerobic digestion	[1.37]
		Squalene	Extraction with supercritical CO_2	[1.38]

The employment of these wastes for the obtaining of multiple added-value compounds, instead of a single product, could increase the economic value of the residue, apart from adjusting the process more to the circular economy and to the zero waste philosophy, since their integral revalorisation is carried out. In the literature, there are some examples in which the integral

revalorisation of different winery wastes such as grape seeds, grape pomace, grape skins or wine lees, between others, has been carried out for the production of multiple added-value compounds. Yedro et al. [1.39], for instance, developed a biorefinery approach for the integral revalorisation of the grape seeds for the obtaining of different added-value products such as, essential oils, polyphenols, sugars and lignin. Firstly, the grape seeds were subjected to a solvothermal extraction using a mixture of ethanol and water for the extraction of essential oils and polyphenols. Then, the solid residue was subjected to different steps of a hydrothermal fractionation-hydrolysis treatment carried out between 150 and 340 ºC for the solubilisation of its hemicellulosic and cellulosic fractions. After this treatment, a liquid phase containing hexoses and pentoses and a solid phase, which corresponded to the Klason lignin, were obtained.

Another winery residue that has been studied for its integral revalorisation under a biorefinery approach is the grape pomace. Martínez et al. [1.40] proposed its employment for the production of polyphenols, volatile fatty acids (VFA), polyhydroxyalkanoates (PHAs) and methane. As it is shown in Figure 1.6, they firstly subjected the grape pomace to a supercritical CO_2 extraction for the isolation of polyphenols. The obtained solid residue was subjected to an anaerobic acidogenic digestion producing volatile fatty acids. Then, an aerobic fermentation of the VFA was carried out using a pure culture of *Cupriavidus necator* obtaining PHAs. The solid phase achieved after the anaerobic acidogenic digestion was subjected to an anaerobic methanogenic digestion for the production of methane.

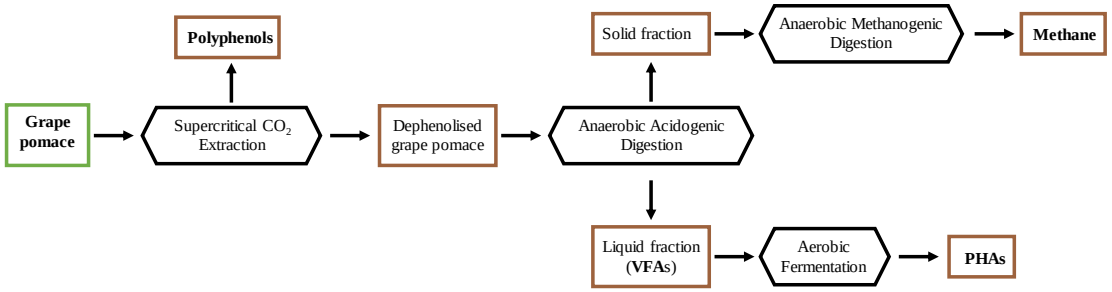


Figure 1.6. Scheme of the biorefinery approach for the integral revalorisation of grape pomace, adapted from Dávila et al. [1.24].

The grape skins are another winery residue whose integral revalorisation has been studied. Mendes et al. [1.41] proposed a biorefinery approach of these wastes for the obtaining of

biocomposites, oleanoic acid and ethanol, as it can be seen in Figure 1.7. Firstly, they subjected the grape skins to consecutive or simultaneous extractions with hexane and water obtaining three different streams: a solid residue, an organic phase and an aqueous phase. The solid residue was used for the production of biocomposites, particularly low-density boards, while the organic phase could be used for the isolation of oleanoic acid. The aqueous phase, on the other hand, was subjected to an acid hydrolysis in order to obtain a liquid phase enriched in hexoses which were fermented by the *Saccharomyces Cerevisiae* 4072 to produce ethanol.

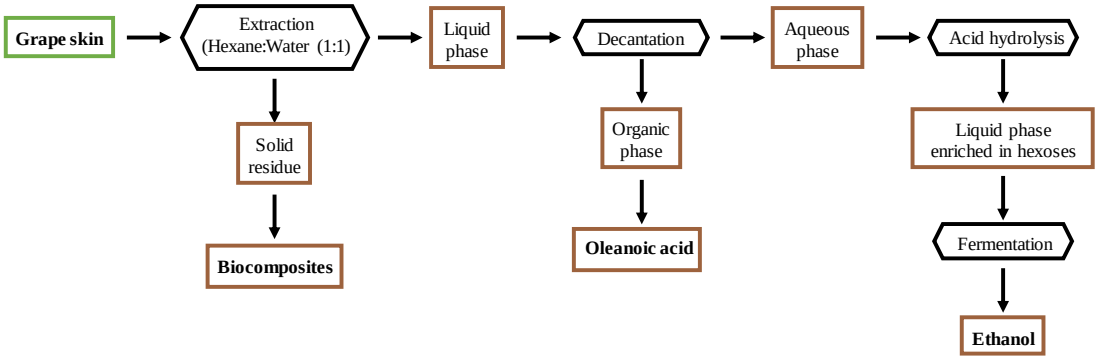


Figure 1.7. Scheme of the biorefinery approach for the integral revalorisation of the grape skin, adapted from Dávila et al. [1.24].

Dimou et al. [1.42] proposed an integral revalorisation of the wine lees for the production of compounds of added-value such as, ethanol, polyphenols, tartrate and poly(3-hydroxybutyrate) (PHB), using the biorefinery approach shown in Figure 1.8. Firstly, the solid and liquid phases of the wine lees were separated by centrifugation, being the latter phase distilled for the obtaining of ethanol and an alcohol-free liquid. The obtained solid phase was subjected to a sequential extraction with a mixture of acetone/water and methanol/water for the isolation of polyphenols. Then, the solid residue was treated with hydrochloric acid for the tartaric acid solubilisation. Finally, the solid recovered after the tartaric acid solubilisation together with the alcohol-free liquid were used in an enzymatic hydrolysis for the production of a nutrient rich fermentation media, which was used for converting glycerol into PHB by *Cupriavidus necator*.

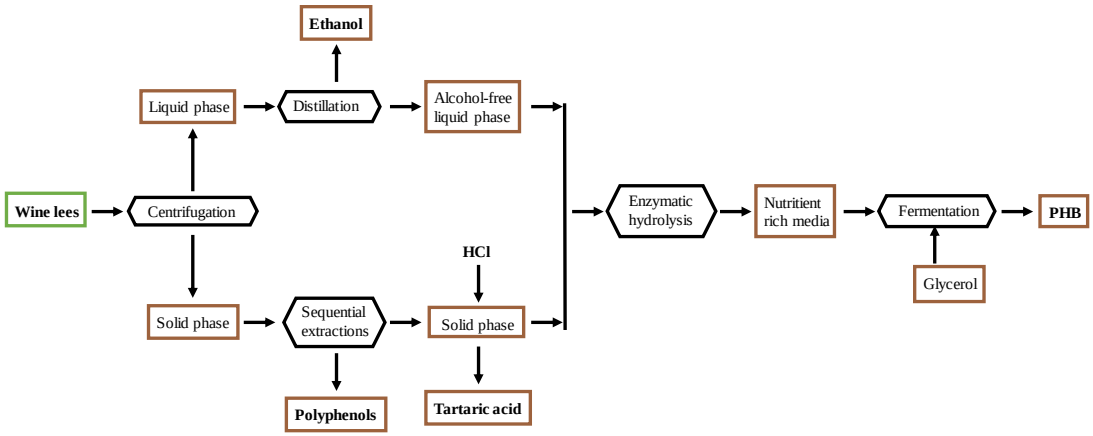


Figure 1.8. Scheme of the biorefinery approach for the integral revalorisation of the wine lees, adapted from Dávila et al. [1.24].

Amendola et al. [1.43] proposed a biorefinery approach for the revalorisation of the grape stalks in order to isolate their hemicellulosic, lignin and cellulosic fractions, as it can be seen in Figure 1.9. Firstly, the grape stalks were subjected to an autohydrolysis treatment, obtaining two streams, a liquid and a solid phase. The monosaccharides and oligomers derived from the solubilisation of the hemicelluloses were precipitated from the liquid phase by the addition of ethanol. The liquid phase recovered after the precipitation with ethanol was acidified for the precipitation of lignin.

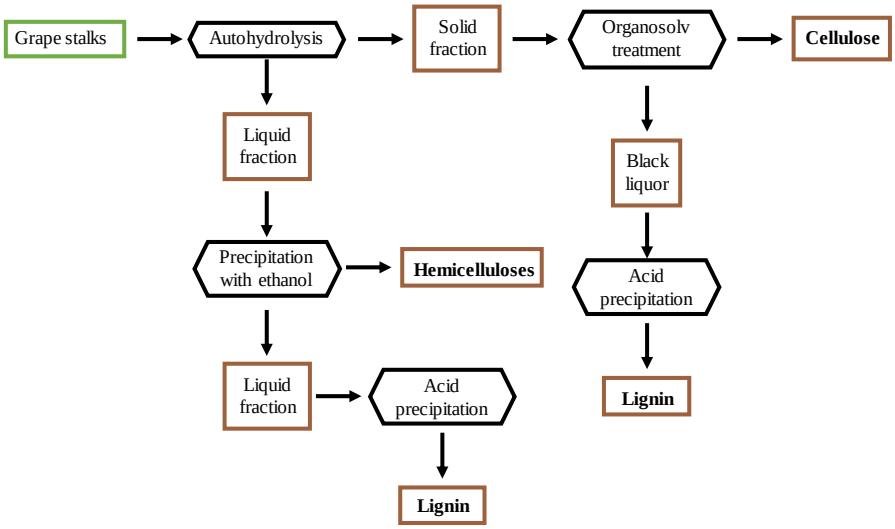


Figure 1.9. Scheme of the biorefinery approach for the integral revalorisation of the grape stalks, adapted from Dávila et al. [1.24].

The autohydrolysed solid was further subjected to an organosolv treatment generating a solid residue enriched in cellulose and a liquid phase denominate as black liquor. The obtained black

liquor was subjected to an acid precipitation in order to isolate the lignin fraction. Compared with the winery residues mentioned above, the integral revalorisation of the grape stalks is not very exploited as their isolated components could be further up-graded in order to obtain products of added-value.

The vine shoots are winery by-products which have been employed by different researchers to obtain different added-value compounds following the biorefinery philosophy. For example, Bustos et al. [1.44] subjected the solid residue obtained from the acid hydrolysis of the vine shoots to a delignification treatment with NaOH for the obtaining of a solid rich in cellulose. This solid was further subjected to a simultaneous saccharification and fermentation (SSF) process with *Lactobacillus rhamnosus* for the production of lactic acid. Max et al. [1.45] studied the employment of the liquid phase recovered after the NaOH treatment of the acid hydrolysed vine shoots for the obtaining of hydroxycinnamic and hydroxybezoic acids.

Bustos et al. [1.46], Portilla et al. [1.47] and Rodríguez-Pazo et al. [1.48] also carried out an acid hydrolysis of the vine shoots obtaining a liquid phase rich in monosaccharides which was further fermented to produce lactic acid. The first ones carried out the fermentation using *Lactobacillus pentosus* obtaining intracellular biosurfactants apart from lactic acid while the second ones employed *Lactobacillus acidophilus* and *Debaryomyces hansenii* for the acquisition of xylitol, microbial biomass and biosurfactants apart from lactic acid. Rodríguez-Pazo et al. [1.47] were able to produce biosurfactants and phenyllactic acid apart from lactic acid by the fermentation of the hemicellulosic sugars contained in the liquid phase using *Lactobacillus pentosus* and *Lactobacillus plantarum.*

Although there are many examples of the revalorisation of vine shoots for the obtaining of different added-value compounds from some of its fractions, there are not examples in which a multi-product biorefinery in cascade for their integral revalorisation is proposed, as it happens with the rest of the winery residues mentioned above. As not all the isolated or obtained fractions are valorised, not all the economic value of the vine shoots is exploited and the proposed biorefinery processes do not fit the circular economy model.

Nevertheless, the research around the employment of the different winery residues under a biorefinery scheme for the obtaining of multiple products of added-value should continue, as the evolution of the technology and the knowledge could permit the obtaining of new added-value products or the employment of more environmentally friendly technologies.

1.4 Main objective of the thesis

The aim of this thesis was to perform an integral revalorisation of winery residues such as the vine shoots, following the biorefinery concept. The biorefinery approach proposed in this study, which is shown in Figure 1.10, follows the circular economy philosophy and it is in line with the sustainable development. It employs green technologies to minimise the waste generation and to obtain multiple added-value products, accomplishing the integral revalorisation of the vine shoots.

In the proposed biorefinery approach, the vine shoots were subjected to different treatments in order to separate their main components, which after being characterised were subjected to up-grading treatments to obtain added-value products. The first stage of the biorefinery scheme proposed was an autohydrolysis pre-treatment to solubilise the hemicellulosic fraction of the vine shoots. The remaining solid was treated using two different routes (second stage of the biorefinery approach) being the separation between the lignin and cellulosic fractions carried out in both of them. The proposed biorefinery approach could permit the obtaining of bioactive extracts, prebiotics, bioethanol, cellulose, which could be used for the generation of biomaterials or platform chemicals, or lignin, which could be used for the production of biomaterials or as a source of phenolic compounds. Some of these products have never been produced from the vine shoots. The procedures used to isolate and up-grade the different fractions of this winery by-product are described in detail during the following chapters.

The environmental impacts derived from the valorisation of vine shoots under a biorefinery approach (Figure 1.10) were evaluated from an environmental perspective using the Life Cycle

Assessment methodology. The evaluation of the routes would permit the identification of environmental hotspots responsible of their largest environmental impacts.

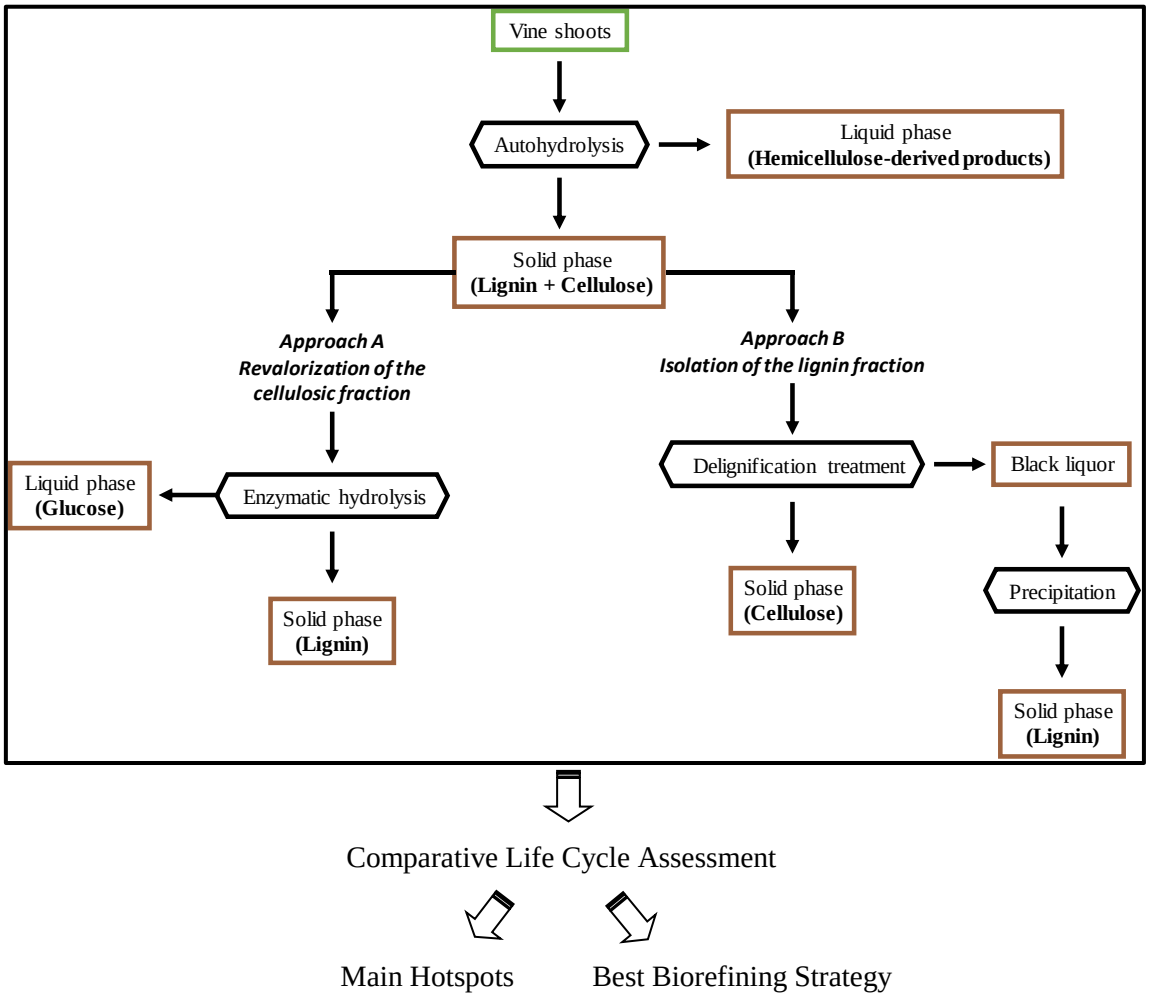


Figure 1.10. Scheme of the biorefinery approach in cascade proposed for the integral revalorisation of the vine shoots.

1.5 References

[1.1] Liguori, R. & Faraco, V. (2016). Biological processes for advancing lignocellulosic waste biorefinery by advocating circular economy. *Bioresource Technology*, *215*, 13-20.

[1.2] Ghatak, H. R. (2011). Biorefineries from the perspective of sustainability: Feedstocks, products and processes. *Renewable and Sustainable Energy Reviews*, *15*(8), 4042-4052.

[1.3] British Petroleum (BP) global. Primary energy statistical review. Retrieved from https://www.bp.com/en/global/corporate/energy-economics/statistical-review-of-world-energy/primary-energy.html (Visited September, 2018)

[1.4] Saha, K., Ra, U. M., Sikder, J., Chakraborty, S., Da Silva, S. S. & Dos Santos, J. C. (2017). Membranes as a tool to support biorefineries: Applications in enzymatic hydrolysis, fermentation and dehydration for bioethanol production. *Renewable Sustainable Energy Reviews, 74,* 873-890.

[1.5] Henrich, E., Dahmen, N., Dinjus, E. & Sauer, J. (2015). The role of biomass in a future world without fossil fuels. *Chemie Ingenieur Technik, 87*(12), 1667–1685.

[1.6] European Commission. Bio-based industries. Retrieved from https://ec.europa.eu/programmes/horizon2020/en/area/bio-based-industries#Article. (Visited September, 2018)

[1.7] Isikgor, F. H. & Becer, C. R. (2015). Lignocellulosic biomass: a sustainable platform for the production of bio-based chemicals and polymers. *Polymer Chemistry, 6*(25), 4497-4559.

[1.8] Gosh, S. K. (2016). Biomass and Bio-waste supply chain sustainability for bio-energy and bio-fuel production. *Procedia Environmental Science, 31,* 31-39.

[1.9] Rombaut, N., Tixier, A. S., Bily, A. & Chemat, F. (2014). Green extraction processes of natural products as tools for biorefinery. *Biofuels Bioproducts & Biorefining, 8*(4), 530-544.

[1.10] Morais, A. R. C. & Bogel-Lukasik, R. (2013). Green chemistry and biorefinery concept. *Sustainable Chemical Processes, 1*(18), 1-3.

[1.11] Perlatti, B., Forim, M. R. & Zuin, V. G. (2014). Green chemistry, sustainable agriculture and processing systems: a Brazilian overview. *Chemical and Biological Technologies in Agriculture, 1*(1), 1-9.

[1.12] FitzPatrick, M., Champagne, P., Cunningham, M. F. & Whitney, R. A. (2010). A biorefinery processing perspective: Treatment of lignocellulosic materials for the production of value-added products. *Bioresource Technology, 101*(23), 8915-8922.

[1.13] Brinchi, L., Cotana, F., Fortunati, E. & Kenny, J. M. (2013). Production of nanocrystalline cellulose from lignocellulosic biomass: Technology and applications. *Carbohydrate Polymers, 94*(1), 154-169.

[1.14] Zuin, V. G. (2016). Circularity in green chemical products, processes and services: Innovative routes based on integrated eco-design and solution systems. *Current Opinion in Green Sustainable Chemistry, 2*, 40-44.

[1.15] Zorpas, A. A., Lasaridi, K., Abeliotis, K., Voukkali, I., Loizia, P., Fitiri, L., … Bikaki, N. (2014). Waste prevention campaign regarding waste framework directive. *Fresenius Environmental Bulletin, 23*(11A), 2876-2883.

[1.16] De Jong, E. & Jungmeier, G. (2015). Biorefinery concepts in comparison to petrochemical refineries. In A. Pandey, R. Höfer, M. Taherzadeh, K. M. Nampoothiri & C. Larroche (Eds.), *Industrial Biorefineries and White Biotechnology* (pp. 3-33). Amsterdam, Netherlands: Elsevier.

[1.17] Cherubini, F., Jungmeier, G., Wellisch, M., Willke, T., Skiadas, I., Van Ree, R. & De Jong, E. (2009). Toward a common classification approach for biorefinery systems. *Biofuels Bioproducts & Biorefining, 3*(5), 534-546.

[1.18] Bio-based Industries Consortium. Mapping European Biorefineries. Retrieved from https://biconsortium.eu/news/mapping-european-biorefineries. (Visited August, 2018)

[1.19] Kamm, B., Gruber, P. R., Kamm, M. (2007). Biorefineries-Industrial processes and products. *Ullmann's Encyclopedia of Industrial Chemistry, 1*, 1-38.

[1.20] Spigno, G., Marinoni, L. & Garrido, G. D. (2017). State of the art in grape processing by-products. In C. M. Galanakis (Ed.), *Handbook of grape processing by-products* (pp. 1-28). Amsterdam, Netherlands: Elsevier.

[1.21] Food and Agriculture Organisation of the United Nations. FAOSTAT. Retrieved from http://www.fao.org/faostat/en/#data/QC. (Visited August, 2018)

[1.22] Teixeira, A., Baenas, N., Dominguez-Perles, R., Barros, A., Rosa, E., Moreno, D. A. & Garcia-Viguera, C. (2014). Natural bioactive compounds from winery by-products as health promoters: a review. *International Journal of Molecular Sciences, 15*(9), 15638-15678.

[1.23] Chouchouli, V., Kalogeropoulos, N., Konteles, S. J., Karvela, E., Makris, D. P. & Karathanos, V. T. (2013). Fortification of yoghurts with grape (*Vitis vinifera)* seed extracts. *LWT-Food Science and Technology, 53*(2), 522-529.

[1.24] Dávila, I., Robles, E., Egües, I., Labidi, J. & Gullón, P. (2017). The biorefinery concept for the industrial valorisation of grape processing by-products. In C. M. Galanakis (Ed.), *Handbook of grape processing by-products* (pp. 29-53). Amsterdam, Netherlands: Elsevier.

[1.25] Nerantzis, E. T. & Tataridis, P. (2006). Integrated enology-Utilization of winery by-products into high added value products. *Journal of Science and Technology, 1*(3), 78-89.

[1.26] Oliveira, D. A., Salvador, A. A. A. S., Smânia, E. F. A., Maraschin, M. & Ferreira, S. R. S. (2013). Antimicrobial activity and composition profile of grape (*Vitis vinífera*) pomace extracts obtained by supercritical fluids. *Journal of Biotechnology, 164*(3), 423-432.

[1.27] Oliveira, M. & Duarte, E. (2016). Integrated approach to winery waste: waste generation and data consolidation. *Frontiers of Environmental Science & Engineering, 10*(1), 168-176.

[1.28] Arvanitoyannis, I. S., Ladas, D. & Mavromatis, A. (2006). Potential uses and applications of treated wine waste. *International Journal of Food Science and Technology, 41*(5), 475-487.

[1.29] Rodríguez, L. A., Toro, M. E., Vazquez, F., Correa-Daneri, M. L., Gouiric, S. C. & Vallejo, M. D. (2010). Bioethanol production from grape and sugar beet pomaces by solid-state fermentation. *International Journal of Hydrogen Energy, 35*(11), 5914-5917.

[1.30] Fabbri, A., Bonidazi, G. & Serranti, S. (2015). Micro-scale energy valorization of grape marcs in winery production plants. *Waste Management, 36,* 156-165.

[1.31] Egües, I., Serrano, L., Amendola, D., De Faveri, D. M., Spigno, G. & Labidi, J. (2013). Fermentable sugars recovery from grape stalks for bioethanol production. *Renewable Energy, 60,* 553-558.

[1.32] Prozil, S. O., Costa, E. V., Evtuguin, D. V., Cruz, L. P. & Domingues, M. R. M. (2012). Structural characterization of polysaccharides isolated from grape stalks of *Vitis vinifera L. Carbohydrate. Research, 356,* 252-259.

[1.33] Alfonso, J., Passos, C. P., Coimbra, M. A., Silva, C. M. & Soares-da-Silva, P. (2013). Inhibitory effect of phenolic compounds from grape seeds (*Vitis vinifera L.*) on the activity of angiotensin I converting enzyme. *LWT-Food Science and Technology, 54*(1), 265-270.

[1.34] Poveda, J. M., Loarce, L., Alarcón, M., Díaz-Maroto, M. C. & Alañón, M. E. (2018). Revalorization of winery by-products as a source of natural preservatives obtained by means of green extraction techniques. *Industrial Crops and Products, 112,* 617-625.

[1.35] Fiori L. (2007). Grape seed oil supercritical extraction kinetic and solubility data: Critical approach and modelling. *Journal of Supercritical Fluids, 43*(1), 43-54.

[1.36] Rajha, H. N., Jaoude, N. A., Louka, N., Maroun, R. G. & Vorobiev, E. (2014). Industrial byproducts valorization through energy saving processes. Alkaline extraction of polyphenols

from vine shoots. *International Conference on Renewable Energies for Developing Countries.* (REDEC 2014) 7038537, 89-94.

[1.37] Da Ros, C., Cavinato, C., Bolzonella, D. & Pavan, P. (2016). Renewable energy from thermophilic anaerobic digestion of winery residue: preliminary evidence from batch and continuous lab-scale trials. *Biomass and Bioenergy, 91,* 150-159.

[1.38] Naziri, E., Glisic, S. B., Mantzouridou, F. T., Tsimidou, M. Z., Nedovic, V. & Bugarski, B. (2016). Advantages of supercritical fluids extraction for recovery of squalene from wine lees. *Journal of Supercritical Fluids, 107,* 560-565.

[1.39] Yedro, E. M., García-Serna, D. J., Cantero, A., Sobrón, F. & Cocero, M. J. (2015). Hydrothermal fractionation of grape seeds in subcritical water to produce oil extract, sugars and lignin. *Catalysis Today, 257*(Part 2), 160-168.

[1.40] Martinez, G. A., Rebecchi, S., Decorti, D., Domingos, J. M. B., Natolino, A., Del Rio, D., … Fava, F. (2016). Towards multi-purpose biorefinery platforms for the valorisation of red grape pomace: production of polyphenols, volatile fatty acids, polyhydroxyalkanoates and biogas. *Green Chemistry, 18*(1), 261-270.

[1.41] Mendes, J. A. S., Xavier, A. M. R. B., Evtuguin, D. V. & Lopes, L. P. C. (2013). Integral utilization of grape skins from white grape pomaces. *Industrial Crops and Products, 49,* 286-291.

[1.42] Dimou, C., Kopsahelis, N., Papadaki, A., Papanikolau, S., Kookos, I. K., Mandala, I. & Koutinas, A. A. (2015). Wine lees valorization: biorefinery development including production of generic fermentation feedstock employed for poly(3-hydroxybutyrate) synthesis. *Food Research International, 73,* 81-87.

[1.43] Amendola, D., De Faveri, D. M., Egües, I., Serrano, L., Labidi, J. & Spigno, G. (2012). Autohydrolysis and organosolv process for recovery of hemicelluloses, phenolic compounds and lignin from grape stalks. *Bioresource Technology, 107,* 267-274.

[1.44] Bustos, G., Moldes, A. B., Cruz, J. M. & Domínguez, J. M. (2005). Production of lactic acid from vine-trimming wastes and viticulture lees using a simultaneous saccharification fermentation method. *Journal of the Science of Food and Agriculture, 85*(3), 466-472.

[1.45] Max, B., Salgado, J., Corte, S. & Domínguez, J. M. (2010). Extraction of phenolic acids by alkaline hydrolysis from the solid residue obtained after prehydrolysis of trimming vine shoots. *Journal of agricultural and food chemistry, 58*(3), 1909-1917.

[1.46] Bustos, G., De la Torre, N., Moldes, A. B., Cruz, J. M. & Domínguez, J. M. (2007). Revalorization of hemicellulosic trimming vine shoots hydrolyzates through continuous production of lactic acid and biosurfactants by *L. pentosus*. *Journal of Food Engineering*, *78*(2), 405-412.

[1.47] Portilla, O. M., Rivas, B., Torrado, A., Moldes, A. B. & Domínguez, J. M. (2008). Revalorisation of vine trimming wastes using *Lactobacillus acidophilus* and *Debaryomyces hansenii*. *Journal of the Science of Food Agriculture*, *88*(13), 2298-2308.

[1.48] Rodríguez-Pazo, N., Salgado, J. M., Cortés-Diéguez, S. & Domínguez, J. M. (2013). Biotechnological production of phenyllactic acid and biosurfactants from trimming vine shoots hydrolyzates by microbial coculture fermentation. *Applied Biochemistry and Biotechnology*, *169*(7), 2175-2188.

La ciencia no es solo una disciplina de razón, sino también de romance y pasión.
-Stephen Hawking

Chapter 2

Autohydrolysis of the vine shoots

2.1 Background

2.1.1 Vine shoots

Vine shoots, which are the raw material used in this thesis, are annual agricultural residues generated during the pruning of the vine. This activity has important implications for the vines themelf, since it affects their form, shape and size, influencing the balance between vegetative and the fruit growth, and consequently the quantity and quality of the grape production [2.1]. Although the amount of vine shoots could fluctuate from year to year, it is estimated that around 21 millions of tons are produced per year worldwide [2.2]. Due to the low-economic value of these residues, they are commonly burned in the fields to prevent the proliferation of phytopathogens [2.3] or they are left in the fields as organic fertilizers [2.4] as it can be seen in Figure 2.1, causing environmental and ecological pollution.

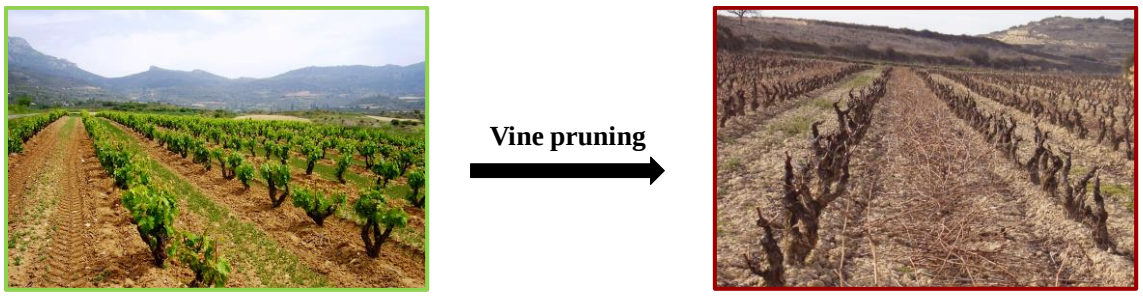


Figure 2.1. Vineyards before and after the vine pruning.

Although the composition of the vine shoots is influenced by the vine variety, the time of pruning or the climate among other factors [2.1], they are mainly composed by cellulose (~35.0 %), hemicelluloses (~20.0 %) and lignin (~27.0 %) [2.5]. Apart from their main components they also contain other minor compounds such as lipids, proteins, minerals [2.6] and water soluble organic low molecular weight compounds, such as stilbenes, flavonoids, phenolic acids, alcohols, terpenes or aldehydes, between others [2.7].

In the recent years, as it has been mentioned in the section 1.3 of the Chapter 1, the employment of the vine shoots under a biorefinery perspective has been gaining attention, due to their low-cost, high abundance and renewability. Their composition could also permit the obtaining of

multiple added-value compounds by fractionating these residues in their main components and by up-grading them, following the philosophy of the circular economy model.

The first fraction isolated from the vine shoots according to the biorefinery scheme proposed in this thesis (Figure 1.10, section 1.4 of the Chapter 1) was the hemicellulosic fraction, as compared with the cellulose and lignin it is the most labile fraction of the lignocellulosic biomass [2.8].

2.1.2 Hemicelluloses

The hemicelluloses are heterogeneous polymers made up of monosaccharides with polymerisation degrees between 80 and 200 [2.9] and they represent between the 15.0 and 35.0 % of the lignocellulosic biomass [2.10]. Their main function is to stabilise the cell wall as they interact with the cellulose by hydrogen bonds and with the lignin by covalent bonds [2.8], as it can be observed in Figure 2.2.

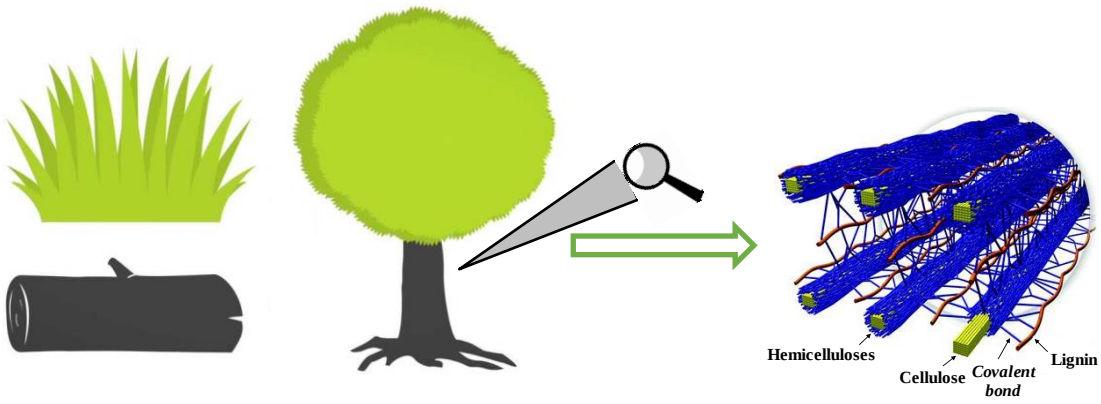


Figure 2.2. Scheme of the internal structure of the biomass, adapted from Brandt et al. [2.11].

The structure of the hemicelluloses consists on a β-(1→4) backbone of glucose, mannose or xylose units with short branches constituted by pentoses, hexoses, acetyl groups and uronic acids, arrange in different proportions. Taking into account their structure, which are represented in Figure 2.3, the hemicelluloses can be classified in the following main groups:

- **Mannans.** The mannans are mainly constituted by a mannose backbone, which can present galactose and acetyl groups as substituents. Depending on their substituents and the composition of their backbone, the mannans can be classified in different sub-groups.

The **linear mannans** present the simplest structure as they are constituted by a mannose backbone without branches. The **galactomannans** contain α-1-6 D-galactose units attach to the mannose backbone. The **glucomannans** are another type of mannans in which the main chain is composed by mannose and glucose units link by a β-1-4 bond, being the mannose to glucose ratio variable with the feedstock [2.12]. The glucomannans are usually straight chains with little branching located in the mannose units and they can also be acetylated [2.13]. The **galactoglucomannans** present a higher branching than the glucomannans as either on glucose or mannose units a α-1-6 D-galactose can be bound, being the acetyl groups also forming part of the side chains. It has been reported that the solubility of the mannans increases with the degree of galactose branching [2.14].

- **Xylans.** The xylans are the most abundant hemicelluloses present in the biomass. They consist on a backbone of β-D-xylose units bind via β-1-4-glycosidic bonds and they can present xylose, arabinose, glucuronic acid, 4-O-methyl ether or acetyl groups as substituents [2.15], being their branching type and degree variable with the feedstock. The **homoxylans** are linear or branched homopolymers in which β-1-4, β-1-3 or a mixture of these two glycosidic bonds maintain xylose units together [2.16]. When the xylose backbone presents arabinoses as substituents bind to the C3 of the xyloses, these xylans are denoted as **arabinoxylans.** Another xylan type are the **glucuronoxylans**, which consist on a xylose backbone substituted on every 10 units with 4-O-methyl-D-glucuronic acid. These hemicelluloses together with the arabinoxylans could be partially acetylated at the C2 or C3 of the xylose units forming the backbone [2.16, 2.17]. It has been reported that the presence of the acetyl groups as substituents increase the solubility of the xylans [2.18]. The **arabinoglucuronoxylans** consist on a xylose backbone with arabinose and glucuronic acids bind to the O2 and O3 positions of the xyloses [2.19]. They could also present coumaric or ferulic acids bind to the C5 position of the arabinose substituents by ether linkages [2.20].
- **Xyloglucan.** The xyloglucans consist on a backbone of β-D-glucoses bind via a β-1-4 glycosidic bond, with xylose substituents bind to the C6 position of some glucose units

[2.21]. These hemicelluloses present a high degree of branching, presenting arabinose and galactose units attach to the xyloses [2.22].

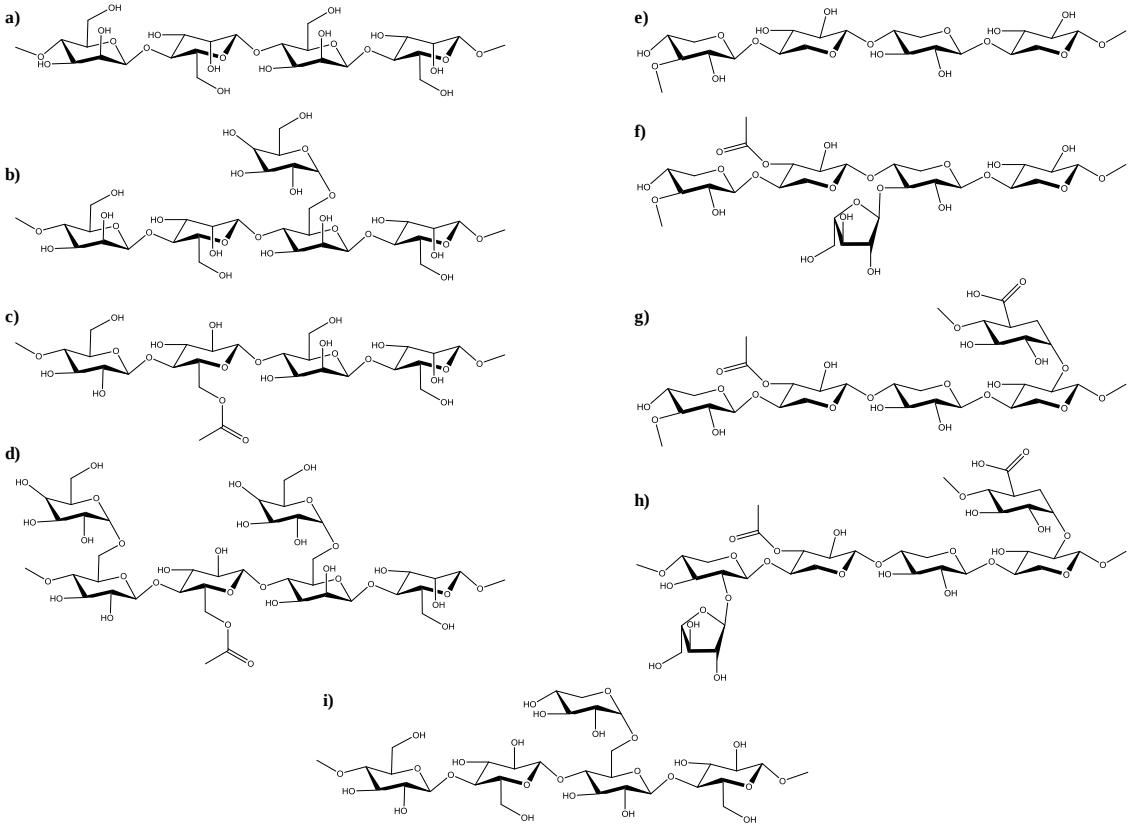


Figure 2.3. Basic structure of the different types of hemicelluloses: (a) linear mannans, (b) galactomannans, (c) glucomannans, (d) galactoglucomannans, (e) homoxylans, (f) arabinoxylans, (g) glucuronoxylans, (h) arabinoglucuronoxylans and (i) xyloglucans.

The hemicellulosic content of the biomass, together with the type of hemicelluloses and their branching degree depend on the feedstock, as it can be observed in Table 2.1. Thus, the characteristics and the potential application of the hemicellulose-derived products would also depend on the feedstock as well as on the isolation procedure and the conditions employed.

Table 2.1. Percentage of the different types of hemicelluloses present in several raw materials, adapted from Girio et al. [2.22] and Poespowati et al. [2.23].

Type of feedstock	Species	Hemicellulosic type	Amount[a] (%)
Softwood	Pine, Spruce, Fir	Galactoglucomannan	10.0-25.0
		Arabinoglucuronoxylan	5.0-10.0
		Glucomannan	2.0-5.0
Hardwood	Birch, Aspen, Oak	Glucuronoxylan	15.0-30.0
		Xyloglucan	2.0-25.0
		Glucomannan	2.0-5.0
Grasses	Switchgrass, *Pennisetum, Miscanthus sinensis*	Glucuronoarabinoxylan	15.0-30.0
		Arabinoglucuronoxylan	5.0-10.0
Cereals	Rice, Wheat, Rye	Glucuronoarabinoxylan	15.0-30.0
		Arabinoxylan	0.15-30.0
		Arabinoglucuronoxylan	5.0-10.0
Green Algae	*Caulerpa, Bryopsis sp.*	Homoxylan	20.0-50.0

[a] oven-dried basis

2.1.2.1 Isolation of hemicelulloses

In the lignocellulosic biomass, hemicelluloses are linked to each other and to cellulose by hydrogen bonds, while they are covalently bind to lignin by ester and ether linkages.

Therefore, in order to isolate the hemicelluloses, mechanical or chemical processes are necessary to break down these interactions [2.24]. Although there are many procedures to isolate hemicelluloses, when choosing one of them there are some aspects to take into consideration. These aspects could be the preservation of the hemicellulosic fraction without altering the structure of the remaining fractions or the prevention of the formation of degradation products, among others. The cost effectiveness and the low energy requirements also have to be considered when selecting an isolation process [2.25].

Some of the most common procedures employed for the isolation of this fraction are detailed below.

- **Alkaline extraction.** During this process the α-ether linkages between the hemicelluloses and lignin, as well as, the ester bonds between lignin and/or hemicelluloses and hydroxycynnamic acids, such as p-coumaric and ferulic acids [2.9], are cleaved in the basic medium created by chemicals such as, CaOH, NaOH, KOH and NH_4OH [2.15]. The alkaline extraction requires mild operating conditions (temperature and pressure), but it is not selective for hemicelluloses as lignin is also solubilised during the treatment. So, to obtain relatively pure hemicelluloses bleached biomass should be used [2.26]. The structure of the native hemicelluloses is also altered during the alkaline extraction, as saponification reactions take place removing their acetyl groups and uronic acids [2.27]. Another disadvantage of this procedure is the formation of irrecoverable salts or the incorporation of salts into the biomass structure [2.28].
- **Organic solvent extraction.** In this treatment, the hemicelluloses together with lignin and the degradation products of the cell wall components are extracted in different proportions [2.9]. Some of the organic solvents most commonly used are ethanol, methanol, glycerol, ethylene glycerol [2.15] or dimethyl sulfoxide (DMSO) [2.9]. However, there are many variants of the organic solvent extraction as apart from using aqueous solutions, the addition of organic acids, such as, acetic acid, formic acid or oxalic acid [2.15], or bases, such as, NaOH, has been studied [2.29]. It has been observed that each kind of solvent have a different effect on the structure of the hemicelluloses. The neutral solvents permit the extraction of the hemicelluloses without altering their structure, while when acid and basic solvents are used a partial depolymerisation of the solubilised hemicelluloses occurs by the cleavage of the glycosidic bonds, as well as by the removal of the ester groups present in the hemicelluloses [2.9]. The employment of organic solvents presents some advantages. They are easily recovered by distillation, they present low environmental impact and the extractions do not require much energy. However, the use of these solvents also has some drawbacks, such as their high price or the extra security measures that has to be applied due to their flammability [2.30].

- **Steam explosion.** During this process the biomass is heated (normally between 210 and 290 ºC) for a short period of time using high-pressure saturated steam (normally between 20 and 50 bars) [2.27]. Under the high pressure, the steam condenses "wetting" the biomass and with the sudden decrease of the pressure, the condensed moisture is evaporated producing the desegregation of the lignocellulosic biomass due to the breakage of inter- and intra-molecular linkages [2.27]. Consequently, the lignin is easily depolymerised while the hemicelluloses are readily hydrolysed [2.9]. Most of the steam explosion treatments produce mainly oligosaccharides along with low quantities of lignin compounds [2.27]. However, in some cases extractions with water and alcohols are necessary to remove the solubilised lignin [2.9].
- **Hot water extraction or autohydrolysis.** In this treatment, the hydrolysis of the hemicelluloses takes place using hot water and working at temperatures between 160 and 240 ºC, with a pressure higher than the saturation point of the water. The high temperature and pressure produce the auto-ionisation of the water leading hydronium ions (H_3O^+) which catalyse the hydrolysis of the glycosidic linkages of the hemicelluloses, removing also part of their uronic acids and acetyl groups. The hydronium ions coming from the acetyl groups also act as catalysts improving the kinetic of the hydrolysis. The uronic acids might also contribute to the formation of hydronium ions although their role is not completely understood [2.27]. Apart from the hydrolysis of the glycosidic bonds of the hemicelluloses the linkages between the hemicelluloses and lignin are also hydrolysed. However, the lignin fraction is not significantly solubilised during this process [2.27], being monosaccharides, oligosaccharides and degradation products, the main products obtained in the autohydrolysis. One of the advantages of this treatment is the employment of mild pH conditions which avoids corrosion problems, as well as, acid recycling and precipitate removal steps [2.27].

From the mentioned methods, the most applied ones are the steam explosion and the autohydrolysis. These treatments permit the selective solubilisation of the hemicelulloses,

producing liquid phases rich in oligomeric and monomeric sugars derived from the total or partial hydrolysis of the hemicelluloses [2.8]. The low quantity of lignin-derived products present in the liquid phases makes it not necessary to carry out a further step for the removal of lignin. Between these two methods, the autohydrolysis is supposed to permit the effective solubilisation of hemicelluloses without removing totally their acetyl and uronic groups [2.31] and without causing significant effects on the structure of the remaining lignin and cellulose fractions [2.32]. Moreover, the autohydrolysis and the steam explosion could be considered as green processes as only water is used, however they present a high energy requirement [2.15].

In order to make the procedures to isolate the hemicelluloses more effective, energy efficient, environmentally friendly and less time consuming, the intensification of these methods by the employment of microwaves and ultrasounds has been studied [2.9, 2.15].

During the procedures assisted by microwaves the biomass and the solvent are electromagnetically irradiated, transforming this electromagnetic energy into heat. Thus, compared with the conventional heating, in which the heat is transferred by convection, the microwave-assisted treatment requires shorter times. Other advantages of the microwaves are the less solvent requirements, the higher extraction rates and the lower cost [2.33]. However, as the microwave-assisted treatments are faster than the conventional ones, it is more difficult to achieve a good extraction yield without extensive degradation of the hemicelluloses and without the dissolution of the lignin and cellulose [2.9].

In ultrasound-assisted isolation procedures, the efficiency of the extraction is improved by the cavitation phenomenon that takes place [2.34]. The cavitation consists on the formation, growth and the collapse of gas bubbles in the liquid medium due to the ultrasound waves [2.34]. This produces a high turbulence and liquid circulation in the media which facilitates the penetration of the solvent into the biomass [2.35]. However, it has to be taken into account that long irradiation times and the high power of ultrasounds could influence or change the structure of the isolated hemicelluloses [2.35].

Examples of the employment of the previously detailed procedures to separate hemicelluloses from different raw materials are collected in Table 2.2.

Table 2.2. Examples of different procedures to solubilise the hemicelluloses from several raw materials.

Raw material	Type of isolation procedure	Conditions	Reference
Wheat straw	Alkaline extraction	0.5 M NaOH at 55 ºC for 2h	[2.36]
Pennisetum sinese Roxb		0.5 % H_2O_2 and 4.0 % NaOH at 75 ºC for 4h, assisted by microwaves	[2.37]
Olive tree pruning		7.5 wt.% NaOH at 50 ºC for 60 min, assisted by ultrasounds	[2.38]
Wheat straw	Solvent extraction	Acetic acid/H_2O (90/10, v/v) at 85 ºC for 4h	[2.39]
Barley straw		Dioxane/H_2O (90/10, v/v) at 80 ºC for 4 h	[2.40]
Banana rachis	Steam explosion	H_2O at 217 ºC and 21.23 bars for 300 s	[2.41]
Eucalyptus globulus		H_2O at 183 ºC and 9.8 bars for 10 min	[2.42]
Eucalyptus globulus	Autohydrolysis	H_2O at 175 ºC for 30 min	[2.43]
Olive tree pruning		H_2O at 50 ºC for 120 min, assisted by ultrasounds	[2.38]
Corn stover and cob		H_2O at 200 ºC for 50 min, assisted by microwaves	[2.44]

Among the different methods described for the solubilisation of the hemicellulosic fraction of the lignocellulosic biomass, in this thesis the autohydrolysis was chosen as the first treatment of the biorefinery scheme proposed for the vine shoots (Figure 1.10, section 1.4 of the Chapter 1).

2.1.2.2 Potential applications of the hemicellulose-derived products isolated from vine shoots

The hemicellulosic fraction of biomass has received much attention in the last decades for its application in different fields, due to its biodegradability, its excellent biocompatibility and its physico-chemical properties [2.9].

In this thesis, since the procedure used to solubilise the hemicellulosic fraction was the autohydrolysis, the main hemicellulose-derived compounds that could be obtained are oligosaccharides with different polymerisation degrees and substituents, followed by monosaccharides and degradation products such as furfural or hydroxymethylfurfural (HMF) [2.31].

The compounds obtained during the autohydrolysis of the vine shoots were assayed as prebiotics, antioxidants and antimicrobials [2.45, 2.46]. However, in addition to the applications evaluated

in this thesis, the hemicellulose-derived compounds could be used to produce many added-value commodities with application in fields, such as medicine, food, cosmetic or pharmaceutical industries, between others, as it can be seen in Table 2.3.

Table 2.3. Added-value products obtained from hemicellulose-derived compounds and their applications.

Feedstock	Hemicelluloses isolation procedure	Added-value product	Fields of application	Reference
Rice	Autohydrolysis	Prebiotics	Food and cosmetic industries	[2.47]
Bamboo	Autohydrolysis			[2.31]
Spruce	Autohydrolysis	Hydrogels	Medicine and environmental protection	[2.48]
Bamboo	Alkaline extraction			[2.49]
Spruce	Autohydrolysis	Films	Food industry	[2.50]
Sugar cane bagasse	Alkaline extraction			[2.51]
Bamboo	Acid hydrolysis	Xilytol	Food, deontological and pharmaceutical industries	[2.52]
Oil palm empty fruit banches	Autohydrolysis			[2.53]
Vine shoots	Acid hydrolysis	Lactic acid	Medicine and food industry or as a platform	[2.54]
Pubescens	Extraction with a GVL/H_2O mixture	Furfural	As additive in fungicides, inks, adhesives or as a platform	[2.55]

As it has been mentioned, one of the applications assessed in this thesis for the hemicellulose-derived compounds is as functional food ingredients due to the potential prebiotic activity of the oligosaccharides. In the literature, there are many examples in which the prebiotic activity of oligosaccharides obtained from different lignocellulosic biomass has been proved [2.45, 2.56]. However, the prebiotic activity of the oligosaccharides isolated from vine shoots has never been assessed at the best of our knowledge.

The prebiotics are defined as "selectively fermented ingredients that allow specific changes, both in the composition and/or activity of the gut microbiota, conferring benefits to the host´s wellbeing and health" [2.57]. The prebiotics are fermented by intestinal microbiota such as bifidobacteria and lactobacilli, generating short chain fatty acids (SCFA) such as propionic,

butyric or acetic acid [2.47], which could influence the bowel functions, calcium absorption, lipid metabolism or could reduce the risk of colon cancer [2.58]. It has been reported that inulin, lactulose or oligosaccharides such as arabinoxylans, galactooligosaccharides (GalactOS), fructooligosaccharides (FOS) or xylooligosaccharides (XOS) present prebiotic activity [2.59-2.61].

As it has been mentioned, during the autohydrolysis treatment, apart from oligosaccharides and monosaccharides, degradation products are also formed. Commonly, the liquid phases obtained during this treatment, also denoted as autohydrolysis liquors or hydrolysates, need to be refined in order to use the oligosaccharides as functional food ingredients, as they have to present a purity between 75.0 and 95.0 % [2.62]. In this thesis, the refining of the autohydrolysis liquors obtained from the vine shoots was carried out using membrane technology followed by ion exchange. The membrane technology is a green process as no reagents are used and it permits the removal of monosaccharides and phenolic derived compounds [2.63].

The second application given to the compounds obtained during the autohydrolysis treatments of the vine shoots is related to the non-saccharides compounds formed during this process. Apart from the solubilisation of the hemicellulosic fraction different secondary processes such as, the removal of extractives, the solubilisation of the acid-soluble lignin, the neutralisation of the ashes, the Maillard reaction or the generation of acetyl groups and carbohydrate degradation products could take place during the autohydrolysis treatment [2.64]. These reactions consequently produce liquid phases containing saccharides and non-saccharides compounds, such as, phenolics and carbohydrate degradation products which could present antioxidant and antimicrobial activities [2.46, 2.65]. In this thesis, the separation of the saccharide and non-saccharide compounds formed during the autohydrolysis treatments of the vine shoots was carried out by a liquid-liquid extraction. The non-saccharide compounds forming part of the extracts could present antioxidant and antimicrobial properties.

2.2 Objectives

The main objective of this chapter was to study the revalorisation of the hemicellulosic fraction of the vine shoots targeting the use of the hemicellulose-derived oligosaccharides as functional food ingredients with prebiotic effect and the use of non-saccharide compounds as food preservatives.

To attain this objective, autohydrolysis treatments at different temperatures were carried out to solubilise the hemicellulosic fraction of the vine shoots. The autohydrolysis liquors were chemically characterised in order to determine the conditions that permitted the obtaining of the highest quantity of oligosaccharides. The liquid phase obtained at the conditions that allowed the maximum yield of oligosaccharides, was refined by membrane technology followed by ion exchange. The refined oligosaccharides were chemical and structurally characterised and their digestibility throughout a simulated gastrointestinal system and their prebiotic activity were tested *in vitro*.

For the production of extracts with bioactive activities, the autohydrolysis liquors obtained at different temperatures were subjected to liquid-liquid extractions. The extracts were chemically characterised and their antioxidant and antimicrobial activities were assessed.

Moreover, in order to carry out an integral revalorisation of the vine shoots the solid phases obtained after the autohydrolysis treatments at different temperatures were chemically characterised, being their treatment and application described throughout the Chapter 3.

The streams obtained in the different treatments or processes carried out in this chapter and the analytics to which the different streams were subjected are depicted in Figure 2.4.

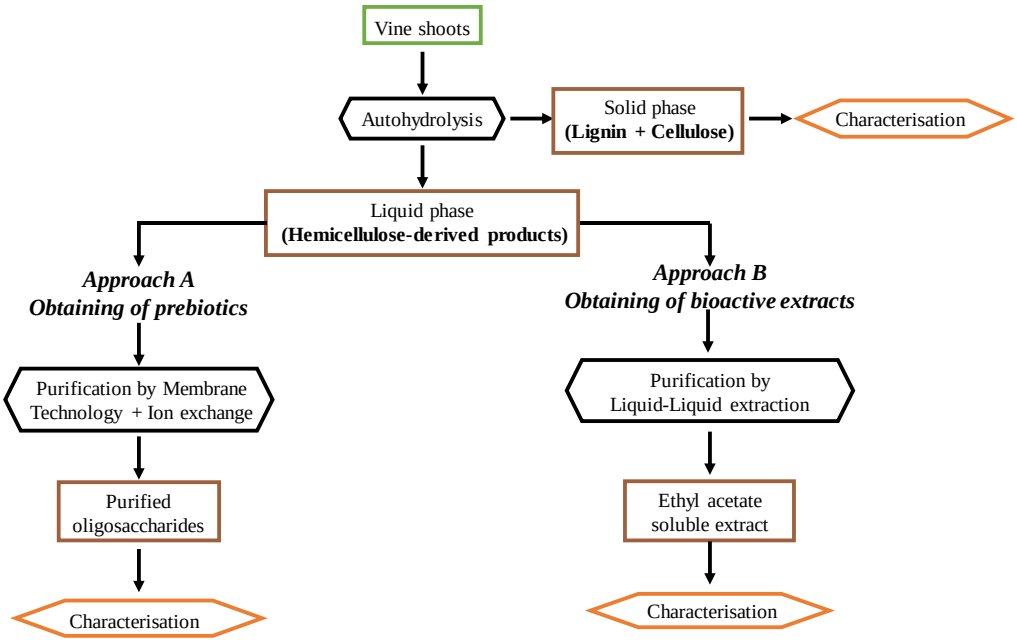


Figure 2.4. Scheme of the processing of the autohydrolysis liquors.

2.3 Materials and methods

2.3.1 Characterisation of the raw material

The vine shoots employed throughout this thesis were pruned from vines of the grape variety Hondarribi Zuri after the vintages of 2014 and 2015 and they were supplied by the local winery Aldako Bodega S. L. in Oiartzun (Basque Country). Once collected, the vine shoots were air-dried, milled and sieved, in order to have a homogenised single lot with a particle size smaller than 0.4 cm. Throughout the whole thesis the vine shoots obtained after the two vintages were used, which explains the slight differences that could be observed in the composition of the starting material used in the different processes.

The vine shoots were characterised according to protocols proposed by the Technical Association of Pulp and Paper Industries (TAPPI) [2.66] and by the National Renewable Energy Laboratory (NREL) [2.67], which are described in detail in the **Appendix I**. The vine shoots were subjected to TAPPI protocols to determine their content in ashes (TAPPI T211-om-93), in ethanol-toluene extractives (TAPPI T204cm-97), in acid-insoluble or Klason lignin (TAPPI T222-om-98) and in

moisture (TAPPI T264-om-97). The holocellulose and the α-cellulose contents were determined by protocols proposed by Wise et al. [2.68] and Rowel [2.69], respectively. The hemicelluloses, glucan and acid-insoluble lignin content of the vine shoots were also analysed by a quantitative acid hydrolysis (QAH), which is a protocol developed by the NREL.

2.3.2 Solubilisation of the hemicellulosic fraction of the vine shoots by autohydrolysis

The solubilisation of the hemicellulosic fraction present in the vine shoots was carried out by non-isothermal autohydrolysis treatments using a temperature range between 180 and 215 ºC. To carry out these treatments the vine shoots and water were mixed using a liquid/solid ratio (LSR) of 8 g/g (in oven-dried basis) in a 1.5 L stainless steel 5100 Parr reactor, in which the temperature was controlled by a Parr PID control. Once reached the desired temperature the reactor was cooled down and the liquid and solid phases were separated by filtration. The autohydrolysis treatments were carried out in duplicates.

The solid phase was washed with water to remove the solubilised hemicelluloses that could remain attached to the solid. Then, it was air-dried, quantified and subjected to a moisture determination (**Appendix I**) to estimate the solubilisation of the substrate during the autohydrolysis treatment. The obtained solid phase was also subjected to a QAH, which is described in the **Appendix I,** to determine its acid-insoluble lignin, hemicelluloses and glucan content. The holocellulose and α-cellulose content of the solid was not analysed by the TAPPI protocols. Nevertheless, the glucan content determined by the NREL protocol in the solid could be mainly attributed to the cellulose, as around 80.0 % of the hemicelluloses of the vine shoots were solubilised during the treatments carried out between 195 and 215 ºC.

The hydrolysates obtained in the autohydrolysis treatments carried out at different temperatures were subjected to the determination of the monosaccharide, oligosaccharide, acetic and galacturonic acids and degradation products (such as furfural and HMF) content. Futhermore, their non-volatile compounds (NVC) and other non-volatile compounds (ONVC) contents were

analysed, using for all the analyses the protocols described by Garrote et al. [2.70], which are collected in the **Appendix II**. The total phenolic and total flavonoids content (TPC and TFC, respectively) of the hydrolysates were also determined, using the protocols described by Singleton and Rossi [2.71] (1965) and Blasa et al. [2.72], respectively (**Appendix II**).

In order to analyse the structure of the compounds present in the hydrolysates, they were characterised by Fourier Transform Infrared (FTIR) spectroscopy and High Performance Size Exclusion Chromatography (HPSEC), being these structural determinations described in the **Appendix II**. The hydrolysates were also subjected to Thermogravimetric Analysis (TGA) to determine their thermal stability (**Appendix II**).

To measure the intensity of the autohydrolysis treatments and to facilitate the comparison of the results obtained with the ones obtained using other reactors, the severity of the treatments was determined. The severity (S_0) takes into account the combined effects of the temperature and the time of the heating and the cooling periods of the non-isothermal treatments [2.73], being it estimated as follows:

$$\boldsymbol{S_0} = log\ R_0 = log\left[R_{0\ Heating} + R_{0\ Cooling}\right] = log\left[\int_0^{t_{Max}} e^{\left(\frac{T(t)-T_{Ref}}{\omega}\right)}dt + \int_{t_{Max}}^{t_F} e^{\left(\frac{T'(t)-T_{Ref}}{\omega}\right)}dt\right]$$

where R_0 is the severity factor, t_{Max} (min) is the time necessary to reach the maximum temperature of each autohydrolysis treatment and t_F (min) is the time required for the entire heating-cooling cycle. T(t) and T'(t) (ºC) are the temperature profiles of the heating and cooling stages, respectively, and ω and T_{Ref} are parameters whose values have been reported in the literature (ω = 14.75 ºC and T_{Ref} = 100 ºC).

2.3.3 Refining of the hydrolysates by membrane technology for the manufacturing of oligosaccharides with prebiotic potential

The obtaining of oligosaccharides with high purity was carried out by refining the autohydrolysis liquors obtained from the vine shoots by membrane technology, specifically nanofiltration, followed by an additional ion exchange step to achieve a further purification. However, only the

hydrolysate obtained during the non-isothermal autohydrolysis of the vine shoots carried out at 200 ºC was refined by membranes, as it presented the highest oligosaccharide content. The refining sequence consisted on a dead-end filtration in concentration mode, followed by a discontinuous diafiltration-concentration and an ion exchange step, as it is depicted in Figure 2.5.

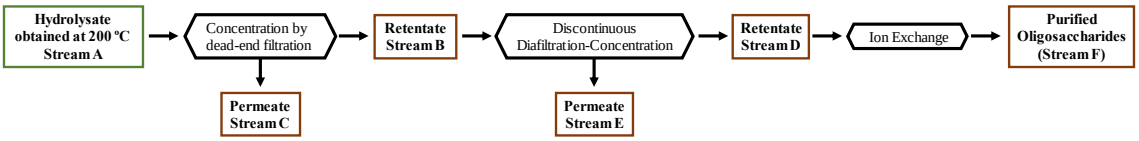


Figure 2.5. Sequence used for the refining of the hydrolysates, modified from Dávila et al. [2.74].

Firstly, the hydrolysate obtained at 200 ºC (stream A) was subjected to a dead-end filtration in concentration mode using an EMD Millipore™ Amicon™ Bioseparations Stirred Cell (EMD Millipore, St Charles, MO, USA) until a Volume Concentration Ratio (VCR) of 3.3 was achieved. The stirred cell was equipped with a 1 kDa cut off regenerated cellulose membrane with an effective membrane area of 45.4 10^{-4} m^2. The pressure in the device was generated by a compressed nitrogen gas cylinder controlled by a regulator and measured by a gauge attached to the inlet line. The liquid phase was continuously stirred with a magnetic stir bar, being thestirring rate maintained constant to avoid the formation of a deep vortex. The obtained retentate (stream B) was subjected to a discontinuous diafiltration-concentration process, using the same membrane and 3 diavolumes, which is defined as the volume ratio between the added water and the initial solution. The diluted sample was concentrated up to a VCR = 5.1, yielding the retentate D. These processes were performed at room temperature and with a transmembrane pressure of 4 bar. The stream D was subjected to an ion exchange process using an IRA-96 resin (from Sigma-Aldrich, St. Louis, MO, USA). The stream D and the resin were maintained in contact overnight at room temperature using gentle agitation and a mass ratio of 15/1 g/g. This process yielded a refined hydrolysate which is mainly composed by oligosaccharides (stream F).

The chemical composition of all the retentates (streams B and D) and of the refined hydrolysate (stream F) was analysed using the procedures described in the **Appendix II**, in particular the determination of their NVC, ONVC and their monosaccharides and oligosaccharides contents

was carried out. The refined hydrolysate (stream F) was subjected to different structural analyses such as, FTIR, Matrix Assisted Laser Desorption/Ionisation Time of Flight Mass Spectrometry (MALDI-TOF), Ultraperformance Liquid Chromatography-Diode Array Detector-Electrospray Ionisation-Mass Spectrometry (UPLC-DAD-ESI-MS) and High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) (described in the **Appendix II)** to elucidate the structure of the oligosaccharides that it contained.

For the application of the oligosaccharides as functional food ingredients, the stream F was subjected to a simulated *in vitro* gastrointestinal digestion, using the protocol described by Gullón et al. [2.75], which is collected in the **Appendix III**. Furthermore, the prebiotic activity of the digested oligosaccharides was studied by subjecting them to the protocol outlined by Gullón et al. [2.45], which is also described in the **Appendix III**.

2.3.4 Liquid-liquid extractions of the hydrolysates for the obtaining of bioactive extracts

The hydrolysates obtained in the non-isothermal autohydrolysis treatments of the vine shoots carried out between 180 and 215 ºC were subjected to liquid-liquid extractions in order to obtain bioactive extracts. The extraction of the hydrolysates was carried out by mixing the liquor with ethyl acetate in a hydrolysate/solvent ratio of 1/3 (v/v). A single extraction stage was performed by stirring the mixture at room temperature for 15 minutes. The immiscible phases were separated by decantation and the organic phase was vacuum evaporated at 40 ºC for the removal of the solvent.

The liquid-liquid extractions were carried out in duplicates, on the one hand to determine the extraction yield and on the other hand to characterise the extracts. To determine the extraction yield, the non-volatile solids present in the extracts were measured by oven-drying at 105 ± 3 ºC the extracts obtained after the vacuum evaporation until a constant weight was achieved. The extracts dried at 105 ºC were not characterised as the high temperatures could have degraded their phenolic compounds, reducing their biological activities. For the characterisation and

determination of the activities of the extracts, the extracts obtained after the vacuum evaporation were dried at 40 ± 5 ºC.

The chemical composition of the ethyl acetate extracts was determined by subjecting them to TPC, and TFC analysis and to qualitative Gas Chromatography/Mass Spectrometry (GC/MS), following the procedures described in the **Appendix IV**. The antioxidant power of the extracts was measured by subjecting them to the α,α-Diphenyl-β-picrylhydrazyl (DPPH) assay [2.76], to the 2,2´-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay [2.77] and to the ferric reducing antioxidant power (FRAP) test [2.78], which detailed procedures are reported in the **Appendix IV**. Another tested biological activity of the extracts was their *in vitro* antimicrobial activity, which was analysed using the procedure proposed by Moreira et al. [2.65]. The procedure used for the determination of the ability of the extracts to inhibit the growth of the following six bacterial strains: *Listeria innocua, Staphylococcus aureus, Escherichia coli, Bacillus cereus, Pseudomonas aeruginosa and Salmonella sp.* is detailed in the **Appendix IV**.

2.4 Results and discussion

2.4.1 Characterisation of the vine shoots

Prior to the fractionation of the main components of the vine shoots, their chemical composition was stablished by subjecting them to different analytical procedures. It was determined that they contained 2.6 ± 0.1 % of ashes and 3.79 ± 0.03 % of extractives (expressed as g/100 g oven-dried vine shoots). According to the NREL protocol, the vine shoots presented 26.3 ± 0.3 % of acid-insoluble lignin, 26.9 % of hemicelluloses and 33.2 ± 3.2 % of glucan (corresponding to a 60.2 % of holocelluloses) (expressed as g/100 g oven-dried vine shoots). The hemicelluloses content was measured as the joint contribution of 12.3 ± 0.7 % of xylan, 1.4 ± 0.2 % of arabinosyl substituents (ArOS), 3.2 ± 0.7 % of galactosyl substituents (GalactOS), 1.6 ± 0.3 % of mannosyl substituents (MaOS), 4.3 ± 0.5 % of acetyl groups (AcOS) and 4.1 ± 0.2 % of galacturonic acids (GaOS) (expressed as g/100 g oven-dried vine shoots). Thus, the composition of the hemicelulloses, in molar ratio, in xylose: arabinose: galactose: mannose: acetyl groups was = 10:

1.7: 2.1: 1.1: 10.7. The composition of the vine shoots determined using the NREL protocol is in agreement with what it has been previously reported by Bustos et al. [2.79] and Moldes et al. [2.5]. However, these authors were not able to observe the presence of other monosaccharides apart from glucose, xylose and arabinose due to the analytical method employed.

The Klason lignin, hemicelluloses and α-cellulose present in the vine shoots were also determined by the TAPPI (T222-om-98) protocol and by the procedures proposed by Wise et al. [2.68] and Rowell [2.69], respectively. According to these procedures, the vine shoots contained 28.4 ± 1.1 % of acid-insoluble lignin, 59.0 ± 0.3 % of holocellulose (31.8 ± 1.2 % of α-cellulose and 27.2 % of hemicelluloses) expressed as g/100 g oven-dried vine shoots. Although the two methods used for the determination of the cellulosic and hemicellulosic fractions consider these fractions differently, the results obtained were comparable. Thus, due to the lower time needed to carry out the quantitative acid hydrolysis (NREL protocol), this procedure was selected as the routine protocol for the determination of the chemical composition of the solid fractions obtained throughout the different treatments.

2.4.2 Solubilisation of the hemicellulosic fraction of the vine shoots by the autohydrolysis treatment

The procedure selected during this thesis for the isolation of the hemicellulosic fraction from the vine shoots was the autohydrolysis, as it has been reported that this process presents a high selectivity towards the solubilisation of this fraction, apart from being one of the greenest procedures [2.15, 2.31]. To study the influence of the temperature on the solubilisation of the hemicelluloses and consequently on the characteristics of the resulting products, the treatments were carried out in a range of temperatures between 180 and 215 ºC under a non-isothermal regimen. In Figure 2.6 the temperature profile of the treatment carried out, for instance at 205 ºC is shown.

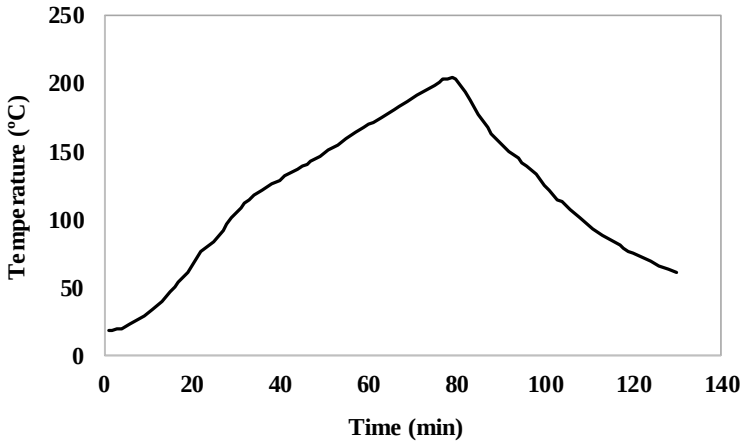


Figure 2.6. Temperature profile of the autohydrolysis treatment of the vine shoots at 205ºC.

The temperature profile of the autohydrolysis treatment permits the determination of the severity of the processes (S_0), making possible the comparison of the obtained results with the ones achieved at similar severities by other authors.

The effectiveness of the autohydrolysis treatment performed at different severities in the removal of the hemicelluloses present in the vine shoots was estimated by considering the solubilisation of the raw material and the chemical composition of the untreated and autohydrolysed vine shoots. All these parameters, together with the severity of the treatments are collected in Table 2.4.

The solubilisation of the vine shoots increased sharply between 180 and 200 ºC, as it can be seen in this Table. Afterwards, the solubilisation continued increasing but with a lower rate, presenting its maximum (40.5 %) at 215 ºC (S_0 = 4.65). Surek and Buyukkileci [2.80] and Moniz et al. [2.81] also observed similar solubilisation percentages (38.2% and 38.5%, respectively) during the autohydrolysis treatments of rye straw and corn straw at S_0 = 4.29 and S_0 = 4.51, respectively.

The increase of the solubilisation of the vine shoots with the temperature is mainly attributed to the removal of the hemicelluloses, as at the harshest conditions (S_0 = 4.65) this fraction was almost completely removed from the vine shoots, being only 6.8 % of the hemicelluloses present in the vine shoots remained in the solid after the treatment. The removal of the hemicelluloses allowed the obtaining of solid fractions mainly composed by acid-insoluble lignin and glucan, as these two fractions are the least susceptible in this process.

Table 2.4. Severity of the autohydrolysis treatments, substrate solubilisation (expressed as g/100 g oven-dried vine shoots), chemical composition of the spent solid (expressed as g/100g oven-dried spent solid) and hemicellulose removal yield (%) obtained at the different temperatures.

	Temperature (ºC)							
	180	**185**	**190**	**195**	**200**	**205**	**210**	**215**
Severity (S_0)	3.29	3.45	3.60	3.82	4.01	4.28	4.47	4.65
Substrate solubilisation	24.2 ± 0.5	27.9 ± 0.5	30.7 ± 0.3	33.8 ± 0.7	38.7 ± 1.1	38.8 ± 0.9	41.1 ± 1.3	40.5 ± 0.4
Fraction								
Glucan	34.0 ± 2.6	32.0 ± 0.4	30.0 ± 0.8	28.0 ± 1.1	27.7 ± 1.7	34.4 ± 1.5	34.6 ± 0.3	34.8 ± 1.9
Xylan	12.6 ± 1.4	8.5 ± 0.3	6.0 ± 0.4	4.2 ± 0.2	3.3 ± 0.4	2.5 ± 0.5	1.8 ± 0.2	1.7 ± 0.3
Arabinosyl substituents	0.91 ± 0.04	0.80 ± 0.01	0.71 ± 0.04	0.37 ± 0.03	ND	ND	ND	ND
Galactosyl substituents	ND	ND	ND	ND	ND	ND	ND	ND
Manosyl substituents	1.5 ± 0.1	1.7 ± 0.2	1.4 ± 0.01	1.2 ± 0.2	1.0 ± 0.3	1.0 ± 0.2	0.60 ± 0.04	0.60 ± 0.08
Acetyl groups	3.4 ± 0.2	3.1 ± 0.2	2.8 ± 0.4	ND	ND	ND	ND	ND
Galacturonic acid	2.7 ± 0.4	2.1 ± 0.2	1.80 ± 0.03	1.5 ± 0.2	1.2 ± 0.1	1.1 ± 0.2	1.0 ± 0.1	0.80 ± 0.03
Klason lignin	34.1 ± 1.6	39.4 ± 2.3	44.0 ± 1.7	49.0 ± 3.5	52.0 ± 1.7	54.0 ± 3.2	55.1 ± 2.2	55.3 ± 1.1
Hemicelluloses removal yield (%)	40.7	56.7	68.1	82.1	87.5	89.6	92.6	93.2

ND: not detected

Surek and Buyukkileci [2.80] also obtained solid residues mainly composed by Klason lignin and glucan (67.9 and 28.3 %, respectively) when they subjected barley husks to autohydrolysis under severe conditions ($S_0 = 4.29$).

The percentage of the acid-insoluble lignin contained in the solid residues increased continuously with the temperature. However, as during the autohydrolysis the reaction media is slightly acid it is possible to have a partial solubilisation of the acid-soluble lignin during the treatments. Conversely to the Klason lignin, the glucan content of the solid residues did not follow an extremely clear trend as it fluctuated slightly with the temperature. These fluctuations could be associated to the procedure used to determine the composition of the solids, as it does not differentiate the glucose present in the hemicelluloses from the one forming part of the cellulose. For a better understanding of what occurs during the autohydrolysis processes the chemical and the structural characteristics of the products solubilised during the treatments were analysed.

2.4.2.1 Characterisation of the hydrolysates obtained at different temperatures

As it is expected, during the autohydrolysis treatments mainly the solubilisation of the hemicellulosic fraction of the vine shoots took place. As it occurred with the composition of the residual solids, the composition of the hydrolysates was also strongly affected by the temperature of the process as it can be seen from the degree of conversion of the polysaccharides present in the vine shoots into their solubilised oligomers and monosaccharides, which are collected in Table 2.5.

Table 2.5. Conversion of the polysaccharides present in the vine shoots into their corresponding derived products.

	Temperature (ºC)							
	180	**185**	**190**	**195**	**200**	**205**	**210**	**215**
Severity (S_0)	3.29	3.45	3.60	3.82	4.01	4.28	4.47	4.65
Substrate into NVC (% of oven-dried weight)	17.4	18.6	22.1	24.8	27.7	26.3	23.0	20.9
Substrate into VC (% of oven-dried weight)	6.8	9.3	8.6	9.0	11.0	12.5	18.2	19.7
Glucan into Glc (%)	0.9	0.9	1.1	0.7	0.8	1.0	1.5	2.6
Xylan into Xyl (%)	0.4	0.5	0.6	1.7	1.9	4.2	6.8	7.0
Arabinosyl substituents into Ara (%)	25.0	26.3	31.4	19.0	21.2	18.3	11.2	10.4
Galactosyl substituents into Ga (%)	6.6	6.7	7.9	6.2	7.0	7.4	9.5	9.2
Manosyl substituents conversion into Man (%)	5.2	4.6	5.5	5.1	5.7	7.6	11.7	17.7
Acetyl groups into acetic acid (%)	12.7	14.2	16.9	34.8	38.8	57.5	75.2	105.8
Galacturonyl substituents into galacturonic acid (%)	12.2	12.2	14.5	13.1	14.6	13.9	7.8	5.8
Glucan into GOS (%)	15.7	15.8	18.9	19.6	21.8	20.5	14.1	9.0
Xylan into XOS (%)	29.0	39.4	46.9	74.5	83.1	79.0	39.2	18.7
Arabinosyl groups into ArOS (%)	37.8	40.1	47.7	38.5	42.9	19.7	16.8	19.7
Galactosyl groups into GalactOS (%)	19.6	18.9	22.6	29.8	33.2	20.0	6.4	ND
Manosyl groups into MaOS (%)	25.6	26.0	30.9	56.0	62.5	50.3	55.8	77.7
Acetyl groups conversion into AcOS (%)	24.3	25.9	30.9	18.5	20.6	17.8	15.5	15.0
Galacturonic acid present in soluble components (% of the initial amount)	24.5	30.6	36.5	60.0	66.9	63.8	38.9	31.8

ND: not detected. Non-volatile content (NVC), Volatile content (VC), Oven-dried (o. d.), Glucose (Glc), Xylose (Xyl), Arabinose (Ara), Galactose (Ga), Mannose (Man), Glucooligosaccharides (GOS), Xylooligosaccharides (XOS), Arabinosyl substituents (ArOS), Galactosyl substituents (GalactOS), Mannosyl substituents (MaOS), Acetyl groups (AcOS).

In this table, not only the hemicellulose-derived products have been taken into account but also the glucan-derived compounds as the autohydrolysis liquors also contained glucooligosaccharides

and glucose. Due to the analytical procedure employed for the characterisation of the solid residues, it was not possible to determine the cellulosic or hemicellulosic origin of the glucan-derived compounds. These compounds could be forming part of the main chain or as substituents of the hemicelluloses or they can be released due to the partial solubilisation in hot water of the cellulose [2.82]. Furthermore, the conversion of the substrate into non-volatile compounds (NVC) increased with the temperature until it reaches its maximum at 200 ºC, then it started to decrease with the increase of the temperature. Martínez et al. [2.83] also observed this trend during the autohydrolysis of sugar beet pulp, and they attributed the increase of the NVC to the solubilisation of hemicelluloses, extractives and other fractions while the decrease of the NVC was attributed to decomposition reactions. Thus, the conversion of the substrate into volatiles suffers a more drastic increase after 200 ºC, as it can be appreciated in Table 2.6 for the concentrations of HMF and furfural in the liquors.

With respect to the polysaccharide-derived products, which are the main constituent of the non-volatile content, it can be appreciated that the conversion of the hemicelluloses of the vine shoots into their corresponding oligomers and substituents increased with the temperature of the treatment until their reached their maximum at 200 ºC. Then, above this temperature, these conversion degrees started to decrease due to a further hydrolysis of the oligomers into their corresponding monosaccharides. Thus, the conversion of the polysaccharides into their corresponding monosaccharides and acids increased slowly with the increase of the temperature until it reached 200 ºC, then their conversion degrees increased more drastically. These behaviours were also observed for the conversion of glucan into the corresponding GOS and glucose.

Jesus et al. [2.84] were able to obtain the maximum XOS content in the autohydrolysis liquors obtained from vine shoots in a treatment carried out in an isothermal regimen and at a similar severity (S_0 = 4.13). However, compared with the results obtained in this thesis they achieved a lower conversion degree of xylan into xylooligosaccharides (65.1 %) that could be associated to the differences between working on an isothermal and non-isothermal regimen, to the differences in the vine specie or to the variations in the climate conditions.

The NVC of the autohydrolysis liquors were not only attributed to polysaccharide-derived compounds, since during the autohydrolysis treatment some secondary processes such as the solubilisation of extractives or acid-soluble lignin could take place. Some of the products generated in these secondary processes were the phenolic and flavonoid compounds as it can be seen in Table 2.6. The presence of the phenolic compounds could also be attributed to the partial depolymerisation of lignin which could occur at harsh autohydrolysis conditions [2.85].

Table 2.6. Degradation compounds concentration and total phenolic and total flavonoid content (TPC and TFC, respectively) present in the autohydrolysis liquors obtained at different temperatures.

	Temperature (ºC)							
	180	**185**	**190**	**195**	**200**	**205**	**210**	**215**
Severity (S_0)	3.29	3.45	3.60	3.82	4.01	4.28	4.47	4.65
Furfural (g/L)	0.02 ± 0.01	0.030 ± 0.003	0.054 ± 0.002	0.142 ± 0.003	0.27 ± 0.02	0.52 ± 0.04	1.19 ± 0.10	1.81 ± 0.07
HMF (g/L)	0.013 ± 0.002	0.014 ± 0.001	0.021 ± 0.003	0.051 ± 0.002	0.08 ± 0.01	0.154 ± 0.003	0.30 ± 0.03	0.50 ± 0.01
TPC[a]	0.88 ± 0.05	1.10 ± 0.03	1.22 ± 0.01	1.30 ± 0.03	1.53 ± 0.03	1.89 ± 0.02	2.01 ± 0.04	2.25 ± 0.04
TFC[b]	0.78 ± 0.03	0.84 ± 0.01	0.87 ± 0.02	0.85 ± 0.01	0.820 ± 0.004	0.99 ± 0.01	1.070 ± 0.002	1.10 ± 0.01

[a] expressed as g gallic acid (GAE)/100 g oven-dried vine shoots), [b]expressed as g RE/100 g oven-dried vine shoots).

The severity of the autohydrolysis treatment produced an increase of the solubilisation of the phenolics and flavonoids compounds of the vine shoots, presenting their maximum concentrations (2.25 g GAE/100 g vine shoot and 1.10 g RE/100 g vine shoots, respectively) in the liquors obtained at the severest conditions (215 ºC, S_0 = 4.65). Jesus et al. [2.84] reported a similar TPC to the one obtained in the autohydrolysis treatment carried out at 205 ºC (S_0 = 4.28) in this thesis (1.88 vs 1.89 g GAE/100g vine shoots, respectively), when they carried out an autohydrolysis treatment of vine shoots in an isothermal regimen working at S_0 = 4.13.

2.4.2.1.1 FTIR analyses of the hydrolysates

In addition to the determination of the chemical composition of the hydrolysates, they were subjected to FTIR analyses in order to corroborate their composition and to have a deeper insight

of their structure. The FTIR spectra of the autohydrolysis liquors obtained at different temperatures are represented in Figure 2.7.

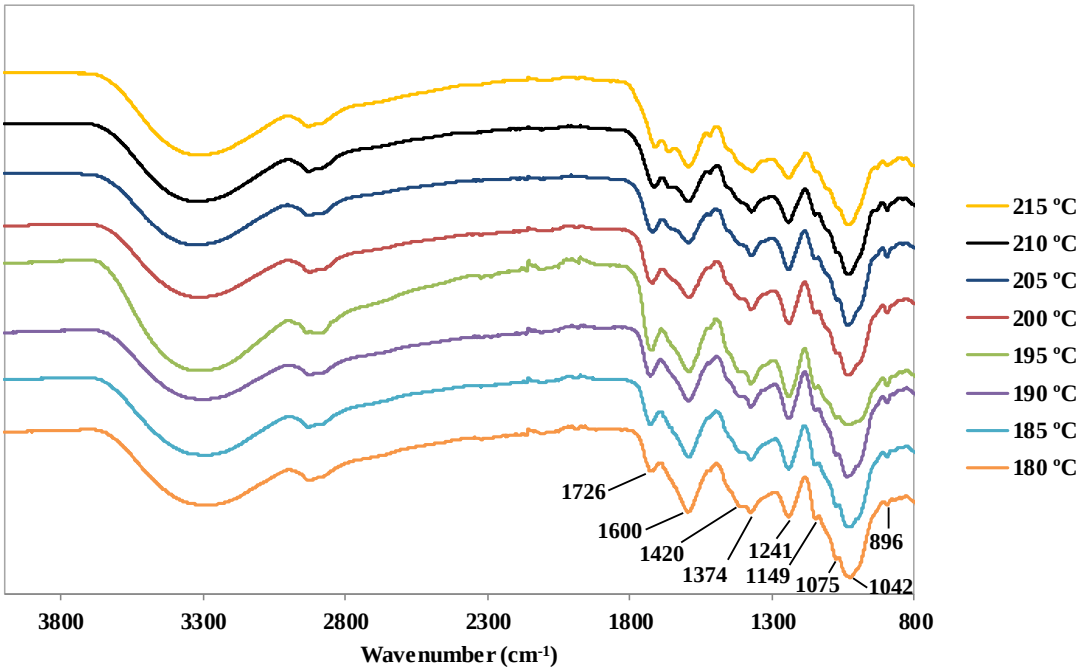


Figure 2.7. FTIR spectra of the hydrolysates obtained during the autohydrolysis treatments of vine shoots carried out at different temperatures.

The band assignation was carried out taking into account what it has been previously reported in the literature for the characterisation of the hemicelluloses [2.86-2.89] and it was observed that all the spectra presented the bands expected for the hemicellulosic polymers. The presence of xylans was corroborated by the band observed at 1042 cm^{-1} which corresponded to the C-O, C-C stretching or to the C-OH bending vibration of the hemicelluloses. The small band observed at 896 cm^{-1} is assigned to the β-glycosydic bonds between the sugar units that form part of the backbone of the hemicelluloses. The small shoulders observed at 1075 and 1149 cm^{-1} confirmed the presence of arabinosyl side chains, corresponding the last band to the glycosidic bond vibration in arabinoxylans. The presence of acetyl substituents was corroborated by the bands that could be appreciated at 1726, 1374 and 1241 cm^{-1} which corresponded to the C=O stretching, to the symmetric CH_3 bending and to the C-O stretching vibrations. The bands observed at 1600 and

1420 cm^{-1} confirmed the presence of galacturonyl substituents as they represent the asymmetric and symmetric (C=O) stretching vibration of the carboxylate groups.

Apart from the typical hemicelluloses´ bands which were observed in the FTIR spectra of all the hydrolysates, a small band could be appreciated at 1656 cm^{-1} in the liquors obtained in the treatments carried out above 205 ºC. This band confirms the presence of a higher content of lignin-derived phenolic compounds in these liquors as it is attributed to the C=C stretching of coniferyl and sinapyl alcohols, which are two of the monomers of lignin.

2.4.2.1.2 HPSEC analyses of the hydrolysates

The influence of the severity of the autohydrolysis treatments in the molecular weight distribution of the polysaccharide-derived products solubilised during the autohydrolysis treatments was assessed by HPSEC.

As it can be seen in Figure 2.8 the temperature at which the autohydrolysis treatment is carried out, and therefore the severity of the process, exerted a high influence on the molecular weight distribution of the obtained polysaccharide-derived products. It was also appreciable that each hydrolysate contained several fractions with polysaccharide-derived products of different molecular weights. Depending on the molecular weight (Mw) of the products contained in each fraction they could be classified as low Mw oligosaccharides (~200 Da), medium Mw oligosaccharides (~10,000 Da) and high Mw oligosaccharides (~100,000 Da) [2.90]. Apart from these fractions, the hydrolysates also contained a small fraction with a Mw lower than 180 Da. Detailed information about the different fractions found in each hydrolysate, including its percentage, the Mw, the number average (Mn) and the polydispersity index (Mw/Mn) is collected in Table 2.7.

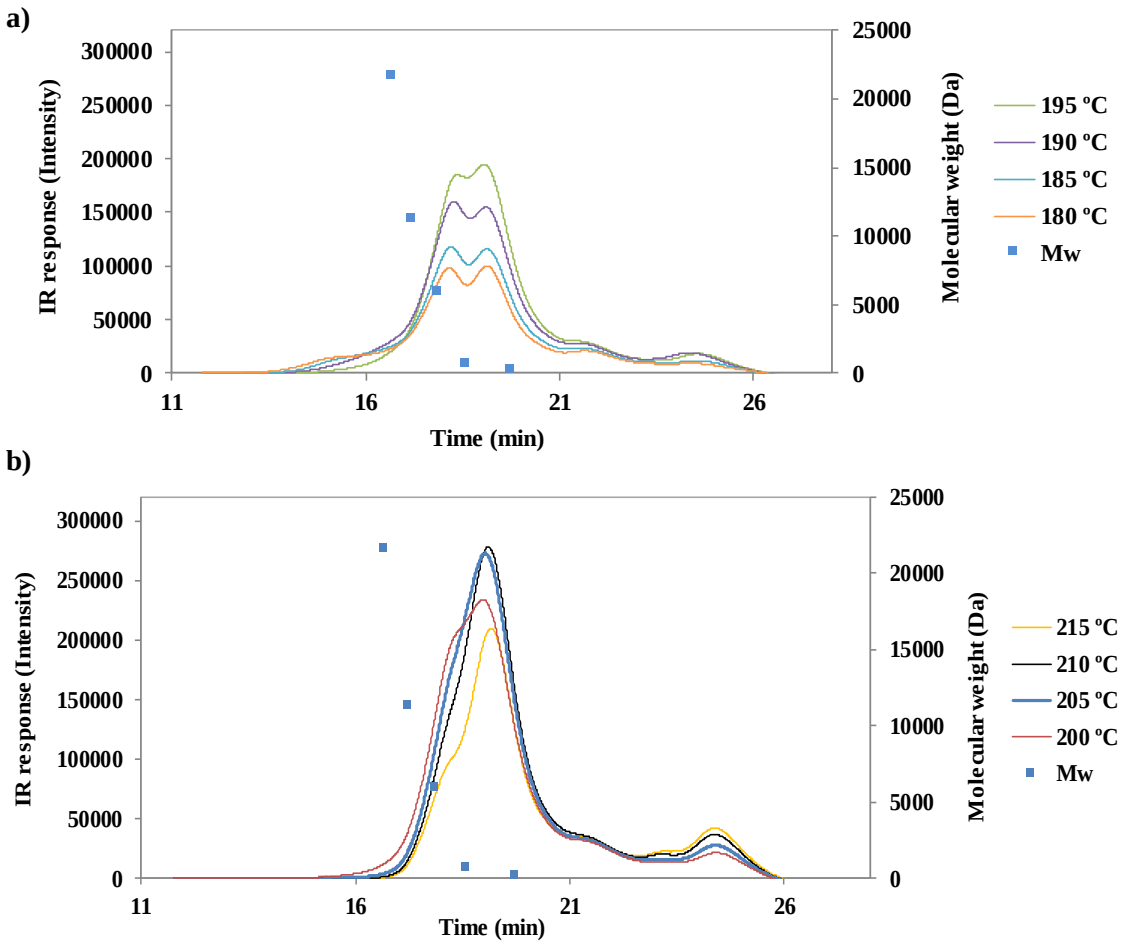


Figure 2.8. HPSEC chromatograms of the hydrolysates obtained at different temperatures: (a) between 180 and 195 ºC, (b) between 200 and 215 ºC.

As it can be seen in this Table, the higher the severity of the autohydrolysis treatment the lower is the molecular weight of the polysaccharide-derived products, being only the hydrolysates obtained at the mildest conditions the ones that contained high Mw polysaccharide-derived products. Then, as the severity of the treatment increased, a decrease was noticed on the percentage of the medium molecular weight fraction present in the autohydrolysis liquors as well as on its molecular weight. For instance, the hydrolysate obtained at 180 ºC contained 44.5 % of a fraction with a molecular weight of 8,318 Da, while the one produced at 215 ºC presented a fraction of 4,026 Da that corresponded to the 20.1 % of the sample. Regarding the low MW polysaccharide-derived products, they were the predominant fraction of the hydrolysates obtained under the severest conditions, since the increase of the severity of the autohydrolysis treatment promote the hydrolysis reactions of the oligosaccharides with high and medium polymerisation

degrees. These trends were also observed by Garrote et al. [2.91] and Wang et al., [2.92] when they analysed by HPSEC the autohydrolysis liquors obtained from *Eucalyptus globulus* and rapeseed straw, respectively, at different severities.

Table 2.7. Percentage, average molecular weight (Mw), number average (Mn) and polydispersity index (Mw/Mn) of the different fractions of polysaccharide-derived products present in the autohydrolysis liquors obtained at different temperatures.

T (ºC)	High molecular weight fraction				Medium molecular weight fraction				Low molecular weight fraction			
	%	Mw (Da)	Mn	Mw/Mn	%	Mw (Da)	Mn	Mw/Mn	%	Mw (Da)	Mn	Mw/Mn
180	6.8	145,770	110,191	1.32	44.5	8,318	3,612	2.49	42.8	455	124	1.59
185	5.9	85,483	124,143	1.35	42.8	6,142	3,262	1.88	42.5	437	107	1.88
190					50.6	10,390	3,153	3.29	44.6	416	124	3.34
195					41.3	6,440	3,150	2.04	54.3	487	130	3.74
200					41.3	4,449	2,698	1.64	54.4	485	169	2.86
205					27.6	3,944	2,958	1.34	68.5	564	178	3.16
210					17.3	4,330	3,556	1.21	78.1	627	199	3.15
215					20.1	4,026	3,239	1.24	75.0	550	170	3.22

The fractions with polysaccharide-derived products with high MW observed in the hydrolysates obtained at 180 and 185 ºC presented a molecular weight higher than what other authors observed for oligosaccharides obtained by autohydrolysis. Wang et al. [2.92], for instance, obtained xylooligosaccharides with Mw of 8430 Da during the autohydrolysis of rapeseed straw at S_0 3.26. Nevertheless, Tunc and Van Heiningen [2.90] also observed the presence of polysaccharide-derived productswith molecular weights between 80,000 and 132,000 Da in the autohydrolysis liquors obtained from a mix of southern hardwoods (sweet and black gum (35.0%), oak (35.0%), maple (15.0%), poplar and sycamore (12.0%) and southern magnolia (3.0%)) under the mildest conditions (150 and 130 ºC, respectively). They attributed these high molecular weights to oligo-glucose or glucooligosaccharodes, which were also subsequently hydrolysed with the increase of the severity of the treatment.

The presence of glucooligosaccharides in the hydrolysates obtained from the vine shoots at different temperatures was observed during their chemical composition analysis. These compounds could be attributed to the presence of glucoxylans in the vine shoots rather than to the amorphous cellulose. Since, the cellulose is only partly soluble in water up to DP 7-8 [2.82, 2.93]

and the polysaccharide-derived products of the hydrolysates obtained at 180 and 185 ºC presented a higher Mw (145,770 and 85,483 Da, respectively).

2.4.2.1.3 TGA analyses of the hydrolysates

The thermostability is an important parameter to take into account for bioactive compounds if they are going to be subjected to sterilisation processes [2.94] or if they are going to be employed during biomaterials synthesis. Taking into account this, the thermal degradation profile of the compounds present in the autohydrolysis liquors was determined by subjecting them to thermogravimetric analyses. The resulting thermogravimetric curves and the first derivative curves are shown in Figures 2.9 and 2.10 (a and b), respectively.

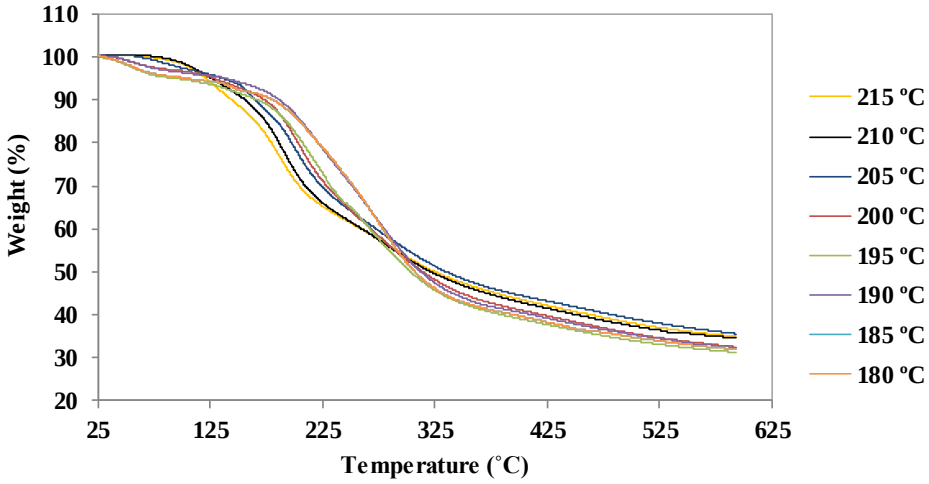


Figure 2.9. Thermogravimetric curves of the hydrolysates obtained at different temperatures.

As it can be seen from Figure 2.9, the thermal stability of the compounds solubilised during the autohydrolysis treatments is strongly affected by the severity of the process, as it has been previously seen for their chemical composition and molecular weight distribution. All the hydrolysates showed the same degradation pattern which presented three stages, but the temperature at which each stage started was influence by the severity of the autohydrolysis process. The second degradation step, for instance, took place at lower temperatures in the case of the hydrolysates obtained at more severe conditions.

The first degradation step took place below 100 ºC and a weight loss between 3.0 and 4.0 % was noticed for all the autohydrolysis liquors. This decrease on the mass was associated to the gradual evaporation of the water contained in the samples. The second degradation step which took place between 100 and 350 ºC (considering all the hydrolysates), is associated to the degradation of the hemicelluloses which is supposed to take place between 200 and 320 ºC [2.95]. The last degradation step started around 375 ºC and it could be attributed to the degradation of the remaining residues into gaseous products such as CO, CO_2, CH_4, CH_3COOH or HCOOH between others [2.92]. However, not all the residues were converted into gases, as at 600 ºC between 31.0 and 35.0 % of char was obtained, which could be attributed to impurities.

The influence of the severity of the autohydrolysis treatment was more noticeable in the second degradation step, which is associated with the hemicelluloses, as it can be observed in the first derivative curves (Figures 2.10a and 2.10b). From these figures it can be noted that the degradation step that occurs between 100 and 350 ºC, is further constitute by different degradation processes, in which the degradation of monosaccharides, branches and backbone of the polysaccharide-derived products took place [2.96]. It could also be noticed that the highest the severity of the autohydrolysis treatment the lower is the temperature at which the degradation process with the highest weight loss rate occurred. Sun et al. [2.97] observed that the thermal stability of the hemicelluloses was related with their molecular weight, appreciating the increase of the thermal stability of the hemicelluloses when their Mw increased. This is much in relation with what it could be observed in Figure 2.10, as the hydrolysate obtained at 180 ºC, which contained the polysaccharide-derived products with the highest MW, presented the degradation process with the highest weight loss rate occurring at the highest temperature (T_{max} = 272 ºC). Conversely, the hydrolysate obtained at 215 ºC, which is mainly constituted by polysaccharide-derived products with low molecular weight, presented at T_{max} = 186 ºC the process with the highest weight loss rate.

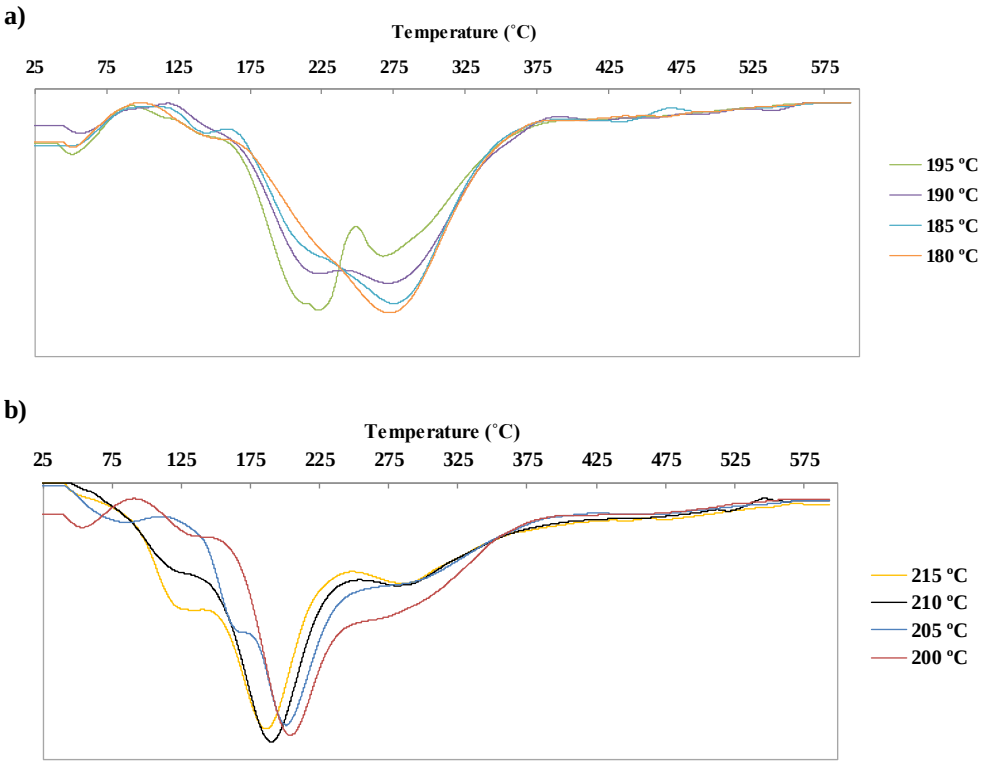


Figure 2.10. Derivative curves of the hydrolysates obtained at different temperatures. (a) between 180 and 195 ºC, (b) between 200 and 215 ºC.

2.4.3 Refining of the hydrolysates by membrane technology for the manufacturing of oligosaccharides

One of the applications given in this thesis to the autohydrolysis liquors , as it has been mentioned above, was the obtaining of functional food ingredients. In the literature, there are many examples of oligosaccharides with prebiotic activity obtained by autohydrolysis treatments of lignocellulosic biomass [2.59, 2.98], but their obtaining from vine shoots has not been exploited yet. However, the autohydrolysis liquors apart from oligosaccharides also contained monosaccharides, carbohydrate degradation products and phenolic and flavonoid compounds, as it has been seen in the section 2.4.2.1. Thus, in order to separate the oligosaccharides from the other compounds without interest for this purpose, the autohydrolysis liquors were refined using membrane technology, as it is a green technology that permits the removal of monosaccharides and other non-volatile compounds (ONVC) or impurities contained in the hydrolysates. It is necessary to highlight that only the hydrolysate obtained during the treatment carried out at 200

ºC was subjected to the refining process, as they contained the highest oligosaccharide content and no very high monosaccharide and degradation product content.

This hydrolysate was subjected to sequential steps of nanofiltration working in concentration and discontinuous diafiltration-concentration mode followed by an ionic exchange process, as it is represented in Figure 2.5. The composition of the retentates obtained in each purification step together with the composition of the refined liquor is reported in Table 2. 8. The composition of the hydrolysate obtained during the autohydrolysis treatment of the vine shoots at 200 ºC that was subjected to the refining process is also shown in Table 2.8.

Table 2.8. Composition of the hydrolysate obtained in the autohydrolysis treatment at 200ºC (stream A), the retentates obtained in the different refining stages (stream B-D) and the refined liquor (stream F), expressed as g/g NVC.

Fraction	Stream			
	A	**B**	**D**	**F/G**
NVC[a]	0.026	0.077	0.044	0.041
Glucose	0.004	0.001	ND	0.003
Xylose	0.041	0.013	0.006	0.008
Arabinose	0.045	0.001	ND	0.001
Glucooliogosaccharides	0.106	0.157	0.170	0.194
Xylooligosaccharides	0.367	0.479	0.514	0.571
Arabinosyl substituents	ND	0.001	0.001	0.004
Galactosyl substituent	0.011	0.012	0.013	0.020
Mannosyl substituents	0.020	0.022	0.023	0.036
Acetyl groups	0.048	0.078	0.097	0.100
Galacturonyl substituents	0.036	0.041	0.044	0.052
ONVC (by difference)	0.321	0.194	0.130	0.009
VC[b] (acetic acid)	0.117	0.038	0.016	0.014

ND: not detected. [a]The NVC was expressed as g. [b]VC: volatile compounds

The vine shoots employed to obtain this hydrolysate and the ones used to analyse the influence of the temperature of the autohydrolysis treatment were acquired after different vintages, thus, differences in the composition of the hydrolysates were observed. This could be attributed to the differences in the composition of the vine shoots due to variations of the weather conditions or in the soil composition.

Regarding the purification process, firstly a nanofiltration operating on concentration mode was carried out until a volume concentration ratio (VCR) of 3 was achieved, obtaining the corresponding retentate B. Comparing the composition of the streams A and B, which is shown in the Table 2.8, it could be appreciated that between 79.3 and 98.0 % of the different monosaccharides (xylose and arabinose, respectively) present in the hydrolysate obtained in the autohydrolysis treatment at 200 ºC (stream A) were removed.

The recovery yields of the oligosaccharides during the first nanofiltration stage were high, varying between 100.0 % for MaOS and AcOS and 71.5 % for GalactOS and galacturonyl substituents. The removal of a high percentage of the monosaccharides and 61.1 % of ONVC of the stream A, leaded to an increase of the total oligosaccharide content from 0.588 g/g NVC (stream A) to 0.790 g/g NVC (stream B).

The second stage of the refining treatment consisted on a nanofiltration working on discontinuous diafiltration-concetration mode. The diafiltration was carried out using 3 diavolumes and then the retentate was concentrated until a VCR of 5.1 was achieved, leading the retentate D. Comparing the composition of the streams B and D it could be seen that 61.8 % of the ONVC and between 73.0 and 81.1 % of the monosaccharides present in the stream B were removed (arabinose and glucose, respectively). The total oligosaccharide content increased during this stage from 0.790 g/g NVC (stream B) to 0.862 g/g NVC (stream D). However, the observed recovery yields were lower than those achieved during the first nanofiltration stage, as in this case it varied between 70.0 % for MaOS and 60.8 % for XOS, GalactOS and galacturonyl substituents.

The stream D was subsequently subjected to an ion exchange process yielding the stream F. In this stage, the monosaccharides present in the stream D were not removed, while a 70.9 % of the ONVC present in the stream D were eliminated. This provoked an increase of the total oligosaccharide content from 0.862 g/g NVC (stream D) to 0.977 g/g NVC (stream F), resulting in high recovery yields which varied between 100.0 % for ArOS, GalactOS and MaOS and 82.6 % for AcOS.

If the whole refining process is considered (the nanofiltration working in concentration and discontinuous diafiltration-concentration mode followed by the ionic exchange process), it could

be appreciated that the total oligosaccharides content increased from 0.588 g/g NVC (stream A) to 0.977 g/g NVC (stream F). This was a consequence of the high ONVC and monosaccharide removal (95.7% and close to 100.0%, respectively) achieved during the refining process. Rivas et al., [2.99] also were able to remove totally the ONVC present in the autohydrolysis liquors obtained from birch wood using a similar refining scheme than the one carried out in this thesis. However, it has to be taken in mind that during the whole refining process oligosaccharides, which were the target compounds of this process, were also removed. The recovery yields of the oligosaccharides considering the streams A and F were: 53.0% for glucooligosaccharides (GOS), 45.3% for XOS, 53.1% GalactOS, 53.7% for ManOS, 60.7% for AcOS and 41.1% for galacturonyl susbtituents.

Apart from the unavoidable oligosaccharides´ losses, the proposed refining scheme permitted the obtaining of a hydrolysate with a 99.0 % of purity, constitutes in a 97.0 % of oligosaccharides, which could present prebiotic activity. The refined oligosaccharides were analysed by different instrumental techniques to elucidate their structure in order to establish a structure-function interrelationship.

2.4.3.1 Elucidation of the structure of the refined oligosaccharides

The composition, branches and polymerisation degree of the oligosaccharides are important characteristics that affect their biological activities. From the data obtained about the chemical composition of the refined oligosaccharides (Table 2.8, Stream F) and from the HPSEC analysis carried out to the autohydrolysis liquor obtained at 200 ºC (Section 2.4.2.1.2.), it is expected that the refined oligosaccharides consist on a mixture of oligomers with different molecular weights, being mainly constituted by xylooligosaccharides with different type of branches.

With the aim of having a more detailed information about their structure, the refined hydrolysates (stream F) were analysed by HPAEC-PAD, MALDI-TOF-MS and UPLC-DAD-ESI-MS.

2.4.3.1.1 HPAEC-PAD analysis of the refined oligosaccharides

The refined oligosaccharides were subjected to HPAEC-PAD analysis using xylooligosaccharides of polymerisation degrees between 2 and 6 as standards. Figure 2.11 displays the elution profile of the oligosaccharides.

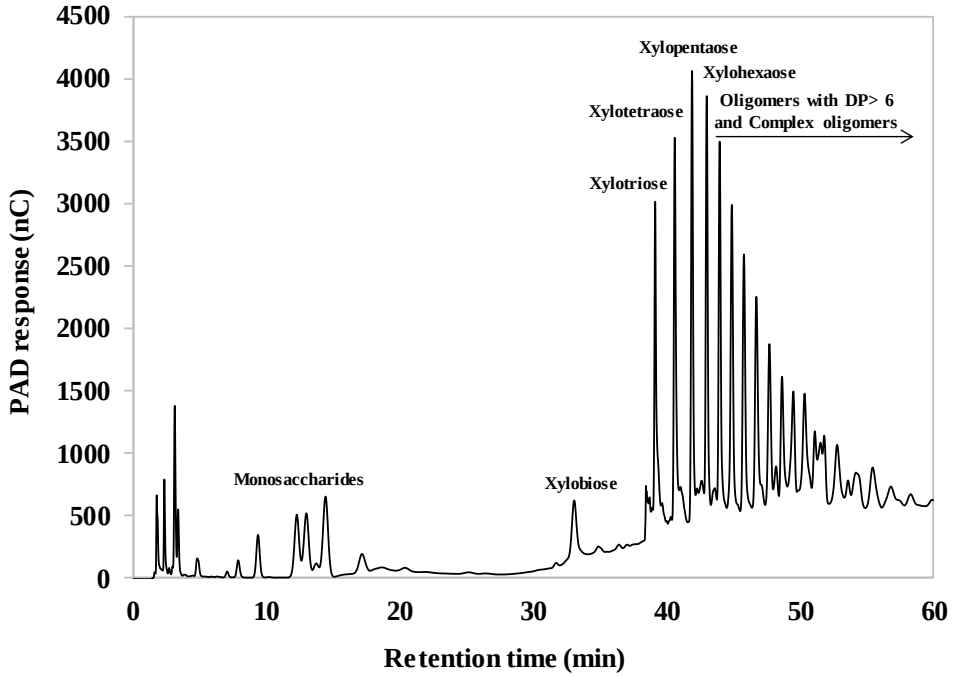


Figure 2.11. HPAEC-PAD elution profile of the refined oligosaccharides (stream F).

Taking into account the HPAEC-PAD elution profile of the refined oligosaccharides, three remarkable zones, corresponding to monosaccharides, linear xylooligosachharides and more complex oligomers could be appreciated, being linear xylooligosaccharides with polymerisation degrees (DP) up to 6 identified. Chen et al. [2.100] obtained xylooligosaccharides with DP between 1 and 9 during the autohydrolysis of *Miscanthus x giganteus*, while Carvalheiro et al. [2.101] obtained xylooligosaccharides with DP larger than 9 during the autohydrolysis of brewery´s spent grains.

For the application of the oligosaccharides as prebiotics, their chain length is a key factor as it could determine in which site of the digestive system occurs their fermentation. It is important for the oligosaccharides to reach distal colonic regions as many colonic diseases, such as the ulceratic colitis and tumors takes place in this region [2.102, 2.103]. Sanchez et al. [2.104] observed that short-chain arabinoxylooligosaccharides were fermented in the proximal colon by

bifidobacteria and lactobacilli, while larger molecules with DP around 29 tend to be fermented in the distal colon.

Thus, as the refined oligosaccharides obtained from the vine shoots contained complex oligosaccharides and linear xylooligosaccharides with DP up to 6, it is presumable that they could present their prebiotic activity in both parts of the gut.

2.4.3.1.2 MALDI-TOF-MS analysis of the refined oligosaccharides

The refined oligosaccharides were analysed by MALDI-TOF-MS in order to have more information about their branching degree and substituents. In Table 2.9 the structures of the oligosaccharide associated to the different mass signals observed during this analysis are collected.

Table 2.9. Results obtained of the MALDI-TOF-MS analysis of the refined oligosaccharides and the possible oligosaccharide assignation.

m/z	Structure	m/z	Structure
702.3	$Pent_5Na$	1255.0	$Pent_3Hex_3MeUrAc_3K$
723.8	$PentHex_2Ac_5K$	1297.6	$Pent_7Ac_3MeUrK$
771.5	$Pent_2Hex_2Ac_3K$	1340.5	$Pent_6Hex_2Ac_4K$
861.6	$Pent3Hex_2Ac_2K$	1369.8	$Pent_9Ac_3MeNa$
885.4	$Pent_5Ac_4K$	1385.6	$Pent_8HexAc_3Na$
903.8	$Pent_3Hex_2Ac_3K$	1428.7	$Pent_8Ac_3MeUrK$
949.0	$Pent_4MeUr_2Na$	1471.2	$Pent_8Ac_4MeUrK$
992.8	$Pent_4Hex_2Ac_2K$	1530.4	$Pent_9Ac_3UrNa$
1008.3	$Pent_7AcNa$	1690.9	$Pent_{10}HexAc_4Na$
1035.0	$Pent_4Hex_2Ac_3K$	1705.1	$Pent_{10}Ac_4UrNa$
1048.3	$Pent_7Ac_2Na$	1775.4	$Pent_7Hex_5Na$
1124.4	$Pent_5Hex_2Ac_2K$	1865.7	$Pent_4Hex_8Na$
1166.8	$Pent_5Hex_2Ac_3K$	2173.8	$Pent_8Hex_3Ac_5MeUr_2Na$
1182.3	$Pent_8Ac_2Na$	2337.3	$Pent_{16}Ac_4K$
1209.3	$Pent_5Hex_2Ac_4K$	2349.9	$PentHex_{12}AcMeUrNa$
1224.6	$Pent_7Ac_2UrNa$	2653.9	$Pent_{17}HexMeUrK$

Pent = pentose; Hex = hexose; Ac = Acetyl group; Ur = uronic acid; MeUr = methyluronic acid.

The structure association was carried out taking into account that the oligosaccharides were detected as sodium or potassium adducts. The results obtained during the HPAEC-PAD analysis of the refined oligosaccharides, suggested that they could be constituted by a mixture of oligomers with different polymerisation degrees. This was corroborated by the results obtained during the MALDI-TOF-MS analysis.

As it can be seen in this Table the refined oligosaccharides were constituted by oligomers highly substituted by acetyl and methylglucuronosyl groups with polymerisation degrees between 3 and 18. Apart from these substituents the identified oligosaccharides were mainly constituted by a higher number of pentoses than hexoses, which could be attributed to xylooligosaccharides presenting galactose, mannose and glucose as substituents (taking into consideration the chemical composition of the oligosaccharides). However, oligosaccharides with a higher number of hexoses than pentoses were also observed. These oligosaccharides could be attributed to acetylated and substituted glucooligosaccharides ([$PentHex_{12}AcMeUrNa$]), as the GOS were the second most abundant oligosaccharides determined in the stream F. The presence of hexoses and pentoses forming part of the main chains could suggest the presence of xyloglucan between the hemicelluloses of the vine shoots.

The results of the MALDI-TOF-MS analysis also suggested the complex and branched structure of the oligosaccharides obtained during the autohydrolysis treatment of the vine shoots, similar to what it has been published for oligosaccharides obtained by the autohydrolysis of oil palm empty fruit bunches [2.105] or corn stover [2.56].

2.4.3.1.3 UPLC-DAD-ESI-MS analysis of the refined oligosaccharides

Apart from analysing the refined oligosaccharides by MALDI-TOF-MS they were also subjected to an UPLC-DAD-ESI-MS analysis to have a deeper insight into their structure. The UV chromatograms of these oligosaccharides, which are shown in Figure 2.12, permitted the identification of five peaks corresponding to different oligosaccharide series. The oligosaccharide series that form part of each of the peaks were tentatively identified being this information collected in Table 2.10.

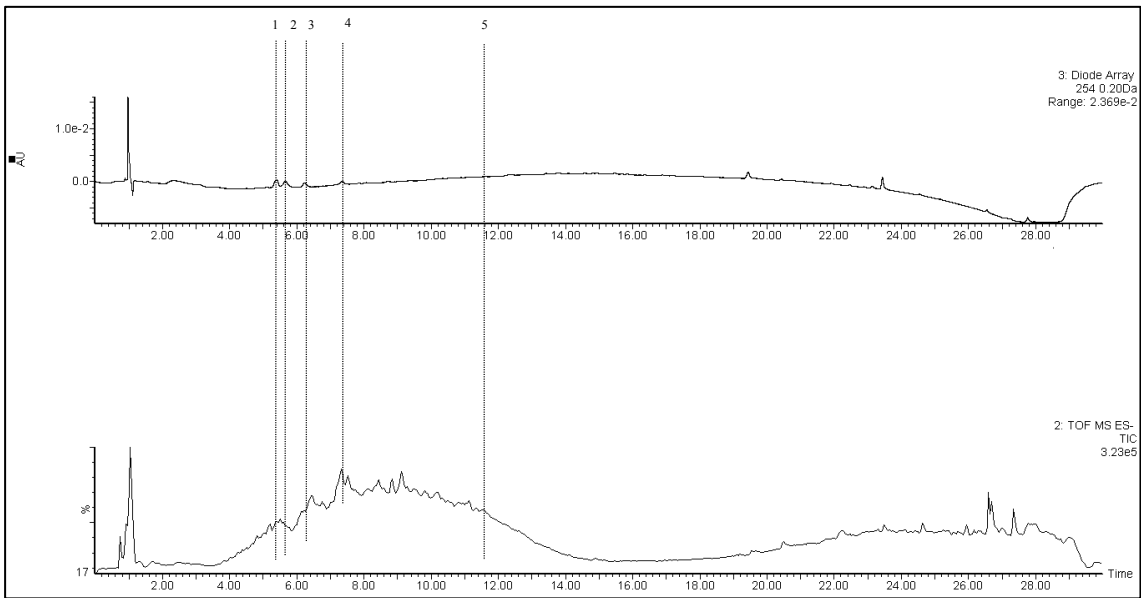


Figure 2.12. UV and MS (in positive ionisation mode) chromatograms of the refined oligosaccharides analysed by UPLC-DAD-ESI-MS.

As it can be seen from the results collected in Table 2.10 the identified oligosaccharides presented polymerisation degrees up to 9, which are lower than the ones observed during the MALDI-TOF-MS analysis. Furthermore, the identified oligosaccharides presented a high substitution pattern, a high acetylation degree and they containeduronic substituents as it was noted during the MALDI-TOF-MS analysis. However, during the UPLC-DAD-ESI-MS analysis glucooligosaccharides were not observed as the identified oligosaccharides were mainly composed by pentoses and not by hexoses.

It could be noticed that the oligosaccharides identified by these two instrumental techniques did not coincide strictly. This could be due to the lack of suitable fragmentation standards which means that there is not enough information to elucidate correctly the structure with these two techniques [2.106]. Nevertheless, the results obtained by MALDI-TOF-MS and UPLC-DAD-ESI-MS corroborate that the refined oligosaccharides obtained during the autohydrolysis treatment of the vine shoots were mainly constituted by highly substituted xylooligosaccharides with different polymerisation degrees.

Table 2.10. Tentative oligosaccharides series in the UPLC-DAD-ESI-MS spectra of the peaks eluted during the UPLC analysis of the refined oligosaccharides.

Peak	m/z	Oligosaccharide series	Peak	m/z	Oligosaccharide series
1	520.94	$[Pent_3Ac_2Na]^+$	**2**	647.98	$[Pent_2UrMeUr]$
	737.97	$[Pent_4Ac_4Na]^+$		779.97	$[Pent_3UrMeUr]$
	869.97	$[Pent_5Ac_4Na]^+$		1133.96	$[Pent_3Ur_3MeUr+2H]^+$
	1001.97	$[Pent_6Ac_4Na]^+$		1398.98	$[Pent_5Ur_3MeUr+3H]^+$
	1265.96	$[Pent_8Ac_4Na]^+$		1818.84	$[Pent_8Ur_3MeUr+3H^+Na^+]$
	1440.95	$[Pent_9Ac_5Na]^+$			
3	647.98	$[Pent_2Hex_2Ac]$	**4**	515.99	$[PentHex_2Ac]$
	779.98	$[Pent_3Hex_2Ac]$		821.97	$[Pent_3Hex_2Ac_2]$
	911.97	$[Pent_4Hex_2Ac]$		953.97	$[Pent_4Hex_2Ac_2]$
	1043.96	$[Pent_5Hex_2Ac]$		1085.96	$[Pent_5Hex_2Ac_2]$
	1349.94	$[Pent_7Hex_2Ac_2]$		1217.95	$[Pent_6Hex_2Ac_2]$
				1349.95	$[Pent_7Hex_2Ac_2]$
				1613.93	$[Pent_9Hex_2Ac_2]$
5	773.97	$[Pent_2Hex_2Ac_4]$			
	905.97	$[Pent_3Hex_2Ac_4]$			
	1211.96	$[Pent_5Hex_2Ac_5]$			
	1343.95	$[Pent_6Hex_2Ac_5]$			
	1475.95	$[Pent_7Hex_2Ac_5]$			
	1517.96	$[Pent_7Hex_2Ac_6]$			

Pent = pentose, Hex = hexose, MeUr = methyluronic acid, Ur = uronic acid, Ac = acetyl groups

2.4.3.2 Determination of the prebiotic activity of the refined oligosaccharides

The rich substitution pattern and the wide range of polymerisation degrees of the refined oligosaccharides obtained during the autohydrolysis of the vine shoots could be beneficial for their biological properties. Since it has been reported that this kind of oligosaccharides present higher bifidogenic properties than the linear ones [2.107].

However, prior to studying the prebiotic activity of the refined oligosaccharides it was necessary to assess their digestibility by subjecting them to *in vitro* simulated gastrointestinal digestion. The

oligosaccharides must remain unaltered by the gastric enzymes nor the gastric pH, as they should reach the gut maintaining their bioactive properties in order to be considered as prebiotics [2.108]. Thus, to observed the stability of the oligosaccharides towards the gastrointestinal digestion the chemical composition of the digested oligosaccharides was determined apart from subjecting them to HPAEC-PAD analysis. During these analyses, it was observed that the release of monosaccharides during the gastric digestion, which was a consequence of the hydrolysis of the oligosaccharides, was less than 3.0 % and that the HPAEC-PAD profile of the digested oligosaccharides was the same as the one obtained from the refined ones (Figure 2.11).

2.4.3.2.1 Analysis of the oligosaccharides consumption during the in vitro fermentation

During the *in vitro* fermentation, oligosaccharides are used as carbon sources by human faecal microbiota to produce short chain fatty acids (SCFAs) which have beneficial activity on the human functions. To determine the consumption of the digested oligosaccharides by the faecal microbiota at determined fermentation times, the carbohydrate composition of the fermentation media was analysed. The carbohydrate content of the fermentation media was analysed at specific fermentation times, being the data collected in the Table 2.11.

Table 2.11. Carbohydrate consumption during the *in vitro* fermentation of the digested xylooligosaccharides obtained from vine shoots (expressed as g/L).

Fraction	Time (h)				
	t = 0	**t = 4**	**t = 10**	**t = 24**	**t = 48**
Glucooligosaccharides (GOS)	1.92 ± 0.05	2.17 ± 0.08	0.95 ± 0.11	0.11 ± 0.04	0.11 ± 0.08
Xylooligosaccharides (XOS)	5.92 ± 0.23	6.10 ± 0.23	3.71 ± 0.21	0.63 ± 0.14	0.62 ± 0.05
Arabinosyl substituents (ArOS)	0.03 ± 0.01	0.040 ± 0.003	0.007 ± 0.003	ND	ND
Galactosyl substituents (GalactOS)	0.77 ± 0.07	0.73 ± 0.03	0.35 ± 0.03	ND	ND
Manosyl substituents (MaOS)	0.47 ± 0.03	0.39 ± 0.01	0.24 ± 0.03	ND	ND
Acetyl groups (AcOS)	1.00 ± 0.04	1.32 ± 0.32	0.23 ± 0.07	0.23 ± 0.02	0.20 ± 0.20
Total oligosaccharides	10.08 ± 0.18	11.34 ± 0.32	5.08 ± 0.75	0.97 ± 0.16	0.648 ± 0.001

ND: not detected.

It could be observed in this Table that during the first 10 h of incubation the oligosaccharides present in the fermentation media were slowly assimilated by the microbiota as only 35.0 % of

the total oligosaccharides present at beginning of the fermentation process were consumed. However, after 24 h of incubation the concentration of the total oligosaccharides decreased drastically, being more than 80.0 % of them consumed, while at the end of the fermentation they were almost completely metabolised.

Moniz et al. [2.109] also observed that the xylooligosaccharides isolated from corn stover by autohydrolysis were not completely consumed before 24 h of fermentation. Conversely, they observed that commercial xylooligosaccharides were almost metabolised after 10 h of fermentation. The slow fermentation rate observed for the xylooligosaccharides obtained from lignocellulosic biomass by autohydrolysis could be attributed to their higher branching degree and to their more complex structure [2.109].

The stability of the refined oligosaccharides obtained during the autohydrolysis of the vine shoots during the gastrointestinal digestion and their slow fermentation rate suggested that they could reach the more distal colon, being able to exert there their prebiotic activity.

2.4.3.2.2 Analysis of the organic acids profile generated during the fermentation of the refined oligosaccharides

The short chain fatty acids (SCFA) generated during the fermentation of the digested oligosaccharides (OS) could exert benefits on different intestinal functions, making evidential their prebiotic potential. In order to compare the organic acid profile generated during the fermentation of the digested oligosaccharides obtained from vine shoots with the profile generated with commercial prebiotic, the *in vitro* fermentation was also carried out using fructooligosaccharides (FOS). In Table 2.12 the profile of the organic acids generated during the fermentation of the digested oligosaccharides and the FOS, together with the pH variation are shown.

During the fermentation of the digested oligosaccharides and FOS, the pH of the fermentation medium decreased with the time due to the formation of organic acids. Differences were observed in the total concentration of SCFA generated during the fermentations of OS and FOS and in their profile. The fermentation of the digested oligosaccharides produced a higher amount of SCFAs

than during the fermentation of the FOS, being a maximum observed after 48 h of incubation (139 vs 102.6 mM). When a medium without carbon source was used (the Control), low amount of SCFAs was obtained as the fermentation of proteins or amino acids by putrefactive bacteria took place.

Regarding the profile of short chain fatty acids generated during the fermentation, differences could be observed when the digested oligosaccharides or FOS were used, as it can be appreciatted in Table 2.12.

Table 2.12. Concentration of the SCFAs (mM) and pH of fermentation media achieved using the digested oligosaccharides from vine shoots (OS) and frutooligosaccharides (FOS) as carbon sources.

Carbon source	Time (h)	pH	Lactic acid	Formic acid	Acetic acid	Propionic acid	Butyric acid	Total SCFA
	0	7.20 ± 0.01	ND	ND	2.0 ± 0.3	ND	ND	2.0
	4	6.90 ± 0.01	0.1 ± 0.1	1.1 ± 0.1	2.2 ± 1.4	0.8 ± 1.3	ND	4.1
OS	10	6.50 ± 0.01	4.5 ± 0.6	9.2 ± 2.5	38.2 ± 2.3	6.7 ± 1.0	1.1 ± 0.4	55.2
	24	5.00 ± 0.01	1.5 ± 0.9	13.1 ± 4.3	81.7 ± 3.0	23.6 ± 4.3	10.3 ± 1.1	128.7
	48	4.8 0 ± 0.01	ND	11.8 ± 5.6	87.5 ± 3.7	26.2 ± 2.7	13.9 ± 3.3	139.4
	0	7.20 ± 0.01	ND	ND	1.3 ± 0.5	ND	ND	1.3
	4	6.9 ± 0.1	2.6 ± 0.7	2.2 ± 1.1	11.8 ± 3.7	0.9 ± 0.4	ND	14.9
FOS	10	6.4 ± 0.1	9.3 ± 2.5	10.2 ± 1.9	35.0 ± 2.4	5.6 ± 0.9	6.4 ± 1.1	57.2
	24	4.7 ± 0.1	8.1 ± 1.4	15.6 ± 3.5	61.0 ± 3.7	12.1 ± 2.5	14.7 ± 2.6	103.4
	48	4.63 ± 0.04	ND	13.9 ± 3.2	61.3 ± 2.7	13.1 ± 1.1	14.2 ± 0.8	102.5
	0	7.20 ± 0.01	ND	ND	1.1 ± 0.6	ND	ND	1.1
	4	7.3 ± 0.1	0.1 ± 0.1	0.06 ± 0.06	0.7 ± 0.2	ND	ND	0.8
Control	10	7.2 ± 0.1	0.1 ± 0.1	3.0 ± 3.0	6.6 ± 1.0	2.3 ± 0.2	0.6 ± 0.2	12.5
	24	6.9 ± 0.1	ND	0.8 ± 0.8	23.1 ± 2.5	4.3 ± 1.1	5.7 ± 1.8	33.9
	48	6.9 ± 0.1	ND	ND	37.0 ± 5.7	7.4 ± 2.4	8.1 ± 2.7	52.5

ND: not detected.

The main metabolic product generated during the fermentation independently of the carbon source or the fermentation time was the acetic acid, which accounted for more than 50.0% of the total SCFAs. The highest acetic acid concentration was achieved after 48 h of incubation, being its concentration 30.0 % higher when the digested oligosaccharides were used (87.5 and 61.3 mM acetate when OS and FOS were used, respectively). The production of acetic acid has been related with several intestinal functions such as the increase of the colonic blood flow or the regulation of the normal epithelial cell division [2.110].

The second most abundant organic acid formed during the *in vitro* fermentation was the propionic acid, which presented its maximum concentration after 48 h of incubation when OS were used as the carbon source (26.23 mM). This acid is supposed to reduce the lipogenesis and the serum cholesterol levels, playing an important role in the prevention of cardiovascular diseases [2.111]. The ratio between the acetic and propionic acids (A/P) could indicate the potential hypolipidemic properties of the prebiotics as it is associated with the reduction of the liver lipids or the synthesis of cholesterol [2.112]. From the data collected in Table 2.12, it could be appreciated that when the FOS were used as carbon sources instead of the digested oligosaccharides a higher A/P ratio was achieved, 4.68 and 3.34, respectively.

Another SCFA generated during the fermentation of the OS and FOS was the butyric acid, which is an anti-inflamatory agent [2.113] and together with the propionic acid it has been reported to help in the prevention and inhibition of colorectal cancer [2.110]. This SCFA was not produced until 10 h of incubation and it presented its highest concentration after 48 h when OS were used and after 24 h when FOS were used, 13.95 mM and 14.67 mM, respectively.

The formic and lactic acids were also produced during the *in vitro* fermentation of the digested oligosaccharides and FOS. The production of these acids was not observed before 4 h of fermentation and their production was higher when fructooligosaccharides were employed being the maximum production achieved after 24 and 10 h, for the formic and lactic acids, respectively. It could be observed that the concentration of lactic acid decreased after 10 h of fermentation being it completely disappeared after 48 h, independently of the employed carbon source. The lactic acid is a transient metabolite produced by bifidobacteria and lactic bacteria which is then converted into other organic acids by other bacteria present in the gut [2.114], explaining this its disappearance.

The SCFAs profile observed during the *in vitro* fermentation of the digested oligosaccharides obtained during the autohydrolysis of the vine shoots is similar to what it has been reported in studies in which *in vitro* fermentation of OS with different structural characteristics were analysed [2.98, 2.105].

2.4.3.2.3 Dynamics of the bifidobacterium population during the in vitro fermentation of the refined oligosaccharides

As it has been seen from the results shown by now, the oligosaccharides obtained during the autohydrolysis of the vine shoots presented prebiotic activity as they were assimilated by the gut microbiota releasing different SCFAs. To observe how these oligosaccharides promote the growth of the *Bifidobacterium* population due to their prebiotic activity, the pellets obtained after the prebiotic activity assessment were analysed by FISH. The growth of the bifidobateria is a key factor in the protection and establishment of the gastrointestinal health [2.98], as it could decrease the susceptibility to diseases such as infections of *Clostridium difficile* and gastroenteritis [2.115]. The bifidogenic potential of the digested oligosaccharides and FOS is illustrated in Figure 2.13.

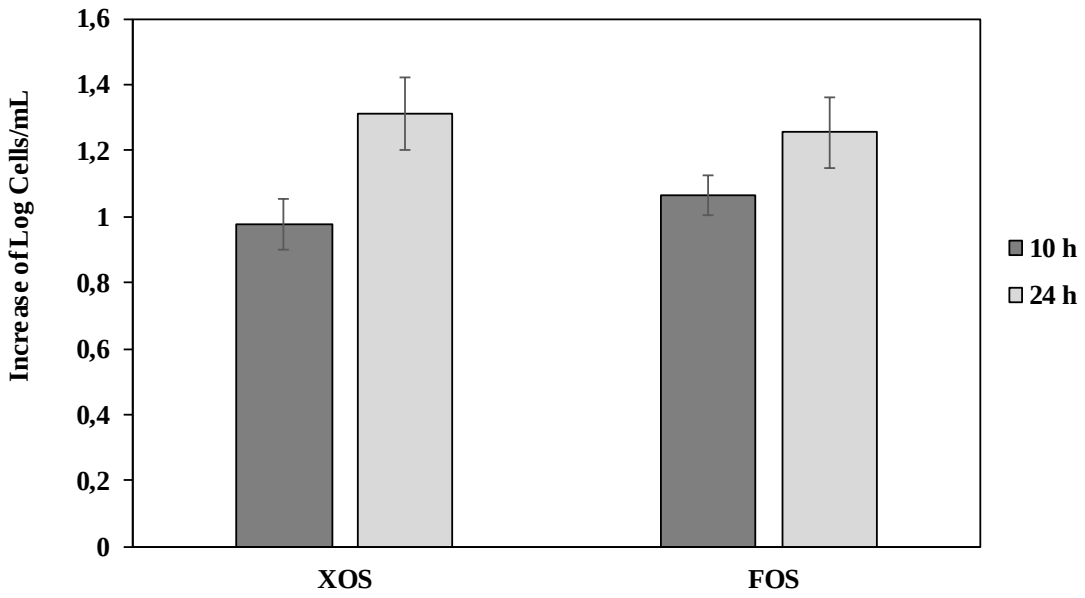


Figure 2.13. Increase of the *Bifidobacterium* population at 10 h and 24 h of fermentation (with respect to time 0 h). The initial bacterial counts was 7.93 ± 0.15. Error bars indicate standard deviations (n = 3).

As it can be appreciated in this Figure, the increase observed in the *Bifidobacterium* population was similar when either OS or FOS were used as carbon sources. An average increase of 14.0 % was observed after 24h of fermentation. Similar observations were reported in the literature when arabinoxilooligosaccharides from wheat bran [2.45] or xylooligosaccharides from corn stover [2.56] were used as carbons sources for *in vitro* fermentation with faecal microbiota.

2.4.4 Liquid-liquid extractions of the hydrolysates for the obtaining of bioactive extracts

Apart from the polysaccharide-derived compounds, the hydrolysates obtained in the autohydrolysis treatments carried out at different temperatures also contained phenolic and flavonoid compounds (Table 2.6), as during these treatments also secondary reactions took place. To recover these bioactive compounds, the hydrolysates were subjected to liquid-liquid extractions with ethyl acetate. These extractions permitted the recovery of compounds solubilised during the autohydrolysis treatments, obtaining extraction yields between 0.95 and 3.80 g extract/100 g oven-dried vine shoots (using the liquors obtained at 180 and 215 ºC, respectively), as it can be seen in Table 2.13.

Table 2.13. Effect of the severity of the autohydrolysis treatments of the vine shoots on the extraction yield (g extract/100 g oven-dried vine shoots) and on the TPC and TFC of the extracts.

	Temperature (ºC)							
	180	**185**	**190**	**195**	**200**	**205**	**210**	**215**
Severity (S_0)	3.29	3.45	3.60	3.82	4.01	4.28	4.47	4.65
Extraction yield	0.95 ± 0.17	1.17 ± 0.16	1.25 ± 0.18	1.30 ± 0.12	1.75 ± 0.21	2.80 ± 0.24	3.50 ± 0.21	3.80 ± 0.19
TPC (g GAE/100 g VS[a])	0.39 ± 0.02	0.52 ± 0.03	0.55 ± 0.08	0.59 ± 0.04	0.84 ± 0.04	1.46 ± 0.02	1.60 ± 0.03	1.62 ± 0.02
TPC (g GAE/ g extract[b])	0.41	0.44	0.44	0.42	0.48	0.52	0.46	0.43
TFC (g RE/100 g VS[a])	0.53 ± 0.06	0.60 ± 0.05	0.66 ± 0.05	0.61 ± 0.01	0.69 ± 0.05	0.83 ± 0.07	0.91 ± 0.07	0.92 ± 0.06
TFC (g RE/ g extract[b])	0.56	0.51	0.53	0.47	0.40	0.30	0.26	0.24

[a]oven-dried vine shoots. [b]oven-dried extracts.

Therefore, the extraction yield was strongly affected by the severity of the autohydrolysis treatment, as the increase of the temperature allowed the obtaining of higher quantities of extracts. Conde et al. [2.46] and Castro et al. [2.116] also observed this trend when they isolated extracts from autohydrolysis liquors of grape pomace and from steam explosion liquors of *Olea Europa* wood, respectively. The first ones were able to isolate between 0.89 and 2.42 g extract/100 g grape pomace, while Castro et al. achieved extraction yields between 1.40 and 2.35 g extract/100 g *Olea Europa* wood.

The obtained extracts were subjected to different analyses in order to assess the influence of the severity of the autohydrolysis treatments on their composition and consequently on their antioxidant and antimicrobial activities.

2.4.4.1 Analysis of the extracts recovered from the hydrolysates

The composition of the extracts recovered from the different hydrolysates in phenolic and flavonoids compounds was analysed, being the results collected in Table 2.13. As it can be appreciated, the phenolic and flavonoid content of the extracts increased with the severity of the autohydrolysis treatment, since they are solubilised from the vine shoots. The increase of these contents was more pronounced for the phenolic compounds than for the flavonoid compounds, as the TPC increased 4.2 times comparing the extracts isolated from the liquors obtained at 180 ºC and 215 ºC, while the TFC only increased 1.7 times. Rostro et al. [2.117], also noticed the influence of the severity of the autohydrolysis treatments on the TPC of the extracts obtained from nixtamalized maize pericarp (NMP), observing variations between 0.04 g GAE/100 g NMP (at S_0 = 3.12) and 0.337 g GAE/100 g NMP (at S_0 = 3.96).

Actually, this trend is associated more with the extraction yield than with the phenolic and flavonoid content of the extracts, as it has been observed that when the phenolic and flavonoid content per gram of extract is considered the extracts isolated from the autohydrolysis liquors obtained at 215 ºC did not present the maximum phenolic and flavonoid content. It has been observed that the phenolic content of the extracts increased with the temperature until 205 ºC (0.52 g GAE/g extract), afterwards the phenolic content of the extract started to decrease. The extract that presented the maximum flavonoid content was the one isolated from the hydrolysate obtained at 180 ºC (0.56 g RE/g extract). Then, the increase of the temperature of this processing provoked a decrease of the flavonoid content of the isolated extracts, observing a TPC of 0.24 g RE/g extract in the extract isolated from the liquor obtained at 215 ºC. Conde et al. [2.46] also observed a decrease of the TPC of the extracts isolated from the autohydrolysis liquors obtained from almond shells, chestnut burs and grape pomace with the increase of the temperature of the

treatment. Gullón et al. [2.118] also observed this behaviour in the extracts obtained from the hydrolysates produced from chestnut shells and they attributed it to the degradation of the solubilised phenolic and flavonoid compounds with the increase of the temperature or to the higher presence of other degradation products.

Another aspect that was appreciated during the liquid-liquid extractions is that they did not remove all the phenolic and flavonoid compounds solubilised during the autohydrolysis process. Comparing the results reported in Tables 2.6 and 2.13 it could be observed that the autohydrolysis liquors presented higher phenolic and flavonoid contents than the extract obtained from the corresponding hydrolysate. Thus, the percentages of the phenolic and flavonoid compounds that were initially present in the liquors and which passed to be part of the extracts were also strongly influenced by the severity of the autohydrolysis treatment, as the phenolic extraction yield increased from 44.3 % at 180 ºC to 72.0 % at 215 ºC. The flavonoid extraction percentage increased from 67.9 % at 180 ºC to 83.6 % at 215 ºC with the increase of the temperature. The differences in the percentages of the phenolic and flavonoid compounds could be attributed to the highest affinity for the organic phase of the phenolic and flavonoid compounds solubilised under more severe conditions.

To have a more detailed vision of the composition of the extracts, they were qualitatively analysed by GC/MS. In addition to phenolic and flavonoid compounds, which have been determined by the TPC and TFC assessments, they could contain degradation products, extractives, waxes, aliphatic fatty acids or esters, between others [2.119]. In Table 2.14 the compounds identified by GC/MS together with their abundance in the extracts are collected. The identification of the compounds was carried out using the NIST library and using literature about the identification of the products generated during autohydrolysis treatments [2.70, 2.116].

The amount of compounds identified in the extracts by GC/MS was quite low, between 10.1 and 38.8 mg compounds/100 g extracts (corresponding to the extracts isolated from the hydrolysates obtained at 180 and 210 ºC, respectively). This could be attributed to the limitations of the technique, which could be the low volatility of the compounds with high molecular weights, the no detection of compounds with molecular weights higher than 800 Da by the mass spectrometer

or the employment of a library which could limit the identification of some compounds. It can be noted that no flavonoid compounds were identified by GC-MS although their presence was observed during the chemical analysis of the extracts.

Table 2.14. Composition of the extracts isolated from the hydrolysates obtained during the autohydrolysis of the vine shoots carried out at different temperatures (mg compound/100 g extract).

Name	t (min)	Main fragments (m/z)	Temperature (ºC)[a]							
			180 (3.29)	**185** (3.45)	**190** (3.60)	**195** (3.82)	**200** (4.01)	**205** (4.28)	**210** (4.47)	**215** (4.65)
Carbohydrate- derived compounds										
Furfural	3.7	96,39,29	ND	ND	ND	ND	ND	1.9	4.6	0.9
5-methyl furfural	5.4	110,53,27	ND	ND	ND	ND	ND	1.2	1.0	ND
5-hydroxymethyl furfural	9.9	97,126,41	ND	ND	ND	4.3	10.3	14.9	15.3	21.2
Lignin-derived compounds										
Phenol	5.6	94,66,39	ND	ND	ND	ND	ND	0.6	ND	ND
Guaiacol	7.3	109,124,81	ND	ND	ND	1.9	2.5	3.3	2.7	2.3
Catechol	9.18	110,64,63	ND	ND	ND	0.9	1.5	1.6	1.8	1.5
Syringol	13.7	154,139,96	ND	ND	ND	1.0	ND	1.1	ND	ND
4-hydroxy benzaldehyde	13.9	121,122,93	ND	ND	ND	ND	ND	0.6	ND	ND
Vanillin	14.9	151,152,109	ND	2.7	3.5	3.2	3.2	3.2	2.9	2.3
Acetovanillone	16.6	151,166,123	ND	2.3	2.2	1.0	1.0	0.8	1.2	0.8
Guaiacylacetone	17.4	137,180,122	ND	3.1	1.9	1.2	1.4	1.1	0.9	1.7
Syringaldehyde	19.4	182,181,167	4.9	5.5	8.0	5.2	5.1	4.4	5.3	3.7
Acetosyringone	20.3	181,196,153	5.2	8.3	12.9	5.1	4.1	3.0	3.1	2.7

[a] inside the brackets the severity associated to each temperature is presented. ND: not detected

The identified compounds could be classified as carbohydrate degradation products and as phenolic compounds derived from lignin. The presence of the lignin-derived compounds could be associated to the acid-soluble lignin that could have been solubilised due to the acid media generated during the autohydrolysis treatment. Moreover, the presence of the lignin-derived compounds could also be associated to the release of the lignin fragments that could remain covalently bound to the hemicellulosic oligomers solubilised during the autohydrolysis due to the acid reaction media [2.120]. It has been reported that the depolymerisation of the lignin fragments into smaller ones could also take place [2.85], being these smaller fragments the ones identified

by GC/MS. Although lignin-derived compounds were present in the extracts, it has been previously seen that the autohydrolysis treatments did not affected highly the lignin fraction present in the vine shoots, similar to what Garrote et al. [2.121] reported.

As it can be seen in Table 2.14, the abundance and the compounds present in the extracts varied strongly with the severity of the autohydrolysis treatments. Between the identified lignin-derived compounds the most abundant ones were acetosyringone, syringaldehyde and vanillin. Castro et al. [2.116] and Garrote et al. [2.70] also observed the presence of vanillin and syringaldehyde between the most abundant lignin-derived compounds of ethyl acetate and dichloromethane extracts obtained from the steam explosion and autohydrolysis liquors of *Olea Europa* wood and corncobs, respectively.

As it occurred with the total phenolic content of the extracts, the maximum of lignin-derived compounds was not observed in the extract isolated from the hydrolysate obtained at the severest conditions (215 ºC). The maximum of lignin-derived compounds were observed in the extract isolated from the autohydrolysis liquor obtained at 190 ºC (28.5 mg compounds/100 g extract) afterwards the presence of the lignin-derived compounds in the extracts decreased with the increase of the temperature. This could be associated with the degradation of the compounds with the temperature or with recondensation reactions that the solubilised lignin breakdown fragments could suffer due to the increase of the severity of the aqueous processing [2.119].

With respect to the carbohydrate-derived compounds, their quantity in the extracts increased with the temperature. They were not observed in the extracts isolated from the liquors obtained below 195 ºC (4.3 mg compounds/100 g extracts), but afterwards their concentration in the extracts increased until they reached their maximum in the extract isolated from the hydrolysate obtained at 215 ºC (22.1 mg compounds/100 g extracts). As it has been mentioned throughout the thesis, these products were formed during secondary reactions at which the dehydration of pentoses and hexoses took place forming furfural and HMF, respectively.

The phenolics, flavonoids and carbohydrate-derived compounds present in the extracts, among others, are expected to influence the antioxidant and antimicrobial activities of the extracts [2.122, 2.123].

2.4.4.2 Antioxidant activity of the extracts isolated from the autohydrolysis liquors

The antioxidant activity of the extracts isolated from the different autohydrolysis liquors was tested by subjecting them to three different assays; the DPPH, the ABTS and the FRAP tests. The results obtained from these tests are collected in Table 2.15 and as it can be observed the severity of the autohydrolysis treatment affected strongly the antioxidant activity of the extracts.

From the data collected in this Table it can be appreciated that the highest the temperature of the autohydrolysis treatment the highest is the amount of antioxidant compounds solubilised from the vine shoots present in the extract. However, this is related with the increase of the extraction yield with the severity of the autohydrolysis process and not with the increase of the amount of antioxidant compounds. As it occurred with the TPC and TFC of the extracts if the specific antioxidant activity was taken into account, being it expressed as the value of Trolox equivalent/g extract, the extract with the maximum antioxidant activity was not the one isolated from the liquor obtained at 215 ºC.

Table 2.15. Antioxidant activity of the extracts obtained from the different autohydrolsis liquors, measured by DPPH, ABTS and FRAP assays.

	Temperature (ºC)							
	180	**185**	**190**	**195**	**200**	**205**	**210**	**215**
Severity (S_0)	3.29	3.45	3.60	3.82	4.01	4.28	4.47	4.65
DPPH (g TE/100 g VS[a])	0.58 ± 0.02	0.66 ± 0.02	0.67 ± 0.01	0.73 ± 0.02	0.82 ± 0.03	0.99 ± 0.03	1.01 ± 0.02	1.05 ± 0.05
DPPH (g TE/ g extract[b])	0.61	0.56	0.54	0.56	0.47	0.35	0.31	0.28
ABTS (g TE/100 g VS[a])	1.58 ± 0.07	2.01 ± 0.22	2.08 ± 0.09	2.35 ± 0.11	3.56 ± 0.19	4.25 ± 0.22	4.45 ± 0.09	4.39 ± 0.10
ABTS (g TE/ g extract[b])	1.66	1.72	1.66	1.81	2.03	1.52	1.27	1.16
FRAP (g TE/100 g VS[a])	0.72 ± 0.07	0.91 ± 0.07	0.89 ± 0.08	0.97 ± 0.06	1.99 ± 0.12	2.29 ± 0.20	2.57 ± 0.14	2.68 ± 0.15
FRAP (g TE/g extract[b])	0.76	0.78	0.71	0.75	1.14	0.82	0.73	0.71

[a]oven-dried vine shoots. [b]oven-dried extracts.

Considering the specific antioxidant activity, differences were observed between the tests. During the DPPH test, it was noticed that the extract recovered from the liquor obtained at 180 ºC

presented the highest specific antioxidant activity (0.61 g TE/g oven-dried extract), then this activity decreased with the increase of the temperature. Conversely, the extract presenting the highest specific radical scavenger capacity in the ABTS and FRAP assays was the one isolated from the liquor obtained at 200 ºC (2.03 g TE/g oven-dried extract and 1.14 g TE/g oven-dried extract, respectively). Afterwards, the increase of the severity of the aqueous process provoked a decrease of the antioxidant activity of the extract. The contradictory results obtained during the different tests could be explained by the fact that each test differs from the others in terms of oxidant, target species, reaction mechanisms and reaction conditions [2.46]. Therefore, it is necessary to carry out more than one antioxidant assay, as the results obtained with a single test would be scarcely representative due to mixed and cooperative mechanisms presented by the antioxidants [2.46].

It has to be remarked that the extract obtained at 200 ºC presented higher antioxidant capacity in the ABTS assay than the one reported by Conde et al. [2.46] for the synthetic antioxidant BHA (3-tert-butyl-4-hydroxyanisole) (1.80 g TE/g).

2.4.4.3 Antimicrobial activity of the extract isolated from the liquors obtained at 200 ºC

The extracts recovered from autohydrolysis liquors could also present antimicrobial activities. However, instead of analysing the antimicrobial activity of the extracts isolated from the liquors at different temperatures against six different microorganisms, only the antimicrobial activity of the extract isolated from the hydrolysate obtained at 200 ºC was analysed. Uniquely the antimicrobial activity of this extract was analysed on the one hand to reduce the number of samples and on the other hand because it was the extract containing the compounds with the highest antioxidant activity during the ABTS and FRAP assays. Aleksic and Knezevic [2.124] suggested that antioxidant compounds could present antimicrobial activity through various mechanisms of action provoking a detrimental impact on the pathogens physiology, which could be the damage on the cell membrane, the coagulation of the cell content, the inhibition of cellular functions or of the enzyme synthesis, between others. As this extract could be used as food

preservative its antimicrobial activity against six microorganisms associated with the spoilage of food products was analysed by the microdilution assay. The determined minimum inhibitory concentration (MIC) and minimum bacterial concentration (MBC) are collected in Table 2.16.

Table 2.16. Minimum inhibitory concentration (MIC, mg/mL) and minimum bacterial concentration (MBC, mg/mL) of the extract isolated form the hydrolysate obtained from the vine shoots at 200 ºC.

Microorganisms	MIC	MBC
Gram (-)		
E. coli	15	20
P. aeruginosa	10	15
Salmonella sp.	10	15
Gram (+)		
B. cereus	10	15
S. aureus	5	10
L. innocua	5	10

As it can be seen in Table 2.16, the extract presented a higher antimicrobial effect against *S. aureus* and *L. innocua* as the lowest concentration of the extract was needed to detect microbicide effects and inhibitory effects on the bacterial growth (MBC = 10 mg/mL and MIC = 5 mg/mL, respectively). Dong et al. [1.125] also observed low MIC and MBC values for *S. aureus* (in the range of 1.25-5.625 mg/mL and 1.5-10 mg/mL, respectively), but in this case for the lignin residue of corn stover obtained in the ethanol production. On the other hand, the *E. coli* was the most resistant microorganism as the highest MIC and MBC values were obtained for it (15 and 20 mg/mL, respectively). It has been reported that the Gram negative compared with the Gram positive present a higher resistance which could be associated to the presence of hydrophobic lipopolysaccharide in the outer membrane that blocks the penetration of the antimicrobial agents [2.65], reducing the sensitivity of these bacteria to the extract.

The antimicrobial activity of the extract could be associated to the different compounds that it contained independently of their carbohydrate or lignin origin, since several authors have reported that the antibacterial activity present of different extracts was associated to the synergic effect of their components [2.65]. Chai et al. [2.126] for instance, reported the antimicrobial activity of

different furan compounds against the *Bacillus subtilis* and *Salmonella* bacteria, while Cava-Roda et al. [2.127] reported the antibacterial activity of vanillin against *Escherichia coli O157:H7* and *Listeria monocytogenes*.

2.5 Conclusions

The non-isothermal autohydrolysis treatments of vine shoots working in a temperature range between 180 and 215 ºC was performed to solubilise their hemicellulosic fraction. The treatments carried out under the most severe conditions (195-215 ºC) permitted the solubilisation of more than 80.0 % of the hemicelluloses present in the vine shoots, obtaining solid residues with around 30.0 % of glucan and 50.0 % of Klason lignin (expressed g/100 g oven-dried spent solid). The composition of the solubilised products depended strongly on the severity of the treatment. The generation of monosaccharides, degradation products, phenolics and flavonoids increased with the severity of the treatment, while the highest conversion of the polysaccharides into oligosaccharides was achieved in the treatment carried out at 200 ºC (0.588 g/g NVC), afterwards it started to decrease. The molecular weight distribution and the thermal stability of the polysaccharide-derived products present in the liquors also were influenced by the severity of the treatments. It was observed that the molecular weight of these products decreased with the increase of the temperature of the aqueous processing. For instance, the main fraction of the polysaccharide-derived products of the liquor obtained at 180 ºC presented a molecular weight of 8,318 Da, while the main fraction of the ones contained in the liquor obtained at 215 ºC presented a MW of 550 Da. Regarding the thermostability of the polysaccharide-derived products it also decreased with the increase of the temperature.

The autohydrolysis liquor obtained at 200 ºC, as it contained the highest amount of oligosaccharides with potential prebiotic activity, was subjected to a membrane purification treatment followed by an ion exchange process in order to refine the oligosaccharides. This purification sequence permitted the removal of 95.7 % of the ONVC of the liquors and closely to 100.0 % of the monosaccharides, obtaining a liquor composed by 97.0 % of oligosaccharides.

These oligosaccharides were constituted by a complex mixture of oligosaccharides, mainly composed by xylooligosaccharides with different polymerisation degrees and highly branched, being acetyl, methylglucuronosyl, mannosyl, galactosyl, glucosyl and arabinosyl the substituents that they presented.

Prior to the assessment of the prebiotic activity of the refined oligosaccharides, they were subjected to an *in vitro* simulated gastrointestinal digestion, and it was concluded that the oligosaccharides could reach the gut without being altered. The digested oligosaccharides were then subjected to *in vitro* fermentation with human faecal microbiota in order to evaluate their prebiotic activity. It was determined that the digested oligosaccharides were slowly consumed by the gut microbiota producing an increase of the bifidobacterium population and releasing an organic acid profile similar to what it was achieved with fructooligosaccharides, which are extensively commercialised prebiotics. Due to the complex structure of the oligosaccharides and their low fermentation rate they could reach distal colon regions presenting their prebiotic activity there.

Moreover, not only hemicellulose-derived products were solubilised during the autohydrolysis treatments of vine shoots as several secondary reactions also took place. These compounds, among which phenolic and flavonoid compounds were present, were extracted by liquid-liquid extractions with ethyl acetate, as they could present bioactive properties. The quantity and composition of the isolated extracts was strongly influenced by the severity of the autohydrolysis treatment, since the composition of the autohydrolysis liquors also varied with the severity. The severest conditions (215 ºC) permitted the obtaining of the highest amount of extracts (3.8 g extract/100g oven-dried vine shoot) containing the highest carbohydrate-derived compounds. The extracts isolated from the liquors obtained using intermediate conditions (205 ºC), contained the highest phenolic compounds (0.52 g GAE/g extract), some of which were identified as lignin-derived compounds, while the highest flavonoid content was observed in the extract isolated from the liquor obtained at 180 ºC (0.56 g RE/g extract). It was noticed that while the concentration of the phenolic and flavonoid compounds present in the extract decreased with the increase of the temperature, the content of the carbohydrate-derived compounds increased with the temperature.

Between the extracts isolated from the liquors obtained at different temperatures, the one isolated from the liquor obtained at 200 ºC presented the highest antioxidant activity during the ABTS and FRAP assays. This extract presented higher antioxidant capacity than the BHA, which is a synthetic antioxidant, during the ABTS assay. Among the different extracts, the antimicrobial activity of the extract obtained from the liquors produced at 200 ºC was assessed, observing that it presented a higher antimicrobial activity against Gram positive microorganisms than against Gram negative microorganisms.

Thus, it could be concluded that the autohydrolyisis could be a suitable procedure to revalorise the hemicellulosic fraction of the vine shoots as a first stage of an integral biorefinery. The treatment carried out at 200 ºC, permitted the obtaining of the maximum amount of oligosaccharides with similar prebiotic activity as commercialised prebiotics and of extracts with similar antioxidant activity as a synthetic antioxidant and with antimicrobial activity against Gram positive and negative microorganisms.

2.6 References

[2.1] Sánchez-Gómez, R., Alonso, G. L., Salinas, M. R. & Zalacain, A. (2017). Reuse of vine-shoots wastes for agricultural purposes. In C. M. Galanakis (Eds.), *Handbook of grape processing by-products* (79-104). Amsterdam: Elsevier.

[2.2] Argun, H. & Onaran, G. (2015). Delignification of vineyard pruning residues by alkaline peroxide treatment. *Industrial Crops and Products, 74*, 697-702.

[2.3] Sánchez, A., Ysunza, F., Beltrán-García, M. J. & Esqueda, M. (2002). Biodegradation of viticulture wastes by Pleurotus: a source of microbial and human food and its potential use in animal feeding. *Journal of Agricultural and Food Chemistry*, *50*(9), 2537-2542.

[2.4] Peralbo-Molina, A. & Luque de Catro, M. D. (2013). Potential of residues from the Mediterranean agriculture and agrifood industry. *Trends in Food Science & Technology*, *32*(1), 16-24.

[2.5] Moldes, A. B., Bustos, G., Torrado & A., Domínguez, J. M. (2007). Comparison between different hydrolysis processes of vine-trimming waste to obtain hemicellulosic sugars for further lactic acid conversion. *Applied Biochemistry and Biotechnololgy, 143*(3), 244-256.

[2.6] Sánchez-Gómez, R., Zalacain, A., Alonso, G. L. & Salinas, M. R. (2014). Vine-shoot waste aqueous extracts for re-use in agriculture obtained by different extraction techniques: phenolic, volatile, and mineral compounds. *Journal of Agricultural and Food Chemistry, 62*(45), 10861-10872.

[2.7] Delgado-Torre, M. P., Ferreiro-Vera, C., Priego-Capote, F., Pérez-Juan, P. M. &Luque de Castro, M. D. (2012). Comparison of accelerated methods for the extraction of phenolic compounds from different vine-shoot cultivars. *Journal of Agricultural and Food Chemistry, 60*(12), 3051-3060.

[2.8] Fortunati, E., Luzi, F., Puglia, D. & Torre, L. (2016). Extraction of lignocellulosic materials from waste products. In D. Puglia, E. Fortunati & J. M. Kenny (Eds.). *Multifunctional Polymeric Nanocomposites based on cellulosic reinforcements* (pp. 1-38). Amsterdam, Netherlands: Elsevier.

[2.9] Peng, F., Peng, P., Xu, F. & Sun, R. C. (2012). Fractional purification and bioconversion of hemicelluloses. *Biotechnology Advances, 30*(4), 879-903.

[2.10] Limayem, A. & Ricke, S. C. (2012). Lignocellulosic biomass for ethanol production: Current perspective, potential issues and future prospects. *Progress in Energy and Combustion Science, 38*(4), 449-467.

[2.11] Brandt, A., Grasvik, J., Hallett, J. P. & Welton, T. (2013). Deconstruction of lignocellulosic biomass with ionic liquids. *Green Chemistry, 15*(3), 550-583.

[2.12] Zia, F., Zia, K. M., Zuber, M., Ahmad, H. B. & Muneer, M. (2016). Glucomannan based polyurethane: A critical short review of recent advances and future perspectives. *International Journal of Biological Macromolecules, 87,* 229-236.

[2.13] Moreira, L. (2008). An overview of mannan structure and mannan-degrading enzyme systems. *Applied Microbiology and Biotechnology, 79*(2), 165-178.

[2.14] Silveira, J. L. M. & Bresolin, T. M. B. (2011). Pharmaceutical use of galactomanans. *Química Nova, 34*(2), 292-299.

[2.15] Naidu, D. S., Hlangothi, S. P. & John, M. J. (2018). Bio-based products from xylan: A review. *Carbohydrate Polymers, 179,* 28-41.

[2.16] Zhou, X., Li, W., Mabon, R. & Broadbelt, L. J. (2016). A critical review on hemicellulose pyrolysis. *Energy Technology*, *5*(1), 52-79.

[2.17] Gröndahl, M., Teleman, A. & Gatenholm, P. (2003). Effect of acetylation on the material properties of glucuronoxylan from aspen wood. *Carbohydrate Polymers, 52*(4), 359-366.

[2.18] Van Dyck, J. S. & Pletschke, B. I. (2012). A review of lignocellulose bioconversion using enzymatic hydrolysis and synergistic cooperation between enzymes-Factors affecting enzymes, conversion and synergy. *Biotechnology Advances, 30*(6), 1458-1480.

[2.19] Luonteri, E., Siika-Aho, M., Tenkanen, M. & Viikari, L. (1995). Purification and characterization of three α-arabinosidases from Aspergillus terreus. *Journal of Biotechnology, 38*(3), 279-291.

[2.20] Kozlova, L., Mikshina, P. & Gorshkova, T. (2012). Glucuronoarabinoxylan extracted by treatment with endoxylanase from different zones of growing maize root. *Biochemistry (Moscow), 77*(4), 395-403.

[2.21] Rose, J. K. (2003). *The plant cell wall*. Florida, US: CRC Press.

[2.22] Gírio, F. M., Fonseca, C., Carvalheiro, F., Duarte, L. C., Marques, S. & Bogel-Lukasik, R. (2010). Hemicelluloses for fuel ethanol: A review. *Bioresource Technology, 101,* 4775-4800.

[2.23] Poespowati, T., Riyanto, A., Hazlan, Mahmudi, A. & Kartika-Dewi, R. (2018). Enzymatic hydrolysis of liquid hot water pre-treated macro-alga (*Ulva lactuca*) for fermentable sugar production. *MATEC Web of Conferences, 156*(01015). Retrieved from https://www.matec-conferences.org/articles/matecconf/pdf/2018/15/matecconf_rsce2018_01015.pdf. (Visited September, 2018).

[2.24] Ebringerova, A. & Heinze, T. (2000). Xylan and xylan derivatives-biopolymers with valuable properties, 1. Naturally occurring xylans structures, isolation procedures and properties. *Macromoecular Rapid Communication, 21*(9), 542–556.

[2.25] Mosier, N., Wyman, C., Dale, B., Elander, R., Lee Y., Holtzapple, M. & Ladisch M. (2005). Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource Technology, 96*(6), 673–686.

[2.26] Farhat, W., Venditti, R., Quick, A., Taha, M., Mignard, N., Becquart, F. & Ayoub, A. (2017). Hemicellulose extraction and characterization in paper coatings and adhesives. *Industrial Crops and Products, 107,* 370-377.

[2.27] Carvalheiro, F., Duarte, L. C. & Gírio, F. M. (2008). Hemicellulose biorefineries: a review on biomass pretreatments. *Journal of Scientific & Industrial Research, 67,* 849-864.

[2.28] Behera, S., Arora, R., Nandhagopal, N. & Kumar, S. (2014). Importance of chemical pretreatment for bioconversion of lignocellulosic biomass. *Renewable and Sustainable Energy Reviews, 36,* 91-106.

[2.29] Shatalov, A. A. & Pereira, H. (2002). Carbohydrate behaviour of *Arundo donax* L. in ethanol-alkali medium of variable composition during organosolv delignification. *Carbohydrate Polymers, 49*(3), 331-336.

[2.30] Maruya, D. P., Singla, A. & Neg S. (2015). An overview of key pretreatment processes for biological conversion of lignocellulosic biomass to bioethanol. *3 Biotech, 5*(2), 597-609.

[2.31] Xiao, X., Bian, J., Peng, X. P., Xu, H., Xiao, B. & Sun, R. C. (2013). Autohydrolysis of bamboo (*Dendrocalamus giganteus* Munro) culm for the production of xylo-oligosaccharides. *Bioresource Technology, 138,* 63-70.

[2.32] Gullón, P., Pereiro, G., Alonso, J. L. & Parajó, J. C. (2009). Aqueous pretreatment of agricultural wastes: Characterization of soluble reaction products. *Bioresource Technology, 100,* 5840-5845.

[2.33] Ganzler, K., Salgó, A. & Valkó, K. (1986). Microwave extraction: a novel simple preparation method for chromatography. *Journal of Chromatography A, 371,* 299-306.

[2.34] Shirsath, S., Sonawane, S. & Gogate, P. (2012). Intensification of extraction of natural products using ultrasonic irradiations-A review of current status. *Chemical Engineering and Processing: Process Intensification, 53,* 10-23.

[2.35] Ebringerová, A. & Hromádková, Z. (2010). An overview on the application of ultrasound in extraction, separation and purification of plant polysaccharides. *Central European Journal of Chemistry, 8*(2), 243-257.

[2.36] Ruzene, D. S., Silva, D. P., Vicente, A. A., Gonçalves, A. R. & Teixeira, J. A. (2008). An Alternative Application to the Portuguese Agro-Industrial Residue: Wheat Straw. *Applied Biochemistry and Biotechnology, 147*(1-3), 85-96.

[2.37] Tian, Z., Chen, J., Ji, X., Lyu, G., Lucia, L. A. & Yang, G. (2017). Fractionation and characterization of three main components from Pennisetum sinese Roxb. (P. sinese) by microwave-assisted H_2O_2-NaOH extraction. *BioResources, 12*(2), 2646-2659.

[2.38] García, A., González Alriols, M., Llano-Ponte, R. & Labidi, J. (2011). Ultrasound-assisted fractionation of the lignocellulosic material. *Bioresource Technology, 102*(10), 6326-6330.

[2.39] Xu, F., Liu, C. F., Geng, Z. C., Sun, J. X., Sun, R. C., Hei, B. H., … Je, J. (2006). Characterisation of degraded organosolv hemicelluloses from wheat Straw. *Polymer Degradation and Stability, 91*(8), 1880-1886.

[2.40] Pisarnitskii, A. F., Rubeniya, T. Y. & Rutitskii, A. O. (2006). Oak wood hemicelluloses extracted with aqueous-alcoholic media. *Applied Biochemistry and Microbiology, 42*(5), 514-518.

[2.41] Tiappi, M. F., Jacquet, N., Vanderghem, C., Aguedo, M., Thomas, H. G., Gerin, P., … Richel, A, (2018). Fractionation and Structural Characterization of Hemicellulose from Steam-Exploded Banana Rachis. *Waste and Biomass Valorization,* https://doi.org/10.1007/s12649-018-0457-9

[2.42] Martin-Sampedro, R., Revilla, E., Villar, J. C. & Eugenio, M. E. (2014). Enhancement of enzymatic saccharification of Eucalyptus globulus: Steam explosion versus steam treatment. *Bioresource Technology, 167,* 186-191.

[2.43] Rigual, V., Santos, T. M., Domínguez, J. C., Alonso, M. V., Oliet, M. & Rodríguez, F. (2018). Evaluation of hardwood and softwood fractionation using autohydrolysis and ionic liquid microwave pretreatment. *Biomass and Bioenergy, 117*, 190-197.

[2.44] Aguilar-Reynosa, A., Romaní, A., Rodríguez-Jasso, R. M., Aguilar, C. N., Garrote, G. & Ruiz, H. A. (2017). Comparison of microwave and conduction-convection heating autohydrolysis pretreatment for bioethanol production. *Bioresource Technology, 243*, 273-283.

[2.45] Gullón, B., Gullón, P., Tavaria, F., Pintado, M., Gomes, A.M., Alonso, J.L. & Parajó, J.C. (2014). Structural features and assessment of prebiotic activity of refined arabinoxylooligosaccharides from wheat bran. *Journal of Functional Foods, 6*(1), 438-449.

[2.46] Conde, E., Moure, A., Domínguez, H. & Parajó, J. C. (2011). Production of antioxidants by non-isothermal autohydrolysis of lignocellulosic wastes. *LWT-Food Science and Technology, 44*(2), 436-442.

[2.47] Gullón, P., González-Muñoz, M. J., Van Gool, M. P., Schols, H. A., Hirsch, J., Ebringerova, A. & Parajó, J. C. (2010). Production, refining, structural characterization and fermentability of rice husk xylooligosaccharides. *Journal of Agricultural and Food Chemistry, 58*(6), 3632-3641.

[2.48] Elgueta, E., Sánchez, J., Dax, D., Xu, C., Willför, S., Rivas, B. & González, M. (2016). Functionalized galactoglucomannan-based hydrogels for the removal of metal cations from aqueous solutions. *Journal of Applied Polymer Science, 133*(41), 44093.

[2.49] Qi, X. M., Chen, G. G., Gong, X. D., Fu, G. Q., Niu, Y. S., Bian, J., … Sun, R. C. (2016). Enhanced mechanical performance of biocompatible hemicelluloses-based hydrogel via chain extension. *Scientific Report, 6*, 33603.

[2.50] Edlund, U., Ryberg, Y. Z. & Albertsson, A. C. (2010). Barrier films from renewable forestry waste. *Biomacromolecules, 11*(9), 2532-2538.

[2.51] Huang, B., Tang, Y., Pei, Q., Zhang, K., Liu, D. & Zhang, X. (2018). Hemicellulose-based films reinforced with unmodified and cationically modified nanocrystalline cellulose. *Journal of Polymers and the Environment, 26*(4), 1625-1634.

[2.52] Miura, M., Watanabe, I., Shimotori, Y., Aoyama, M., Kojima, Y. & Kato, Y. (2013). Microbial conversion of bamboo hemicellulose hydrolysate to xylitol. *Wood Science and Technology, 47*(3), 515-522.

[2.53] Harahap, B. M. & Kresnowati, M. T. A. P. (2018). Moderate pretreatment of oil palm empty fruit bunches for optimal production of xylitol via enzymatic hydrolysis and fermentation. *Biomass Conversion and Biorefinery, 8*(2), 255-263.

[2.54] Bustos, G., De la Torre, N., Moldes, A. B., Cruz, J. M. & Domínguez, J. M. (2007). Revalorization of hemicellulosic trimming vine shoots hydrolyzates through continuous production of lactic acid and biosurfactants by *L. pentosus*. *Journal of Food Engineering, 78(2),* 405-412.

[2.55] Luo, Y., Li, Z., Zuo, Y., Su, Z. & Hu, C. (2017). A simple two-step method for the selective conversion of hemicellulose in pubescens to furfural. *ACS-Sustainable Chemistry & Engineering, 5*(9), 8137-8147.

[2.56] Buruiana, C. T., Gómez, B., Vizireanu, C., & Garrote, G. (2017). Manufacture and evaluation of xylooligosaccharides from corn stover as emerging prebiotic candidates for human health. *LWT - Food Science and Technology, 77,* 449-459.

[2.57] Roberfroid, M. (2007). Prebiotics: The concept revisited. *The Journal of Nutrition, 137*, 830S-837S.

[2.58] Rycroft, C. E., Jones, M. R., Gibson, G. R. & Rastall, R. A. (2001). A comparative in vitro evaluation of the fermentation properties of prebiotic oligosaccharides. *Journal of Applied Microbiology, 91*(5), 878-887.

[2.59] Gómez, B., Veiga, A., Parajó, J. C. & Alonso, J. L. (2015). Production, purification, and in vitro evaluation of the prebiotic potential of arabinoxylooligosaccharides from Brewer´s spent grain. *Journal of Agricultural and Food Chemistry, 63*(38), 8429-8438.

[2.60] Cummings, J., Macfarlane, G. & Englyst, H. (2001). Prebiotic digestion and fermentation. *The American Journal of Clinical Nutrition, 73*, 415S-420S.

[2.61] Mei, G., Carey, C., Tosh, S. & Kostrynska, M. (2011). Utilization of different types of dietary fibres by potential probiotics. *Canadian Journal of Microbiology, 57*(10), 857-865.

[2.62] Moure, A., Gullón, P., Domínguez, H. & Parajó, J. C. (2006). Advances in the manufacture, purification and applications of xylo-oligosaccharides as food additives and nutraceuticals. *Process Biochemistry, 41*(9), 1913-1923.

[2.63] Gullón, P., González-Muñoz, M. J., Domínguez, H. & Parajó, C. (2008). Membrane processing of liquors from Eucalyptus globulus autohydrolysis. *Journal of Food Engineering, 87*(2), 257-265.

[2.64] Vegas, R., Alonso, J. L., Domínguez, H. & Parajó, J. C. (2005). Manufacture and refining of oligosaccharides from industrial solid wastes. *Industrial & Engineering Chemistry Research, 44*(3), 614-620.

[2.65] Moreira, D., Gullón, B., Gullón, P., Gomes, A. & Tavaria, F. (2016). Bioactive packaging using antioxidant extracts for the prevention of microbial food-spoilage. *Food & Function, 7*(7), 3273-3282.

[2.66] TAPPI Standards 2007, Test methods of the Technical Association of the Pulp and Paper Industry, Atlanta.

[2.67] Sluiter, A., Hames, B., Ruíz, R., Scarlata, C., Sluiter, J., Templeton, D. & Crocker, D. (2008). *Determination of structural carbohydrates and lignin in biomass*. Retrieved from https://www.nrel.gov/docs/gen/fy13/42618.pdf (Visited September, 2018).

[2.68] Wise, L. E., Murphy, M. & D'Adieco, A. A. (1946). Chlorite holocellulose, its fractionation and bearing on summative wood analysis and on studies on the hemicelluloses. *Paper Trade Journal, 122*(2), 35-43.

[2.69] Rowell, R. (1983). The chemistry of solid wood. Based on short course and symposium sponsored by the Division of Cellulose. *Paper and Textile Chemistry at* 185th meeting of the American Chemical Society, Seattle, Washington.

[2.70] Garrote, G., Falqué, E., Domínguez, H. & Parajó, J. C. (2007). Autohydrolysis of agricultural residues: Study of reaction by products. *Bioresource Technology, 98*(10), 1951-1957.

[2.71] Singleton, V. L. & Rossi, J. A. (1965). Colorimetric of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture, 16*, 144-158.

[2.72] Blasa, M., Candiracci, M., Accorsi, A., Piacentini, P. M., Albertini, M. C. & Piatti, E. (2005). Raw Milleflori honey is packed full of antioxidants. *Food Chemistry, 97*(2), 217-222.

[2.73] Vargas F., Domínguez E., Vila C., Rodríguez A. & Garrote G. (2015). Agricultural residue valorization using a hydrothermal process for second generation bioethanol and oligosaccharides production. *Bioresource Technology, 191*, 263-270.

[2.74] Dávila, I., Gullón, B., Alonso, J. L., Labidi, J., Gullón, P. (2019). Vine shoots as new source for the manufacture of prebiotic oligosaccharides. *Carbohydrate Polymers, 207*, 34-43.

[2.75] Gullón, B., Pintado, M. E., Barber, X., Fernández-López, J., Pérez-Álvarez, A. & Viuda-Martos, M. (2015). Bioaccessibility, changes in the antioxidant potential and colonic fermentation of date pits and apple bagasse flours obtained from co-products during simulated *in vitro* gastrointestinal digestion. *Food Research International, 78*, 169-176.

[2.76] Von Gadow, A., Joubert, E. & Hansmann, C. F. (1997). Comparison of the antioxidant activity of aspalathin with that of other plant phenols of Rooibos tea (Aspalathus linearis), α-tocopherol, BHT and BHA. *Journal of Agricultural and Food Chemistry, 45*(3), 632-637.

[2.77] Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M. & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology & Medicine, 26*(9-10), 1231-1237.

[2.78] Benzie, I. F. F. & Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of antioxidant power: the FRAP assay. *Analytical Biochemistry, 239*(1), 70-76.

[2.79] Bustos, G., Moldes, A. B., Cruz, J. M. & Domínguez J. M. (2004). Production of fermentable media from vine-trimming wastes and bioconversion into lactic acid by *Lactobacillus pentosus*. *Journal of the science of food and Agriculture 84*(15), 2105-2112.

[2.80] Surek, E. & Buyukkileci, A. O. (2017). Production of xylooligosaccharides by autohydrolysis of hazelnut (*Corylus avellana* L.) shell. *Carbohydrate Polymers, 174*, 565-571.

[2.81] Moniz, P., Pereira, H., Quilhó, T. & Carvalheiro, F. (2013). Characterisation and hydrothermal processing of corn straw towards the selective fractionation of hemicelluloses. *Industrial Crops and Products, 50*, 145-153.

[2.82] Sixta, H. (2006). Chemical pulping. In H. Sixta (Ed.), *Handbook of pulp. Vol. 1* (pp. 3-19). Weinheim, Germany: WILEY-VCH.

[2.83] Martínez, M., Gullón, B., Schols, H. A., Alonso, J.L. & Parajó, J. C. (2009). Assessment of the production of oligomeric compounds from sugar beet pulp. *Industrial and Engineering Chemistry Research,* 48(10), 4681–4687.

[2.84] Jesus, M. S., Romaní, A., Genisheva, Z., Teixeira, J. A. & Domingues, L. (2017). Integral valorization of vine pruning residue by sequential autohydrolysis stages. *Journal of Cleaner Production, 168*, 74-86.

[2.85] Leschinsky, M., Zuckerstätter, G., Weber, H. K., Patt, R. & Sixta, H. (2008). Effect of autohydrolysis of Eucalyptus globulus wood on lignin structure. Part 1: comparison of different lignin fractions formed during water prehydrolysis. *Holzforschung*, *62*(6), 645–652.

[2.86] Jiang, H., Chen, Q., Ge, J. & Zhang, Y. (2014). Efficient extraction and characterization of polymeric hemicelluloses from hybrid poplar. *Carbohydrate polymers, 101*(1), 1005-1012.

[2.87] Egües, I., González Alriols, M., Herseczki, Z., Marton, G. & Labidi, J. (2010). Hemicelluloses obtaining from rapeseed cake residue generated in the biodiesel production process. *Journal of Industrial and Engineering Chemistry*, *16*(2), 293-298.

[2.88] Fang, J. M., Sun, R. C. & Tomkinson, J. (2000). Isolation and characterization of hemicelluloses and cellulose from rye straw by alkaline peroxide extraction. *Cellulose*, *7*(1), 87-107.

[2.89] Gullón, P., González-Muñoz, M. J., Van Gool, M. P., Schols, H. A., Hirsch, J., Ebringerova, A. & Parajó, J. C. (2011). Structural features and properties of soluble products derived from Eucalyptus globulus hemicelluloses. *Food Chemistry, 127*(4), 1798–1807.

[2.90] Tunc, M. S. & Van Heiningen, A. R. P. (2011). Characterization and molecular weight distribution of carbohydrates isolated from the autohydrolysis extract of mixed southern hardwoods. *Carbohydrate Polymers, 83*(1), 8-13.

[2.91] Garrote, G., Kabel, M. A., Schols, H. A., Falqué, E., Domínguez, H. &, Parajó J. C. (2007). Effects of Eucalyptus globulus Wood Autohydrolysis Conditions on the Reaction Products. *Journal of Agricultural and Food Chemistry, 55*(22), 9006-9013.

[2.92] Wang, Z. W., Zhu, M. Q., Li, M. F., Wang, J. Q., Wei, Q. & Sun, R. C. (2016). Comprehensive evaluation of the liquid fraction during the hydrothermal treatment of rapeseed straw. *Biotechnology for Biofuels, 9*(142), 142-158.

[2.93] Gray, M. C., Converse, A. O. & Wyman, C. E. (2003). Sugar Monomer and Oligomer Solubility. Data and Predictions for Application to Biomass Hydrolysis. *Applied Biochemistry and Biotechnology, 105*(1-3), 179-193.

[2.94] Hajji, M., Hamdi, M., Sellimi, S., Ksouda, G., Laouer, H., Li, S. & Nasri, M. (2019). Structural characterization, antioxidant and antibacterial activities of a novel polysaccharide from Periploca laevigata root barks. *Carbohydrate Polymers, 206*, 380-388.

[2.95] Egüés, I., Eceiza, A. & Labidi, J. (2013). Effect of different hemicelluloses characteristics on film forming properties. *Industrial Crops and Products, 47*, 331-338.

[2.96] Yang, H. Y., Song, X. L., Yuan, T. Q., Xu, F. & Sun, R. C. (2011). Fractional Characterization of Hemicellulosic Polymers Isolated from *Caragana korshinskii Kom. Industrial & Engineering Chemistry Research, 50*(11), 6877-6885.

[2.97] Sun, X. F., Sun, R. C., Lu, Q., Xu, F. & Lin, L. (2002). Fractional isolation and physicocheical characterization of hemicelluloses by a two-stage treatment from *Haloxylon ammodendron* and *Elaegnus angustifolia*. *Journal of Agricultural and Food Chemistry, 50*(22), 6400-6407.

[2.98] Ruíz, E., Gullón, B., Moura, P., Carvalheiro, F., Eibes, G., Cara, C. & Castro, E. (2017). Bifidobacterial growth stimulation by oligosaccharides generated from olive tree pruning biomass. *Carbohydrate Polymers, 169*, 149-156.

[2.99] Rivas, S., Santos, V. & Parajó, J. C. (2017). Aqueous fractionation of hardwood: selective glucuronoxylan solubilisation and purification of the reaction products. *Journal of Chemical Technology, 92*(2), 367-374.

[2.100] Chen, M. H., Bowman, M. J., Dien, B. S., Rausch, K. D., Tumbleson, M. E. & Singh, V. (2014). Autohydrolysis of *Miscanthus x giganteus* for the production of xylooligosaccharides (XOS): Kinetics, characterization and recovery. *Bioresource Technology*, *155*, 359-365.

[2.101] Carvalheiro, F., Esteves, M. P., Parajó, J. C., Pereira, H. & Gírio, F. M. (2004). Production of oligosaccharides by autohydrolysis of brewery´s spent grain. *Bioresource Technology, 91*(1), 93-100.

[2.102] Gullón, B., Gómez, B., Martínez-Sabajanes, M., Yáñez, R., Parajó, J. C. & Alonso, J. L. (2013). Pectic oligosaccharides: Manufacture and functional properties. *Trends in Food Science & Technology*, *30*(2), 153-161.

[2.103] Al-Tamimi, M., Palframan, R., Cooper, J., Gibson, G. & Rastall, R. (2006). In vitro fermentation of sugar beet arabinan and arabino-oligosaccharides by the human gut microflora. *Journal of Applied Microbiology*, *100*(2), 407-414.

[2.104] Sanchez, J. I., Marzorati, M., Grootaert, C., Baran, M., Van Craeyveld, V., Courtin, C. M., … Van de Wiele, T. (2009). Arabinoxylan-oligosaccharides (AXOS) affect the protein/carbohydrate fermentation balance and microbial population dynamics of the Simulator of Human Intestinal Microbial Ecosystem. *Microbial biotechnology*, *2*(1), 101-113.

[2.105] Ho, A. L., Kosik, O., Lovegrove, A., Charalampopoulos, D., & Rastall, R. A. (2018). In vitro fermentability of xylo-oligosaccharide and xylo-polysaccharide fractions with different molecular weights by human faecal bacteria. *Carbohydrate Polymers*, *179*, 50-58.

[2.106] Martín, M. C. & Ballesteros, M. (2010). Espectrometría de masas y análisis de biomarcadores. *MONOGRAFÍA XXX: Biomarcadores: Analítica, diagnóstico y terapéutica*. Retrieved from http://www.analesranf.com/index.php/mono/article/view/1066 (Visited October, 2018).

[2.107] Moura, P., Cabanas, S., Lourenço, P., Gírio, F., Loureiro-Dias, M. C. & Esteves, M. P. (2008). *In vitro* fermentation of selected xylo-oligosaccharides by piglet intestinal microbiota. *LWT-Food Science and Technology*, *41*(10), 1952-1961.

[2.108] Gibson, G. R., Hutkins, R., Sanders, M. E., Prescott, S. L., Reimer, R. A., Salminen, S. J., … Reid, G. (2017). Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. *Nature Reviews Gastroenterology and Hepatology*, *14*(8), 491-502.

[2.109] Moniz, P., Ho, A. L., Duarte, L. C., Kolida, S., Rastall, R. A., Pereira, H. & Carvalheiro, F. (2016). Assessment of the bifidogenic effect of substituted xylo-oligosaccharidesobtained from corn straw. *Carbohydrate Polymers, 136,* 466-473.

[2.110] Sharma, M., & Shukla, G. (2016). Metabiotics: one step ahead of probiotics; an insight into mechanisms involved in anticancerous effect in colorectal cancer. *Frontiers in Microbiology, 7,* 1940-1955.

[2.111] Hosseini, E., Grootaert, C., Verstraete, W., & Van de Wiele, T. (2011). Propionate as a health-promoting microbial metabolite in the human gut. *Nutrition Reviews, 69*(5), 245-258.

[2.112] Den Besten, G., Lange, K., Havinga, R., Van Dijk, T. H., Gerding, A., Van Eunen, K., … Reijngoud D. J. (2013). Gut derived short-chain fatty acids are vividly assimilated into host carbohydrates and lipids. *American Journal of Physiology-Gastrointestinal and Liver Physiology, 305*(12), G900-G910.

[2.113] Zeng, H., Lazarova, D. L., & Bordonaro, M. (2014). Mechanisms linking dietary fiber, gut microbiota and colon cancer prevention. *World Journal of Gastrointestinal Oncology, 6*,41-51.

[2.114] Hughes, S. A., Shewry, P. R., Gibson, G. R., McCleary B. V. & Rastall, R. A. (2008). In vitro fermentation of oat and barley derived beta-glucans by human faecal microbiota. *FEMS Microbiology Ecology, 64*(3), 482-493.

[2.115] Woodmansey, E. J., McMurdo, M. E. T., Macfarlane, G. T. & Macfarlane, S. (2004). Comparison of compositions and metabolic activities of fecal microbiotas in young adults and in antibiotic-treated and nonantibiotic-treated elderly subjects. *Applied and Environmental Microbiology, 70*(10), 6113-6122.

[2.116] Castro, E., Conde, E., Moure, A., Falqué, E., Cara, C., Ruíz, E. & Domínguez, H. (2008). Antioxidant activity of liquors from steam explosion of Olea europea wood. *Wood Science and Technology, 42*(7), 579–592.

[2.117] Rostro, M., Sánchez-González, M., Rivas, S., Moure, A., Domínguez, H. & Parajó, J. C. (2014). Non-isothermal autohydrolysis of nixtamalized maize pericarp: Production of nutraceutical extract. *LWT-Food Science and Technology, 58*(2), 550-556.

[2.118] Gullón, B., Eibes, G., Dávila, I., Moreira, M. T., Labidi, J. & Gullón, P. (2018). Hydrothermal treatment of chestnut shells (Castanea sativa) to produce oligosaccharides and antioxidant compounds. *Carbohydrate Polymers, 192,* 75-83.

[1.119] Garrote, G., Cruz, J. M., Domínguez, H. & Parajó, J. C. (2008). Non-isothermal autohydrolysis of barley husks: Product distribution and antioxidant activity of ethyl acetate soluble fractions. *Journal of Food Engineering, 84*(4), 544-552.

[2.120] El Hage, R., Chrusciel, L., Desharnis, L. & Brosse, N. (2010). Effect of autohydrolysis of *Miscanthus x giganteus* on lignin structure and organosolv delignification. *Bioresource Technology, 101*(23), 9321-9329.

[2.121] Garrote, G., Domínguez, H. & Parajó, J. C. (2001). Interpretation of deacetylation and hemicellulose hydrolysis during hydrothermal treatments on the basis of the severity factor. *Process Biochemistry, 37*(10), 1067-1073.

[2.122] Zhao, L., Chen, J., Su, J., Li, L., Hu, S., Li, B., … Chen, T. (2013). In vitro antioxidant and antiproliferative activities of 5-hydroxymethylfurfural. *Journal of Agricultural and Food Chemistry, 61*(44), 10604–10611.

[2.123] Witzler, M., Alzagameem, A., Bergs, M., El Khaldi-Hansen, B., Klein, S. E., Hielscher, D., … Schulze, M. (2018). Lignin-Derived Biomaterials for Drug Release and Tissue Engineering. *Molecules, 23*(8), 1885-1907.

[2.124] Aleksic, V. & Knezevic, P. (2014). Antimicrobial and antioxidative activity of extracts and essential oils of *Myrtus communis* L. *Microbiology Research, 169*(4), 240–254.

[1.125] Dong, X., Dong, M., Lu, Y., Turley, A., Jin, T. & Wu, C. (2011). Antimicrobial and antioxidant activities of lignin from residue of corn stover to ethanol production. *Industrial Crops and Products, 34*(3), 1629–1634.

[1.126] Chai, W. M., Liu, X., Hu, Y. H., Feng, H. L., Jia, Y. L., Guo, Y. J., … Chen, Q. X. (2013). Antityrosinase and antimicrobial activities of furfuryl alcohol, furfural and furoic acid. *International Journal of Biological Macromolecules, 57,* 151–155.

La ciencia se compone de errores, que a su vez, son los pasos hacia la verdad.
-Julio Verne

Chapter 3

Revalorisation of autohydrolysed solids

3.1 Background

During the autohydrolysis treatment of the vine shoots the hemicellulosic fraction was almost completely solubilised, whereas little alterations were caused in their lignin and cellulosic fractions, being them recovered in the solid fractions [3.1]. This aqueous pre-treatment modifies the native structure of the vine shoots facilitating the subsequent separation of the remaining two fractions. It has been reported that the autohydrolys isincreases the solubility of the lignin in a subsequent delignification process [3.2]. Furthermore, the autohydrolysis is also supposed to increase the cellulose digestibility [3.3], as the hemicelluloses, which tend to act as physical barriers preventing the approach of the enzymes to the cellulose, have been removed [3.4].

Throughout this chapter the processes used for the fractionation and revalorisation of the autohydrolysed solids have been described, as well as, the characteristics of the isolated cellulose and lignin fractions. Likewise, the results obtained from the different approaches used to fractionate these solids are discussed.

3.1.1 Cellulose

Cellulose is the most abundant and important component of the lignocellulosic biomass, although its abundance depends on the feedstock. It constitutes around 30.0 % of herbaceous plants, and between 40.0 and 50.0 % of wood [3.5]. This biopolymer is produced by photosynthesis and it provides the strength and stability to the cell walls of the biomass [3.6]. It is a non-branched polymer composed by long chains of D-glucose bound via β-1,4-glycosidic bonds. The cellulose chains present polymerisation degrees around 10,000, which could vary with the feedstock [3.6]. This biopolymer presents intra-chain hydrogen bonds between the oxygen atoms and the hydroxyl groups of adjacent glucose molecules. It also presents intermolecular hydrogen bonds and Van der Waals forces that permit the parallel stacking of multiple cellulose chains forming elementary fibrils [3.7]. The intra-chain and intermolecular hydrogen bonds between the cellulose chains are represented in Figure 3.1.

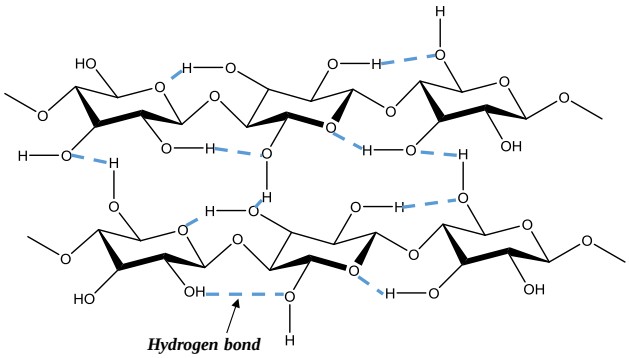


Figure 3.1. Cellulose structure and its intra-chain and intermolecular hydrogen bonds.

The cellulose elementary fibrils are constituted by crystalline and amorphous regions. In the first region, the cellulose chains present a highly ordered structure, while in the amorphous region the structure of the fibrils is disordered [3.7]. These fibrils are further aggregated into larger microfibrils which could be organised in lattices in the cell walls, presenting a highly crystalline structure. This high organisation makes the cellulose resistant to some reagents and insoluble in water [3.8].

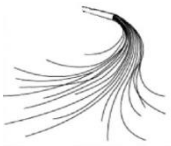
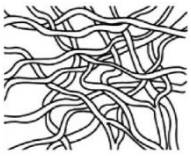
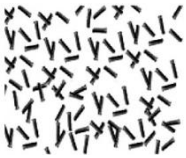
Taking into account the dimension, the extraction method employed and its origin, the structure of the cellulose can be classified in the following categories [3.6], which are represented in Table 3.1:

- **Celullose microfibrils (MFC).** They consist on a bunch of stretched cellulose chains and they are the smallest structural unit of plant fibres [3.9]. They are long, flexible and they present a length normally on the micrometer scale and a diameter size between 3 and 100 nm [3.6]. They contain alternate crystalline and amorphous domains and they could be formed by delamination of wood pulp through mechanical pressure before and/or after a chemical or enzymatic treatment [3.9].
- **Celullose nanofibers (NFC).** They are made of aggregates of cellulose microfibrils with a length of several micrometers and a diameter size between 20 and 50 nm [3.6]. They present a network structure and as the MFC, they contain crystalline and amorphous regions [3.10]. The nanofibers could be obtained by extracting them with an enzymatic

pre-treatment followed by a high-shear mechanical treatment from secondary cell wall fibres [3.6].

- **Cellulose nanocrystals (CNC).** They are formed by rigid rod-like particles that present between 5 and 70 nm of width and between 100 nm and several micrometres of length [3.11]. These particles are made of 100.0 % cellulose and they present a high crystallinity degree, between 54.0 and 88.0 % [3.7]. The nanocrystals are generally obtained by a pre-treatment in which the cellulose is isolated followed generally by a hydrolysis to remove the amorphous regions of the cellulose [3.11].

Table 3.1. Structures of the micro- and nano-cellulose particles, adapted from Khalil et al. [3.9].

Celullose structure		Diameter (nm)	Length
Microfibrils		3-100	Several micrometers
Nanofibers		20-50	Several micrometers
Nanocrystals		5-70	Between 100 nm and several micrometers

3.1.2 Lignin

Lignin is a heterogeneous aromatic polymer that constitutes between 20.0 and 30.0 % of woody plants providing them with rigidity, impermeability and resistance to microbial attack and oxidative stress [3.6, 3.12]. This biopolymer is an amorphous network of three phenyl propane units: p-coumaryl alcohol (H), synapyl alcohol (S) and coniferyl alcohol (G), which are represented in Figure 3.2. These phenyl propane units are randomly bound together by different ether linkages, such as β-O-4´ and α-O-4´, and by carbon-carbon bonds such as, β-β´, β-5´ and 5-5´, which are formed by laccases and peroxidases [3.13]. Apart from the intramolecular bonds,

lignin is also bound to hemicelluloses acting as a glue that gathers all the structural fractions of the lignocellulosic biomass together [3.14].

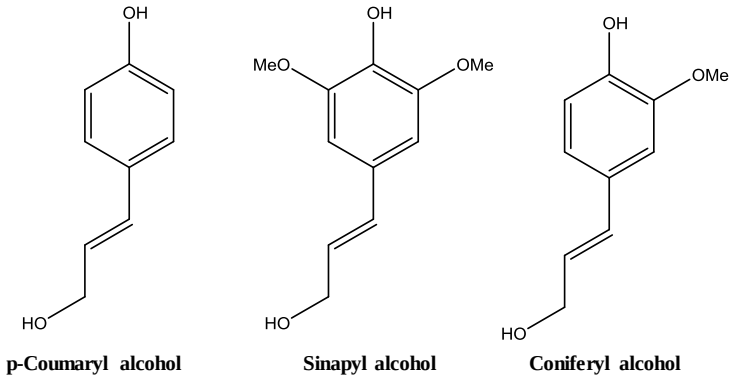


Figure 3.2. Structure of the monomers that form part of the lignin.

The determination of the lignin structure still remains a challenge as it has been observed that its features vary with the isolation procedure [3.15]. Nevertheless, different models have been developed to illustrate its macromolecular structure, since in 1977 Adler presented a structure for a softwood lignin [3.16, 3.17], being one of these proposed structures represented in Figure 3.3. The lignin content, as well as, its composition and structure are not only affected by the type of lignocellulosic feedstock but also by the extraction procedure, which makes it difficult to elucidate the original structure of the lignin in biomass. Softwoods, for instance, present lignins mainly composed by guaiacyl units, which come from the coniferyl alcohol, while hardwoods present lignins with different ratios of guaiacyl and syringyl units, being the latest one coming from the sinapyl alcohols. Herbaceous species or grasses, on the other hand, contain lignins composed by different ratios of guaiacyl, syringyl and p-hydroxyphenyl units, which come from the p-coumaryl alcohol [3.18].

The differences of the lignin constituents also affect its extraction easiness. The lignins mainly composed by coniferyl alcohols, as the one present in the softwoods, are more difficult to extract than the ones present in hardwood and herbaceous species. The coniferyl alcohols present an unsubstituted C5 that tends to be forming part of C-C interunits linkages making the depolymerisation of this type of lignins more difficult. Conversely, the lower condensation degree and the lower branching degree of the lignins containing synapyl alcohols facilitate the extraction

of the hardwood and herbaceous lignins [3.18]. Grabber et al. [3.19] also reported the influence of the constitution of the lignin on its solubility in acid, neutral or alkaline media.

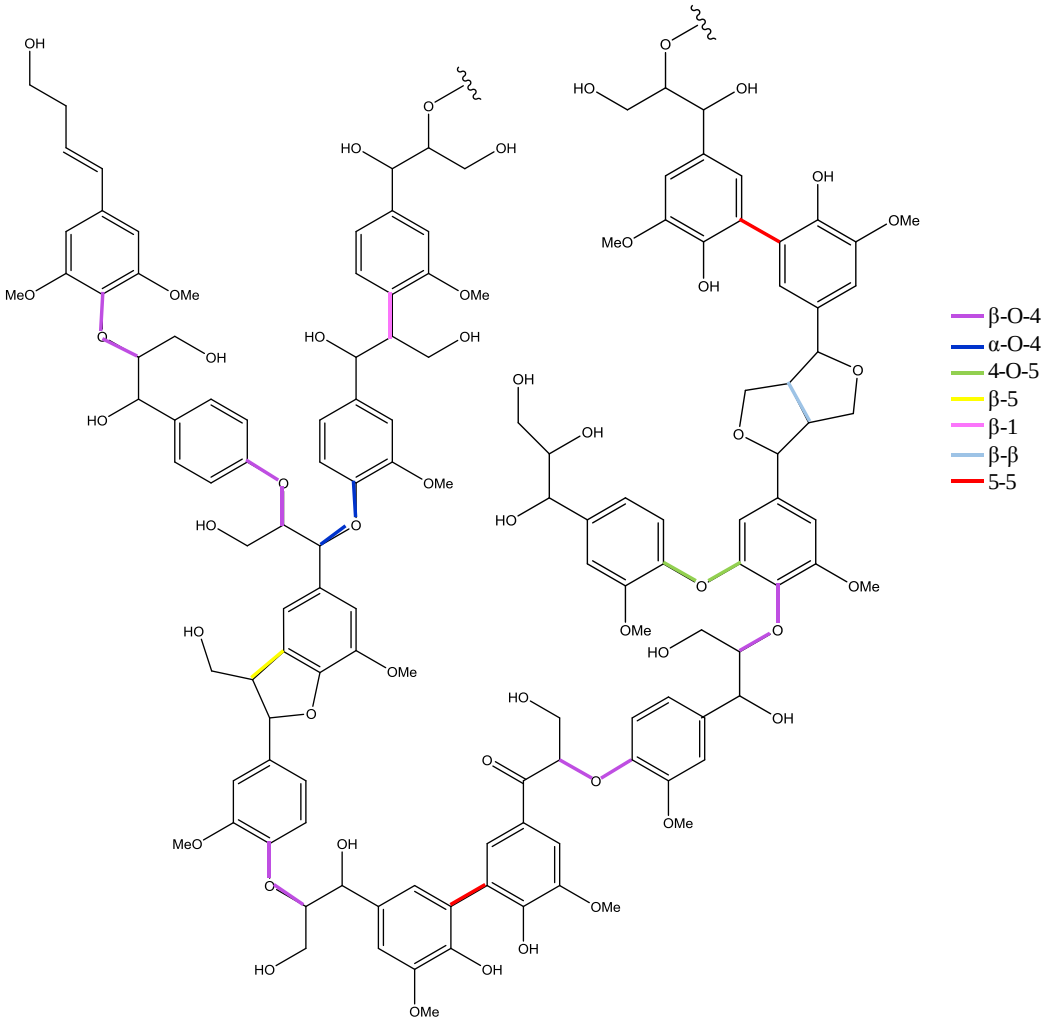


Figure 3.3. Structure of the lignin macromolecule, adapted from the American Chemistry Society [3.20] and the different ether and C-C bonds between the lignin units.

3.1.3 Separation of the cellulose and lignin from the autohydrolysed solid

To carry out an integral revalorisation of biomass, the cellulose and lignin that remained in the autohydrolysed solid should be separated, preferably by removing one of them without affecting the other one, so as to up-grade the two of them. When selecting the separation or extraction procedure the cost effectiveness and the energy requirements also have to be taken into account [3.21]. The different procedures that could be used to separate the cellulose and lignin from

lignocellulosic feedstocks can be classified as biological, chemical and physicochemical. Table 3.2 collects the effects, advantages and drawbacks of the different methods that could be employed for the separation of these two fractions from a hemicelluloses-free solid.

The chemical processes described in this Table could be intensified by the employment of microwaves, pulsed-electric-fields, ultrasounds or by the combination of microwaves and ultrasounds, being the effect caused by the microwaves and ultrasounds described in the Section 2.1.2.1. When the pulsed-electric-fields are applied the biomass is treated with two electrodes to which voltage pulses are applied with an electrical field strength of 0.1-80 kV/cm for very short times [3.22]. This could facilitate delignification procedures and enzymatic hydrolysis as it provokes the disruption of the biological membrane and local structural changes [3.23, 3.24].

In this thesis, chemical delignifications and enzymatic hydrolysis were used to disjoin the cellulose and lignin present in the autohydrolysed solids. Among the different chemical delignification treatments that could be used, alkaline, organosolv and acetosolv conditions were assessed to remove the lignin fraction from the autohydrolysed vine shoots due to the wide experience of the research group in these procedures. The lignin removal during the alkaline process is attributed to the cleavage of the ester bonds and the glycosidic side chains of the lignin [3.25]. In the organic solvent extraction, also denoted as organosolv treatment, carried out in this case with ethanol, the hydroxyl groups of the alcohols are supposed to attack the acid-ester bonds along with the ether linkages of the lignin components [3.26]. The lignin removal during the acid-catalysed treatment, also denoted as acetosolv due to the employment of acetic acid, occurs due to the breakage of the α-aryl ether and β-aryl ether bonds present in the lignin structure [3.27].

Table 3.2. Details of the procedures used for the separation of the cellulosic and lignin fractions considering a hemicellulosic-free solid.

Treatment	Reagents	Effect	Advantage	Disadvantage	Reference
Chemical treatment					
Acid	H_2SO_4, HCl, H_3PO_4, HNO_3, maleic acid, oxalic acid and heteropolyacids	Hydrolysis of the amorphous regions of the cellulose	Enzymatic hydrolysis is sometimes not required to yield fermentable sugars	Hazardous, toxic, and highly corrosive reagents	[3.22, 3.31]
Alkaline	NaOH, KOH, $Ca(OH)_2$, Na_2CO_3, NH_3	Lignin removal and cellulose swelling and decrystallisation	Mild temperature and pressure, low sugar degradation and easy recovery and removal of alkalis	Long reaction time and the requirement of highly concentrated solutions	[3.31]
Oxidation	H_2O_2, O_2, O_3, air	Disruption of the lignin structure, releasing low Mw degradation products	Mild and environmentally friendly conditions and high selectivity	High cost	[3.32]
Organosolv	MeOH, EtOH or γ-valerolactone, between others and their aqueous solutions	Hydrolysis of lignin into low Mw components which are soluble in organic solvents	Easy recovery of the organic solvents by distillation	High cost, handling of harsh organic solvents	[3.33]
Ionic liquids	[Amim]Cl, [$C_n$mmim]Cl, [C_2mim][OAc] or [Ammim]Br between others	Dissolution of the lignin and cellulose crystallinity reduction	Low vapour pressure, mild reaction conditions and low toxicity	High cost, complexity of synthesis and high loading of ionic liquid	[3.22, 3.34]
Eutectic liquids	Choline chloride	Lignin removal	Green solvent, biodegradable and biocompatible pre-treatment	Poor stability under high pre-treatment temperatures	[3.35]
Physico-chemical treatment					
Steam explosion	H_2O	Partial conversion of the lignin to soluble low Mw fractions	Low capital investment and environmental impact and moderate energy requirements	Less effective for softwood	[3.31, 3.36]
Supercritical CO_2	CO_2(g), H_2O	Almost complete removal of the lignin and cellulose crystallinity reduction	High efficiency and the employment of green solvents	High operating pressures	[3.31]
Ammonium recycle percolation process	5.0-15.0 wt.% liquid ammonia	Almost complete solubilisation of the lignin fraction, maintaining the cellulose almost intact	High efficiency and selectivity towards the lignin fraction	High energy demand and high ammonia loadings	[3.37]
Biological treatment					
Microorganisms	White-rot fungi and actynomycetes	Lignin depolymerisation and disintegration	Mild and environmentally friendly conditions and high selectivity	Low efficiency and low degradation rate	[3.12, 3.38]
Enzymes	Cellulase and β-glucosidase	Hydrolysis of the cellulose	Mild and environmentally friendly conditions and high selectivity	High price of the enzymes	[3.39]

The intensification of the most effective chemical delignification treatment was also carried out by assisting it with microwaves and ultrasounds, as these techniques could permit the enhancement of the lignin removal making at the same time the treatment more environmentally friendly [3.28, 3.29, 3.30].

Regarding the procedures that could be used to remove the cellulosic fraction, in this thesis the enzymatic hydrolysis was chosen. Compared with the acid treatment, which could also be used to hydrolyse this fraction, the enzymatic hydrolysis employs milder conditions, avoiding corrosion problems and the formation of degradation products [3.39]. The enzymes used in this thesis were endo- and exo-glucanases and β-glucosidase. The endo-glucanase acts on the amorphous regions of the cellulose hydrolysing the β-1-4 linkages randomly, while the exo-glucanase acts on the chain ends in a processive way, producing mainly cellobiose [3.39]. Since the cellobiose inhibits cellulase activity, the β-glucosidase was used to hydrolyse the cellobiose to glucose [3.40]. Commonly the enzymatic hydrolysis is carried out with solids which have been previously delignified [3.41, 3.42], as during this treatment the cellulose surface area and the porosity of the solid increases, making the cellulose more accessible [3.31, 3.43]. Nevertheless, there are also examples in which the enzymatic hydrolysis has been carried out with solids obtained after autohydrolysis treatments without being delignified [3.44, 3.45]. Therefore, in this thesis to assess the effect of the delignification on the enzymatic digestibility of the solid phases, the delignified and not delignified autohydrolised solids were subjected to enzymatic hydrolyses. The isolation of the lignin and the cellulose is an important stage of a biorefinery approach as these fractions could be used for the obtaining of multiple added-value compounds with applications in many different fields.

3.1.4 Revalorisation of lignin and cellulose

The obtaining of added-value products from cellulose and lignin could improve the economic viability of the biorefinery approach.

Lignin could present many different applications as it can be seen in Figure 3.4, however its potential application depends on the feedstock, the isolation procedure and the post-treatment employed, as its physico-chemical features are influenced by all of these aspects [3.46]. One of the applications that could be given to lignin is as a carbon source for the obtaining of energy [3.47]. The macromolecular structure of lignin also permits its employment as a filler in different polymer composites such as, phenol/formaldehyde resins, polyurethanes, polyvinylchlorides, polypropylenes, polyethylenes, polylactides or epoxy resins between others [3.46, 3.48]. Due to the aromatic character of this biopolymer, it has been used as a source of aromatic compounds [3.49] which could be further modified to produce important industrial organic chemicals such as hydrocarbons, alcohols, polyols, ketones, acids, or phenol derivatives, between others [3.50].

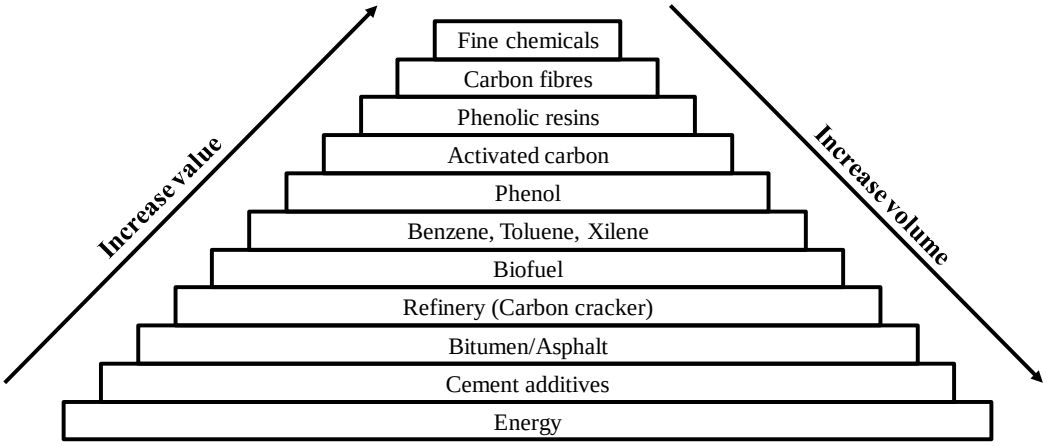


Figure 3.4 Applications of the lignin fraction, adapted from Sahoo et al. [3.46].

Regarding the cellulosic fraction, its applications varied significantly depending on its structure, since its glucose monomers, microfibrils, nanofibers or nanocrystals do not present the same applications. The cellulose nanofibers, for instance, have been used to produce composites to be used in flexible circuits or solar panels [3.51], while the cellulose nanocrystals have been widely used in medicine for wound dressing, cartilage/bone regeneration, dental repairs or as cancer curing drugs [3.52]. The high application of the cellulose nanocrystals in medicine is associated to their biocompatibility, tissue-friendliness, non-toxic nature or their antimicrobial effects [3.53]. The glucose monomers obtained via enzymatic or chemical hydrolysis from cellulose could be

used for the production of bioethanol or building blocks, such as lactic acid, hydroxymethylfurufural or itaconic acid between others [3.44, 3.54-3.56].

In this thesis, the cellulosic fraction of the vine shoots was revalorised by the production of bioethanol, while although different procedures were assessed for the isolation of their lignin fraction, its applications were not explored.

3.2 Objectives

The main objective of this chapter was to study different methods to revalorise the cellulosic and lignin fractions of the vine shoots, which remained almost unaltered after the autohydrolysis of these residues. The revalorisation was carried out following two different routes, as it is depicted in Figure 3.5. In the first route (A), on the one hand the cellulosic fraction was solubilised by subjecting the autohydrolysed vine shoots to an enzymatic hydrolysis, and on the other hand a Simultaneous Saccharification and Fermentation (SSF) process was carried out followed by a delignification treatment in order to yield bioethanol and lignin. In the second route (B) the order of the stages was changed, firstly the delignification was carried out followed by an enzymatic hydrolysis.

Moreover, in each route several aspects were assessed. For the route A, the influence of the severity of the autohydrolysis treatment on the enzymatic digestibility of the autohydrolysed solids was studied. Furthermore, the solid with the highest enzymatic digestibility was subjected to a simultaneous saccharification and fermentation (SSF) process for the conversion of its cellulosic fraction into bioethanol.

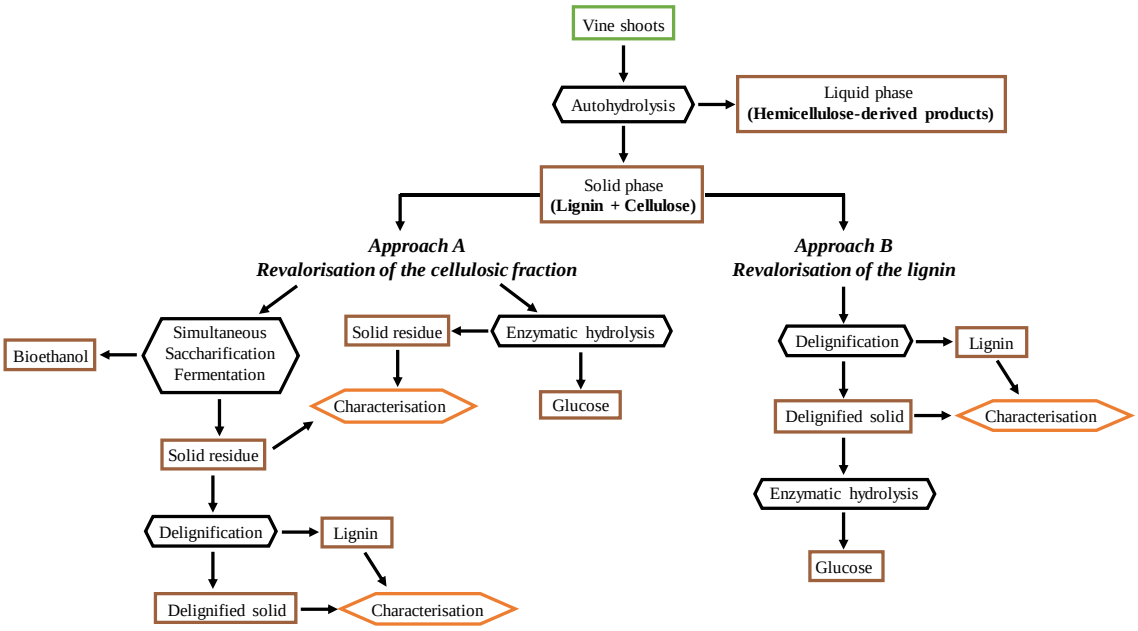


Figure 3.5 Scheme of the processing of the autohydrolysed vine shoots following two different routes.

For the route B, the efficiency of different delignification procedures on the removal of lignin from the autohydrolysed solid obtained in the treatment that provided the highest concentration of oligosaccharides was studied. The influence of the different delignification procedure on the features of the isolated lignins was also assessed. The delignification procedures that were studied were the organosolv, acetosolv and alkaline delignifications, being the intensification of the last procedure by microwaves and ultrasounds also analysed. Since the intensification of the alkaline delignification treatment by microwaves could make this process greener, it was optimised to maximise the lignin removal with the minimum cellulose loss using the most environmentally friendly conditions as possible. Furthermore, the influence of the delignification process in a subsequent enzymatic hydrolysis of the delignified solid was also studied, in order to observe the effect not only of the removal of the hemicellulosic fraction but also of the removal of the lignin on the enzymatic digestibility.

Another aspect that was studied was the influence of the stages that integrate the cascading processing to which the vine shoots were subjected until the obtaining of bioethanol (Figure 3.5) on the characteristics of the lignin. Thus, lignin was isolated from the untreated vine shoots, the autohydrolysed solid and the solid obtained after the SSF process by the most efficient

delignification procedure. This analysis can be useful to determine the step of the biorefinery scheme in which is more suitable to extract the lignin in function of the application to which it will be intended.

3.3 Materials and methods

3.3.1 Approach A: Revalorisation of the cellulosic fraction of the autohydrolysed vine shoots

One of the approaches used to revalorise the autohydrolysed solids is the employment of their cellulosic fraction for the production of bioethanol. Therefore, the influence of the severity of these hydrothermal treatments on the enzymatic digestibility of the solids was assessed with the aim of establishing the autohydrolysis conditions that permit the highest conversion of cellulose into glucose and therefore the highest bioethanol production.

The solid phases obtained in the non-isothermal autohydrolysis treatments of the vine shoots carried out at different temperatures were subjected to enzymatic hydrolysis (EnzH). These solids were used wet in order to preserve their structure open, which facilitates the access of the enzymes to the cellulosic structure.

The enzymatic hydrolysis was carried out by mixing the solid with water in an Erlenmeyer flask using a LSR of 20 g/g (in oven-dried basis) and using a 0.05 N citric acid-sodium citrate buffer to adjust the pH to 4.85. The enzymes added to the mixture were the commercial preparation of *Trichoderma reesei* cellulases "Celluclast 1.5", which was used in a cellulase/solid ratio (ESR) of 20 FPU/g (in oven-dried basis) and a β-glucosidase "Novozyme 188" supplied by Novozyme (Madrid, Spain), which was employed in a β-glucosidase/cellulase ratio (Cb/Cl) of 10 IU/FPU. Once the enzymes were added, the mixture was incubated at 48.5 ºC in a Unimax 1010 shaker equipped with an incubator 1000 (Heidolph) with an orbital agitation of 150 rpm.

After 0, 5, 11, 24, 32, 48, 72 and 96 h of incubation an aliquot of the enzymatic media was taken, centrifuged and its phases were separated by filtration. The liquid phase obtained at each

incubation time was subjected to monosaccharide quantifications using the Aminex HPX-87H column and the method described in the **Appendix II**.

The enzymatic hydrolyses were carried out once while the different analyses were carried out in duplicates.

3.3.1.1 Bioethanol production from the autohydrolysed solid obtained at 210 ºC

Among the different obtained autohydrolysed solids, the one obtained at 210 ºC presented the highest enzymatic digestibility. This solid was subjected to a SSF process for the production of bioethanol.

The yeast used in the SSF process was *Saccharomyces cerevisiae* (DSM 70449) which was grown in an Erlenmeyer flask containing the nutrients, which consisted on a solution with 10 g/L glucose, 5 g/L peptone, 3 g/L malt extract and 3 g/L yeast extract. The yeast was incubated at 32 ºC for 24 h in a Unimax 1010 shaker equipped with an incubator 1000 (Heidolph) with an orbital agitation of 200 rpm.

To carry out the SSF process the solid was mixed with water in a LSR of 10 g/g (in oven-dried basis) in a 100 mL Erlenmeyer flask and it was autoclaved at 121 ºC for 15 min. The working volume was 50 mL at pH 5. Compared with the EnzH during the SSF higher solid loadings were used (LSR of 20 g/g vs 10 g/g (in oven-dried basis)) to achieve higher glucose concentrations in the fermentation media.

The SSF process was started by adding 5 mL of the nutrient media previously sterilised, 5 mL of the yeast inoculum, the cellulose "Celluclast 1.5" in an ESR of 20 FPU/g and the β-glucosidase "Novozyme 188" in a Cb/Cl of 10 IU/FPU to the Erlenmeyer flask containing the solid. The samples were incubated at 35 ºC for 48 h with an orbital agitation of 120 rpm. At the end of the incubation, the liquid and solid phases were separated by filtration and they were processed as it was done with the liquid and solid phases obtained during the enzymatic hydrolysis (Section 3.3.1). The determination of ethanol in the liquid phase was carried out by HPLC using the

Aminex HPX-87H column and the method described in the **Appendix II** for the determination of monosaccharides, acids and degradation products in the autohydrolysis liquors.

The SSF process and the different analyses were carried out in duplicates.

3.3.2 Approach B: Recovery of the lignin fraction from the autohydrolysed solid obtained at 200 ºC

The second approach used to revalorise the autohydrolysed solid, was the recovery of their lignin fraction. During the delignification treatments two separate streams were obtained, on the one hand lignin which could be used for the synthesis of materials or for obtaining aromatic compounds and on the other hand a solid enriched in cellulose. This solid could be used to obtain dissolutions enriched in glucose whicht could be further employed for the production of bioethanol.

3.3.2.1 Delignification alternatives of the autohydrolysed solid

Different delignification procedures were tested in order to remove the lignin fraction from the autohydrolysed solids, being their efficiency and their influence on the lignin structure also analysed. The evaluated delignification procedures were the organosolv, acetosolv and alkaline treatments following the procedures described by Toledano et al. [3.57], Erdocia et al. [3.58] and Bustos et al. [3.59], respectively, after carrying out some modifications. The intensification of the alkaline treatment employing ultrasounds and microwaves was also studied.

Among the different autohydrolysed solids, only the one obtained in the treatment carried out at 200 ºC was subjected to the different delignification procedures, since this autohydrolysis conditions permitted the achievement of the highest oligosaccharide concentration and the extracts with the highest bioactive properties. The delignification treatments were carried out using the autohydrolysed solid wet, in order to preserve its structure open.

The organosolv and acetosolv delignifications were carried out by mixing the autohydrolysed solid with a 50.0 wt.% ethanol solution in a LSR of 8 g/g (in oven-dried basis) and with a solution

containing 65.0 wt.% acetic acid and 0.073 wt.% HCl in a LSR of 12 g/g (in oven-dried basis), respectively. The treatments were carried out in a 1.5 L stainless steel Parr reactor at 200 ºC and 130 ºC, respectively, being the temperature controlled by a Parr PID control. Once the target temperature was reached, it was maintained for 90 min and after being the reactor cooled down, the solid and liquid phases were separated by filtration. Then, 4 volumes of acidified water for the organoslv delignification and 4 volumes of water in the case of the acetosolv delignification were added to the liquid phase (also denoted as black liquors) in order to precipitate the lignin solubilised during these treatments. The precipitated lignins were isolated by filtration, neutralised with water and air-dried.

To carry out the alkaline delignification the autohydrolysed solid was mixed with a 12.0 wt.% NaOH solution in a LSR of 10 g/g (in oven-dried basis). The mixture was autoclaved at 124 ºC for 105 minutes without stirring. One of the alkaline delignification treatments was carried out assisted by ultrasounds, so prior to the autoclaving stage the mixture was sonicated 30 minutes using an Elmasonic S 70H sonicator working with an ultrasonic power of 150 W. After the autoclaving stage, the liquid and solid phases were separated by filtration. The obtained black liquor was acidified with 96.0 wt.% H_2SO_4 until pH 2 was reached and the precipitated lignin was separated by filtration, neutralised with water and air-dried.

The organosolv, acetosolv lignins and the ones obtained in the alkaline delignification treatments with and without the assistance of ultrasounds were subjected to different analytical and instrumental techniques. The carbohydrate, acid-insoluble lignin and ash content of the isolated lignins was determined using protocols described by Erdocia et al. [3.58] and Liu et al. [3.60], after carrying out some modifications. These procedures are described in detail in the **Appendix V**. The acid-soluble lignin forming part of the isolated lignin was analysed by the TAPPI protocol (TAPPI UM250 um-83) [3.61], which is also detailed in the **Appendix V**. The lignins were also subjected to FTIR, HPSEC and Pyrolysis-Gas Chromatography/Mass Spectrometry (Py-GC/MS) analyses, being their detailed procedures collected in the **Appendix V**.

Regarding the solid phases obtained after these four delignification treatments, they were washed with the solution employed during the delignification treatment and afterwards, they were washed

with water until their neutralisation. The solid phases were then air-dried, quantified and subjected to moisture determinations and to quantitative acid hydrolysis (**Appendix I**) in order to estimate the solubilisation of the substrate during the treatment and the composition of the delignified solids.

All the delignification treatments were carried out in duplicates, while the chemical analyses were carried out in triplicates.

3.3.2.2 Intensification of the alkaline delignification of the autohydrolysed solid assisted by microwaves

To reduce the environmental impacts that the alkaline delignification of autohydrolysed vine shoots could cause, the intensification of this treatment with microwaves was studied. The treatment was optimised using an experimental design procedure in order to achieve the maximum lignin removal with the minimum cellulose loss.

The autohydrolysed solid was mixed with a NaOH solution with concentrations between 0.2 and 6.0 wt.% using a LSR of 10 g/g (in oven-dried basis) in a 70 mL PrepPlus® reactor. The experiments were carried out in a CEM-Mars microwave system using temperatures between 50 and 150 ºC and reaction times ranging from 0 to 1 h. The temperature was controlled by a fibre optic temperature sensor and 5 min were used in all the experiments to reach the target temperature. Once cooled down, the solid and liquid phases were separated by filtration. The solid phase was washed with a solution with the same NaOH concentration as the one used during the delignification treatment, and then it was neutralised with water. Afterwards, the delignified solid was air-dried, quantified and subjected to moisture analysis and to quantitative acid hydrolysis (**Appendix I)** in order to determine the solubilisation of the substrate during the delignification treatment and its composition.

The experiments were planned according to a 2 level 3 factor Box-Wilson Central Composite Face Centred (CCF, α: ±1) design. This is a 2^k factorial design, where 2^k indicates the number of runs for the single factorial design and *k* the number of studied variables or factors; in this case, as 3 operating variables were studied, 8 runs were carried out. In addition, 6 axial experiments

were performed to analyse non-linear effects and interactions according to the CCF design and 4 replicates at the centre point were carried out to determine the experimental error. The operating conditions of the performed experiments are collected in Table 3.3.

Table 3.3. Operating conditions of the experiments carried out in the experimental design of the microwave-assisted alkaline delignification of the autohydrolysed solid obtained at 200 ºC.

Exp.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
T (ºC)	50	150	50	150	50	150	50	150	100	100	100	100	50	150	100	100	100	100
t (min)	0	0	60	60	0	0	60	60	30	30	30	30	30	30	0	60	30	30
wt% NaOH	0.2	0.2	0.2	0.2	6.0	6.0	6.0	6.0	3.1	3.1	3.1	3.1	3.1	3.1	3.1	3.1	0.2	6.0

The results obtained in the experimental design were analysed by an analysis of variance (ANOVA) working with a 95.0 % confidence interval, as this permits the determination of the operating variables and interactions that significantly influence the response variables.

Once the experimental conditions that permitted the maximum lignin removal with the minimum cellulose loss were determined, the experiment was performed. The delignified solid obtained at the optimum conditions was air-dried, quantified and subjected to moisture analysis and to quantitative acid hydrolysis (**Appendix I**). The lignin isolated at the optimum conditions was subjected to the same analysis as the lignins isolated with the different delignification procedures and to ^{13}C-NMR and ^{1}H-NMR analyses, being all the procedures described in the **Appendix V**.

All the delignification treatments were carried out in duplicates, while the chemical analyses were carried out in triplicates.

3.3.2.3 Enzymatic hydrolysis of the autohydrolysed solid with and without alkaline delignification treatment

The influence of the delignification treatment on the enzymatic digestibility was assessed, as lignin could reduce the activity of the cellulases. To do so, the autohydrolysed solid obtained at 200 ºC was subjected to an alkaline delignification treatment and the resulting solid as well as the autohydrolysed solid were subjected to enzymatic hydrolysis using the procedure described previously in the Section 3.3.1. In this case, an ESR of 25 FPU/g (in oven-dried basis) and a Cb/Cl

of 5 IU/FPU were used. Only the delignified solid obtained in the alkaline delignification treatment carried out without any assistance was subjected to an enzymatic hydrolysis, since between the delignified solids obtained by not intensified delignification treatments this was the one with the lowest lignin content.

The enzymatic hydrolysis was carried out in duplicates, while the chemical analyses were carried out in triplicates.

3.3.2.4 Study of the influence of each biorefinery stage on the structural features of the isolated lignin

To study how the different stages of the biorefinery scheme proposed for the vine shoots (Figure 1.10) could influence the structure of the lignin fraction, the untreated vine shoots, the autohydrolysed solid obtained at 210 ºC and the solid obtained after the SSF process were subjected to an alkaline delignification without any assistance. Among the different delignification treatments, only this one was used, as it was the most effective delignification treatment that employs the lower energy requirement and because the assistance of this procedure by microwaves was carried out afterwards. The obtained delignified solids and the alkaline lignins were subjected to the same analyses mentioned in the Section 3.3.2 for the different delignified solids and isolated lignins.

All the delignification treatments were carried out in duplicates, while the chemical analyses were carried out in triplicates.

3.4 Results and discussion

3.4.1 Route A: Revalorisation of the cellulosic fraction of the autohydrolysed solid

One of the approaches used to revalorise the solids obtained during the autohydrolysis treatments of the vine shoots was the employment of their cellulosic fraction for the production of bioethanol. However, prior to the bioethanol production, the influence of the severity of this processing on

the enzymatic digestibility of the autohydrolysed solids was studied in order to determine the autohydrolysed solid with the highest digestibility. This solid would be the most suitable for the production of bioethanol.

As it has been seen throughout the Section 2.4.2, the increase of the severity of the autohydrolysis treatments promoted the removal of the hemicellulosic fraction of the vine shoots, making the obtained solid richer in glucan and lignin, as it can be seen in Table 3.4. The NREL procedure employed for the determination of the composition of the solid determines the glucan content [3.62], which could be mainly attributed to the cellulosic content as great part of the hemicelluloses were removed during the autohydrolysis treatments. Thus, the estimations in this Section were carried out considering the glucan content.

Table 3.4 Composition of the solids obtained during the autohydrolysis treatments carried out at different temperatures (expressed as percentage of dry spent solid).

Fraction	Temperature (ºC)							
	180	**185**	**190**	**195**	**200**	**205**	**210**	**215**
Severity (S_0)	3.29	3.45	3.60	3.82	4.01	4.28	4.47	4.65
Glucan	27.2 ± 0.2	28.9 ± 0.6	28.7 ± 0.5	31.0 ± 0.6	31.2 ± 0.8	32.49 ± 0.02	34.8 ± 0.4	33.7 ± 1.2
Klason lignin	44.7 ± 0.4	46.4 ± 0.1	50.5 ± 0.4	50.9 ± 2.1	53.5 ± 0.9	52.5 ± 2.9	54.5 ± 0.8	55.1 ± 0.9
Xylan	13.1 ± 0.4	11.1 ± 0.1	9.2 ± 0.4	6.9 ± 0.6	5.4 ± 0.1	4.6 ± 0.2	3.0 ± 0.3	2.5 ± 0.3
Acetyl substituents	2.8 ± 0.1	1.8 ± 0.3	1.8 ± 0.1	1.4 ± 0.1	1.0 ± 0.2	ND	ND	ND

ND: not detected.

As it can be seen in this Table, the lignin content of the autohydrolysed solids increased continuously with the severity of the autohydrolysis treatment. Conversely, the glucan content of the solids increased until the treatment carried out at 210 ºC, afterwards its content in the solid start to decrease due to the possible degradation of the glucan. Nevertheless, it has to be taken into account that the composition of the autohydrolysed solids collected in this Table differs slightly from the composition of the solids obtained during the autohydrolysis of the vine shoots shown in Table 2.4 of the Section 2.4.2. This could be a consequence of the employment of vine shoots obtained in a different vintage for the assessment of the enzymatic hydrolysis.

3.4.1.1 Enzymatic hydrolysis of the autohydrolysed solids

The enzymatic hydrolysis is a process that presents a high selectivity for the cellulosic fraction, leaving the lignin fraction almost unaltered. During this hydrolysis no degradation products are generated so it is not necessary to carry out a purification treatment prior to the fermentation stage for the production of bioethanol [3.39]. Nevertheless, the effectiveness of the removal of the cellulosic fraction from the autohydrolysed solids depended strongly on the severity of the autohydrolysis treatment as it can be appreciated in Figure 3.6, which reflects the glucose concentration in the enzymatic media generated during the enzymatic hydrolysis of the different autohydrolysed solids.

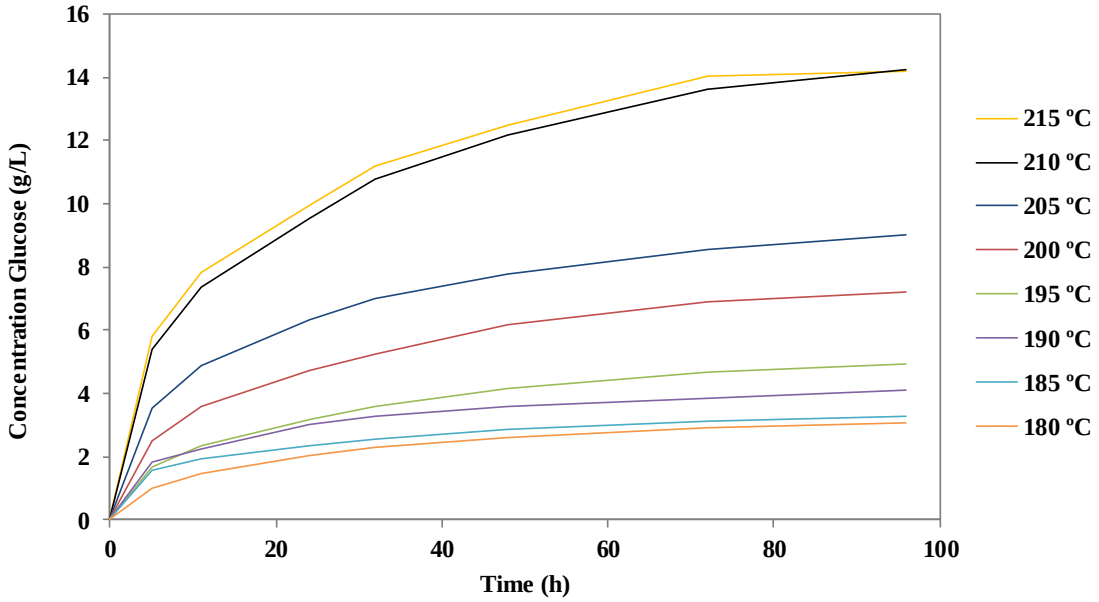


Figure 3.6. Glucose concentration profile during the enzymatic hydrolysis of the different autohydrolysed solids.

As it is better appreciated in the glucose concentration profiles obtained during the enzymatic hydrolysis of the autohydrolysed solids generated at the severest conditions (215 ºC (S_0 = 4.65) and 210 ºC (S_0 = 4.47)), the glucose release occurred in two steps. During the first 24 h a fast initial glucose release was observed while afterwards the glucose was released slowlier, similar to what it was described by Requejo et al. [3.63].

From the concentration of the glucose released during the enzymatic hydrolysis of the different autohydrolysed solids the glucan to glucose conversion (GC, glucose/potential glucose in percentage) can be calculated. The GC is defined as the ratio between the glucose generated in the enzymatic hydrolysis and the potential glucose contained in the solids. The potential glucose in the solids is estimated by considering the total conversion of the glucan into glucose without degradation.

By fitting the variation of the GC with the time to the hyperbolic model proposed by Holtzapple et al. [3.64], the maximum possible enzymatic conversion (GC_{Max}) could be estimated by the following equation:

$$CG_t = CG_{MAX} \cdot \frac{t}{t + t_{1/2}}$$

where GC_t is the glucan to glucose conversion at time t, GC_{MAX} (%) is the maximum glucan to glucose conversion at an infinite reaction time, and $t_{1/2}$ (h) is the time needed to achieve 50.0 % of the GC_{MAX}. In Table 3.5 the GC_{MAX} values obtained for each autohydrolysed solids, together with the $t_{1/2}$, which measures the kinetic of the enzymatic hydrolysis, and the R^2 values, which showed the adjustment of the experimental data to the proposed model [3.44], are collected.

Table 3.5. Maximum glucan to glucose conversion (GC_{MAX}), time needed to achieve half of GC_{MAX} ($t_{1/2}$) and coefficient of determination R^2 estimated during the enzymatic hydrolysis of different autohydrolysed solids.

Temperature (ºC)	**180**	**185**	**190**	**195**	**200**	**205**	**210**	**215**
GC_{MAX} (%)	22.4	21.6	26.8	32.2	48.8	55.5	81.2	81.1
$t_{1/2}$ (h)	17.26	7.02	8.75	15.20	15.29	11.64	11.65	10.19
R^2	0.992	0.979	0.988	0.988	0.987	0.988	0.999	0.992

As it is reflected in Figure 3.7, which represents the estimated GC_{MAX} and the experimental results, and as it is shown by the R^2 values collected in Table 3.5, the experimental data adjusted correctly to the hyperbolic model. Furthermore, the estimated GC_{MAX} values for the enzymatic hydrolysis of each autohydrolysed solid were quite close to the glucan to glucose conversion

achieved after 96 h of enzymatic hydrolysis, suggesting that the prolongation of the saccharification time would not produce an improvement of the glucan conversion.

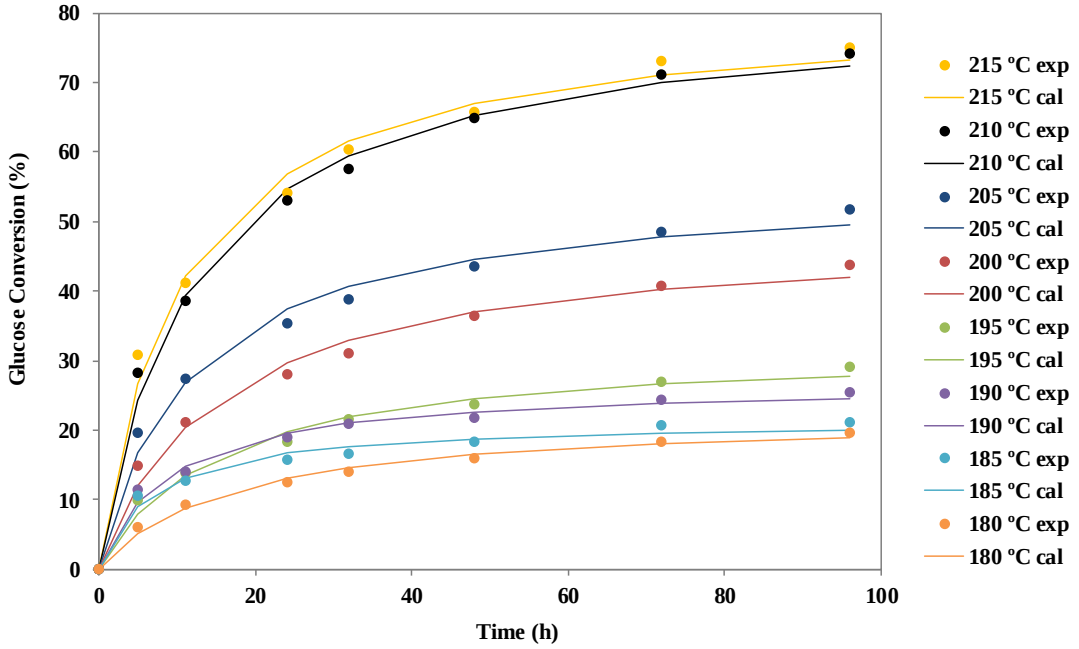


Figure 3.7. Glucan to glucose conversion profile experimentally and theoretically determined during the enzymatic hydrolysis of the different autohydrolysed solids.

The enzymatic digestibility of the autohydrolysed solids increased with the severity of the treatment as it can be appreciated from the GC and from GC_{MAX} values shown in Figure 3.7 and in Table 3.5, respectively. The solids obtained in the treatments carried out under the severest conditions (215 ºC (S_0 = 4.65) and 210 ºC (S_0 = 4.47)) presented the highest susceptibility towards the enzymatic hydrolysis as they permitted the achievement of the highest glucan to glucose conversion. The higher susceptibility of these solids towards the enzymatic hydrolysis is related to their lower hemicellulosic content, as they tend to block the approach of the enzymes to the cellulose [3.4]. The highest lignin content of these solids did not seem to influence strongly the glucan to glucose conversion, although it is supposed to decrease the efficiency of the enzymatic hydrolysis by blocking the approach of the enzymes to the cellulose and by binding irreversibly to the cellulases through hydrophobic interactions [3.65]. The low influence of the lignin content in the enzymatic hydrolysis could be attributed to the structural changes that the autohydrolysis treatment provokes on the vine shoots. The increase of the severity of the autohydrolysis treatment

is associated to the raise of the porosity of the substrate which could facilitate the enzymatic action [3.66]. Michelin and Teixeira [3.45] and Dominguez et al. [3.67] carried out autohydrolysis treatments of brewer´s spent grain at S_0 = 4.13 and of *Paulownia tomentosa* at S_0 = 4.43, respectively, prior to the enzymatic saccharification. They achieved glucan to glucose conversions of 76.1 % and 86.0 % operating with a solid loading of 5 %(w/v), 15 FPU/g and 1 IU/FPU and with a solid loading of 9.0 % (w/v), 10 FPU/g and 10 IU/FPU, respectively. The GC that they obtained was similar to the one achieved using the autohydrolysed solid obtained at the harsher conditions (74.0 % at S_0 = 4.47 and 75.0 % at S_0 = 4.65).

3.4.1.2 Bioethanol production from the autohydrolysed solid obtained at 210 ºC

The production of bioethanol from the autohydrolysed solid was carried out by a simultaneous saccharification fermentation (SSF) process. In this case, the enzymatic hydrolysis of the cellulosic fraction of the biomass is carried out in the presence of fermentative microorganisms, which uses the released glucose for the production of bioethanol and for their growth [3.68].

Among the different autohydrolysed solids only the one obtained in the treatment carried out at 210 ºC was subjected to the SSF process. Although this solid did not present the highest enzymatic digestibility, since the one obtained at 215 ºC presented a higher glucan to glucose conversion (74.0 % vs 75.0 %), the difference in the GC did not justify the higher energy requirements of carrying out the autohydrolysis treatment at higher temperature. In addition, the autohydrolysed solid obtained at 210 ºC presented the highest glucan content as it can be seen in Table 3.4.

To make the production of bioethanol more economically viable a higher solid loading than the one used during the enzymatic hydrolysis of the different autohydrolysed solids was employed, a LSR of 10 g/g (in oven-dried basis) instead of 20 g/g (in oven-dried basis). The employment of higher solid loadings favours the production of bioethanol, however, it has to be taken in mind that it could also decrease the bioethanol yield due to poor mass transfer [3.69].

The amount of bioethanol produced from the autohydrolysed solid obtained at 210 ºC after 48 h of SSF process was 13.3 g ethanol/L, which corresponded to a conversion of glucan to ethanol

(EC) of 67.4 %. This parameter represents the percentage of ethanol obtained with respect to the ethanol that could be produced by the stoichiometric conversion of the glucan present in the substrate. Requejo et al. [3.63], achieved a similar EC (70.0 %) carrying out the SSF of autohydrolysed olive tree trimmings using a LSR of 8 g/g (in oven-dried basis) and an ESR of 10 FPU/g.

3.4.2 Route B: Revalorisation of the lignin fraction of the autohydrolysed solids

The second route that could be used to revalorise the autohydrolysed solids is by exploiting their lignin fraction. The isolation of this fraction was carried out using different chemical treatments that do not affect in high degree to the cellulosic fraction of the autohydrolysed solid, which could be further used for the production of bioethanol as it has been seen in the Section 3.4.1.2. Among the autohydrolysed solids obtained at different temperatures, only the one obtained during the treatment carried out at 200 ºC was subjected to different delignification treatments in order to perform an integral revalorisation of the vine shoots.

The autohydrolysed solid obtained at 200 ºC was subjected to organosolv, acetosolv and alkaline delignification treatments being the latest one also intensified by the application of ultrasounds (US). Table 3.6 gathers together the experimental conditions used in each treatment, the composition of the solid phase obtained and the substrate solubilised after each delignification process. This data permits the determination of the delignification yield and the percentage of the cellulose lost during the treatment.

As during the autohydrolysis treatment carried out at 200 ºC more than 80.0 % of the hemicelluloses present in the vine shoots were removed, the glucan content determined in this autohydrolysed solid and in the delignified solids has been considered as cellulose content.

Table 3.6. Substrate solubilisation (expressed as g/100 g oven-dried autohydrolised solid), composition of the autohydrolysed solid after being delignified by the different treatments (expressed as percentage of dry spent solid) and the percentages of the delignification yield and the cellulose loss.

	Autohydrolysis	Organosolv	Acetosolv	Alkaline	Alkaline + US
Temperature (ºC)	200	200	130	124	124
Reagent	H_2O	50.0 wt.% EtOH	65.0 wt.% acetic acid + 0.073wt.% HCl	12.0 wt.% NaOH	12.0 wt.% NaOH
Time (min)	-	90	90	105	105 + 30 min US at room tempreature
Substrate solubilisation (%)	-	17.8 ± 0.2	29.7 ± 0.1	53.0 ± 1.3	58.9 ± 0.3
Klason lignin	46.8 ± 2.25	49.7 ± 0.3	49.9 ± 0.2	32.2 ± 0.1	28.7 ± 0.2
Glucan	39.9 ± 0.39	44.6 ± 1.2	39.5 ± 0.6	69.4 ± 1.5	65.5 ± 0.7
Xylan	4.90 ± 0.02	ND	2.5 ±0.1	ND	ND
Acetyl substituents	0.85 ± 0.04	ND	ND	ND	ND
Delignification yield	-	12.6	25.0	67.7	74.8
Cellulose loss	-	8	30.3	18.3	32.6

ND: not detected.

Jiménez et al. [3.70] carried out the delignification of untreated vine shoots using EtOH, ethylene-glycol, NaOH and Kraft conditions. They achieved a delignification yield of 26.1 % when the organosolv delignification was carried out, while when alkaline and Kraft delignifications were carried out the delignification yields increased to 61.9 and 75.3 %, respectively.

Nevertheless, although Kraft delignification could be more efficient than the alkaline hydrolysis, the latest one permitted the obtaining of purer cellulose [3.71]. Furthermore, the alkaline delignification presents advantages for the employment of the obtained solid in enzymatic hydrolysis, as it provokes the decrease of the crystallinity and polymerization degrees of the cellulose and the increase of its surface area, facilitating the enzymatic digestibility of the cellulosic fraction [3.59, 3.72].

Regarding the alkaline delignification of the autohydrolysed solid obtained at 200 ºC, it could be appreciated that the assistance of the treatment by ultrasounds enhanced the delignification yield but it also favoured the cellulose loss, provoking the increase of these parameters in a 7.0 and 14.3 %, respectively, compared with the not assisted alkaline treatment. The raise of the

delignification yield caused by the employment of ultrasounds was also observed by Sun and Tomkinson [3.28], when they carried out the alkaline delignification of wheat straw. They noticed an increase of 5.2 % of the delignification yield when the sonication time was increased from 5 to 35 min and they associated it to the mechanical actions of the ultrasounds on the cell walls. This could facilitate the access of the reagents and the extraction of the lignin fraction in a subsequent treatment. Nevertheless, the exact mechanism of the ultrasound-assisted alkaline delignification still remains unclear [3.73].

In Table 3.6, it can also be appreciated that the cellulosic fraction is also affected during the delignification treatments. The lower cellulose loss was observed during the organosolv treatment (8.0 %), although it has been reported that it only solubilise lignin and hemicelluloses [3.33]. Conversely, the acetosolv process was among the not assisted delignification treatments the one in which the highest cellulose loss was observed (30.3 %). This could be related to the employment of dilute acids under severe conditions. Although the acetosolv treatment has been proposed as a method to solubilise lignin [3.60] it could also be considered as an acid pre-treatment used to hydrolyse amorphous cellulose [3.31]. Regarding the alkaline delignifications, 32.6 and 18.3 % of the cellulose present in the autohydrolysed solid was solubilised during the treatments carried out with and without the ultrasound assistance, respectively. This could be due to the peeling reactions that the cellulose could suffer in basic medium at 120-130 ºC [3.74]. These reactions provoke the removal of between 50 and 60 glucose units depending on the conditions [3.2]. The higher cellulose loss observed in the ultrasound-assisted alkaline delignification could be attributed to the mechanical action of the ultrasounds but also to the solubilisation of the amorphous cellulose, which is more susceptible to be solubilised by sonication-assisted treatments [3.75].

3.4.2.1 Intensification of the alkaline delignification of the autohydrolysed solid by the employment of microwaves

Among the different not intensified delignification treatments to which the autohydrolysed solid obtained at 200 ºC was subjected the alkaline delignification was the one that permitted the

highest lignin removal. To make this process more effective and greener by reducing its operating time and the amount of reagent employed, the alkaline delignificationit was assisted by microwaves instead of carrying it out with conventional heating. An experimental design of this treatment was performed to maximise the lignin removal and to minimise the cellulose loss by optimising the temperature, reaction time and NaOH concentration used. The employment of microwaves has some advantages compared with the conventional heating, as it could be its faster heat transfer or its more energy efficient heating mechanism [3.76].

The composition of the delignified solids together with the delignification yields and cellulose loss percentages obtained during each microwave-assisted alkaline delignification treatment are collected in Table 3.7. From this Table it could be appreciated that the experimental conditions employed during the delignification treatment influence strongly the amount of lignin and cellulose removed. The determined delignification yields varied from 9.3 to 89.2 % while the percentage of cellulose loss varied between 0.0 and 34.5 %.

The Pareto percentages of the operating variables and their linear and quadratic interactions, which are shown in Table 3.8, indicate their influence on the delignification yields and on the cellulose loss. From this Table it could be appreciated that the NaOH concentration exerted the strongest influence on the two response variables (19.0 and 28.0 % on the delignification yield and on the cellulose loss percentage, respectively), while the temperature has a stronger influence on the delignification yield than on the cellulose loss percentage, 16.0 vs 12.0 %. The reaction time has a significant influence on the delignification yield (8.0 %) while is its quadratic interaction which influenced strongly the cellulose loss percentage (22.0 %).

Figure 3.8 reflects the effect of the operating conditions and the most important interactions detected during the ANOVA analysis of the determined delignification yields and cellulose loss percentages. Figures 3.8.a and 3.8.b show the effects of the temperature on the delignification yield for the lowest (0.2 wt.%) and the highest (6.0 wt.%) NaOH concentrations and the shortest (0 min) and longest (60 min) reaction times, while Figures 3.8.c and 3.8.d show the same effects for the cellulose loss percentage.

Table 3.7. Experimental conditions and the results obtained in the different experiments carried out during the experimental design of the microwave-assisted alkaline delignification.

Exp.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
T (ºC)	50	150	50	150	50	150	50	150	100	100	100	100	50	150	100	100	100	100
t (min)	0	0	60	60	0	0	60	60	30	30	30	30	30	30	0	60	30	30
wt.% NaOH	0.2	0.2	0.2	0.2	6.0	6.0	6.0	6.0	3.1	3.1	3.1	3.1	3.1	3.1	3.1	3.1	0.2	6.0
Substrate solubilisation (%)	6.2	11.4	8.6	12.43	23.3	33.6	17.1	60.2	28.2	29.5	33.9	32.6	17.6	53.2	18.4	32.6	13.7	28.0
Lignin (wt. %)	49.74 ± 0.24	50.76 ± 0.64	48.54 ± 0.42	47.99 ± 2.31	46.96 ± 2.45	43.74 ± 0.04	42.95 ± 1.25	13.98 ± 0.75	43.56 ± 2.64	42.61 ± 0.65	44.10 ± 2.08	42.68 ± 0.41	45.09 ± 0.72	25.84 ± 0.02	44.23 ± 1.43	45.73 ±3.25	46.60 ± 0.19	43.83 ±2.04
Cellulose (wt. %)	40.57 ± 4.23	38.35 ± 0.23	37.68 ± 0.58	39.51 ± 0.41	36.78 ± 1.50	42.71 ± 0.02	39.92 ± 0.77	66.87 ± 0.66	37.70 ± 2.54	40.32 ± 0.80	38.02 ± 3.61	36.34 ± 0.28	36.33 ± 0.99	55.52 ± 2.10	39.17 ± 1.58	43.38 ±3.53	41.02 ± 0.58	39.54 ±0.53
Delignification yield (%)	9.3	13.6	13.8	17.4	30.0	43.6	30.8	89.2	39.2	41.6	43.4	44.1	27.8	76.5	29.9	40.1	21.9	38.7
Cellulose loss (%)	0.0	10.1	7.8	6.3	24.5	24.1	11.4	28.7	27.5	23.9	32.7	34.5	19.9	30.4	14.5	21.8	5.2	23.8

Table 3.8. Relative influence of the operating variables on the analysed response variables.

	R^2	I. Term	A (T)	B (t)	C (wt.% NaOH)	AB	AC	BC	A^2	B^2	C^2	ABC	A^2B	A^2C	AB^2	AC^2	B^2C	BC^2	A^2B^2
Delignification yield (%)	0.997	42.34	24.34 (16.0)	6.49 (8.0)	8.42 (19.0)	5.51 (6.0)	8.01 (9.0)	4.77 (5.0)	9.20 (2.0)	-7.94 (8.0)	-12.69 (8.0)	5.70 (6.0)	n.s	9.01 (4.0)	-14.38 (7.0)	n.s	n.s	n.s	n.s
Cellulose loss (%)	0.907	28.13	3.59 (12.0)	n.s	8.31 (28.0)	n.s	n.s	n.s	n.s	-10.01 (22.0)	-13.62 (16.0)	3.67 (11.0)	n.s	n.s	n.s	n.s	n.s	n.s	9.62 (9.0)

n.s: Non significant with 95.0 % confidence.

Response: I. Term + A (T) + B (t) + C (wt.% NaOH) + AB + AC + BC + A^2 + B^2 + C^2 + ABC + A^2B + A^2C + AB^2 + AC^2 + B^2C + BC^2 + A^2B^2

Numbers in bracket correspond to the Pareto percentage which measures the influence of each interaction on the response variable. Pareto values represent the percentage of the orthogonal estimated total value.

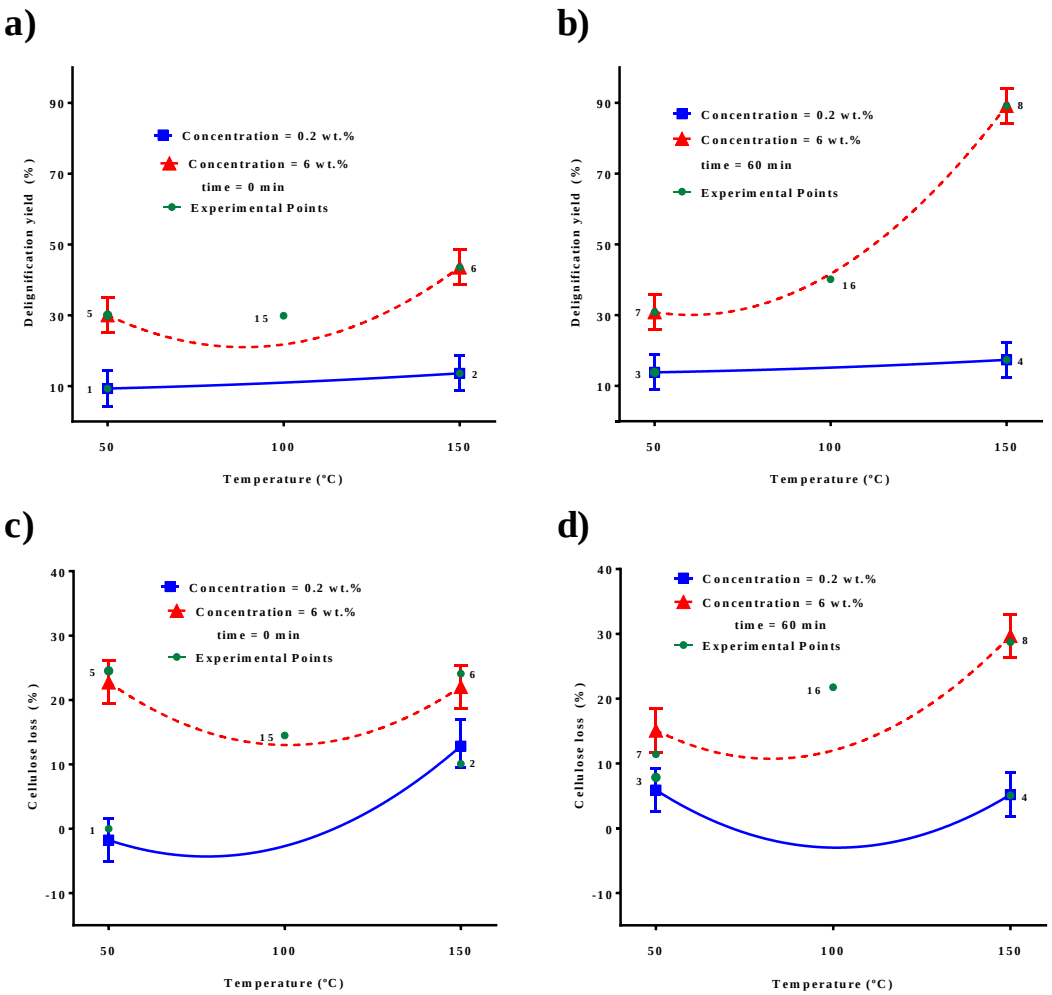


Figure 3.8. Effect of the temperature and NaOH concentration on the deliginification yield (a/b) and cellulose loss percentage (c/d) when different reaction times (t=0 min and t=60 min) are used.

Comparing Figures 3.8.a and 3.8.b it can be appreciated that the effect of the temperature on the delignification yield depends on the concentration of NaOH used, being this effect better appreciated when long reaction times were employed. It could be observed that when the lowest NaOH concentration was used (0.2 wt.%) the reaction time nor the temperature had a significant effect on the delignification yield. This suggests that it is necessary to have a minimum amount of NaOH to promote the solubilisation of the lignin fraction by increasing the temperature or the reaction time [3.77].

Conversely, when the highest NaOH concentration was employed the delignification yield increased with the temperature being it more pronounced when long reaction times were used. In particular, the delignification yield varied from 30.0 % at 50 ºC to 44.0 % at 200 ºC when short

reaction times were used, while it varied from 30.0 % at 50 ºC to 89.0 % at 200 ºC when long reaction times were employed. This could suggest the synergic interaction of the three operation variables similar to what it was observed by McIntosh and Vancov [3.78] when they studied the delignification of wheat straw. However, during the microwave-assisted alkaline delignification treatments not only the lignin was removed from the autohydrolysed solid obtained at 200 ºC as its cellulose fraction is also partially removed, as it can be seen from Figures 3.8.c and 3.8.d and from the results shown in Table 3.7.

Comparing these Figures it could be appreciated that the effects of the temperature and reaction time on the cellulose loss percentage depended on the NaOH concentration. It can be appreciated that when the lowest NaOH concentration (0.2 wt.%) and the shortest reaction time (0 min) were used the cellulose loss percentage started to increase with the temperature once 100 ºC were reached, passing from around 0.0 % at 100 ºC to 13.0 % at 200ºC. This increase could be associated with the peeling reactions of the cellulose that tend to start taking place around 120 ºC [3.74]. Conversely, when the longest reaction time was used, the cellulose loss was not significantly affected by the temperature.

When the highest NaOH concentration (6.0 wt.%) was used independently of the reaction time or the temperature, the cellulose loss percentage was higher than when the lowest NaOH concentration was used. At high NaOH concentration and short reaction time, the cellulose loss percentage was not affected by the increase of the temperature. While the increase of the temperature from 100 to 150 ºC when the highest NaOH concentration and the longest reaction times (60 min) were used, provoked an increase of the cellulose loss percentage from 12.0 % to 28.0 %, respectively. This increase in the cellulose loss percentage could be related with the peeling reaction which might be promoted by high NaOH concentration (6.0 wt.%), high temperatures and long reaction times [3.74].

The constructed experimental design apart from studying the influence of the operating variables on the delignification yield and on the cellulose loss percentage permitted the prediction of the optimum operating variables, since it presents a R^2 value higher than 0.9 for the two studied response variables as it can be seen in Table 3.8. The optimum operating conditions were

estimated as a result of the compromise between the lignin and cellulose removal and the severity of the operating conditions. In order to optimise the treatment, a relative importance was given to the operating and response variables grading them with values between 1 and 5, as it can be seen in Table 3.9. In particular, a higher importance was given to minimise the cellulose loss percentage (5) and to maximise the delignification yield (4) than to making the operating conditions milder (3).

Table 3.9. Theoretical optimization of the microwave-assisted alkaline delignification treatment: operating variables and response variables.

Variable	Objective	Interval of variation	Relative importance (1-5)	Optimum predicted value
Operating variables				
Temperature (°C)	Minimise	50-150	3	150
Time (min)	Minimise	0-60	3	30
NaOH (wt.%)	Minimise	0.2-6.0	3	6.0
Response variables				
Delignification yield (%)	Maximise	0.0-100.0	4	88.8 ± 7.7
Cellulose loss (%)	Minimise	0.0-100.0	5	26.4 ± 11.1

The determined optimum operating conditions (6.0 wt.% NaOH for 30 min at 150 ºC) predicted the removal of the 88.0 % of the lignin present in the autohydrolysed solid with an avoidable cellulose loss percentage of 26.4 %, as it is shown in the Table 3.9. These predictions were experimentally corroborated and non-statistically significant differences (p-value > 0.05) were found between the predicted and the experimental values. The solid delignified during the microwave-assisted treatment carried out under the optimum conditions contained 56.9 ± 0.4 wt.% of cellulose and 21.8 ± 1.3 wt.% of lignin. Taking also into account that during the treatment 42.9 % of the autohydrolysed solid was solubilised, it was estimated that 81.8 % of the lignin and 34.6 % of the cellulose present in the autohydrolysed solid were removed.

Therefore, it was observed that the assistance of the alkaline delignification treatment by microwaves could improve the delignification yield, using half of the NaOH concentration and 28.0 % of the reaction time used during the alkaline delignification treatment carried out by conventional heating (Section 3.4.2), despite of the use of higher temperatures.

3.4.2.2 Characterisation of the lignins obtained by different delignification procedures

As the extraction procedure influences the physico-chemical features of the isolated lignin [3.79], the ones obtained during the organosolv and acetosolv treatments as well as those obtained during the alkaline delignifications with and without the assistance of ultrasounds and microwaves were analysed in detail.

The different lignins were subjected to several chemical analyses in order to determine their composition, as it is one of the most important features and it could provide verification that the precipitated solid is indeed lignin. Thus, during the acidification of the black liquors obtained during the alkaline delignification, the repolymerisation and precipitation of the aromatic species takes place leading an insoluble solid, being these species coming from the solubilisation and transformation of the lignin and carbohydrates present in the feedstock [3.80].

As it can be seen in Table 3.10 all the lignins isolated from the autohydrolysed solid obtained at 200 ºC presented a high acid-insoluble lignin content and therefore a high purity, independently of the procedure employed for their isolation.

Table 3.10. Composition and purity (expressed as g/100 g oven-dried lignin) of the lignins isolated from the autohydrolysed solid obtained at 200ºC using different delignification procedures.

Fraction	Organosolv	Acetosolv	Alkali	Alkali + US	Alkali + Mw
Klason lignin	81.0 ± 4.4	82.3 ± 1.9	79.6 ± 0.7	88.6 ± 0.9	93.0 ± 0.9
Acid-soluble lignin	1.12 ± 0.01	1.2 ± 0.1	1.66 ± 0.04	1.9 ± 0.1	1.6 ± 0.2
Glucose	0.36 ± 0.05	0.57 ± 0.01	0.84 ± 0.02	1.3 ± 0.1	1.2 ± 0.1
Xylose	0.30 ± 0.05	0.43 ± 0.02	4.2 ± 0.1	5.4 ± 0.1	1.58 ± 0.04
Ash	8.7	4.6	2.6	0.1	0
Lignin purity (%)	82.1	83.5	81.3	90.5	94.6

The high purity of these lignins and their low carbohydrate content was a consequence of the autohydrolysis treatment to which the vine shoots were subjected prior to the delignification, as during this treatment around 87.0 % of their hemicelluloses was removed. Nevertheless, the

glucose and xylose content determined in the different lignins could be associated with the co-precipitation of lignin with the solubilised carbohydrates.

Zamudio et al. [3.81], for instance, observed that the acid-insoluble lignin content in the black liquors obtained during the alkaline delignification of autohydrolysed *Paulownia* was 21.5% higher than in the black liquors obtained by the same procedure from untreated *Paulownia*. They attributed this to the removal of the hemicelluloses during the autohydrolysis treatment [3.81]. Carvalho et al. [3.82] also were able to obtain a lignin with a high purity (91.6 %) and only 1.9 % of glucose and 0.4 % of xylose from acid pretreated coffee (*Coffea Arabica)* husk by an alkaline delignification.

3.4.2.2.1 Py-GC/MS analyses of the lignins isolated by different delignification procedures

The obtained lignins were subjected to Py-GC/MS analyses in order to determine qualitatively the phenolic compounds that form part of their structure; however, it has to be taken into account that the determined compounds do not need to be forming part of the lignin structure. The compounds were identified using the NIST library and what it has been reported in the literature for the pyrolysis of lignin [3.83-3.85]. The pyrolysis products determined in each lignin together with their relative abundance is shown in Table 3.11. The identified compounds were categorised as lignin-derived and carbohydrate-derived compounds, being the first ones sub-classified as guaiacyl-derivates (G), p-coumaryl-derivates (H), syringol-derivates (S) and catechol-derivates (C) [3.86]. The first three groups are associated to the three phenyl propane units that constitute the lignin, while the catechol-derivates are supposed to be formed by the degradation of syringyl-derivates due to the high pyrolysis temperatures [3.87].

As it can be seen in this Table, the distribution of the compounds obtained during the pyrolysis analyses depended on the assessed lignin. The highest lignin-derived products content was obtained during the pyrolysis of the acetosolv lignin and of lignin isolated by the not intensified alkaline treatment (78.8 and 74.0 %, respectively).

Table 3.11. Distribution of the main compounds obtained in the pyrolysis of the lignins isolated by the different delignification procedures (expressed as g/100g lignin).

Compound	rt (min)	Main fragments (m/z)	Origin	Organosolv	Acetosolv	Alkaline	Alkaline + US	Alkaline + Mw
Toluene	3.94	91,92,65	-	1.00	1.03	0.58	0.57	0.77
Furfural	4.86	96,95,67	Carbohydate	ND	ND	0.99	1.06	ND
Phenol	7.29	94,66,65	H	4.47	10.53	4.23	3.52	5.44
2-methyl phenol	8.49	108,107,79	H	0.75	2.16	0.64	0.56	1.37
4-methyl phenol	8.82	107,108,77	H	4.22	8	3.24	2.91	3.98
Guaiacol	9.12	109,124,81	G	5.95	13.98	4.94	4.03	6.26
4-ethyl phenol	10.48	107,122,77	H	ND	1.17	ND	0.73	ND
4-methyl guaiacol	11.10	138,123,95	G	6.68	15.45	7.42	4.76	6.46
Catechol	11.22	110,64,63	C	1.01	ND	1.70	2.97	2.14
3-methoxy catechol	12.92	140,125,97	C	5.13	3.07	6.48	4.73	5.28
4-ethyl guaiacol	13.43	137,152,122	G	2.39	4.84	3.16	2.83	3.97
4-methyl catechol	13.77	124,123,78	C	0.88	0.45	1.99	1.76	1.26
Syringol	15.73	154,139,111,96	S	8.22	6.63	8.67	7.29	7.71
3,4-dimethoxy phenol	15.97	154,139,111,151	S	2.39	ND	2.42	1.89	1.57
Vanillin	17.00	151,152,81	G	ND	ND	0.61	0.90	ND
Cis-Isoeugenol	17.07	164,149,77	G	ND	ND	0.60	0.61	0.76
Isovanillin	17.28	151,152,81	G	1.79	0.79	ND	ND	ND
4-methyl syringol	17.95	168,153,125	S	ND	ND	10.83	ND	ND
Acetovanillone	18.68	151,166,123	G	0.74	0.43	1.17	1.38	0.98
4-ethyl syringol	19.37	167,182,168	S	ND	ND	3.79	3.31	ND
Guaiacylacetone	19.46	137,180,122	G	ND	ND	0.97	0.85	0.70
4-vinyl syringol	20.01	180,165,137	S	ND	ND	6.76	4.76	ND
4-allyl syringol	20.56	194,91,119	S	0.66	0.81	0.83	0.76	0.70
Homosyringalde-hyde	20.66	167,196,168	S	ND	ND	0.72	ND	ND
Syringaldehyde	21.40	182,181,167	S	1.42	ND	1.17	3.17	ND
Acetosyringone	22.34	181,196,153	S	1.23	ND	1.65	3.08	0.50
Total siryngyl derivates			S	13.92	7.44	36.84	24.24	10.48
Total guaiacyl derivates			G	17.55	35.49	18.87	15.39	19.13
Total p-coumaryl derivates			H	9.44	32.39	8.11	7.72	10.79
Total catechol derivates			C	7.02	3.52	10.16	9.46	8.68

Conversely, the lowest lignin-derived products content was observed in the organosolv lignin and in the alkaline lignin isolated by the microwave-assisted process (47.9 and 49.1 %, respectively). In the case of the alkaline lignin isolated with the assistance of microwaves, its low lignin-derived products content could be attributed to a more condense structure of this lignin which hinders its

pyrolysis. Its more condense structure could be associated to the repolymerisation reactions that could have taken place during the delignification treatment.

The organosolv lignin surprisingly presented a lower lignin-derived content than what it was expected as this delignification procedure is supposed to provide relatively pure lignins[3.88]. Constant et al. [3.88], for instance, obtained organosolv lignins from untreated wheat-straw, poplar and spruce which contained 80.8, 77.5 and 79.6 % of lignin-derived products content. Sequeiros et al. [3.18] also isolated organosolv lignins from untreated olive tree pruning containing 98.0 % of lignin-derived products.

It is clear from the data shown in Table 3.11 that the distribution of the pyrolysis products generated from each lignin is non-identical, due to the different lignin isolation procedure employed. The most abundant products were the guaiacyl-derivates, except in the alkaline lignins obtained with and without the assistance of ultrasounds which presented a higher number of syringyl-derived compounds. The most abundant products determined in all the lignins were phenol, guaiacol, 4-methyl guaiacol, 3-catechol and syringol.

The catechol-derived compounds identified during the pyrolysis could have been formed during the pyrolysis, while the p-coumaryl-derived products could be formed by demethoxylation reactions of the aromatic ring of guaiacyl or syringyl units or by the cracking of C-C inter unit bonds between the p-hydroxyphenyl units of the lignin as Shen et al. [3.89] suggested.

These analyses demonstrate that in spite of having lignins with similar acid-insoluble lignin content, between 80.0 and 93.0 %, their phenolic composition varied highly with the employed isolation procedure.

3.4.2.2.2 FTIR analyses of the lignins isolated by different delignification procedures

The FTIR analysis of the isolated lignins permits the determination of the principal functional groups and units forming part of their structure. The FTIR spectra of each lignin, which are illustrated in Figure 3.9, presented the characteristic bands of the lignins isolated by different procedures from other raw materials, although their intensity varied. These variations could suggest that the abundance of some functional groups depended on the procedure employed for

the isolation of the lignin. García et al. [3.90] also observed differences in the intensities of the bands present in lignins isolated using autohydrolysis, organosolv and alkali treatments from *Miscanthus sinensis*. The assignation of the bands was carried out taking into account what it has been published about the FTIR analysis of lignin [3.58, 3.88].

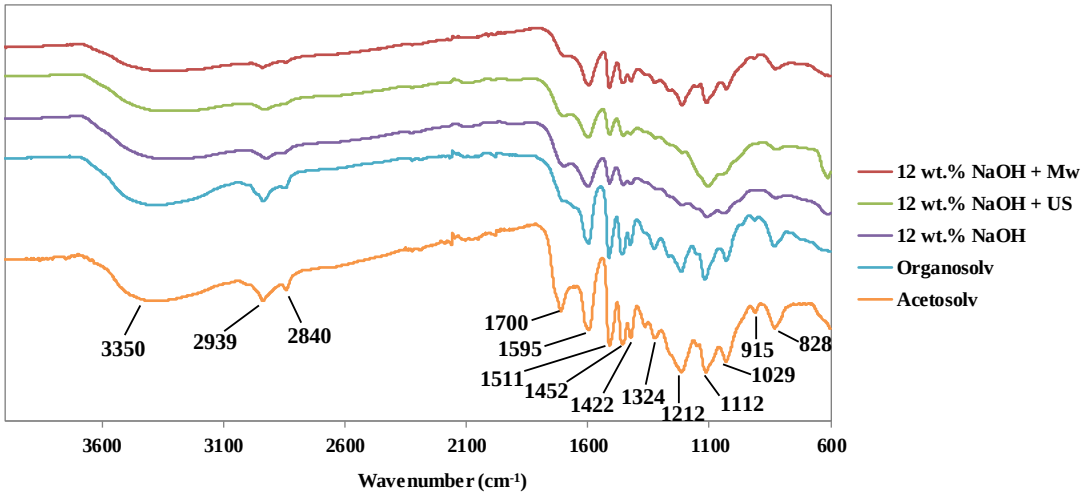


Figure 3.9. FTIR spectra of the lignins isolated by different delignification procedures.

All the spectra presented a wide band at 3350 cm^{-1} which corresponded to the O-H stretching vibration of aromatic and aliphatic hydroxyl groups. The bands observed at 2939, 2840 and 1452 cm^{-1} corresponded to the C-H stretching vibration in methyl and methylene groups and to the C-H bending vibrations of the same groups, respectively. All the lignins presented the bands attributed to the characteristic vibrations of the aromatic skeleton of the lignin at 1595, 1511 and 1422 cm^{-1}. The first band is associated with the aromaticity, while the last two are associated with the presence of guaiacyl units, corresponding the latest one to the C-H deformation in guaiacyl rings. The bands observed at 1029 and 915 cm^{-1} also confirmed the presence of guaiacyl units in the lignin structure as they are attributed to their C-H bond deformation and C-H out of plane bending. The presence of syringyl units in the isolated lignins was corroborated by the bands observed at 1324 and 1212 cm^{-1} which corresponded to the syringyl and condensed guaiacyl rings breathing vibrations [3.91] and to the C-O vibrations in these units. Moreover, bands associated

only to the syringyl rings were also observed at 1112 and 828 cm^{-1}, being them associated to the C-H deformation and to the C-H out of plane bending vibration of these rings, respectively.

The band observed at 1700 cm^{-1}, which corresponded to the stretching vibration of non-conjugated carbonyl groups with the aromatic lignin skeleton, was the one presenting the highest intensity variation between the different lignins. This band was very intense for the lignin isolated by the acetosolv procedure, being this intensity variation associated to the esterification during this treatment of some of the hydroxyl groups present in the lignin [3.92].

According to the FTIR analyses all the lignins isolated from the autohydrolysed solid were constitute by syringyl and guaiacyl units. Thus, the catechol and p-coumaryl-derivate compounds determined during the pyrolysis analysis could have been generated due to the high temperature of the pyrolysis treatment [3.87, 3.89].

3.4.2.2.3 HPSEC analyses of the lignins isolated by different delignification procedures

The molecular weight distribution of lignin is an important feature to take into consideration for its application [3.93], as it is a factor that limits the solubility of this biopolymer in all the common solvents at room temperature [3.94]. The lignins isolated by different extraction procedures were analysed by HPSEC, being their average molecular weight (Mw), number-average (Mn) and polydispersity index (Mw/Mn) shown in Table 3.12. The molecular weight distribution of the different lignins is also plotted in Figure 3.10.

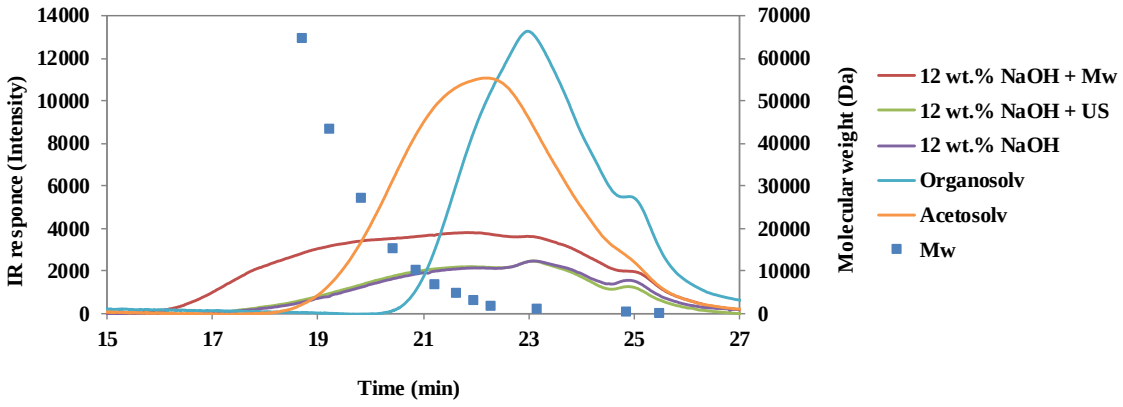


Figure 3.10. Molecular weight distribution profile of the lignins isolated from the autohydrolysed solid using different delignification procedures.

As it can be seen in this Figure the procedure employed for the isolation of the lignin fraction influenced strongly the molecular weight distribution of this biopolymer. The lignins isolated by the organosolv and acetosolv treatments presented a lower molecular weight than the three isolated alkaline lignins, as it can be appreciated in Table 3.12. Nitsos et al. [3.95] also observed that the alkaline lignins extracted from softwood and hardwood presented higher molecular weight than the organosolv lignins extracted from the same raw materials. Constant et al. [3.88], for instance, obtained an organosolv lignin from wheat straw with a molecular weight (1960 Da) similar to the one observed in the organosolv lignins isolated from autohydrolysed vine shoots. Erdocia et al. [3.58] obtained an acetosolv lignin from olive tree pruning with a higher molecular weight (16416 Da) than the one observed in the acetosolv lignin isolated from the autohydrolysed vine shoots.

Table 3.12. Average molecular weight (Mw), number-average (Mn) and polydispersity index of the lignins isolated from the autohydrolysed solid using different procedures.

	Organosolv	**Acetosolv**	**Alkali**	**Alkali + US**	**Alkali + Mw**
Mw (Da)	1902	6579	12,159	14,465	38,371
Mn (Da)	915	1639	1297	1349	1453
Mw/Mn	2.08	4.01	9.37	10.72	26.41

Regarding the alkaline lignins, their molecular weight varies between 12,159 and 38,371 Da, presenting some of these lignins a molecular weight similar to the one observed by Toledano et al. [3.96] and Coral Medina et al. [3.49] when they analysed the alkaline lignins obtained from olive tree pruning and oil palm empty fruit (10875 and 12580 Da, respectively). The higher molecular weight of the alkaline lignins compared with the organosolv and acetosolv lignins could be attributed to the dissolution of larger lignin molecules, as Li et al. [3.97] suggested, which was also reflected on the higher delignification yields observed during the alkaline treatments. Furthermore, the high MW of these lignins could also be due to condensation reactions that could have been taking place during the alkaline delignification treatments [3.98]. Among these lignins, the ones obtained by the microwave- and ultrasound-assisted treatments presented the highest

molecular weights, due to the positive effect of these intensification techniques on repolymerisation reactions [3.28, 3.99].

Regarding the polydispersity index, the alkaline lignins presented higher values due to their more heterogeneous distribution. This could be attributed to the possible breaking-up of high molecular weight lignin fractions in alkaline media [3.100].

3.4.2.2.4 NMR analyses of the lignin isolated by the microwave-assisted alkaline delignification treatment

In order to elucidate the structure and composition of the isolated lignins, the one obtained during the microwave-assisted alkaline delignification treatment was subjected to ^{13}C- and ^{1}H-NMR analyses. Only this lignin was analysed by NMR as it was the one obtained by the most promising delignification procedure. These analyses were carried out without acetylating the lignin as few differences between the results obtained with and without acetylated lignins have been reported [3.49]. The signals observed in the ^{13}C- and ^{1}H-NMR spectra were attributed using the assignation carried out by Coral Medina et al. [3.49], Santos et al. [3.91], She et al. [3.98] and Sun et al. [3.100] between others.

In Figure 3.11, which represented the ^{13}C-NMR spectra of this alkaline lignin, some of the characteristic signals of the lignin structure can be appreciated. The signal observed at 191.7 ppm corresponded to the carbonyl groups present in a benzaldehyde structure [3.101], while the one noticed at 175 ppm was attributed to aliphatic COOR structure which could have been produced during the oxidation of lignin. Nevertheless, this latest signal could also indicate the presence of proteins, hemicelluloses or ester-linked fatty/hydroxyl acids.

The ^{13}C-NMR spectra region between 150 and 100 ppm is associated to the aromatic region, being the signal observed at 153 ppm attributed to the C_3 and C_5 in etherified syringyl units. At 148.5 ppm a signal corresponding to the C_3 and C_5 in syringyl units (phenolic) and to the C_3 and C_5 in guaiacyl units was appreciated. The signals present at 135 ppm and 130 ppm were associated to the C_1 in etherified guaiacyl units [3.102] and to the C-α and C-β bonds in the Ar-CH=CH-CH_2OH structures of guaiacyl and syringyl units.

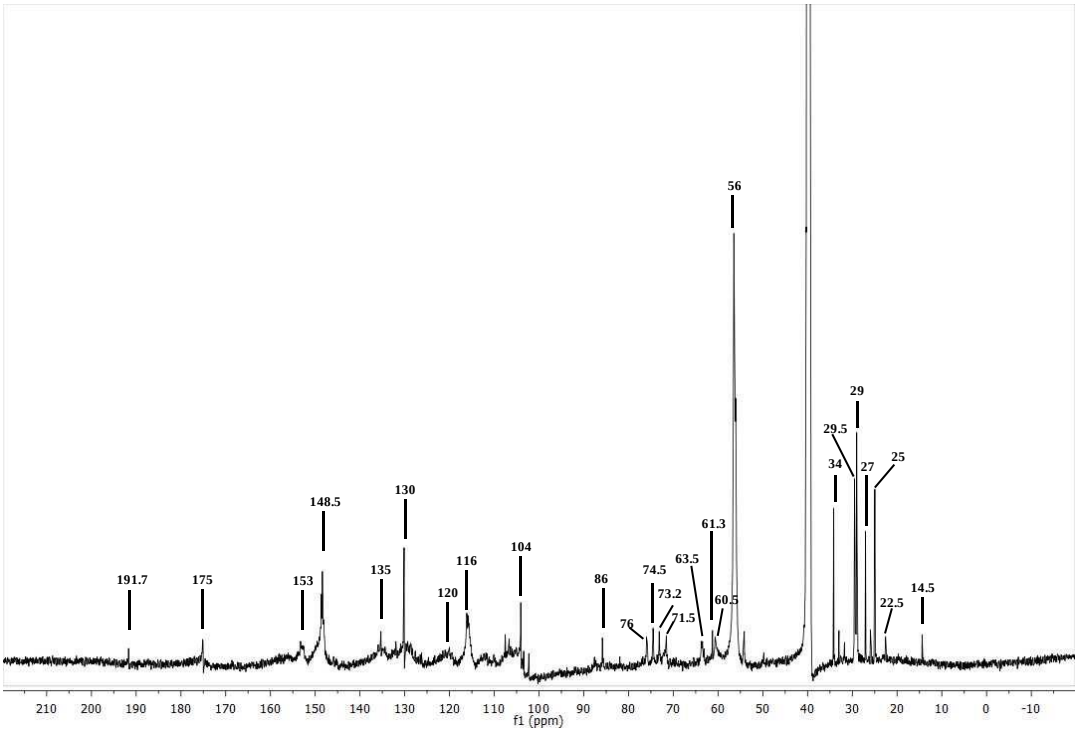


Figure 3.11. ^{13}C-NMR spectra of the lignin isolated by the microwave-assisted alkaline delignification at the optimum conditions from the autohydrolysed solid obtained at 200 ºC.

Apart from these signals that were characteristic of the lignin structure, the ones observed at 120 ppm, 116 ppm, 104 ppm corresponded to the C_6 in guaiacyl units, C_5 in guaiacyl units and C_2 and C_6 in syringyl units, respectively.

Between 95 and 50 ppm the oxygenated aliphatic region of the ^{13}C-NMR spectrum could be observed, being the lignin inter-unit linkages appreciated in this region. The signal observed at 86 ppm and at 56 ppm corresponded to the CH-α in syringyl units [3.102] and to the carbon in Ar-OCH_3 structures, respectively. According to Erdocia et al. [3.58] between 80-70 ppm and between 70-60 ppm the signals associated to the C-α in β-O-4 guaiacyl and syringyl units and to the C-γ in cinnamyl alcohol units could be appreciated. The signals that were observed between 40 and 10 ppm accounted for aliphatic and propylic side chains and they are a fingerprint of the lignin structure.

Figure 3.12 represents the ^{1}H-NMR spectra of the lignin isolated by the microwave-assisted alkaline delignification at the optimum conditions. The complex multiplet observed between 6.5 and 7.5 ppm was attributed to the aromatic protons in syringyl and guaiacyl units, but it could also correspond to p-hydroxyphenyl units and to p-coumarates and ferulates [3.100].

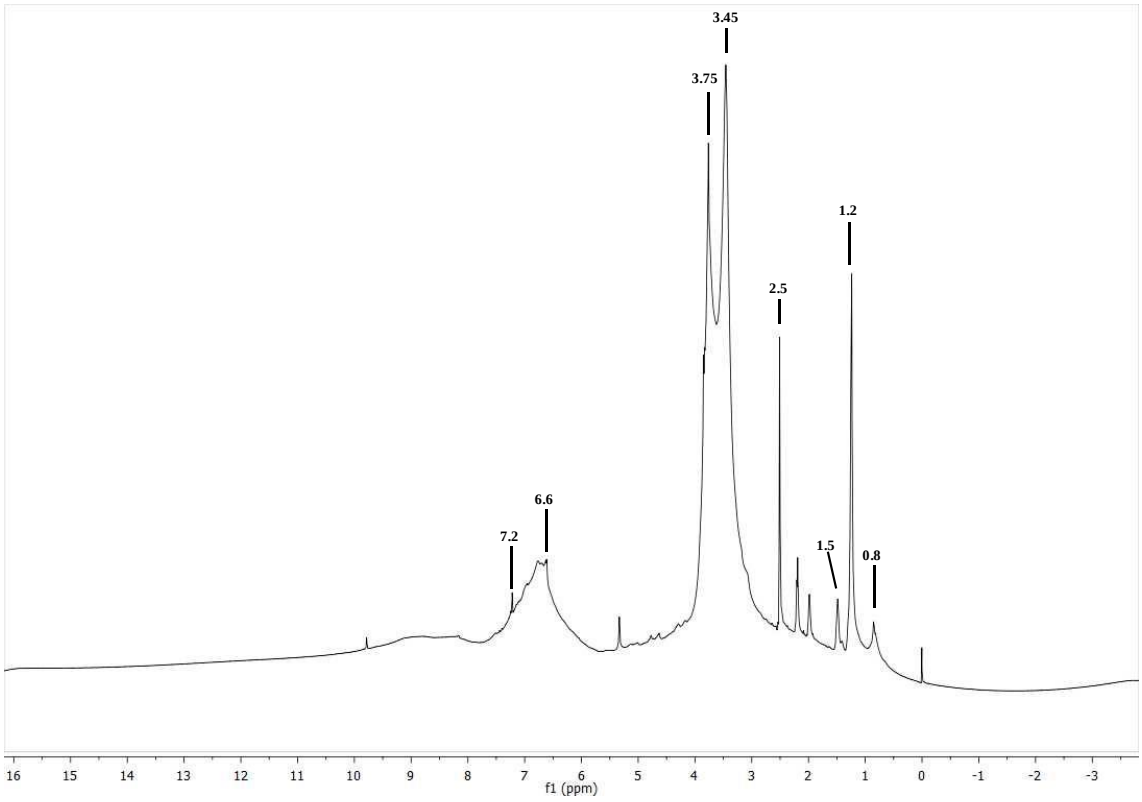


Figure 3.12. [1]H-NMR spectra of the lignin isolated by the microwave-assisted alkaline delignification at the optimum conditions from the autohydrolysed solid obtained at 200 ºC.

The signals observed at 3.75 and below 1.5 ppm were associated to methoxyl protons and to protons in aliphatic and methylene saturated groups, respectively, while the one observed at 0.8 ppm corresponded to the protons of the methyl group in aliphatic saturated structures. The signal associated to dimethylsulfoxide, which was used as the solvent, was observed at 2.5 ppm while the signal observed at 3.45 ppm was attributed to the protons of the water contained in the solvent.

3.4.2.3 Enzymatic hydrolysis of the solid obtained after the delignification of the autohydrolysed solid obtained at 200 ºC

The absence of the hemicellulosic fraction in the autohydrolysed solids seem to improve the enzymatic hydrolysis up to a conversion of glucan to glucose of 75.0 % when the autohydrolysed solid obtained at 215 ºC was employed, as it has been seen in the Section 3.4.1. However, this conversion is not efficient enough to industrialise the process. Thus, as the lignin also affects the enzymatic hydrolysis of the cellulose [3.65], the influence of the removal of this fraction on the enzymatic hydrolysis of the autohydrolysed solid was assessed.

As it is wanted to integrally revalorise the vine shoots, the autohydrolysed solid obtained at 200 ºC was subjected to an enzymatic hydrolysis using an ESR of 25 FPU/g and a β-glucosidase/cellulase ratio of 5 IU/FPU. Only this solid was employed, as this temperature of the autohydrolysis treatment permitted the maximum formation of oligosaccharides and the obtaining of the most bioactive extracts. The solid obtained after the alkaline delignification of the autohydrolysed solid obtained at 200 ºC, was also subjected to an enzymatic hydrolysis under the same conditions. In this case, the alkaline delignification was carried out without any assistance, as the optimisation of the alkaline delignification was carried out afterwards.

It was observed that after 96 h of enzymatic hydrolysis 11.0 g glucose/L and 39.6 g glucose/L were achieved when the autohydrolysed and the delignified solids were employed. This corresponded to a glucan to glucose conversion of 49.5 % and close to 100.0 %, respectively. This confirmed that the removal of the lignin together with the removal of the hemicelluloses provokes an enhancement of the enzymatic hydrolysis, as it was expected. It has been reported that aside from removing the lignin fraction, the alkaline delignification also provokes the swelling of the solid, leading to an increase of the internal surface area, a decrease of the crystallinity of the cellulose. Furthermore, it promotes the separation of the structural linkages between lignin and carbohydrates [3.103], favouring all these changes the enzymatic saccharification.

3.4.2.4 Study of the influence of each biorefinery stage on the structural features of the isolated lignins

As it has been observed up to now, the lignin together with the hemicelluloses influence the enzymatic digestibility of the vine shoots, being the removal of these fractions essential steps in the proposed biorefinery approach of the vine shoots (Figure 1.10, Section 1.5). The removal of lignin could be carried out before or after the removal of the hemicelluloses or after the revalorisation of the cellulose by the production of bioethanol. Thus, the characteristics of the lignins isolated from the untreated vine shoots (VS), the autohydrolysed solid obtained at 210 ºC (AH) and the spent solid obtained in the SSF process (SFF) were studied, since the feedstock from

which the lignin is isolated could also influence its features. The composition and structural characteristics of these lignins could allow establishing the step of the biorefinery processing sequence of the vine shoot (Figure 1.10) in which is better to remove the lignin fraction. As the delignification procedure employed also affects the features of the lignin, the isolation procedure used for the three different feedstocks was the conventional alkaline delignification.

In Table 3.13 the influence of the alkaline delignification treatment on the three different solids could be appreciated as their composition prior and after the delignification treatment is presented. As it can be appreciated, the delignification yield is not significantly influenced by the employed feedstock as it varied from 60.3 to 67.7 %. However, during the delignification treatments not only the lignin fraction of the different solids was affected, since the hemicellulolosic fraction was also solubilised. For instance, the hemicellulosic content of the vine shoots was reduced from 18.9 to 4.8 % after the delignification treatment. Apart from being solubilised during the alkaline delignification treatments, the hemicelluloses are also partially deacetylated [3.104].

Table 3.13. Substrate solubilisation (expressed as g/100 g oven-dried vine shoots), composition of the different solids prior and after the delignification treatment (expressed as percentage of dry spent solid) and the percentages of the delignification yield and cellulose loss.

	Prior to the delignification			**After being delignified**		
	Untreated VS	**Autohydrolysed VS**	**SSF residue**	**Untreated VS**	**Autohydrolysed VS**	**SSF residue**
Substrate solubilisation	-	-	-	57.5 ± 0.5	46.8 ± 0.5	60.9 ± 2.7
Fraction						
Xylan	9.7 ± 0.2	2.9 ± 0.3	2.6 ± 0.7	4.8 ± 0.4	ND	ND
Arabinosyl substituents	1.5 ± 0.1	ND	ND	ND	ND	ND
Acetyl groups	4.2 ± 0.3	ND	0.4 ± 0.1	ND	ND	ND
Galacturonyl substituents	3.51 ± 0.03	ND	ND	ND	ND	ND
Glucan	26.3 ± 0.2	33.3 ± 0.4	16.0 ± 1.9	54.6 ± 0.5	52.2 ± 3.1	27.4 ± 1.2
Klason lignin	33.5 ± 0.6	54.5 ± 0.8	68.5 ± 0.4	31.3 ± 0.8	33.1 ± 1.3	60.7 ± 0.1
Delignification yield (%)				60.3	67.7	65.4
Glucan loss (%)				11.8	16.6	33.0

ND: not detected.

The cellulosic fraction of the three solids was also affected during the alkaline delignification treatments due to the peeling reactions that it suffers under these conditions. It can be appreciated that with the increase of the number of treatments to which the vine shoots were subjected the higher was the quantity of glucan lost during the delignification. This could be explained because of the structural alteration caused to the vine shoots during the autohydrolysis treatment and SSF process.

It has been observed that although the same delignification procedure was used its effect was different depending on the feedstock used which could be translated in differences between the features of the lignins.

3.4.2.4.1 Chemical composition of the lignins isolated from different feedstocks

The lignins isolated from the untreated vine shoots (VSL), the autohydrolysed vine shoots obtained at 210 ºC (AHL) and the spent solid obtained in the SSF process (SSFL) were subjected to different chemical analyses in order to determine their content in carbohydrate, ashes and acid-soluble and insoluble lignin, being their composition shown in Table 3.14.

Table 3.14. Chemical composition of the lignins isolated from the three different feedstocks (expressed as g/100 g oven-dried lignin).

Fraction	VSL	AHL	SSFL
Acid-insoluble lignin	48.8 ± 3.3	70.2 ± 5.9	87.8 ± 0.2
Acid-soluble lignin	3.1 ± 0.1	2.38 ± 0.07	1.21 ± 0.05
Glucose	2.30 ± 0.02	2.025 ± 0.002	2.38 ± 0.02
Xylose	29.7 ± 0.2	3.1 ± 0.1	2.9 ± 0.1
Ash	ND	ND	0.26
Lignin Purity (%)	48.9	72.6	89.0

ND: not detected.

The lignin isolated from the untreated vine shoots presented the highest carbohydrate and the lowest acid-insoluble lignin content, as during the alkaline treatments the lignin and hemicellulosic fractions could be solubilised [3.6]. The carbohydrates solubilised during the alkaline treatments could be trapped during the subsequent precipitation stage or they could

remain covalently bound to the lignin [3.105], being forming part of the precipitated solid. Thus, the obtaining of lignin from pre-treated biomass permits the production of lignin with a higher purity. Similar trends were observed by Wang and Chen [3.106] when they studied the influence of a steam explosion treatment on the composition of the black liquor and consequently on the purity of the precipitated lignin. They appreciated an increase of the lignin present in the black liquor from 53.7 % to 93.0 % and a decrease from 45.8 % to 4.6 % of their carbohydrate content when prior to the alkali delignification of corn stalk a steam explosion treatment was carried out. Between the two lignins obtained from the pre-treated vine shoots, the one obtained after the SSF process presented the highest purity. Lee et al. [3.107] also recovered an alkaline lignin with a similar purity (84.5 %) from the residue generated during the bio-ethanol production from oak wood.

3.4.2.4.2 FTIR analyses of the lignins isolated from different feedstocks

The alkaline lignins isolated from the three solids were analysed by FTIR to determine their main functional groups and composition. The lignins, independently of the feedstock form which they were isolated, presented the characteristic bands of the lignin structure that have been detailed in the Section 3.4.2.2.1.

However, the differences in the composition of the lignins also affected the intensity of some of the bands observed in the spectra shown in Figure 3.13. For instance, it could be appreciated that the band observed at 1030 cm^{-1} presented a higher intensity in the spectra of the lignin isolated from the untreated vine shoots than in the spectra of the other two lignins. The higher intensity of this band could be associated to the fact that it does not only correspond to the C-H bond deformation in guaiacyl units but it is also attributed to the stretching and bending vibrations of C-O, C-C and C-OH and glycosidic C-O-C bonds present in hemicelluloses [3.108].

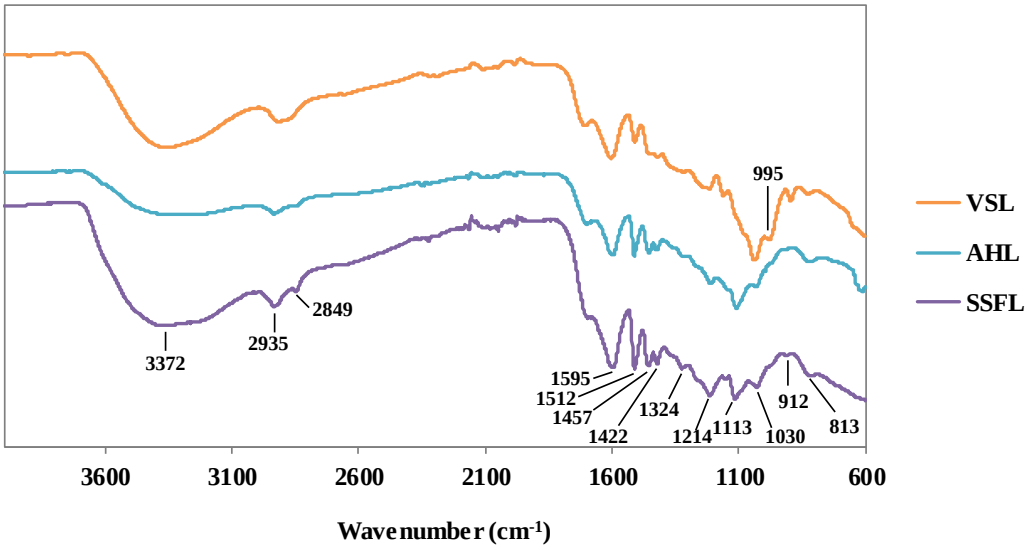


Figure 3.13. FTIR spectra of the alkaline lignins isolated from the different feedstock obtained through the biorefinery sequence.

The intensity of the band present at 912 cm^{-1} was also higher for the lignin isolated from the untreated vine shoots as apart from corresponding to the C-H out of plane bending of guaiacyl rings it is also associated to the presence of β-glycosidic linkages between xylopyranoses [3.108]. Furthermore, apart from the change of the intensity of these bands, the FTIR spectra of the lignin isolated from the untreated vine shoots presented a new band at 995 cm^{-1} which represented the C-O stretching in the hemicelluloses [3.109].

The lower intensity of the band observed at 1030 and 912 cm^{-1} and the absence of the band observed at 995 cm^{-1} in the spectra of the lignins isolated from the autohydrolysed vine shoots and SSF confirmed their lower hemicelluloses content.

3.4.2.4.3 Py-GC/MS analyses of the lignins isolated from different feedstocks

The FTIR analyses permitted the perception of the guaiacyl and syringyl units forming part of the structure of the three lignins, however in order to have more information about the structure of the lignins they were analysed by Py-GC/MS. The pyrolysis products, which were identified using the NIST library and the literature about the lignin pyrolysis [3.83-3.85] together with their relative abundance are collected in Table 3.15.

Table. 3.15. Distribution of the main compounds obtained in the pyrolysis of the alkaline lignins isolated from the different feedstock obtained in the different biorefinery stages (expressed as g/100 g lignin).

Compound	Rt (min)	m/z	Origin	% VSL	% AHL	% SSLF
Toluene	4.03	91, 92, 65	-	ND	0.69	0.63
Furfural	4.97	96, 95, 67	Carbohydrate	8.13	0.65	0.34
Furfuryl alcohol	5.21	98, 97, 81	Carbohydrate	0.60	ND	ND
Phenol	7.45	94, 66, 65	H	3.30	4.57	4.7
***o*-cresol**	8.65	108, 107, 77	H	0.69	1.04	1.02
***p*-cresol**	8.98	107, 108,77	H	3.25	3.68	3.98
Guaiacol	9.28	109, 124, 81	G	3.01	4.03	4.47
2,4-dimethyl phenol	10.29	107, 122, 121	H	ND	ND	0.85
4-ethyl phenol	10.47	107, 122, 77	H	0.67	ND	ND
2-ethyl phenol	10.67	107, 122,77	H	ND	0.74	ND
4-methyl guaiacol	11.33	138, 123, 95	G	ND	7.85	5.48
Catechol	11.43	110, 64, 53	C	1.93	ND	3.47
3-methyl catechol	13.07	124, 78,123	C	ND	1.17	ND
3-methoxy catechol	13.25	140, 125, 97	C	2.32	4.23	6.93
4-ethyl guaiacol	13.77	137, 152, 122	G	3.34	2.27	2.45
4-methyl catechol	14.16	124, 123, 78	C	ND	2.27	2.26
3,4-dimethoxy phenol	15.99	154,139,111,151	S	0.86	ND	2.8
Syringol	16.01	154, 139, 111,96	S	2.39	6.63	7.72
Vanillin	17.15	151, 152, 81	G	ND	0.82	0.97
Acetovanillone	18.90	151, 166, 123	G	ND	1.47	1.26
4-vynil syringone	20.20	180, 165, 137	S	2.36	5.36	6.09
4-allyl syringol	20.75	194, 91, 119	S	ND	0.69	0.82
Syringaldehyde	21.58	182, 181, 111	S	0.7	1.21	0.9
Acetosyringone	22.53	181, 196, 153	S	0.68	1.52	1.24
Total syringyl derivates			S	7.0	15.4	19.6
Total guaiacyl derivates			G	6.4	16.4	14.6
Total p-coumaryl derivates			H	7.9	10.0	10.6
Total catechol derivates			C	4.3	7.7	12.7

ND: not detected.

The identified pyrolysis products came mainly from the degradation of the lignin and carbohydrates present in the isolated lignin. The one obtained from the untreated vine shoots presented the lowest lignin-derived products content (25.5 %) and the highest carbohydrate-derived products content (8.73 %). Conversely, the lignins isolated from the autohydrolysed solid and the spent solid obtained in the SSF process presented higher lignin-derived products content (49.6 and 57.4 %, respectively) and lower carbohydrate-derived products content (0.7 and 0.3 % respectively) than the VSL.

Among the lignin-derived products, although their relative abundance varied, almost the same products were determined in the lignins isolated from the pre-treated vine shoots, with the exception of some compounds such as 2,4-dimethyl phenol, 4-ethyl phenol, 2-ethyl phenol, 3-methyl catechol, catechol and 3,4-dimethoxy phenol. The most abundant lignin-derived products also varied between the lignins isolated from the untreated and from the pre-treated vine shoots. The most abundant products obtained from the untreated vine shoots were 4-ethyl guaiacol, phenol and *p*-cresol, while the most abundant ones determined in the lignins isolated from the pre-treated vine shoots were syringol, 4-vynil syringone, 4-methyl guaiacol and 3-methoxy catechol.

When comparing the type of lignin-derived products obtained from the three lignins, it was noticed that the content of the compounds of p-coumaryl, catechol and syringol type increased with the successive treatments to which the vine shoots was subjected. However, this trend was not followed by the guaiacol type compounds content, as their content is slightly higher for the AHL than for the SSFL.

Although catechol and p-coumaryl type compounds were determined by the Py-GC/MS analysis, they were not observed during the FTIR analysis, so their presence could be associated to their formation during the pyrolysis [3.87, 3.89]. Regarding the carbohydrate-derived products obtained from the three lignins, the lignin isolated from the untreated vine shoots presented the highest content, being the furfural the most abundant compound.

This is in accordance with what it was observed during the purity analysis of the different lignins. Thus, taking into account their chemical constitution, it seemed that it is better to carry out the

delignification treatment of solids from which the hemicelluloses have been removed, as they present lower carbohydrate impurities.

3.4.2.4.4 HPSEC analyses of the lignins isolated from different feedstocks

The lignins isolated from the three different solids were analysed by HPSEC in order to determine their molecular weight distribution, being their average molecular weight (Mw), number average (Mn) and polydispersity index (Mw/Mn) collected in Table 3.16.

As it has been seen up to now, the treatments to which the vine shoots used for the lignin extraction had been subjected influenced strongly the lignin features and their molecular weight distribution is not an exception as it can be seen in Figure 3.14. Taking into account the data shown in Table 3.16 and Figure 3.14, it can be appreciated that the lignin isolated from the untreated vine shoots presented a small fraction (14.3 %) with a molecular weight extremely higher (154,927 Da) than the Mw of the fractions of the lignins isolated from the other two solids.

Such a high molecular weight could correspond to polysaccharides since during the alkaline delignification they could remain covalently bound to the lignin [3.88]. The absence of this fraction in the other lignins indicated that these compounds were solubilised or removed during the autohydrolysis treatment.

Without taking into consideration this high molecular weight fraction present in the lignin isolated from untreated vine shoots, the lignin isolated from the spent solid obtained in theSSF process presented the highest molecular weight, as around 60.7 % of it presented an average molecular weight of 55987 Da. After the SSFL, the lignin isolated from the autohydrolysed solid presented the highest molecular weight. The higher molecular weight of the SSFL could be associated with the easier cleavage of the inter-bonds and ether linkages of the lignin as a consequence of the autohydrolysis and enzymatic hydrolysis treatments which also favoured repolymerisation reactions [3.110, 3.111].

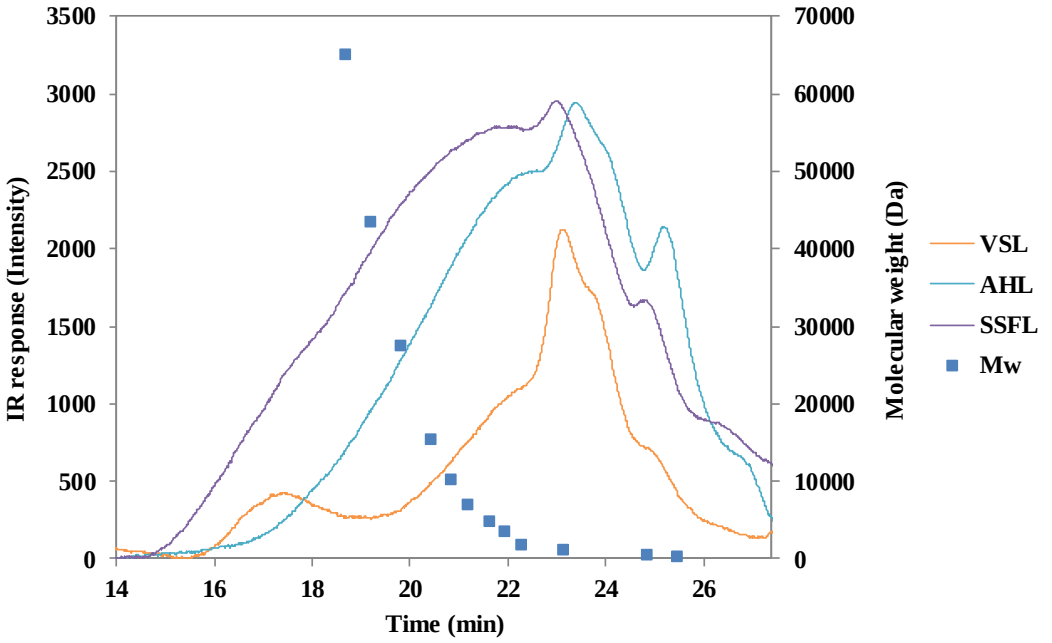


Figure 3.14. Molecular weight distribution of the alkaline lignins isolated from the different feedstock obtained in the different biorefinery stages.

The polydispersity index followed the same trend as the molecular weight distribution, confirming the condensation reactions that could have taken place during isolation of the lignin obtained after the bio-ethanol production from the autohydrolysed vine shoots, as according to Tolbert et al. [3.112] these aspects are related.

Nevertheless, in spite of the very high molecular weight and polydispersity index of the lignins isolated from the spent solid generated in the SSF process, Qiao et al. [3.113] reported that these kind of lignins present better chemical reactivity than either lignosulfonate or Kraft lignins. The lignins isolated from the solid residue generated during the bioethanol production have been employed for the production of polyurethanes, epoxy resins, copolymers for the heavy metals ions biosorption [3.113].

Table. 3.16. Molecular weight average (Mw), number average (Mn) and polydispersity index (Mw/Mn) of the alkali lignins isolated from the solids obtained in the different biorefinery stages.

	VSL						AHL						SSFL					
%	14.3	29.6	29.4	19.9	6.9	Whole	49.3	19.8	13.7	15.1	2.1	Whole	60.7	25.2	7.5	5.4	1.2	Whole
Mw (Da)	154927	9009	1420	692	364	25327	29807	1759	818	355	192	15212	55987	1195	362	236	280	34348
Mn (Da)	120509	5108	1339	667	356	1298	8360	1649	788	362	191	1136	10035	981	353	35235	277	1248
Mw/Mn	1.28	1.76	1.06	1.038	1.02	19.51	3.56	1.07	1.04	1.06	1.01	13.39	5.57	1.22	1.02	1.00	1.009	27.52

3.5 Conclusions

Two different routes were assessed to revalorise the lignin and cellulose that remained in the vine shoots after their autohydrolysis treatment. The first route consisted on solubilising firstly the cellulosic fraction of the autohydrolysed vine shoots by a SSF process for the production of bioethanol and then the lignin fraction of the obtained solid by a delignification treatment. In the second route, the order of these procedures was inverted, firstly a delignification treatment was carried out followed by an enzymatic hydrolysis.

In order to determine the most appropriate autohydrolysed vine shoots to be used for the production of bioethanol, the autohydrolysed solids obtained at different temperatures were subjected to enzymatic hydrolysis. It was observed that the enzymatic digestibility of the solid increased with the severity of the treatment due to the removal of the hemicellulosic fraction. The highest glucan to glucose conversion (75.0 %) was obtained with the autohydrolysed solid obtained at 215 ºC (S_0 = 4.65). Due to the high digestibility of the solids obtained under the severest conditions of the autohydrolysis treatment, their employment for the obtaining of bioethanol was assessed. The production of bioethanol was performed using the solid from the autohydrolysis treatment carried out at 210 ºC (4.47), being possible to achieve a glucan to bioethanol conversion of 67.4%.

To determine the best procedure to remove the lignin fraction from the autohydrolysed solid, according to what was proposed in the second route, different delignification treatments were carried out. Among the assessed treatments, it was observed that higher delignification yields were achieved with alkaline conditions (67.7 %) compared with organosolv or acetosolv treatments, in which only 12.6 and 25.0 % of the lignin present in the autohydrolysed solid was removed. It was observed that the delignification yield attained using alkaline conditions could be increased by employing intensification techniques such as the ultrasounds or the microwaves, obtaining better results with the last intensification technique. The employment of microwaves permitted the reduction of 72.0 % of the reaction time and 50.0 % of the amount of reagent used during the alkaline delignification carried out with conventional heating, obtaining at the same

time a 81.8 % delignification yield at the optimum conditions. Although the lignins obtained with the different delignification procedures presented purities between 80.0 and 90.0 %, their molecular weight distribution and their phenolic composition was quite different. Regarding the molecular weight of the lignins, the ones obtained by alkaline delignifications presented higher molecular weight and polydispersity index being theses parameters increased when ultrasounds or microwaves were applied. It was also observed that the ratio between the syringyl- and guaiacyl-derived compounds present in the lignin varied strongly with the delignification procedure.

By carrying out the delignification of the autohydrolysed solid obtained at 200 ºC prior to an enzymatic hydrolysis, a glucan to glucose conversion close to 100.0 % was achieved, which was higher than the conversion achieved when the autohydrolysed vine shoots without being delignified was subjected to an enzymatic hydrolysis. Therefore, for an efficient enzymatic hydrolysis it has been seen the necessity of removing the hemicelluloses and lignin from the vine shoots. Furthermore, it was observed the suitability of removing firstly the hemicellulosic fraction, as the lignins obtained from autohydrolysed vine shoots presented a higher purity than the one isolated from untreated vine shoots.

Thus, from the results presented in this chapter it could be concluded that the most suitable approach to accomplish an integral revalorisation of the vine shoots consisted on a multi-cascade processing that would incorporate an autohydrolysis treatment, as the first stage of the biorefinery, followed by an alkaline delignification and a subsequent enzymatic hydrolysis process. Alternatively, the last stage also could comprise a SSF to obtaining bio-ethanol.

3.6 References

[3.1] Gullón, P., Pereiro, G., Alonso, J. L. & Parajó, J. C. (2009). Aqueous pretreatment of agricultural wastes: Characterization of soluble reaction products. *Bioresource Technology, 100,* 5840-5845.

[3.2] Sixta, H. (2006). Chemical pulping. In H. Sixta (Ed.), *Handbook of pulp. Vol. 1* (pp. 3-19). Weinheim, Germany: WILEY-VCH.

[3.3] Yan, L., Zhang, L. & Yang, B. (2014). Enhancement of total sugar and lignin yields through dissolution of poplar wood by hot water and dilute acid flowthrough pretreatment. *Biotechnology for fuels, 7*(1), 76.

[3.4] Zhang, J., Tang, M. & Viikari, L. (2012). Xylans inhibit enzymatic hydrolysis of lignocellulosic materials by cellulases. *Bioresource Technology, 121*, 8-12.

[3.5] Sundarraj, A. A. & Ranganathan, T. V. (2018). A review on cellulose and its utilization from agro-industrial waste. *Drug Invention Today, 10*(1), 89-94.

[3.6] Fortunati, E., Luzi, F., Puglia, D. & Torre, L. (2016). Extraction of lignocellulosic materials from waste products. In D. Puglia, E. Fortunati & J. M. Kenny (Eds.), *Multifunctional Polymeric Nanocomposites based on cellulosic reinforcements* (pp. 1-38). Amsterdam, Netherlands: Elsevier.

[3.7] Moon, R. J., Martini, A., Nairn, J., Simonsen, J. & Youngblood, J. (2011). Cellulose nanomaterials review: structure, properties and nanocomposites. *Chemical Society Reviews, 40*(7), 3941-3994.

[3.8] Fleming, K., Gray, D. G. & Matthews, S. (2001). Cellulose Crystallites. *Chemistry-A European Journal, 7*(9), 1831-1836.

[3.9] Khalil, H. P. S. A., Davoudpour, Y., Islam, M. N., Mustapha, A., Sudesh, K., Dungani, R. & Jawaid, M. (2014). Production and modification of nanofibrillated cellulose using various mechanical processes: A review. *Carbohydrate Polymers, 99*, 649-665.

[3.10] Missoum, K., Belgacem, M. N. & Bras, J. (2013). Nanofibrillated Cellulose Surface Modification: A Review. *Materials, 6*(5), 1745-1766.

[3.11] Brinchi, L., Cotana, F., Fortunati, E. & Kenny, J. M. (2013). Production of nanocrystalline cellulose from lignocellulosic biomass: Technology and applications. *Carbohydrate Polymers, 94*(1), 154-169.

[3.12] Agbor, V. B., Cicek, N., Sparling, R., Berlin, A. & Levin, D. B. (2011). Biomass pretreatment: Fundamentals toward application. *Biotechnology Advances, 29*(6), 675-685.

[3.13] Bauer, S., Sorek, H., Mitchell, V. D., Ibáñez, A. B. & Wemmer, D. E. (2012). Characterization of *Miscanthus giganteus* Lignin Isolated by Ethanol Organosolv Process under Reflux Condition. *Journal of Agricultural and Food Chemistry, 60*(33), 8203-8212.

[3.14] Naidu, D. S., Hlangothi, S. P. & John, M. J. (2018). Bio-based products from xylan: A review. *Carbohydrate Polymers, 179,* 28-41.

[3.15] Sun, R. C., Fang, J. M. & Tomkinson, J. (2000). Delignification of rye straw using hydrogen peroxide. *Industrial Crops and Products, 12*(2), 71–83.

[3.16] Adler E. (1977). Lignin chemistry –past, present and future. *Wood Science and Technology, 11*(3), 169-218.

[3.17] Stewart, J. J., Akiyama, T., Chapple, C., Ralph, J. & Mansfield, S. D. (2009). The effects on lignin structure of overexpression of ferulate 5-hydroxylase in hybrid poplar. *Plant Physiology, 150*(2), 621-635.

[3.18] Sequeiros, A. & Labidi, J. (2017). Characterization and determination of the S/G ratio via Py-GC/MS of agricultural and industrial residues. *Industrial Crops and Products, 97,* 469-476.

[3.19] Grabber, J. H. (2005). How Do Lignin Composition, Structure, and Cross-Linking Affect Degradability? A Review of Cell Wall Model Studies. *Crop Science, 45*(3), 820-831.

[3.20] American Chemistry Society. Molecule of the week archive: Lignin. Retrieved from https://www.acs.org/content/acs/en/molecule-of-the-week/archive/l/lignin.html. (Visited October 2018).

[3.21] Mosier, N., Wyman, C., Dale, B., Elander, R., Lee Y., Holtzapple, M. & Ladisch M. (2005). Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource Technology, 96*(6), 673–686.

[3.22] Hassan, S. S., Williams, G. A. & Jaiswal, A. K. (2018). Emerging technologies for the pretretament of lignocellulosic biomass. *Bioresource Technology, 262,* 310-318.

[3.23] Janositz, A., Semrau, J. & Knorr, D. (2011). Impact of Pulsed Electric Fields (PEF) on post-permeabilization processes in plant cells. *Innovative Food Science and Emerging Technologies, 12,* 269–274.

[3.24] Kumar, A. K. & Sharma, S. (2017). Recent updates on different methods of pretreatment of lignocellulosic feedstocks: a review. *Bioresources and Bioprocessing, 4*(1), 7.

[3.25] Sarkar, N., Ghosh, S. K., Bannerjee, S. & Aikat K. (2012). Bioethanol production from agricultural wastes: an overview. *Renewable Energy, 37*(1), 19–27.

[3.26] Lee, H. V., Hamid, S. B. A. & Zain, S. K. (2014). Conversion of lignocellulosic biomass to nanocellulose: structure and chemical process. *The Scientific World Journal, 2014*, 631013.

[3.27] Zhao, X. & Liu, D. (2012). Kinetic Modeling and Mechanisms of Acid-Catalyzed Delignification of Sugarcane Bagasse by Aqueous Acetic Acid. *BioEnergy Research, 6*(2), 436-447.

[3.28] Sun, R.C. & Tomkinson, J. (2002). Comparative study of lignins isolated by alkali and ultrasound-assisted alkali extractions from wheat straw. *Ultrasonics Sonochemistry*, *9*(2), 85–93.

[3.29] Singh, R. D., Bhuyan, K., Banerjee, J., Muir, J. & Arora, A. (2017). Hydrothermal and microwave assisted alkali pretreatment for fractionation of arecanut husk. *Industrial Crops and Product*, 102, 65-74.

[3.30] Avelino, F., Da Silva, K. T., De Souza Filho, M. D. S. M., Mazzetto, S. E. & Lomonaco, D. (2018). Microwave-assisted organosolv extraction of coconut shell lignin by Brønsted and Lewis acids catalysts. *Journal of Cleaner Production, 187*, 785-796.

[3.31] Kumar, R., Sharma, R. K. & Singh, A. P. (2017). Cellulose based grafted biosorbents - Journey from lignocellulose biomass to toxic metal ions sorption applications - A review. *Journal of Molecular Liquids, 232*, 62-93.

[3.32] García-Cubero, M. A. T., González-Benito, G., Indacoechea, I., Coca, M. & Bolado, S. (2009). Effect of ozonolysis pretreatment on enzymatic digestibility of wheat and rye straw. *Bioresource Technology, 100*, 1608-1613.

[3.33] Zhao, X., Cheng, K. & Liu, D. (2009). Organosolv pretreatment of lignocellulosic biomass for enzymatic hydrolysis. *Applied Microbiology and Biotechnology*, *82*(5), 815–827.

[3.34] Sun, N., Rodríguez, H., Rahman, M. & Rogers, R. D. (2011). Where are ionic liquid strategies most suited in the pursuit of chemicals and energy from lignocellulosic biomass? *Chemical Communications, 47*, 1405-1421.

[3.35] Zhang, C. W., Xia, S. Q., Ma, P. S. (2016). Facile pretreatment of lignocellulosic biomass using deep eutectic solvents. *Bioresource Technology, 219*, 1–5.

[3.36] Pielhop, T., Amgarten, J., Von Rohr, R. & Studer, M. H. (2016). Steam explosion pretreatment of softwood: the effect of the explosive decompression on enzymatic digestibility. *Biotechnology for Biofuels*, *9*(1), 152.

[3.37] Da Costa Sousa, L., Chundawat, S. P., Balan, V. & Dale, B. E. (2009). Cradle-to-grave assessment of existing lignocellulose pretreatment technologies. *Current Opinion in Biotechnology*, *20*(3), 339–347.

[3.38] Tuomela, M., Vikman, M., Hatakka, A. & Itävaara, M. (2000). Biodegradation of lignin in a compost environment: a review. *Bioresource Technology*, *72*, 169–183.

[3.39] Kumar, D. & Murthy, G. S. (2016). Enzymatic Hydrolysis of Cellulose for Ethanol Production: Fundamentals, Optimal Enzyme Ratio, and Hydrolysis Modeling. In V. K. Gupta (Ed.), *New and future developments in Microbial Biotechnology and Bioengineering*. *Microbial cellulose system properties and applications* (pp. 65-78). Amsterdam, Netherlands: Elsevier.

[3.40] Rosales-Calderon, O., Trajano, H. L. & Duff, S. J. (2014). Stability of commercial glucanase and β-glucosidase preparations under hydrolysis conditions. *Peer J, 2014*(1), e402.

[3.41] Espirito Santo, M., Rezende, C. A., Bernardinelli, O. D., Pereira, N., Curvelo, A. A. S., De Azevedo, E. R., … Polikarpov, I. (2018). Structural and compositional changes in sugarcane bagasse subjected to hydrothermal and organosolv pretreatments and their impacts on enzymatic hydrolysis. *Industrial Crops and Products, 113*, 64-74.

[3.42] Wu, X., Huang, C., Zhai, S., Liang, C., Huang, C., Lai, C. & Yong, Q. (2018). Improving enzymatic hydrolysis of wheat straw through sequential autohydrolysis and alkaline post-extraction. *Bioresource Technology*, *251*, 374-380.

[3.43] Zhou, Z., Lei, F., li, P. & Jiang, J. (2018). Lignocellulosic biomass to biofuels and biochemical: A comprehensive review with a focus on ethanol organosolv pretreatment technology. *Biotechnology and Bioengineering*, *115*(11), 2683-2702.

[3.44] Vargas, F., Domínguez, E., Vila, C., Rodríguez, A. & Garrote, G. (2015). Agricultural residue valorization using a hydrothermal process for second generation bioethanol and oligosaccharides production. *Bioresource Technology*, *191*, 263-270.

[3.45] Michelin, M. & Teixeira, J. A. (2016). Liquid hot water pretreatment of multi feedstocks and enzymatic hydrolysis of solids obtained thereof. *Bioresource Technology*, *216*, 862-869.

[3.46] Sahoo, M., Seydibeyoglu, M. O., Mohanty, A. K. & Misra, M. (2011). Characterization of industrial lignins for their utilization in future value added applications. *Biomass and Bioenergy*, *35*, 4230-4237.

[3.47] Li, X. H. & Wu, S. B. (2014). Chemical structure and pyrolysis characteristics of the soda-alkali lignin fractions. *BioResources, 9*(4), 6277-6289.

[3.48] Lora, J. H. & Glasser, W. G. (2002). Recent Industrial Applications of Lignin: A Sustainable Alternative to Nonrenewable Materials. *Journal of Polymers and the Environment, 10*(1/2), 39-48.

[3.49] Coral Medina, J. D., Woiciechowski, A., Zandona Filho, A., Noseda, M. D., Kaúr, B. S. & Soccol, C. R. (2015). Lignin preparation from oil palm fruit bunches by sequential acid/alkaline treatment -a biorefinery approach. *Bioresource Technology, 194*, 172-178.

[3.50] Hatakeyama, H. & Hatakeyama, T. (2010). Lignin structure, properties and applications. *Advances in Polymer Science, 232*(1), 1-63.

[3.51] Giri, J. & Adhikari, R. (2013). A brief review on extraction of nanocellulose and its application. *BIBECHANA, 9*, 81-87.

[3.52] Halib, N., Perrone, F., Cemazar, M., Dapas, B., Farra, R., Abrami, M., … Grazzi, M. (2017). Potential applications of nanocellulose-containing materials in the biomedical field. *Materials, 10*(8), 977.

[3.53] Tayeb, A. H., Amini, E., Ghasemi, S. & Tajvidi, M. (2018). Cellulose nanomaterials-Binding properties and applications: A review. *Molecules, 23*(10), 2684.

[3.54] Sánchez, C., Serrano, L., Llano-Ponte, R. & Labidi, J. (2014). Bread residues conversión into lactic acid by alkaline hydrothermal treatments. *Chemical Engineering Journal, 250*(15), 326-330.

[3.55] Su, Y., Brown, H. M., Huang, X., Zhou, X. D., Amonette, J. E. & Zhang, Z. C. (2009). Single-step conversion of cellulose to 5-hydroxymethylfurfural (HMF), a versatile platform chemical. *Applied Catalysis A: General, 361*(1-2), 117-122.

[3.56] Regestein, L., Klement, T., Grandes, P., Kreyenschulte, D., Heyman, B., Maßmann, T., … Büchs, J. (2018). From beech wood to itaconic acid: case study on biorefinery process integration. *Biotechnology for fuels, 11*(279), 1-11.

[3.61] TAPPI Standards 2007, Test methods of the Technical Association of the Pulp and Paper Industry, Atlanta.

[3.62] Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D. & Crocker, D. (2008). *Determination of structural carbohydrates and lignin in biomass*. Retrieved from https://www.nrel.gov/docs/gen/fy13/42618.pdf (Visited September, 2018)

[3.57] Toledano, A., Erdocia, X., Serrano, L. & Labidi J. (2013). Influence of extraction treatment on olive tree (Olea europea) pruning lignin structure. *Environmental Progress and Sustainable Energy*, *32*(4), 1187-1194.

[3.58] Erdocia, X., Prado, R., Corcuera, M. T. & Labidi, J. (2014). Effect of different organosolv treatments on the structure and properties of olive tree pruning lignin. *Journal of Industrial and Engineering Chemistry*, *20*(3), 1103-1108.

[3.59] Bustos, G., Moldes, A.B., Cruz, J.M. & Domínguez, J.M. (2005). Production of lactic acid from vine-trimming wastes and viticulture lees using a simultaneous saccharification fermentation method. *Journal of the Science of Food and Agriculture,* 85(3), 466–472.

[3.60] Liu, J., Pan, Y., Yao, C., Wang, H., Cao, X. & Xue, S. (2015). Determination of ash content and concomitant acquisition of cell compositions in microalgae via thermogravimetric (TG) analysis. *Algal Research*, *12*, 149–155.

[3.63] Requejo, A., Peleteiro, S., Rodríguez, A., Garrote, G. & Parajó, J. C. (2012). Valorization of residual woody biomass (Olea europea trimmings) based on aqueous fractionation. *Journal of Chemical Technology and Biotechnology*, *87*(1), 87-94.

[3.64] Holtzapple, M. T., Caram, H. S. & Humphrey, A. E. (1984). A comparison of two empirical models for the enzymic hydrolysis of pretreated poplar wood. *Biotechnology and Bioengineering*, *26*(8), 936-941.

[3.65] Hu, F., Jung, S. & Ragauskas, A. (2013). Impact of pseudolignin versus dilute acid-pretreated lignin on enzymatic hydrolysis of cellulose. *ACS Sustainable Chemistry and Engineering*, *1*(1), 62–65.

[3.66] Rocha, G. J. M., Silva, V. F. N., Martín, C., Gonçalves, A. R., Nascimento, V. M. & Souto-Maior, A. M. (2013). Effect of xylan and lignin removal by hydrothermal pretreatment on enzymatic conversion of sugarcane bagasse cellulose for second generation ethanol production. *Sugar Tech*, *15*(4), 390–398.

[3.67] Domínguez, E., Romaní, A., Domingues, L. & Garrote, G. (2017). Evaluation of strategies for second generation bioethanol production from fast growing biomass Paulownia within a biorefinery scheme. *Applied energy*, *187*, 777-789.

[3.68] Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y. Y., Holtzapple, M. & Ladisch, M. (2005). Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource Technology, 96*, 673-686.

[3.69] Xu, Y., Li, J., Zhang, M. & Wang, D. (2018). Modified simultaneous saccharification and fermentation to enhance bioethanol titers and yields. *Fuel, 215*, 647-654.

[3.70] Jiménez, L., Angulo, V., Ramos, E., De la Torre, M. J. & Ferrer, J. L. (2006). Comparison of various pulping processes for producing pulp from vine shoots. *Industrial Crops and Products, 23*(2), 122-130.

[3.71] Karlström, K., Sjögren, B., Vorwerg, W. & Volkert, B. (2014). Sulphur-free cooking for value added cellulose. *Cellulose Chemistry and Technology, 48*(9-10), 781-786.

[3.72] Taherzadeh, M. J. & Karimi, K. (2007). Enzyme-based hydrolysis processes for ethanol from lignocellulosic materials: a review. *BioResources, 2*(4), 707-738.

[3.73] Singh, S., Bharadwaja, S. T. P., Yadav, P. K., Moholkar, V. S. & Goval, A. (2014). Mechanistic investigation in ultrasound-assisted (alkaline) delignification of parthenium hysterophorus biomass. *Industrial & Engineering Chemistry Research, 53*(37), 14241-14252.

[3.74] Xu, H., Li, B. & Mu, X. (2016). Review of alkali-based pretreatment to enhance enzymatic saccharification for lignocellulosic biomass conversion. *Industrial and Engineering Chemistry Research, 55*, 8691-8705.

[3.75] Eblaghi, M., Niakousari, M., Sarshar, M. & Mesbahi, G. R. (2016). Combining ultrasound with mild alkaline solutions as an effective pretreatment to boost the release of sugar trapped in sugarcane bagasse for bioethanol production. *Journal of Food Process Engineering, 39*(3), 273-282.

[3.76] Aguilar-Reynosa, A., Romani, A., Rodriguez-Jasso, R. M., Aguilar, C. N., Garrote, G., Ruiz, H. A. (2017). Microwave heating processing as alternative of pretreatment in second generation biorefinery: an overview. *Energy Conversion and Management, 135*, 50–65.

[3.77] Monte, L. S., Escócio, V. A., De Sousa A. M. F., Furtado, C. R. G., Leite M. C. A. M., Visconte L. L. Y. & Pacheco E. B. A. V. (2018). Study of time reaction on alkaline pretreatment applied to rice husk on biomass component extraction. *Biomass Conversion and Biorefinery, 8*(1), 189-197.

[3.78] McIntosh, S. & Vancov, T. (2011). Optimisation of dilute alkaline pretreatment for enzymatic saccharification of wheat straw. *Biomass and Bioenergy, 35*(7), 3094-3103.

[3.79] Tejado, A., Peña, C., Labidi, J., Echeverria, J. M. & Mondragon, I. (2007). Physico-chemical characterization of lignins from different sources for use in phenol-formaldehyde resin synthesis. *Bioresource Technology, 98*, 1655-1663.

[3.80] Yao, K., Wu, Q., An, R., Meng, W., Ding, M., Li, B. & Yuan, Y. (2018). Hydrothermal pretreatment for deconstruction of plant cell wall: Part I. Effect on lignin-carbohydrate complex. *AIChE Journal*, *64*(6), 1938-1953.

[3.81] Zamudio, M. A. M., Alfaro, A., De Alva, H. E., García, J. C., García-Morales, M. & López, F. (2015). Biorefinery of paulownia by autohydrolysis and soda-anthraquinone delignification process. Characterization and application of lignin. *Journal of Chemical Technology and Biotechnology, 90*(3), 534-542.

[3.82] Carvalho, F., Srinivas, K., Helms, G. L., Isern, N. G., Cort, J. R., Gonçalves, A. R. & Kiær B. (2018). Characterization of coffee (Coffea Arabica) husk lignin and degradation products obtained after oxygen and alkali addition. *Bioresource Technology, 257*, 172-180.

[3.83] Del Río, J. C., Gutiérrez, A., Hernando, M., Landín, P., Romero, J. & Martínez, A. T. (2005). Determining the influence of eucalypt lignin composition in paper pulp yield using Py-GC/MS. *Journal of Analytical and Applied Pyrolysis, 74*(1-2), 110–115.

[3.84] Nunes, C. A., Lima, C. F., Barbosa, L. C. A., Colodette, J. L., Gouveia, A. F. G. & Silvério, F. O. (2010). Determination of eucaliptus spp lignin S/G ratio: A comparison between methods. *Bioresource Technology, 101*, 4056–4061.

[3.85] Fernández-Rodríguez, J., Gordobil, O., Robles, E., González-Alriols, M. & Labidi, J. (2017). Lignin valorization from side-streams produced during agricultural waste pulping and total chlorine free bleaching. *Journal of Cleaner Production, 142*(Part 2), 2609–2617.

[3.86] Wang, S., Ru, B., Lin, H., Sun, W. & Luo, Z. (2015). Pyrolysis behaviors of four lignin polymers isolated from the same pine wood. *Bioresource Technology, 182*, 120–127.

[3.87] Ma, Z., Sun, Q., Ye, J., Yao, Q. & Zhao, C. (2016). Study on the thermal degradation behaviors and kinetics of alkali lignin for production of phenolic-rich bio-oil using TGA-FTIR and Py-GC/MS. *Journal of Analytical and Applied Pyrolysis, 117*, 116–124.

[3.88] Constant, S., Wienk, H. L. J., Frissen, A. E., De Peinder, P., Boelens, R., Van Es, D. S., … Bruijnincx, P. C. A. (2016). New insights into the structure and composition of technical lignins: a comparative characterization study. *Green Chemistry, 18*, 2651–2665.

[3.89] Shen, D., Liu, G., Zhao, J., Xue, J., Guan, S. & Xiao, R. (2015). Thermo-chemical conversion of lignin to aromatic compounds: Effect of lignin source and reaction temperature. *Journal of Analytical and Applied Pyrolysis, 112*, 56-65.

[3.90] García, A., Toledano, A., Andrés, M. A. & Labidi, J. (2010). Study of the antioxidant capacity of *Miscanthus sinensis* lignins. *Process Biochemistry, 45*(6), 935-940.

[3.91] Santos, J. I., Fillat, U., Martín-Sampedro, R., Eugenio, M. E., Negro, M. J., Ballesteros, I., … Ibarra, D. (2017). Evaluation of lignins from side-streams generated in an olive tree pruning-based biorefinery: Bioethanol production and alkaline pulping. *International Journal of Biological Macromolecules, 105*(Pt 1), 238-251.

[3.92] Villaverde, J. J., Li, J., Ek, M., Ligero, P. & De Vega, A. (2009). Native lignin structure of miscanthus x giganteus and its changes during acetic and formica acid fractionation. *Journal of Agricultural and Food Chemistry, 57*(14), 6262-6270.

[3.93] Andrianova, A. A., Yeudakimenka, N. A., Lilak, S. L., Kozliak, E. I., Ugrinov, A., Sibi, M. P., Kubátová. (2018). Size exclusion chromatography of lignin: The mechanistic aspects and elimination of undesired secondary interactions. *Journal of Chromatography A, 1534*, 101-110.

[3.94] Wang, H., Pu, Y., Ragauskas, A. & Yang, B. (2019). From lignin to valuable products–strategies, challenges, and prospects. *Biresource Technology, 271*, 449-461.

[3.95] Nitsos, C., Stoklosa, R., Karnaouri, A., Vörös, D., Lange, H., Hodge, D., … Christakopoulos, P. (2016). Isolation and Characterization of Organosolv and Alkaline Lignins from Hardwood and Softwood Biomass. *ACS Sustainable Chemistry and Engineering, 4*, 5181-5193.

[3.96] Toledano, A., Erdocia, X., Serrano, L. & Labidi, J. (2012). Influence of the extraction treatment on olive tree (Olea europaea) pruning lignin structure. *Environmental Progress and Sustainable Energy, 32*(4), 1187-1194.

[3.97] Li, H. Y., Sun, S. N., Wang, C. Z. & Sun, R. C. (2015). Structural and dynamic changes of lignin in eucalyptus cell walls during successive alkaline ethanol treatments. *Industrial Crops and Products, 74*, 200–208.

[3.98] She, D., Xu, F., Geng, Z. C., Sun, R. C., Jones, G. L. & Baird, M. S. (2010). Physicochemical characterization of extracted lignin from sweet sorghum stem. *Industrial Crops and Products, 32*(1), 21-28.

[3.99] Hussin, M. H., Rahim, A. A., Ibrahim, M. N. M. & Brosse, N. (2013). Physicochemical characterization of alkaline and ethanol organosolv lignins from oil palm (Elaeis guineensis) fronds as phenol substitutes for green material applications. *Industrial Crops and Products, 49*, 23-32.

[3.100] Sun, S. N., Li, M. F., Yuan, T. Q., Xu, F. & Sun, R. C. (2012). Sequential extractions and structural characterization of lignin with ethanol and alkali from bamboo (neosinocalamus affinis). *Industrial Crops and Products, 37*, 51–60.

[3.101] Holtman, K. M., Chang, H. M., Jameel, H. & Kadla, J. F. (2014). Quantitative ^{13}C-NMR characterization of milled wood lignins isolated by different milling techniques. *Journal of Wood Chemistry and Technology, 26*(1), 21-34.

[3.102] Sun, X. F., Jing, Z., Fowler, P., Wu, Y. & Rajaratnam, M. (2011). Structural characterization and isolation of lignin and hemicelluloses from barley straw. *Industrial Crops and Products, 33*(3), 588-598.

[3.103] Kang, K. E., Jeong, G. T. & Park, D. H. (2012). Pretreatment of rapeseed straw by sodium hydroxide. *Bioprocess and Biosystems Engineering, 35*(5), 705–713.

[3.104] Ebringerová, A. & Heinze, T. (2000). Xylan and xylan derivatives - biopolymers with valuable properties, 1. Naturally occurring xylans structures, isolation procedures and properties. *Macromolecular Rapid Communications, 21*(9), 542-556.

[3.105] Huijgen, W. J. J., Telysheva, G., Arshanitsa, A., Gosselink, R. J. A. & De Wild, P. J. (2014). Characteristics of wheat straw lignins from ethanol-based organosolv treatment. *Industrial Crops and Products, 59*, 85-95.

[3.106] Wang, G. & Chen, H. (2014). Carbohydrate elimination of alkaline-extracted lignin liquor by steam explosion and its methylolation for substitution of phenolic adhesive. *Industrial Crops and Products, 53*, 93-101.

[3.107] Lee, S. J., Kim, H. J., Cho, E. J., Song, Y. & Bae, H. J. (2015). Isolation and characterization of lignin from the oak wood bioethanol production residue for adhesives. *International Journal of Biological Macromolecules, 72*, 1056-1062.

[3.108] Sun, S., Li, M., Yuan, T., Xu, F. & Sun, R. (2012). Effect of ionic liquid pretreatment on the structure of hemicelluloses from corncob. *Journal of Agricultural and Food Chemistry, 60*(44), 11120-11127.

[3.109] Pandey, K. K. & Theagarajan, K. S. (1997). Analysis of wood surfaces and ground wood by diffuse reflectance (DRIFT) and photoacoustic (PAS) fourier transform infrared spectroscopic techniques. *Holz als Roh- und Werkstoff, 55*(6), 383–390.

[3.110] Obama, P., Ricochon, G., Muniglia, L. & Brosse, N. (2012). Combination of enzymatic hydrolysis and ethanol organosolv pretreatments: Effect on lignin structures, delignification yields and cellulose-to-glucose conversion. *Bioresource Technology, 112*, 156-163.

[3.111] Kaparaju, P. & Felby, C. (2010). Characterization of lignin during oxidative and hydrothermal pretreatment processes of wheat straw and corn stover. *Bioresource Technology, 101*, 3175-3181.

[3.112] Tolbert, A., Akinosho, H., Khunsupat, R., Naskar, A. K. & Ragauskas, A. J. (2014). Characterization and analysis of the molecular weight of lignin for biorefining studies. *Biofuels, Bioproducts and Biorefinery, 8*(6), 836-856.

[3.113] Qiao, W., Li, S. & Xu, F. (2016). Preparation and characterization of a phenol-formaldehyde resin adhesive obtained from bio-ethanol production residue. *Polymers and Polymer Composites, 24*(2), 99-105.

El futuro pertenece a aquellos que entienden que hacer más con menos es compasivo, próspero, duradero, más inteligente y más competitivo.
-Paul Hawken

Chapter 4

Life Cycle Assessment of the vine shoots biorefinery

4.1 Background

Throughout the Chapters 2 and 3 different procedures to carry out an integral revalorisation of vine shoots have been proposed being their main components, isolated, characterised and revalorised, except the lignin which was only characterised. Based on the results obtained in each fractionation step, the most suitable process sequence of a biorefinery approach for the integral revalorisation of the vine shoots was determined. Nevertheless, the realistic feasibility of the proposed vine shoots biorefinery in terms of its environmental impact has to be taken into account, by subjecting it to a Life Cycle Assessment.

4.1.1 Life Cycle Assessment (LCA)

The Life Cycle Assessment is a systematic procedure internationally standardised that permits the quantification of all the relevant emissions, resources consumed, impacts on the environment and health and resource depletion issues throughout a product´s life cycle [4.1, 4.2]. The life cycle of a product considers from the raw material acquisition, through production, use, end-of-life-treatment and recycling up to the disposal of the remaining wastes [4.1].

The LCA provides complete, objective and transparent information about the impact of the life cycle of a product on the environment, permitting the determination of the problematic processes or stages also denoted as hotspots. This information allows the proposal of actions that could reduce the environmental impact or at least improve the situation.

Although in 1990 the Society of Environmental Toxicology and Chemistry (SETAC) held LCA workshops and identified various stages of the LCA framework, it was not until 1997 when the ISO developed an Environmental Management series of norms that a global LCA standard became available [4.3].

This standardised procedure is applied in many sectors such as the industry, the administration or by NGOs and it is also widely employed for assessing the environmental impact of biorefineries. The LCA has been employed, for instance, in biorefinery approaches in which bioethanol was produced from different feedstock such as corn [4.4], sweet sorghum stem [4.5] or sweet potatoes

[4.6], in order to determine the green house gases (GHG) emissions or other environmental impacts such as the acidification or the eutrophication [4.7]. There are also examples in which more complex biorefineries in which apart from bioethanol other co-products are obtained from lignocellulosic biomass. Bello et al. [4.8] assessed the impact of the production of bioethanol, acetic acid, lignin and furfural from hardwoods on different environmental aspects such as the climate change, the ozone depletion or the acidification. González-García et al. [4.9] for instance, applied the LCA during the production of oligosaccharides from sugar beet pulp in order to compare the effect of the autohydrolysis or the enzymatic hydrolysis on different environmental aspects such as, acidification, global warning or marine aquatic ecotoxity, between others.

4.2 Objectives

The main objective of this chapter is to subject the biorefinery approach proposed in Figure 4.1 to a Life Cycle Assessment in order to study the environmental impact of its different stages. The proposal of the biorefinery approach for the vine shoots has been carried out taking into account the results obtained throughout the thesis. However, some of the stages considered during the LCA were not the same as the ones used in the thesis, since some of the treatments were not carried out before the LCA. Thus, the conditions of some of the treatments were taken from bibliography, while the results obtained in these stages were estimated.

The proposed biorefinery scheme for the integral revalorisation of the vine shoots consisted on an autohydrolysis treatment of the vine shoots, as a first stage treatment. The liquid phase obtained in this hydrothermal treatment was subjected to a liquid-liquid extraction for the obtaining of antioxidants followed by a purification stage using membrane technology for the achievement of refined oligosaccharides. On the other hand, the solid phase obtained in the autohydrolysis treatment was delignified leading lignin and a solid residue which was used in an enzymatic hydrolysis being the leading glucose fermented for the production of bioethanol.

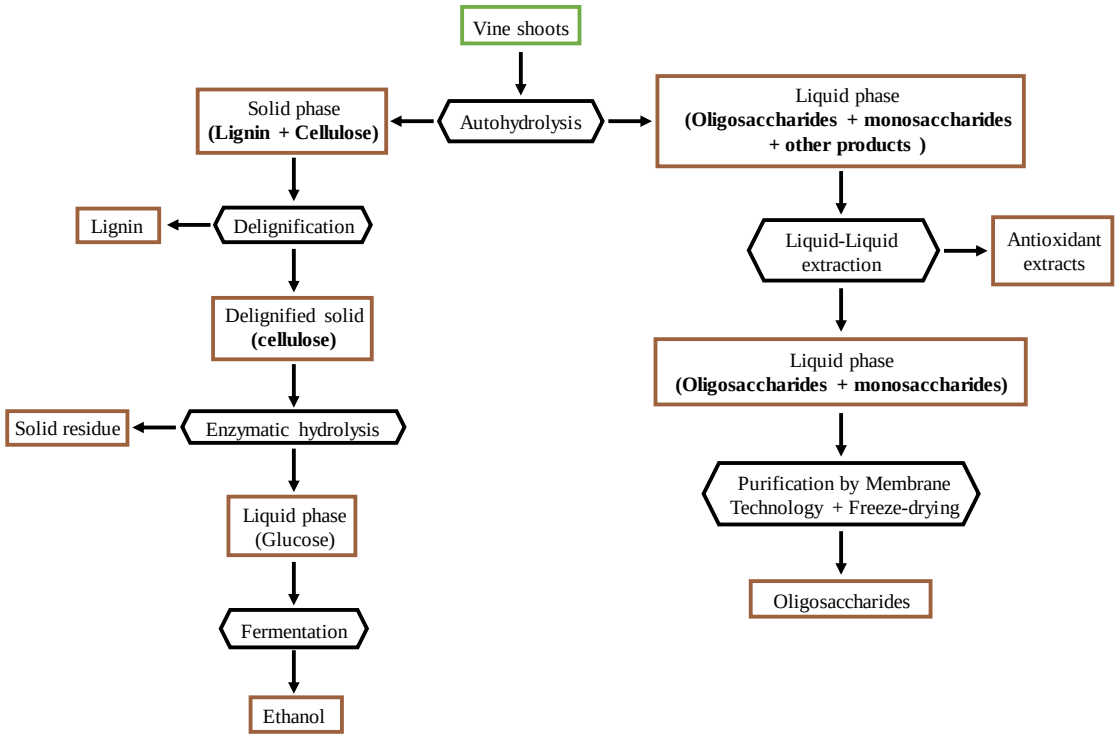


Figure 4.1. Biorefinery approach for the integral revalorisation of the vine shoots analysed by LCA.

Although the conditions used for the LCA of this biorefinery approach were not in some stages the optimum ones determined throughout this thesis, this assessment would permit the determination of the hotspot stages which would need to be improved.

4.3 Materials and methods

The Life Cycle Assessment of the proposed biorefinery approach for the vine shoots was carried out considering the environmental impacts generated from the extraction of the raw material to the production of the target compounds, which consisted on a cradle-to-gate approach. The functional unit employed is based on the amount of biomass valorised per batch, in particular 100 Kg of oven-dried vine shoots provided by a local farmer. The consideration of a reference unit based on the amount of the revalorised by-product is commonly used in LCA of waste management systems [4.9]. The proposed biorefinery approach, also denoted as Scenario in the LCA terminology, was considered as a multi-outputs system in which more than one product that could be used for further applications were obtained. However, due to the employed of this

functional unit no allocation has been needed and a system expansion approach has also been considered.

4.3.1 Description of the valorising scenario

The proposed valorisation scenario was constituted by different subsystems in which a different process takes place, as it can be appreciated in Figure 4.2. As it has been commented some of these processes employed conditions determined throughout this thesis while others have been taken from bibliographic data. Nevertheless, each subsystem will be described briefly.

The first subsystem (SS1) considered the autohydrolysis treatment of the vine shoots with water in a stainless steel Parr reactor working with a LSR of 8 g/g (in oven-dried basis) in a non-isothermal regimen at 200 ºC (15.54 bar), as it has been described in detail in the Section 2.3.2. The process related to the vine shoots production has not been considered within the system boundaries as the environmental impacts that it causes could be attributed to the grape harvesting, thus the allocation criteria was not applied in this study. It was considered that vine shoots of the optimum size (≈ 0.4 mm) were directly supplied by a local farmer.

Once the autohydrolysis was finished the reaction media was cooled down during 10 min by internal refrigeration and the solid and liquid phases were separated by centrifugation (SS2). The obtained liquid phase was extracted with ethyl acetate in a single extraction stage at room temperature with 15 min of stirring using a hydrolysate:solvent ratio of 1:3 (v/v) (SS3), being this process described in more detail in the Section 2.3.4 . The obtained organic phase was vacuum evaporated at 40 ºC in order to obtain an extract rich in antioxidants and to recover the solvent (SS4).

The obtained aqueous phase was concentrated by a membrane processing technology (SS5) previously described by González-García et al. [4.10]. Briefly, the concentration stage was carried out using a regenerated cellulose membrane of 1 KDa cut off (Millipore) working at a pressure of 4 bar at room temperature until an overall retentate to feed ratio of 1/4 (v/v) was achieved.

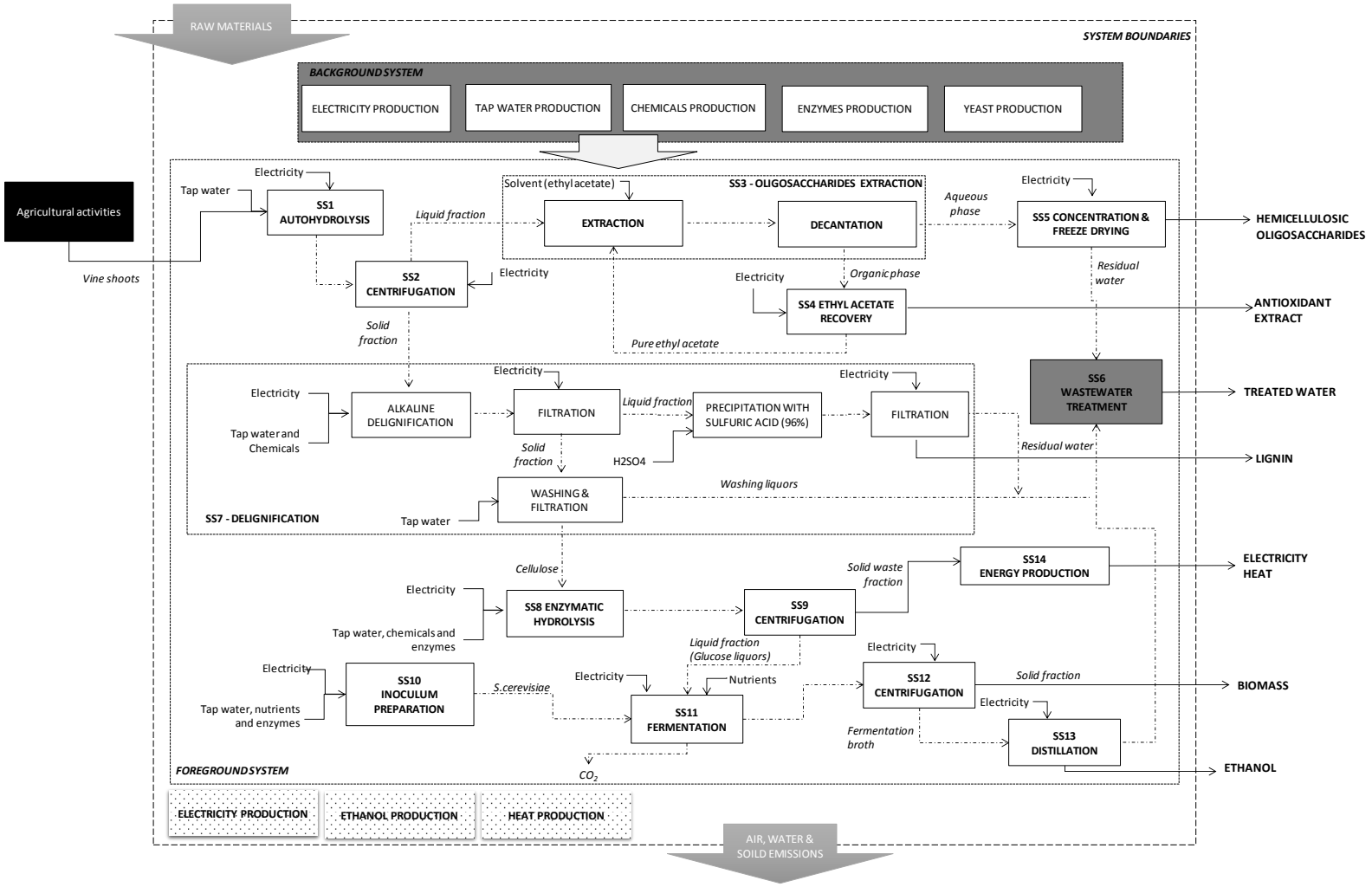


Figure 4.2. Valorisation scenario proposed for the vine shoots.

Then, the retentate was freeze-dried obtaining a solid containing the refined oligosaccharides, while the permeate was sent to a wastewater treatment unit (SS6).

Conversely, the solid obtained in the autohydrolysis treatment was subjected to an alkaline delignification (SS7), as it is described in the Section 3.3.2.1. Briefly, the autohydrolysed solid was mixed in a LSR of 10 g/g (in oven-dried basis) with an aqueous solution of 12.0 wt.% NaOH and the obtained mixture was autoclaved at 124 ºC (pressure = 2.32 bar) during 105 min. The obtained solid and liquid phases were separated by vacuum filtration. The delignified solid was washed with water until its neutralisation and then it was filtered. The obtained black liquor was acidified with 96.0 wt.% H_2SO_4 until pH 2 and the precipitated lignin was recovered by filtration and washed several times with acidified water and then with water.

The delignified solid was then subjected to an enzymatic hydrolysis (SS8) to obtain glucose which could be further employed for the production of bioethanol. Briefly, the delignified solid was mixed with water using a LSR of 30 g/g and 25 FPU/g (in oven-dried basis) of the cellulase (Celluklast 1.5L) and 5 IU/FPU of the β-glucosidase were added. The enzymatic hydrolysis was carried out at 48 ºC for 48 h and 0.05 N citric acid-sodium citrate buffer was used to maintain the pH of the enzymatic medium at 4.85. The obtained solid and liquid phases were separated by centrifugation (SS9), being the recovered solid residue considered as an additional product to be used for the production of energy. Thus, the production of energy (SS14) was also considered in this scenario. An avoided product approach was used with the production of the same amount of electricity (considering the Spanish electricity profile) and heat (considering the natural gas as a source) in a combined heat and power unit.

The liquid phase obtained in the enzymatic hydrolysis was subjected to an alcoholic fermentation (SS11) to convert the glucose into bioethanol and CO_2. To achieve this aim, firstly the inoculum was prepared (SS10) by growing the yeast *Saccharomyces cerevisiae* (DSM 70449) in a medium containing 10 g/L glucose, 5 g/L peptone, 3 g/L malt extract and 3 g/L yeast extract for 24 h at 30 ºC and 120 rpm. Once, the cells were grown, they were separated by centrifugation, resuspended in a phosphate buffer and inoculated in the medium (to achieve a 2.5 g yeast/L in

oven-dried basis) together with the nutrient without glucose (SS11). The fermentation was carried out for 48 h at 35 ºC and 120 rpm.

Once the fermentation was carried out, the yeast cells and the medium containing bioethanol were separated by centrifugation (SS12). In order to obtain bioethanol for chemical uses a distillation was carried out to purify the fermentation broth (SS13). The energy consumption of the distillation stage was taken from a database of Aspen Plus®. As it was carried out with the solid obtained after the enzymatic hydrolysis (SS9) the bioethanol was also considered with the avoided product approach. Thus, the avoided ethanol production was also taken into acount during the environmental impact evaluation.

4.3.2 Inventory data acquisition

To carry out a consistent LCA a high quality Life Cycle Inventory (LCI) data has to be collected. Particularly, the direct inputs and outputs of each subsystem, such as, the electricity requirements of the equipments, the chemical doses, the tap water and the nutrients and enzymes employed were taken from the experimental procedures carried out and they were measured and expressed as the average data per batch (100 kg oven-dried vine shoots). The energy requirements of the employed equipments were estimated taking into account the power of the equipments and their time of employment. The detailed information about the direct inputs is collected in Table 4.1 while the information regarding the direct outputs is collected in Table 4.2.

The data corresponding to the composition of the vine shoots and of the products obtained in the autohydrolysis and alkaline delignification treatments were the ones determined throughout the thesis, being the composition of the autohydrolysis liquor obtained at 200 ºC expressed as g/L collected in Table 4.3. Meanwhile the outputs obtained during the oligosaccharide purification, enzymatic hydrolysis and fermentation were estimated based on the experience of the group, as the conditions employed were not the same as those used throughout this thesis.

Table 4.1. Relevant inventory data (per functional unit) of the direct inputs considered during the vine shoots revalorisation into high added-value products.

Inputs	Quantity	Inputs	Quantity
Autohydrolysis (SS1)		*Enzymatic hydrolysis (SS8)*	
Vine shoots	100 Kg (oven-dried basis)	Electricity (Agitation)	5056 MJ
Tap water	795 Kg	Tap water	758.2 Kg
Electricity (Reactor)	1763 MJ	β-Glucosidase	6.18 Kg
Centrifugation (SS2)		Celluclast	11.20 Kg
Electricity (Centrifuge)	2.43 MJ	Citrate buffer	46.68 Kg
Oligossacharide extraction (SS3)		*Centrifugation (SS9)*	
Ethyl acetate[a]	1964 Kg	Electricity (Centrifuge)	2.61 MJ
Electricity	20.4 MJ	*Inoculum preparation (SS10)*	
Ethyl acetate recovery (SS4)		Electricity (Fermenter)	334.5 MJ
Electricity	943 MJ	Tap water	93.0 Kg
Concentration and freeze drying (SS5)		Nutrients	2.00 Kg
Electricity (Nanofiltration)	408.9 MJ	*Fermentation (SS11)*	
Electricity (Freeze-dryer)	4523 MJ	Electricity (Fermenter)	5856 MJ
Wastewater treatment plant (SS6)		Nutrients	28.0 Kg
Wastewater	21,927 Kg	*Centrifugation (SS12)*	
Delignification (SS7)		Electricity (Centrifuge)	167.9 MJ
Electricity (Autoclave)	8505 MJ	*Distillation (SS13)*	
Tap water (Delignification)	429.4 Kg	Electricity (Distiller)	6364 MJ
NaOH	7.51 Kg	*Energy production (SS14)*	
H_2SO_4	94.0 Kg	Solid waste fraction (from SS9)	8.40 Kg (oven-dried basis)
Tap water (Filtration)	32.0 Kg		
Electricity (Filtration)	5.72 MJ		
Tap water (Washing)	20.644 Kg		

With respect to the background activities such as the production of the electricity, the chemicals, nutrients and tap water the information were taken from Ecoinvent database® [4.11]. The data regarding the production of electricity considered the average electricity generation and import/export data obtained in Spain in 2014 [4.12]. The data regarding the electricity and heat produced by the conversion of the solid fraction obtained in the enzymatic hydrolysis into energy (SS14) were taken considering the data given by Dones et al. [4.13]. While the data about the

enzyme production were taken with respect to the data given by Nielsen and Wenzel [4.14] and Feijoo et al. [4.15].

Table 4.2. Outputs obtained during the vine shoots revalorisation into high added-value products.

Components	Composition
Antioxidant extract (from SS4)	2.8 Kg
Ethyl acetate (from SS4)	1964 Kg
Waste stream into air (from SS5)	113.1 Kg
Purified hemicellulosic oligosaccharides (from SS5)	17.9 Kg
Treated water (from SS6)	21,927 Kg
Cellulose (from SS7)[a]	17.0 Kg d.m
Lignin (from SS7)[a]	16.0 Kg d.m.
Glucose liquors (from SS9)[b]	933.3 Kg
Ethanol (from SS13)	10.7 Kg
Electricity (from SS14)	13.1 kWh
Heat (from SS14)	25.2 kWh

[a]d.m.: dry matter, [b]22.39 Kg glucose

Furthermore, the bioethanol obtained during the fermentation (SS13) could be used for the generation of energy and chemicals, thus a system expansion perspective was estimated. The production of this amount of ethanol by a conventional way was considered as "avoided product", using the data provided by Jungbluth et al. [4.16]. This perspective was also applied for the energy produced from the solid residue obtained in the enzymatic hydrolysis (SS9), being the data corresponding to the obtained heat and electricity production considered as the heat generated from natural gas as fossil source [4.17] and the electricity achieved in a national grid as it has been mentioned above.

Regarding the wastewater treatment unit in which the wastewater produced in the different subsystems was treated, inventory data provided by the Ecoinvent database ® [4.17] were considered, as this stage was not carried out experimentally.

The detailed description of the data obtained from the Ecoinvent database ® is collected in Table 4.4.

Table 4.3. Composition of the liquors (expressed as g/L) obtained during the autohydrolysis treatment (SS1).

Components	Composition
Glucose	0.32
Xylose	0.28
Arabinose	0.37
Galactose	0.26
Mannose	0.11
Galacturonic acid	0.72
GOS	8.64
XOS	12.22
ArOS	0.74
GalactOS	1.25
MaOS	1.22
AcOS	3.30
GaOS	1.05
Acetic acid	1.99
Hydroxymethylfurfural	0.08
Furfural	0.27
Total phenolic content	2.05
Total flavonoid content	1.05

Table 4.4. Detailed information of the main Ecoinvent database® processes.

Input	Process
Electricity (CHP unit)	Electricity, high voltage {ES}|heat and power co-generation, wood chips, 6667 kW, state-of-the-art 2014| Alloc Rec, U
Electricity (avoided)	Electricity, medium voltage {ES}| market for| Alloc Rec, U
Heat (avoided)	Heat, central or small-scale, natural gas {RE}| market group for| Alloc Rec, U
Tap water	Tap water {Europe without Switzerland}| market for| Alloc Rec, U
Inorganic chemical	Chemical, inorganic {GLO}| market for chemicals, inorganic| Alloc Rec, U
Wastewater treatment	Wastewater, average {CH}| treatment of, capacity 1E9l/year| Alloc Rec, U
Ethanol (avoided)	Ethanol, without water, in 99.7 % solution state, from fermentation {GLO}| market for| Alloc Rec, U
Ethyl acetate	Ethyl acetate {GLO}| market for| Alloc Rec, U
Sodium hydroxide	Sodium hydroxide, without water, in 50.0 % solution state {GLO}| market for| Alloc Rec, U
Sulfuric acid	Sulfuric acid {GLO}| market for| Alloc Rec, U
Buffer	Chemical, inorganic {GLO}| market for chemicals, inorganic| Alloc Rec, U

4.3.3 Impact assessment methodology

Once the scenario was described considering all the inputs and outputs, the environmental impact of the different subsystems forming part of the scenario was analysed. The impact assessment methodology employed was the CML 2001 method v2.05 as all the impact categories that it considers give a full overview of the environmental effect caused by the stages involved in the revalorisation scheme of the vine shoots. This method restricts quantitative modelling to early stages in the cause-effect chain limiting the uncertainty.

The results provided by this method are classified in midpoint categories according to commonly accepted groupings or to common mechanisms [4.18]. The impact categories considered during the evaluation of the biorefinery approach used to revalorise the vine shoots were: the abiotic depletion potential (ADP), acidification potential (AP), eutrophication potential (EP), global warming potential (GWP), ozone layer depletion potential (ODP), human toxicity potential (HTP), freshwater aquatic ecotoxicity potential (FEP), marine aquatic ecotoxicity potential (MEP), terrestrial ecotoxicity potential (TEP) and photochemical oxidation potential (POP).

4.4 Results and discussion

4.4.1 Environmental impact of the vine shoots biorefinery

Throughout this thesis, a biorefinery approach was proposed to revalorise the vine shoots (Figure 4.1) obtaining added-value products such as oligosaccharides with prebiotic potential, antioxidant, bioethanol and lignin. The environmental impact of this biorefinery approach was assessed by determining the effect of each subsystem on the different impact categorise evaluated. The influence of each subsystem on the impact category is reflected in Figure 4.3.

Although all the steps or subsystems considered in the biorefinery approach proposed for the revalorisation of the vine shoots have an effect on the different impact categories, the stage with the strongest influence was the SS8 (enzymatic hydrolysis), followed by the SS7 (delignification), SS11 (fermentation) and SS13 (distillation).

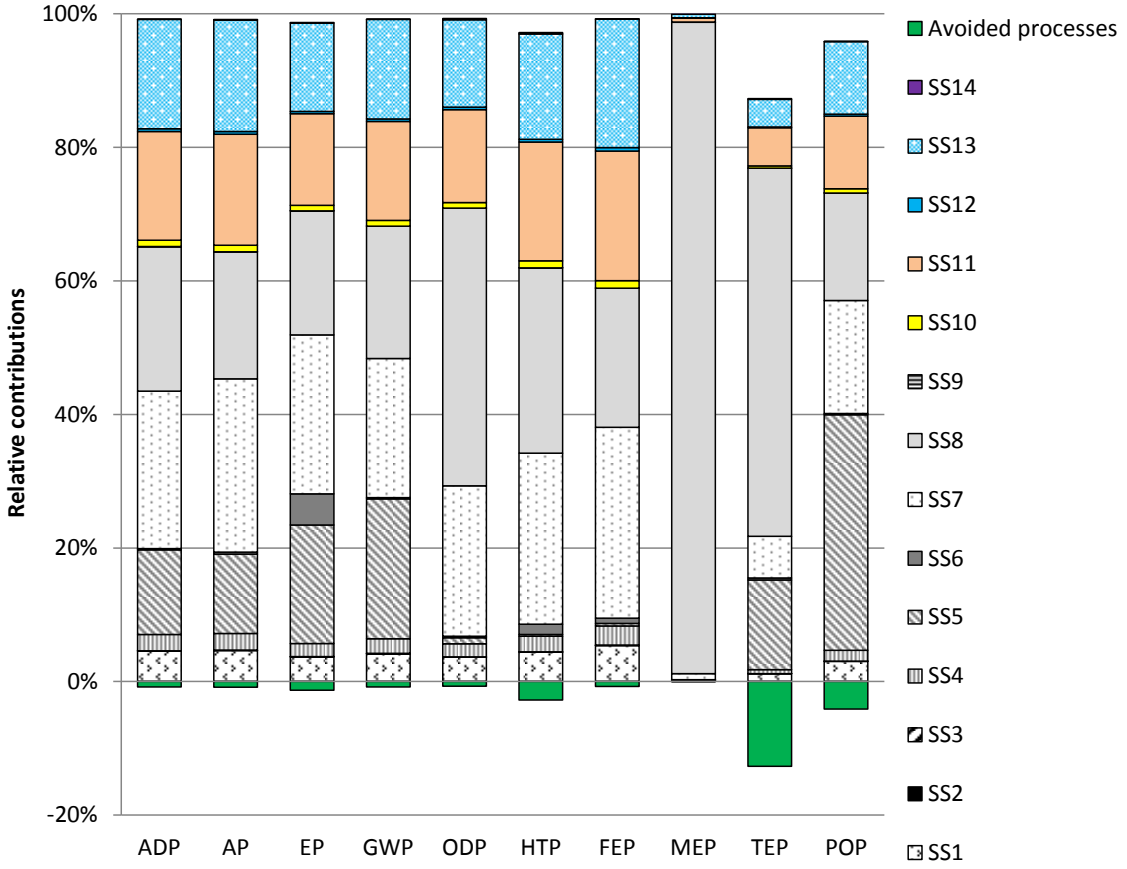


Figure 4.3. Distribution of environmental impacts per subsystems involved in the revalorisation of the vine shoots.

It has to be also noticed that the consideration of avoided processes, which consisted on the employing the bioethanol and the solid phase obtained during the enzymatic hydrolysis for the production of energy, have a positive effect on the environmental impact being it more appreciated in some categories.

Regarding the enzymatic hydrolysis process (SS8), the energy requirement for the agitation and the employment of the enzymes were the hotspots as they were the processes causing the strongest environmental impact as it can be seen in Figure 4.4. In most of the impact categories, the energy requirements presented the highest impact, but the production of the enzymes is almost the only responsible of the effect on the marine aquatic and terrestrial ecotoxicity (MEP and TEP).

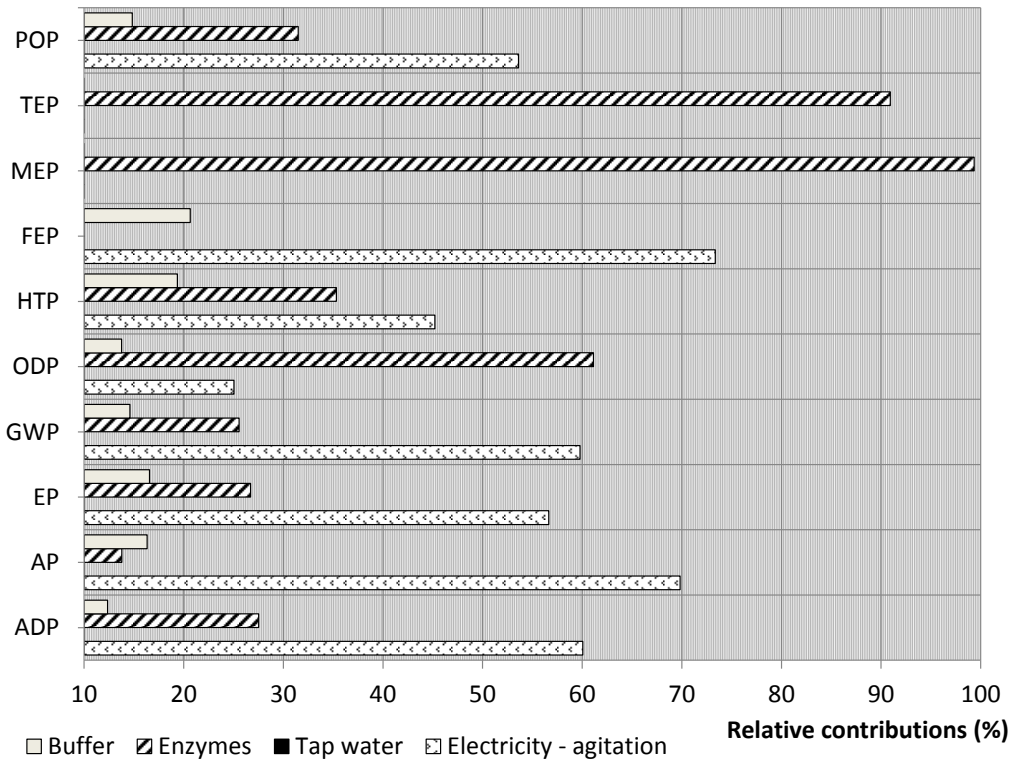


Figure 4.4. Distribution of the environmental impacts per key factors involved in the enzymatic hydrolysis (SS8).

The high impact of the enzyme production is associated with the employment of large amounts of energy and chemicals [4.15]. The high environmental impacts of the subsystems SS7, SS11 and SS13 were associated to the high energy requirements of this procedures, as it can be seen in Figure 4.5, for the delignification stage.

From the obtained results, it can be supposed that the improvement of the energy consumption and of the efficiency of the energy production could reduce the environmental impact of the biorefinery approach proposed for the revalorisation of the vine shoots. Thus, employing renewable resources for the production of energy instead of fossil resources could also benefit its environmental impacts.

The biorefineries compared with the fossil fuel refineries have a lower environmental impact due to their adjustment to the "closing the loop" strategy which is supposed to reduce the waste produced, improve the environment profiles and increase the economic profits [4.19]. Nevertheless, in the environmental assessment of the biorefinery proposed for the revalorisation of the vine shoots it was observed that to achieve the "closing the loop" strategy the environmental

impact increased as the increase of the number of stages produce a raise of the chemicals and energy requirements.

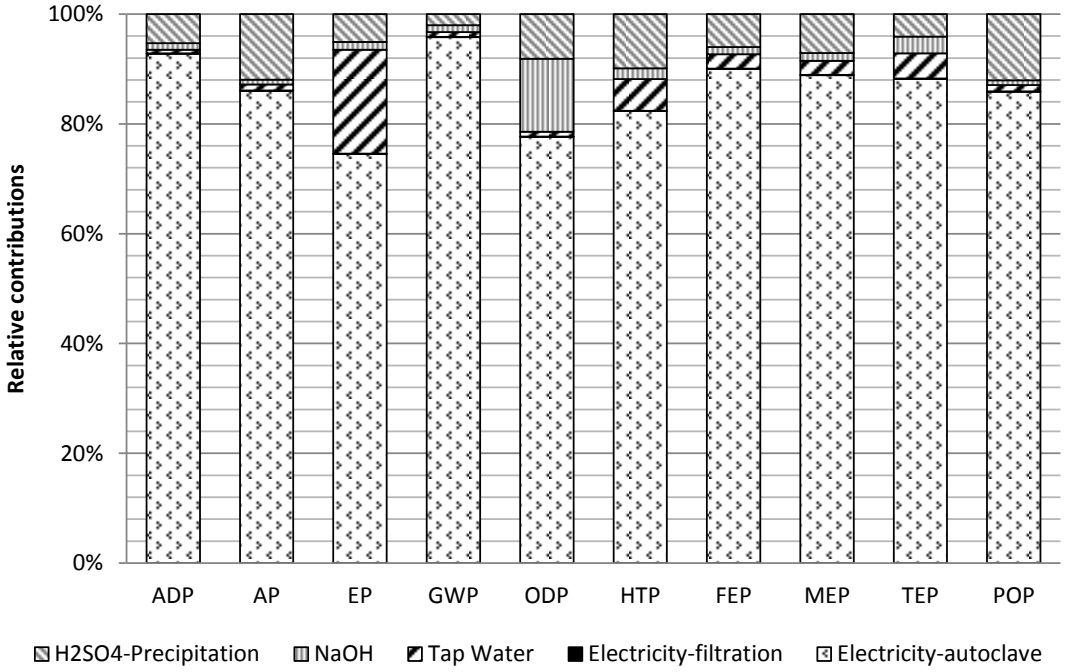


Figure 4.5. Distribution of the environmental impacts per key factors involved in the alkaline delignification process (SS7).

Nevertheless, in the environmental assessment of the biorefinery proposed for the revalorisation of the vine shoots it was observed that to achieve the "closing the loop" strategy the environmental impact increased as the increase of the number of stages produce a raise of the chemicals and energy requirements. However, these observations could be related with the functional unit employed if the functional unit considers the production of the different added-value compounds and their potential revenues associated to their market, the environmental burdens would improved [4.9, 4.10]. In Table 4.5 the market price of the different added-value products obtained during the valorisation of the vine shoots is collected.

Thus, the environmental impact of the proposed biorefinery approach for the revalorisation of the vine shoots could be carried by reducing for instance the energy requirements of the different subunits that form part of the biorefinery approach by the intensification of some of the stages for instance.

Table 4.5. Market prices of the added-value products obtained during the revalorisation of the vine shoots.

Added-value product	Market price	Reference
Antioxidant extract	167.3 €/g	[4.9]
Refined hemicellulosic oligosaccharides	36.3 €/g	[4.20]
Cellulose	686 €/t	[4.21]
Lignin	302 €/t	[4.21]
Ethanol	0.64 €/Kg	[4.22]
Electricity	0.140 €/kWh	[4.23]
Heat	0.016 €/kWh	[4.23]

The assistance of the delignification stage by microwaves, for instance, could reduce the energy requirements of this treatment as although higher temperatures were needed to achieve promising results compared with the conventional heating the reaction time and the amount of the reagent employed were reduce in high degree. The obtaining of added-value products with higher market price, than the ethanol for instance, could also improve the economical viability of the proposed approach.

4.5 Conclusions

The biorefinery approach employed for the revalorisation of the vine shoots was subjected to a Life Cycle assessment in order to evaluate its environmental impact and to determine its hotspots. It was observed that this biorefinery approach was not as green as expected and research is still needed to improve its environmental impacts.

The main hotspots of the proposed biorefinery approach were the enzymatic hydrolysis, delignification, fermentation and distillation processes, due to their high energy requirements. Thus, the employment of more energy efficient procedures as the intensification of the delignification treatment by microwaves or the performance of the simultaneous saccharification and fermentation instead of carrying out the two steps separately could reduce the environmental impact associated with the high energy requirements.

Another aspect that could promote the revalorisation of the vine shoots by a biorefinery approach could be the obtaining of products with higher market values, as although the biorefineries have environmental impact they are still lower compared with the ones generated in fossil fuel refineries.

4.6 References

[4.1] International Standard (ISO) 14040 (2006). *Environmental management-Life cycle assessment-Principles and framework.* Second edition. Geneva (Switzerland).

[4.2] Institute for Environment and Sustainability (2010). *International Reference Life Cycle Data System (LCD) handbook: General guide for Life Cycle Assessment-Detailed guidance.* First edition. Luxembourg: Publications Office of the European Union.

[4.3] International Council of Chemical Associations. How to know if and when it´s time to commission a Life Cycle assessment. Retrieved from https://www.icca-chem.org/wp-content/uploads/2016/05/How-to-Know-If-and-When-Its-Time-to-Commission-a-Life-Cycle-Assessment.pdf (Visited December, 2018).

[4.4] Pieragostini, C., Aguirre, P. & Mussati, M. C. (2014). Life cycle assessment of corn-based ethanol production in Argentina. *Science of the Total Environment, 472*, 212–25.

[4.5] Wang, M., Chen, Y., Xia, X., Li, J. & Liu, L. (2014). Energy efficiency and environmental performance of bioethanol production from sweet sorghum stem based on life cycle analysis. *Bioresource Technology, 163*, 74–81.

[4.6] Wang, M., Shi, Y., Xia, X., Li, D. & Chen, Q. (2013). Life-cycle energy efficiency and environmental impacts of bioethanol production from sweet potato. *Bioresource Technology, 133*, 285–92.

[4.7] Singh, A., Pant, D., Korres, N. E., Nizami, A. S., Prasad, S. & Murphy, J. D. (2010). Key issues in life cycle assessment of ethanol production from lignocellulosic biomass: challenges and perspectives. *Bioresource Technology, 101*, 5003–5012.

[4.8] Bello, S., Ríos, C., Feijoo, G. & Moreira, M. T. (2018). Comparative evaluation of lignocellulosic biorefinery scenarios under a life-cycle assessment approach. *Biofuels, Bioproducts and Biorefining*,*12*(6), 1047-1064.

[4.9] González-García, S., Gullón, B. & Moreira, M. T. (2018). Environmental assessment of biorefinery processes for the valorization of lignocellulosic wastes into oligosaccharides. *Journal of Cleaner Production, 172*, 4066-4073.

[4.10] González-García, S., Lacoste, C., Aicher, T., Feijoo, G., Lijo, L. & Moreira, M. T. (2016). Environmental sustainability of bark valorisation into biofoam and syngas. *Journal of Cleaner Production, 125*, 33-43.

[4.11] Wernet, G., Bauer, C., Steubing, B., Reinhard, J., Moreno-Ruiz, E. & Weidema, B. (2016). The ecoinvent database version 3 (part I): overview and methodology. *The International Journal of Life Cycle Assessment, 21*(9), 1218–1230.

[4.12] Red Eléctrica de España 2014. Avance del informe del Sistema Eléctrico Español 2014. Retrieved from https://www.ree.es/sites/default/files/downloadable/avance_informe_sistema_electrico_2014b.pdf (Visited November 2018).

[4.13] Dones, R., Bauer, C., Bolliger, R., Burger, B., Faist Emmenegger, M., Frischknecht, R., … Tuchschmid, M. (2007). Life cycle inventories of energy systems: results for current systems in Switzerland and other UCTE countries. *Ecoinvent Report No. 5*. Swiss Centre for Life Cycle Assessment, Dübendorf, Switzerland.

[4.14] Nielsen, P. H. & Wenzel, H. (2007). Environmental assessment of Ronozyme (R) p5000 CT phytase as an alternative to inorganic phosphate supplementation to pig feed used in intensive pig production. *The International Journal of Life Cycle Assessment, 12*, 514–520.

[4.15] Feijoo, S., González-García, S., Lema, J. M. & Moreira, M. T. (2017). Life cycle assessment of β-galactosidase enzyme production. *Journal of Cleaner Production, 165*, 204-212.

[4.16] Jungbluth, N., Chudacoff, M., Dauriat, A., Dinkel, F., Doka, G., Faist-Enmenegger, M., … Sutter, J. (2007). Life cycle inventories of bioenergy. Ecoinvent Report No 17. Swiss Centre for Life Cycle Assessment, Dübendorf, Switzerland.

[4.17] Doka, G. (2007). Life cycle inventories of waste treatment services. *Ecoinvent Report No. 13*. Swiss Centre for Life Cycle Assessment, Dübendorf, Switzerland.

[4.18] Guinée, J. B. (2002). Handbook on life cycle assessment operational guide to the ISO standards. *The International Journal of Life Cycle Assessment, 7*(5), 311-313.

[4.19] Liguori, R. & Faraco, V. (2016). Biological processes for advancing lignocellulosic waste biorefinery by advocating circular economy. *Bioresource Technology*, *215*, 13–20.

[4.20] Megazyme, 2016. Retrieved from https://www.megazyme.com (Visited November 2018).

[4.21] Mercopress, 2006. Retrieved from http://en.mercopress.com/2006/08/24/cellulose-prices-attheirhighestin-five-years (Visited July, 2016).

[4.22] Joelsson, E., Erdei, B., Galbe, M. & Wallberg, O. (2016). Techno-economic evaluation of integrated first- and second-generation ethanol production from grain and straw. *Biotechnology for Biofuels*, *9*, 1–16.

[4.23] Gealia Nova, 2016. Retrieved from http://www.gealianova.com/secciones/geotermia/documentos/comparativa.pdf (Visited July, 2016).

Solo atravesando la noche se llega
a la mañana.

-J. R. R. Tolkien

Chapter 5

Conclusions, future work and published research

CONCLUSIONS

In this work, an integral revalorisation of the vine shoots was carried out through a biorefinery approach with the aim of obtaining added-value products from each of their fractions, instead of burning or using the vine shoots as fertilizers.

The biorefinery scheme that permitted the greatest revalorisation of the main constituents of the vine shoots obtaining products with high purity and added-value, consisted on an autohydrolysis followed by a delignification and an enzymatic hydrolysis. During the aqueous processing between 40.0 and 93.0 % of the hemicelluloses present in the vine shoots were solubilised, depending on the employed conditions, obtaining oligosaccharides as the main products. The obtained oligosaccharides consisted on a mixture of xylooligosaccharides of different polymerisation degrees, highly acetylated and with a high number of branches. The autohydrolysis liquor obtained at the optimum conditions after being refined by membrane technology contained 97.0 % of oligosaccharides with a prebiotic potential similar to commercialised prebiotics such as the fructooligosaccharides. The high polymerisation degree and the branches would permit the oligosaccharide reach the distal colon conducting there their prebiotic activity, due to their low digestibility and fermentation rate. Thus, the oligosaccharides of vine shoots could be suitable candidates to be employed as functional food ingredients.

During the autohydrolysis secondary reactions apart from the hemicelluloses solubilisation take place, being possible to isolate extracts with bioactive properties. These extracts contained phenolic and flavonoid compounds apart from carbohydrate-derived compounds, being their abundance and constitution affected by the severity of the hydrothermal treatment. The extracts isolated from the autohydrolysis liquor obtained at the optimum conditions were the ones presenting the highest specific antioxidant activity. It presented a higher radical scavenger capacity during the ABTS assay than the synthetic antioxidant BHA. However, this extract did not only present antioxidant activity it presented also antimicrobial effects and it had an inhibitory effect on the growth of Gram positive and negative bacteria. Thus, due to their antioxidant and

antimicrobial activity the extracts isolated from the autohydrolysis liquor obtained from the vine shoots could be used as food preservatives.

Furthermore, the aqueous processing leaded a solid residue enriched in lignin and cellulose which was subjected to a delignification prior to an enzymatic hydrolysis. Although conversion of the glucan to glucose up to 75.0 % was achieved when the solid obtained in the autohydrolysis carried out at the severest conditions was employed, due to the removal of the hemicellulosic fraction, it was observed that the employment of delignified autohydrolysed solids permitted the achievement of conversion close to 100.0 %.

Among the different delignification procedures employed it was observed that the alkaline delignifications permitted the achievement of higher delignification yields. The intensification of the alkaline delignification by microwaves permitted the achievement of higher delignification yields compared with conventional heating (81.8 vs 67.7 %) using half of the reagent dose and 28.0 % of the reaction time employed with the conventional heating. Independently on the delignification method the isolated lignins presented a high purity, due to the removal of the hemicellulosic fraction from the vine shoots in a previous stage, but differences in the composition and structure of the lignins were observed. For instance, the alkaline lignins presented a higher molecular weight and polydispersity index than the organosolv and acetosolv lignins. Thus, the applicability of each lignin could vary depending on its features.

As it was seen that the autohydrolysed solid with the higher digestibility could be employed for the obtaining of ethanol, achieving a glucan to ethanol conversion of 67.4 %, it could be expected that the employment of delignified autohydrolysed solids would improve the conversion yield due to the higher digestibility of these solids. The residue obtained after the ethanol production stage could also be used for the obtained of lignin with promising characteristic as it was seen.

The environmental impacts of a biorefinery approach similar to the one proposed for the vine shoots were evaluated by a Life Cycle assessment observing that it hotspots were the delignification, enzymatic hydrolysis and fermentation stages, due to their high energy requirements. Thus, it could be expected that the assistance of the delignification process by microwaves and carrying out the enzymatic hydrolysis and the fermentation stages

simultaneously could reduce the environmental impact of the proposed biorefiney approach for the revalorisation of the vine shoots.

So still research need to be carried out on the one hand to increase the number of added-value products obtained from the vine shoots and to reduce the number of isolation processes and to make them greener, in order to promoted the biorefinery of winery by-products such as the vine shoots.

FUTURE WORKS

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

- The intensification of the autohydrolysis treatment by the employment of microwaves.
- Study the environmental impact of a biorefinery approach of the vine shoots in which the enzymatic hydrolysis and fermentation are carried out simultaneously and in which the autohydrolysis and delignification treatments are assisted by microwaves.
- To carry out the fractionation of the vine shoots in a single procedure exploiting each of their fractions using intensification techniques.
- Study the influence of the features of the lignins isolated in the different stages of the biorefinery approach in the characteristic of different biomaterials.
- Employ the delignified vine shoots for the obtaining of different products from the cellulosic fraction chemically or biotechnologically.

PUBLISHED RESEARCH

The research carried out during this thesis led to various published works (2 books chapters and papers in scientific journals). The obtained results have also been presented in various conferences.

Book chapters

Dávila I., Robles E., Egües I., Labidi J., Gullón P. 2017
The biorefinery concept for the industrial valorization of grape processing by-products, 29-53.
In: HANDBOOK OF GRAPE PROCESSING BY-PRODUCTS-SUSTAINABLE SOLUTIONS (Elsevier). ISBN: 978-012809870-7

Gullón B., **Dávila I.**, Garcia-Torreiro M., Yáñez R., Labidi J., Gullón P. 2017.
Production and emerging applications of bioactive oligosaccharides from biomass hemicelluloses by hydrothermal processing, 253-283.
In: HYDROTHERMAL PROCESSING IN BIOREFINERIES: PRODUCTION OF BIOETHANOL AND HIGH ADDED-VALUE COMPOUNDS OF SECOND AND THIRD GENERATION BIOMASS. (Springer International Publishing), ISBN: 978-331956457-9

Papers in Scientific Journals

(JC: Journal Category, JR: Journal Ranking)

Dávila I., Remón J., Gullón P., Labidi J., Budarin V. 2019.
Developing new sustainable vineyard management practices: Selective fractionation of vine shoots via microwave-assisted alkaline delignification. Journal of Cleaner Production (Submitted).
Impact factor: 5.65
JC: Green & Sustainable Science & Technology
JR: 6/33

Dávila I., Gullón B., Labidi J., Gullón P. 2019.
Multiproduct biorefinery from vine shoots: bio-ethanol and lignin production. Renewable Energy (Submitted).
Impact factor: 4.9
JC: Green & Sustainable Science & Technology
JR:7/33

Dávila I., Gullón B., Alonso J. L., Labidi J., Gullón P. 2019.
Vine shoots as new source for the manufacture of prebiotic oligosaccharides. Carbohydrate polymers 207, 34-43.
Impact factor: 5.158

JC: Polymer Science
JR: 7/87

Gullón P., Gullón B., **Dávila I.**, Labidi J., Gonzalez-Garcia S. 2018.
Comparative environmental Life Cycle Assessment of integral revalorization of vine shoots from a biorefinery perspective. Science of the Total Environment 624, 225 - 240.
Impact factor: 4.9
JC: Environmental Science
JR: 22/229

Dávila I., Gullón P., Labidi J., Gullón P. 2018
Assessment of the influence of the temperature in the microwave-assisted alkaline delignification of vine shoots. Chemical Engineering Transactions 70, 1687-1692.
Impact factor: 0.29
JC: Chemical Engineering
JR: 275/677

Gullón B., Eibes G., **Dávila I.**, Vila C., Labidi J., Gullón P. 2017.
Valorization of vine shoots based on the autohydrolysis fractionation optimized by a kinetic approach. Industrial and Engineering Chemistry Research 56, 14164-14171.
Impact factor: 2.84
JC: Chemical Engineering
JR: 34/135

Gullón B., Eibes G., Moreira M. T., **Dávila I.**, Labidi J., Gullón P. 2017.
Antioxidant and antimicrobial activities of extracts obtained from the refining of autohydrolysis liquors of vine shoots. Industrial Crops and Products 107, 105-113.
Impact factor: 3.18
JC: Agricultural Engineering
JR: 3/14

Dávila I., Robles E., Ándres M. A., Gullón P. 2017.
Delignification alternatives of spent solid from autohydrolysis of vine shoots. Chemical Engineering Transactions 57, 85-90.
Impact factor: 0.29
JC: Chemical Engineering
JR: 275/677

Dávila I., Gullón P., Andrés M. A., Labidi J. 2017.
Coproduction of lignin and glucose from vine shoots by eco-friendly strategies: Toward the development of an integrated biorefinery. Bioresource Technology 244, 328-337.
Impact factor: 5.65
JC: Agricultural Engineering
JR: 1/14

Dávila I., Gordobil O., Labidi J., Gullón P. 2016.
Assessment of suitability of vine shoots for hemicellulosic oligosaccharides production through aqueous processing. Bioresource Technology 211, 636-644.
Impact factor: 5.65
JC: Agricultural Engineering
JR: 1/14

Contributions at international scientific congresses

Dávila I., Gullón P., Labidi J., Budarin V.
Assessment of the influence of the temperature in the microwave-assisted alkaline delignification of vine shoots.
Pres 2018. Prague (Czech Republic) 25-29/08/2018.
Poster

Dávila I., Gullón B., Gullón P., Labidi J.
Assessment of the vine shoots as renewable source to obtain second generation bio-ethanol
ICOME 2018. San Sebastián (Spain) 30/04-4/05/2018
Poster

Dávila I., Labidi J., Gullón P.
Development of a sustainable biorefinery based on winemaking by-products
ECO-BIO 2018. Dublin (Ireland) 4-7/03/2018.
Poster

Dávila I., Robles E., Andrés M. A., Gullón P.
Delignification alternatives of spent solid from autohydrolysis of vine shoots.
13th International Conference on Chemical and Process Engineering. Milan (Italy) 25-31/05/2017

Oral communication

Dávila I., Gullón B., Egües I., Labidi J., Gullón P.
Antioxidant activity of waste fractions from autohydrolysis liquors of vine shoots.
6th EuCheMS Chemistry Congress. Seville (Spain) 11-15/09/2016
Poster

Dávila I., Gullón P., Labidi J.
Preliminary assessment of the susceptibility to the enzymatic hydrolysis of spent solids.
from aqueous pretreatment of vine shoots.
14th European Workshop on Lignocellulosics and Pulp. Autrans (France) 28/06-01/07/2018
Poster

Dávila I., Gullón P., Labidi J.
Sequential organosolv delignification of spent solids from aqueous pretreatment of vine shoots: an approach to improve their susceptibility to the enzymatic saccharification.
COST Action FP1306 "Valorization of lignocellulosic biomass side streams for sustainable production of chemicals, materials & fuels using low environmental impact technology". Dubrovnik (Croatia) 4-6/04/2016.
Oral communication

Dávila I., Gullón P., Egües I., Labidi J.
Oligosaccharides from vine shoots by biorefinery approach.
1st International Symposium on the Valorization of wine-making by-products for food sector. Torraza, Coste-Pavia (Italy) 2/10/2015.
Oral communication

Dávila I., Gullón P., Egües I., Labidi J.
Non-isothermal autohydrolysis of vine shoots: a biorefinery approach.
18th ISWFPC- International Symposium on Wood, Fibre and Pulping Chemistry. Viena (Austria) 9-11/09/2015.
Poster

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APPENDIX I

ANALYTICAL PROCEDURES FOR THE CHARACTERISATION OF THE BIOMASS AND THE SOLID FRACTIONS

This appendix collects the procedures used for the chemical characterisation of lignocellulosic biomass. The chemical composition of the raw material (vine shoots) and solid phases obtained after the different evaluated treatments was determined by protocols developed by the Technical Association of Pulp and Paper Industries (TAPPI) [A.I] and the National Renewable Energy Laboratory (NREL) [A.II].

The NREL protocol was used to determine the hemicelluloses, glucan and acid-insoluble lignin content of the raw material and of the different solid phases. Furthermore, the raw material was subjected to protocols proposed by Wise et al. [A.III], Rowell [A.IV] and to a TAPPI protocol for the analysis of the holocelluloses, α-cellulose and acid-insoluble lignin content, respectively.

Each analysis was carried out in triplicates being the results given in oven-dried basis as the arithmetic average value and the standard deviation.

Conditioning of the biomass (TAPPI T257 cm-85)

This section describes procedures of drying, size reduction and representative sampling applied to the biomass before subjecting it to the different treatments and compositional analyses.

The homogeneity of the raw material is an important characteristic to take into consideration when working with it, as its particle size could influence the surface area of the biomass and therefore the impregnation rate of the reagents could vary. According to the TAPPI standards, the particle size of the biomass should be between 0.4 and 0.25 cm, while for the quantitative acid hydrolysis (QAH), following the protocol described by the NREL, the particle size of the biomass should be smaller than 0.5 mm.

In this thesis, once the raw material was air-dried, it was milled, using a Retsch SM 2000 cutting mill, and sieved in order to have a particle size smaller than 0.4 cm, while only for the protocol

proposed by the NREL the solid was milled and sieved again in order to have a particle size smaller than 0.5 mm.

Moisture content determination (TAPPI T264 cm-97)

Biomass samples can contain large and varying amounts of moisture, which can change quickly when exposed to air. Thus, the moisture of the samples when they are in equilibrium with the environment was determined. The determined moisture content has to be taken into account in the subsequent analyses, as the results of chemical analyses of the biomass are typically reported on an oven-dried basis, in order to be meaningful. The procedure used for the moisture determination consists of:

- Weight accurately 1 ± 0.001 g of the sample (m_0) in a previously tared recipient (m_1). The recipient has to be previously dried in an oven at 105 ± 3 ºC for 6 h and cooled down in a desiccator.
- Introduce and keep the recipient containing the sample in an oven at 105 ± 3 ºC for 24 h.
- After this time, introduce the recipient with the sample in a desiccator until it cools down to room temperature. Then, weight the recipient with the sample (m_2) until the weight of the sample is constant to ± 0.2 mg.

The moisture content of the sample (H) is determined as follows:

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

Determination of the ash content of biomass (TAPPI T211 om-93)

The ash content of biomass could be attributed to the mineral matter of the raw material, to the metallic matter from piping and machineries, to various residues from the chemicals used in its manufacture, and/or to the filling, coating, pigmenting or to other added materials. The ashes or the inorganic materials of the biomass can be classified as structural or extractable. The structural ash is bound in the physical structure of the biomass, while the extractable ash can be removed by washing or extracting the biomass.

According to the TAPPI protocol the ash content of the biomass is defined as the solid material that remains after igniting the biomass at 525 ºC and it was determined as follows:

- Weight accurately 1 ± 0.001 g of the sample (m_0) in a previously tared crucible (m_1). The crucible has to be previously ignited in a muffle furnace at 525 ± 25 ºC for 30-60 min, cooled down slightly and introduced in a desiccator.
- Introduce the crucible containing the sample in a muffle furnace and raise the temperature slowly to 525 ± 25 ºC so that the sample is carbonized without flaming.
- Keep the crucible with the sample in the muffle furnace at 525 ± 25 ºC for 3h.
- After this time, let the muffle furnace cool down somewhat, remove the crucible containing the sample and placed it in a desiccator until it cools down to room temperature.
- Then, weight the crucible with the sample (m_2) until the weight of the sample is constant to ± 0.2 mg.

  The ash content (AC) of the sample is estimated as follows:

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

Determination of solvent extractives in biomass (TAPPI T204 cm-97)

The solvent extractives are associated to different type of compounds present in the lignocellulosic biomass, such as fatty acids, resins or waxes, between others. As these compounds do not form part of the cell walls of the biomass, they could be separated by extraction. This should be carried out prior to the determination of the acid-insoluble lignin, holocelluloses, hemicelluloses, α-cellulose or glucan content of biomass, since the extracts could interfere with these analyses. The procedure used for the determination of the solvent extractives consists of:

- Weight accurately 4.0 ± 0.001 g of the sample (m_0) in an extraction thimble and place another thimble on top of this to avoid any sample loss.
- Place the extraction thimble containing the sample in position in the Soxhlet apparatus. Fill the extraction flask with 150 mL of an ethanol-toluene mixture (1:2 v/v). The

extraction flask has to be previously weighted (m_1) after having it dried in an oven at 105 ± 3 ºC for 6 h and cooled it down in a desiccator.

- 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 sample for not less than 24 extractions over a 4-5 h period.
- After this time, remove the flask from the apparatus and partially evaporate the solvent of the extraction flask reaching a volume of 20-25 mL.
- Then, introduce the extraction flask in an oven at 105 ± 3 ºC until the extraction mixture is evaporated.
- Once the extracts are dried, introduce the extraction flask in a desiccator until it cools down to room temperature. Then, weight the extraction flask containing the extracts (m_2) until the weight of the sample is constant to ± 0.2 mg.

The solvent extractives content (SEC) of the sample is estimated as follows:

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

The determination of the solvent extractives was only carried out for the raw material, as during the hydrothermal treatment almost all the extractives present in the raw material are removed.

Determination of the acid-insoluble lignin content of biomass (TAPPI T222 om-98)

The lignin is constituted by an acid-soluble and an acid-insoluble fraction being the latest one determined in this protocol. The acid-insoluble or Klason lignin is defined as an insoluble compound in 72.0 wt.% H_2SO_4. During this protocol, the cellulosic and hemicellulosic fractions are hydrolysed and solubilised together with the acid-soluble lignin, while the Klason lignin is recovered as a solid residue. However, it has to be taken into account that the ashes present in the biomass are not solubilised in this procedure, so since they will remain as part of the acid-insoluble material their content has to be deducted. The TAPPI protocol used to determination the Klason lignin content of biomass consists of:

- Weight accurately 1 ± 0.001 g of the extractive-free sample (m_0) in a 1000 mL beaker.
- Add 15 mL of 72.0 wt.% H_2SO_4 to the beaker containing the sample and stir the mixture with a glass rod in order to have an homogeneous mixture.
- Keep the beaker at room temperature for 2 h and stir the sample frequently during this time to ensure the complete solution.
- Add about 300 to 400 mL of water to a round bottom flask and transfer the content of the beaker to the flask. Then, rinse and dilute the content of the beaker with water to a total volume of 575 mL in order to have a solution with 3.0 wt.% H_2SO_4.
- Boil the mixture for 4 h using a reflux condenser to avoid sample loss.
- After this time, separate the mixture contained in the flask by filtration using a Gooch crucible with a grade 3 pore size and wash the solid residue with hot water until it is neutralised. The Gooch crucible should have been previously dried in the oven at 105 ± 3 ºC for 6 h, cooled down in a desiccator and weighted (m_1).
- Dry the Gooch crucible containing the solid residue in an oven at 105 ± 3 ºC for 24 h.
- After this time, introduce the Gooch crucible with the solid residue in a desiccator to cool it down to room temperature. Then, weight it (m_2) until the weight of the sample is constant to ± 0.2 mg.

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

$$\boldsymbol{Acid-insoluble\ lignin\ content}\ (\%) = \left(\frac{(m_2 - m_1)}{m_0 \cdot \frac{\left(100 - H(\%)\right)}{100}} \cdot \left(100 - SEC\ (\%)\right) \right) - AC(\%)$$

Determination of the holocellulosic content of biomass (Wise et al., 1946)

The holocellulose comprises the water insoluble carbohydrates present in the lignocellulosic biomass, being it constituted by the sum of the hemicellulosic and cellulosic fractions. The determination of the holocellulose was carried out by a procedure proposed by Wise et al. [A.III] which consists on a delignification process in acid medium using sodium chlorite. In this process

the lignin is solubilised remaining the carbohydrates unchanged. The procedure used to determine the holocellulosic content of biomass consists of:

- Weight accurately 2.5 ± 0.001 g of an extractive-free sample (m_0) in a 250 mL beaker and add 80 mL of hot distilled water.
- Introduce the beaker containing the mixture in a water bath at 70 ºC for 6-8 h and stir it periodically in order to have a homogeneous sample.
- Every hour, add 0.5 mL of glacial acetic acid and 2.6 mL of 25.0 wt.% $NaClO_2$ to the beaker.
- After the 6-8 hours period, keep the beaker in the water bath at 70 ºC for another 12 h without carrying out any addition.
- Then, separate the solid and the liquid phases by filtrating the mixture using a Gooch crucible with a grade 2 pore size and wash the solid phase with hot water until it is neutralised. The Gooch crucible should have been previously dried in an oven at 105 ± 3 ºC for 6h, cooled down in a desiccator and weighted (m_1).
- Dry the Gooch crucible containing the solid phase in an oven at 105 ± 3 ºC for 24 h.
- Then, introduce the Gooch crucible with the sample in a desiccator to cool it down to room temperature and weight it (m_2) until the weight of the sample is constant to ± 0.2 mg.

The holocellulosic content of the sample is calculated as follows:

$$\textbf{\textit{Holocellulosic content}}\ (\%) = \left(\frac{(m_2 - m_1)}{m_0 \cdot \frac{\left(100 - H(\%)\right)}{100}} \cdot \left(100 - SEC\ (\%)\right) \right)$$

Determination of the α-cellulose and the hemicellulosic content of biomass (Rowell 1983)

There is a TAPPI protocol for the determination of the α, β and γ-cellulose but it is specific to paper pulp and special emphasis is placed on this. Therefore, in this thesis the determination of the cellulosic and hemicellulosic content of the lignocellulosic biomass was carried out using the

protocol proposed by Rowell [A.IV]. In this method, only the α-cellulose is determined, being it considered as the fraction of the holocelluloses that remains insoluble after the treatment with NaOH and acetic acid. The α-cellulose is the main cellulose type in lignocellulosic biomass. The hemicellulosic fraction, on the other hand, is considered as the fraction of the holocelluloses solubilised in this procedure. As only the α-cellulose is determined by this protocol there could be some errors during the estimation of the cellulosic content of the lignocellulosic biomass as the β and γ-celluloses are considered as hemicelluloses. The procedure used for the determination of the α-cellulose and the hemicelluloses consists of:

- Weight accurately 2.0 ± 0.001 g of dry holocellulose (m_0) in a 100 mL beaker. The holocellulose used has to be extracted by the methodology described in the previous section (Determination of the holocellulosic content).
- Add 10 mL of a solution of 17.5 wt.% NaOH to the beaker containing the sample, using continuous stirring.
- Add three times 5 mL of a solution of 17.5 wt.% NaOH to the beaker waiting 5 minutes between additions. After the last addition, let the alkali solution react with the sample at room temperature for 30 minutes.
- After this time, stop the reaction by adding 33 mL of distilled water to the beaker containing the sample and wait 1 h.
- Then, separate the solid and liquid fractions by filtration using a Gooch crucible of a grade 2 pore size and wash the solid with 100 mL of a solution of 8.3 wt.% NaOH and twice with distilled water. The Gooch crucible should have been previously dried in an oven at 105 ± 3 ºC for 6 h, cooled down in a desiccator and weighted (m_1).
- Add 15 mL of a solution of 10.0 % (v/v) acetic acid to the Gooch crucible containing the sample. After 3 minutes remove it by filtration and wash the solid residue with hot water until it is neutralised.
- Dry the Gooch crucible containing the solid residue in an oven at 105 ± 3 ºC for 24 h.

- Then, introduce the Gooch crucible with the solid residue in a desiccator to cool it down to room temperature and weight it (m_2) until the weight of the sample is constant to ± 0.2 mg.

The α-cellulose content is estimated as follows:

$$\boldsymbol{\alpha - Cellulosic\ content}\ (\%) = \frac{(m_2 - m_1)}{m_0 \cdot \frac{\left(100 - H(\%)\right)}{100}}$$

The hemicellulosic content is calculated as the difference between the initial holocellulosic content and the determined α-cellulose content of the sample.

$$\boldsymbol{Hemicellulosic\ content}\ (\%) = Holocellulosic\ content\ (\%) - \alpha - Cellulosic\ content\ (\%)$$

Quantitative acid hydrolysis (NERL)

This protocol developed by the NERL [A.II] permits the determination of the structural carbohydrates and the acid-insoluble lignin present in extractives-free lignocellulosic biomass. This procedure consists in two consecutive acid hydrolyses, being the first one used to convert the cellulosic and hemicellulosic polymers into oligomers and the second one to convert the oligomers into monomers. The determination of the concentration of the monosaccharides at the end of the hydrolysis permits the estimation of the hemicelluloses and the glucan content of the lignocellulosic biomass. After the hydrolysis a solid residue is obtained which corresponds to the Klason lignin, but which could also contain ashes. The main drawback of this procedure is that it does not permit the estimation of the cellulose itself as the glucan content determined in the biomass could belong both to the cellulosic and hemicellulosic fractions. The quantitative acid hydrolysis of biomass samples was carried out as follows:

- Weight accurately 0.25 ± 0.001 g of the sample (m_0) in a test tube. The particle size of the sample should be below 0.5 mm and the moisture of the sample (H) has to be previously determined.

- Add 2.5 mL of 72.0 wt.% H_2SO_4 to the test tube containing the sample and stir it in order to have an homogeneous mixture. Then, introduce the test tube in a water bath at 30 ºC and keep it there for 1 h, stirring it periodically.
- After this hour, add distilled water to the test tube in order to stop the reaction. Then, transfer the content of the test tube to a previously tared pressure flask and add distilled water until the weight of the whole mixture is 74.33 g (m_1), which corresponds to a concentration of 4.0 wt.% H_2SO_4.
- Note down the weight of the pressure flask with the sample (m_2) and autoclave it for 1 h at 121 ºC.
- After this time, cool down the pressure flask by introducing it in a water bath and once it is cold, note down its weight again (m_3). This would permit the estimation of the sample losses that could occur during the second hydrolysis stage.
- Separate the mixture contained in the pressure flask by filtration using a Gooch crucible with a grade 3 pore size. The Gooch crucible should have been previously dried in an oven at 105 ± 3 ºC for 6h, cooled down in a desiccator and weighted (m_4).
- Dry the Gooch crucible containing the solid residue in an oven at 105 ± 3 ºC for 24 h.
- After this time, introduce the Gooch crucible with the solid phase in a desiccator to cool it down to room temperature. Then, weight it (m_5) until the weight of the sample is constant to ± 0.2 mg.
- Analyse the liquid phase obtained after the filtration by High Performance Liquid Chromatography (HPLC) in order to determine 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. However, the methods and the columns used for the determination of the monosaccharides, the acids and the degradation products were different. The determination of the monosaccharides (glucose, xylose, arabinose, galactose and mannose) was carried using a Transgenomic

CARBOSep CHO-682 column working with a flow rate of 0.4 mL water/min at 80 ºC and eluting 40 µL of the sample, after neutralising it with $BaCO_3$. The determination of the acetic and galacturonic acids, furfural and HMF was carried using an 300 x 7.8 mm Aminex HPX-87H column (Bio-Rad Laboratories, USA) working with a flow rate of 0.6 mL/min at 50 ºC and eluting 20 µL of the sample with a mobile phase of 0.005 M H_2SO_4.

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

$$\textbf{\textit{Klason lignin content}}\ (\%) = \left(\frac{m_5 - m_4}{m_0 \cdot \frac{(100 - H(\%))}{100}} \cdot (100 - SEC\ (\%)) \right) - AC(\%)$$

The glucan and xylan content and the content of arabinosyl (ArOS), mannosyl (MaOS), galactosyl (GalactOS) and acetyl groups (AcOS) and galacturonic acids (GaAc), which could be present as substituents of the hemicelluloses, is estimated as follows:

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

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

where the parameters F describes the degradation of the carbohydrates and C_{est} takes into account the increase of the molecular weight of the monosaccharide during the hydrolysis. These two parameters are standardised for the different monosaccharides and their value are collected in the Table A.1. [X] is the concentration (g/L) of the monosaccharide or acids determined during the HPLC analysis, ρ is the density of the liquid phase obtained in the QAH and it is considered 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 hemicelullosic content was determined as follows:

$$\textbf{\textit{Hemicellulosic content}}\ (\%) = xylan\ (\%) + ArOS\ (\%) + MaOS\ (\%) + GalactOS\ (\%) + AcOS\ (\%) + GaAc\ (\%)$$

Table A.1. Values of the F and C_{est} parameters for the different monosaccharides, the acetic acid and the galacturonic acid.

	Glucan/Manosyl/Galactosyl/ substituents	**Xylan/Arabinosyl substituents**	**Acetyl substituents**	**Galacturonic acid substituents**
	$CG_n/CMa_n/CGa_n$	CX_n/CAr_n	CG_A	CG_{GaAc}
	Glucose/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 II

PROCEDURES FOR THE CHARACTERISATION OF THE LIQUORS OBTAINED DURING THE AUTOHYDROLYSIS OF THE VINE SHOOTS

This appendix collects the procedures used for the chemical and structural characterisation of the liquid phases obtained during the autohydrolysis treatments of the vine shoots carried out at different temperatures.

The chemical characterisation of the liquors was carried by determining their non-volatile content (NVC), their other non-volatile content (ONVC) and their content in monosaccharides, oligosaccharides, acids and degradation products, being these analyses carried out following the procedures described by Garrote et al. [A.V]. The total phenolic and total flavonoid content (TPC and TFC, respectively) of the hydrolysates were also determined using the Folin-Ciocalteau (FC) method [A.VI] and the procedure described by Blasa et al. [A.VII], respectively. These analyses were carried out in triplicates.

The structural characteristics of the compounds present in the hydrolysates were determined by subjecting them to instrumental analytical techniques such as HPSEC, FTIR, TGA, HPAEC-PAD, MALDI-TOF and UPLC-DAD-ESI-MS. Prior to the FTIR, TGA, HPAEC-PAD, MALDI-TOF and UPLC-DAD-ESI-MS analyses the hydrolysates were freeze-dried, in order to facilitate their handling and analysis.

Determination of the monosaccharide, acids and degradation products content

The monosaccharide content of the liquors together with the acetic and galacturonic acids and the degradation products (furfural and hydroxymethylfurfural) generated during the autohydrolysis were determined by HPLC. The method employed for the determination of these compounds was the same as the one used for their determination in the liquid phases obtained in the QAH of the biomass, which is described in the **Appendix I**. Briefly, the monosaccharides (xylose, arabinose, glucose, mannose and galactose) were quantified using a Transgenomic CARBOSep CHO-682

column after diluting and neutralising the sample, while the acids and degradation products were quantified using an Aminex HPX-87H column.

Determination of the oligosaccharides content

The oligosaccharides, which are constituted by monosaccharides, acetyl groups and/or uronic acids, are the main products obtained in the solubilisation of hemicelluloses by an autohydrolysis treatment. The oligosaccharides can not be quantified by HPLC, so in order to quantify them the hydrolysates were subjected to a hydrolysis to convert the oligosaccharides into monosaccharides. Thus, the increase of the concentration of the monosaccharides and acids after the hydrolysis is attributed to the quantity of oligosaccharides, which is expressed as its equivalent of monosaccharides. The procedure used to quantify the oligosaccharides was modified from the one described by Garrote et al. [A.V], as instead of 20 minutes, 30 minutes were necessary for the complete hydrolysis of the oligosaccharides. The procedure used for the determination of the oligosaccharides consists of:

- Weight between 1 and 5 ± 0.001 g of the liquors (m_0) in a previously tared pressured flask.
- Add water and 96.0 wt. % H_2SO_4 to the pressured flask until a solution of 25 g containing 4.0 wt.% H_2SO_4 is obtained (m_1).
- Note down the weight of the pressure flask containing the sample (m_2) and autoclave it for 30 min at 121 ºC.
- After 30 minutes, cool down the pressure flask by introducing it in a water bath and once it is cold, note down its weight again (m_3). This permits the estimation of the sample losses that occurred during the autoclaving stage.
- Subject the obtained sample to the HPLC analyses described for the characterisation of the composition of the liquid phases obtained during the QAH (**Appendix I)** in order to quantify the monosaccharides and acetic and uronic acids that it contained.

The quantity of glucoologosaccharides (GOS), xylooligosaccharides (XOS) and the content of arabinosyl (ArOS), mannosyl (MaOS), galactosyl (GalactOS), acetyl groups (AcOS) and galacturonic acids (GaAc) as substituents is estimated as follows:

$$[\boldsymbol{GOS/XOS/ArOS/MaOS/GalactOS/AcOS/GaAc}]\ (\mathrm{g/L}) = \left(\frac{m_1}{m_0} \cdot \left(\frac{m_1 - (m_2 - m_3)}{m_1}\right) \cdot [X]\right) - [X_0]$$

where [X] is the concentration (g/L) of the monosaccharides or acids determined during the HPLC analysis of the hydrolysed liquors. [X_0] is their concentration (g/L) in the liquors prior to their hydrolysis, also determined by HPLC as it has been described in the **Appendix II** for the determination of the monosaccharides, acids and degradation product content of the liquors.

Determination of the non-volatile content (NVC)

The non-volatile content (NVC) of the hydrolysates corresponds to the solid content of the liquors. This solid content is constituted by saccharides (including monosaccharides, oligosaccharides and their substituents) and non-saccharide compounds (denoted as other non-volatile content (ONVC)), which could be phenolic compounds, for instance. The procedure used for the determination of the non-volatile content consists of:

- Weight accurately 1.5 ± 0.001 g of the liquor (m_0) in a previously tared recipient (m_1). The recipient has to be previously dried in the oven at 105 ± 3 ºC for 6 h and cooled down in a desiccator.
- Introduce and keep the recipient containing the sample in the oven at 105 ± 3 ºC.
- From time to time, introduce the recipient with the sample in a desiccator until it cools down to room temperature and weight it (m_2) until the weight of the sample is constant to ± 0.2 mg.

The non-volatile content (NVC) of the liquors is determined as follows:

$$\boldsymbol{Non-volatile\ content}\ (g\ NVC/g\ hydrolysate) = \frac{(m_2 - m_0)}{(m_1 - m_0)}$$

The other non-volatile content (ONVC) of the hydrolysates corresponds to the difference between the NVC and the saccharide content (including monosaccharides, oligosaccharides and their substituents) of the liquors. The ONVC of the liquors is calculated as follows:

$$\textbf{\textit{Other non} - \textit{volatile content}}\ (g\ ONVC/\ g\ hydrolysate) = \left(\frac{NVC - \frac{\sum X_{id}}{\rho}}{NVC}\right) \cdot 100$$

where X_{id} is the concentration (g/L) of the identified saccharide compounds and ρ is the density of the liquor (aprox. 1000 g/mL).

Determination of the total phenolic content (TPC)

The autohydrolysis liquors could present phenolic compounds as the low pH achieved in the treatments could facilitate the solubilisation of the acid-soluble lignin. The estimation of the total phenolic content of the hydrolysates was carried out according to the Folin-Ciocalteau (FC) method [A.VI]. The FC is a bright yellow reagent that presents a different colour depending of its oxidation state. When it is oxidised the FC is colourless while when it is in contact with reducing agents it presents a dark blue colour (with a maximum absorbance at λ = 750 nm). The FC method has been widely used for the spectrophotometric determination of phenolic compounds due to their capacity of reducing this reagent.

Prior to the determination of the total phenolic content of the hydrolysates, a calibration curve has to be constructed in order to interpret the absorbance measurements, and to transform them in total phenolic content, expressed as gallic acid equivalent (C_{GAE}, mg/L). The calibration curve was constructed using six aqueous solutions of gallic acid with concentrations between 100 and 500 ppm.

The determination of the phenolic compounds present in the liquors and of the gallic acid present in the calibration solutions was carried out as follows:

- Place 300 µL of the hydrolysate or the calibration solution in a test tube.
- Add 2.5 mL of a 1/10 (v/v) aqueous solution of the Folin-Ciocalteau reagent to the test tube and stir it for 1 min using a vortex.

- Add 2 mL of a solution of 7.5 % (w/v) Na_2CO_3 to the test tube and stir it for another minute with the vortex.
- Cork the test tube with parafilm, cover it with aluminium foil and keep it in a thermostatic bath at 50 ºC for 5 minutes.
- 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 Shimadzu UV-1800 spectrophotometer.

In order to estimate the absorbance associated only with the reduced FC reagent a blank solution was used. This solution was prepared following the same procedure as the ones described above but adding water instead of the hydrolysates or the calibration solutions.

Determination of the total flavonoid content (TFC)

The obtained autohydrolysis liquors could also present flavonoids due to the solubilisation of extracts during the treatments. The estimation of the total flavonoid content of the hydrolysates was carried out according to the method described by Blasa et al. [A.VII]. This method is based on a spectrophotometric analysis using aluminium trichloride.

In a basic medium the aluminium cation forms stable complexes with the flavonoids, producing a change of the colour of the medium, from colourless to pink (with a maximum absorbance at λ = 510 nm). 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 total flavonoid content of the liquors, a calibration curve using rutin as reference and distilled water as solvent was constructed. The calibration curve permits the interpretation of the absorbance measurements, and their transformation in total flavonoid content (expressed as rutin equivalent (mg/L)). The calibration curve was constructed using six solutions containing rutin between 0 and 700 ppm.

The determination of the flavonoids present in the hydrolysates and of the rutin present in the calibration solutions was carried out as follows:

- Place 1 mL of the hydrolysate or the calibration solution in a test tube.
- Add 0.3 mL of 5.0 % (w/v) $NaNO_2$ to the test tube and stir it for 1 min using a vortex.
- Wait 5 minutes, add 0.3 mL of 10.0 % (w/v) $AlCl_3$ to the test tube and stir it for another minute with the vortex.
- Wait 6 minutes and add 2 mL of 1 N NaOH to the test tube.
- Wait 5 minutes and measure the absorbance of the sample at 510 nm. In this thesis, the absorbance measurements were carried out in a Shimadzu UV-1800 spectrophotometer.

In order to estimate the absorbance associated only with the aluminium-flavonoid complex a blank solution was used. This solution was prepared following the described procedure but adding water instead of the hydrolysates or the calibration solutions.

High Performance Size Exclusion Chromatography (HPSEC) analysis

The autohydrolysis liquors were analysed by HPSEC analysis to evaluate the molecular weight distribution of the saccharide compounds present in the hydrolysates and to estimate their average molecular weight (Mw), number average (Mn) and polydispersity index (Mw/Mn). These analyses were carried in a Jasco LC Net II/ADC chromatograph equipped with a refractive index detector and a photodiode array detector. The chromatographic separation was carried out by an Agilent Aquagel-OH column with a mixed-H phase and a particle size of 8µm, working at 40 ºC with a flow rate of 0.6 mL/min. 40 µL of the hydrolysate were eluted using a solution of 0.005N H_2SO_4 as the mobile phase.

The calibration of the HPSEC was carried out using pullulan polysaccharides with molecular weights between 180 to 805,000 Da, supplied by Agilent.

Freeze-drying of the hydrolysates

To have more sensible FTIR, TGA, HPAEC-PAD, MALDI-TOF and UPLC-DAD-ESI-MS analyses the autohydrolysis liquors were freeze-dried in order to concentrate the compounds that

they contained. Firstly the hydrolysate was kept at -20 ºC and once it was frozen, it was introduced in a Christ Alpha 1-4 LD plus freeze-dryer (Biolock Scientific). The sample was kept in the freeze-dryer until the water contained in the ice crystals was sublimated.

Fourier Transform Infrared (FTIR) spectroscopy analysis

The chemical structure of the compounds present in the liquors was evaluated by analysing the freeze-dried hydrolysates by a PerkinElmer Spectrum Two FT-IR spectrometer. The FTIR spectra were recorded between 4000-800 cm^{-1}, accumulating a total of 8 scans in transmision mode with a resolution of 4 cm^{-1}.

Thermogravimetric analysis (TGA)

The thermal stability of the compounds present in the hydrolysates was assessed by subjecting the freeze-dried hydrolysates to thermogravimetric analyses using a Mettler Toledo TGA/SDTA 851 thermogravimetric analyser. Between 3 and 5 mg of the sample were tested under a nitrogen atmosphere using a heating rate of 10 ºC/min to increase the temperature from 25 to 600 ºC.

High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) analysis

The polymerisation degree (DP) of the oligosaccharides present in the freeze-dried hydrolysates was determined by subjecting them to HPAEC-PAD analyses. The determination was performed using an ICS3000 instrument (Dionex, Sunnyvale, USA) equipped with a 250 ×2 mm CarboPac PA-1 column (from Dionex). The sample was eluted in a non-isocratic mode being the mobile phases used; water (phase A), a solution containing 0.2 M NaOH and 2 M NaOAc (phase B) and a solution containing 0.2 M NaOH (phase C). The elution pattern used was the following one; during the first 30 min the employed mobile phase was composed by 88.0 % A and 12.0 % C, from 30-35 min 50.0 % A and 50.0 % C, from 35-40 min 2.5 % B and 47.5 % of C, from 40-50

min 4.5 % B and 45.5 % C, from 50-65 min 6.5 % B and 43.5 % C, from 65-66 min 26.0 % B and 24.0 % C and from 66-95 min 50.0 % A and 50.0 % B.

The calibration of the equipment was carried out using xylooligosaccharide standards with DP between 2 and 6, provided by Megazyme.

Matrix Assisted Laser Desorption-Ionisation Time of Flight Mass Spectrometry (MALDI-TOF) analysis

The freeze-dried hydrolysates were analysed by MALDI-TOF to determine the molecular weight distribution of the oligosaccharides present in the hydrolysates. The analyses were carried out in an Ultraflex workstation of Bruker Daltonics (Bremen, Germany) working in positive ion mode. The data were acquired as the sum of 100 shots using 100 ns of delayed extraction time and 25 kV of accelerating voltage. To carry out the analysis 1 µL of an aqueous solution containing 1mg/mL of the freeze-dried hydrolysates and 0.1% (v/v) of trifluoroacetic acid (TFA) was mixed with 1 µL of a matrix solution of 10 mg/mL of 2,5-dihydrobenzoic acid (DHB) in 50.0 % (v/v) acetonitrile. The calibration of the MALDI-TOF was carried out using xylooligosaccharides standards with DP between 2 and 6 supplied by Megazyme.

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

The freeze-dried liquors were subjected to UPLC-DAD-ESI-MS analyses to obtain more information about the composition of the oligosaccharides and to complement the information obtained by other structural analyses carried out. The analyses were carried out in an UPLC (Acquity, Waters) equipped with a diode array detector and using a 100 x 2.1 mm C18 column with 1.7 µm particle size (Acquity, Waters). 5 µL of a methanol solution containing the freeze-dried hydrolysates in a concentration of 500 mg/L were eluted using a flow rate of 0.3 mL/min at 40 ºC. The elution of the sample was carried out using a non-isocratic method being the mobile phases used; an acidified water solution containing formic acid (0.1 % (v/v)) (phase A) and

methanol (phase B). From 0 min to 2 min 99.0 % of the mobile phase A was used, then until the minute 28 the mobile phase contained 99.0 % of B and then until the minute 30 the mobile phase was composed by 99.0 % of the mobile phase A. The DAD recorded UV spectrums from 200 to 500 nm, while the chromatograms were registered at 254, 280 and 320 nm. The mass spectrometry analyses were carried out using a LCT Premier XE (Waters) fitted with an electrospray ionization interface. The ESI-MS was performed using scans from m/z 100 to 2000, working in positive ionization mode and with a capillary voltage and cone voltage of 2000 and 100 V, respectively.

APPENDIX III

PROCEDURES FOR THE EVALUATION OF THE DIGESTIBILITY AND THE PREBIOTIC ACTIVITY OF OLIGOSACCHARIDES

This appendix gathers together the procedures used to determine the digestibility and the prebiotic activity of the refined oligosaccharides obtained from the autohydrolysis liquor produced at 200 ºC after their purification by membrane technology and ion exchange (stream F). The digestibility of these oligosaccharides was determined by simulating an *in vitro* gastrointestinal digestion using the protocol described by Gullón et al. [A.VIII]. Their prebiotic activity was tested by subjecting them to the protocol described by Gullón et al. [A.IX] in which an *in vitro* fermentation with human faecal microbiota is carried out. All the analyses were performed in triplicates, being the results given, in oven-dried basis, as the arithmetic average value and the standard deviation.

Prior to these analyses, the refined oligosaccharides (Stream F) were freeze-dried in order to concentrate these compounds. The employed procedure was the same as the one described in the **Appendix II** for the freeze-drying of the liquors obtained during the autohydrolysis treatments.

Evaluation of the *in vitro* digestibility of the refined oligosaccharides

The digestibility of the refined oligosaccharides (stream F) after freeze-drying them, was tested by subjecting them to an *in vitro* digestion that simulates the conditions of each gastrointestinal stage [A.VIII]. This protocol describes the conditions used to simulate the three different stages of the digestion: the mouth, the stomach and the small intestine. The digestions were carried out using enzymes while the absorption and the mechanical agitation was simulated using dialysis tubing and peristaltic movements.

The procedure used to simulate the mouth digestion consists of:

- Mix 2.4 g of the freeze-dried oligosaccharides with 9 mL of water and 1 mL of simulated saliva. Prepare the simulated saliva by diluting 100 U/ml α-amylase (provided by Sigma

Aldrich) in 1 mM $CaCl_2$. Add 1M $NaHCO_3$ to adjust the pH of the mixture to 6.9. The saliva solution simulates the mastication stage.

- Crush the mixture for 2 minutes.
- Then, transfer the sample to a glass screw topped bottle and incubate it at 37 ºC for 5 min in a water-shaking bath at 50 rpm.

The gastric digestion was simulated as follows:

- Decrease the pH of the solution obtained after the mouth digestion until pH 2 with 6M HCl.
- Add 1 mL of pepsin dissolved in 10 mL of 0.1 M HCl to the acidified sample.
- Incubate the sample for 2 h in a water-shaking bath at 37 ºC and 50 rpm.

The procedure used to simulate the small intestine digestion conditions consists of:

- Increase the pH of the solution obtained after the gastric digestion until pH 7 with 6 M NaOH.
- Add 2.5 mL of pancreatin (provided by Sigma Aldrich) dissolved in 10 mL of 0.5 M $NaHCO_3$ and 2.5 mL of a bile salt mixture (provided by Sigma Aldrich) dissolved in 10 mL of 0.5 M $NaHCO_3$.
- Incubate the sample for 2 h in a water-shaking bath at 37 ºC and 50 rpm.
- After the 2h of incubation, heat the sample at 100 ºC for 5 min in order to inactivate the enzyme.
- Once the solution is cooled down, transfer it to a dialysis membrane of 1 kDa molecular weight cut-off and dialyse it overnight at 25 ºC in a solution of 10 mM NaCl to remove low molecular weight digestion products.
- Subject the dialysed solution to an HPAEC-PAD analysis and to HPLC analyses to determine its monosaccharide and oligosaccharides content, using the procedures described in the **Appendix II**.

Assessment of the prebiotic activity of the digested oligosaccharides

The prebiotic activity of the digested oligosaccharides (stream F) after freeze-drying them, was evaluated by an *in vitro* fermentation of the oligosaccharides using human faecal microbiota [A.IX]. The fermentation of the oligosaccharides promoted the growth of the bifidobacteria present in the microbiota and the generation of short chain fatty acids (SCFA) and lactic acid as fermentation products. In this thesis, the faecal microbiota was obtained from three healthy human volunteers, who did not consumed antibiotics or probiotics nor did have any digestive diseases during the three months previous to the study. Apart from the digested oligosaccharides, fructooligosaccharides (FOS) from chicory (Sigma-Aldrich Co., St. Louis, USA) were also subjected to the in vitro fermentation in order to compare the results achieved when oligosaccharides obtained from vine shoots were used with commercialised prebiotics.

Prior to the fermentation stage, the faecal inoculum and the fermentation media which contains the digested oligosaccharides or the FOS were prepared separately. All the additions and inoculations were carried out inside an anaerobic cabinet (5.0 % H_2, 10.0 % CO_2 and 85.0 % N_2). In this thesis, a Model Bactron IV anaerobic cabinet (Hel-Lab, USA) was used.

The faecal inoculum was prepared as follows:

- Prepare a reduced physiological salt solution (RPS) that contains 0.5 g/L of cysteine-HCl and 8.5 g/L of NaCl and presents a pH of 6.8.
- Dissolve the faecal sample with RPS until a concentration of 100 g faeces/L is achieved. The faeces should have been collected in sterile vials, kept in an anaerobic cabinet and used within a maximum of 2 h after their collection.

The fermentation media was prepared as follows:

- Prepare a solution containing 5 g/L of trypticase soya broth without dextrose (supplied by BBL, Lockeysville, USA), 5 g/L of bactopeptone (Becton-Dickinson, Ammersham, Buckinghamshire, UK), 0.5 g/L of cysteine-HCl, 1.0 % (v/v) of a trace mineral solution and 0.2 % (v/v) of 0.5 g/L of resazurin solution. To this solution also 1.0 % (v/v) of a salt solution A containing 100 g/L of NH_4Cl, 10 g/L of $MgCl_2 \cdot 6\ H_2O$ and 10 g/L of $CaCl_2 \cdot$

2 H_2O and 0.2 % (v/v) of a salt solution B containing 200 g/L of $K_2HPO_4 \cdot 3\ H_2O$ were added. This solution consists in the nutrient medium solution.

- Adjust the pH of the nutrient medium solution to pH 6.8 with a NaOH solution.
- Deoxygenate the nutrient medium solution by bubbling it with a constant stream of nitrogen overnight. When the anaerobic conditions are achieved the pink medium solution passed to be colourless due to the reduction of resazurin, which acts as a redox indicator.
- Dispense 8 mL of the nutrient medium into airtight anaerobic culture tubes and seal them with aluminium caps.
- Add aseptically the stock solution of Yeast Nitrogen Base (YNB) and the a solution containing the oligosaccharides (FOS or the digested oligosaccharides) to the anaerobic culture tubes containing the nutrient medium, in order to have a concentration of 5 g YNB/L and 10 g oligosaccharides/L in the medium. Previously, concentrated aqueous solutions of the YNB (x60), of the digested oligosaccharides (x130) and of the fructooligosaccharides (x130) are prepared and filtered-sterilized into sterile airtight serum bottles.

Once the faecal inoculum and the fermentation medium were prepared, the determination of the prebiotic activity was analysed as follows:

- Inoculate 0.2 mL of the solution containing the faecal slurry in a concentration of 2.0 % (v/v) to the prepared fermentation medium containing the YNB and the digested oligosaccharides or FOS. A negative blank was also prepared by adding the faecal slurry to solution to which no carbon sources were added.
- Incubate the mixture at 37 ºC for 33 h, without agitation.
- At determined fermentation times, take an aliquot from the mixture and centrifuge it in order to separate the cells and the supernatant.
- Analyse the supernatant by HPLC in order to determine the monosaccharides present in the supernatant and the short chain fatty acids (SCFA) and the lactate generated during

the fermentations. In this thesis, the HPLC method used for these analyses was the one described in the **Appendix I** for the determination of the composition of liquid phases obtained after the quantitative acid hydrolysis. The SCFA were analysed by the Aminex HPX-87H column.

- Analyse the oligosaccharide content of the supernatant following the procedure described in the **Appendix II** for the determination of the oligosaccharides content of the autohydrolysis liquors.
- Subject the cells or the particulate matter to a Fluorescence *In Situ* Hybridisation (FISH) analysis in order to quantify selected bacterial groups.

In this thesis, the FISH analysis was carried out using a synthetic oligonucleotide probe labelled with fluorescent dye Cy3. The probe employed (probe Bif164) targets specific regions of the 16S rRNA gene of the *Bifidobacterium* genus.

The FISH analyses were carried out as follows:

- Mix 375 µL of the slurry containing the particulate matter with 1125 µL of a solution of 4.0 % (w/v) of filtered paraformaldehyde.
- Maintain the mixture overnight at 4 ºC in order to have the cells fixed.
- Centrifuge the cells and wash them twice with a filtered phosphate-buffered saline (PBS) solution.
- Suspend the washed cells in a mixture of 150 µL of PBS and 150 µL ethanol and store them at -20 ºC for at least 1 h.
- Add 16 µL of the fixed cells, after warming them up, 64 µL of distilled water and 200 µL of a filtered hybridisation buffer to a 1.5 mL microcentrifuge tubes. This prepared solution consists on the hybridisation mixture. The hybridization buffer has to be previously prepared and it contains 40 mM Tris-HCl at pH 7.2, 1.8 M NaCl and 20 mL/L of a solution of 100 g sodium dodecyl sulphate/L.
- Add 5 µL of a solution containing the nucleotide probe in a concentration of 50 ng/µL and 45 µL of above hybridisation mixture to a 0.5 mL microcentrifuge tubes.

- Shake the mixture contained in the 0.5 mL microcentrifuge tubes and incubate it overnight at 50 ºC.
- Add 20 µL of a solution containing 4´,6-diamino-2-phenylindole (DAPI) in a concentration of 500 ng/mL and 5 mL of a pre-warmed hybridisation buffer, which contained 20 mM Tris-HCl and 0.9 M NaCl, to a 10 mL microcentrifuge tube.
- Add the previously prepared hybridisation mixture, shake it and incubate the mixture at 50 ºC for 30 min.
- Filter the samples through a 0.20 µm pore size filters under vacuum.
- Place the filter on a slide, add 5 µL of an antifade reagent, which consist on polyvinyl alcohol mounting medium with DABCO antifading, and cover the filter with a coverslip.
- Store the slides in the dark at 4 ºC for a maximum of 3 days and during this time examine them with a Olympus BX41 epifluorescence microscope equipped with a Fluor 100 lents.

APPENDIX IV

PROCEDURES FOR THE CHARACTERISATION OF THE ETHYL ACETATE SOLUBLE EXTRACTS OBTAINED FROM THE AUTOHYDROLYSIS LIQUORS

This appendix brings together the procedures used for the chemical characterisation of the ethyl acetate soluble extracts and for the assessment of their biological activities. These extracts were obtained by a liquid-liquid extraction of the hydrolysates generated during the autohydrolysis treatments of the vine shoots carried out at different temperatures.

The chemical characterisation of the extracts was carried out by subjecting them to a total phenolic content (TPC) and to a total flavonoid content (TFC) determination by the Folin-Ciocalteau (FC) method [A.VI] and by the protocol described by Blasa et al. [A.VII], respectively. The extracts were also qualitatively analysed by GC/MS in order to identify some of the compounds present in the extracts.

The biological properties tested on the extracts were their antioxidant and their antimicrobial activities. The antioxidant activity was determined by the 2,2´-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), (α,α-Diphenyl-β-picrylhydrazyl) (DDPH) and Ferric Reducing Antioxidant Power (FRAP) assays following the methodology described by Re et al. [A. X], Von Gadow et al. [A. XI] and Benzie and Strain [A. XII]. The antimicrobial activity of the extracts was determined using a microdilution assay following the method described by Moreira et al. [A.XIII]. The analyses were carried out in triplicates, being the results expressed as the arithmetic average value and the standard deviation.

Determination of the total phenolic content (TPC)

The total phenolic content of the ethyl acetate soluble extracts was determined following the method developed by Singleton and Rossi [A.VI]. The Folin-Ciocalteau method has been described in detail in the **Appendix II** for the chemical characterisation of the hydrolysates. Briefly, 300 µL of an ethanol solution containing 250 ppm of the extracts were introduced in a

test tube. Then, 2.5 mL of a solution of the Folin-Ciocalteau reagent (1/10 (v/v)) and 2 mL of 7.5 % (w/v) Na_2CO_3 were added to the test tube. After incubating the samples for 5 min in a water bath at 50 ºC their absorbance at 760 nm was measured using a Shimadzu UV-1800 spectrophotometer. To interpret the absorbance measured in the samples and to transform it in the total phenolic content a calibration curve was constructed using ethanolic solutions containing between 0 and 320 ppm of gallic acid. The blank solution in this case was prepared with ethanol instead of distilled water.

Determination of the total flavonoid content (TFC)

The determination of the flavonoid content of the extracts was carried out by the procedure developed by Blasa et al. [A.VII], which has been detailed during the determination of the TFC of the hydrolysates in the **Appendix II**. Briefly, 1 mL of an ethanol solution containing 480 ppm of the ethyl acetate soluble was introduced in a test tube. Then, 0.3 mL of 5.0 % (w/v) $NaNO_2$, 0.3 mL of 10.0 % (w/v) $AlCl_3$ and 2 mL of 1N NaOH were added to the test tube. After keeping the sample 5 minutes at room temperature, its absorbance at 510 nm was measured using a Shimadzu UV-1800 spectrophotometer. In order to interpret the absorbance measurements and to determine the total flavonoid content a calibration curve using ethanolic solutions containing between 0 and 550 ppm of rutin was constructed. The blank solution was prepared using ethanol instead of distilled water.

Qualitative analysis of the extracts by Gas Chromatography/Mass Spectrometry (GC/MS)

The identification of the compounds present in the extracts was carried out by subjecting them to a GC/MS analysis. An ethyl acetate solution containing 0.02 % (w/v) of the extract was analysed in an Agilent Technologies 7890A gas chromatograph (GC) coupled to an Agilent Technologies 5975C mass spectrometer (MS) using He as the gas carrier with a flow of 1 mL/min. 1 µL of the sample was introduced in the GC in split mode and it was passed through a 30 m x 0.25 mm x

0.25 µm film thickness HP-5MS (5.0 % phenyl-methylpolysiloxane) column. The GC method used for the separation of the compounds present in the extract consisted on increasing the temperature from 50 ºC to 120 ºC (5 min) at 10 ºC/min, then to 280 ºC (8 min) at 10 ºC/min and finally to 300 ºC (2 min) at 10 ºC/min. The mass spectrometer worked in electron ionization (IE) mode applying an electron energy of 69.9 eV. The identification of the compounds was carried out by comparing the mass spectra of each compound with the National Institute of Standards Library (NIST) and with compounds reported in the literature. The peak area ratio of a specific compound can reflect its concentration as its chromatographic peak area is correlated linearly with its quantity [A. XIV]. The peak area ratio was calculated for the compounds with chromatographic peak areas higher than 0.4 % and the sum of these peak areas were normalized to 100.0 % to determine the relative abundant of each compound.

Determination of the antioxidant activity of the extracts by the ABTS assay

One of the procedures used for the determination of the antioxidant activity of the ethyl acetate soluble extracts was the ABTS assay [A.X]. In this assay, the capacity of the extract to reduce the ABTS radical is measured spectrophotometrically. When the ABTS is oxidized or it is as a radical it presents a dark blue colour (with a maximum absorbance at λ = 734 nm), while when it is reduced it becomes colourless. The ABTS radical is formed by the oxidation of the anionic species resulted from the dissolution of the 2,2´-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt and it is very sensitive to the light and to the presence of heavy metals.

Prior to the determination of the capacity of the extracts to reduce the ABTS radical a calibration curve was constructed using phosphate buffer saline (PBS) solutions containing between 25 and 500 ppm of Trolox. The calibration curve permits the interpretation of the absorbance measurements in order to transform them in Trolox equivalent antioxidant capacity, expressed as Trolox equivalent (mg/L).

The procedure used for the ABTS assay consists of:

- Prior to the analysis of the samples and calibration solutions, prepare the ABTS radical solution by mixing 7 mM ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and left the mixture for 12-16 h in the darkness at room temperature to ensure the radical full formation.
- Afterwards, dilute the ABTS radical solution with PBS to have an absorbance of 0.70 at 734 nm. In this work, the absorbance measurements were carried out in a Shimadzu UV-1800 spectrophotometer.
- Add to a test tube 20 µL of an ethanolic solution containining 450 ppm of the extract or 20 µL of the calibration solutions.
- Then, add 2 mL of the diluted ABTS radical solution to the test tube and wait 6 minutes.
- After this time, measure the absorbance of the samples at 734 nm.

In order to observe the reduction of the absorbance produced only by the Trolox or by the extracts, a blank solution was prepared using ethanol instead of the sample or of the calibration solutions.

Determination of the antioxidant activity of the extracts by the DDPH assay

Another procedure used for the determination of the antioxidant activity of the extracts was the DPPH assay [A.XI]. As it occurs with the ABTS assay, this test also consists on a spectrophotometrical analysis of the capacity of the extract to reduce the DPPH radical. The DPPH is a dark powder composed by stable radical molecules and once it is dissolved it presents a violet colour (with a maximum absorbance at λ = 515 nm). However, when the DPPH is neutralized by another radical or it is reduced, the solution changes the colour from violet to pale yellow.

Prior to subjecting the extracts to the DPPH assay, a calibration curve using ethanolic solutions containing between 0 and 100 ppm of Trolox was constructed. The calibration curve permitted the interpretation of the absorbance measurements in order to transform them in Trolox equivalent antioxidant capacity, expressed as Trolox equivalent (mg/L).

The procedure used for the DPPH assay consists of:

- Place 50 µL of an ethanolic solution that contains 450 mg/L of the extracts or 50 µL of the calibration solutions in a test tube.
- Add to the test tube 2 mL of an ethanolic solution that contain the 0.06 mM DPPH and stir it for 1 min using a vortex.
- Cork the test tube with parafilm, cover it with aluminium foil and keep it in the dark at room temperature for 16 minutes.
- After this time, measure the absorbance of the sample at 515 nm. In this thesis, these measurements were carried out in a Shimadzu UV-1800 spectrophotometer.

A blank solution that contained ethanol instead of the calibration solutions or the solutions containing the extracts was subjected to the DPPH assay in order to observe only the reduction of the absorbance produced by the Trolox or by the extracts.

Determination of the antioxidant activity of the extracts by the FRAP assay

The last procedure used for the determination of the antioxidant activity of the extracts was the FRAP assay [A.XII]. As it occurs with the described methods for the determination of the antioxidant activity, this assay consist on a spectrophotometrical analysis of the capacity of the extract to reduce the Fe^{3+} cation to Fe^{2+}. The Fe^{3+} forms an stable colourless complex with the 2,4,6-tripyridyl-s-triazine (Fe^{3+}-TPTZ) in an acid medium however in the presence of antioxidants the Fe^{3+} is reduced and the (Fe^{2+}-TPTZ) complex is formed, which presents a blue colour (with a maximum absorbance at $\lambda = 593$ nm).

As it was done in the previous antioxidant assays before subjecting the extracts to the FRAP assay, a calibration curve was constructed using methanolic solutions containing between 0 and 350 ppm of Trolox. The results were expressed as Trolox equivalent (mg/L).

The procedure used for the FRAP assay consists of:

- Prior to the analysis of the samples and of the calibration solutions, prepare the FRAP reagent solution by mixing 25 mL of a 0.3 M acetate buffer (pH 3.6), 2.5 mL of a 10 mM solution of 2,4,6-tripyridyl-s-triazine in 40 mM HCl and 2.5 mL of 20 mM $FeCl_3 \cdot 6\ H_2O$.

- Afterwards, place 100 µL of the calibration solutions or 100 µL of a methanolic solution containing 500 ppm of extracts in a test tube.
- Add 3 mL of the FRAP reagent to the test tube and stir it for 1 min using a vortex.
- Then, incubate the test tube in a water bath at 50 ºC for 20 minutes.
- After this time, cool down the test tube at room temperature and measure the absorbance of the sample at 593 nm. In this thesis, the measurements were carried out in a Shimadzu UV-1800 spectrophotometer.

A blank solution prepared with methanol was also subjected to the FRAP assay.

Determination of the *in vitro* antimicrobial activity of the extracts

The antimicrobial activity of the extracts obtained from the different hydrolysates against six different bacterial strains was determined using the procedure described by Moreira et al. [A. XIII]. The bacterial strains used were *Listeria innocua* (NCTC 10528), *Staphylococcus aureus* (isolated from food sample, accession number 18N, collection from CINATE), *Escherichia coli* (ATCC 25922), *Bacillus cereus* (DSM 4313), *Pseudomonas aeruginosa* and *Salmonella sp.* (isolated from food source, internal collection from CINATE). The antimicrobial activity of the extracts was estimated by the determination of their minimum inhibitory concentration (MIC) and their minimum bactericidal concentration (MBC). The MIC is considered as the lowest extract concentration needed to inhibit microbial growth after 24h of incubation at 37 ºC. The MBC, on the other hand, is the lowest extract concentration necessary to prevent the bacterial growth, being the initial viability also reduced by at least 99.9% after 24h of incubation at 37 ºC.

To carry out the determination of the *in vitro* antimicrobial activity of the extracts, firstly the microorganisms were cultured.

The culture of the microorganisms was carried out as follows:

- Reactivate the microorganism by inoculating it to sterile Mueller-Hinton broth (MHB). In order to store the microorganisms they have to be mixed with glycerol at 15.0 % (v/v) and stored in criovials at -80 ºC until use.

- Let the inoculum reactivate by incubating it at 37 ºC for 10-12 h under aerobic conditions without shaking.
- Then, transfer an aliquot of the culture to a tube containing fresh MHB and incubate it overnight at 37 ºC.

Once the microorganisms were cultured, different samples in which the bacterial growth reduction was analysed were prepared. These samples were the followings:

- A positive control which corresponds to a MHB solution of the inoculum containing DMSO in a concentration of 5.0 % (v/v).
- Negative controls which correspond to MHB solutions containing 5.0 % (v/v) of DMSO and between 5 and 20 mg/mL of the extracts. These solutions represent the abiotic controls.
- The test samples correspond to MHB solutions of the inoculum containing 2.0 % (v/v) of MHB solutions containing between 5 and 20 mg/mL of the extracts. In each solution the tested bacteria presents a population of 10^6 CFU/mL.

All the samples were incubated at 37 ºC for 24 h. The positive and negative controls are used to check potential contaminations and for comparative purposes.

To determine the MIC and MBC of the extracts, the bacterial growth reductions were determined by comparing the viable cell counts between the positive control and the test samples after 0 and 24h of incubation. The bacterial growth reduction was evaluated as follows:

- Dilute 100 µL of the test samples or of the positive control with peptone water through serial decimal dilutions.
- Place 20 µL of each diluted sample on a MH agar and incubate them at 37 ºC for 48h.
- Count the viables using the drop count method described by Miles et al. [A.XV] after 0 and 24h of incubation.

APPENDIX V

PROCEDURES FOR THE CHARACTERISATION OF LIGNIN

This appendix gathers the procedures used to characterise chemically and structurally the lignins isolated by different delignification treatments. The chemical composition of the lignin was determined by measuring its monosaccharide, ash and acid-soluble and acid-insoluble lignin content. The monosaccharide and acid-insoluble lignin content were determined following the protocol described by Erdocia et al. [A.XVI], after being modified. The acid-soluble lignin and the ash content were determined by the TAPPI (TAPPI UM250 um-83) protocol [A.I] and by modifying the protocols described by Liu et al. [A.XVII], respectively. These analyses, except the determination of the ash content, were carried out in triplicates, being the results expressed as the arithmetic average value and the standard deviation.

Furthermore, the lignin was subjected to different instrumental analyses such as FTIR, Py-GC/MS, HPSEC, TGA, ^{13}C-NMR and ^{1}H-NMR in order to elucidate better its structure and to have more information about its composition.

Determination of the ash content of lignin

The ashes consist on the inorganic matter present in the lignin isolated from biomass and its presence is typically attributed to the procedure used in its isolation. In this case, instead of using the procedure detailed in the **Appendix I** for the determination of the ash content in biomass a modification of the procedure proposed by Liu et al. [A.XVII] was used. Instead of carrying out the determination using a muffle furnace a thermogravimetric analysis was carried out, as it permits the employment of milligrams of sample. To analyse the ash content in lignin, between 3 and 5 mg of the isolated lignin were introduced in a TGA/SDTA RSI analyzer 851 (Mettler Toledo) working under an air atmosphere and using a heating rate of 10 ^{O}C/min to increase the temperature from 25 ^{O}C to 800 ^{O}C.

The determination of the ash content (AC (%), expressed as g/100 g oven-dried vine shoots) is important for the estimation of the acid-insoluble lignin content of the isolated lignin, as the ashes are not solubilised during the acid hydrolysis treatment and they contribute in the gravimetric determination of the acid-insoluble lignin.

Determination of the acid-insoluble lignin and carbohydrate content of lignin

The determination of the chemical composition of the lignins isolated by the different treatments is an essential analysis as it permits the determination of its purity, which is estimated as the contribution of the acid-soluble and acid-insoluble lignin. The isolated lignins could contain carbohydrates as impurities, since during the delignification treatment cellulose and hemicelluloses could be partially solubilised and trapped in the lignin during the precipitation stage or they could remain covalently link to the lignin [A.XVIII]. The determination of the acid-insoluble lignin and carbohydrate content was carried out by modifying the procedure described by Erdocia et al. [A.XVI], as instead of having the samples at 100 ºC for 3h, they were autoclaved at 121 ºC for 1h, obtaining comparable and more precise results.

The procedure used to determine the acid-insoluble and carbohydrate content of the isolated lignins is the same as the quantitative acid hydrolysis used for the determination of the chemical composition of the biomass (**Appendix I**). However, to carry out the second hydrolysis stage the sample has to be less diluted as the concentration of H_2SO_4 in the has to be 12.0 wt.% instead 4.0 wt.%. In this thesis, the carbohydrates present in the lignin were determined by analysing the resulting liquid phases using an 300 x 7.8 mm Aminex HPX-87H column (Bio-Rad Laboratories, USA), as in almost all the cases the lignins have been extracted from autohydrolysed solids from which the hemicelluloses have been removed.

The estimation of the acid-insoluble or Klason lignin was carried as follows:

$$\textbf{\textit{Klason lignin content}}\ (\%) = \left(\frac{m_5 - m_4}{m_0 \cdot \frac{(100 - H(\%))}{100}} \cdot 100 \right) - AC(\%)$$

The estimation of the carbohydrate present in the isolated lignin was carried out as follows:

$$\boldsymbol{Glucan/xylan/ArOS}\ (\%) = F\ \cdot C_{est} \cdot \frac{[X]}{\rho} \cdot \frac{P}{m_0 \cdot \left(\frac{100 - H(\%)}{100}\right)} \cdot\ 100$$

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

A more detailed explanation of these calculations is collected in the **Appendix I**.

In order to determine the acid-soluble lignin present in the isolated lignin, the volume of the liquid phase obtained from the above treatment has to be measured (V_F).

Determination of the acid-soluble lignin content of the isolated lignin (TAPPI UM250 um-83)

In the estimation of the purity of the lignin the acid-soluble lignin is also taken into account, as due to the acid conditions used in the determination of the acid-insoluble lignin part of the isolated lignin could have been solubilised.

The determination of the acid-soluble lignin content on the isolated lignin consists of:

- Dilute 75 times an aliquot of the liquid phase obtained during the determination of the acid-insoluble lignin using a solution of 1M H_2SO_4.
- Measure the absorbance of the sample at 205 nm, using the solution of 1M H_2SO_4, as the blank. The absorbance measured should be between 0.1 and 0.8. In this thesis, the absorbance measurements were carried out in a Shimadzu UV-1800 spectrophotometer.

The acid-soluble lignin was estimated as follows:

$$\boldsymbol{Acid - soluble\ lignin}\ (\%) = \frac{Abs_{205\ nm} \cdot DF\ \cdot V_F}{\varepsilon\ \cdot DM \cdot l} \cdot 100$$

where $Abs_{205\ nm}$ is the absorbance measured at 205 nm (relative to a 1M H_2SO_4 at 205nm) and DF is the dilution factor. ε is the extinction coefficient of the lignin at 205 nm (110 L/g·cm), DM is the weight of the sample used in the acid hydrolysis (in oven-dried basis) and l is the path length, typically 1 cm.

The purity of the isolated lignin is estimated as follows:

$$\boldsymbol{Purity}\ (\%) = Acid - soluble\ lignin\ (\%) + Acid - insoluble\ lignin\ (\%)$$

Fourier Transform Infrared (FTIR) spectroscopy analysis

The chemical structure of the lignin was evaluated by analysing it by a PerkinElmer Spectrum Two FT-IR spectrometer. The FTIR spectrums were recorded between 4000-600 cm^{-1}, accumulating a total of 8 scans in transmission mode with a resolution of 4 cm^{-1}.

High Performance Size Exclusion Chromatography (HPSEC) analysis

The molecular weight distribution of the lignins was determined by subjecting it to HPSEC analysis, being their average molecular weight (Mw), number average (Mn) and polydispersity index (Mw/Mn) studied. Prior to the HPSEC analysis, a solution containing 3 g lignin/L in dimethylformamide (DMF) with 0.1 % of lithium bromide was prepared. 20 µL of this sample were analysed by a Jasco LC Net II/ADC chromatograph equipped with a refractive index detector and two PolarGel-M columns (Varian Polymer Laboratories) in series. The sample was eluted with the DMF solution at 40 ºC using a flow rate of 0.7 ml/min.

The calibration of the HPSEC was carried out using polystyrene standards with molecular weights between 62,500 and 266 g/mol, provided by Sigma Aldrich. Since, these standards were used the results obtained by the HPSEC analysis are orientative and only serve for the comparative analysis carried out throughout this thesis.

Pyrolysis-Gas Chromatography/Mass Spectrometry (Py-GC/MS) analysis

The Py-GC/MS analysis of the lignin provides information about its structure, as the pyrolysis products represent in higher or lower degree the structural units that constitute the original lignin structure [A.XIX]. However, it has to be taken into account, that these products could not be forming part of the lignin, as during the pyrolysis the structure of the lignin is changed. The isolated lignin was analysed by a 5150 Pyroprobe pyrolyzer (CDS Analytical In., Oxford, PA)

connected to an Agilent 6890 gas chromatograph coupled to an Agilent 5973 (Agilent Technologies In., USA) mass spectrometer. The gas chromatograph employed He as the carrier gas and it was equipped with a 30 m x 0.25 mm x 0.25 µm film thickness HP-5MS ((5.0 % phenyl)-methylpolysiloxane) column. The pyrolysis was performed at 600 ºC (15 s) reached using a heating rate of 20 ^{O}C/ms and the interface was kept at 260 ^{O}C. The chromatographic method used for the separation of the pyrolysis products was the same as the one detailed for the qualitative analysis of the extracts, which is described in the **Appendix IV**.

^{13}C and ^{1}H Nuclear Magnetic Resonance (^{13}C- and ^{1}H-NMR) analysis

The NMR analyses of the lignin provide information about its chemical structure, as it permits to determine qualitatively some important building blocks of its structure. To carry out the analysis 40 mg of lignin dissolved in deuterated dimethyl sulfoxide (DMSO-d6) were analysed by a Bruker Avance 500 MHz equipped with a z-gradient BBI probe working at 30 ºC. The spectral width used was 25,000 Hz for the ^{13}C and 5,000 Hz for the ^{1}H analysis.

REFERENCES IN APPENDIX

[A.I] TAPPI Standards 2007, Test methods of the Technical Association of the Pulp and Paper Industry, Atlanta.

[A.II] Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D. & Crocker, D. (2008). *Determination of structural carbohydrates and lignin in biomass*. Retrieved from https://www.nrel.gov/docs/gen/fy13/42618.pdf

[A.III] Wise, L. E., Murphy, M. & D'Adieco A. A. (1946). Chlorite holocellulose, its fractionation and bearing on summative wood analysis and on studies on the hemicelluloses. *Paper Trade Journal, 122*(2), 35-43.

[A.IV] Rowell, R. (1983), The chemistry of solid wood. Based on short course and symposium sponsored by the Division of Cellulose. *Paper and Textile Chemistry at* 185th meeting of the American Chemical Society, Seattle, Washington.

[A.V] Garrote, G., Falqué, E., Domínguez, H. & Parajó, J. C. (2007). Autohydrolysis of agricultural residues: Study of reaction by products. *Bioresource Technology*, 98, 1951-1957.

[A.VI] Singleton, V. L. & Rossi, J. A. (1965). Colorimetric of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture, 16*, 144-158.

[A.VII] Blasa, M., Candiracci, M., Accorsi, A., Piacentini, P. M., Albertini, M. C. & Piatti, E. (2005). Raw Milleflori honey is packed full of antioxidants. *Food Chemistry, 97*(2), 217-222.

[A.VIII] Gullón, B., Pintado, M. E., Barber, X., Fernández-López, J., Pérez-Álvarez, A. & Viuda-Martos, M. (2015). Bioaccessibility, changes in the antioxidant potential and colonic fermentation of date pits and apple bagasse flours obtained from co-products during simulated *in vitro* gastrointestinal digestion. *Food Research International, 78*, 169-176.

[A.IX] Gullón, B., Gullón, P., Tavaria, F., Pintado, M., Gomes, A.M., Alonso, J.L. & Parajó, J.C. (2014). Structural features and assessment of prebiotic activity of refined arabinoxylooligosaccharides from wheat bran. *Journal of Functional Foods, 6*(1), 438-449.

[A.X] Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M. & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology & Medicine, 26*(9-10), 1231-1237.

[A.XI] Von Gadow, A., Joubert, E. & Hansmann, C. F. (1997). Comparison of the antioxidant activity of aspalathin with that of other plant phenols of Rooibos tea (Aspalathus linearis), α-tocopherol, BHT and BHA. *Journal of Agricultural and Food Chemistry, 45*(3), 632-637.

[A.XII] Benzie, I. F. F. & Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of antioxidant power: the FRAP assay. *Analytical Biochemistry, 239*(1), 70-76.

[A.XIII] Moreira, D., Gullón, B., Gullón, P., Gomes, A. & Tavaria, F. (2016). Bioactive packaging using antioxidant extracts for the prevention of microbial food-spoilage. *Food & Function, 7*(7), 3273-3282.

[A.XIV] Chen, L., Wang, X., Yang, H., Lu, Q., Li, D., Yang, Q. & Chen, H. (2015). Study on pyrolysis behaviours of non-woody lignins with TG-FTIR and Py-GC/MS. *Journal of Analytical and Applied Pyrolysis, 113*, 499-507.

[A.XV] Miles, A. A., Misra, S. S. & Irwin, J. O. (1938). The estimation of the bactericidal power of the blood. *The Journal of Hygiene, 38*(6), 732-749.

[A.XVI] Erdocia, X., Prado, R., Corcuera, M. T. & Labidi, J. (2014). Effect of different organosolv treatments on the structure and properties of olive tree pruning lignin. *Journal of Industrial and Engineering Chemistry, 20*(3), 1103-1108.

[A.XVII] Liu, J., Pan, Y., Yao, C., Wang, H., Cao, X. & Xue, S. (2015). Determination of ash content and concomitant acquisition of cell compositions in microalgae via thermogravimetric (TG) analysis. *Algal Research, 12*, 149–155.

[A.XVIII] Huijgen, W. J. J., Telysheva, G., Arshanitsa, A., Gosselink, R. J. A. & De Wild, P. J. (2014). Characteristics of wheat straw lignins from ethanol-based organosolv treatment. *Industrial Crops and Products, 59*, 85-95.

[A.XIX] Sequeiros, A. & Labidi, J. (2017). Characterization and determination of the S/G ratio via Py-GC/MS of agricultural and industrial residues. *Industrial Crops and Products, 97*, 469-476.

List of tables in appendix

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Appendix I

The employment of wastes generated by agricultural activities such as the viticulture for the obtaining of high-added value products could produce economic profits and contribute to the establishment of bio-based economy. In this context, the integral revalorisation of a winery waste such as the vine shoots was proposed for the obtaining of energy and multiple added-value products. The environmental impact of the revalorisation of these feedstocks was evaluated.

This work has been carried out thanks to the financial support of the Basque Government

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