Bruno Miguel da Silva Pedras

Licenciado em Bioquímica

Valorization of grape pomace through hot compressed water extraction/hydrolysis

Dissertação para obtenção do Grau de Mestre em Biotecnologia

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FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

Setembro 2015

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Resumo

Nos próximos anos é esperado que o consumo mundial de energia aumente bastante devido às economias emergentes. É portanto necessário aumentar a utilização de energias renováveis. A biomassa é o única fonte de carbono renovável que existe em larga escala para ser usada como fonte de energia.

As massas vínicas são um dos resíduos agro-industriais que existem em maior quantidade em todo o mundo, sendo um boa fonte de biomassa.

O objectivo deste trabalho consiste na valorização das massas vínicas de uva branca (WWGP) e uva tinta (RWGP), através da extração de compostos fenólicos com actividade antioxidante, bem como da extracção/hidrólise dos carbohidratos, usando água subcrítica (HCW). Este trabalho foca-se principalmente na optimização do processo para WWGP, enquanto para RWGP apenas foi testado um conjunto de condições. As temperaturas usadas foram 170, 190 e 210 °C para WWGP, e 180 °C para RWGP. Os caudais de água usados foram de 5 e 10 mL/min, e a pressão foi sempre mantida a 100 bar.

Antes de usar HCW foi feita um caracterização química dos dois resíduos, que revelou que a WWGP tem um grande teor de açúcares livres (40%, aproximadamente), essencialmente glucose e frutose, enquanto a RWGP tem teores mais elevados de lípidos, proteínas, lenhina e açúcares estruturais.

Para a WWGP, os melhores resultados foram obtidos a 210 °C e 10 mL/min: rendimento em compostos solúveis (69 wt.%), extracção de fenóis (26.2 mg/g) e recuperação de açúcares (49.3 wt.% face a 57.8% existentes). Para a RWGP as condições não foram optimizadas (180 °C e 5 mL/min), sendo os valores do rendimento em compostos solúveis (25 wt.%), extracção de polifenóis (19.5 mg/g) e recuperação de açúcares (11.4 wt.% face a 33.5% existentes) muito mais baixos.

Foi também determinada a actividade antioxidante dos extractos obtidos em cada ensaio, sendo o melhor resultado para a WWGP, nomeadamente nos extractos obtidos a 210 °C (EC_{50}=20.8 µg/mL, em que EC_{50} = concentração que induz metade do efeito máximo; EC_{50} = 22.1 µg/mL para a RWGP, a 180 ºC).

Palavras-chave: biomassa, massas vínicas, água subcrítica, polifenóis, carbohidratos, actividade antioxidante

Abstract

The world energy consumption is expected to increase strongly in coming years, because of the emerging economies. Biomass is the only renewable carbon resource that is abundant enough to be used as a source of energy

Grape pomace is one of the most abundant agro-industrial residues in the world, being a good biomass resource.

The aim of this work is the valorization of grape pomace from white grapes (WWGP) and from red grapes (RWGP), through the extraction of phenolic compounds with antioxidant activity, as well as through the extraction/hydrolysis of carbohydrates, using subcritical water, or hot compressed water (HCW). The main focus of this work is the optimization of the process for WWGP, while for RWGP only one set of parameters were tested. The temperatures used were 170, 190 and 210 °C for WWGP, and 180 °C for RWGP. The water flow rates were 5 and 10 mL/min, and the pressure was always kept at 100 bar.

Before performing HCW assays, both residues were characterized, revealing that WWGP is very rich in free sugars (around 40%) essentially glucose and fructose, while RWGP has higher contents of structural sugars, lignin, lipids and protein.

For WWGP the best results were achieved at 210 °C and 10 mL/min: higher yield in water soluble compounds (69 wt.%), phenolics extraction (26.2 mg/g) and carbohydrates recovery (49.3 wt.% relative to the existing 57.8%). For RWGP the conditions were not optimized (180 °C and 5 mL/min), and the values of the yield in water soluble compounds (25 wt.%), phenolics extraction (19.5 mg/g) and carbohydrates recovery (11.4 wt.% relative to the existing 33.5%) were much lower.

The antioxidant activity of the HCW extracts from each assay was determined, the best result being obtained for WWGP, namely for extracts obtained at 210 °C (EC_{50}=20.8 μg/mL; EC_{50} = half maximum effective concentration; EC_{50} = 22.1 μg/mL for RWGP, at 180 ºC).

Keywords: Biomass, grape pomace, hot-compressed water, phenolic compounds, carbohydrates, antioxidant activity

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DPPH - 2,2-diphenyl-1-picrylhydrazyl

EIA - Energy Information Administration

GP - grape pomace

HCW - hot compressed water

OECD - Organization for Economic Co-operation and Development

P - Pressure

RWGP - red wine grape pomace

T - Temperature

TPC - total phenolic content

WWGP - white wine grape pomace

1 State of the art

1.1 Biorefinery concept

Energy resources will play an important role in the world's future [1]. Today there is great concern about energy resources and when global oil production will reach its peak. Most predictions indicate that this will occur in the early twenty-first century, and that afterwards production will decrease but demand will rise, leading to a dramatic increase in price. According to the U.S. Energy Information Administration (EIA), world energy consumption is expected to increase strongly, around 50% between 2009 and 2035, because of the emerging economies outside the OECD (Organization for Economic Cooperation and Development). These nations are expected to account for 84% of growth in world energy use, while developed OECD nations are expected to account for only 14%. And unless the use of renewable energies rises, fossil fuels will account for 90% of the increase in world energy consumption [2]. These projections leads to increasing interest from industries to seek sustainable and alternative sources for energy [3].

Energy sources are divided in three categories: fossil fuels, renewable sources and nuclear sources. The fossil fuels include coal, petroleum and natural gas. The world energy markets are very dependent on fossil fuels as sources of energy, fuels and chemicals, and since millions of years are needed to form them on Earth, their reserves are finite at human scale and subject to depletion [1]. The renewable sources already used are wind, sun, water and biomass. Nuclear sources consist in nuclear fission and fusion.

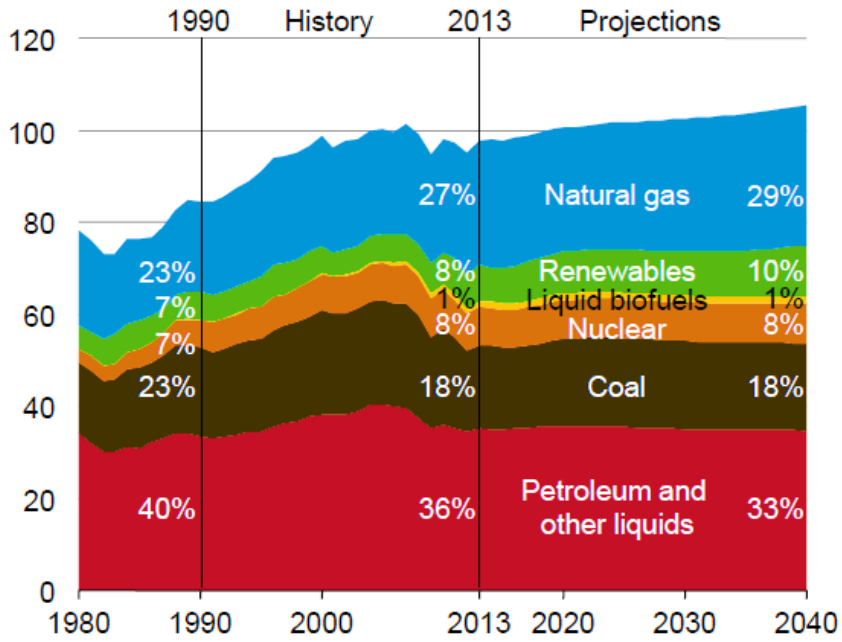

Figure 1.1 - World primary energy consumption in the U.S. between 1980 and 2040 [4] .

Petroleum is the most frequently used raw material, which is a problem because its amount is limited, and also because it is not environmentally friendly [5]. With global demand for energy increasing, carbon dioxide emissions are expected to reach a new record, increasing from 31Gt in 2011 to nearly 37 Gt in 2035 [6], and resulting in global warming, also caused by CH_4 and N_2O emissions. This, as well as the accumulation of plastics in landfill sites, acid rain, smog in high industrialized areas, and ground water pollution, are all due to the use of oil and other fossil fuels [3]. The concerns over climate change and energy security are the main drivers to encourage the growth of renewable energy, which already contributes 13% to global energy supply, of which around 10% comes from bioenergy [6] – energy content of solid, liquid or gaseous products from biological raw materials, or biomass.

Biomass in the only naturally-occurring, energy-containing carbon resource that is large enough to be used as a substitute for fossil fuels, because it has intrinsic chemical energy content [1] and is a green and sustainable supply of energy. Biomass is composed of a variety of forestry and agricultural processing resources, industrial processing resources, municipal solid and urban wood residues. The forestry resources are the residues from the harvesting of forest products, fuel wood extracted from forestlands, residues generated at primary forest product processing mills, and forest resources that could become available due to initiatives to reduce fire hazards and improve forest health. The agricultural resources are grains used for biofuels production, animal residues, and crop residues as cotton, sugarcane, rice, fruit, and nut fruit. Industrial process resources are primary and secondary wood processing mill residues, pulping liquors and food and feed processing residues. Municipal and urban wood residues include a

variety of materials, such as yard and tree trimmings, land-clearing wood residues, wooden pallets, packaging materials, and construction and demolition debris [7].

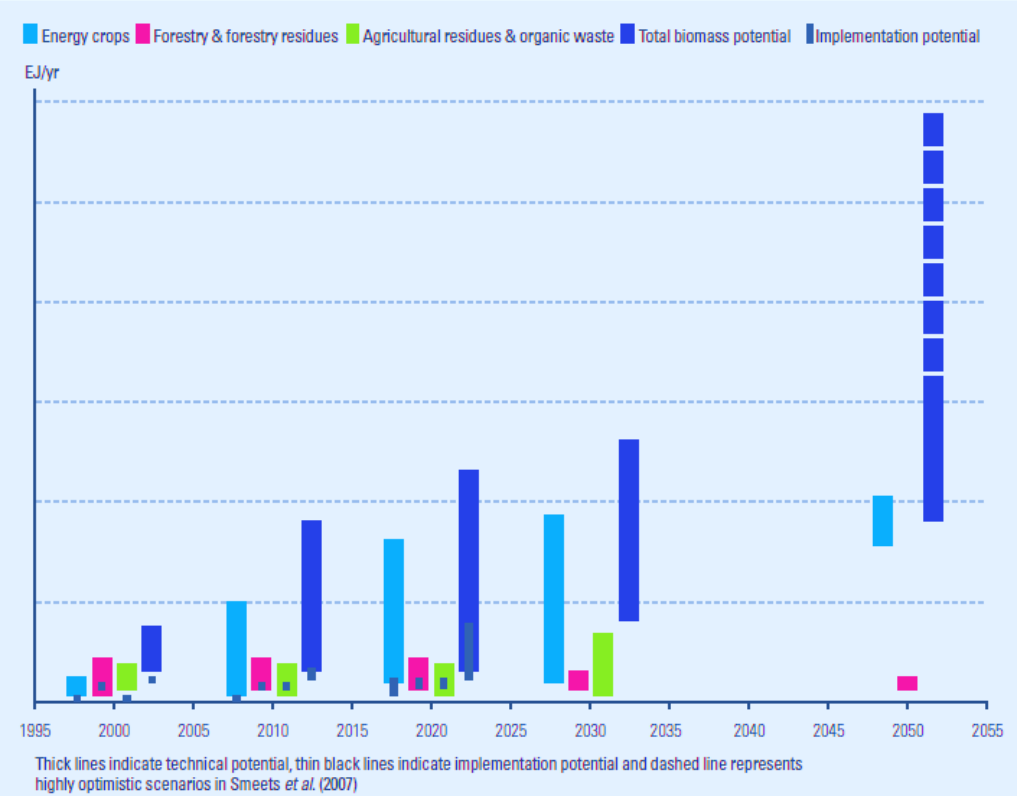

Figure 1.2 - Biomass potential in Europe [8].

With the amount of available biomass, a shift could be made in the development of processes to generate products needed at large scale with low price, such as products now being made through oil refineries. In classic refineries, the raw material is petroleum, used to produce a variety of products that are sold directly or transformed into value-added products, such as plastics or fibers. In the U.S., 17% of products derived from petroleum are classified as chemicals. If these chemicals could be obtained from renewable sources like biomass, it would be good for the environment and the dependence from fossil fuels would decrease [9]. This is the biorefinery concept, using a combination of processes in the production of fuel, energy and value-added products from biomass [3].

The term biorefinery is widely used nowadays, but it covers a range of industrial realities, and several definitions for the term biorefinery have been developed over the last few years. But the core concept is the conversion of biomass into several product streams – materials, chemicals, energy, food and feed – and the integration of various technologies and processes in the most sustainable way. The EIA has the following definition: "Biorefining is the sustainable processing of biomass into a spectrum of marketable products and energy" [8].

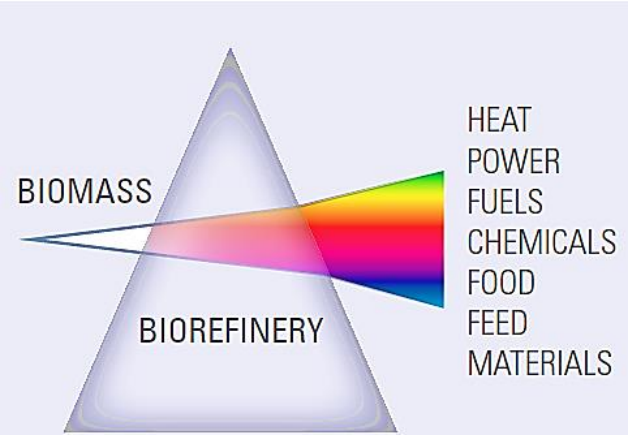

Figure 1.3 - Biorefinery concept representation [8].

Currently research is being developed around three biorefinery systems. The first is the "Whole Crop Biorefinery", using cereals or maize as raw material, the second is the "Green Biorefinery", using "nature-wet" biomasses, such as green grass or immature cereal, and the third is the "Lignocellulose Feedstock Biorefinery", using "nature-dry" raw material, such as cellulose-containing biomass and wastes. Lignocellulose material is very abundant, because it is present in all vegetable cells. Waste biomass from some process can be raw material for another process, and thus a low-cost resource can be a good resource for the production of products of interest and added-value.

The next figure shows various products that can be obtained from this so rich and abundant material.

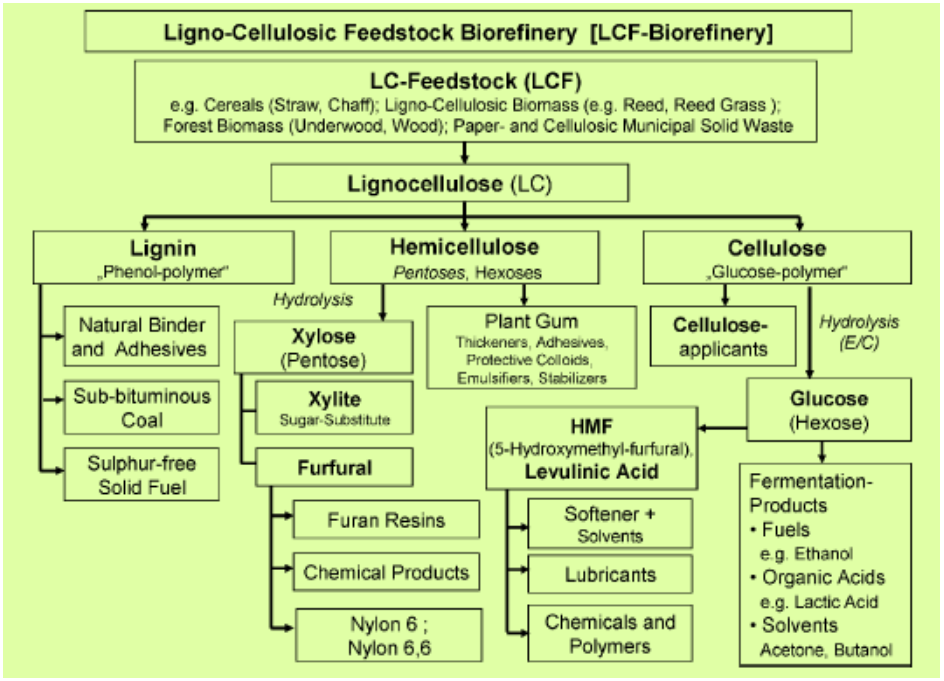

Figure 1.4 - Lignocellulose feedstock biorefinery [5].

The main technologies used in biorefineries are extraction, biochemical and chemical processes. It is important that the strengths and weaknesses of the used technologies are recognized to enable the integration of different technologies and feedstocks, and to maximize the diversity of applications and products formed [10].

The utilization of biomass as carbon feedstock instead of petroleum is only part of the sustainable process concept. To protect the environment during the development of biorefineries, the Green Chemistry principles have to be followed. In particular, it must be avoided the use or generation of environmentally harmful and hazardous chemicals. The aim of combining biorefinery with green chemistry is the production of green and sustainable chemical products. When a new process is developed, it should aim at using sustainable feedstocks, during the production and during the product life cycle, energy demands should be minimized, safer processes should be used, hazardous chemical use and production avoided, and the final product should be non-toxic, environmentally friendly and easily recyclable with minimum production of waste [3].

1.2 Agro-industrial residues. Carbohydrates

Most of the biomass used comes from agro-industrial residues, because these are renewable, low-cost, and the most abundant resource on earth. They are constituted by various agricultural and food industry wastes, deriving from the processing of a particular crop or animal product, usually by an agricultural firm.

The materials included are mainly molasses, bagasse, oilseed cakes and maize milling by-products. Crop residues include all agricultural residues such as straw, stem, stalk, leaves, husk, shell, peel, lint, seed, pulp, cotton, groundnut, jute, legumes, coffee, cacao, olive, tea, fruits and palm oil [11].

There are various types of agro-industrial residues, and for each type there are more appropriate treatments. In this context, these residues can be divided into two groups: Dry residues and Wet residues. Dry residues include field and seed crops, which are materials remaining above the ground after harvesting, fruit and nut crops that are mainly orchard pruning and brushes, vegetable crops that consist in vines and leaves remaining after harvesting, and nursery crops that are essentially the pruning and trimmings taken from plants during their growth and preparation for market. The wet residues are constituted by animal slurry, farmyard manure and grass silage [11].

Agro-industrial residues are mainly composed by lignocellulose, a compact, partially crystalline structure consisting in cellulose, hemicellulose and lignin. Cellulose is a regular, linear, homopolymer made of D-glucose monomers linked by β-(1→4) glycosidic linkages.

Cellulose is the dominant structural polysaccharide. Hemicellulose is a polysaccharide made of various sugar monomer units, such as xylose, galactose, mannose, arabinose, and also glucose, which makes it non-crystalline. The function of hemicellulose is binding cellulose and lignin. Lignin is a random, three-dimensional phenyl-propanoic polyphenol [12]. Lignin found in nature is made of three monomers: coniferyl alcohol, sinapyl alcohol and p-coumaryl alcohol, arranged in an irregular structure that provides strength and resistance to enzymatic degradation [11].

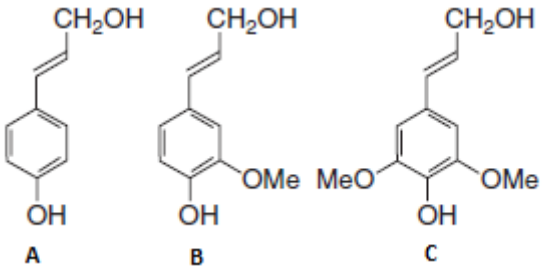

Figure 1.5 - The constituents of lignin: p-coumaryl alcohol (A), coniferyl alcohol (B) and sinapyl alcohol (C) [11].

Table 1.1 shows the composition of common agro-industrial residues.

Table 1.1 - Composition of a wide range of agro-industrial residues [11].

Agro-industrial residues	Lignin (wt.%)	Cellulose (wt.%)	Hemicellulose (wt.%)
Corn cobs	6.1-15.9	33.7-41.2	31.9-36.6
Sugarcane bagasse	10-20	40-41.4	27-37.5
Wheat straw	8.9-17.3	32.9-50	24-35.5
Rice straw	9.9-2.4	36.2-47	19-24.5
Corn stalks	7-18.4	35-39.6	16.8-35
Barley straw	13.8-14.5	33.8-37.5	21.9-24.7
Rye straw	19.0	37.6	30.5
Oat straw	17.5	39.4	27.1
Flax	22.3	34.9	23.6
Soya stalks	19.8	34.5	24.8
Sunflower stalks	13.4	42.1	29.7
Vine shoots	20.3	41.1	26.0
cotton stalks	21.5	58.5	14.4
Sunflower seed hulls	29.4	24.1	28.6
Thistle	22.1	31.1	12.2

Microorganisms can produce a variety of products through glucose fermentation, and enzymatic saccharification is one of the best strategies to achieve this due to its low energy requirement and low level of pollution caused. The problem with agro-industrial residues is that cellulose in the lignocellulosic structure is not immediately available for hydrolysis, due to its binding to hemicellulose and enclosure by lignin. Basically cellulose forms a skeleton surrounded by hemicellulose, which works as matrix with lignocellulose encrusted. It is suggested that covalent cross-linking between lignin and polysaccharides are behind the remarkable stability of lignocellulosic materials against biological attack.

To make cellulose accessible to enzymes, a pre-treatment is required. This process has to be effective and economic, and various techniques have been studied. An appropriate pre-treatment has to disrupt hydrogen bonds in crystalline cellulose, break down the cross-linked matrix of hemicellulose and lignin, and increase the porosity and surface area of cellulose for enzymatic hydrolysis [13]. The next figure represents schematically the pre-treatment of lignocellulosic materials.

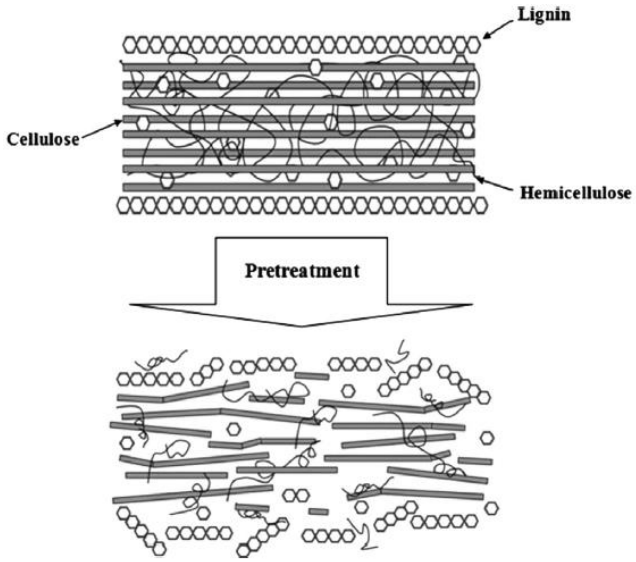

Figure 1.6 - Representation of the pre-treatment effect on lignocellulose [13].

This pre-treatment can be physical, chemical, biological or enzymatic, as represented in Table 1.2.

Table 1.2 - Different pre-treatments for lignocellulosic materials [11].

Pre-treatment	Examples	Effect of Pre-treatment
Physical	Milling	Fine, highly decrystalized structure
	Steam Explosion, Steaming traetment	Increased pore size/hemicellulose-hydrolysis
	Hydrothermal	Hemicellulose hydrolysis, alteration in properties of cellulose and lignin
	Irradiation	Depolymerization
Chemical	NaOH, NH_3, H_2O_2, Peroxyformic acid, Organosolvents	Lignin/hemicellelose degradation
	Peroxymonosulphate	Activates delignification
Biological	White-rot fungi (*Bjerkendraadusta,Phanerochaete chysoporium, Cerporiopsis subvermispora),* Specific bacteria	Lignin degradation
Enzymatic	Lignin Peroxidases (LiP, MnP, laccase)	Selective lignin/hemicellulose degradation

1.3 Valorization of agro-industrial residues

The research and development of valorization processes for agro-industrial residues has been the object of much attention. This growing interest is due to the increased necessity to take into consideration aspects like pollution of the environment and economy. There is the need for new methods and policies for waste handling and treatment, for the recovery, bioconversion and utilization of valuable residues that in addition to creating pollution and being potentially hazardous, can be recycled or be raw materials for conversion into added-value products.

Many products made from fossil resources can be replaced by others made from greener, bio-based technologies. The problem is that these new products only have market interest if they are cheaper than the petroleum derived products. In fact, in almost all the cases the developed technologies make the final product more expensive owing to the costs of the processes. The new product also has to prove that it performs at least as well as the petroleum-based product, with lower environmental impact [14].

Historically, bio-based chemical producers have targeted high-value fine, or specialty, chemicals markets, often where specific functionality played an important role. But the low price of crude oil has acted as a barrier to bio-based commodity chemical production and producers focused on the specific attributes of bio-based chemicals, due to high production costs.

The foreseen increase in oil prices, the consumer demand for environmentally friendly products, population growth and limited supplies of non-renewable resources give now new opportunities for bio-based chemicals and polymers.

The transition to a bio-based economy is driven by an over-dependency of many countries on fossil fuel imports, the anticipation that oil, gas, coal and phosphorus will reach a peak production in the near future, the need of countries to start using different energy sources, the global warming issue and the desire to reduce greenhouse gases production, as well as the need to stimulate regional and rural development. The production of bio-based products (chemicals, materials) can be made from single processes. However the production in integrated biorefinery processes, producing both bio-based products and secondary energy carriers (fuel, power, heat), as in oil refineries, is apparently a more efficient approach for the sustainable valorization of biomass resources in a bio-based economy.

The main driver for biorefineries development and implementation is the transportation sector because of the large quantities of fuel needed every day, mainly in heavy duty road transport and in aviation, which makes necessary the integration of fuels from renewable sources (biofuels). The problem with biofuels is their production cost, which is higher than for oil fuels. A very promising approach to reduce biofuel production costs is using integrated biorefinery processes. In a biorefinery biofuel is produced but also are value-added products from biomass resources in a very efficient approach, because the added-value of the co-products makes possible to produce fuels with lower costs that make them viable. An example is the production of ethanol through fermentation, where the required sale price of ethanol was € 775 per ton. But with the split of carbohydrate stream, 80% for ethanol and 20% for lactic acid production, the required sale price of ethanol was reduced to € 545 per ton. Another example is biodiesel production, which had a required sale price of € 765 per ton, but in an integrated process, producing also epichlorohydrin from glycerol – a by-product of biodiesel production – came down to a sale price of € 735 per ton [14].

With the diversity of industrial biorefinery processes, raw materials and bio-based products, EIA created eight different platforms: Syngas platform, Biogas platform, C6 and C5/C6 sugar platform, Plant-based oil platform, Algae oil platform, Organic Solutions Platform, Lignin Platform and Pyrolysis oil platform [14].

In the C6 and C6/C5 sugar platform, six-carbon sugars can be accessed from sucrose or through cellulose or starch hydrolysis to give glucose, which serves as feedstock for many

fermentation processes to access a variety of chemical building blocks. Fermentation has been used extensively by the chemical industry to produce many products, in the scale of 8 million ton per year. The six- and five-carbon sugars are produced from hemicellulose hydrolysis, which can lead to the similar products through fermentation processes.

In addition to being used for fermentation, six- and five-carbon sugars can undergo selective dehydration, hydrogenation and oxidation reactions to give useful products, such as sorbitol, furfural, glucaric acid, hydroxymethylfurfural and levulinic acid. These products have a wide range of applications in the pharmaceutical and food Industries. For example, over a million ton of sorbitol are produced per year for use as food ingredient, personal care ingredient (toothpaste) and for industrial use [14].

These technologies have a promising future because of their sustainability based on the use of wastes as raw material, adding-value to them, reducing their accumulation in the environment, and at the same time making them a source of revenue, as opposed to spending money treating them as residues from industries. The processes described above involved fermentative processes to develop added-value products from lignocellulosic materials. But these materials also contain bioactive molecules, which can be obtained using extraction processes [15]. The two approaches can be integrated, whereby bioactive compounds are first extracted, and then the resulting biomass, essentially carbohydrates and lignin, can be used to obtain other products.

Residues from the food and agricultural industries have great potential to be raw materials for biorefineries because they are a rich source of carbohydrates and bioactive molecules.

A good example is the valorization of apple pomace. Apple pomace is a left-over solid residue obtained after extraction of apple juice, consisting in 25-30% of the total processed fruits. Several million tons of apple pomace are generated worldwide, and there is a cost associated with its treatment. But apple pomace contains numerous phytochemicals in the form of simple sugars, pectin, dietary fibers and natural antioxidants. Apple pomace is being studied as substrate for microbial growth in the production of value-added products, such as organic acids, enzymes, single cell proteins, low alcoholic drinks, ethanol, biogas, pigments and baker's yeast. In addition to fermentative processes, another focus is the isolation of bioactive molecules from apple pomace, namely pectin extraction, and then dietary fiber, natural antioxidants and aromatic compounds. Other applications of apple pomace, as textile dye removal, heavy metal absorbent and protein stabilizer, were also studied [15].

Another example are mango seeds, which are available in large quantities in tropical countries and are simply discarded or used as animal feed. Mango seeds are a natural and renewable resource for extraction of cellulose nanocrystals, which can be used in the production

of new polymeric materials. This adds value to an agro-industrial residue, making it an alternative to fossil resources [16].

Also tomato is one of the most widely cultivated vegetable crops in Mediterranean countries. During tomato processing into products such as tomato juice, paste, purée, ketchup sauce and salsa, a by-product known as tomato pomace is produced, representing 4% of fruit weight. This residue is rich in nutrients and can be used as a fiber, protein and fat source [17].

In this work, the aim is the valorization of grape pomace from white and red wine processes. Grape pomace is rich in bioactive compounds, such as antioxidants that could have application in the pharmaceutical, food and cosmetic industries, as well as being rich in carbohydrates that can be hydrolysed and processed by microorganisms into other products, such as carotenoids [18].

1.4 Grapes and the wine making process

Grapes of *Vitis vinifera* are one of the world's largest fruit crops in the world, with more than 70 million tons produced per year [19]. According to the OIV report, in 2014 45% of produced grapes were used as unpressed grapes, 83% to be consumed as fresh grapes and the rest as dried grapes. The remaining 55% of grape production were pressed and used for wine production. In 2014, over 50% of the world vineyard was accounted for by 5 countries, namely Spain (14%), China (11%), France (10%), Italy (9%) and Turkey (7%). Portugal occupied the 8th place. About 41% of world grapes were produced in Europe, 29% in Asia and 21% in America, the biggest world producer being China, followed by the U.S., France and Italy. In China almost all grapes are used as fresh grapes, while in European countries, namely France, Italy and Spain, and in the U.S., grapes are mostly used for wine production. In 2014, France was the world biggest wine producer, with 46.7 millions of hectoliters (17%), followed by Italy (16%), Spain (15%) and the U.S. (8%). Portugal was the 11th world producer, with 6.2 millions of hectoliters [20].

Wine making is considered as much an art as a science. Basically, wine is the product of total or partial alcoholic fermentation of fresh grapes with a minimum alcohol percentage of 8.5%. There are three types of wines: still wine, fortified wine and sparkling wine. Still wine has the simplest making process, consisting in normal fermentation after grape pressing. Still wine has no gas. It can be white, rosé or red, depending on the production method and grapes used. There are basically two methods for making wine: white and red. These have a major difference, which is that in the white method the grape juice is decanted before fermentation, while in the red method the seeds, skins and pulp are removed only after fermentation in order to maintain grapes' pigments and tannins in the wine. Red wine is made through the red method

using red grapes, white wine through the white method but can use white or red grapes, and rosé wine can be made from both white and red grapes through the white method, but the skins stay a short time in contact with the juice to transfer some pigments to the wine. Sparkling wine has a similar process, but has a final step of fermentation in the bottle, creating carbon dioxide bubbles. Fortified wines are made by adding alcohol during the fermentation process, which inhibits the fermentation, thus creating a sweeter wine since not all the sugar has been transformed into alcohol. In Portugal there are famous fortified wines, such as Port wine, Madeira wine and Moscatel [21].

Figure 1.7 - The three different types of still wine: white, red and rosé wines [21].

1.5 Grape pomace

The wine-making process generates grape pomace as by-product, which is estimated to represent about 20% by weight of the grapes [22]. Grape pomace is the residue that remains after pressing grapes for obtaining juice. In the last few years, a lot of research has been done on industrial applications of grape pomace, such as the isolation of nutritional ingredients, dietary fibre, production of citric acid, tartaric acid, ethanol, grape seed oil, natural food colorants and compounds for therapeutic use, in order to add value to this residue [23–26].

Grape pomace consists of stalks (30%), seeds (30%) and skin and pulp (40%) [23]. Marc is the name given to grape skin and pulp. Grape pomace is used in the preparation of spirits – "Grappa" or "Marc" – in cattle feeding, soil conditioning, as fertilizer or is simply dumped in disposal sites. The disposal of grape pomace is a problem for wineries, because it represents thousands of tons, and if not treated, grape pomace can create various environmental hazards, from surface and ground water pollution to foul odors that attract flies, pests and can easily spread diseases. Certain compounds from grape skin and pulp can cause oxygen decrease in the soil and infiltrate surface, soil and ground waters.[27] Saturated markets and stricter requirements have brought about the decline of the utilization of grape pomace for making spirits, in the past

few years [28]. This meant that wine producers were no longer able to generate revenues from their grape pomace, and instead had to pay to dispose of great amounts of waste.

Figure 1.8 - Dry grape pomace from red wine (A) and white wine (B) production.

Today it is known that grape pomace is an important source of phenolic compounds [23]. These have been the center of attention of recent studies due to their relation with the beneficial effects attributed to a moderate consumption of wine [29], and will be referred later.

Grape pomace is a rich source of sugars with various applications. These sugars are in a complex lignocellulosic matrix of around 34-50% cellulose, 19-34% hemicellulose and 11-30% lignin [30]. Grape pomace has a high content of dietary fiber, representing 80% of dry matter, of which insoluble fiber accounts for the major part, followed by protein.

Grape skins are composed of cellulose, hemicellulose, pectin and lignin arranged in a complex matrix, cell walls having the greater amounts of phenolic constituents. Approximately 50% of the grape skins fraction is cellulose.

The grape seed is a complex matrix comprising around 40% fiber, 16% oil, 11% proteins and 7% complex phenols, sugars and mineral salts. A product of interest from grape seed in addition to phenolic compounds is grape seed oil, which represents 8-22% of the seed and has a content of over 86% of unsaturated fatty acids, such as linoleic acid [31]. Other fatty acids include oleic acid, palmitic acid and stearic acid. Grape seed oil is marketed as low cholesterol frying oil. Grape seed extracts have been reported to have various pharmacological applications, most of them attributed mainly to phenolic compounds [23]. But grape seed also contains non-phenolic antioxidants, such as tocopherols and β-carotene, both vitamins that are potent antioxidants and are critical to human health.

1.6 Phenolic Compounds

Phenolic compounds represent one of the most numerous, important [32] and widely distributed groups of natural products in the plant kingdom. They are synthesized during the normal development of the plant, as well as in response to different situations, such as stress and UV radiation [19]. Phenolic compounds exhibit a wide range of physiological properties, such as anti-allergenic, anti-artherogenic, anti-inflammatory, anti-microbial, anti-oxidant, anti-thrombotic, cardioprotective and vasodilatory effects. They are normally associated with the health benefits derived from consuming high levels of fruits and vegetables [33].

In this context, grape pomace is an underused residue of the wine making process, because during vinification only 30-40% of phenolic compounds are extracted, depending on grape varieties, vineyard location and technological parameters of wine making (destemming, crushing, maceration and pressing) [34]. For this reason, there is great interest in the exploitation of this grape by-product to obtain potentially bio-active compounds. Winery residues could thus be an alternative source for obtaining natural antioxidants (any substance that when present at low concentrations, compared with those of the oxidizable substrate, significantly delays or inhibits oxidation of this substrate [35]) that can be considered completely safe in comparison with the synthetic ones that are now largely used in the food industry but have reported undesirable effects on the enzymes of human organs [29].

Structurally, phenolic compounds comprise an aromatic ring, bearing one or more hydroxyl substituents, and range from simple phenolic molecules to highly polymerized compounds. Most naturally occurring phenolic compounds are present as conjugates with mono- and polysaccharides, linked to one or more of the phenolic groups, and may also occur as functional derivatives, such as esters and methyl esters [33].

Five major classes of polyphenols can be found in food: Phenolic acids, flavonoids, tannins, lignans and stilbenes.

Phenolic acids are phenols with a carboxylic group and consist of two subclasses, namely hydroxybenzoic and hydroxycinnamic acids. Hydroxybenzoic acids include gallic, p-hydroxybenzoic, protocatechuic, vanillic and syringic acids, which have in common the C6-C1 structure. Hydroxycinnamic acids, on the other hand, are aromatic compounds with a three-carbon side chain (C6-C3), the most abundant being p-coumaric, ferulic, caffeic and sinapic acids [33].

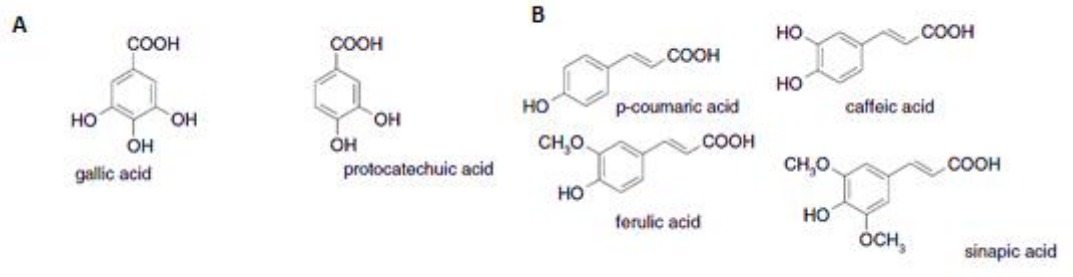

Figure 1.9 - Representation of some phenolic acids: a - hydroxybenzoic acids and b - hydroxycinnamic acids [33].

Flavonoids are the largest group of plant phenolics and the best studied polyphenols group. Flavonoids are low molecular weight compounds consisting in a diphenyl propane (C6-C3-C6) skeleton [36]. Essentially the structure consists of two aromatic rings 1 and 2, joined by a bridge of three carbons in the form of a heterocyclic ring 3 (figure 1.10). The variations in ring 3 result in the major flavonoid classes, which are flavonols, flavones, flavanones, isoflavones, flavanonols and anthocyanidins and chalcones [31]. Flavones and flavonols are the most widely occurring and structurally diverse [33].

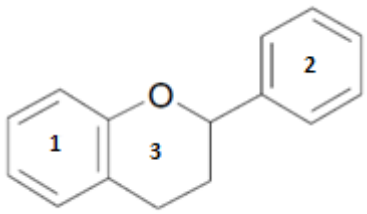

Figure 1.10 - Main structure of flavonoids [33].

The two main types of tannins are condensed tannins and hydrolysable tannins. Condensed tannins (proanthocyanidins, also called procyanidins or oligomeric procyanidins) are the major phenolic compounds found in grapes. When in contact with salivary proteins, they are responsible for the astringency of fruits [32]. Grape seed oil has high amounts of tannins, 1000 times higher than other seed oil. It is reported that all the acylated procyanidins found in grape seed are esters of gallic acid.

Lignans consist of two phenylpropanoid moieties connected via their C8-side chain carbons. They are not very abundant in fruit.

The main stilbenes found in food of plant origin are resveratrol and its glycosides. Resveratrol is a phytoalexin produced in plants in response to pathogen attack and is a naturally occurring fungicide [31]. Resveratrol is the common term for 3,5,4'-hydroxystilbene, and it can exist in both *cis* and *trans* isomeric forms, the *trans* form being the most commonly found in

plants, and better studied [37]. It is an important polyphenol found in grape skins and seeds, largely in red ones. Resveratrol can also be found in peanuts, berries and rhubarb.

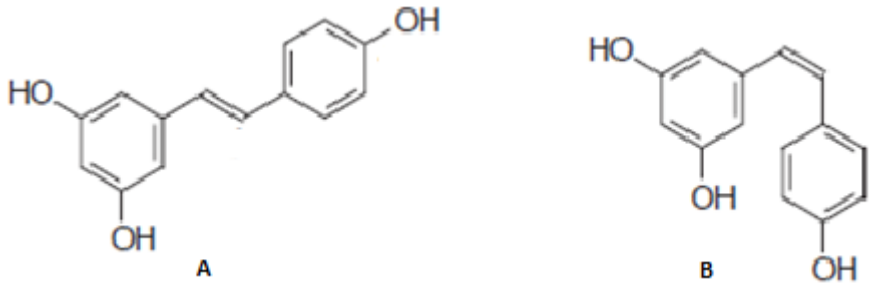

Figure 1.11 - The two isomeric forms of resveratrol: *trans* (A) and *cis* (B) [37].

Although phenolics are present in all plant foods, some fruits, such as grapes, are extremely rich in these bioactive compounds, present usually in skins, seeds and short stems. Grape pomace is rich in extractable phenolic compounds, the main ones being anthocyanins, catechins, procyanidins, flavonol glycoside, phenolic acids, and stilbenes. Their amount can vary considerably. An example are anthocyanins, which exist in great amount in red grape pomace but not in white grape pomace, which in turn is very rich in flavanols. Grape pomace also contains high amount of non-extractable polyphenols, such as polymerized condensed tannins and some polyphenols that form complexes with fiber [31].

The antioxidant activity of phenolic compounds is due to their ability to scavenge free radicals, donate hydrogen atoms or electrons, or chelate metal cations. These properties are directly linked with their structure. In the case of phenolic acids, the antioxidant activity depends of the number and position of the hydroxyl groups in relation to the carboxyl group. E.g. the antioxidant activity of trihydroxylated gallic acid is high [33].

Hydroxycinnamic acids exhibit higher antioxidant activity than hydroxybenzoic acids, which could be associated to the CH=CH-COOH group that can easily donate the proton and stabilize the radical in comparison with the -COOH group.

In flavonoids, the relation between structure and antioxidant activity is more complex, but the principles are the same. Antioxidant activity is determined by changes in rings 2 and 3 (figure 1.10), and when there exist double bonds in ring 3 and/or hydroxyl groups in rings 2 or 3, the activity is higher [33].

The possible health benefits derived from phenolic compounds depend on their absorption and metabolism, determined by their structure, conjugation with other phenolics, degree of glycosylation, molecular size and solubility [33].

Polyphenols have known health promoting effects and other properties in different biological and food systems, related with antioxidant characteristics as reducing agents, inhibiting and delaying lipid oxidation in diverse food systems [19]. They also have a positive influence on cardiovascular health and some types of cancer [35]. Resveratrol, for example, can be beneficial for human health, has low toxicity in humans and is used to prevent lipid oxidation.

Studies also have revealed that resveratrol inhibits the growth of human breast and prostate cancer cell lines, and induces differentiation and apoptosis of the HL-60 myeloid leukemia cell lines. There is growing evidence that resveratrol also can prevent or delay hearth diseases, ischemic and chemically induced injuries, pathological inflammation and viral infections [38].

The cancer prevention mechanism of food polyphenols has been extensively studied and many chemo preventive polyphenols may interrupt or reverse the carcinogenesis process by acting on intracellular signaling molecules network [31].

Studies show that grape's polyphenols contribute to cholesterol and triacylglycerol reduction and can inhibit the oxidation of LDL (low-density lipoprotein), thus reducing the risk of heart disease. Also experimental studies indicate that grape polyphenols could reduce atherosclerosis by a number of mechanisms, including inhibition of oxidation of LDL and other favorable effects on cellular redox state. Procyanidins present in grape pomace inhibit human endothelial NADPH oxidase, the enzyme responsible for the increased production of reactive oxygen species [31].

Phenolic compounds also have been shown to have antimicrobial activity. For example resveratrol has been reported to have strong antifungal and antibacterial activities.

The extraction step is very important for the recovery, isolation and identification of phenolic compounds. Solid-liquid extraction is the most common technique for obtaining phenolic-rich extracts from grape pomace, whereby the analytes contained in the solid matrix migrate to the solvent that is in contact with the matrix. The efficiency of the process can be controlled by concentration gradients, solvent type, particle size, temperature and extraction time. The solvent plays a key role, the most used solvents being ethanol, methanol, acetone, water and also acidified solutions. Solid-liquid extraction is time-consuming and requires relatively large quantities of solvents. Also it can lead to great losses in polyphenols recovery.

A more recent technique for extracting phenolic compounds is supercritical fluid extraction, which uses supercritical fluids, such as CO_2, to extract compounds from the matrices. The characteristics of supercritical fluids will be summarized in the next section. Another technique is accelerated solvent extraction. This consists in using the conventional solvents but applying high temperatures and pressures, in order to increase the extraction efficiency due to deeper penetration of the solvent in the sample matrix pores. Other emerging techniques include enzymatic release, which is a kind of pretreatment that can improve the efficiency in water extractions due to increased accessibility of the phenolic compounds. Another recent technology is high-voltage discharges, which due to the creation of a high voltage between two electrodes, cause particle fragmentation and cell structure damage, accelerating the extraction of intracellular compounds [35]. Other techniques are microwave-assisted extraction and ultrasound-assisted extraction, which consist in using microwaves or ultrasounds to cause cell disruption, increasing mass transfer and facilitating solvent access [39].

In this work, another extraction technique is going to be used – hot-compressed water extraction – which also leads to good yields and uses only water.

1.7 Hot-compressed water

The high potential of subcritical and supercritical fluids has been leading to extensive research since the late 70's of last century [40].

The critical point is the endpoint of the vaporization curve of a substance. At the critical point, the density of the gas and liquid are identical. Above its critical point, a substance is said to be a supercritical fluid. Supercritical fluids present a combination of properties of gases and liquids, such as low viscosity, high diffusivity, good solvation ability, making them suitable for the development of new processes that cannot be carried out by conventional liquids and gases [41]. Close to the critical point, small changes in temperature and pressure of a supercritical fluid allow the adjustment of its solvation ability, which can facilitate separation processes and the recovery of solutes. Supercritical fluids are used for the extraction of bioactive compounds as an alternative to organic solvents, thus avoiding unfavorable environmental impact. Recent studies showed that at industrial scale supercritical fluid extraction is an efficient and cost effective technique for the extraction of carotenoids [42].

The most common solvent used in supercritical fluid extraction is carbon dioxide because it is inert, non-flammable and non-toxic. However, to dissolve highly polar compounds, such as fatty acids and sterols, very high pressure is needed. To decrease pressure, a small quantity of cosolvent like water or ethanol can be used [42].

Hot-compressed water (HCW) or subcritical water is a highly promising, energy-efficient and environmentally benign technique for extraction processes, such as polyphenol extraction from biomass [43].

Water is nonflammable, nontoxic, readily available, safer and a truly environmentally friendly solvent. Its properties can be manipulated by changing temperature, in the range between 100°C and 374°C [44], applying sufficient pressure to maintain it in the liquid state.

Subcritical water is not a physically defined state, but rather a region bellow the critical point and above the triple point of water (fig. 1.12). The term "subcritical water" was created to distinguish processes performed below the critical point but above the vaporization curve, from processes with supercritical water [45]. Henceforth, the designation HCW will be used.

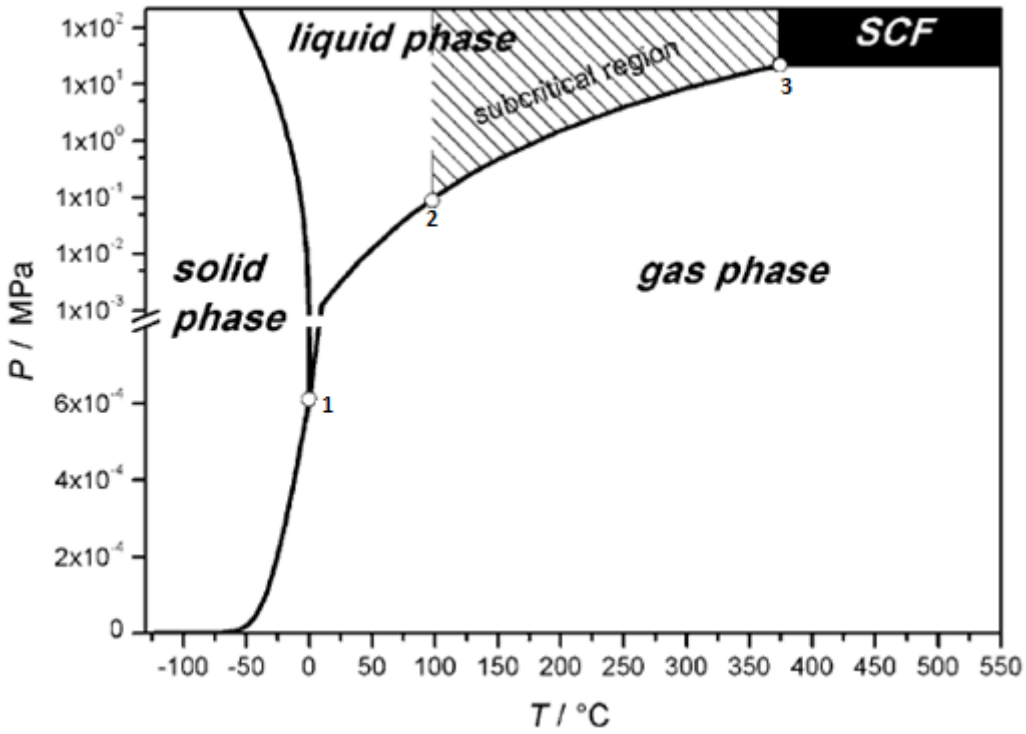

Figure 1.12 - Phase diagram of water indicating the subcritical region, the triple point (1), boiling point at ambient pressure (2) and critical point (3) [45].

HCW has lower viscosity and surface tension than water at ambient temperature, which increases mass transfer rates from plant tissue matrices [46]. The dielectric constant of HCW (water polarity) decreases abruptly with increasing temperature, due to hydrogen bond dissociation. Thus the solubility of ionic molecules decreases, while the solubility of hydrophobic molecules increases. This enables the extraction of compounds that are normally extracted with organic solvents such as ethanol or methanol. At near critical and supercritical conditions gases like O_2, N_2, CO, NH_3 and CO_2 are completely miscible with water [40]. Thus changing water conditions from ambient to supercritical changes its character from a solvent for ionic species to a solvent for non-ionic species. This effect is one of the major drawbacks of processing residues, since salts dissolved in the feed stream may precipitate and eventually block the reactor. This can be avoided by increasing the pressure because at moderate and high pressures, the solubility of salts does not drop to zero [40].

Ionic strength is another property of water that changes. As temperature increases, the ionic product of water, K_W, increases, by three orders of magnitude from $K_W = 10^{-14}$ at 25 °C to $K_W = 10^{-11}$ at 300 °C [47]. Above this temperature the ionic product decreases again, to values below $K_W = 10^{-20}$. K_W increases lead to increasing H^+ and OH^- concentrations, making water a more reactive medium, thus behaving as reagent as well as solvent. An example is the hydrolysis of cellobiose to the respective monomers [45].

These properties give HCW a wide range of applications, such as in this work, where water is used as solvent to extract value-added compounds with better yields than conventional methods, as well as to hydrolyze lignocellulosic material into polysaccharides. Despite incomplete hydrolysis of lignocellulose, HCW is an efficient pretreatment for enzymatic hydrolysis, which leads to full hydrolysis into monosaccharides.

When using HCW but especially supercritical water, corrosion has to be taken into consideration. Reactors made of inert materials must be used, such as stainless steel [45].

Table 1.3 - Water properties at different conditions [45].

	Ambient temperature	**Subcritical water**	**Supercritical water**
Temperature (°C)	0-100	100-374	>374
Vapor pressure (MPa)	0.003 (24 °C)	0.1 (100 °C)-22.1 (374 °C)	>22.1
Aggregate state	liquid	liquid	no phase separation
Density (g/cm^3)	0.997 (25 °C)	0.958 (101 °C; 0.11 Mpa)	between gas-like and liquid-like densities, for example 0.252 (410 °C, 30 MPa)
Viscosity (µPas)	L: 884 G: 9.9 (25 °C)	L: 277 G: 12.3 (101 °C)	low
		L: 50.4 G: 30.7 (101 °C)	
Heat capacity C_p ($Jg^{-1}K^{-1}$)	L: 4.2 G:2.0 (25 °C)	L: 4.2 G:2.1 (101 °C)	1300(400 °C; 25 MPa)
		L: 69 G:145 (371 °C)	
Dielectric constant	78.5 (25 °C; 0.1 MPa)	27.1 (250 °C; 5 MPa)	5.9 (400 °C; 25 MPa)
		18.2 (330 °C; 30 MPa)	10.5 (400 °C; 50 MPa)
Compressibility	No	slightly increased, but still a liquid 27.1 (at 370 °C)	yes
Ionic product K_w	10^{-14} (increasing to 10^{-12} at 100 °C)	increases from 10^{-12} (101 °C) to 10^{-11} (300 °C)	strongly decreasing to below 10^{-20} (400 °C) and below 10^{-23} (550 °C); increases slightly with P

Several studies have been done on polyphenols extraction using HCW, and it is reported that this process is more efficient than conventional hydro-alcoholic or methanol extraction at atmospheric pressure [46]. Some examples are the extraction of polyphenols from potato peel [48], bitter melon [49], rosemary plants [50], rice bran [51] and pomegranate [52].

Examples of other applications are the extraction of dietary fiber, cellulose, hemicellulose and pectin from citrus junos peel [53], reducing sugars, amino acids and proteins from rice bran and soybean meal [54], and production of glucose and xylose from depolymerization of cellulose and hemicellulose from rape seed straw [55].

2 Materials and methods

2.1 Chemical characterization

2.1.1 Grape pomace and GP powder

The residues used in this work - white and red grape pomace - were provided by a Portuguese wine producer. They were lyophilized (lyophilizer CHRIST ALPHA 1-4, Braun Biotec International), by first freezing them using liquid nitrogen, and then submitting them to vacuum for 3 days. They were then allowed to warm to room temperature, milled to ca. 2 mm particle sizes, and the resulting grape pomace powder – GP powder – was stored in the freezer. The water content of each residue as provided was determined gravimetrically. The water content of GP powder was also determined gravimetrically, after keeping the material in an oven at 105 °C overnight.

2.1.2 Protein determination

The nitrogen content of GP powder was determined by elementary analysis performed at Laboratório de Análises, REQUIMTE-LAQV. To determine protein content, a nitrogen-to-protein conversion factor of 6.25 was used [56].

2.1.3 Ash determination

For the determination of the ash content, 0.8 g of GP powder were weighed in a porcelain crucible and placed in a muffle at 550 ºC. After 6 hours the crucible was removed and placed in a desiccator to cool down. After that, it was weighed and through mass difference ash content was determined [57].

2.1.4 Phenolic compounds extraction (Hydro-alcoholic extraction)

To 1 g of GP powder was added 20 mL of (25:75, v/v) ethanol:water. The mixture was incubated at 50 °C for 18 hours, under constant magnetic stirring (150 rpm). After filtration, to the filtered liquid obtained was added 5 mL of diethyl ether (SIGMA Aldrich 99.8%), under stirring. The well stirred mixture was allowed to rest for 10 min, for phase separation to occur, and the ether fraction was separated. The extraction procedure – ether addition, equilibrium,

phase separation – was done three times, and the three ether fractions were collected together. Na_2SO_4 (ca. 50 mg) were added to this organic phase and the solvent was evaporated under nitrogen. The remaining solid was dissolved in methanol:water (1:1, v/v), after which the mixture was filtered [58]. The solid recovered (Na_2SO_4) was discarded, and the solution was used for the quantification of phenolics.

2.1.5 Carbohydrates and lipid content

Before carbohydrate analysis, 2 g of GP powder were defatted through Soxhlet extraction, for 4 hours, using 65 mL of *n*-hexane. The residue was dried overnight at 40 °C, to remove the solvent, after which it was weighed. The solvent in the solution was removed through evaporation, and the remaining oil was weighed. The two measurements of the fat content of GP powder were in good agreement.

Defatted GP powder (0.8 g) was extracted with 40 mL of a (80:20, v/v) ethanol:water, in an ultrasonic bath for 15 minutes at room temperature. The extraction was terminated by centrifugation (10000 rpm, for 10 min, at 4 °C). The process was repeated three times. The three supernatants were combined and ethanol was evaporated at 50 °C, under vacuum, in a rotary evaporator. The remaining solution was diluted with 80 mL of water [59], and used for carbohydrate analysis.

The remaining residue – defatted GP powder, after removal of soluble carbohydrates – was dried at 40 °C overnight. To hydrolyze the insoluble, structural carbohydrates, to 0.3 g of this residue were added 3 mL of 72% (w/w) H_2SO_4. The mixture was incubated in a water bath at 30 °C, under stirring, for 1 hour, after which the mixture was diluted to 4% (w/w) by adding 84 mL of water, and incubated at 121 °C in a silicone bath, under stirring, for 1 hour. The mixture was then filtered and the supernatant analyzed to quantify carbohydrates [60].

The solid remaining after acid hydrolysis was washed with water, dried at 105 ºC overnight, and weighed. Its ash and nitrogen contents were determined as already indicated. The amount of Klason lignin was obtained by subtracting resistant protein and acid insoluble ash from the weight of dry residue [59].

2.2 HCW apparatus and reaction conditions

For hydrolysis reactions with HCW, the apparatus represented in figure 2.1 was used. It comprises a pump, connected to a distilled water container, which pumps water through a high pressure tube connected to the reactor. Before reaching the reactor, water passes through a filter,

and is heated by a heating wire connected to a temperature controller. Pressure is measured with a pressure indicator. The body of the reactor is a 51 cm long, 5 cm external diameter, 2.6 cm internal diameter stainless steel tube. The reactor is placed in an electric oven with temperature control. The reactor is filled with GP powder, kept between porous discs. The water leaving the reactor passes through a filter. Its temperature and pressure are monitored, as done for the water inlet stream. The pressure of the system is controlled by a Back Pressure Regulator (BPR; Tescom Europe®, 26-1000). The outlet stream is collected for analysis. The valves and fittings used are from HIP and SWAGELOK.

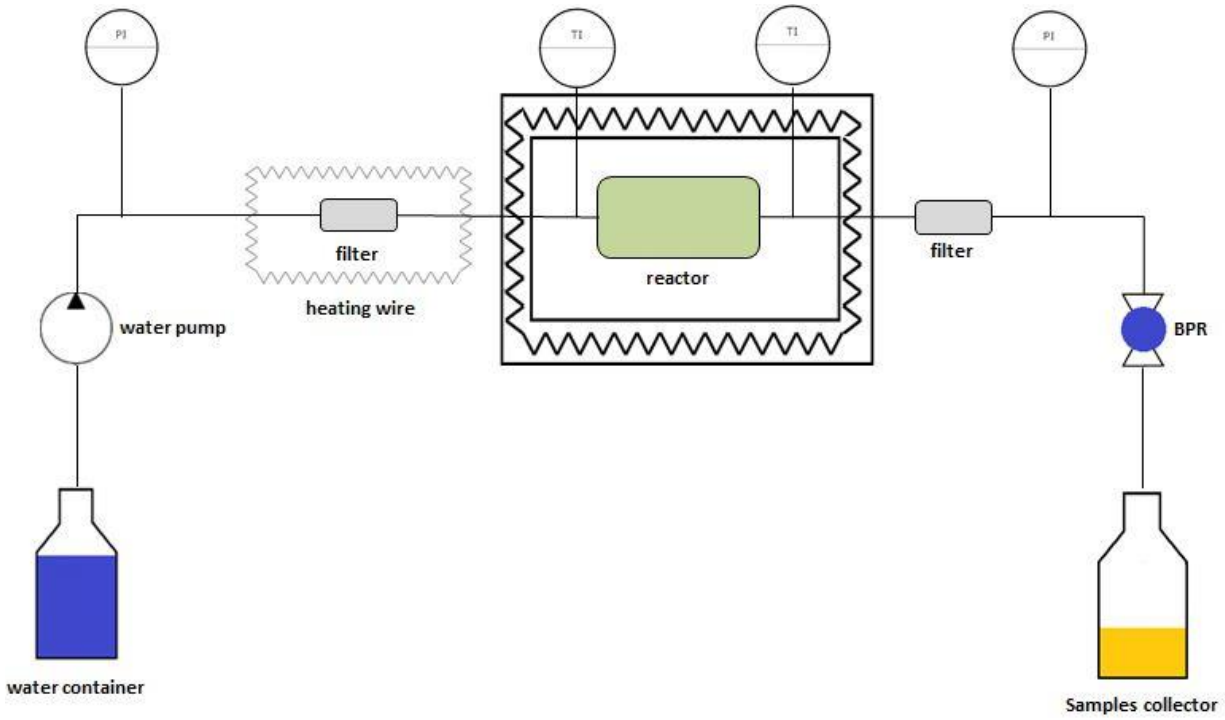

Figure 2.1 - Schematic of the HCW experimental set-up. PI is a pressure indicator and TI a temperature indicator. The BPR is a back pressure regulator.

Figure 2.2 - HCW apparatus used for extraction and hydrolysis of GP powder.

As seen earlier, the two parameters that influence the properties of HCW are pressure and temperature. In all the assays the pressure was kept constant, at 100 bar. Four temperatures were used: 170, 180, 190 and 210 ℃. The water flow used was 5 or 10 mL/min.

To perform an experiment, the pump is turned on with the flow rate selected, and the BPR set for 100 bar. When the pressure reaches 100 bar, the water heating wire and the oven are turned on, and sample collection starts. Schott flasks are used. The first sample is collected until the temperature of the water exiting the reactor reaches 50 ºC, the second as the outlet stream temperature varies from 50 to 130 ℃, and the third as temperature varies from 130 to the maximum temperature (one of the four T values indicated above). From then on temperature is kept constant for 30 minutes, and samples are collected in falcon tubes every 5 min. Samples are stored at 4 ℃. Each sugar-rich liquor sample is used for quantification based on colorimetric methods. 10 mL of each sample are lyophilized to obtain what will henceforth be called GP extract, which is further used for quantification based on HPLC analysis.

2.3 Colorimetric quantification methods

2.3.1 Total Carbohydrates analysis

This method is used to quantify the reducing sugar content of the sugar-rich liquors, using a calibration curve built with D(+)-glucose monohydrate (SIGMA Aldrich) solutions. These were prepared from a 3 g/L stock solution, with concentrations of 1, 0.5, 0.25, 0.1, 0.05, 0.025 g/L in milli-Q water. The blank was milli-Q water.

To 500 µL of either a standard solution or sugar-rich liquor were added 1.5 mL of H_2SO_4 (Panreac 96%) and 300 µL of a 5% (w/v) aqueous solution of phenol (Sigma Aldrich 99-100%). The resulting mixtures were well stirred. After incubation for 5 min at 90 ºC in an Accu BlockTM Digital Dry Bath, the mixtures are well stirred, and cooled to room temperature by immersion in a water bath. Absorbance is measured at 490 nm with a DU®800 Spectrophotometer from Beckman Coulter, Brea, USA. The results obtained are expressed in g/L glucose equivalent [61].

2.3.2 Total phenolic content - Folin-Ciocalteu method

This method was used for the determination of the total phenolic compounds content of the sugar-rich liquors, using a calibration curve built with gallic acid monohydrate (Sigma 98%). Standard solutions of gallic acid were prepared from a 5 g/L stock solution, with concentrations of 10, 25, 50, 100, 150, 250, 500 mg/L in milli-Q water. The blank was milli-Q water.

Before determination of phenolics, it was necessary to perform a step of protein precipitation due to their interference with phenolics quantification. To 800 µL of sugar-rich liquor were added 120 µL of 100% (w/v) trichloroacetic acid (Scharlau 99.5%). The mixture was stirred well, and stored for 5 min at -20 °C, and then at 4 °C for 15 min. After centrifugation (12000 g, 15 min) (Heraeus sepatech, Biofuge 13 Centrifuge), the precipitate was discarded [62].

To 20 µL of the recovered supernatant, as well as to standard gallic acid solutions, were added 1.58 mL of distilled water, and 100 µL of Folin-Ciocalteu reagent (MERK). The mixtures were well stirred and incubated for about 5 min at room temperature. Then it was added 300 µL of sodium carbonate solution (Sigma), followed by incubation at 40 °C for 30 min, in an Accu BlockTM Digital Dry Bath. Absorbance was measured at 750 nm (it should be 765 nm, but due to spectrophotometer limitations it was measured at 750nm) with DU® 800 Spectrophotometer

from Beckman Coulter, Brea, USA. The concentration of the samples was determined using the gallic acid calibration curve, and expressed in mg/L GAE (gallic acid equivalent) .

2.4 HPLC methods

2.4.1 HPLC method for phenolic compounds analysis

HPLC analysis allows the identification and quantification of individual phenolic compounds in GP extracts. The analysis was performed with a Thermo Scientific, Finnigan Surveyor Autosampler Plus, Finnigan Surveyor LC Pump Plus HPLC, and the software used for data treatment was ChromQuest 5.0.

The method used has an injection volume of 5 μL and flow rate of 1 mL/min. The absorbance is measured at two different wavelengths: 280 nm and 320 nm, with an Accela UV/Vis Detector. The column (C18 BDS HYPERSIL; 250 mm length; 4 mm I.D) was kept at a constant temperature of 40 ºC. The mobile phase is a mixture of two eluents, A and B. Eluent A is water with 2% (v/v) of glacial acetic acid (Scharlau 99.8%) and eluent B is (50:50, v/v) water:acetonitrile (Sigma Aldrich 99.93%) with 0.5% (v/v) of acetic acid. The gradient program was 10% B to 32.5% (25 min), 32.5% B to 10% B (2 min) and 10% B stable (3 min).

Standard curves were built for gallic acid, syringic acid, vanillic acid, catechin and vanillin. All the standards used were from Sigma Aldrich 98-99% [58]. Stock solutions were prepared with a concentration of 1000 ppm of those compounds in (50:50, v/v) water:methanol. The calibration curves covered concentrations between 1 ppm and 500 ppm.

For the identification of flavonoids, more specifically quercetin, another method was used. This method has an injection volume of 10 μL and flow rate of 0.5 mL/min, kept stable for the duration of the run (15 min). The mobile phase was (60:40:0.4, v/v) milli-Q water:methanol (Sigma 99.8%):phosphoric acid. The absorbance was measured at 269 nm with the same detector, and the column was kept at a constant temperature of 40 ºC [63]. Samples were prepared by dissolving GP extract in (50:50, v/v) water:methanol, to a concentration of 5000 ppm.

In both methods, the concentration of each compound was calculated through the respective calibration curve.

2.4.2 HPLC method for carbohydrates analysis

HPLC was used to identify and quantify glucose, fructose, galactose, arabinose, mannose, xylose, sucrose and fucose in GP extracts. All the analyses were performed at Laboratório de Análise, LAQV-REQUIMTE, with a Dionex ICS-3000 system, with electrochemical detection, using a 4x50 mm Thermo BioLC Dionex AminoTrap pre-column and a 4x250 mm Thermo Dionex CarboPac SA10 column, at a constant temperature of 40 ºC. A 1 mM NaOH solution was used as mobile phase, at a constant flow rate of 1.2 mL/min. Calibration curves were built for the monosaccharides (25, 50, 75, 100, 150, and 250 ppm). Standard solutions of the latter were submitted to treatment with sulphuric acid to correct for losses due to destruction of sugars during dilute acid hydrolysis [60], and 4% (w/w) sulphuric acid was used for dilution to build calibration curves.

2.5 Antioxidant activity. DPPH (2,2-diphenyl-1-picrylhydrazyl) assay

To evaluate the antioxidant activity of the GP extracts, the DPPH assay was used. The DPPH radical is a stable radical, with a deep purple color, which becomes colorless or changes to yellow when neutralized. This assay is based on the measurement of the loss of DPPH color at 517 nm after reaction with solutions prepared from GP extract, and the reactions are monitored using a spectrophotometer.

The stock solution is prepared by dissolving 24 mg of DPPH (Sigma) in 100 mL of methanol, and storing at -20 °C for a minimum of 2 hours. This solution is then diluted: to 10 mL are added 45 mL of methanol, and the absorbance is measured at 517 nm. If the absorbance is above 1,1, more methanol needs to be added, until absorbance reaches a value near 1. To 4 mL of the solution with absorbance near 1, are added 150 μL of solutions of extract in (50:50, v/v) water:methanol (range of concentrations between 50 and 5000 mg/L). The blank is obtained by adding 150 μL of (50:50, v/v) water:methanol to 4 mL of the DPPH solution with absorbance near 1. The mixtures are well stirred and stored for 40 min in amber vials, in the dark, to prevent the interference of light with the radicals.

After the 40 min period, the samples are well mixed and the absorbance is measured at 517 nm. To evaluate the inhibition of the free radical by each sample, the following equation was used, where A_{DPPH} is the absorbance of the blank (DPPH solution) and A_{sample} is the absorbance of the sample with extract (DPPH solution + solution of GP extract):

$$\%\ of\ inhibition = \frac{A_{DPPH} - A_{sample}}{A_{DPPH}}$$

To evaluate the antioxidant activity of GP extracts, the half maximum effective concentration (EC_{50}) was calculated from the inhibition curves obtained.

3 Results and discussion

3.1 Chemical characterization

The GP residue was provided by a wine producer and arrived at our laboratory the day after its recovery from the wine making process. The composition of this residue was determined using several methods. The first step was drying the residue to obtain a dry weight basis. Through lyophilization it was determined that GP, as provided by the winery, has around 60 wt.% of water. As for the GP powder obtained upon lyophilization, its water content is below 0.5%.

The major components identified in both residues – GP powder from red wine making (RWGP) and GP powder from white wine making (WWGP) – were proteins, lipids, ash (which results from inorganic salts), lignin and carbohydrates (Table 3.1).

In both types of residues, the dominant component are the carbohydrates, as expected. The quantification of carbohydrates required the separation of free sugars, which are reducing sugars directly available in GP, from structural sugars, which are present as part of the structure of cellulose and hemicellulose (Table 3.2).

Comparing the two residues, WWGP has a higher content in free sugars, over 40%, while RWGP has only about 7%. This difference is due to the fact that RWGP suffered fermentation during the wine making process, which consumed the free sugars present in grapes, while WWGP was not fermented. In both cases, the biggest contribution to free sugars are glucose and fructose, whose ratio in mature grapes right before harvesting is close to 1 [64].

The most abundant structural carbohydrate is glucose because cellulose in formed only by this sugar, whereas hemicellulose is formed by a variety of sugars, as seen in the tables. RWGP is richer in structural sugars (around 27%) than WWGP (around 16%), which agrees with reports in the literature [59].

Ash, protein, lipids and lignin have higher amounts in RWGP, as observed by other authors [65].

Table 3.1 - Composition of GP powder, in wt.%. Prefixes: RW = red wine; WW = White wine.

Component	wt.%	
	RWGP	**WWGP**
Protein	14.7 ± 1.2	3.9 ± 0.7
Lipids	11.9 ± 1.0	7.3 ± 0.5
Ash	7.0 ± 0.4	4.9 ± 0.1
Carbohydrates	33.5 ± 0.4	57.8 ± 2.4
Lignin	30.3 ± 1.4	16.5 ± 1.5

Table 3.2 – Carbohydrates identified and quantified in GP powder.

	Component	wt.%	
		RWGP	**WWGP**
free sugars	total	6.9 ± 0.3	41.8 ± 2.2
	glucose	2.8	22.2
	fructose	3.1	17.9
	fucose	1.0	1.5
	sucrose	0	0.2
structural sugars	total	26.6 ± 0.7	16.0 ± 0.2
	glucose	15.0	7.8
	galactose	2.2	1.8
	arabinose	2.7	2.0
	mannose	2.3	1.0
	fucose	0.3	0.3
	xylose	4.1	3.0

As the main objective of this work is to extract phenolic compounds from GP, it was also performed the extraction of these compounds through a conventional technique, namely hydro-alcoholic extraction (Table 3.3).

The results reveal low contents of total phenolic compounds (TPC), which represent around 1 mg per g of RWGP and about three times less in WWGP. Some compounds were identified and quantified by HPLC. HPLC analysis revealed that the most abundant phenolic identified in GP powder is gallic acid. In RWGP, it were also identified vanillic acid, syringic acid and quercetin, and in WWGP vanillic acid, catechin and vanillin.

The higher TPC values suggest that there exist other compounds that were not identified by HPLC, due to lack of standards that could help in that identification. Another reason might be the difference between the pH of the samples and of the standards used, because at different

pH values the absorbance of phenolic compounds will be different [66]. Thus some compounds could be present in the samples in different forms than in the standards, such as glycosylated forms of quercetin [67], not allowing their quantification. The amount of TPC is determined by the colorimetric method. However, it relies on a calibration curve built with gallic acid. Thus the discrepancy between the TPC and the total amount of phenolics measured by HPLC is not due to lack of agreement between the two methods, as confirmed by other authors [68].

The TPC values given in Table 3.3 will be used for reference throughout this work.

Table 3.3 – Phenolic compounds identified and quantified in GP powder, given in µg of each compound per g of GP powder. TPC = Total phenolic compounds; GA = Gallic acid; VA = Vanillic acid; SA = Syringic acid.

	content ($\mu g/g_{GP\ powder}$)	
	RWGP	**WWGP**
TPC	803.6 ± 44.4	265.9 ± 2.1
GA	39.1 ± 2.5	10.2 ± 1.5
VA	14.2 ± 2.1	3.7 ± 0.9
SA	35.4 ± 1.4	0
Catechin	0	6.4 ± 0.5
Quercetin	2.2 ± 0.1	0
Vanillin	0	3.5 ± 0.2

3.2 Hot-compressed water extraction/hydrolysis

3.2.1 Process efficiency

In this work it was studied the influence of temperature and water flow rate on the extraction/hydrolysis efficiency of HCW for WWGP. RWGP had already been studied in our lab, at small scale (up to 3 g GP). The aim here was to scale-up the process through an increase in the amount of GP powder used, of two orders of magnitude. Only one set of experimental parameters were tested in this case.

In all the assays it was observed a change in the color of the samples recovered along the extraction process. In the beginning, they were clear, slightly yellow in the case of WWGP, and red in the case of RWGP. As the extraction proceeded, the color of the samples accentuated. And when temperature increased to its maximum value, the samples gained turbidity and became yellow/brown.

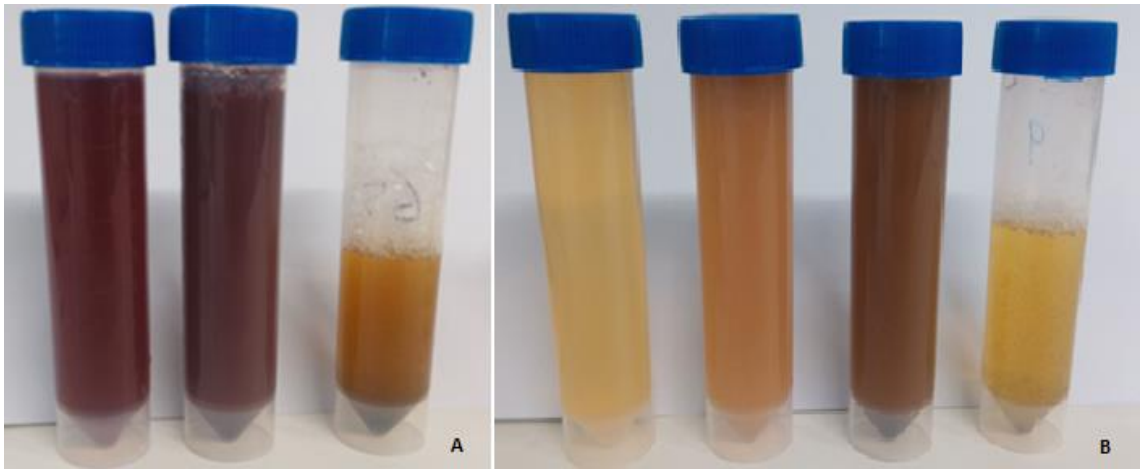

Figure 3.1 - Samples recovered along HCW assays with RWGP (A) and WWGP (B).

To evaluate the influence of temperature on the extraction/hydrolysis of WWGP, assays were performed reaching a maximum temperature of 170, 190 and 210 °C. Biomass conversion is given by the weight difference of GP powder placed in the reactor and GP residue that is recovered from the reactor at the end of an experiment. The yield of water soluble compounds is measured by the amount of extract obtained; the extract is obtained by lyophilizing the sugar-rich liquor collected throughout the whole assay.

Table 3.4 – Conversion of GP powder and yield of water soluble compounds at different HCW assay conditions. P = 100 bar.

	Conditions			
	Temperature (°C)	Flow rate (mL/min)	Yield (%)	Conversion (%)
WWGP	170	10	61	79
	190	10	65	80
	210	10	69	85
	190	5	41	63
RWGP	180	5	25	51

Conversion increases with the increase in temperature, as expected, from around 79% at 170 ºC to around 85% at 210 ºC. With the increase in temperature, the ionic product of water increases, and water becomes a stronger catalyst for the hydrolysis of biomass. A higher water flow rate is more efficient for the conversion of biomass than a lower one. One possible explanation is that at lower water flow rates, the GP powder is less compact and water can follow preferential paths that avoid the GP powder bed.

In all the assays there exists a large difference between the amount of biomass that disappears from the reactor, as measured by biomass conversion, and the amount of compounds that are recovered from the reactor, as measured by the yield of water soluble compounds,

which ranges from 14% to 25%. This difference can be explained by the production of volatile compounds that are released during the process and are not taken into account in yield calculations. Another factor could be the cooling of the system after the end of the assay. The last sample is collected at the end of the 30 min period at maximum temperature. The heating system is then turned off. To cool the system faster, the water flow is raised up to 50 mL/min. The cooling phase lasts approximately 1 hour, during which time some compounds can be extracted. This is taken into account when the GP residue remaining in the reactor is weighed, but the compounds extracted during the cooling phase are not taken into account in the quantification of the water soluble compounds.

The best yield of water soluble compounds was achieved at 210 °C (69%), around 9% higher than at 170 °C (Table 3.4). These differences could be related not only with temperature, as seen above for conversion, but also with the duration of the process. The heating time increases when the target temperature is higher, and thus assays performed at higher temperatures are longer, and a higher amount of extract is recovered.

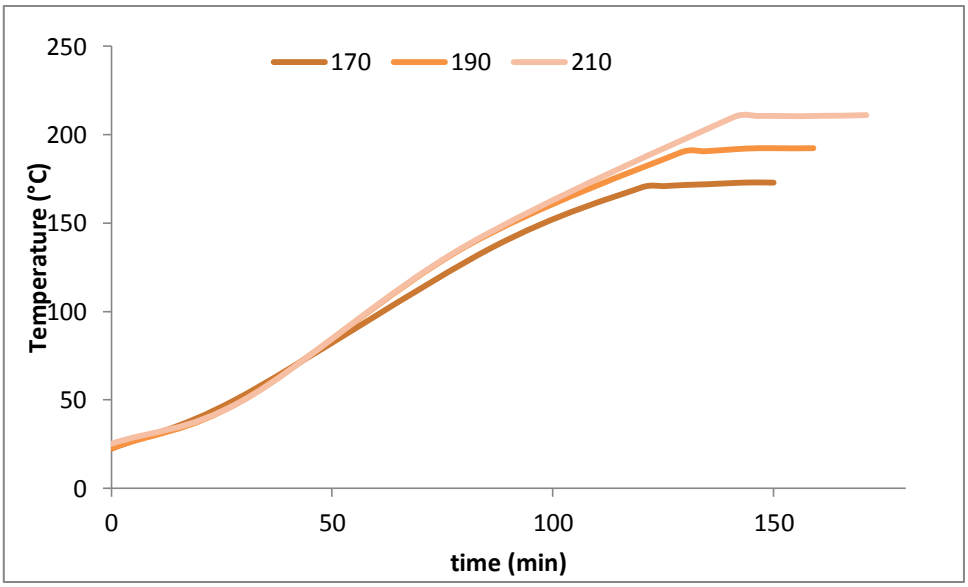

Figure 3.2 - Temperature increase over time for three assays with different maximum temperatures. Water flow rate = 10 mL/min, P=100 bar.

The change in the water flow rate had a big influence in the yield, as seen in two assays performed at 190 °C, where water flow rates of 10 and 5 mL/min led to yields of 65 and 41%, respectively. This difference may be due to the high amounts of free, water soluble sugars in WWGP (Table 3.2). At low flow rates, water saturates and cannot extract more sugars.

The extraction from RWGP reached much lower conversion and yield. The temperature used (180 °C) was one of the temperatures used in previous work done with the small reactor, at

which higher yields were obtained. One problem could be the water flow used, 5 mL/min, which in the large reactor configuration did not lead to results as high as with 10 mL/min.

Carbohydrates are the major component of GP (Table 3.1). Comparing the assays with WWGP with the assay with RWGP, the lower conversion and yield obtained with RWGP can be due to the lower amount of carbohydrates present in this residue, which is nearly half of the amount of carbohydrates in WWGP.

The extraction curves for WWGP at three different temperatures suggest that up to 50 °C, the extraction of free sugars is the main process that occurs (Figure 3.3). WWGP is very rich in free sugars (Table 3.2), immediately available. This could explain the sharp increase in the amount of water soluble compounds recovered at the beginning of the assay. From then on, the increase in extraction yield with time is much more moderate, as temperature increases beyond 100 °C and water reaches the subcritical region. In this region, water acquires different properties, becoming also a reagent. The extraction is slower because for structural carbohydrates to be released, the hydrolysis of polymers like cellulose and hemicellulose must take place. With the dissolution of biomass, the extraction of less polar compounds, such as polyphenols, begins.

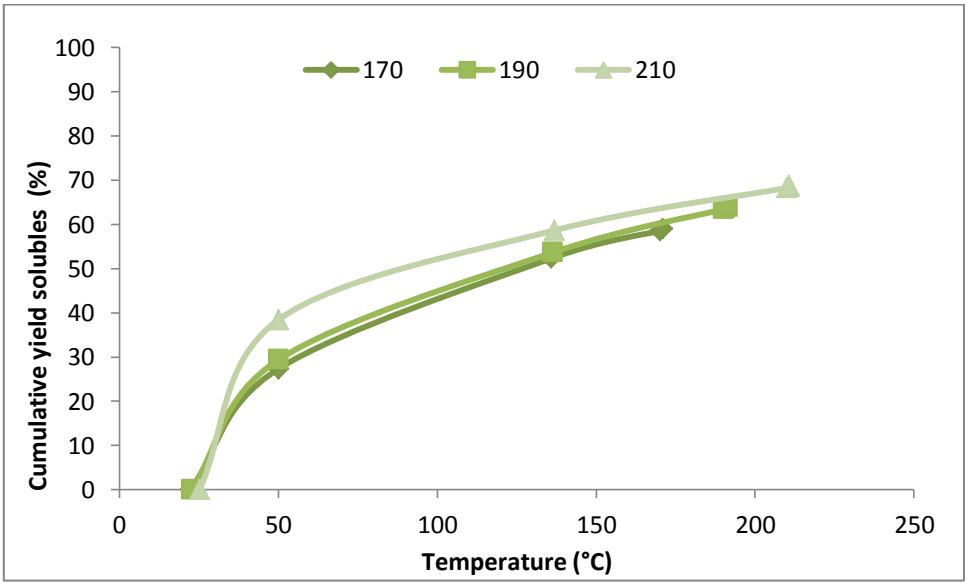

Figure 3.3 - Cumulative yield of water soluble compounds for three extractions performed at 170, 190 and 210 °C, using a water flow rate of 10 mL/min, at 100 bar.

The extraction/hydrolysis profile against time is specific to the different residues (Fig. 3.4 and 3.5). As analyzed earlier, for WWGP there is a first stage in the assay where the extraction yield increases more pronouncedly with time. In the case of RWGP, it is found that the extraction yield is constant during the process until the target temperature is reached. This supports the conclusion that the extraction of free sugars is behind the sharp increase in extraction yield for WWGP at the first stage of the assay.

For both residues, once the target temperature is reached, the process can be finished because the extraction yield stabilizes, and it is disadvantageous to continue with the process due to the energy costs involved.

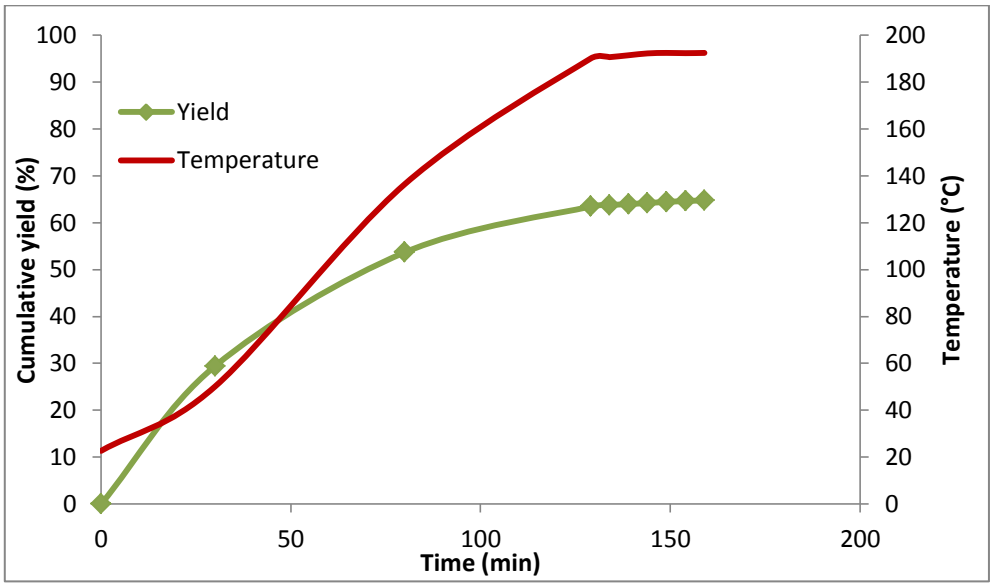

Figure 3.4 - Cumulative yield of water soluble compounds for an extraction performed with WWGP at 190 °C, using a water flow rate of 10 mL/min, at 100 bar.

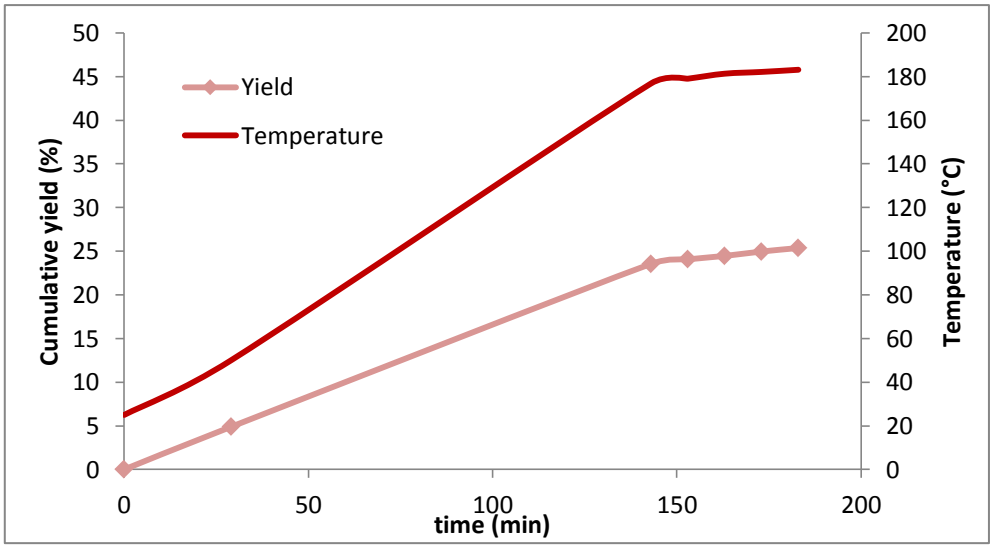

Figure 3.5 - Cumulative yield of water soluble compounds for an extraction performed with RWGP at 180 °C, using a water flow rate of 5 mL/min, at 100 bar. The maximum value of the Y-axis is half the value in Figure 3.4.

3.2.2 Chemical composition analysis

3.2.2.1 Polyphenolic content

To evaluate the extraction of phenolic compounds from the GP residues, it was performed the quantification of TPC, using the Folin-Ciocalteau method (Table 3.5). For WWGP, the highest TPC obtained was 26.2 mg/g of GP powder, obtained at 210 °C. The results at 170 and 190 °C, obtained with same water flow rate, are very similar, and around 10 mg/g lower. The change in water flow rate to 5 mL/min led to a slight decrease in TPC.

For RWGP, the highest TPC obtained was 19.6 mg/g of GP powder at 180 ºC, which is higher than the TPC obtained for WWGP at 190 °C. However, the two results cannot be compared because of the different composition of the residues.

But the most striking finding is that for both residues the results obtained with HCW are approximately two orders of magnitude higher than the results obtained with hydro-alcoholic extraction (Table 3.3), a conventional method for chemical characterization. This difference can be explained by a lower efficiency of the hydro-alcoholic extraction due to the fact that part of these phenolic compounds are entrapped within the lignocellulosic structure, which makes their extraction difficult. Therefore the conventional extraction process does not allow the evaluation of the potential of the residues as a source of phenolic compounds.

Nevertheless the results are too different. When HCW is used, the lignocellulosic structure is hydrolyzed and in addition to non-structural phenolic compounds, which become more accessible, there may also be degradation of lignin, one of the components of lignocellulose that is rich in polyphenols. This is suggested by the lignin content of the remaining residue after HCW treatment – 8.6 wt.% (referred to GP powder; Table 3.1) in the case of WWGP (210 ℃), and 25.5% in the case of RWGP. These results show that for WWGP, around half of the lignin (8.6% compared to 16.5%) resisted HCW treatment, whereas in the case of RWGP most of the lignin resisted HCW attack, which could be related to the lower temperature of the assay. In any case, the values given in Table 3.5 for TPC fall within the range of values obtained by other authors, for example around 25 mg/g for RWGP and 12 mg/g for WWGP [59]. These values seem to indicate that RWGP has a higher content of phenolic compounds than WWGP. This agrees with the values in Table 3.5, extracts from RWGP having higher content of phenolics than extracts from WWGP, when considering a similar temperature of HCW treatment.

To identify some of the individual compounds present in the GP extracts, it was also performed HPLC analysis. For both residues, this analysis led to the same compounds that have been previously identified after hydro-alcoholic extraction, gallic acid being the most abundant compound. As seen previously, the total obtained from HPLC analysis is much lower than that obtained by colorimetric TPC determination.

Table 3.5 – Yields of extraction of phenolic compounds for WWGP and RWGP, at different conditions. P = 100 bar. Cat = Catechin; Qrc = Quercetin; Van = Vanillin. Other abbreviations as in Table 3.3.

	Conditions		content ($mg/g_{GP\ powder}$)						
	T (°C)	Flow rate (mL/min)	TPC	GA	VA	SA	Cat	Qrc	Van
WWGP	170	10	16.7	0.20	0.04	0	0.03	0	0.02
	190	10	16.8	0.24	0.01	0	0.06	0	0.02
	210	10	26.2	0.36	0.03	0	0.20	0	0.04
	190	5	14.4	0.25	0.01	0	0.08	0	0.02
RWGP	180	5	19.5	0.51	0.01	0.02	0	0.01	0

Figures 3.6 and 3.7 show the evolution of TPC content of the sugar-rich liquors over time and as a function of temperature, for WWGP and RWGP. In both cases, the extraction of phenolics follows closely the increase in temperature, the linear variation of temperature being accompanied by a linear variation of TPC, which nearly stabilizes when the target temperature is reached.

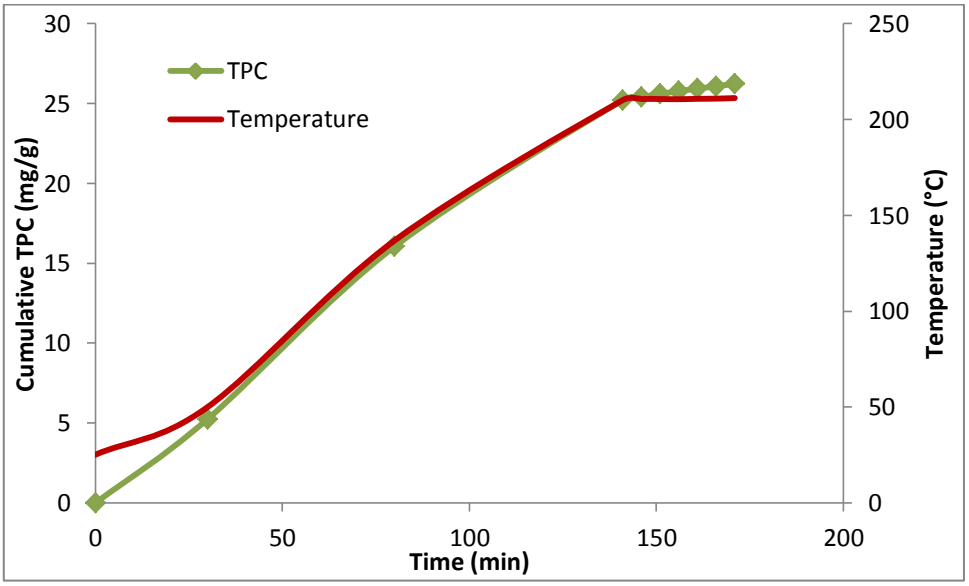

Figure 3.6 - Accumulation of phenolic compounds extracted over time for WWGP, at 210 °C and 10 mL/min water flow rate. P = 100 bar.

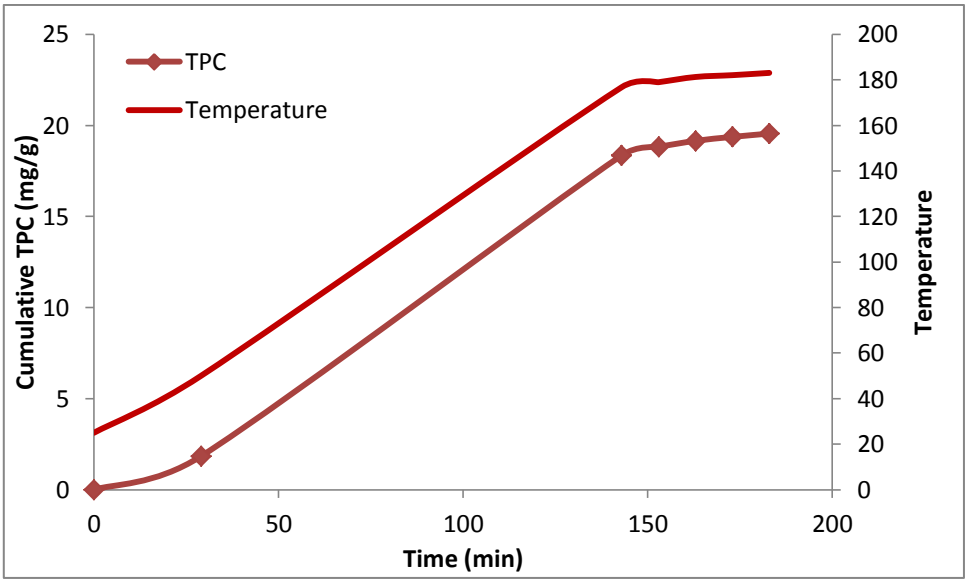

Figure 3.7 - Accumulation of phenolic compounds extracted over time for RWGP, at 180 °C and 5 mL/min water flow rate. P = 100 bar.

As referred earlier, after temperature reaches its maximum value, samples are collected every 5 min. All samples yielded extracts with similar TPC values, which are the highest values measured in any extract collected throughout the assay. This confirms that higher temperatures lead to extracts richer in phenolics. As expected, the extraction of phenolic compounds is favored at subcritical water conditions, due to the lower polarity of water that increases the solubility of these compounds.

Table 3.6 – TPC of extracts obtained from liquor samples collected at the highest assay temperatures. P = 100 bar.

	Conditions		TPC
	T (°C)	Flow rate (mL/min)	($mg/g_{extract}$)
WWGP	170	10	52.8
	190	10	100.1
	210	10	164.7
	190	5	85.4
RWGP	180	5	89.0

3.2.2.2 Carbohydrates content

As observed earlier, GP residues are a rich source of sugars. To assess the ability of HCW to extract carbohydrates, it was used the colorimetric method, which quantifies all the recovered sugars, using a glucose calibration curve, and the HPLC method, which allows the identification and quantification of single monosaccharides.

The values of total reducing sugars recovered when treating WWGP increase as temperature increases from 170 to 210 ºC, the value at 210 ºC being around 10% higher than at 170 ºC (Table 3.7). This may be explained by the increase in the reactivity of water. Water flow rate is also seen to have great impact on the amount of carbohydrates recovered. Comparing these results with the total amount of carbohydrates determined in GP powder (Table 3.1), it can be observed that HCW treatment under the best conditions allowed the recovery of 49.3 g sugar per 100 g GP powder, which corresponds to a recovery of 85.3% of the existing carbohydrates.

As expected, the amount of reducing sugars recovered from RWGP was lower. This residue has a lower amount of carbohydrates than WWGP, and in addition it has already been referred that the assays with RWGP were not optimized.

Table 3.7 - Reducing sugars recovered at different conditions when using WWGP and RWGP. P = 100 bar.

	Conditions		Reducing sugars recovered (wt.%)
	T (°C)	Flow rate (mL/min)	
WWGP	170	10	40.1
	190	10	41.2
	210	10	49.3
	190	5	32.8
RWGP	180	5	11.4

In the case of WWGP, which has a high amount of free sugars, the amount of carbohydrates recovered increases pronouncedly at first, and then increases more moderately. In the case of RWGP, which has most of the carbohydrates in the lignocellulosic structure, the amount of carbohydrates recovered increases at constant rate throughout the assay (Figures 3.8 and 3.9).

Figure 3.8 shows that the best conditions for saccharides hydrolysis and extraction is until 130-140 °C, because after that temperature range is reached the change in the amount of carbohydrates collected is minimal. This could be due to the fact that at higher temperatures, the degradation of the monosaccharides occurs, to give compounds such as 5-HMF and furfural.

Therefore, when the main goal of the extraction is only the recovery of reducing sugars,

it is not necessary to reach higher temperatures. However, in this work the main objective was the recovery of phenolic compounds, which requires higher temperatures to be solubilized.

It can also be seen in figures 3.8 and 3.9 that the first extracts recovered are practically composed by reducing sugars only. In the case of WWGP, carbohydrates account for around two thirds of the extracts collected afterwards (about half in the case of RWGP). It is known that the extracts have phenolic compounds in their composition, but their amounts are not significant compared with the carbohydrate content. Therefore the extracts must have other components, such as protein or inorganic material, to account for the difference between the total amount of water soluble compounds recovered and the total amount of carbohydrates recovered. To clarify this further analysis would need to be done, such as nitrogen determination, or ashing to determine the inorganic material.

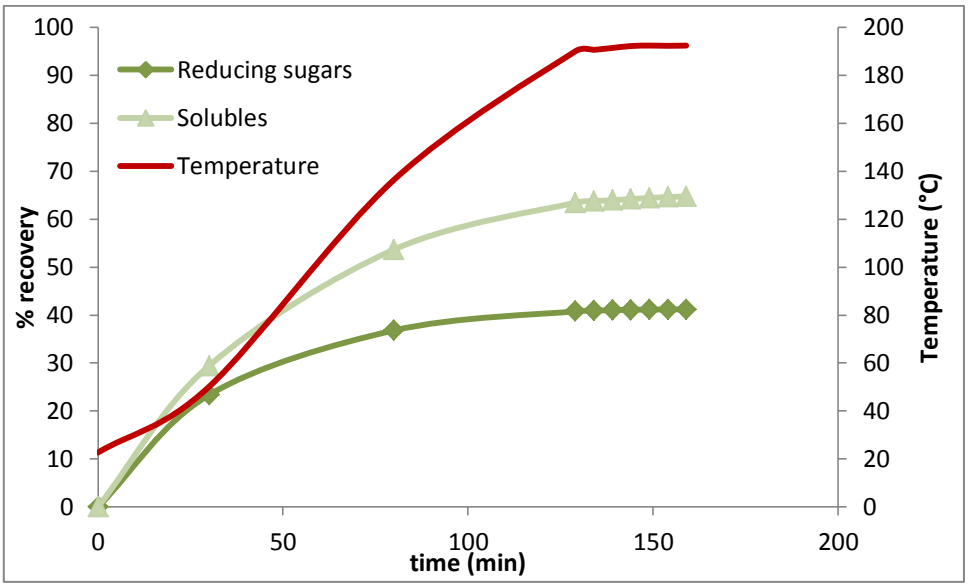

Figure 3.8 - Cumulative amounts of reducing sugars and water soluble compounds (measured in extracts), relative to the amount of GP powder used in the assay with WWGP. T = 190 °C, water flow rate = 10 mL/min. P = 100 bar.

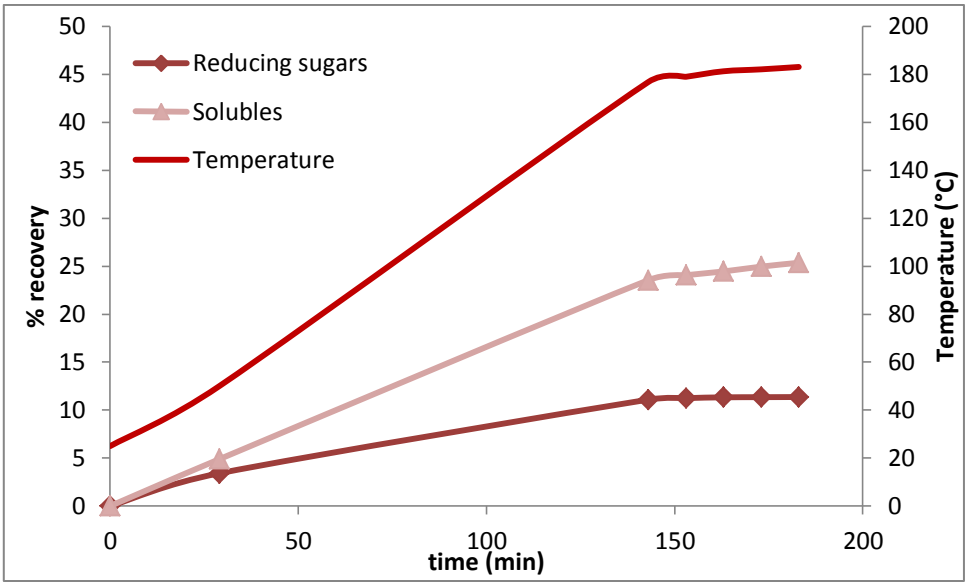

Figure 3.9 – Cumulative amounts of reducing sugars and water soluble compounds (measured in extracts), relative to the amount of GP powder used in the assay with RWGP. T = 180 °C; Water flow rate = 5 mL/min; P = 100 bar.

The colorimetric method used quantifies all the reducing sugars present in the samples, but they could be from monosaccharides, disaccharides or tri-saccharides. It also quantifies the derivatives 5-HMF and furfural. For the analysis of the monosaccharides present in the GP extracts, HPLC analysis was performed (figures 3.10, 3.11 and 3.12).

In the case of WWGP, in the first two extracts collected, the first one until temperature reached 50 ºC, and the second as temperature varied from 50 to 130 ºC, the only monosaccharides detected were glucose and fructose. It was to be expected that the first extracts would have essentially these two monomers, which were present in large amounts as free sugars in WWGP. However, in the second extract, collected when temperature varied from 50 °C to 130 °C, it was expected that other sugars would be detected, since hydrolysis of biomass starts when water becomes HCW. However, that only happened when temperature varied from 130 °C to 190 ºC, and compared to glucose and fructose, the amounts of other sugars were minimal.

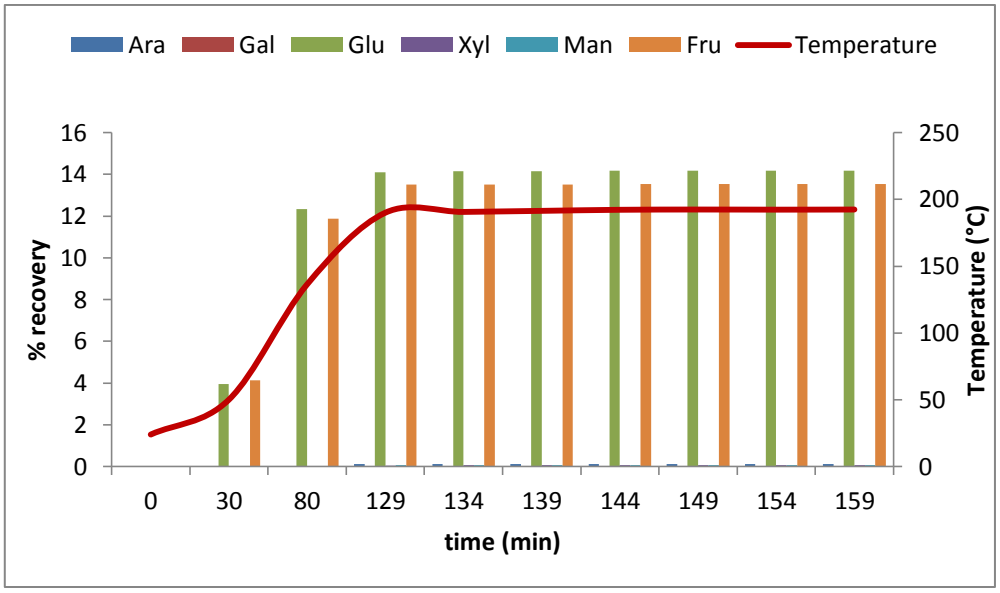

Figure 3.10 – Cumulative amounts of monosaccharides recovered in samples collected throughout the assay with WWGP (each data point is a sum value calculated for samples collected since the beginning of the assay, up to the time indicated in the X-axis). The % values given compare directly with the % values given in Table 3.2. Values given for arabinose (Ara), galactose (Gal), glucose (Glu), xylose (Xyl), mannose (Man) and fructose (Fru). T = 190 °C; Water flow rate = 10 mL/min and P = 100 bar.

The existence of fructose in the final extracts was not expected, because unlike glucose, which exists as free sugar in WWGP but also as the building block of cellulose, fructose exists only as free sugar, and does not form part of the lignocellulosic structure. The high levels of fructose detected in the extracts could be from the remaining soluble fructose, since only around 14% was extracted from out of the 17.9% given in Table 3.2 for WWGP, but could also result from the Lobry de Bruyn–Alberda van Ekenstein transformation (LBET), which consists in the conversion of D-glucose into D-fructose, the reverse reaction being much slower. This normally occurs under room temperature and at high pH, but the increased ionic product of HCW at the temperatures reached in the assays could trigger this reaction even at neutral pH [45].

The other sugars detected are shown in Fig. 3.11. Arabinose is the most abundant, followed by xylose and mannose. Xylose is the most abundant sugar monomer in GP powder after glucose and fructose, and thus the data for the extracts suggests that some degradation occurs. There is evidence that HCW can recover xylose from rye straw [30]. The small amount of these monomers indicates that HCW hydrolysis is not efficient to obtain monosaccharides, but it is important to note that the depolymerization that occurs during HCW hydrolysis of biomass releases mostly disaccharides and oligosaccharides, and these are not detected by the HPLC technique used in this work. A full analysis would require complete hydrolysis, which could be achieved with an enzymatic treatment. HCW is only a pre-treatment that allows the lignocellulose depolymerization, keeping sugars in oligomers to protect from degradation.

Once the maximum temperature of the assay was achieved, no variations were detected in the cumulative amounts of monosaccharides measured in the extracts, indicating that carbohydrate extraction stabilized.

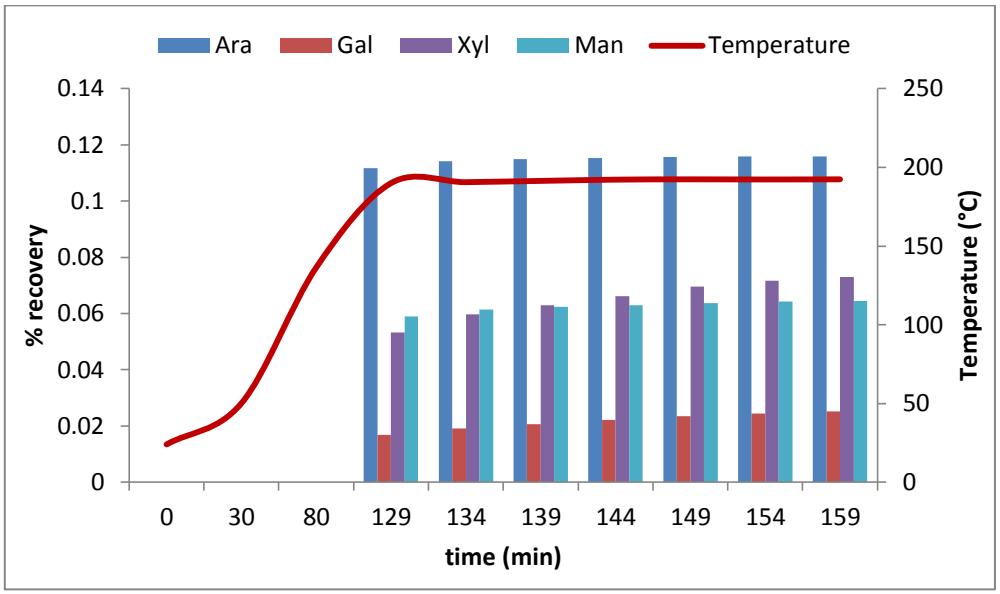

Figure 3.11 - Cumulative amounts of monosaccharides recovered in samples collected throughout the assay with WWGP (each data point is a sum value calculated for samples collected since the beginning of the assay, up to the time indicated in the X-axis). The % values given compare directly with the % values given in Table 3.2. T = 190 °C, water flow rate = 10 mL/min and P = 100 bar

In the case of RWGP, the results were a little different. The first extract only revealed the presence of glucose and fructose, but in small amounts (around 0.1 and 0.15% respectively). The second sample already revealed the presence of other monosaccharides, namely arabinose, galactose, xylose and mannose, resulting from the hydrolysis of hemicellulose. The two most abundant were arabinose and xylose. Although the wt.% of xylose in GP powder was almost twice that of the other less abundant sugars, the extracts obtained had a content of xylose that was about half of that for arabinose. In extracts from RWGP, the relative amount of fructose is much higher than that of glucose, which is even more surprising than what was found for the glucose/fructose ratio in the case of extracts from WWGP, again suggesting that the LBET transformation took place.

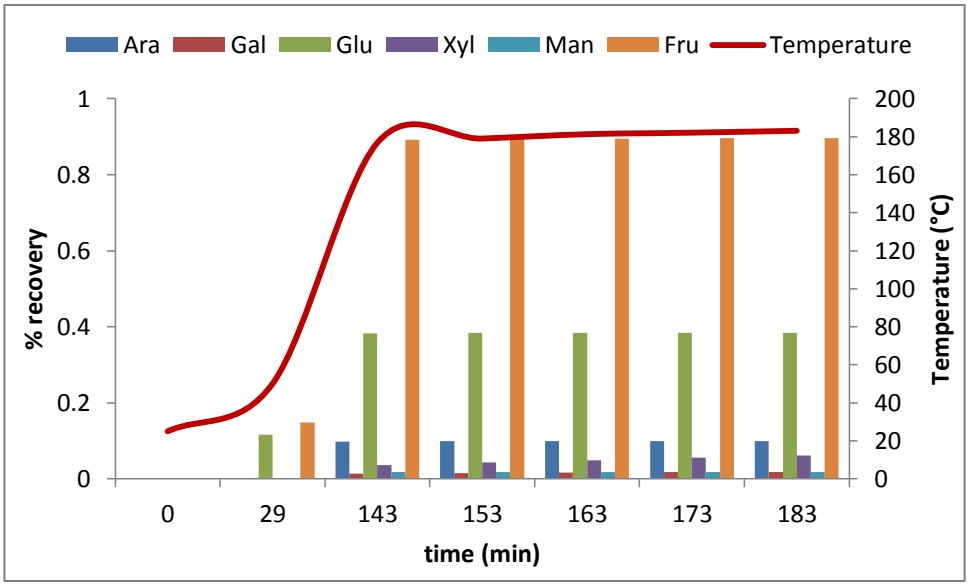

Figure 3.12 - Cumulative amounts of monosaccharides recovered in samples collected throughout the assay with RWGP (each data point is a sum value calculated for samples collected since the beginning of the assay, up to the time indicated in the X-axis). The % values given compare directly with the % values given in Table 3.2. T = 180 °C, water flow rate = 5 mL/min and P = 100 bar.

In conclusion, and as shown in Figure 3.13, for WWGP at 190 °C and 10 mL/min, around 65 wt.% of GP powder (25 % for RWGP) used appears in the form of water soluble compounds. GP powder has around 58 wt.% of carbohydrates (34 % for RWGP), and the total amount of the latter that are recovered in an assay, as part of the extracts obtained, corresponds to around 41 wt.% of GP powder (11 % for RWGP). This represents a recovery of nearly 71 wt.% of the total amount of carbohydrates in GP powder from WWGP (32 % for RWGP). The total amount of monosaccharides recovered in the extract corresponds to around 28 wt.% of GP powder (1.5 % for RWGP), which can be explained by the fact that the carbohydrates in WWGP are mostly free sugars, available as sugar monomers. This data indicates, as discussed earlier, that polysaccharides are the major part of the GP extracts. The extracts also have phenolic compounds, in low amounts, as well as lipids, which will be addressed later.

From the recovered sugars in the GP extracts, about 68% were identified as monomers in the case of WWGP, a value that went down to 13% in the case of RWGP. This could be explained by the higher content of free sugars of WWGP. The remainder of the recovered sugars could be more complex sugar molecules resulting from the hydrolysis and soluble in the liquors, such as di-, or trisaccharides.

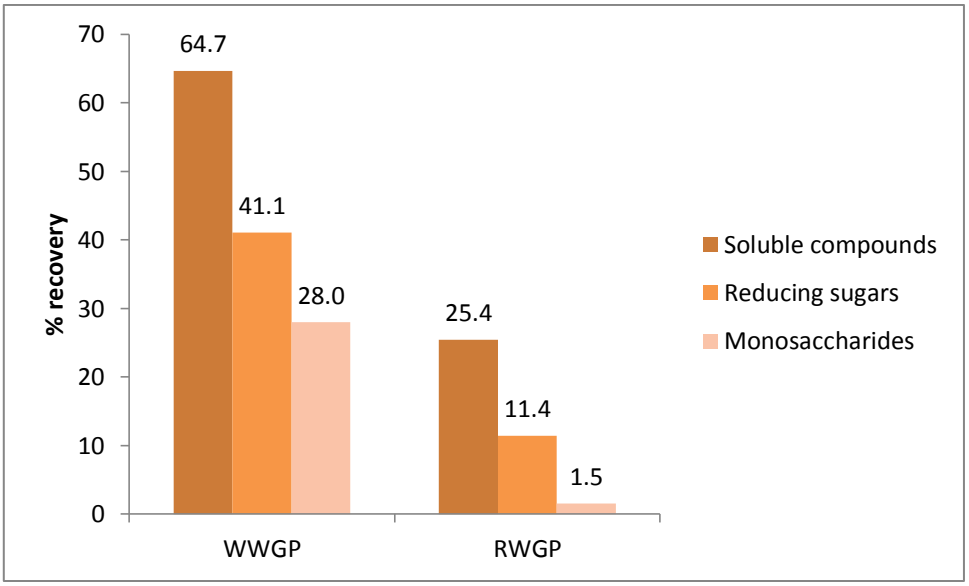

Figure 3.13 – Cumulative amounts of water soluble compounds, reducing sugars, and monosaccharides recovered in samples collected throughout the assay with WWGP or RWGP. % recovery is measured relative to GP powder. For WWGP, T = 190 °C, Water flow rate = 10 mL/min. For RWGP, T = 180 °C and Water flow rate = 5 mL/min, P = 100 bar.

For the sake of closing mass balances it was performed acid hydrolysis of the residues that were recovered from the reactor after the HCW process. Comparing these results with the reducing sugars extracted (Table 3.7), there is a gap between the two sets of values, whose sum should be equal to the total amount of reducing sugars in the GP powder (Table 3.1). In the case of WWGP, the sugars not quantified amount to around 6.3% of GP powder at 210 °C (difference between 57.8% in Table 3.1., and 49.3% in Table 3.7 + 2.2% in Table 3.8), which was the assay where more sugars were extracted, mainly free sugars. For RWGP, the loss of carbohydrates amounted to around 13.5% of GP powder (difference between 33.5% in Table 3.1., and 11.4% in Table 3.7 + 8.6% in Table 3.8), but the recovery in the residue was higher due to the higher content in structural sugars of RWGP relative to WWGP.

As referred earlier, during the process there could occur a loss of sugars by degradation into other compounds that were not detected, and are not quantified by the colorimetric method, or more probably, there are more sugars being extracted during the cooling phase at the end of process, where a much higher water flow is used for almost an hour. If this is the case, then the balance for the sugars cannot be correctly done.

Table 3.8 - Content in reducing sugars of the residue that remained in the reactor at the end of the HCW process. The % values given compare directly with the % values given in Table 3.1. P = 100 bar.

	Conditions		Reducing sugars (wt.%)
	T (°C)	Flow rate (mL/min)	
WWGP	170	10	3.7
	190	10	3.4
	210	10	2.2
	190	5	5.6
RWGP	180	5	8.6

3.2.2.3 Lipid content

The content in lipids of the GP residue that remains in the reactor after the extraction/hydrolysis was determined through Soxhlet extraction with *n*-hexane. This procedure had two objectives: to calculate how much oil (lipids) HCW is able to extract, and to evaluate the potential of those final residues as a source of oil after HCW treatment.

The results obtained are given in table 3.9.

Table 3.9 - Oil content of the GP residue that remained in the reactor after HCW treatment (RR) and what that amount represents relative to the amount of oil in the GP powder (GPR).

	Conditions		Oil content (wt.%)	
	T (°C)	Flow rate (mL/min)	RR	GPR
WWGP	170	10	17.0	3.6
	190	10	20.3	4.0
	210	10	20.9	3.2
	190	5	13.3	4.9
RWGP	180	5	21.2	10.2

In the case of WWGP, the oil content in the residues that remain in the reactor increases as the temperature of the assay increases. The increase in temperature brings about a decrease in polarity of HCW, and it might be expected that water would be a better solvent for oil, leading to lower levels of oil in the residue left in the reactor. However, the increase in temperature is accompanied by an increase in biomass conversion. The balance between these two effects leads to a higher concentration of oil in the residue left after HCW extraction (up to 20% oil content)

when compared to the GP powder (around 7% oil content; table 3.1), increasing temperature accentuating this effect. The influence of the water flow rate should be similar (lower biomass conversion).

Around 43% of the oil in GP powder from WWGP is not extracted by HCW (around 3% of the total weight of GP powder used). From this data it can be concluded that the residues treated with HCW have good potential as a source of lipids, which could be recovered by oil extraction with another green solvent, such as supercritical CO_2.

Before being used as oil source, it is necessary to evaluate the oil composition, for example the fatty acid profile, as well as the existence of other compounds of interest, such as antioxidant compounds that were not extracted by HCW due to their polarity.

In the case of RWGP, the results obtained indicate that only a small amount of the oil present in GP powder (11.9 wt.%) was extracted by HCW (the equivalent to around 1% of the total weight of GP powder used). Again, the residue remaining in the reactor is much richer in oil than the original material.

3.2.3 Antioxidant Activity (DPPH assay)

The antioxidant activity of different GP extracts was evaluated through their action in DPPH radical inhibition. The extracts selected for the study were those that had higher TPC, which were those collected at the highest temperatures. The liquors collected once the temperature in the reactor reached the maximum value were added together and lyophilized, and the resulting extracts were tested.

In table 3.10 it is shown the half maximal effective concentration (EC_{50}) for each extract. The maximum antioxidant activity for WWGP was achieved with extracts collected at 210 °C, which are the extracts with higher contents of phenolic compounds.

Figure 3.14 shows the influence of extract concentration on its antioxidant activity. The highest antioxidant activity observed for all the assays was around 92-93% for a concentration of extract of around 55 µg/mL (in the 4.15 mL solution used for the DPPH assay) for an assay temperature of 210 °C.

Figure 3.15 shows that indeed, in the case of extracts from WWGP, an inverse relationship is obtained for TPC and EC_{50} values, which indicates that HCW extraction at higher temperatures produces extracts with higher antioxidant activity. The changes in water flow rate in this case do not lead to significant differences in antioxidant activity, which may be due to the fact that the corresponding TPC values were similar.

In the case of RWGP, it was obtained an EC_{50} of 22.1, and maximum antioxidant activity was reached at around 150 µg/mL, with approximately 94% of DPPH radical inhibition.

Interestingly, the RWGP extract obtained at 180 ºC, with a TPC of 87.8 mg/g, had a higher antioxidant activity than the WWGP extract obtained at 190 ºC, with a TPC content of around 87.4 mg/g. This data indicates that the antioxidant activity is not only related with the total phenolic content, but also with the different phenolic compounds that are present in each extract. Different compounds have different antioxidant activities [69] and different EC_{50} values.

As shown earlier (Table 3.5), the composition of the two extracts is different. The presence of quercetin, which is known to possess high antioxidant activity [70], was only detected in RWGP extracts, which might help explain why those extracts have comparatively a higher antioxidant activity.

Table 3.10 - Half maximal effective concentration of extracts obtained from sugar-rich liquors collected after maximum temperature was reached in every assay.

	Conditions		EC_{50}	TPC
	T (°C)	Flow rate (mL/min)	(µg/mL)	($mg/g_{extract}$)
WWGP	170	10	72.3	44.4
	190	10	35.1	87.4
	210	10	20.8	113.4
	190	5	39.8	79.0
RWGP	180	5	22.1	87.8

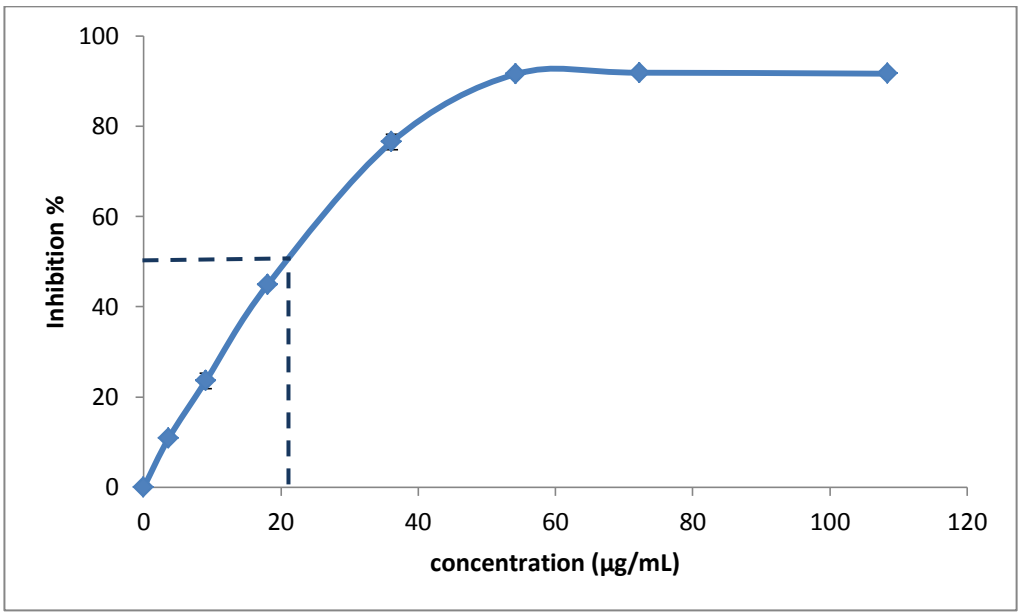

Figure 3.14 - Antioxidant activity of the extracts obtained from liquors collected at 210 °C and water flow rate of 10 mL/min. P = 100 bar. The lines represent the EC_{50} value.

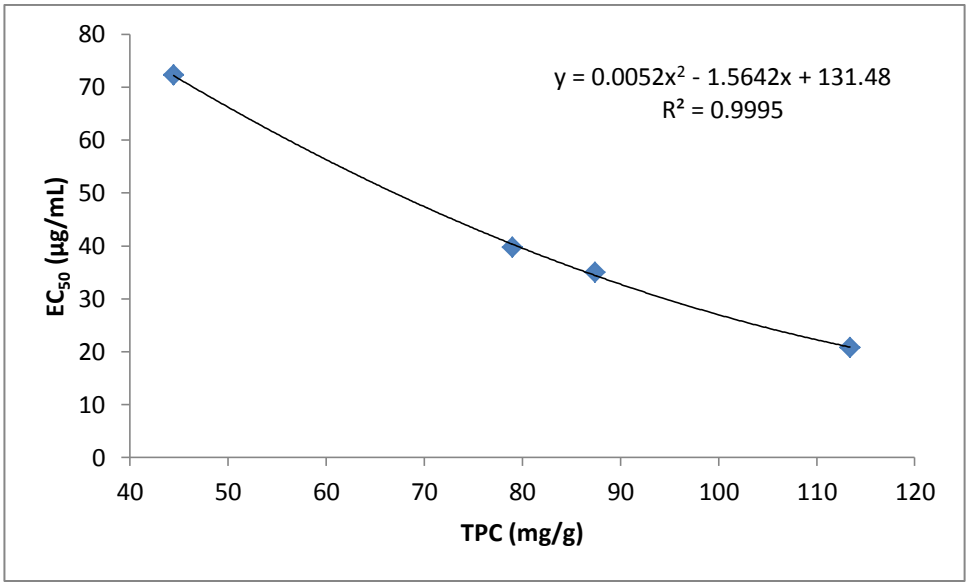

Figure 3.15 - Relationship between half maximal effective concentration and TPC of WWGP extracts, obtained from liquors collected at different conditions.

4 Conclusions

The aim of this work was the valorization of grape pomace, one of the most abundant agro-industrial residues, using HCW for extraction/hydrolysis of value-added products. Special attention was given to grape pomace from white winemaking (WWGP), and only one set of conditions was tested for grape pomace from red wine making (RWGP), which had been the object of an earlier study in the host laboratory.

Before starting HCW treatments, it was performed a chemical characterization of WWGP and RWGP. It was observed that WWGP has a high content of free sugars, around 40%, while RWGP has a comparatively higher content of structural sugars, lipids, proteins, lignin and ash.

RWGP was seen to have a three times higher content in phenolic compounds than WWGP, and among those a few were identified, namely gallic acid and vanillic acid in both, syringic acid and quercetin in RWGP, and vanillin and catechin in WWGP. From the total phenolic content only a small part was identified by HPLC, due to the use of a limited number of standards, or to pH variations that led to forms of phenolics that eluded the method of detection.

In addition to phenolic compounds, grape pomace is a source of carbohydrates. To evaluate the influence of temperature and water flow rate in the extraction/hydrolysis of carbohydrates, three temperatures (170, 190, 210 °C) and two water flow rates (5 and 10 mL/min) were tested, best results being obtained at 210 °C, 10 mL/min, and a pressure of 100 bar: yield in water soluble compounds of 69%, and biomass conversion of 85%. The assay with RWGP, aimed at scaling-up the process already studied (180 °C, 5 mL/min), led to a very low yield in water soluble compounds (25%), and the process must still be optimized.

The highest content of phenolic compounds was achieved in extracts obtained at 210 °C for WWGP and 180 ºC for RWGP: 26.2 and 19.5 mg/g, respectively (expressed per g of the original residue, on a dry basis). The content of phenolic compounds of the GP extracts obtained depends on temperature, and is little affected by water flow rate. Extracts richer in phenolic compounds are obtained once the temperature of the assay reaches its maximum value. The most abundant phenolic compound identified was gallic acid (0.36 mg/g for WWGP at 210 °C, and 0.51 mg/g for RWGP at 180 °C). The amounts of total phenolic compounds (TPC) measured in the extracts are much higher than those obtained in chemical characterization, possibly due to the fact that HCW treatment leads to the depolymerization of lignocellulosic material, making available phenolic compounds that were trapped in the structure, and additionally may lead to lignin degradation. The latter was shown to occur to some extent, as seen by the content in lignin of the residue remaining in the reactor after the HCW process.

Lignin is composed by phenolic compounds, and thus lignin degradation could contribute to the TPC values obtained.

From carbohydrates analysis it was found that the highest recovery of carbohydrates in the liquors obtained occurred in assays performed at 210 °C (49.3%; 11.4% for RWGP, at 180 ºC). In this case the water flow rate proved to have a larger influence than temperature, a lower water flow rate leading to lower yields in water soluble compounds. It was also concluded that the first samples collected are rich in sugars due to the extraction of free sugars, the following samples having comparatively lower carbohydrate contents. HPLC analysis confirmed that the first samples collected in the assays have only glucose and fructose. At higher temperatures, when water becomes more reactive, are other monosaccharides detected, namely coming from hemicellulose. It is interesting to note the increase in fructose which was not expected because this is not a structural sugar, unlike glucose monomers which are the building blocks of cellulose. The high amounts of fructose detected can be explained by the Lobry de Bruyn–Alberda van Ekenstein transformation of glucose into fructose. Through the quantification of the sugars of the GP residue that remains in the reactor after HCW treatment, it was concluded that around 15% of sugars are not detected, either because of degradation in the reactor, or because of extraction during the cooling phase after the end of the process. The latter could be monitored in future assays.

It was also analyzed the lipid content of the GP residue that remained in the reactor, being observed that for WWGP, lipid extraction is higher (around 50% of the total amount present in the original residue) than for RWGP (1%). However, it is interesting to note that in both cases, the final residues are much richer in oil than the original residues.

To evaluate the potential of the GP extracts their antioxidant activity was evaluated, being observed for WWGP that with the increase in the temperature of the assay, the antioxidant activity increases, which leads to a lower half maximal effective concentration of the extracts. This fact is related to the phenolic content of the extracts that are richer in such compounds at higher temperatures. Another conclusion is that the RWGP extracts have a higher antioxidant activity than WWGP extracts, because the same TPC led to a much lower EC_{50}, a fact that must be related to the antioxidant activity of the different compounds present in the extracts.

5 Future work

- Optimize HCW conditions for red wine grape pomace extraction/hydrolysis;

- Perform supercritical CO_2 extraction from the GP residue that remained in the reactor after HCW treatment;

- Continue with antioxidant activity tests for WWGP extracts and develop an application in the cosmetics, food or pharmaceutical industry;

- Perform enzymatic hydrolysis of the extracts to convert remaining oligosaccharides to sugar monomers;

- Perform microorganism growth studies with the extracts from WWGP richer in carbohydrates, to evaluate their potential as alternative carbon source, and develop another application by producing added-value products from microbial activity.

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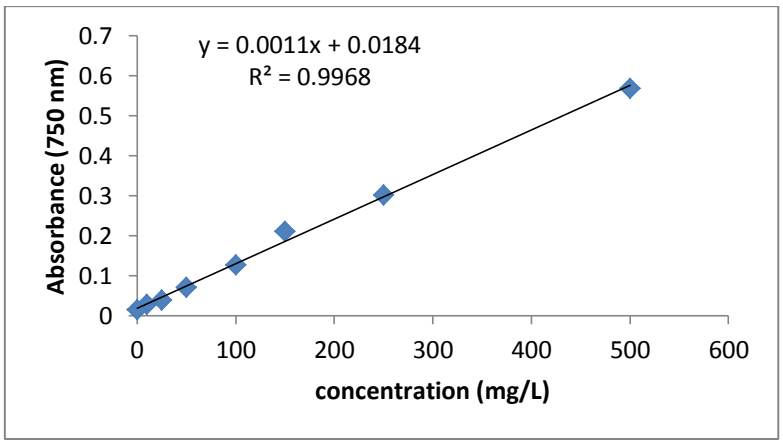
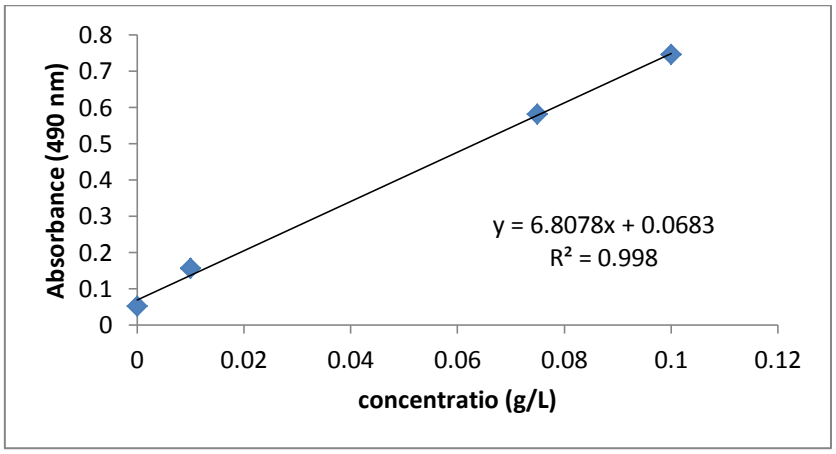
7 Appendix

Colorimetric methods

Gallic acid standard curve

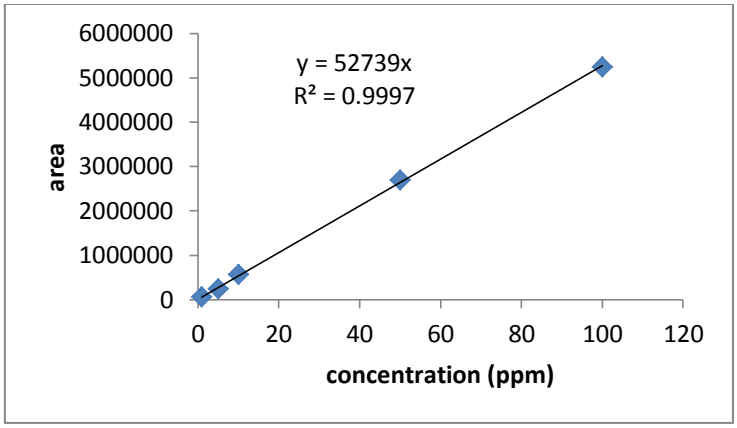


Glucose calibration curve

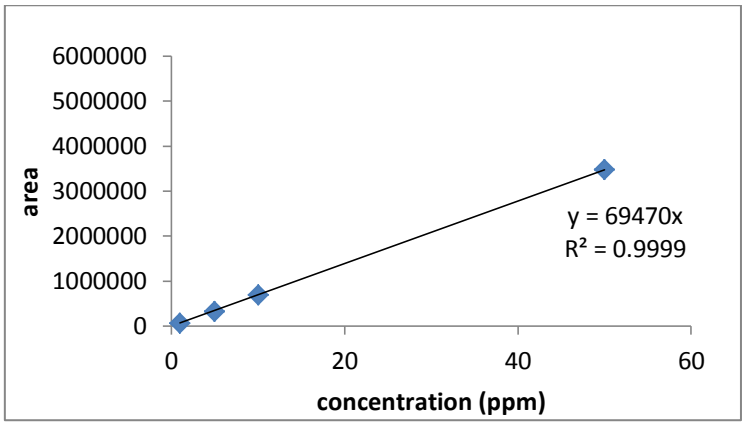


HPLC methods

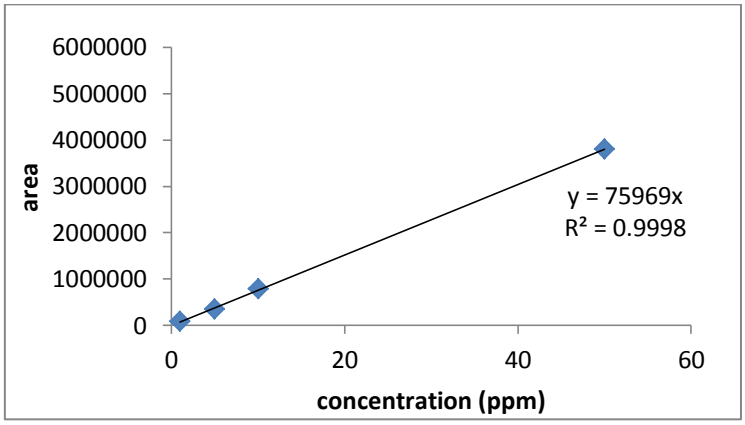
Gallic acid calibration curve



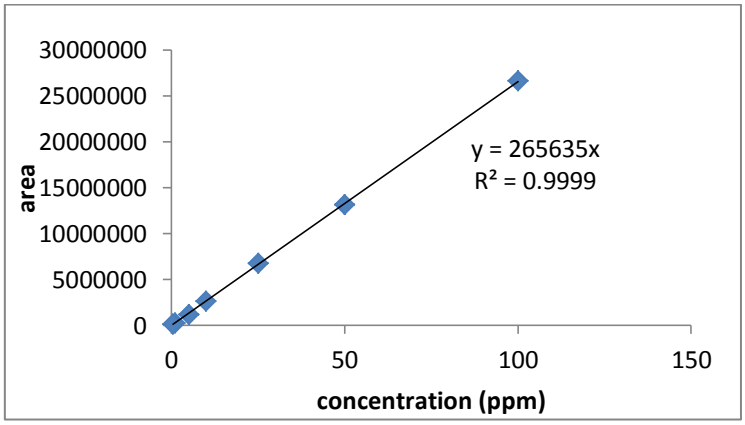
Vanillic acid calibration curve



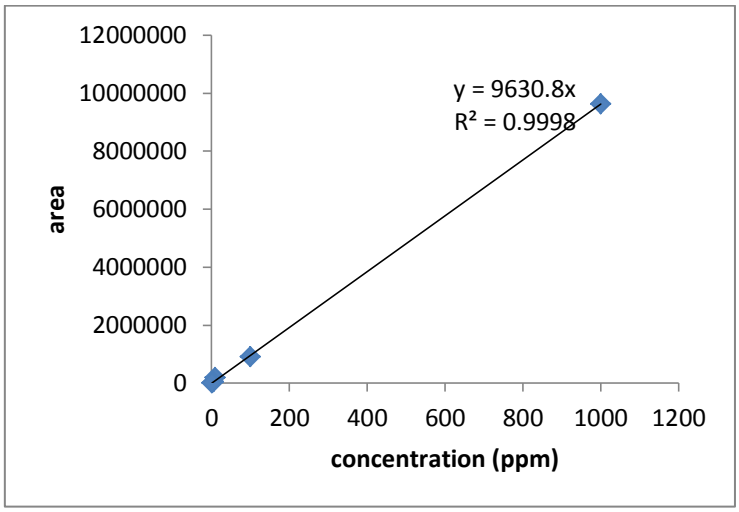
Syringic acid calibration curve



Quercetin calibration curve



Catechin calibration curve



Vanillin calibration curve