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LICENCIADA EM BIOQUÍMICA

Valorization of grape pomace residues integrating hot compressed water with biotechnology

DISSERTAÇÃO PARA OBTENÇÃO DO GRAU DE MESTRE EM BIOTECNOLOGIA

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VALORIZATION OF GRAPE POMACE RESIDUES INTEGRATING HOT COMPRESSED WATER WITH BIOTECHNOLOGY

To the school of life and RRP.

Nature does nothing in vain.

(Aristotle)

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"Eis o meu segredo: só se vê bem com o coração.
O essencial é invisível aos olhos."

Antoine de Saint-Exupéry

Por mais que sejamos engenhosos na arte da escrita, as palavras nunca são suficientemente completas para transmitir um sentimento genuíno.

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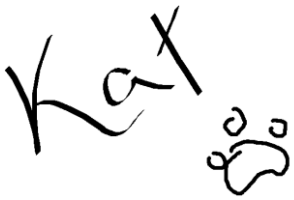
"Quem me conhece de verdade,

sabe o significado do meu silêncio."



RESUMO

O objetivo do presente trabalho consiste no desenvolvimento de um processo de valorização do bagaço de uva (GP), um dos resíduos agroindustriais mais abundantes no mundo. Foram testados dois caminhos de valorização distintos através da utilização de água subcrítica tanto como meio de reação como agente de extração.

Enquanto os compostos polifenólicos podem ser extraídos usando água subcrítica, as estruturas de polissacáridos podem ser obtidos na sua forma mais simples a partir da reação de hidrólise utilizando água subcrítica como solvente e reagente. Os carbohidratos obtidos foram, posteriormente, utilizados como fontes de carbono para o crescimento de leveduras oleaginosas.

As experiências de hidrólise foram realizadas a uma pressão constante de 100 bar, um caudal de 2-5 ml / min de água e a temperaturas máximas de 150 ºC a 300 ºC.

O maior rendimento de hidrólise, 90%, foi obtido a 300 °C, no entanto, apenas 30% da massa inicial foi recolhida na forma de produtos solúveis. Este resultado foi semelhante para todas as temperaturas estudadas. As quantidades máximas de furfural e 5-HMF foram obtidas a 250ºC e 200 ºC, respetivamente. A quantidade total de monossacáridos recuperados nos hidrolisados variou entre 7 - 9%.

As experiências de extração foram realizadas em simultâneo com as reações de hidrólise. O total de compostos fenólicos (TPC) para as temperaturas de 150ºC e 180ºC foram de 497 µg / g $_{\text{resíduo inicial}}$ e 506 µg / g $_{\text{resíduo inicial}}$, respetivamente. No entanto, a análise de HPLC revelou um teor máximo de apenas 30 µg / g $_{\text{resíduo inicial}}$ relativo ao ácido gálico obtido a 180 °C.

Os hidrolisados foram, posteriormente, utilizados como fontes alternativas de carbono para o crescimento de leveduras oleaginosas do género *Rhodotorula* e *Rhodosporidium*. As melhores taxas de crescimento foram obtidas para os hidrolisados recolhidos a 150 ºC e 180 ºC por *Rhodotorula babjevae* (0,05 h^{-1} e 0,04 h^{-1}) e *Rhodotorula yarrowii* (0,04 h^{-1} e 0,04 h^{-1}), respetivamente.

Termos chave: bagaço de uva, água subcrítica (HCW), polifenóis, hidrolise, leveduras oleaginosas

ABSTRACT

The main goal of the present work is the development of a process for the valorisation of grape pomace (GP), one of the most abundant agroindustrial residues in the world. Different valorisation pathways were studied using hot compressed water (HCW) as reaction medium and as extraction agent.

While polyphenolic compounds could be extracted using HCW, simple saccharide structures could be obtained from the hydrolysis reaction using HCW as both solvent and reactant. The saccharides obtained were used as carbon sources for oleaginous yeast growth.

Hydrolysis experiments were performed at constant pressure, 100 bar, a flow rate of 2 – 5 ml/min of water and maximum temperature between 150ºC - 300 ºC.

Highest hydrolysis yield of *ca.* 90% was obtained at 300 ºC, however only about 30% of the initial mass was obtained as soluble products. This result was similar for all temperatures studied. Accordingly to chemical composition of hydrolysates higher yields of furfural and 5-HMF were obtained at 250ºC and 200ºC respectively. Amount of total monosaccharides recovered in the hydrolysate varied between 7 - 9%.

The extraction experiments were carried out simultaneously to the hydrolysis reactions. Total phenolic contents (TPC) of 497 $\mu g/g_{initial\ residue}$ and 506 $\mu g/g_{initial\ residue}$ were obtained at 150ºC and 180ºC respectively. Nevertheless by HPLC analysis only 30 $\mu g/g_{initial\ residue}$ was obtained at 180ºC. The antioxidant with the highest content identified was gallic acid.

Afterwards hydrolysates were used as carbon sources by oleaginous yeast of genus *Rhodotorula* and *Rhodosporidium.* The best growth rates were achieved with hydrolysates collected at 150ºC and 180ºC by *Rhodotorula babjevae (*0,05 h^{-1} and 0,04 h^{-1}) and *Rhodotorula yarrowii (*0,04 h^{-1} and 0,04 h^{-1}).

Keywords: grape pomace, hot compresses water (HCW), polyphenols, hydrolysis, oleaginous yeasts

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NOMENCLATURE

CO_2 – carbon dioxide
GP – grape pomace
HCW – subcritical water / hot compressed water
HPLC – high performance liquid chromatography
K – Kelvin
MPa – mega Pascal
OD – optic density
ppm – parts per million
SCF – super critical fluids
TAG – triacylglyceride
TPC – total phenolic content
YNB – yeast nitrogen based

OVERVIEW

"In the beginning there was nothing, which exploded."
— Terry Pratchett, Lords and Ladies

1. Chapter: OVERVIEW

1.1 - Biorefinery concept

Human evolution, not just like a biological specie, but firstly as a complex organized society, would be unimaginable without energy. It's a broad concept that reveals in many forms and is originated by innumerable fonts that has an undeniable role starting from our day by day basic need, to inaccessible hunger of our technological progresses.

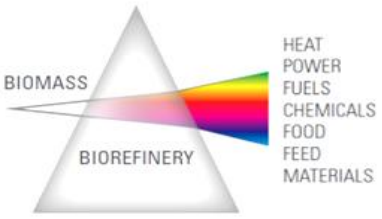

Figure 1.1 - Schematic representation of biorefinery concept.

Last century statistical analysis shows an exponential raise of two main variables that affects directly world energy needs: technological development of industrialized countries and demographical grows of developing regions. This results in an exponential growth of our energy needs (Fig. 1.2) which is not sufficiently followed by a strong renewable energy sources development in order to relieve classic fossil fuel that provides for centuries a solid energy base to our society since the first Industrial Revolution.

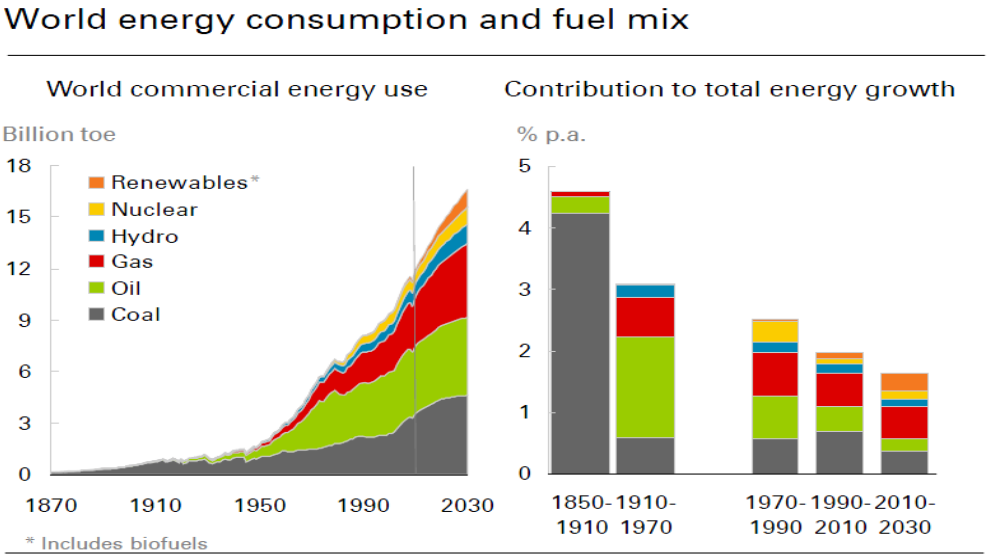

Figure 1.1 - The growing needs for alternative energy sources.
The decreasing use of coal firstly due to the appearance of oil and gas in the second middle of the last century and the increasing interest in the renewable sources that marks the beginning of the second millennium.[1]

Shifting society's dependence from petroleum-based to renewable biomass-based resources is considered as the key to the development of a sustainable industrial society and the effective management of greenhouse gas emissions.[2] Changing these tendencies, the future world energy economy will likely be based on a wide range of alternative energy platforms: wind, water, sun, nuclear fission and fusion, as well as biomass. Similarly, the production of chemicals will increasingly depend on biomass, particularly derived from plants.[3] These results in bio-based chemicals with the same functionality and performance will represent a "green" alternative to traditional petroleum-based ones.

The biorefinery concept development is related with the increasing importance of biomass in the energy field. It's widely used nowadays and it covers a range of industrial realities. Therefore, several definitions have been developed over the last few years according to the context, making this term a subject to debate, but the core concept embedded in all definitions is that the overall goal of the biorefinery production approach 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.[4] In other words, bioconversion processes development allows to almost obsolete the term ''waste", in its application to biomass processing, since each production stream has the potential to be converted into a by-product stream rather than waste streams.[3] There are three different product categories derived from biomass: biofuels (e.g. biodiesel and bioethanol), bio-energy (e.g. heat and power) and bio-based chemicals and materials (e.g. antioxidants and biopolymers). The biorefinery concept is then analogous to today's petroleum refineries that produce multiple fuels, power and chemical products from petroleum. [35]

A biorefinery approach is a multi-step process (Fig. 1.3). First of all, due to inherently complex and heterogeneous in composition of the biomass it typically needs pre-treatment to turn the raw material more amenable for further processing. Following pre-treatment, the biomass components are subject to a combination of biological and/or chemical treatments attending to final products required (Fig. 1.3). The outputs from this step (e.g. specialty chemicals or reducing sugars) could be converted to chemical building blocks for further processing uses, to polymers ready for market use and to a fuel/energy sources.[3]

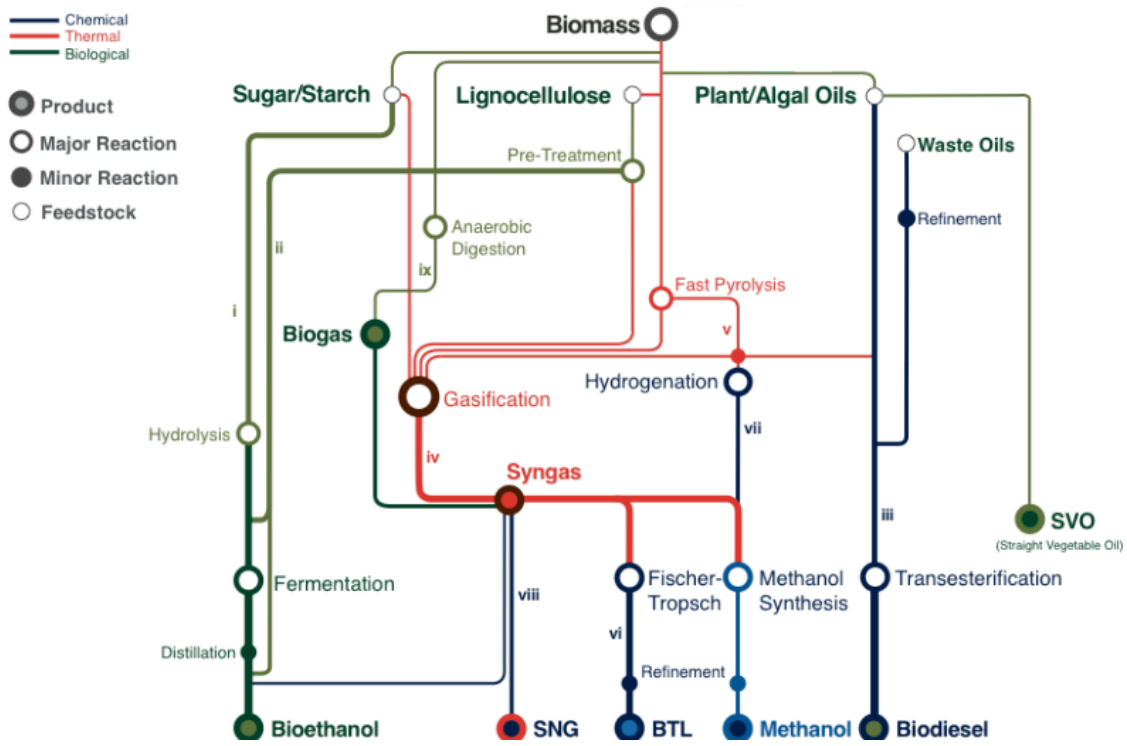

Figure 1.2 - A schematic representation of the complexity and diversity of biorefinery processes. Principal outfits such as Bioethanol, Synthetic Natural Gas (SNG), Biomass-to-Liquid (BTL), Methanol, Biodiesel and Straight Vegetable Oil (SVO). Processes designed by Latin numbers are: i. Fermentation of Sugar/Starch Crops; ii. Fermentation of Lignocellosic Biomass; iii. Transesterification of Triglycerides; v. Gasification: Formation of Syngas; v. Fast Pyrolysis; vi. Fischer-Tropsch Synthesis; vii. Hydrogenation; viii. Conversion of Syngas to Methane; x. Anaerobic Digestion. [5]

Unfortunately, the relatively low energy content, seasonality and discrete geographic availability of biomass feedstock have been noted as principal barriers to the large volume demands for energy and fuel. In contrast, the development of bio-sourced chemical products requires far lower fraction of biomass to satisfy demand. Consequently, there has been an increase in lignocellulosic biomass processing research, focusing particularly on agricultural and forestry residues, due to its low cost, abundance and renewability. That new perspective brought the topic of processing bio-sourced materials to the forefront of sustainable engineering research of the last 15 years resulting in the considerable number of critical research reviews about lignocellulosic biomass pretreatment [6], hydrolysis [7], enzymatic and microbial conversion [8], as well as biomass processing approaches [9] [10]and future biorefining perspectives. [11]

Regarding the upgrading from the research field to the Chemical industry perspective, replacing selected chemical intermediates in current product portfolios of the chemical companies have more advantages comparing to the full building of an entire biorefinery. Using this strategy, biorefinery implementation is mostly driven by economics (not regulation) and sustainability concerns. Therefore in the future industry reality may emerge three following types of companies[5]:

• Traditional chemical companies that replace fossil chemicals with green alternatives within already existing product lines;
• New companies focused on the production of completely novel products out of biomass; however, the main challenges will be to integrate these new molecules into existing value chains, and the related long commercialization process;
• Companies that deliver technologies like cell factories designed by metabolic pathway engineering, with the potential to deliver both existing and novel chemical compounds into the value chain without producing the chemicals themselves; for many of these companies, a royalty-based business model would be preferred.

Nowadays, the implementation in industry of biorefinery processes is still in a nascent state, with most second-generation biorefinery plants (using cellulosic material). The landscape of active small companies is rather scattered and fragmented, but there are an increasing number of large investors starting to be interesting in this subject. EU, for example, has discussed its future perspectives in the biorefinery field in "European Biorefinery Vision for 2030"; similar studies are carried out through over the industrialized world.

1.2 – Agro industrial residues

The agro-industrial residues consist of many and varied wastes from agriculture and food industry.[12] They represent a main source of biomass due to its low cost, abundance and renewability. In the European land exploitation perspective, agriculture sector hold on the second place covering about 40% of total EU land; almost the same area is covering by forestry (circa 42%). Consequently, a wide variety of biomass is available and that, which is potentially available for non-food use, comes mostly from the agro-forestry sector.[4]

Statistically, cereals are the major arable European crop accounting for about 59 million hectares, with one sixth of this area used for wheat. Rapeseed and sugar beet also represent the main source of biomass for non-food use, predominantly to produce biofuels along with other crops that represent minor fractions.[4]

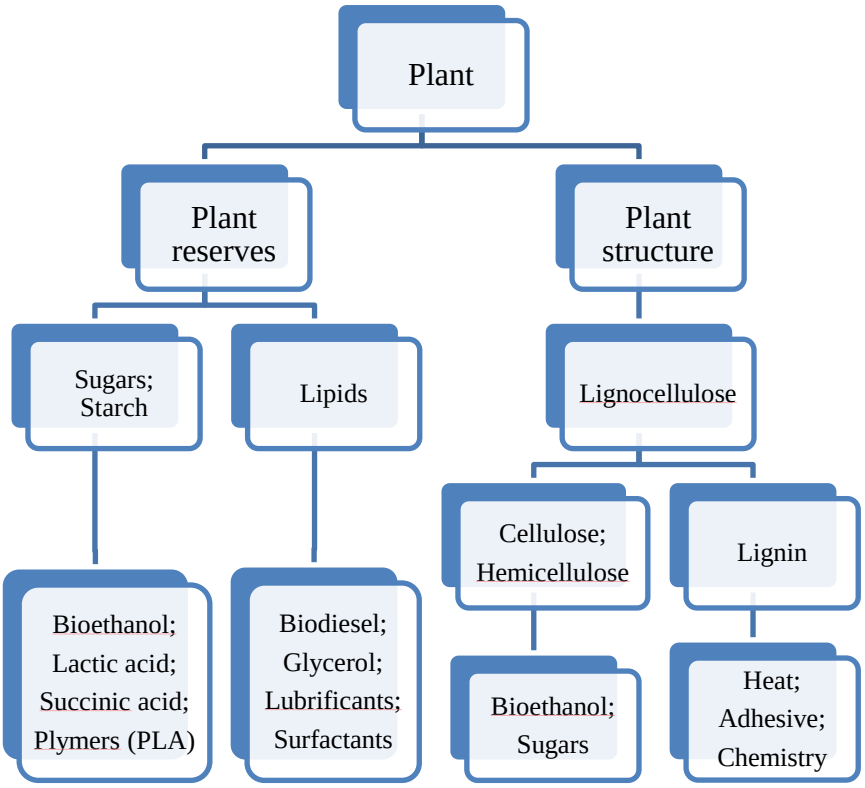

Figure 1.3 - Theoretical example of possibility of the whole plant valorisation in biorefinery, based on the function of plant components used as raw material. (adopted from [13])

This data reveal that the development of the European Biobased Economy already assumed that a large part of the material generated in both the food and non-food value chains should be used as raw materials to develop processing systems capable to produce by-products with more added-value. Currently, agricultural residues are mainly used for livestock feed and litter as well as to maintain soil fertility. However, European policy is starting to focus on transformation in the large scale of these residues into added-value biomaterials, biobased chemicals and biofuels, or its use as a local source of power or biogas.

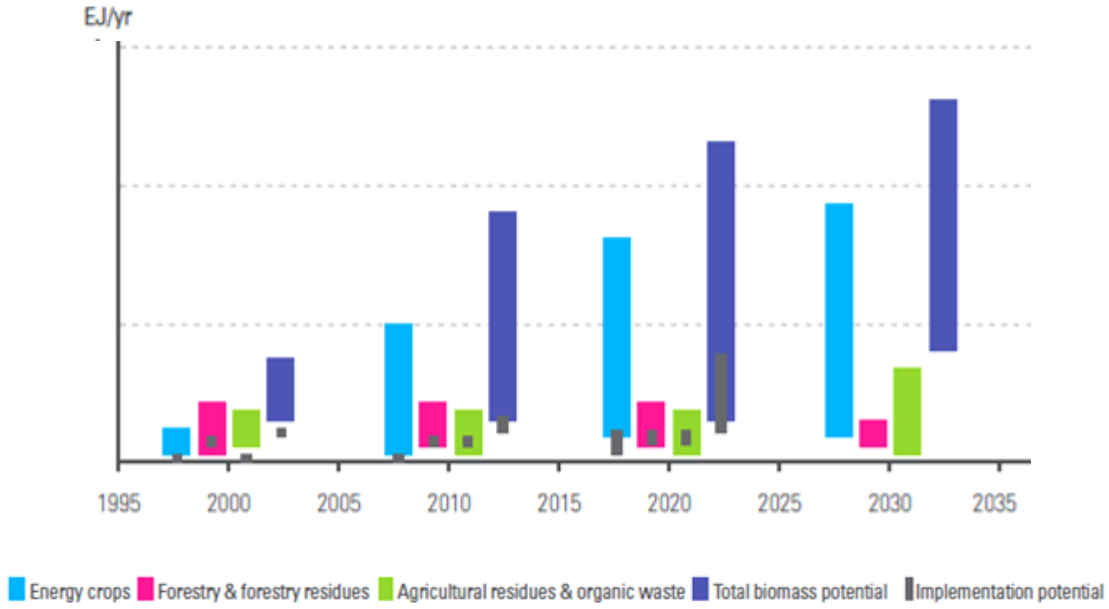

Figure 1.4 - This diagram shows a summary of biomass potential at European level (by sector and in total) in the past, present and future perspective.
European policy focuses essentially on energy crops (agricultural biomass resources such as sugar- and starch derived biomass (e.g. sugar beet, wheat and maize), oilseed derived biomass (e.g. rapeseed, sunflower)and lignocellulosic crops (C4-grasses or short rotation coppice)that will significantly contribute to the growth of total biomass potential.(adapted from [4])

The main characteristic of agro-industrial residues is its lignocellulosic nature. They are composed by three principal polymers: cellulose, hemicellulose and lignin and in a minor extent by structural proteins, lipids and ash. [14] (fig.1.6)

Cellulose is composed by D-glucose units condenced through β(1→4)-glycosidic bonds and also linked by hydrogen bonds established between the multiple hydroxyl groups presented on the structure. The resulting *microfibrils* have a high tensile strength and its semicrystalinity confers resistance to hydrolysis due to absence of water in the structure and the strong bond between the glucose chains prevents hydrolases act.[15]

Hemicellulose principal constituents are xylose, arabinose, galactose and manose that present crosslinking with glycans. It contains most of the D-pentose sugars from which xylose is, in most cases, present in the largest amount. Typically, hemicellulose comprises between 20 to 50% of the lignocellulose polysaccharides. It's a random, highly heterogeneous, amorphous and easily hydrolysed structure. Hemicellulose serves as a connection between the lignin and the cellulose fibbers and gives more rigidity to the cellulose – hemicelluloses – lignin network. [13] Hemicellulose and cellulose constitute 13–39% and 36–61% respectively, of the total dry matter, respectively. [12]

Lignin is one of the most abundant organic polymers on Earth, exceeded only by cellulose. It's a relatively hydrophobic and aromatic structure made of racemic units of phenylpropanoid from which sinapyl alcohol, coniferyl alcohol and p-coumaryl alcohol are most abundant (fig. 1.5). The phenolic structure of this polymer confers resistance to enzymatic digestion; as a result its disruption represents the main target of raw material pretreatments before enzymatic hydrolysis.[16]

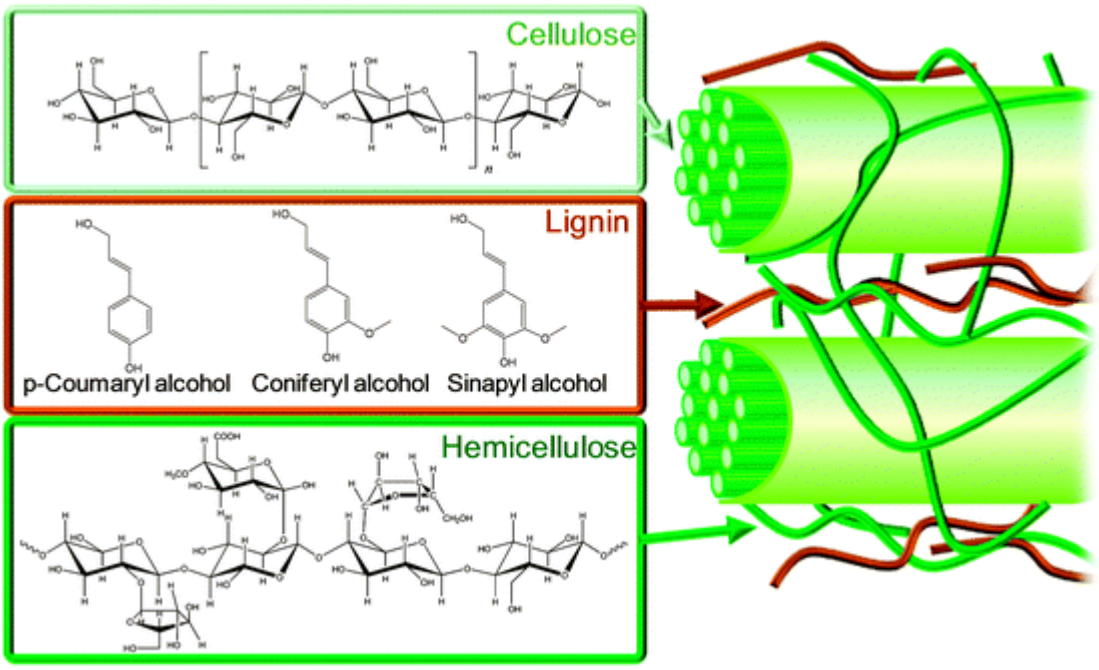

Figure 1.5 - Schematic structure representation of lignocellulosic biomass with cellulose, hemicellulose, and lignin represented. [17]

Due to its complex, heterogeneos structure lignocellulose has been selected during the natural evolution to be the key element of plant structure and consequently to be resistant to biotic and abiotic stresses wich force the need of pretreatment to turn it more amenable for further

processing. There are several methods (fig. 1.6) as treatment with diluted acid (chemical), grinding (physical) or use of hot compressed water (physical and chemical), among others, which were developed to fractionate lignocellulosic biomass allowing a flexible, integrated processing approach and/or creating an access to substrate for enzymes and micro-organisms and enhance its digestibility.

Table 1.1 - Examples of some conventional pre-treatment methods. [12]

Pre-treatment	Examples	Effect of Pre-treatment
Physical	Milling	Fine, highly decrystalized structure
	Steam Explosion, Steaming treatment	Increased pore size/hemicellulose-hydrolysis
	Hydrothermal	Hemicellulose hydrolysis, alteration in properties of cellulose and lignin.
	Irradiation	Depolymerization
Chemical	NaOH, NH_3, H_20_2 Peroxyformic acid, Organosolvents	Lignin/ hemicellulose degradation
	Peroxymonosulphate	Activates deliginification
Biological	White-rot fungi (*Bjerkendra adusta, Phanerochaete chysoporium, Ceriporiopsis subvermispora*) Specific bacteria	Lignin degradation
Enzymatic	Lignin Peroxidases (LiP, MnP, laccase)	Selective lignin/hemicellulose degradation

STATE OF THE ART

"The most incomprehensible thing about the world is that it is at all comprehensible."
— Albert Einstein

2. Chapter: STATE OF THE ART

2. 1 – Residue valorisation: Added value by-products

The majority of organic chemicals and polymers are still derived from fossil based feedstocks, predominantly oil and gas. Non-energy applications account for around 9% of all fossil fuel (oil, gas, coal) use and 16% of oil products.[18] From a technical point of view, almost all industrial materials made from fossil resources could be substitutted by their bio-based counterparts. However, the cost of bio-based pruduction in many casses exceeds the cost of petrochemical production. Also new products must be proven to perform at least as good as the petrochemical equivalent and to have lower environmental impact. Historically, bio-based chemical producers have high value fine or specialty chemical markets, often where a specific functionality played an important role. But the recent climb in oil process, the consumer demand for environmentally friendly products, population grow and limited suplies of non-renewable resources, have now opened a new window of opportunity for bio-based chemicals and polymers. Thus, the integration of biorefinery processes in the co-production of value-added bio-based products (chemicals, materials, food, feed) and secondary energy carriers (fuels, power, heat) is a promissing and efficient approach for the sustainable valorisation of biomass resources in a future biobased economy. Statisticaly, production costs of biofuels could be reduced almost 30% by the co-production of added-value products making the "integrated biorefinery" the main goal of the future biobased industry. [18]

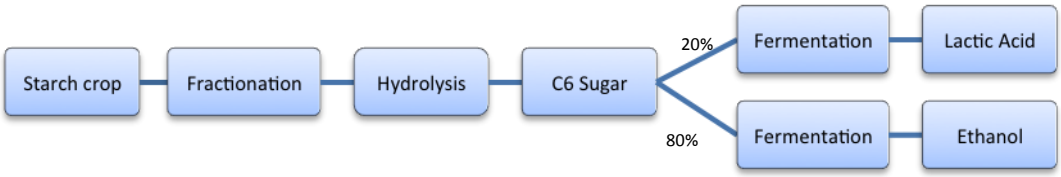

Figure 2.1 - An exaple of integrated ethanol/lactic acid biorefinery production process that reduces final ethanol sale price to about 500€ per tonne from original 700€ per tonne[18].

Due to diversity of industrial biorefinery processses mainly of the original raw materials and final bio-based products, the International Energy Agency nominate eight different platforms from which C6 and C6/C5 Sugar Platform represents particular interest since it covers one of the main objectives of this work. Six carbon sugar platform's main building block is glucose resulting from hydrolysis of starch or cellulose. While mixed six and five carbon platforms are produced from the hydrolysis or dilute acid pre-treatment of hemicelluloses.

Dilute acid hydrolysis (with sulphuric, phosphoric, maleic or oxalic acids) of hemicellulose leads essentially to high yields of xylose (nearly 95%) [14]. Unfortunately, this process may originate undesirable products catalyzing the dehydration of xylose to furfural, which can then form polymers and humins in acidic solutions. Hydrolysis of cellulose may be achieved enzymatically (more selective) or chemically (lower cost).[19] The acid-catalyzed hydrolysis of cellulose typically involves a mineral acid catalyst, but at concentrations higher than those used for hemicelluloses decomposition. As an alternative strategy, cellulose can be processed in an aqueous solution containing a dilute acid at higher temperature to convert the cellulose into equimolar amounts of levulinic and formic acids, passing through glucose and HMF as intermediates [3] [20] [21]. The dilute acid approach is typically favoured due to the lower acid concentrations being less corrosive on equipment and lower acid costs. [14] Several studies are also reporting the possibility of hydrolysis of cellulose by Hot Compressed Water (HCM).[22]

Six and five carbon sugars obtained from hydrolysed cellulose and hemicellulose may serve as a feedstock for fermentation processes originating a variety of important chemicals (Tab. 2.1) or can be chemically converted by dehydration, hydrogenation or oxidation reactions, as examples, to give useful products such as: sorbitol, gluconic acid, furfural, hydnroxymethylfurfural (HMF), among others. All these products have a large application spectrum in Pharmaceutical and Food Industries. For instance over a million tonnes of sorbitol is produced per year as a food ingredient, personal care component (e.g. toothpaste) and industrial use.

Table 2.1 – Global market for fermentation derived fine chemicals. [18]

Chemical	2009 $ millions	2013 $ millions
Amino Acids	5,410	7,821
Enzymes	3,200	4,900
Organic Acids (Lactic acid 20%)	2,651	4,036
Vitamins and related compound	2,397	2,286
Antibiotics	1,800	2,600
Total	15,901	22,351

Economical sustentability and market needs cleary justify intense research and integration of these biorefinery processes. In the other hand, modern biotecnology will allow industry to target new and previosly discharged abandoned products. Coupled with the increase of fossil feedstock costs, cost reduction in the production of traditional fermentation products, such as ethanol and lactic acid, will allow derivative products to capture new or increased market shares and also to allow previosly discharged products such as butanol to re-enter the market.[18]

Not only new chemicals, but also new renewable sources of raw materials have been the focus of the current research on this field. The possible alternative/complementary way to obtain added-value byproducts is its extraction. Idealy, the combination of two pathways: primary extraction of important bioactive molecules and then hydrolysis of polissacharide structures, could considerably improve production yield. Promising candidats are residues provided from food industry with high economical impacts and also with a high accessibility considering a large annual production. The growth of horticulture industries worldwide has generated huge quantities of fruit wastes (25%-40% of the total fruits processed).[23] These residues are generally a good source of carbohydrates, especially cell wall polysaccharides and other functionally important bioactive molecules such as proteins, vitamins, minerals and natural antioxidants.[23]

One of the main examples of horticulture industry residues valorisation are tomatoes. They are one of the major vegetable crops all over the world. Tomato pomace - an inexpensive by-product of tomato manufacturing, left over after processing tomatoes for juice, ketchup, soup, etc., is one of promising examples of new renewable biomass sources. That low cost, abuntant biomass used predomenantly in pet and lifestock food is a good source of dietary fiber, B and A vitamins, lycopene and β-carotene among others value-aded products. [24] [25]Apple pomace is another great example. It's obtained as a by-product during the processing of apple fruits for juice, cider or wine preparation. Owing to the high carbohydrate content, apple pomace is used as a substrate in a number of microbial processes for the production of organic acids, enzymes, single cell protein, ethanol, low alcoholic drinks and pigments but also to the extraction of value added products such as dietary fibre, protein, natural antioxidants, biopolymers, pigments and compounds with unique properties. [23] [26] This work was focused on the valorisation of grape residues with high by-products potential as extractable antioxidants such as: gallic acid, tannins, anthocyanins that could use in pharmaceutical and food industry, and also grape pomace is rich in polysaccharides that could be hydrolysed in simpler structures as used by yeast, for example, for fatty acid production.[27]

However, the central dogma is still the stability, safety and economic feasibility of the processes/products development.

Supercritical fluid extraction is an environmentally benign alternative to conventional industrial solvent extraction. Its main advantage is the absence of toxic residues between products. One of the most frequently used supercritical fluids is carbon dioxide. In addition to the advantage of being neither toxic nor flammable, carbon dioxide is also available at low cost and high purity. Because of its moderate critical temperature, CO_2 can be used to extract thermally labile compounds (e.g. polyphenols). [28]

2.2 – Grape pomace

Grape is a fruiting berry of the deciduous woody vines of the botanical genus *Vitis*. Grapes are a type of fruit that grow in clusters of 15 to 300, and can be crimson, black, dark blue, yellow, green, orange and pink. Today, grape is one of the most abundant world crops mostly cultivated in Europe (~ 40%) followed by Asia (~ 30%) and America (~ 20%).[29] In Portugal, it represents a third major crop cultivated annually in considerable amounts of 920 thousands of tonnes (in 2011) making it a promising renewable biomass source to biorefinery integration processes.[29]

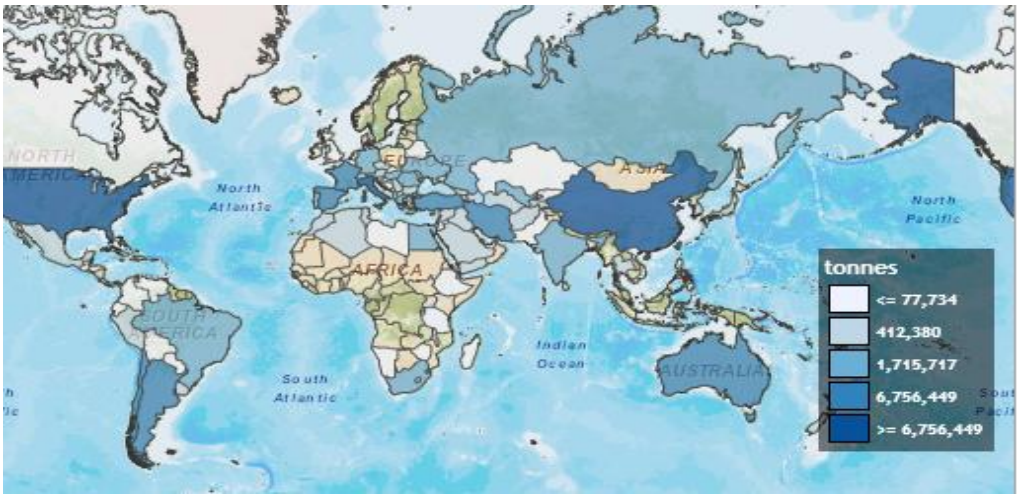

Figure 2.2 - Worldwide production of grapes. In Portugal between 0,4 - 1,7 millions of tonnes were produced in 2011.[29]

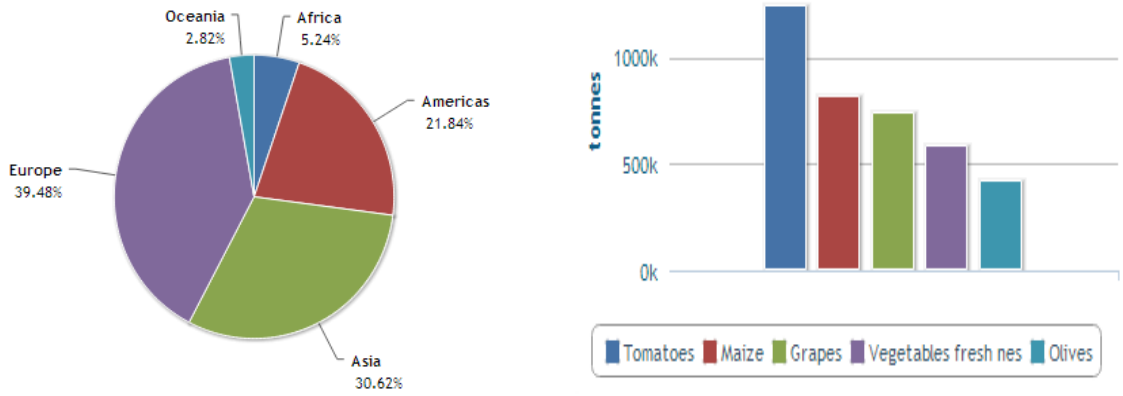

Figure 2.3 - Europe the main world grape producer followed by Asia and America. In Portugal grapes are a third most abundant crop. [29]

Grape conquered the world due the wine making. Archaeological evidence suggests that the earliest known production of wine, made by fermenting grapes, took place between the Caucasus and the Middle East, with evidence of winemaking at different sites dated from 6000 BC in Georgia, 5000 BC in Iran, and 4100 BC in Armenia. Other notable areas of wine

production have been discovered in Greece and date back to 4500 BC. The same sites also contain the world's earliest evidence of crushed grapes. A winemaking press found in 2011 in Armenia has been dated to around 4100 BC.[30]

2.2.1 – Wine making

Wine making is a complex process full of traditions and secrets sacredly guarded by winemakers all over the world. There are three main gropes of wines: still wine, sparkling wine and fortified wine. Between still wines could be identified three different types with consequently three winemaking methods (Fig. 2.6): red wine, white wine and rose.[31] Still wines production uses fermentation process to transform grape juice into wine. If wine is produced through fermentation off skins (named white method), peeled and slightly smashed grapes are used. This method is used for white and rosé wines. If, on the other hand, it is important to maintain the grapes' pigments and tannins, the wine is produced through fermentation on skins (common in reds and rosés made from the red method). The raw material used on this thesis was obtained from the residues of red wine method after "pressing step" so this via of wine making is more interesting to our study. Several possible grape pomace pre-treatment processes are described next of some processes that wine residue "suffered" before its arrival to our laboratory[31]:

Destemming/Crushing

Destemming is the process of totally or partially removing the stems from the grapes. In the production of white or rosé wines, total destemming is usually used. Afterwards, the grapes may or not be crushed. Crushing splits the grapes' skin to release the pulp and juice. When done manually, the process may split the skin or simply crack it. When done mechanically, crushing depends on the distance between the machine's rolls (the smaller the distance, the greater the crushing). Visual aspect of grape pomace residue used in this work suggests that it was subjected to partial destemming or wasn't destemmed at all. (Fig. 2.4)

Figure 2.4 – Visual aspect of GP used in this work. Non crushed stems could be visualised.

Alcoholic fermentation

Red wine is made through fermentation "on skins" (Fig. 2.5), which means that the fermenting must is in contact with the solid parts of the bunches (seeds, skins, and sometimes even stems). The fermentation process, conversion of grapes sugar into alcohol and carbon dioxide by yeasts, takes place in a stainless steel, cement or wooden fermentation tanks.

Figure 2.5 – Grapes fermenting during wine making. (adapted from[31])

Pumping-Over

Grapes' solid parts have a tendency to go up to the surface during fermentation process and it is necessary to mix them with the liquid at the bottom of the fermentation tanks with the help of a pumping system. The advantages of pumping-over are, among others, the homogeneous distribution of yeast and temperature.

Pressing

Unlike white wines, red wines are pressed after fermentation. This happens because the grapes' skins and seeds should be in contact with the must to impart colour and other properties to it. During this process grapes are crushed to release liquid from the berries.

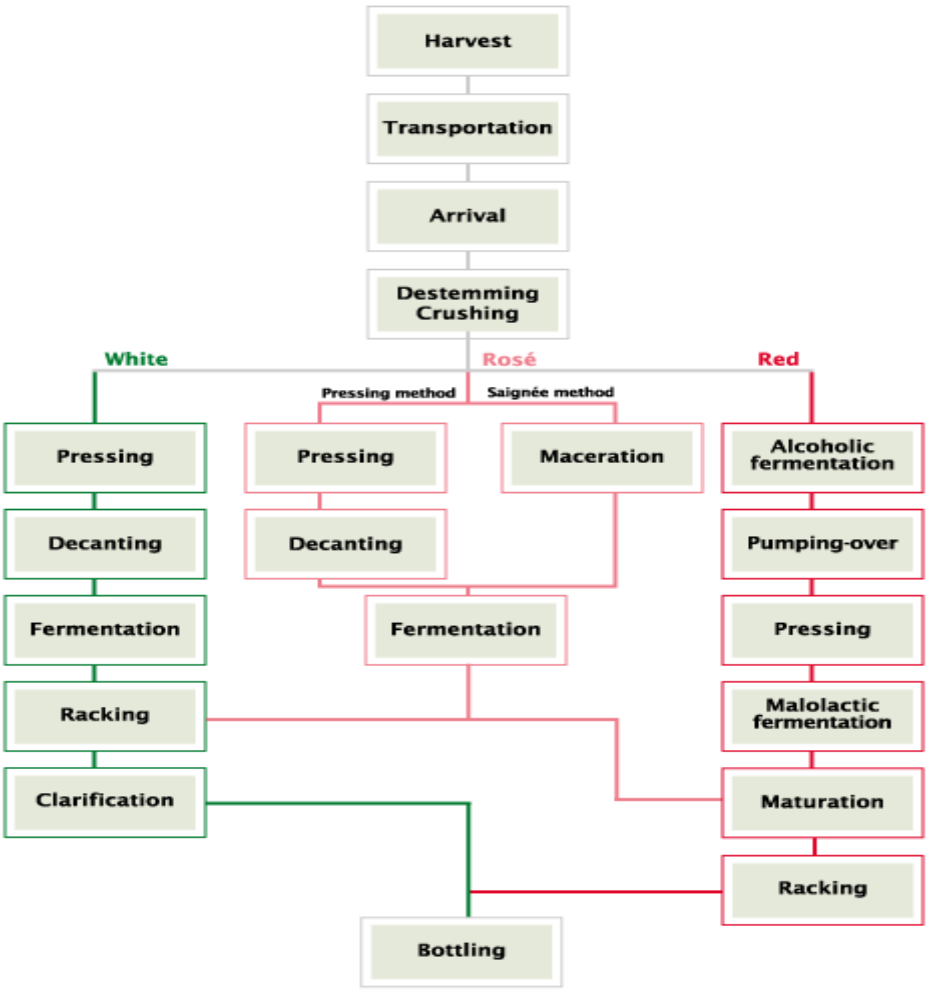

Figure 2.6 - Main steps of Still wines production into three different via. (adapted from [31])

The residue obtained, after pressing is named a grape pomace (GP) which it's a main by-product of all wine industry. It's an extremely heterogeneous mixture consists mainly of peels (skins), seeds and stems and accounts for about 20–25% of the weight of the grape crushed for wine production.[32] Grape seed is rich in extractable phenolic antioxidants such as phenolic acid, flavonoids, procyanidins and resveratrol, while grape skins contain abundant anthocyanins.[33] [34] The health benefits of GP polyphenols have been the great interest of researchers, food industry and nutraceutical industry. In addition to phenolic antioxidants, GP also contains significant amounts of lipid, proteins, nondigestible fibre and minerals. Grape seeds contain 13–19% oil, which is rich in essential fatty acids, about 11% protein, 60–70% of non-digestible carbohydrates [32], and non-phenolic antioxidants such as beta-carotene. [35]

2.2.2 – Polyphenol composition of GP

GP is rich in extractable phenolic antioxidants (10–11% of dryweight).[36] Anthocyanins, catechins, procyanidins, flavonol glycosides, phenolic acids and stilbenes are the principal phenolic constituents found in GP. [37] The polyphenol composition of each part of the GP differs and varies depending on the varieties of grapes and is influenced by the growing location, climate, maturity and the time of fermentation. [38]

Phenolic compounds are the secondary metabolites of plants. Chemically, they can be defined as substances possessing an aromatic ring bearing one or more hydroxyl groups, including their functional derivatives. Polyphenols are compounds that have more than one phenolic hydroxyl group attached to one or more benzene rings. Four major classes of polyphenols found in foods are phenolic acids, flavonoids, lignans and stilbenes.[39] The main bioactive compounds responsible for many reported health benefits of wine and wine by-products consist of antioxidant phenolics such as phenolic acids, anthocyanins, procyanidins and reveratrol.[27]

One of the major stilbenoids found in grape skins and seeds is resveratrol. It's a phytoalexin produced in the plant in response to pathogen attack.[40] It has a low toxicity in humans and is a naturally occurring fungicide. A certain amount of resveratrol from grapes is transferred to wine during winemaking. There is a growing evidence that resveratrol can prevent or delay the onset of various cancers, heart diseases, pathological inflammation and viral infections.[41] As a chemoprevention agent, resveratrol has been shown to inhibit tumour initiation, promotion and progression. [42] [43]

The largest and best studied polyphenols are the flavonoids. Based on their molecular structures, flavonoids are divided into seven subclasses: flavones, flavanones, flavonols, isoflavones, anthocyanidins/anthocyanins, flavanols (or catechins and procyanidins) and chalcones.[44] Another group of flavonoids, which are not included in this classification, are proanthocyanidins, also called, procyanidins, condensed tannins or oligomeric procyanidins.[32] The last ones particularly have many health benefits such as antimutagenic and anticarcinogenic activity, antioxidant and anti-inflammatory activities, prevention and delay of cardiovascular diseases. [45]

Phenolic acids are phenols that possess carboxylic acid functional group and are divided into hydroxycinnamic acids and hydroxybenzoic acids. The hydroxycinnamic acids are more common than hydroxybenzoic acids, and they mainly include gallic acid, p-coumaric, caffeic, chlorogenic acid and ferulic acids from which gallic acid achieve greater amounts in the GP. [46]

Not only health benefits turn polyphenols an attractive value-added target, its antioxidant properties fiend inumerous applications in the food industry. The antioxidative characteristics have been widely studied, including scavenging of free radicals, inhibition of lipid oxidation and reduction in hydroperoxide formation.[47] Polyphenols are proven to be strong antioxidants against lipid oxidation in food system. Grape seed phenolic extracts (GSE) were reported to inhibit lipid oxidation in meat products such as raw and cooked beef products, reducing rancid flavour development without altering the pH and other meat characteristics.[32] Anthocyanins from grapes and are used as food colorants. [48] Food products fortified with plant extracts containing polyphenols are beverages including water or tea-based drinks and dairy products such as yoghurt. [32]

Unfortunately, the production of purified polyphenols extract still remains costly and organic solvents such as ethanol, ethyl acetate and acetone are usually used.[49] Attending to the generation of environmental problems due to the use of organic solvents some efforts have been made to avoid its use.

Table 2.2 – Extraction yields of total phenolic content of grape pomace. (adapted from [50] [51])

Total phenolic content	% (g phenolic compounds/ g total residue)
Subcritical water extract	3,08
Ethanol:water 1:1 extract	2.89
Methanol extract	2.77
Ethanol extract	1.93
Acetone extract	1.57

New processes recently developed for polyphenols/anti-oxidants extraction employ supercritical solvents such as CO_2 with the best extraction conditions 30-40ºC and circa 100bar [52]; the use of HCW achieve the best results using conditions of 140ºC and 100bar (Tab. 2.2); [50] and even magnetic nanoparticles moulds to highly selective extraction of resveratrol from aqueous solution. HPLC analysis reveals that the different extraction systems modify the phenolic composition of the extract quantitatively and not qualitatively.[51] There are a few studies of quantitative phenolic composition of grape pomace as a winery waste and its phenolic content appears in the literature as a TPC (total phenolic content) as a gallic acid equivalent. (Tab. 2.2)

2.2.3 – Grape pomace polysaccharide content

Grape pomace is a rich polysaccharide lignocellulosic residue composed by three principal polymers: cellulose (~ 21%), hemicellulose (~ 12%) [53] and lignin (~ 38%).[54]

Table 2.3 - The main sugars and its extimated content in the grape pomace after fermentation.
It is noteworthy that the polysaccharide content is influenced by the growing location, climate, maturity, the time of fermentation, etc. (adapted from [54])

The main sugars	% of dry matter
Rhamnose	0.5
Fucose	1.24
Arabinose	2.07
Xylose	1.70
Mannose	1.52
Glucose	14.01
Galactose	1.60
Total	22.64

However, as it's explained before, the residue used in this work was sujected to alcoholic fermentation. During this biological process performed by yeasts in the absence of oxigen; sugars, essentially glucose and fructose, are converted into cellular energy. Ethanol and carbon dioxide are produced as metabolic waste products of this process. Thus, our residue has lower polysacharide contents than the original grape without any treatment. Fermentig sugars results from enzimatic clevage of the large pollysacharid polimes, but, as it is explained in the Agro industrial residues chapter, lignocellulosic structure is too complex to be cleaved off by the yeasts. Our principal aim in this work is to hydrolyse those structures to recover maximum of reduced sugars that weren't used during fermentation.

2.2.4 – Grape's seed oil

The oil content of grape seeds was reported in range of 11.6–19.6% depending on the variety and maturity of grapes. Major fatty acids of grape's seed oil are linoleic (66.76 – 73.61%) acid, oleic acid (17.8 – 26.5), palmitic acid (6.35–7.93%) and stearic acid (3.64 – 5.26%), respectively. [55]

The antioxidant and fatty acid compositions of grape's seed oil and thus its nutritional and cosmetic properties may be significantly affected by the grape variety, growing conditions, oil extraction methods and degree of refining. Several studies reveal the possibility of its extraction with supercritical CO_2. However, owing to the high cost of supercritical fluid extraction, commercial grape seed oil is mainly produced by traditional oil extraction methods such as hydraulic press and solvent extraction. [32]

2.2.5 – Grape's seed protein

There are relatively few studies about grape seed protein. GPs/seeds are not considered as an important protein source such as vegetables and nuts, although grape seeds contain 11–13% proteins. Its total protein content and the amino acid composition may vary significantly depending on the variety of grape, location and fertilisation conditions. Amino acid analyses reveal that the most abundant are glycine, glutamic acid and aspartic acid. However, grape seed protein was considered as non-digestible or resistant protein. This is most likely attributed to the strong interaction between protein and tannins (inhibitors of digestive enzymes). The digestibility of purified grape seed protein has not been reported. [32]

As we can see grape has a very complex chemical composition. Rich in such variety of groups of value-added by-products like polysaccharides, phenolic compounds and fatty acids, which are beneficial to human health and have promising applications in the pharmaceutical and food industry; it represents an unquestionable potential to a biorefinery system. Otherwise, it's economically sustainable and renewable residue due the tireless world marked needs of wine.

2.3 – Supercritical fluids and Hot Compressed Water

The interest in the application of supercritical and subcritical fluids and particularly hot pressurized and supercritical water started in the late 70's of last century awake by the first oil crisis and environmental concerns. Renewed interest appeared related with several other fields such as alternative fuels, coal and biomass conversion and waste disposal. Inherent physical and chemical properties of supercritical fluids allow the development of new processes and products.[56]

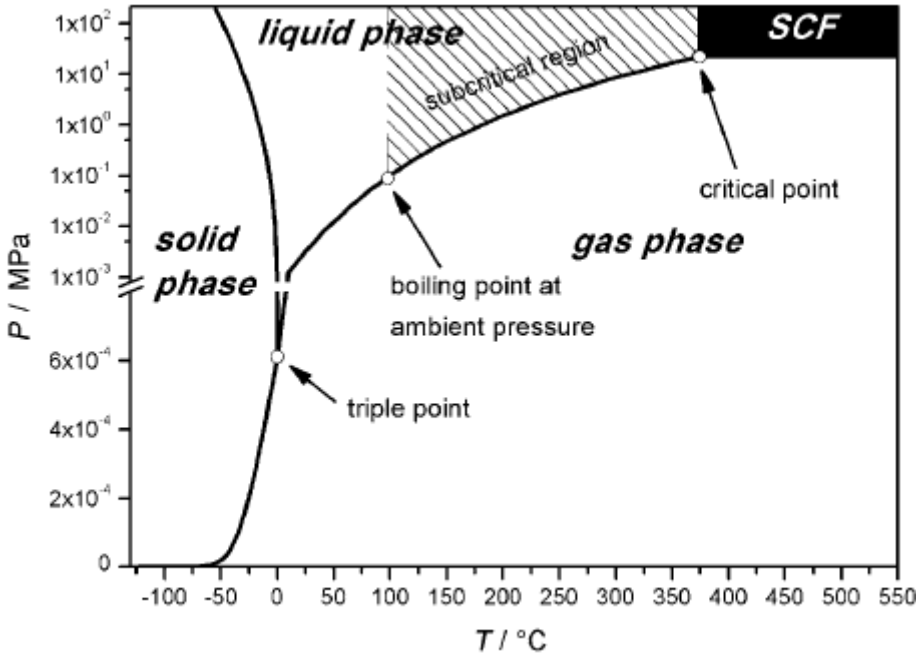

Figure 2.7 - Phase diagram of water. Characteristic points as well as the subcritical region are indicated.[57]

Supercritical fluids (SCF) are any fluid above its critical temperature and pressure where only exists a single supercritical phase with a very particular characteristics. Thus they are characterized by having properties between gas and liquid like viscosities decrease, better mass transference and solvating properties similar to a wide range of organic solvents.

SCF are commonly used for the extraction of natural compounds. Recent studies showed that in industrial scale, supercritical fluid extraction is an efficient and cost effective technique for recovery of phenolic compounds from biomass.[58] The most common SCF are water, CO_2, acetone, alcohols as methanol and ethanol, alkanes like methane, ethane and buthane and also some unsaturated hydrocarbons such as ethylene and propylene, among others.[59] (Tab. 2.4)

Table 2.4 - Supercritical points of the most common SCF.[59]

Fluid	Temperature (K)	Pressure (MPa)
Water	647	22,06
CO_2	304.1	7,38
Methanol	512.6	8,09
Methane	190.7	4,6
Propane	369.6	4,25
Acetone	508.1	4,7

Carbon dioxide is one of the most common solvent for the supercritical fluid extraction process due to its inert, non flammable and non-toxic properties. Very high pressure is therefore, required to dissolve highly polar compounds such as fatty acids and sterols in CO_2.[58] Addition of small quantity of a co-solvent such as water and ethanol to supercritical CO_2 or use of the solvents such as water is an alternative approach for improving the efficiency of the process for the extraction of polar compounds at lower pressures. [58]

Water is considered a "green" solvent, highly available and environmentally benign. It is also largely used, at its supercritical or subcritical state, in industry mostly for extraction of value-added compounds and to promote hydrolysis reaction.[56] However the use of supercritical water needs high additional cost due to it corrosivity and also due to higher temperatures and pressure values are required. Subcritical water reveals more promising candidate to industrial uses because it represents similar characteristics that a supercritical water but is economically advantageous due to amenable conditions needed.

Hot compressed water – liquid water below its supercritical temperature and pressure condition reveals many promising new characteristics opening new opportunities to its use in a variety of chemical reactions. Water from ambient to supercritical conditions changes its character from a solvent for ionic species to a solvent for non-ionic species. [56] This means that with increasing temperature, the nature of reactions supported by HCW changes: below the critical temperature and at high pressures, the ionic product is up to three orders of magnitude higher than under ambient conditions, which means that water is an acid/base catalyst precursor. The pH-value decreases by 3 units, providing much more ions for acid catalyzed reactions. [56] Moreover, the not too low (circa 30-20 in the range of 250ºC-300ºC and 50-300bar) relative dielectric constant seems to enhance ionic reaction. This region is used for a variety of synthesis reactions, but also for some degradation reactions such as biomass liquefaction. Electrochemical properties, e.g. dipole moment decreases from the high value at ambient conditions, but water in the critical

region is still as polar as acetone. On the other hand HCW also exhibits the properties of a non-polar solvent from the macroscopic point of view. The solubility behaviour of water changes tremendously in the neighbourhood of the critical temperature. [56]At relatively low pressures, with increasing temperature, the solubility of salts drops to practically zero. This effect is one of the major drawbacks of processing waste, since salts dissolved in the feed stream could precipitate and eventually block the reactor. But at the other hand at relatively moderate and high pressures, the solubility remains constant or is even increasing. Viscosity is of the order of a normal gas and the diffusion coefficient is at least one order of magnitude higher than that of a liquid. Solubility of water for gases is high in the critical region. At near critical and supercritical conditions water and gases like O_2, N_2, NH_3, CO, CO_2 are completely miscible. [56]

Thus depending on the conditions of temperature and pressure, HCW supports either free radical or polar and ionic reactions. This means that HCW is an "adjustable" solvent or "turning solvent". [60] That "adjustability" of HCW represents the main goal of this work that focuses not only onto hydrolysis of GP lignocellulosic structure to achieve higher yields of reduced sugars, but also onto extraction of value-added compounds. The extraction coupled with hydrolysis provides better exploration of the residue potential not only due to variety of recovered products, but also due to economical advantage from the use of only one fluid. Currently HCW is used for the extraction of phenolic compounds from different kind of residues such as: pomegranate, [61] rice bran, [62] extraction of protein and amino acids from deoiled rice bran, [63] bean dregs waste [64] among others.

2.4 – Oleaginous Yeasts

From the beginnings of his history, Human has learned how to employ microorganisms for his particular benefits. This "symbiosis" between two almost opposed life forms regarding to its complexity, being human one of its most complex representatives when microorganism, on the other hand, represents life seemliest forms, can be traced to prehistoric times to the first leavened bread development. Thus fermentation capacity of the yeasts became one of the most explored bioprocess giving rise to many of new products that had graced "our" tables such as: alcoholic beverages (wine, beer, cider), sour foods (sauerkraut, yogurt) and bread among others.

Figure 2.8 - Examples of some products (wine, beer, yogurt and bread) that results from fermentation capacity of the yeasts.

Nowadays with the constant advances in the industry field and a growing worry about environment conditions the use of microorganisms is not limited to the food industry. Green Chemistry concept defined as the "design of chemical products and processes to reduce or eliminate the use and generation of hazardous substances" aims to employ microorganisms to convert waste materials, predominantly residues (such as agro industrial residues), into valuable products using the living cells as the "catalyst". [65]

Microorganisms can be categorized into heterotrophs and autotrophs depending on the energy source used for growth. Heterotrophic microorganisms, unlike the autotrophs, cannot fix carbon and uses organic carbon (glucose, starch, proteins, lipids, etc.). The conversion processes such as brewing, aerobic digestion, fermentation, and composting converts organic substrates to a variety of useful products at mesophilic temperatures (typically 20–40ºC) within a limited pH range (typically pH 3–8). [66]

Within the group of heterotrophic microorganism are the yeasts, which are probably one of the earliest domesticated organisms still widely used in the biorefinery industry. Yeasts are unicellular, eukaryotic microorganisms from the kingdom of Fungi, with circa 1,500 species

currently described. [67] Yeasts can be found in a wide range of natural habitats such as plant leaves and flowers, soil and salt water. They are also found on the skin surfaces and in the intestinal tracts of warm-blooded animals, where they may live symbiotically or as parasites. To classify such a numerous group essentially uses characteristics of the cell structure, ascospore, colony and also physiological characteristics. One of the better known characteristics is the ability to ferment sugars for the production of ethanol being *Saccharomyces cerevisiae* the most well studied member of that group.[68]

Due to finality of the present work, that consists in the valorisation of agro industrial residues through production of the value added by-products, more attention was given to a restrict group of yeasts named oleaginous yeast group. The main characteristic of those yeasts is its ability to accumulate great amount of lipid, up to 70%, of the total dry cell weight under certain conditions.[69] The production of microbial oils - otherwise referred as Single Cell Oils (SCO) - is now an economic reality representing a viable alternative to plant oils and fats. The first commercial production of an SCO happened in 1985 but unfortunately it only lasted for 6 years and was closed down as no longer cost-effective. This oil was produced using *Mucor circinelloides* and was designed to be rich in the polyunsaturated fatty acid (PUFA) and c-linolenic acid (GLA; 18:3, n-6) for use as an alternative to the then expensive evening primrose oil (believed to have antitumor activity). But the growing interest in PUFAs during the late 1980s and early 1990s due to its nutritional importance especially in the development of newly-born babies such as DHA (an omega-3) or arachidonic acid among others, made SCOs to re-emerge as economically viable alternative to its production. These PUFAs do not occur to any extent in plant oils and could only be obtained from marine animal sources. Consequently a large market is available to ensure their worldwide sales and so to cover the costs of its production. [70]

There are two different biochemical pathways to the lipid accumulation in the cells. The fundamental difference between them occurs on the level of the substrate used. *De novo* lipid accumulation pathway uses hydrophilic substrates (polysaccharides, organic acids, ethanol, etc.) when *ex novo* lipid accumulation pathway uses hydrophobic substrates (vegetable oils, fatty by-products or wastes such as crude fish oils, fatty esters, etc.).[71] In the former case, lipid production is a secondary anabolic activity occurring after nitrogen had been depleted from the growth medium.

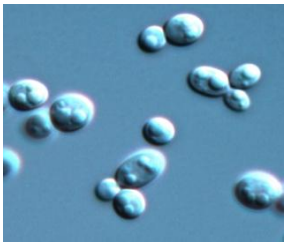

Figure 2.9 - Lipid particles of *Rhodotorula mucilaginosa* (amplification 100x2)

In a series of research works, it has been demonstrated that the oleaginous microorganisms do not possess a hyperactive system of fatty acid biosynthesis, but they are capable of producing in significant amounts of acetyl-CoA, the percursor of fatty acid biosynthesis issued by the intermediate cellular metabolism.[71] Acetyl-CoA generates cellular fatty acids that are then esterified with glycerol generating structural (phospholipids, sphingolipids, etc) and reserve (mainly triacylglycerides - TAGs) lipids. The major industrial applications related with the de novo accumulation of storage lipid in yeasts refer to the production of ‘‘nonspecified’’ lipids that could be further converted into FAMEs (Fatty Acid Methyl Esters that represents 2nd generation bio-diesel). As an example: *Rhodotorula graminis* that is used for production of second generation biodiesel.[72] On the other hand, ex novo lipid production is a growth associated process occurring simultaneously with cell growth, being entirely independent from nitrogen exhaustion from the culture medium. The free-fatty acids are incorporated by the active transport inside the microbial cell. The incorporated fatty acids are either dissimilated for growth needs or become a substrate for endocellular bio-transformations during which a ‘‘new’’ fatty acid profiles, that didn’t exist previously in the substrate, are synthesised. [71]

Once synthesized, yeast TAG are deposited in organelles known as lipid particles. (fig. 2.9) These particles are characterized by a rather simple structure consisting of a highly hydrophobic core of neutral lipids surrounded by a phospholipid monolayer with only a small amount of proteins embedded. [73]

In the present work several species of *Rhodotorula* and *Rhodosporidium* isolated essentially from fruits were used. These species were chosen because the residue used was grape pomace and because they assimilates diverse carbon sources from the simple monosaccharides to more complex structures which is an important characteristic due to heterogeneity of the residue used in the present work. [74] All species used in this work are aerobic which means obligatory presence of the oxygen for it grows. *Rhodotorula spp.* is a common environmental yeast that can be found in air, soil, lakes, ocean water, milk, and fruit juice. Colony colour can vary from being cream coloured to orange/red/pink or yellow. It is able to scavenge nitrogenous compounds from the environment remarkably well, growing even in air which has been carefully cleaned of any fixed nitrogen contaminants. [75] *Rhodosporidium spp.* could also be isolated from the fruits. Both genera are also known as red yeast due the presence of the red pigment. [76] Chemical substances responsible for the pigmentation of *Rhodotorula spp.* and *Rhodosporidium spp.* are carotenoids. They are natural pigments that have been used commercially as food colorants, animal feed supplements, nutraceuticals and other applications.[28]

Thus *Rhodotorula spp.* and *Rhodosporidium spp.* reveal be a very interesting choice because they don't just produces economically sustainable compounds like TAG's, but also are the natural source of another added-value compounds such as carotenoids.

METHODS

"The good thing about science is that it's true whether or not you believe in it."
— Neil deGrasse Tyson

3. Chapter: METHODS

3.1 – Hydrolysis reaction

The residue was dry in a stove (by the mass difference it was concluded that initially it had 30% of water) and then GP was crushed to making the mixture morphologically more homogeneous.

In the literature hydrolysis reaction is described as extremely fast. The main variables that influence the yield and the time of hydrolysis are temperature and pressure, which directly influence state and characteristics of water. Previous studies performed by our team reviled that since it's guaranteed that water is in its subcritical conditions the variation of pressure doesn't induce any significant changes on final results. Therefore we've chosen to maintain constant pressure (≈100bar) through all reactions changing solely the maximum temperature of the media.

Taking into account the use of semi-continuous reactor to perform this study the flow rate of water also emerged as a third important variable that reflects time contact between water and solid residue inside the reactor (about 2g).

Temperature control was another limitation factor. To perform the study of temperature effect on the hydrolysis yields five different temperatures were chosen: 150ºC, 180 ºC, 200 ºC, 250 ºC and 300 ºC (apparatus operational maximum temperature). But as shown at the scheme (pag. 31) those temperatures are achieved after preheating of the experimental set-up. There are two heating serpentines controlled manually during reaction processes. It means that preheating time differs in each experiment, essentially due to room temperature changes and initial water temperature. To minimize the pre-heating effect, a constant flow 2ml/min was maintained to avoid residue degradation that would occur in the absence of water. Pre-heating time starts when temperature reaches 50 ºC. That empirical value was chosen as a "zero point" to all performed reaction to allow comparison of different data. During that preheating time (first step of the process) all samples were constantly collected.

After the maximum temperature was achieved, the reaction enters in its "second phase", the phase of hydrolysis. The temperature is maintained constant for an hour. Due to the manual control mentioned above, temperature during this phase may fluctuate up to ± 5ºC. These oscillations are more evident at higher temperatures. During that hour the flow rate was raised to 5ml/min to avoid degradations of biomass by reducing residue/HCW contact and also due to previous studies performed by the group that showed 5ml/min been the optimum flow rate to higher reaction yields.

To estimate hydrolysis reaction yield of every assay were registered initial mass of the residue inserted into reactor and the mass of the residue that remained at the end of the reaction.

All the samples were collected at falcon tubes of 14ml, filtered with paper filter (FILTER-LAB) and stored at 4ºC. Part of these solutions was used directly to perform colorimetric test. Tree replicates of 2 ml of every sample were lyophilised in the Lyophilizer CHRIST ALPHA 1-4, B.Braun Biotec International., during 48h. There were estimated an average mass of resulted powder to calculate mass variation of water soluble compounds along time and also the total amount of water soluble compounds obtained by assay.

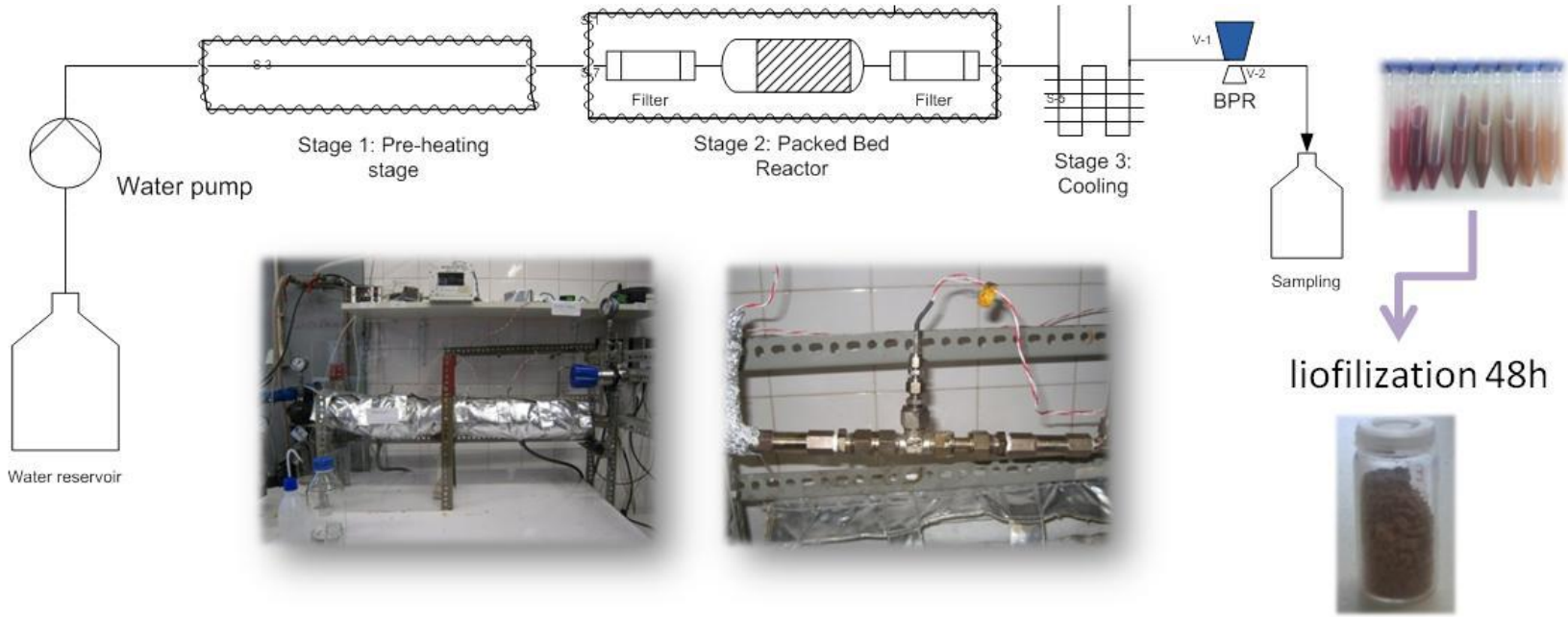

Figure 3.1 - Experimental set-up.

3.2 – Colorimetric tests

3.2.1 Folin – Ciocalteu colorimetric test [77] [78]

This colorimetric test has the purpose to quantify Total Phenolic Content (TPC) of GP hydrolysates into gallic acid equivalent.

Calibration standard curve was performed with five different concentrations of Gallic Acid monohydrate (Sigma 98%) (50, 100, 150, 250 and 500 mg/L) and for blank solution was used Mili-Q water. Aqueous solutions of glucose and bank prepared as aqueous samples of GP hydrolysates described below without protein precipitation step.

Aqueous samples of GP hydrolysates from each assay were used to precipitate proteins that could interfere with the method. At a eppendorf tube to 800µl of the sample were added 120µl of Trichloroacetic Acid reagent (Scharlau 99,5%). These eppendorfs were stored at -20ºC for 5min, then at 4ºC for 15min and centrifuge (Heraeus sepatech, Biofuge 13 Centrifuge) for 15 min at 12 000rpm. To 20µl of resulted supernatant were added 1,58ml of Mili-Q water, 100µl of Folin-Cicalteu reagent (MERK) and the resulted solution were stored at room temperature for 8min. After that were added 300µl Sodium Carbonate (Sigma) solution (200g/L) and incubated in water bath at 40ºC for 30min. The absorbance were measured at 750nm (it should be 765nm but due to spectrophotometer limitation it was measured at 750nm) with DU® 800 Spectrophotometer from Beckman Coulter, Brea, USA.

3.2.2 Colorimetric carbohydrate analysis (modified) [79]

This colorimetric test has the purpose to verify the potential of GP hydrolysates in reduced sugars and derived compounds.

Calibration standard curve was performed with eight aqueous solutions of 5-HMF monohydrate (SIGMA Aldrich) ((0,005; 0,025; 0,05; 0,1; 0,15; 0,2; 0,25; 0,3 g/L) and for blank solution was used Mili-Q water. Aqueous solutions of 5-HMF and bank prepared as aqueous samples of GP hydrolysates described below.

To 500 µl of aqueous samples of GP hydrolysates were added 1,5ml of H_2SO_4 acid (Panreac 96%) and 300 µl of 5% of phenol (SIGMA Aldrich 99-100%) in water. After incubating for 5 min at 90ºC into Accu Block™ Digital Dry Bath samples were cooled to room temperature into water bath and absorbance measured at 490 nm with DU® 800 Spectrophotometer from Beckman Coulter, Brea, USA.

3.3 – HPLC analysis

3.3.1 Carbohydrate analysis

The HPLC analysis has purpose to identify and quantify monosaccharides presented in GP hydrolysates.

The analysis was performed with Chromatographo HPLC Thermo Scientific, Finnigan Surveyor Autosampler Plus, Finnigan Surveyor LC Pump Plus, Finnigan Surveyor RI Plus Detector. Software used to data treatment was ChromQuest 5.0.

Column used was HyperRez XP Carbohydraten Ca^{++} with dimensions 300x7.7 mm at constant temperature 85ºC. As mobile phase was used Mili-Q water at flow rate of 0,6 ml/min.

There were expected low concentrations of monossacarides in GP residue so the Method of Standard Addition Calibration was used. Standard curves were performed to glucose, fructose, arabinose, xylose and galactose (99% SIGMA Aldrich) with 100 ppm as a standard concentration (25; 50; 75; 100; 150; 250 and 500 ppm)

There were prepared two solutions of standards due the fact that fructose and galactose retention times are too closed to arabinose and xylose respectively. The solution A contained 500 ppm of glucose, xylose and arabinose. The solution B contained 500 ppm fructose and galactose.

Samples of GP hydrolysates HPLC analysis were prepared with the powder resulted from lyophilisation in Mili-Q water and filtered with NY Filters (0,20 µm GVS SpA). It was used 400 µl of aqueous GP sample and 100 µl of standard solution so that the final concentration of standard was 100 ppm and concentration of GP hydrolysate 4000 ppm.

3.3.2 Phenolic analysis [80]

The HPLC analysis has purpose to identify and quantify individual phenolic compounds in the GP hydrolysis extract.

The analysis was performed with Chromatographo HPLC Thermo Scientific, Finnigan Surveyor Autosampler Plus, Finnigan Surveyor LC Pump Plus. Software used to data treatment was ChromQuest 5.0.

The method used has an injection volume of 10 µl and flow rate of 1 ml/min. The absorbance is measured at two different wavelengths: 280nm and 320nm with Accela UV/Vis Detector. The column (BDS HYPERSIL C18; length 250mm; I.D. 4mm.) was at a constant temperature of

40ºC. The mobile phase is a mixture of two eluents, A and B. Eluent A is Mili-Q water with 2% (v/v) of acetic acid glacial (Scharlau 99,8%) and eluent B is a mixture of water and acetonitrile (CALO ERBA 99,9%) (50:50) with 0,5% (v/v) of acetic acid. The gradient program was as follows: 10% B to 55% B (50 min), 55% B to 100% B (10 min), 100% B to 10% B (5 min).

Standard curves were performed to 5-HMF (25; 50; 100; 150; 200; 300; 500 and 1000 ppm), Furfural and Chlorogenic acid, (25; 50; 75; 100; 150; 200; 250; 300; 500 and 1000 ppm) Gallic acid (50; 100; 200; 300; 400; 500 and 1000 ppm), Resveratrol (5; 10; 15; 20; 25 and 30 ppm), Caffeic acid and *p*-Coumaric acid (25; 50; 75; 100; 125; 150; 200 and 250 ppm), Ferulic acid (50; 100; 150; 200; 250 and 300 ppm) in Mili-Q water. All the standards used were from SIGMA Algrich 98-99%.

Samples of GP hydrolysates were prepared with lyophilized powder in Mili-Q water with concentration 5000ppm.

3.3.3 Extraction methods of phenolic compounds

1. Hydro-alcoholic extraction 1g of GP residue was added to 20 ml of water:ethanol (Scharlau 99,5%) mixture (75:25 v/v) and stored for 18h at 50ºC with constant magnetic agitation (150 rpm).

2. Extraction with acetone:water [81] 1g of GP residue was extracted twice for 15 min with 10ml of water:acetone (SIGMA Aldrich 99,5%) mixture (20:80 v/v) containing HCl (Scharlau 38%) (0,1:10 v/v) to prevent oxidation of the polyphenols in an ultrasonic bath at room temperature and then stirred for 30 min on a magnetic agitation. After centrifugation (3000 rpm for 10 min) the supernatants from both extractions were combined and made up to final volume of 25 ml with Mili-Q water and filtered with paper filter.

3. Extraction with citric acid 1g of GP residue was added to 10 ml of aqueous solution of citric acid (Scharlau 99,8%) (3g/L) and stored for 30 min at 40ºC with constant magnetic agitation (150 rpm). Samples were filtered with NY Filters (0,20 µm GVS SpA).

   Sample preparation to HPLC analysis: [80]

   To the extracts obtain from the methods described above were added 5 ml of diethyl ether (SIGMA Aldrich 99,8%) for 10 min (3 times). The organic phase was dried over with Na_2SO_4, the solvent was evaporated under nitrogen and the dry residue obtained was dissolved in methanol:water (1:1 v/v) and filtered with NY Filters (0,20 µm GVS SpA).

3.4 – Oleaginous yeast growth

All the yeasts used in this work were supplied by the Portuguese Yeasts Culture Collection (PYCC), Caparica, Portugal.

Table 3.1 - Yeasts strains used in the pre-selection path of the work.

Species name	Origin
Rhodosporidium babjevae	Blackberry
Rhodotorula sp.	Honey
Rhodotorula sesimbrana	Wood
Rhodotorula bacarum	black currants
Rhodotorula babjevae	white grapes
Rodotorula yarrowii	Strawberry

The growth experiments were performed in microplates (96 well Nuclon™ Delta Surface). The maximum liquid volume used was 200µl to provide headspace necessary to cells respiration attending to the fact that all stains used are aerobic microorganisms.

Minimal media (YNB) (Difco™ Yeast Nitrogen Base) were prepared ten times concentrated as recommended by the producer company with 6,7g of YNB, 100 ml of sterile water and 5g of carbon source. During experimental work there were used several carbon sources, but its final concentration in the media were maintained the same.

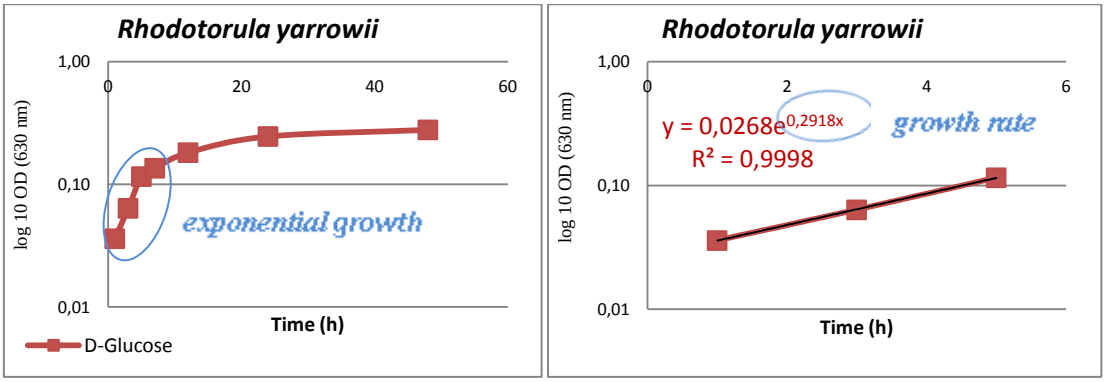

Figure 3.2 - Example of growth curve and growth rate calculation of *Rhodotorula yarrowii* in the minimum growth medium with D-Glucose as the only carbon source.

Culture growths were performed at 25ºC with constant agitation. Microplates were stored inside a plastic box filled with wet cotton to prevent evaporation. Experimental data were collected every two hours at 630 nm with DO detector (Stat Fax – 2100, AWARENESS TECNOLOGY INC.) during 48h. After that, microplates were sealed with porous membrane (INUC™) to

prevent evaporation, but allow oxygen entry. The last OD was taken past 72 hours of incubation. As a blank solution was used YNB with glucose, in the case of GP hydrolysates used as carbon sources there were performed blank solution (YNB with GP hydrolysate) to each one of them.

There were selected three points from the region of exponential growth, as it is represented in the figure 3.2, and growth rates were calculated by the formula $(\log OD - \text{Log } OD_0)/t = \mu/\ln 2$.

RESULTS AND DISCUSSION

"If we knew what it was we were doing, it would not be called research, would it?"
— Albert Einstein

4. Chapter: RESULTS AND DISCUSSION

4.1 – Study of hydrolysis efficiency with HCW

To evaluate temperature influence on the hydrolysis efficiency, samples of liquid phases (aqueous solution of extracted and hydrolysed compounds) were collected in 14 ml falcon tubes. In all assays was noted visual colour difference between the samples been the first sample always more colourless, then the colour was intensifying from the bright wine colour to the dark brown and finally the last simples were light brown and almost colourless. That visual difference noted means *apriori* that chemical composition of samples varies along time and that there is fractionation of the extracted/hydrolysed compounds. The visual aspect of powders resulted from the lyophilisation of the liquid samples also remains different from sample to sample, as it could be seen in the figure 4.2, following the pattern of liquid phases.

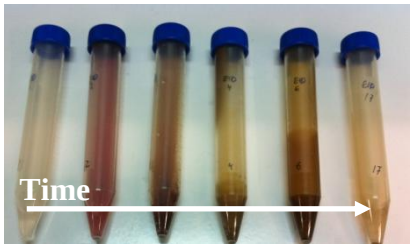

Figure 4.1 - Example of samples collected during hydrolysis assay.

Figure 4.2 - Example of powders resulted from the lyophilisation.

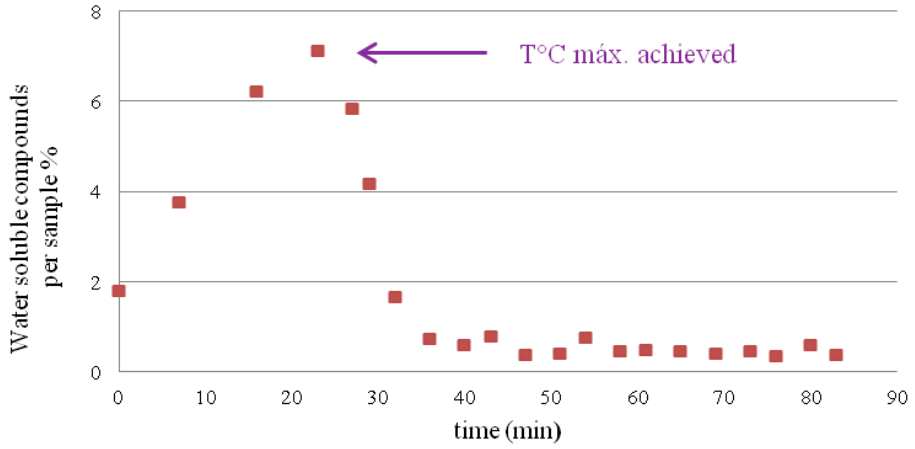

Figure 4.3 - Example of variation of water soluble compounds mass (%) per sample along time (250ºC, 100bar, 2 -5 ml/min).

The mass percentage of the water soluble compounds ($\frac{lyophilised\ mass\ per\ sample}{initial\ mass\ of\ the\ residue} \times 100\%$) increases along time until reaching its maximum value that overlaps with the moment when maximum temperature is reached. After that it decreases drastically for about ten minutes, after which only about 1% of solubilised mass is recovered in each sample till the end of the experiment. (Fig. 4.3)

This behaviour confirms the high rate of hydrolysis reaction mentioned in the literature. With increasing temperature glycosidic bonds, between the polysaccharides of the lignocellulosic structure of the residue are broken, resulting in chemically simpler structures which are soluble in water. From figure above it can be observed that hydrolysis occurs mainly when temperature is increasing, which is in line with the high reaction rates mentioned before. After that point there is a drastic decrease in the recovered soluble compounds followed by no considerable variation on the mass recovered. It can be concluded that temperature defines the amount of hydrolysable material per assay and that after the maximum temperature is reached the reaction has ended. The hydrolysed mass recovered afterwards is due to the residence time of hydrolysed solution in the line after the reactor.

That variation follows the colour pattern of the simples mentioned before. Thus the colour intensity is proportional to the amount of soluble compounds. The pattern of variation of the water soluble compounds mass percentage by sample along time is similar for all obtained hydrolysates independently of the maximum temperature used. (Fig 4.4)

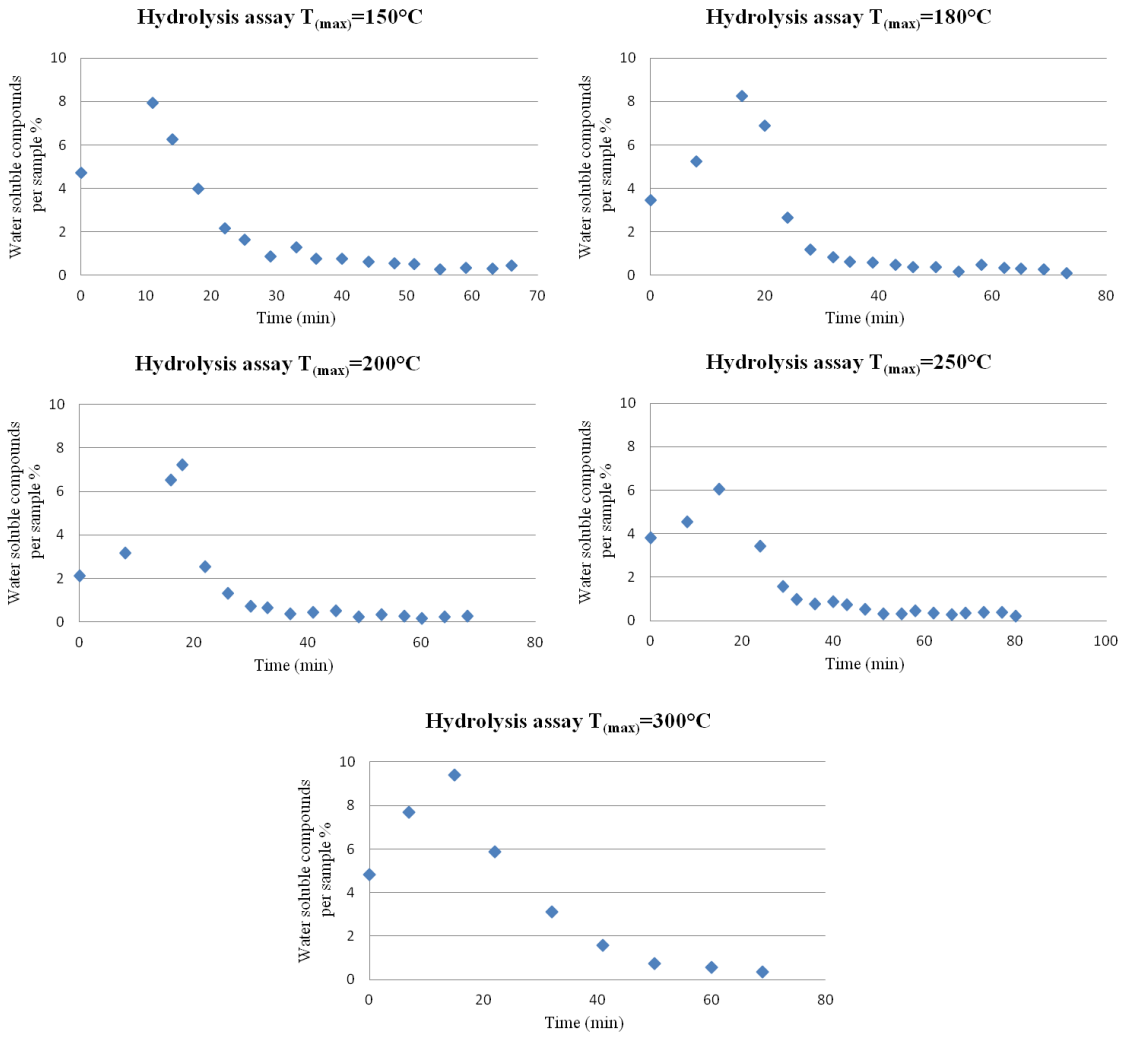

Figure 4.4 - Water soluble compounds mass variation (%) per sample from different hydrolysis assays along time (100bar, 2-5 ml/min).

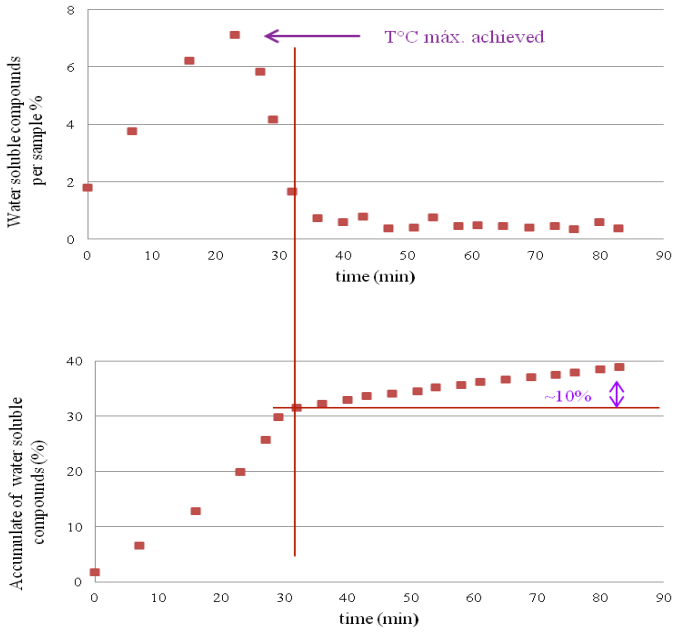

Figure 4.5 - An example of the mass variation (%) per sample of water soluble compounds and accumulated water soluble compounds (%) along time (250ºC, 100bar, 2 - 5 ml/min).

As it is shown in the figure above there is an important correlation between the temperature and recovered material during hydrolysis reaction. Accumulated water soluble compounds data (the sum of water soluble compounds mass percentage per sample) confirms that until maximum temperature is reached water soluble compounds percentage increases exponentially and then during an hour increases only by ≈ 10%. This behaviour is similar in all performed assays. (Fig 4.6) It is important data from the economical point of view of the whole process; it is extremely disadvantageous prolonging so much the reaction time, because the amount of final product doesn't explain energetic costs associated to the temperature and pressure maintenance.

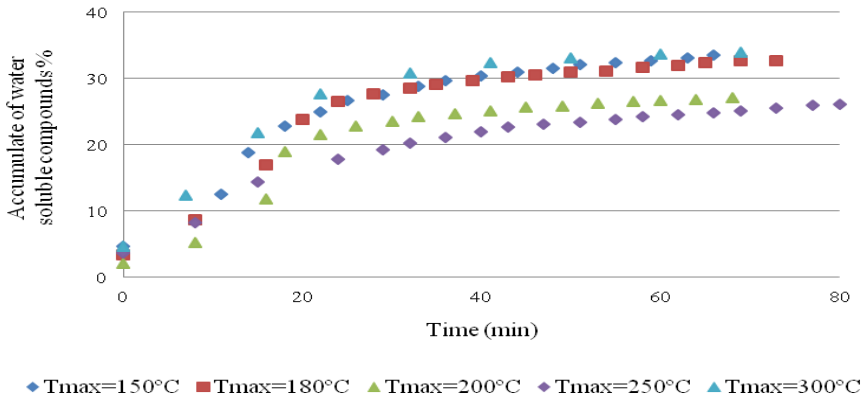

Figure 4.6 - Accumulate biomass variation (%) of water soluble compounds from hydrolysis assays performed at five maximum temperatures (150 – 300ºC, 100bar, 2 -5 ml/min) along time.

Comparing the biomass conversion percentage of hydrolysis reactions $(1 - \frac{final\ mass\ of\ the\ residue}{initial\ mass\ of\ the\ residue} * 100\%)$ performed at five different temperatures it confirms that higher temperatures lead to higher yields. That's because temperature express thermal energy and, naturally, higher amounts of energy promote hydrolysis by breaking more glycosidic bonds. Hydrolysis performed at 300°C reveals higher yield. (Fig 4.7)

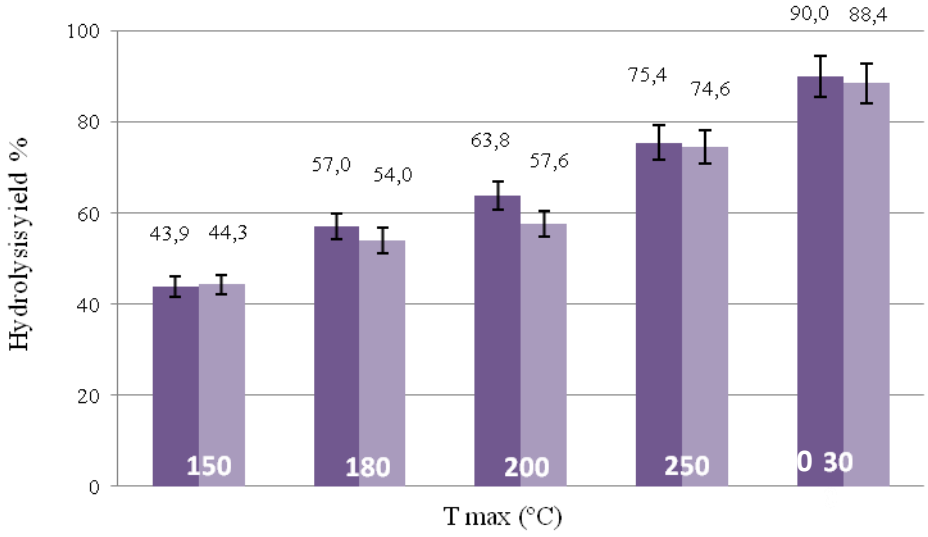

Figure 4.7 - Biomass conversion (%) of hydrolysis assays performed at five temperatures with its replicates (150 – 300ºC, 100bar, 2 – 5ml/min).

But on the other hand, contrary to what would be expected, higher temperatures doesn't induce higher amounts of water soluble compounds ($\frac{total\ lyophilised\ mass}{initial\ mass\ of\ the\ residue} \times 100\%$). Actually, as it could be seen in the figure 4.8, excepting the data corresponding to assays performed at 250°C as a maximum temperature, there is no significant difference between the results obtained.

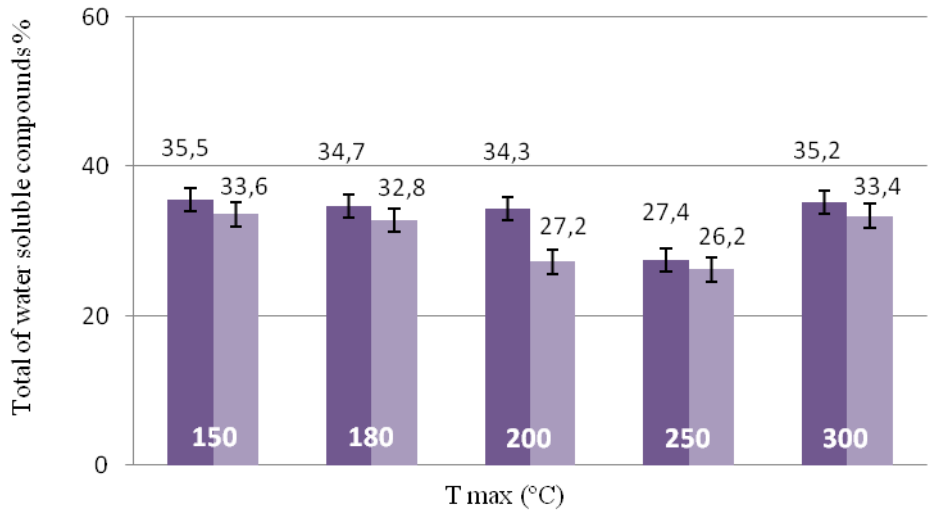

Figure 4.8 - Total percentage of water soluble compounds recovered from hydrolysis assays performed at five temperatures (150 – 300ºC, 100bar, 2 – 5 ml/min) with its replicates.

First of all disagreement between hydrolysis efficiency data and amounts of the recovered material soluble in water could be explained by degradation of the residue. The thermal degradation could result in ashes (that could precipitate and been removed afterwards from the samples during filtration), proteins (that could be precipitated by the tannins present in the GP residue) and furfural (product of thermal degradation of polysaccharides that sublimates during the lyophilisation process). Higher temperatures lead to severe degradation process that is confirmed by the decrease of the biomass conversion percentage noted on the 250°C assays. The sudden increase of the amount of the soluble material on the samples related to 300°C assays could be explain by the increase of the hydrolysis of more complex structures. As it was explain before, GP residue is constituted by three polymeric structures with different morphologic and chemical characteristics and also with the different resistance to hydrolysis process. Cellulose is "protected" by hemicellulose and lignin that enfolds it. So, to reach cellulose it is necessary to hydrolyse the hemicellulose, meaning that more energy is needed. The maximum temperature used in the present study probably provided enough energy to hydrolyse not only greater amounts of hemicellulose but also cellulose that became more vulnerable. That could be confirmed, for example by TOC (total organic carbon) analysis of the residue that remained in the reactor after hydrolysis assays is ended. Comparing this data with the original TOC values of the residue before hydrolysis and having into account that grape pomace has about 21% of cellulose and 12% of hemicellulose (% in dry sample) [53] it would be possible to estimate the amount of cellulose hydrolysed at 300ºC.

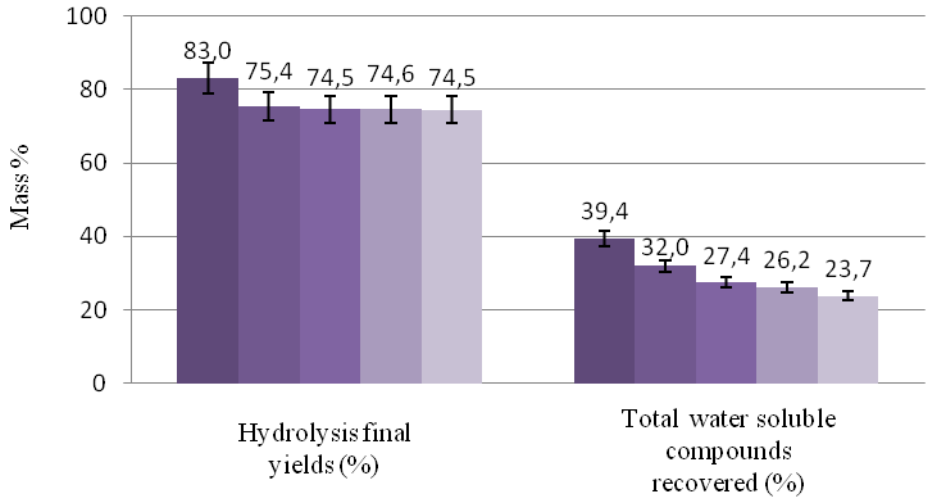

Figure 4.9 - Hydrolysis final yields (%) and total water soluble compounds mass recovered (%) from five assays performed at the same maximum temperature of 250ºC (100bar, 2 – 5 ml/min).

It is also important to note the heterogeneity of the residue and its influence in the hydrolysis performance. Five assays were carried out at the same maximum temperature of 250ºC. As is could be noted in the figure above there is no significant difference between the amount of the converted biomass during hydrolysis reaction. Only the data related to the first experience (83% of converted biomass and 39,4% of total recovered water soluble compounds) stands out from the overall picture. During that first assay the temperature showed abnormal fluctuations, reaching 258ºC as maximum temperature. That data is essential to confirm again the importance of temperature control during hydrolysis reactions. Contrarily to biomass conversion data the amount of recovered soluble compounds between experiments is not so similar. That means that the hydrolysis yield is constant, but the chemical composition of the recovered product is variable. As it was mentioned before, different parts of the residue (ex. seeds and stems) have different chemical composition. At such a small experimental scale (only *ca.* 2g of residue used per assay) a small variation on the amount of grape constituents could results in the significant variation on the composition of the recovered product, for example presence of higher amounts of volatile compounds would result in the lower rates of recovered material.

The composition of the recovered hydrolysates at different temperatures was determined by HPLC analysis and colorimetric assays. It should be recalled that the final goal of this project is not only to develop an economically advantageous hydrolysis process, but also to obtain the final product with the higher amounts of reduced sugars (to be used as a carbon source to the yeast growth) and extractable compounds (such as antioxidants).

4.2 – Study of the chemical composition of GP hydrolysates

4.2.1 – Composition in degradable compounds

One of the most prominent products described in literature that result from degradation of biomass, namely monosaccharide species, during subcritical water processing is 5-HMF. [57] It is originated via dehydration of glucose. This compound is considered a promising future platform for numerous chemical processes. Most important is the perspective to serve as a precursor for "green fuels" for the automotive industry. It could serve directly or via its derivatives such as 5-(chloromethyl)furfural (CMF), for production of fuels such as ethoxymethylfurfural (EMF) or dimethylfuran. For the chemical industry it has been shown that furandicarboxylic acid, an oxidation product of 5-HMF, can be used as a monomer for the substitution of terephthalic acid in the production of polyesters. Furthermore, even antitumor properties have been ascribed to 5-HMF. [57]

There were performed HPLC analyses to identify the presence and amounts of 5-HMF in GP hydrolysates. As it could be seen in the figure below, where is represented the variation of 5-HMF in µg / g $_{\text{initial residue}}$. The recovered amount of that compound is dependent on temperature. It was expected that the amount of 5-HMF would be higher at 300ºC whereas its maximum amounts is observed at hydrolysate from 200ºC assay.

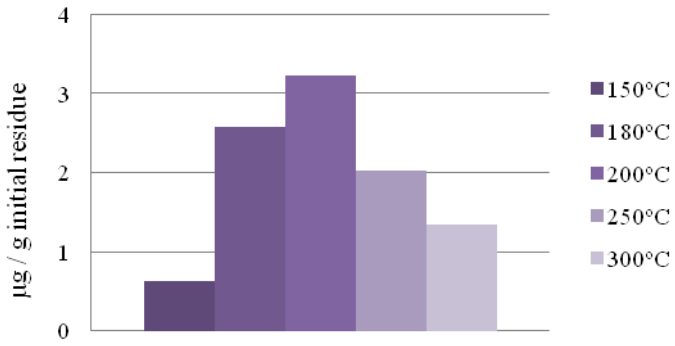

Figure 4.10 - Variation of 5-HMF amounts with increasing temperature (100bar, 2 – 5 ml/min).

However these results are not contradictory to those described in the literature. 5-HMF is also described as easily decomposable, yielding degradation products such as levulinic acid, formic acid, 1,2,5-benzenetriol, as well as soluble and insoluble polymers. The higher yields were reported when biomass was subjected to subcritical water at 240ºC during 200s. In the present work to achieve temperatures above 200ºC about 10-15 min are necessary. During that period 5-HMF could be partially degraded into compounds mentioned above (it wasn't perform any test to identify its presence) shifting the maximum yield peak to 200ºC.

Furfural was another compound, derived from a C5-sugar fragmentation followed by dehydration process, which was identified by HPLC analysis. Having into account reported toxicity of this product to the yeast growth [82] and reminding that one of the main objectives of this work is to use GP hydrolysates as carbon source to oleaginous yeast growth, identification of this compound was extremely important. The amount of furfural revealed to be substantially lower than amount of 5-HMF, but on the contrary of 5-HMF variation behaviour the amounts of furfural increase with temperature (decreasing slightly in the hydrolysate from 300ºC assay). (Fig 4.11)

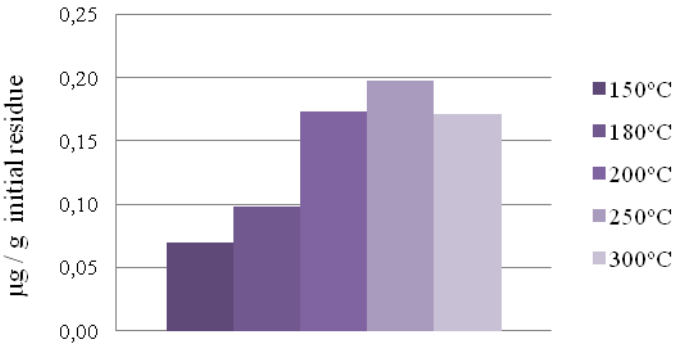

Figure 4.11 - Variation of furfural amounts with increasing temperature (100bar, 2 – 5 ml/min).

In the course of the experimental work was verified the volatility of this compound when the samples of GP hydrolysates were subjected to lyophilisation and evaporation process. Which means that the initial amounts of furfural in the liquid phase of hydrolysates (HPLC analysis were carried out with lyophilized powders) are substantially higher. But there wasn't performing any study to verify its initial amounts. However, having into account that all samples were lyophilized at the same experimental conditions, it can be assumed that the behaviour of the amount of furfural with temperature increasing would be remaining similar to the behaviour from the figure above.

4.2.2 – Polysaccharide content in GP hydrolysates

To evaluate the potential of the GP hydrolysates in the sugar content colorimetric assays of different samples were performed at its liquid phase (meaning samples that were directly collected during hydrolysis assays). Attending to the variation along time of the recovered soluble compounds data, which could be seen in the figure 4.12, it seemed unnecessary to find out chemical composition of samples which total soluble mass content was about 1%. So on, by each of five assays (according to five temperatures used) were carried out colorimetric test of 10 first samples and one from the region of 1% recovered mass as is exemplified in the figure below.

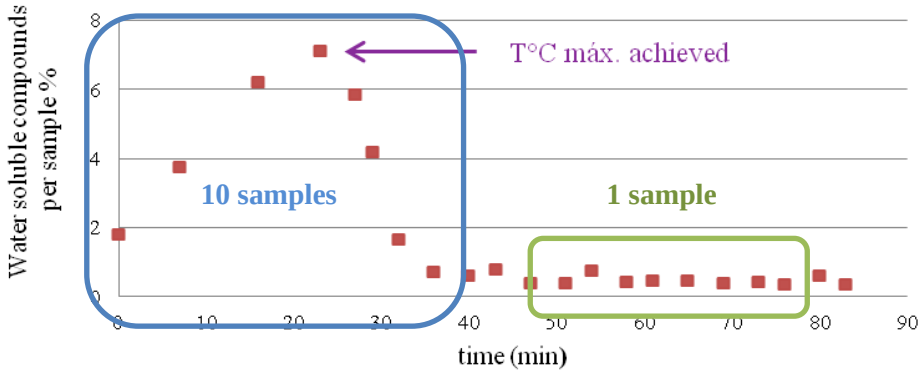

Figure 4.12 - An example of samples selection to performing colorimetric tests.

Calibration standard curves for this method were performed with glucose. During the work it was found that experimental data are influenced by the presence of compounds derived from thermal decomposition of the main monomers such as glucose and fructose that originates essentially furfural and 5-HMF respectively. Concentration of these compounds were confirmed and discussed in the previous chapter; however HPLC analysis were carried out with lyophilised samples when colorimetric method was performed with the samples before its lyophilisation, so the amount of furfural in these samples is unknown but it is significantly higher than that was determined by HPLC.

As it can be seen on the figure below polysaccharides and its derivates content shows the same behaviour of the water soluble compounds mass variation. That means that the amount of carbohydrates is directly proportional to amount of soluble compounds and consequently the higher concentration in polysaccharides and its derivates were found in the samples collected when the maximum temperature was achieved.

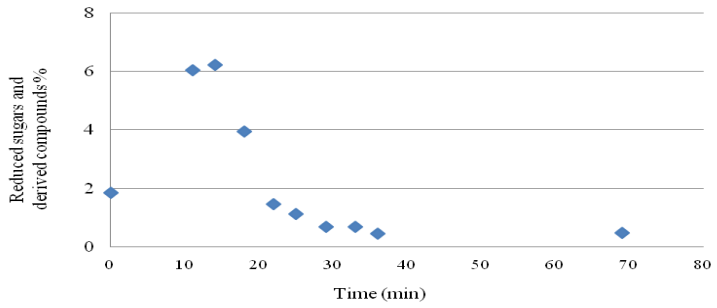

Figure 4.13 - An example of variation of the mass (%) of reduced sugars and derived compounds along time (150ºC, 100bar, 2 – 5 ml/min).

The variation behaviour into amount of total polysaccharides and derived compounds was also similar between assays. (Fig 4.15) This was expected since the pattern of variation of soluble mass compounds was also similar between all hydrolysis assays. As it was mentioned before, furfural concentration in hydrolysates increases with the temperature and the amount of 5-HMF showed to be higher into samples from the assays performed at 200-300ºC. However at the same time as the concentration of degradation compounds increases the composition into polysaccharide content naturally decreases. There is a balance between hydrolysis and decomposition of the GP residue that couldn't be analysed by colorimetric data. That method was performed only with the aim to estimate the potential of hydrolysates into carbohydrate composition. According to data from the figure 4.14 the amount of total polysaccharides and derived compounds increases with the temperature showing higher rates for the 200ºC hydrolysis assay and then decreases drastically.

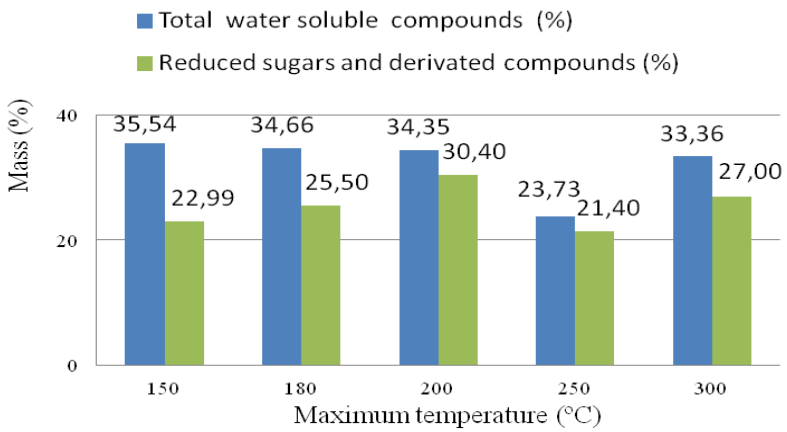

Figure 4.14 - Comparing of total water soluble compounds mass (%) and the amount of total polysaccharides and derived compounds (%) per temperature assays (150 – 300ºC, 100 bar, 2 – 5 ml/min).

The values presented in fig. 4.14 resulted from the difference between the amount of 5-HMF determined from the colorimetric method and the amount analysed by HPLC. Also it should be noted that experimental data of total water soluble compounds and total polysaccharides

content, essentially in the assays performed at 200-250ºC, is very similar suggesting that almost the whole water soluble mass is represented by monosaccharides and its derivates. That is not possible because further on we prove that hydrolysates also contain polyphenols. Such discrepancy between results could be justified by the amounts of furfural. Water soluble compounds data was determined by the sum of total recovered powder per sample after lyophilisation and the furfural, that disappears (mostly) during this process, is not included into these data. To get more precise data about hydrolysates composition HPLC analysis were performed.

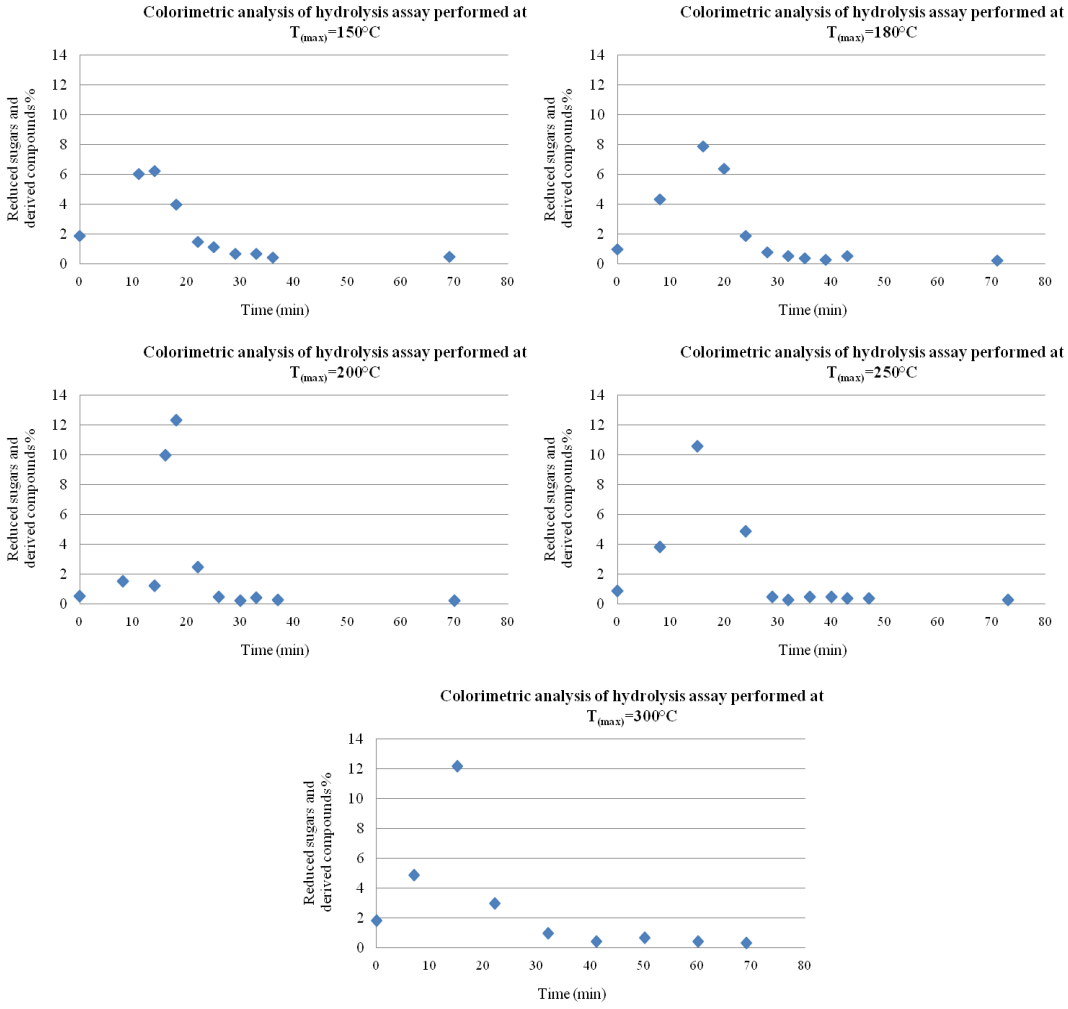

Figure 4.15 - Variation of total polysaccharides and derived compounds along time relatively five hydrolysis assays performed at different temperatures (150 – 300ºC, 100 bar, 2 – 5 ml/min).

HPLC analyses were performed with the same samples that showed higher amounts of soluble compounds (from the region selected in the figure 4.12 with blue square). To compare data from different assays the amount of identified monosaccharides is presented by $\frac{(mg) of\ monosaccharide}{(g) of\ the\ residue}$. There were identified five sugars mentioned in the literature as the main monomers present in the grape pomace. Fructose and glucose are two hexoses that could

exist as residual sugars and as building blocks of cellulose (glucose) and hemicellulose (glucose and fructose). [83] Hemicellulose also contains pentoses, essentially xylose, arabinose and galactose. [53] Therefore, two main groups of monomeric structures should result from the hydrolysis process: the extracted saccharides (residual sugars) and monomers resulted from hydrolysis of polysaccharides (cellulose and hemicellulose). The amount of the latter should naturally increase with the temperature. Experimental data confirms variation of monomer composition in hydrolysates that resulted from assays performed at different temperatures. It means that fractionation of monosaccharides is possible.

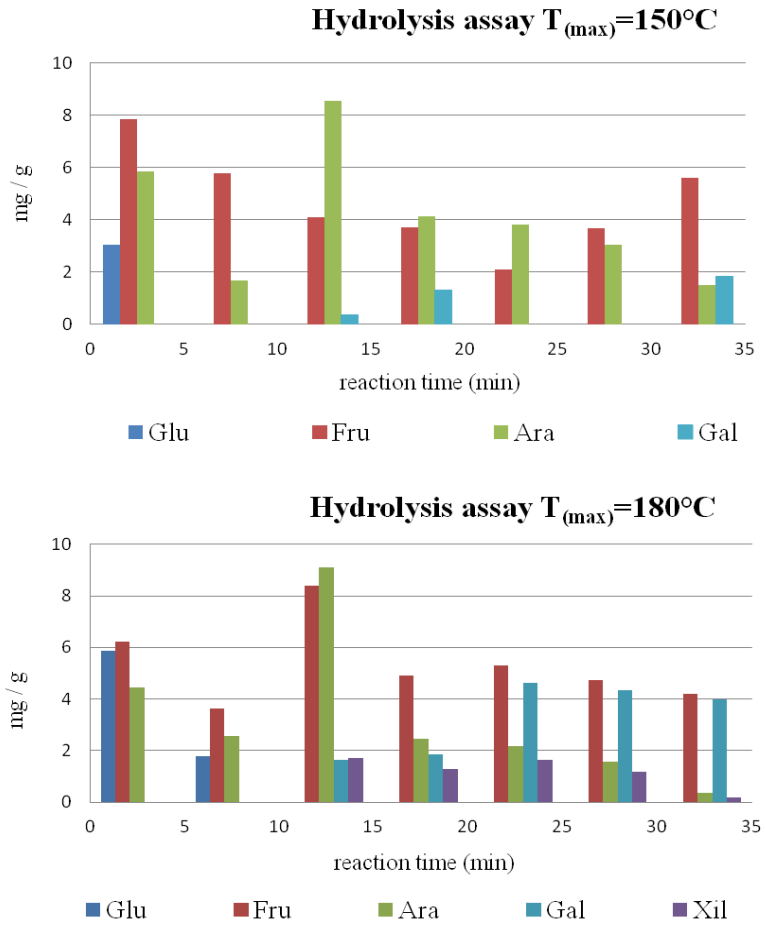

Figure 4.16 - HPLC analysis of chemical composition of GP hydrolysates (150-180ºC, 100bar, 2 – 5 ml/min).

As it could be seen in the figure above at the composition of hydrolysis assays performed at lower temperatures such as 150-180ºC it predominates essentially fructose and arabinose. Relatively low amounts of glucose, identified solely in the first samples of these two assays, suggest that it is part of residue sugars and that it was extracted from the residue and didn't result from hydrolysis. There was no xylose identified at 150ºC and only small amounts of galactose are present. However with increasing temperature the amount of xylose and galactose also increases which means that higher amounts of hemicellulose are hydrolysed. Also it could

be noticed from the figure above that glucose was identified in some samples that were collected at 200ºC and higher temperatures. As it was mentioned before, glucose in GP residue is a part of hemicellulose and cellulose, however the variation of the concentration of glucose isn't similar to gradual variation of the concentrations of xylose and galactose that comes from hemicellulose. Glucose peaks appear in the firsts samples, as in the assays performed at 150-180ºC and then suddenly emerge in the samples collected at higher temperatures. As it is known from the structure of the residue, to hydrolyse cellulose it is necessary to firstly hydrolyse a significant amount of hemicellulose that covers cellulosic structures protecting them. Sudden appear of glucose suggests that at 200ºC and higher temperatures there were hydrolysed sufficient amount of hemicellulose to achieve cellulose structures from which glucose comes from.

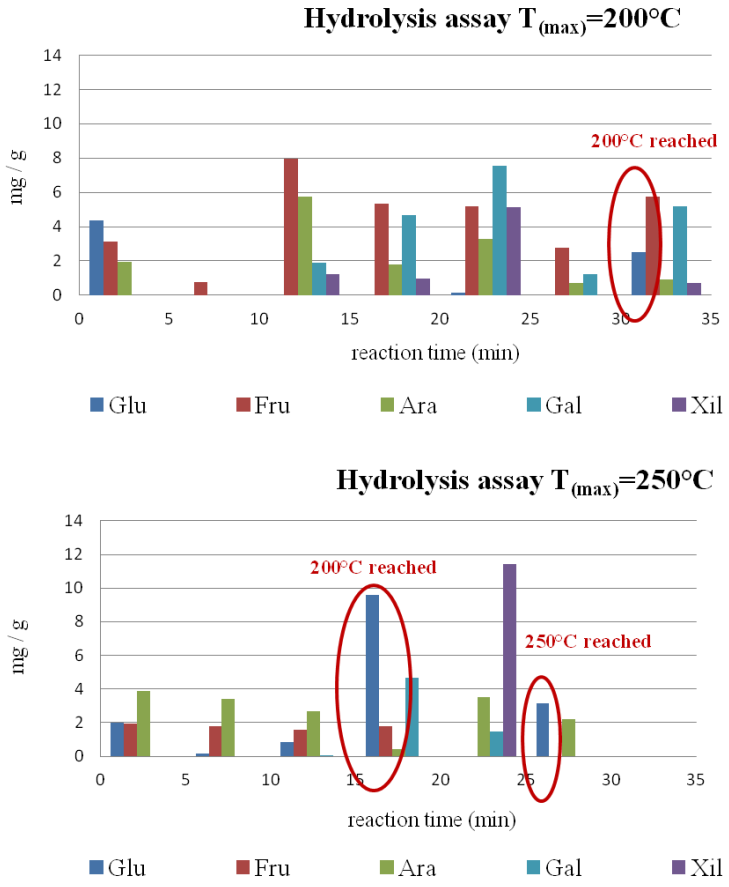

Figure 4.17 - HPLC analysis of chemical composition of GP hydrolysates (200-250ºC100bar, 2 – 5 ml/min).

Relatively small amounts of glucose comparing to more significant amounts of fructose in the composition on the samples collected at temperatures below 200ºC could also be explain by the Lobry de Bruyn-Alberda van Ekenstein transformation (LBET). Through this process D-glucose could be converted to D-fructose. It normally occurs at the room temperature at high pH, however in subcritical water at high temperatures and increasing pKw values LBET occurs at

lower pH. Thus subcritical water treatment of glucose yields measurable amounts of fructose, whereas for the processing of fructose only a negligible glucose formation has been reported. [57]

The composition of hydrolysate from assay performed at 300ºC as maximum temperature is not as rich in monomers diversity as hydrolysates obtained at lower temperatures. As it results from data on the figure below, per sample in media were identified only two monomers with amounts mostly lower than 5mg/g of the residue. However the final total mass of identified monomers related to that assay is in the line with the results of assays performed at lower temperatures (Tab. 4.1). Once again it confirms that there is a tenuous balance between hydrolysis and decomposition of the residue. In one hand glucose that appears in the samples collected at temperatures above 200ºC suggest increasing cellulose hydrolysis. On the other hand the increase of the total mass of monomers is negligible, indicating the simultaneous occurrence of degradation.

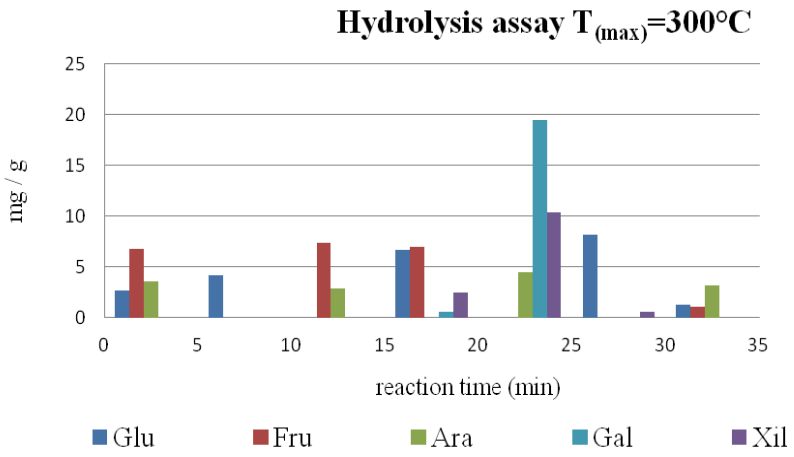

Figure 4.18 - HPLC analysis of chemical composition of GP hydrolysates (300ºC100bar, 2 – 5 ml/min).

Relatively to the total amount of monomers identified per assay once again they are in agreement with the total mass of soluble compounds data. As it could be seen in the table below, there is no significant difference between experiments performed at 180ºC, 200ºC and 300ºC. The lower amounts of monomers were identified in the assay performed at 250ºC, which showed also lower total soluble compounds data. More significant difference between total monomeric and total soluble compounds data is noted in the assay performed at 150ºC.

Table 4.1 - HPLC analysis of total amounts of monomers per hydrolysis assays performed at five temperatures (150 - 300ºC100bar, 2 – 5 ml/min).

	Total amount of monomers per assay (mg / g of the residue)	Total amount of monomers per assay (%)
T=150ºC	67,97	6,80
T=180ºC	90,06	9,01
T=200ºC	80,72	8,07
T=250ºC	56,46	5,65
T=300ºC	93,22	9,32

As it was already discussed there is a minimal concentration of furfural and 5-HMF in the hydrolysate resulted from the assay performed at 150ºC and consequently the influence of these two compounds on the colorimetric and total soluble compounds data could be despised. So from the figure below results that 35,5% of the total initial mass of the residue are water soluble compounds that were extracted/hydrolysed during the process. Almost 30% of total initial mass are polysaccharides that exist as simple monomers and as more complex structures, that is almost 64,5% of the total recovered (water soluble compounds). The missing 35,5% could be extractable antioxidants what would be verified lately through colorimetric and HPLC analysis. From the total initial mass of the residue used in this assay 6.8% are monosaccharides, that is circa 30% of the total polysaccharide content of hydrolysates. Another 70% should be composed by more complex structures resulted from hydrolysis and unidentifiable by HPLC technique such as dimmers, trimmers and more complex polysaccharide structures. That data demonstrates perfectly all the complexity and chemical heterogeneity of the hydrolysates of GP residue.

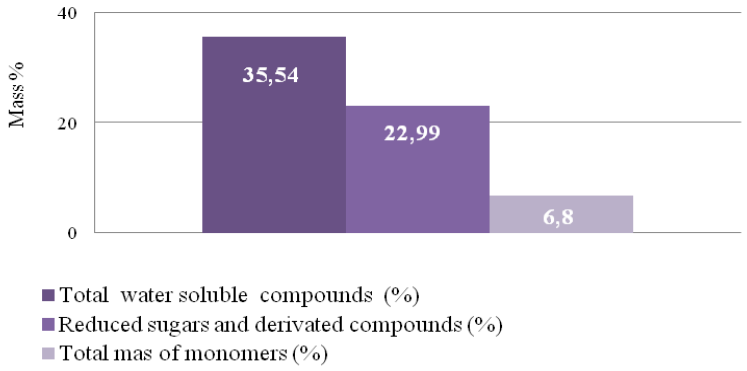

Figure 4.19 - Composition of hydrolysate from the assay performed at 150ºC, 100 bar, 2 – 5 ml/min.

Another important conclusion that could be drawn from HPLC data is about morphologic and consequently chemical heterogeneity of GP residue. As it was already mentioned the first samples of every performed assay are collected at 50ºC. That means that all are resulted from the same experimental conditions and as a result its chemical composition should be very similar. The data from figure 4.20 disprove that suggestion. In fact the data related to each assay seems to be completely random. The only evident similarity is the presence of the same three monosaccharides (glucose, fructose and arabinose). Limitation of the experimental set-up does not allow establishing a more rigorous control on the initial data so these variations affect the whole experimental data as it could be seen in the figure 4.21. Increasing the scale of the reactor for example could significantly reduce the influence of morphological heterogeneity of the residue.

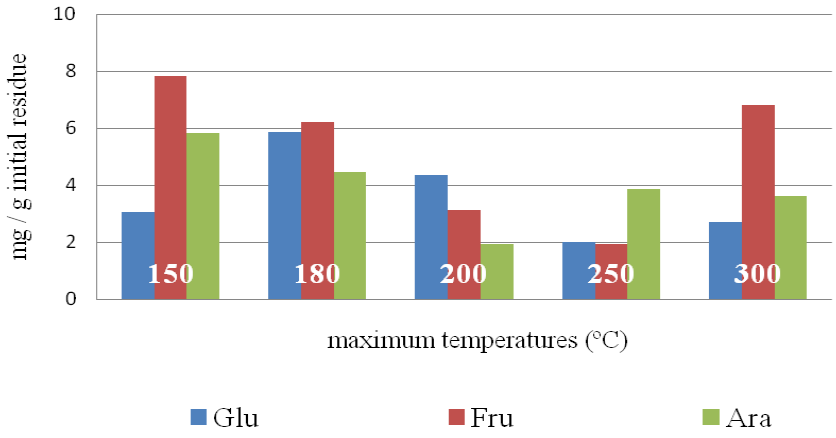

Figure 4.20 - Composition of first samples from five different assays performed at five temperatures (150 - 300ºC, 100 bar, 2 – 5 ml/min).

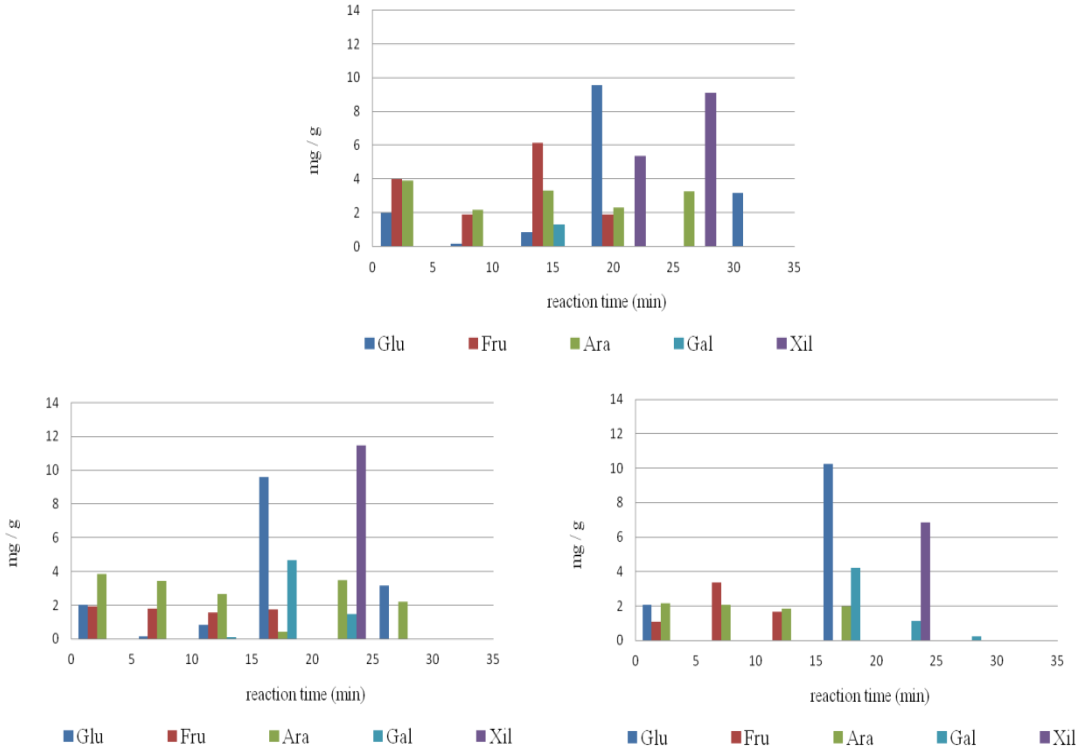

Figure 4.21 - Composition in monosaccharides mg/g of the initial residue of three different assays performed at the same experimental conditions (250ºC, 100bar, 2 – 5 ml/min).

As it could be seen in the figure above three replicates of the assay performed at the same experimental conditions reveal evident variation in the chemical composition of collected samples. However, once again, the three replicates have an important data in common, significant amounts of xylose appear solely at higher temperatures and glucose, as it was discussed previously; suddenly emerge at the temperatures above 200ºC.

4.2.3 – Polyphenolic content of GP hydrolysates

To evaluate the potential of GP hydrolysates in polyphenols colorimetric analysis were performed of the same samples that were used in the previous studies. That colorimetric test gives an estimative of the total phenolic content (TPC) in the samples. The colour intensity (blue) is directly proportional to the concentration on polyphenols. As it could be seen in the figure below there is a direct correlation between the colour intensity of the samples collected during hydrolysis assay and the same samples used in the colorimetric test. That's because the colour of grapes (essentially of grape skins) is due to the pigments presence named anthocyanins. [58]

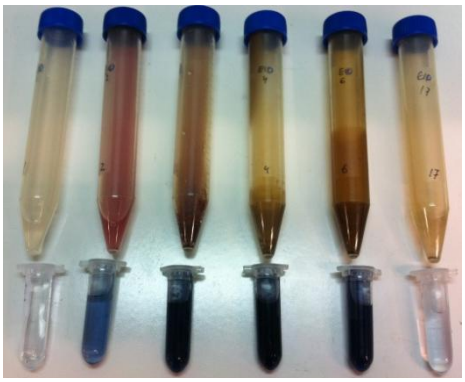

Figure 4.22 - Visual aspect of the liquid phase of hydrolysates collected during 150ºC assay (above) and the same simples during colorimetric test to determine TPC (below).

Once again the variation of TPC per sample along time shows the same behaviour of variation of the water soluble compounds recovered per sample, confirming high yields of the process. Both, hydrolysis and extraction, happens during the pre-heating period and finish almost immediately after maximum temperature is achieved. TPC per sample is much lower comparing to saccharide content. When the amount of sugars were quantified in milligrams the TPC recovered from the same amount of GP residue (1g) is thousand times lower. Having into account the scale quantified material (micrograms) it could be assumed that there is no significant difference between TPC data from essays performed at lower temperatures (Fig. 4.24). In the literature higher amounts of polyphenols from grape pomace are extracted at 200ºC. (ref. na intr) However from the experimental data of Folin-Ciocalteu colorimetric test higher amounts of TPC were clearly identified in the 300ºC assay (Fig. 4.24). Colorimetric tests don't provide sufficiently accurate data to discuss it rigorously. The Folin-Ciocalteu method does not allow the identification of individual compounds and tend to overestimate TPC due to interference of reducing substances such as sugars, aromatic amines, sulfur dioxide, ascorbic acid, organic acids, as well as nonphenolic organic substances that react with the Folin-Ciocalteu reagent. [84] Its principal aim is to evaluate the potential of the material to perform later, if necessary, more rigorous analysis such as HPLC for example.

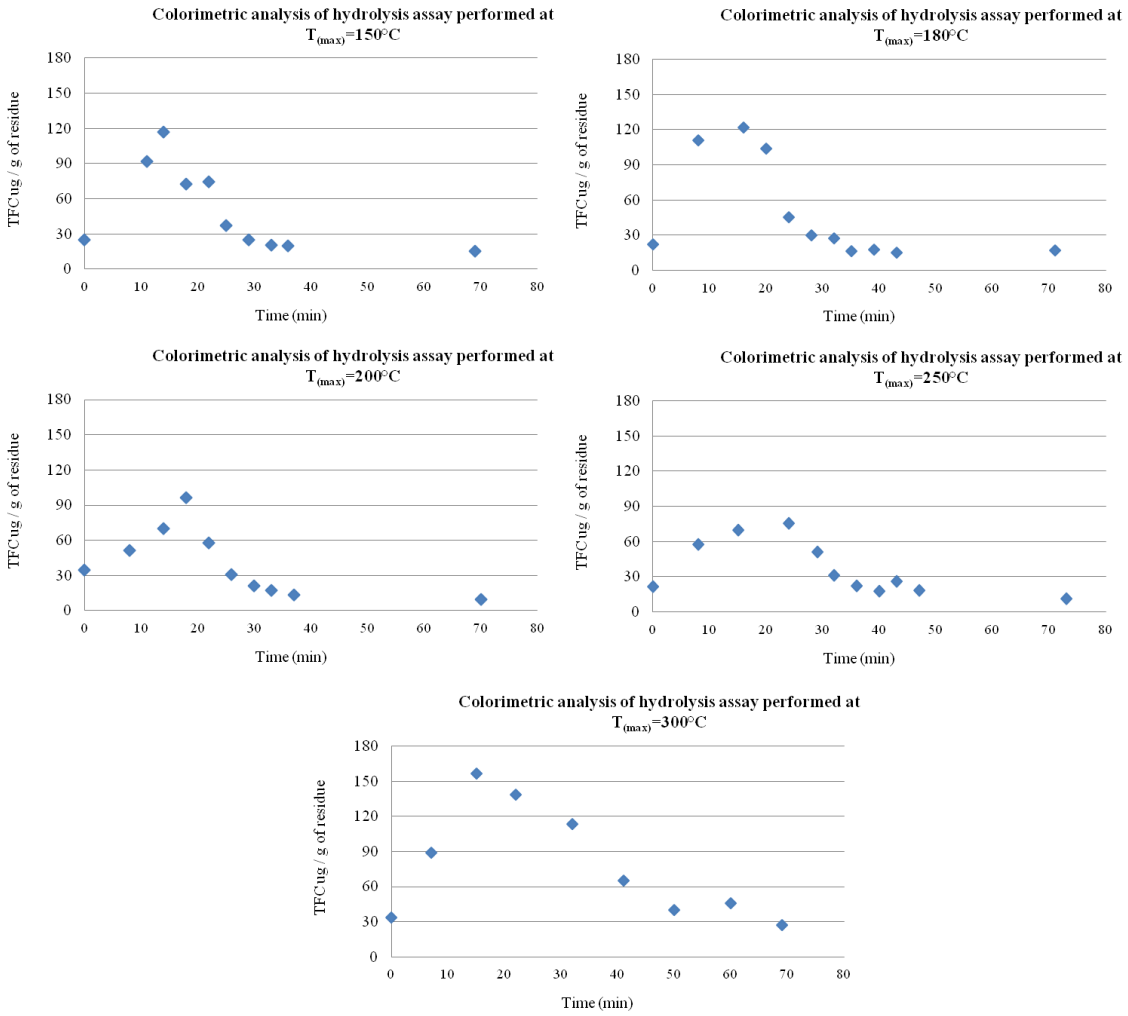

Figure 4.23 - Variation of TPC (µg of TPC / g of residue) per sample along time in the five hydrolysis assays performed at different maximum temperatures (150 – 300ºC, 100 bar, 2 – 5 ml/min).

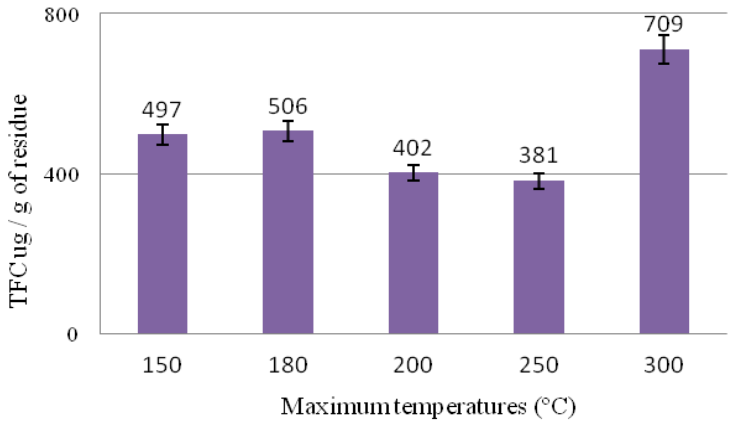

Figure 4.24 - Variation of TPC (µg of TPC / g of residue) per assays performed at different maximum temperatures (150 – 300ºC, 100 bar, 2 – 5 ml/min).

To perform HPLC analysis of GP hydrolysates six compounds were selected related in the literature as more common and more abundant in the grape pomace residue such as: gallic acid caffeic acid, p-coumaric acid, chlorogenic acid, ferulic acid and resveratrol. All can be used in pharmaceutical industry as natural antioxidants or as ingredients of functional food. [46] Gallic acid is from the group of hydroxybenzoic acids (C_6-C_1 structure); p-coumaric acid, caffeic acid and ferulic acid are hydroxycinnamic acids (C_6-C_3 structure); chlorogenic acid is the ester of caffeic acid and resveratrol is a stilbenoid. All these compounds are non-flavonoids.

There were identified substantially higher amounts of gallic acid in comparison to the other antioxidants detected with higher yield achieved at 180ºC. Gallic acid is widely distributed throughout the plant kingdom, where it is present either in free form or, more commonly, as a constituent of tannins, namely gallotannin. It has a great economic importance due to its numerous applications in the pharmaceutical industry. Regarding its biological activity, gallic acid exerts anti-bacterial, anti-viral, anti-inflammatory and antioxidant effects, and anti-melanogenic activity via the inhibition of tyrosinase activity. It also inhibits high fat diet-induced dyslipidaemia, hepatosteatosis and oxidative stress and mutagenic effects of benzidine, a human bladder carcinogen. Anti-cancer activity of gallic acid has been reported in various cancer cells, such as leukemia, oral tumor and esophageal. [85]

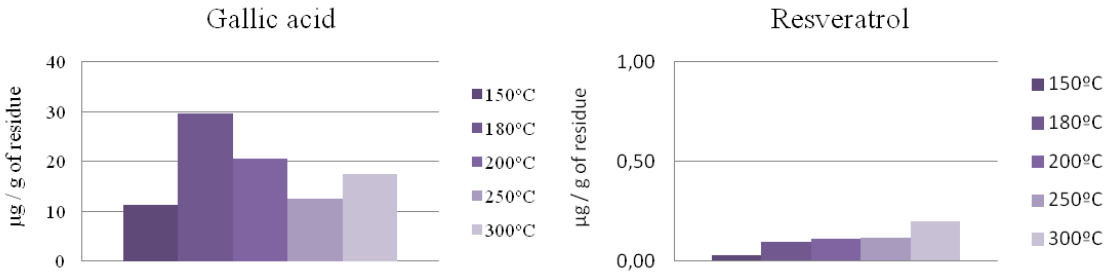

Figure 4.25 - Variation of gallic acid amounts and resveratrol identified in five hydrolysates performed at different temperatures (150 – 300ºC, 100 bar, 2 – 5 ml/min).

It is reported in literature that grapes are very rich in resveratrol, but experimentally, as it could be seen in the figure above, only small amounts of that compound were identified in GP hydrolysates. It is also known that resveratrol, during red wine making process, more specifically during fermentation, is transferred to wine. That does not happen for example during white wine making because white grapes pomace is separated from the grape juice before fermentation process. There is no data about amount of resveratrol that remains in the red grape pomace after fermentation. Best extraction yields of resveratrol from grape skins (that were not fermented) described in literature were achieved with ethanol/water (80:20 v/v) maintained at 60 °C for 30 min. [86] The same method applied to GP residue used in the present

work had not revealed any presence of resvetatrol. It also was not identified in the analysis of the precipitate as it will be described later.

The amounts of other four phenolic acids that were analysed by HPLC are lower than 1 µm/g $_{\text{initial residue,}}$ except for p-coumaric acid that showed lightly higher yield at higher temperatures. (Fig. 4.26) Such low mass rates extracted during the process could be explained by the thermal degradation of these acids mentioned in the literature. [87] However there is no significant difference between amounts of these acids extracted at higher and lower temperatures.

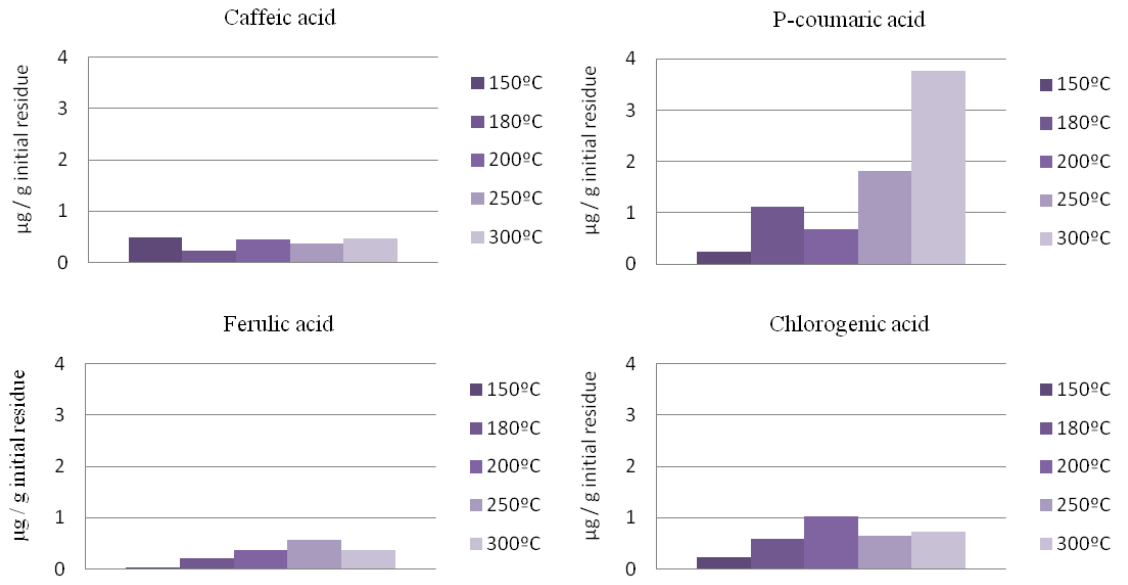

Figure 4.26 - Variation of four phenolic acids amounts identified in five hydrolysates performed at different temperatures (150 – 300ºC, 100 bar, 2 – 5 ml/min).

To evaluate the extraction of phenolic compounds with HCW, other established methods of extraction of phenolic compounds were experimented, such as, extraction with water:ethanol (75:25), water:acetone (20:80) and citric acid (3g/L). However, as it could be seen in the table below, it also revealed low rates of resveratrol and four phenolic acids (ferulic, chlorogenic, caffeic and *p*-coumaric) that suggest that GP residue initially had low amounts of these compounds. It is to remember that GP residue was suggested to fermentation process and that resveratrol and phenolic acids (in which grapes are rich) are important compounds into wine composition being responsible by its flavour, colour and health benefits. Wine making process, more concretely fermentation, is a hidro-alcoholic extraction method of phenolic compounds from grapes to the wine. [46] Low rates of these compounds into residue means high hidro-alcoholic extraction rates achieved during wine making process.

Table 4.2 - Results of phenolic compounds extraction perfermed with diferente methods.

	µg / g initial residue			
	Water : ethanol (75:25)	Water : acetone (20:80)	Citric acid (3g/L)	HCW
Ferulic acid	0.01	0.15	0.01	0,57
Chlorogenic acid	0.05	0.47	0.08	1,02
Caffeic acid	0.60	1.67	0.01	0.44
***p*- Coumaric acid**	0.25	0.32	0.10	3.76
Resveratrol	0.02	0.10	0.01	0.20

It is reported in literature that phenolic acids were extracted with HCW at 140ºC, 100bar with a yield of 36mg/g of the residue.[58] There are other antioxidants equally important as anthocyanins, catechins, procyanidins and tannins that could be extracted with HCW and Folin-Ciocalteu data confirms the presences of phenolic compounds into GP hydrolysates. Although in this work we were not able to confirm the presence of these phenolic groups. Nevertheless, taking into account that anthocyanins and tannins are the main compounds responsible for the wine colour and that the samples of performed extractions with HCW, etanol, acetone and citric acid (see figure below) have the characteristic wine colour, we believe that these compounds are present in the residue and that its identification should be one of the promising topics of the future work.

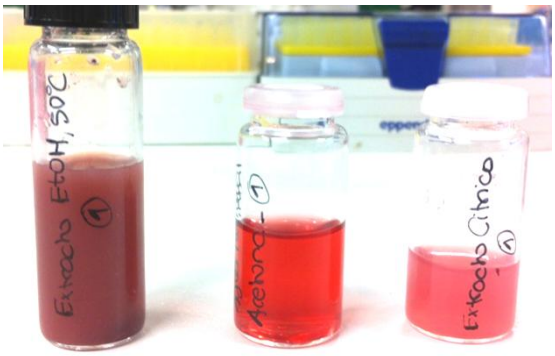

Figure 4.27 - An example of samples resulted from extraction methods performed with the initial GP residue and 1 – ethanol; 2 – acetone; 3 – citric acid. Colour reflects different amounts of extracted tannins and anthocyanins.

4.3 Future work

- Perform acid hydrolysis of the initial residue to characterise its initial potential into reduced sugars;
- Scale up the hydrolysis reaction apparatus to minimise the effect of morphological and chemical heterogeneity of the residue on the experimental data;
- Perform HPLC analysis to identify other phenolic groups extracted during the process such as anthocyanins, catechins, and tannins. Evaluate its application into food and pharmaceutical industries without any additional extraction process from the aqueous solution.
- As the main alternative to lyophilisation, to use membrane technology to concentrate aqueous solution collected during hydrolysation process.

OLEAGINOUS YEASTS

"The most exciting phrase to hear in science, the one that heralds the most discoveries, is not "Eureka!" but 'That's funny…"
— Isaac Asimov

5 Chapter: OLEAGINOUS YEASTS

The aim of this second part of the present work was to:

- Select species of oleaginous yeast based on their growth performance on 31 different carbon sources (mono-, di-, trisaccharides and more complex compounds);
- Confirm the possibility of using GP hydrolysates as the unique carbon source to be assimilated by the pre-selected oleaginous yeasts;
- Identify the best GP hydrolysates to be used as a carbon source to be assimilated by the pre-selected species.

As there are no studies performed with the *Rhodotorula spp.* and *Rhodosporinium spp.* yeasts in the GP hydrolysates, we have opted to choose six species (Tab 5.1) extracted essentially from fruits (attending to the origin of residue) and *Saccharomyces cerevisiae. Although* S.*cerevisiae* is a fermenting, not oleaginous, yeast , it was included in the screen due the fact that it is one of the most intensively studied eukaryotic organisms in molecular and cell biology. When final valuable by-products of *Rhodotorula spp.* and *Rhodosporinium spp.*, as it was mentioned, are TAG's and carotenoids, *S.cerevisiae* could originate valuable biofuel such as ethanol.

Table 5.1 - Yeasts strains used in the pre-selection path of the work.

Species name	Extracted from
Rhodosporidium babjevae	blackberry
Rhodotorula sp.	honey
Rhodotorula sesimbrana	wood
Rhodotorula bacarum	black currants
Rhodotorula babjevae	white grapes
Rodotorula Yarrowii	strawberry

Due to limitations of the experimental set-up and several methods applied to GP hydrolysates for quantification of sugars and phenolic compounds presented in the samples, it left over only small amount of each hydrolysate sample. Thus, before using GP it was performed a preliminary survey to test yeasts for its potential to assimilate a variety of carbon sources.

5.1 – Screening with 31 different carbon sources

To perform better the yeasts strain screening, it was prepared 31 different minimal media with only one available carbon source in each of them. In this way, it was guaranteed that the growth could only be possible if the yeast assimilates the only sugar presented in the medium. Basing on the initial composition of GP residue, it was selected 31 different saccharides. It was selected the most common monomers in which the presence in the hydrolysates of six of them were previously confirmed by HPLC analysis (glucose, fructose, mannose, galactose, xylose and arabinose), several di- and trisaccharides, and more complex compounds which structure resulted from the combination of the main monomers such as cellobiose (disaccharide) that consists of two glucose molecules or maltotriose (trisaccharide) that consists of three of glucose molecules, among others (attachments pag. 89). Thus, even without data of the exact composition of GP hydrolysates, carbon sources were chosen with the aim to approach to the reality.

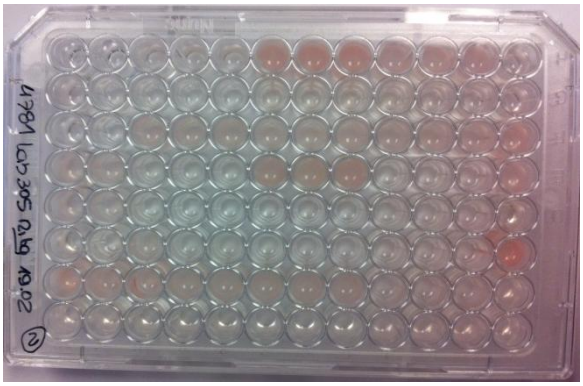

Figure 5.1 - Visual aspect of inoculated microplate (96 well). The colour visible in some wells is a natural colour of yeast due to the presence of carotenoids.

In order to compare yeasts' response to different carbon sources, growth rates based on the optical density variations (DO) along time (that is directly proportional to microorganisms' concentration) were calculated. Graphically, growth rate corresponds to the slope of the exponential growth zone: $(\log OD - \text{Log } OD_0)/t = u/\ln 2$ (Fig. 5.2).

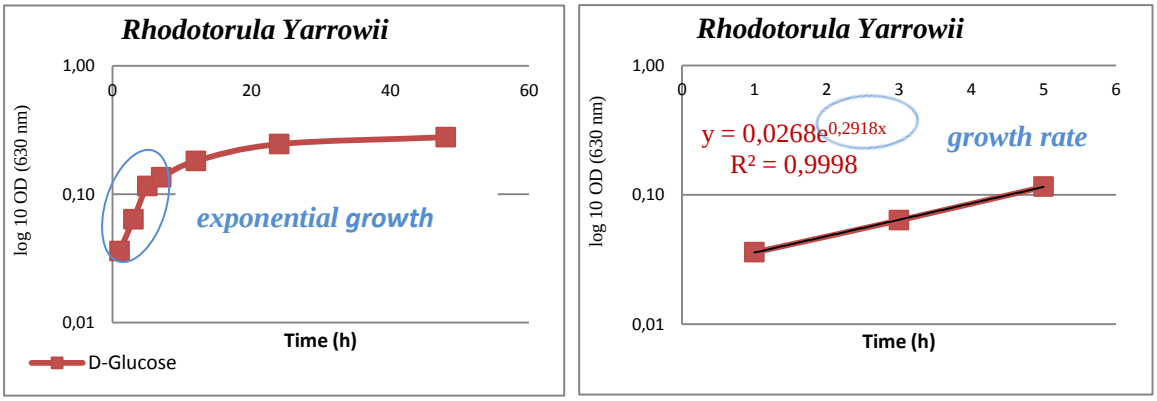

Figure 5.2 - - Example of growth curve and growth rate calculation for *Rhodotorula yarrowii* in the minimum growth medium with D-Glucose as the only carbon source.

The main selection criteria of the most suitable yeasts, attending to the finality of this study, was higher growth rates in the presence of six monomers identified previously by HPLC in the composition of GP hydrolysates. This way, from the seven pre-selected strains, stood out four, stated in the table below. Attending to the fact that desired final products are essentially fatty acids accumulated in the cells, it is important to achieve high yeast biomass that will consequently result in high fatty acids amounts. It is economically favourable achieve higher concentration in the shortest period of time, because time reflects amounts of substrate needed, occupational period of reactor and a consequent growing maintenance cost. Thereby greater growth rates leads to economically benefice process.

Table 5.2 - Growth rates for the four yeasts that showed best performance in the presence of six carbon sources (standard deviation ± 0,03h^{-1}).

	Growth rates μ (h^{-1})			
	Sacchacaromyces cerevisiae	***Rhodotorula sp.***	***Rhodotorula babjevae***	***Rodotorula yarrowii***
Glucose	0,28	0,17	0,27	0,29
Fructose	0,25	0,17	0,27	0,40
Mannose	0,27	0,17	0,10	0,22
Galactose	0,31	0,36	0,24	0,42
L-Arabinose	-	-	0,30	0,38
Xylose	-	0,03	0,29	0,10

However, it is also important to take into account the response of the yeasts to the more complex compounds that are surely presented in GP hydrolysates, because in such heterogeneous and complex residue as grape pomace is, the hydrolysis reaction would never be

complete and the final product would be composed by simple and complex carbohydrates and compounds resulted from its degradation. So, higher the variety of carbon sources which yeasts are able to assimilate, the higher would be its probability to assimilate GP hydrolysates. The strains that have assimilated a wider range of carbon sources were those four that showed higher growth rates in the presence of six monomers (attachments).

5.2 – Screening with mixtures of carbon sources

As it was mentioned before, the behaviour of microorganisms in the presence of more than one carbon source simultaneously is not necessarily equal to that they show in the presence of the only one carbon source. Thus the next step of the yeasts' screening was the study its growth rates in the medium with the mixture of carbon sources. Having into account the preliminary data of monomeric composition of different GP hydrolysates, namely proportions between glucose, fructose, arabinose, galactose and xylose, four different growth medium related to four GP hydrolysates were prepared (hydrolysates related to 250ºC and 300ºC showed similar composition). And, also attending to the possible influence of the glucose on the response of the yeast to reaction medium, another four mixtures, identical to the previous but with glucose were prepared (Tab. 5.3). To compare results of that experiment with the data from the previous screening the same final carbon source concentration of 5g/L was used. That concentration resulted from the sum of different carbon sources according to established proportion between monomers in different mixtures that could be seen in the table 5.3. . Thus, the amount of each monomer in the mixture is even lower than 5g/L. In other words, the main difference between mixtures was its composition in monomers and not in the final concentration of total sugars. The experimental method used to perform the experiments using carbon source mixtures was equal to the method used for the screening with only one carbon source.

Table 5.3 - Proportion between monomers used in each of eight mixtures with the final carbon source concentration of 5g/L. Fru – fructose, Ara – arabinose, Glu – glucose, Gal – galactose, Xyl – xylose.

Related GP hydrolysates	Monomers and its proportion	
T=150ºC	Fru/Ara (1:1)	Glu/Fru/Ara (1:1:1)
T=180ºC	Fru/Ara/Gal (1: 0,5: 0,25)	Glu/ Fru/Ara/Gal (1:1: 0,5: 0,25)
T=200ºC	Fru/Ara/Gal/Xyl (1: 0,5: 0,5: 0,25)	Glu/Fru/Ara/Gal/Xyl (1:1: 0,5: 0,5: 0,25)
T=250ºC	Fru/Ara/Gal/Xyl (1:1:1:1)	Glu/Fru/Ara/Gal/Xyl (1:1:1:1:1)

The results showed no significant difference between all eight mixture mediums as it could be seen by the similarity of growth rates data (Tab. 5.4) and by the growth curves.

Table 5.4 - Growth rates of four yeasts in the presence of different mixtures of monomers used as carbon sources (standard deviation ± 0,03h^{-1}).

	Growth rates μ (h^{-1})	
Yeast	Mixtures without glucose	Mixtures with glucose
S.cerevisiae	0,20	0,13
Rhodotorula sp.	0,11	0,18
Rhodotorula babjevae	0,12	0,16
Rodotorula Yarrowii	0,18	0,13

First of all, it should be noted that, despite of the validation of growth data by tree replicates made in each experiments, both screenings (with the only carbon source and with the mixtures) are not very strict, because the aim of these studies was essentially verify the assimilation of carbon sources by pre-selected yeasts. To make more accurate study of cultures' growth it would be necessary to perform a scale up. However, this method and this experimental scale allow the conclusion that, similar to the screening with only one carbon source, all four strains showed a positive response to the mixtures of monomers and that there is no influence of glucose presence.

As these results confirmed, four pre-selected stains not only assimilated six mono-saccharides identified in GP hydrolysates, but also showed positive response for the assimilation of these compounds present as a mixture solution. Thus, it was the time to perform the study using hydrolysates as only available carbon sources.

5.3 – GP hydrolysates as carbon sources to yeasts growth

There were determined a total amount of monosaccharides in each hydrolysate and the minimum growth mediums were prepared with lyophilised powders in order to maintain the concentration of 5g/L (in carbon source) used in all previous studies. Thus, the main differences between growth media are: proportion between monomeric carbohydrates, presence of other complex sugars and the amounts of 5-HMF and furfural (main compounds resulted from thermal degradation of carbohydrates discussed in the previous chapter).

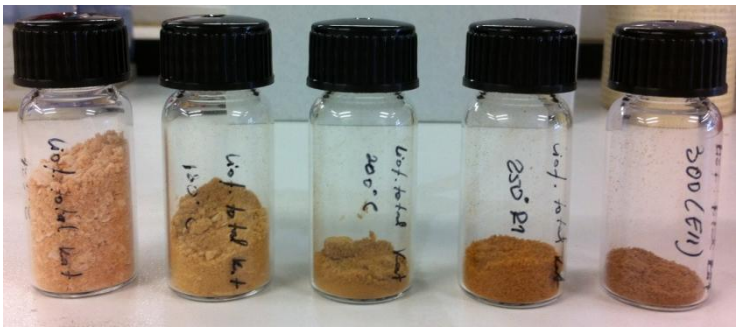

Figure 5.3 - Visual aspect of powders of GP hydrolysates obtained at different temperatures (from left to right: 150 ºC; 180 ºC; 200 ºC; 250 ºC; 300 ºC) after lyophilisation.

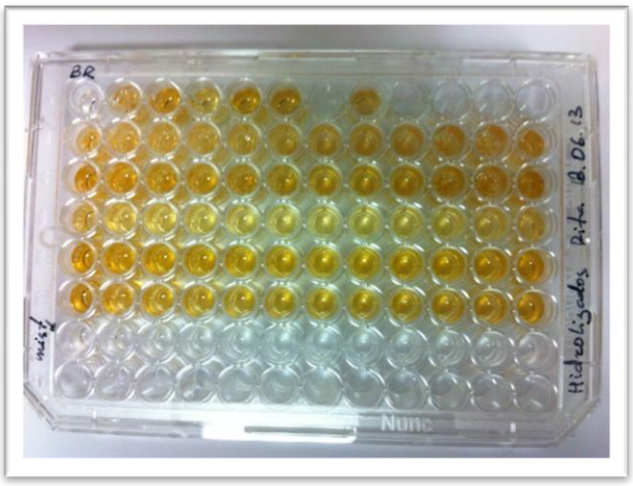

Figure 5.4 - Visual aspect of microplate (96 well) with GP hydrolysates used as carbon sources for yeast growth.

All four pre-selected strains showed a similar tendency in the assimilation of hydrolysates which could be considered as a very promising result, because it allows the conclusion of which hydrolysates is/are more suitable to be used as the main carbon source to these yeasts. If each strain showed a preference by only one hydrolysate, different from which the other strains had

proffered, it would be impossible to conclude about the viability of the whole process of the residue valorisation through the via proposed in this work.

As it could be seen by growth curves in the 5.5, the best assimilating rates were reached by all strains in the presence of GP hydrolysate obtained at 150ºC and the lower or absent growth were achieved at the presence of GP hydrolysate obtained at 300ºC. Also it should be noted that GP hydrolysate obtained at 180ºC gain clearly the second place of the best assimilated carbon source. Higher final OD's in the presence of almost all hydrolysates (except the one obtained at 300ºC) were achieved by *Rhodotorula babjevae* that, curiously, was extracted from white grapes that could have influenced its assimilation capacity. The worse results according to final OD's belong to *S.cerevisae*.

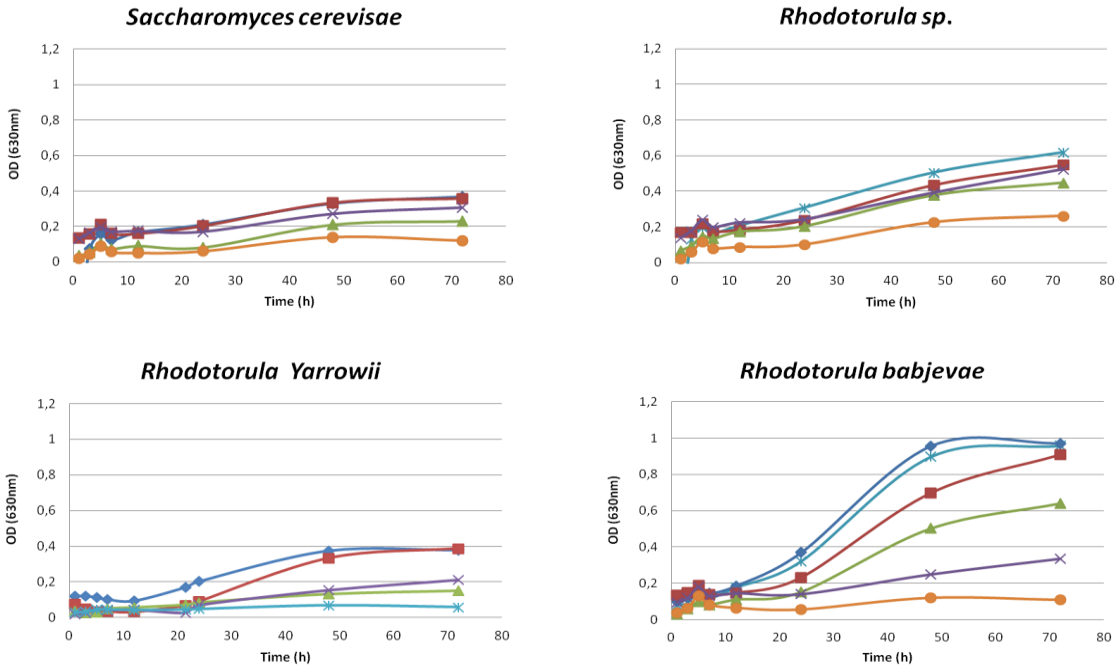

Figure 5.5 - Growth curves of four yeasts in the presence of five GP hydrolysates:
– 150ºC; – 180ºC; –200ºC; – 250ºC; – 300ºC.

Also it could be note that in some cases such as *Rhodotorula babjevae* in the presence of GP hydrolysate obtained at 150ºC or *Rhodotorula yarrowii* in the presences of hydrolysates obtained at 150ºC and 180ºC there were reached equilibrium phase after an exponential growth. That means that in those cases the carbon source was already consumed or at least the simplest metabolisable carbon sources were. It would be possible fundament this hypothesis scaling-up the experiment and making continuous analysis not only at the level of OD variation, but also at

the level of the substrate consumption verifying for example monomeric content by the HPLC analysis.

The growth rates presented in the table above confirm the "leadership" of *Rhodotorula babjevae* related to the assimilation of saccharides presented in the GP hydrolysates as a unique carbon sources available to its growth. The best growth rate was achieved in the presence of GP hydrolysate obtained at 150ºC. The second place for the ability to assimilate GP hydrolysates, according to the growth rates, belongs to *Rhodotorula yarrowii*. To obtain more accurate analysis it would be necessary to perform the culture growth in a major scale (ex. Erlenmeyer flask) for a longer period of time with a more strict control of agitation. However according to the objectives of this study these results, although they are not highly accurate, are perfectly acceptable and provide information that permit to draw the plan of future works.

Table 5.5 - Growth rates of four pre-selected strains in the presence of GP hydrolysates as a unique available carbon source.

	Growth rates μ (h^{-1})			
Hydrolysates named by temperature	***Sacchacaromyces cerevisiae***	***Rhodotorula sp.***	***Rodotorula Yarrowii***	***Rhodotorula babjevae***
T=150ºC	0,02	0,02	0,04	0,05
T=180ºC	0,02	0,02	0,04	0,04
T=200ºC	0,02	0,02	0,02	0,04
T=250ºC	0,02	0,01	0,03	0,02
T=300ºC	0,01	0,02	0,01	0,01

In sum, despite of positive results shown by *Rhodotorula babjevae* and *Rhodotorula Yarrowii* in this last experiment, the growth rates in the presence of hydrolysates are substantially lower than those achieved in the presence of unique monosaccharide and in the presence of the mixtures of carbon sources (ex. *Rhodotorula babjevae* with glucose carbon source: 0,27 h^{-1}; in the mixture with glucose: 0,16 h^{-1}; in the GP hydrolysates obtained at 150ºC: 0,05 h^{-1}). As it was verified previously, at the different proportions in which monosaccharides are present in the hydrolisates obtained at different temperatures, they have no influence in the yeasts' response since its final concentration is normalized. And in the last study with hydrolysates as carbon sources for yeasts' growth the final concentration in monomer was equal for all hydrolysates. So there is another cause that prevents more considerable growth. For example the presence of polysaccharides which influence wasn't studied, it was only verified that four pre-selected strains assimilates some complex sugars in the absence of other carbon sources.

Other possible influence could be originated by the furfural presence. Furfural is described in the literature as toxic compound that, depending on its concentration in the culture medium, can inhibit cells growth rate. It has revealed to be highly toxic to the growth and fermentation of microorganisms for ethanol production, *Rhodotorula toruloides* for example, was highly sensitive to furfural decreasing by 60% its biomass accumulation in the presence of 1g/L of furfural. (Zhao. Effect of some inhibitors) In the present work, the amount of furfural in the hydrolysates increases with the temperature at which hydrolysis reaction is performed (except hydrolysate obtained at 300ºC, furfural amount in this sample is lower than in the sample of 200ºC). That could suggest that lower growth rates in the presence of hydrolysates obtained at higher temperatures could be directly proportional to furfural concentration. However, as it was mentioned before, the amount of furfural presented in the liquid phase of hydrolysates disappears during freeze-drying process and to prepare growth medium it was used freeze-dried powder. According to HPLC data, samples obtained after the hydrolysis reaction could contain $14*10^{-6}$ g/L to $50*10^{-6}$ g/L. Nevertheless, as no any toxicity tests were performed, there is no data to confirm resistance of pre-selected strains to the present amounts of furfural. It has not been performed the quantification of furfural amount presented in the liquid phase of hydrolysates collected during hydrolysis performed by HCW, but its amounts are higher than in the lyophilised powder. Thus, the residue shouldn't be used directly in the growth media.

Lignocellulosic materials (aromatic, polyaromatic, phenolic compounds) released from lignin during its hydrolysis also shows capability to strongly inhibit microorganism growth. Higher temperatures are needed to lignin degradation [16] so higher amounts of these compounds should be present in hydrolysates obtained at 250ºC e 300ºC. That fact could also justify lower growth rates achieved.

5.4 – Future work

- Perform studies of toxicity of GP hydrolysates, essentially for furfural, 5-HMF and compounds resulted from lignin decomposition;
- Perform culture growth in a higher scale (ex. Erlenmeyer flask) with hydrolysates as carbon sources ensuring better agitation and control of evaporation process. Perform that experience during at least three days controlling OD variation and carbohydrates composition of hydrolysates (by HPLC analysis) along time;
- Perform yeast culture growth in a batch with one/two hydrolysates that showed best growth rates;
- Verify lipid accumulation by yeasts, determining its amount (ex. Bligh and dyer Method). [88] and analyzing its composition in TAG's, for instance, by GC analysis. [89]

CONCLUSIONS

"Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less."
— Marie Curie

6 Chapter: CONCLUSIONS

The main objective in this work was the valorisation of one of the most abundant agroindustrial residues, grape pomace (GP) using HCW technology. GP is almost an inexhaustible residue due to the constant demand of one of its main final products – the wine.

There are two main pathways to GP valorisation: 1) the extraction of value-added by-products such as antioxidants and 2) the hydrolysis of complex polysaccharide structures. Antioxidants have a promising market in the food, cosmetic and pharmaceutical industry, whereas simpler saccharides resulted from hydrolysis could be applied as the main carbon sources to the yeast growth. The latter are able to produce value-added products such as carotenoids and omega-3 fatty acids to be used in the food and pharmaceuticals industry. The main goal is to integrate these two pathways into one continuous "green" process.

Gallic acid was the most abundant antioxidant present in the residue studied. The best extraction yield of gallic acid (30µg /g $_{\text{initial residue}}$) was achieved at 180ºC (100bar). It has several applications in the pharmaceutical industry due to its anti-bacterial and anti-inflammatory effect and also its anti-cancer activity. It is economically important compound and "amenable" condition of its extraction further contributes to the economic viability of the process. Also it has been proved through Folin-Ciocalteu tests (with promising TPC rates of 500µg/ g $_{\text{initial residue}}$ achieved at 150-180ºC) that the extracted material from GP residue is rich in other phenolic compounds which identification represents one of the main topics of the future work.

Hydrolysis reaction performed at five different temperatures showed that at higher temperatures almost 90% of total initial residue mass was hydrolysed. However the yield of recovered water soluble material, revealed no significant variation remaining at yields of about 30% independently on the temperature. This fact was attributed to the tenuous balance that exists between hydrolysation and decomposition of biomass during experiment. Although recovered mass rates are similar its chemical composition is not. This means that in terms of recovered mass yield there is no need to work at higher temperatures turning the process economically more advantageous. On the other hand the main goal of hydrolysis of GP is not only to achieve higher yields of hydrolysed material, but to obtain a chemically favourable substrate to been used as the main carbon source to the yeast growth.

Final composition of hydrolysates in terms of monosaccharides, obtained at different temperatures, show no significant difference varying between 7-9%. Nevertheless they reveal differences in terms of proportion between sugars, essentially glucose and xylose, which increasing with temperature. However those variations, as was experimentally demonstrated, have no significant influence in the growth rates of selected strains of oleaginous yeasts. Also

the low amounts of monosaccharides achieved during hydrolysis are not an impediment to use GP hydrolysates as carbon sources. It has been proved that yeast can assimilate more complex structures such as disaccharides, trisaccharides and even more complex compounds.

It is necessary to perform more test of the chemical composition of GP hydrolysates, essentially to identify the presence of toxic compounds that could prevent yeast growth. But with the experimental data presented in this work it could be confirmed that GP hydrolysates were assimilated by oleaginous yeast. The best growth rates were achieved with hydrolysates collected at 150ºC and 180ºC by *Rhodotorula babjevae (*0,05 h^{-1} and 0,04 h^{-1}) and *Rhodotorula yarrowii (*0,04 h^{-1} and 0,04 h^{-1}) confirming that nor into extraction pathway nor during hydrolysis there is no need to operate at temperatures above 200ºC.

The process of GP valorisation proposed in the present work was performed at laboratorial scale and several steps must be optimised. Experimental data proves the viability of this process. It also shows that there is no need to reach temperatures above 200 ºC and pressures higher than 100 bar. The high reaction rates decrease substantially the time of the process. Those experimental conditions reduce the maintenance cost turning economically favourable valorisation process.

7 Chapter: REFERENCES

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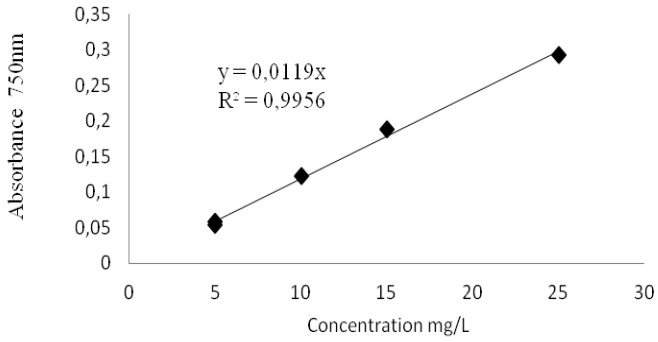
APPENDIX

Colorimetric tests

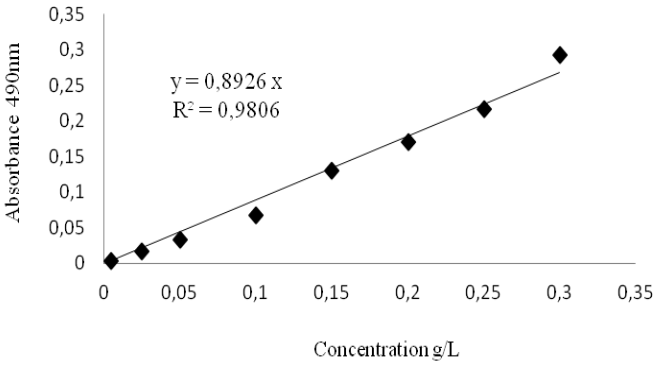
- *Folin – Ciocalteu colorimetric test*

  Gallic acid standad curve



- *Colorimetric carbohydrate analysis (modified)*

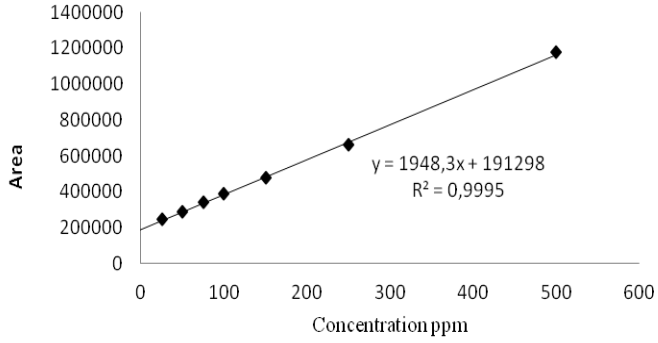
  Glucose standard curve



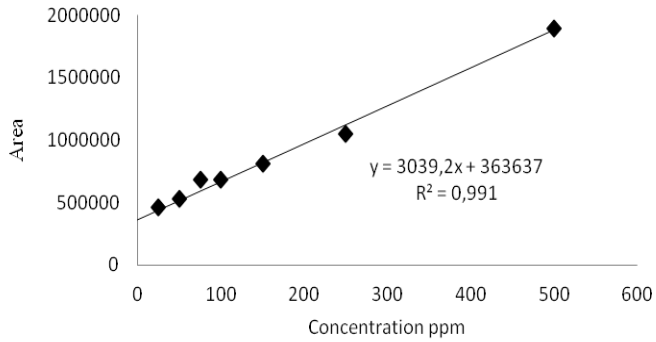
HPLC analysis

- *Carbohydrate analysis*

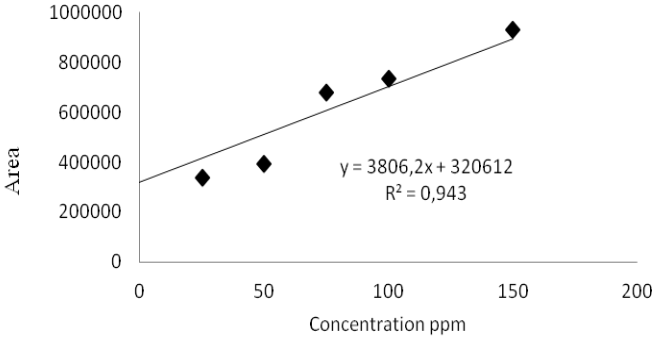
  Glucose standard



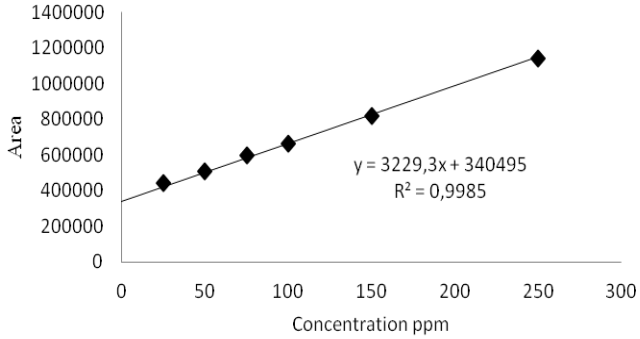
Xylose standard



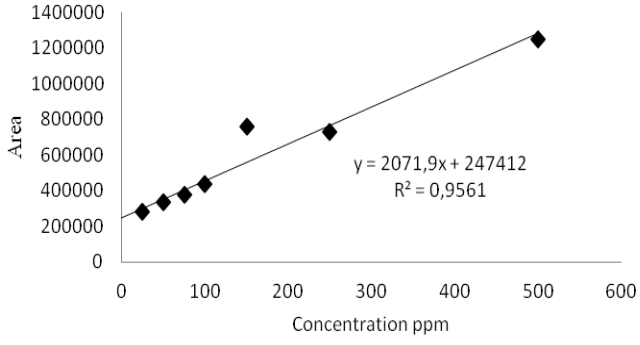
Fructose standard



Arabinose standard

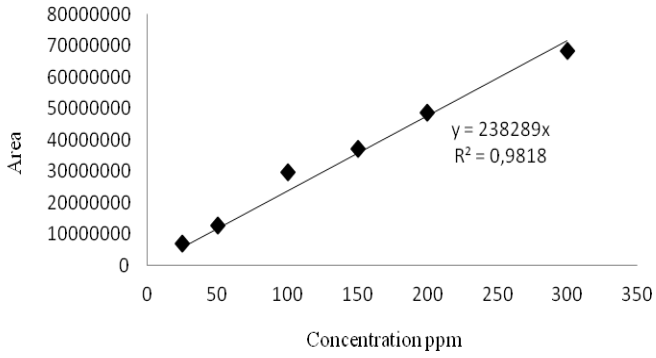


Galactose standard

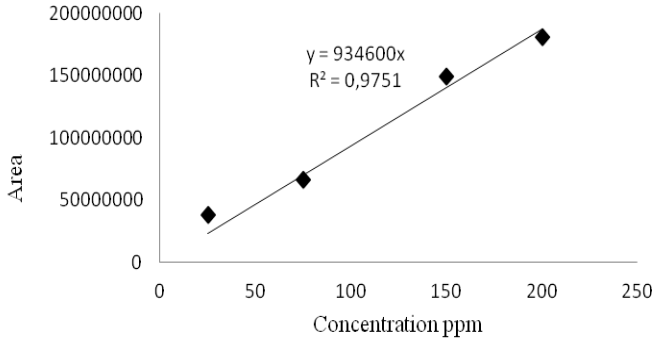


- *Phenolic analysis*

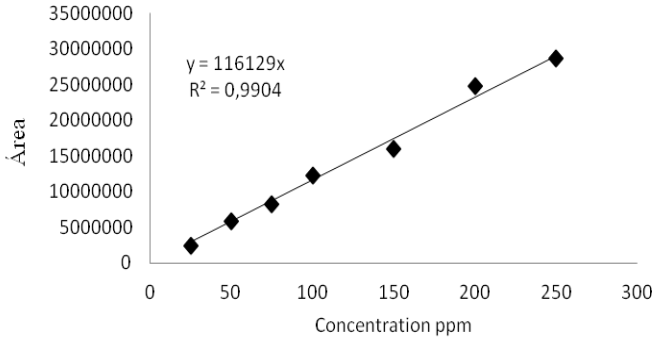
5-HMF standard



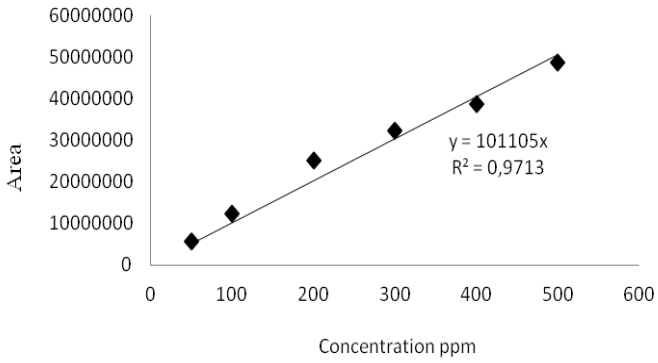
Furfural standard



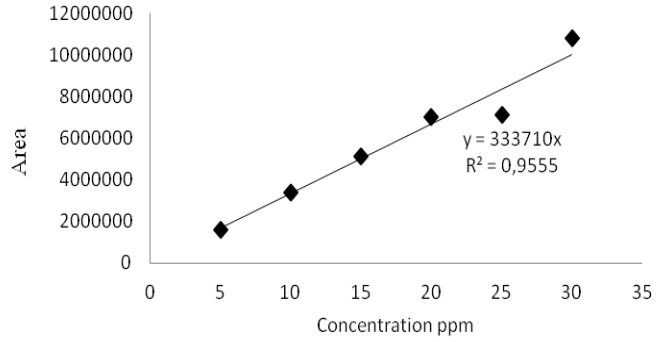
Chlorogenic acid standard



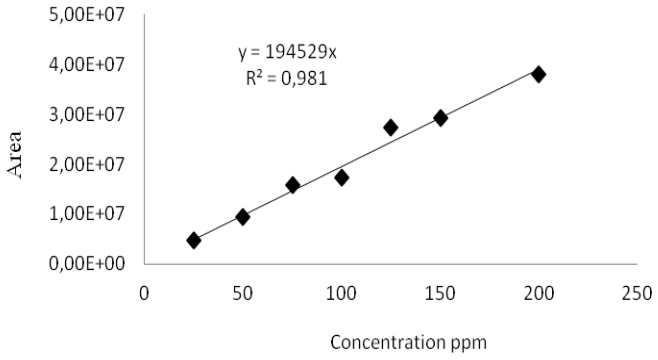
Gallic acid standard



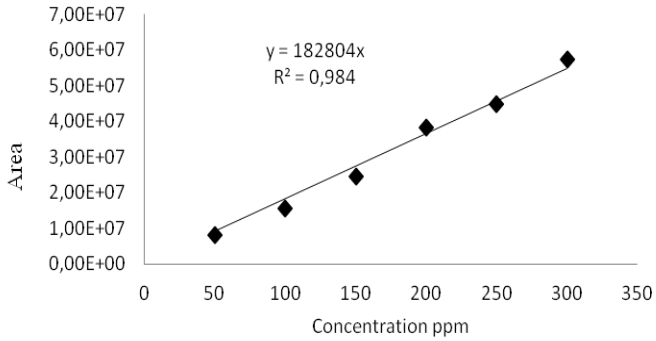
Resveratrol standard



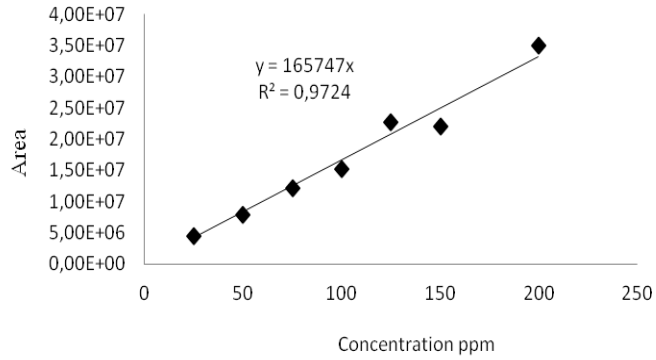
Caffeic acid standard



Ferrulic acid standard



p-Coumaric acid standard



- **OLEAGINOUS YEAST GROWTH**

Carbon Source		Rhodosporidium babjevae	Saccharomyces cerevisae	Rhodotorula sp
		μ (h^{-1})	μ (h^{-1})	μ (h^{-1})
Monomers	Glucose	0,05	0,28	0,17
Hexoses	Frutose	0,05	0,25	0,18
	Manose	-	0,28	0,18
	Galactose	-	0,32	0,36
	Rhamnose	-	-	-
	Tagatose	-	-	-
	Sorbose	-	-	-
Pentoses	L-Arabinose	-	-	-
	D-Arabinose	-	-	-
	Xylose	-	-	0,04
	L-Fucose	-	-	-
	D-Fucose	-	-	-
	D-Ribose	-	-	0,06
Disaccharides	Celobiose	-	-	0,05
	Lactose	-	-	0,08
	Maltose	-	-	0,06
	Melibiose	-	-	0,05
	Sucrose	0,05	-	0,08
	L-α-Trehalose	-	-	0,06
	D-Turanose	0,10	-	0,07
Trissacharides	Maltotriose	-	-	0,11
	Rafinose	0,10	-	0,09
	Melizitose	-	-	0,07
Complex compounds	Arabinogalactan	-	-	0,11
	Cellulose	-	-	0,08
	Xylan	-	-	0,06
	L-Arabinitol	-	-	0,18
	Galactitol	-	-	0,09
	D-Manitol	-	-	0,10
	D-Sorbitol	-	-	0,21
	xilitol	-	-	0,08

Carbon Source		Rhodotorula sesimbrana	Rhodotorula bacarum	Rhodotorula babjevae	Rodotorula yarrowii
		μ (h^{-1})	μ (h^{-1})	μ (h^{-1})	μ (h^{-1})
Monomers	Glucose	0,35	0,04	0,27	0,29
Hexoses	Frutose	0,32	0,04	0,27	0,40
	Manose	0,35	0,02	0,11	0,22
	Galactose	-	0,02	0,24	0,42
	Rhamnose	-	0,05	0,24	0,08
	Tagatose	0,02	0,04	0,18	0,36
	Sorbose	0,05	0,05	0,28	0,09
Pentoses	L-Arabinose	-	0,05	0,30	0,38
	D-Arabinose	-	0,06	0,13	0,19
	Xylose	-	0,17	0,30	0,11
	L-Fucose	-	0,09	0,13	0,43
	D-Fucose	-	0,05	0,17	0,32
	D-Ribose	-	0,07	0,17	0,20
Disaccharides	Celobiose	0,17	0,04	0,21	0,21
	Lactose	0,19	0,02	0,21	0,21
	Maltose	-	0,03	0,21	0,21
	Melibiose	0,24	0,04	0,21	0,21
	Sucrose	-	0,06	0,21	0,21
	L-α-Trehalose	0,18	0,05	0,21	0,21
	D-Turanose	-	0,02	0,21	0,21
Trissacharides	Maltotriose	-	0,06	0,25	0,25
	Rafinose	-	0,06	0,24	0,24
	Melizitose	-	0,06	0,38	0,38
Complex compounds	Arabinogalactan	-	0,06	0,25	0,25
	Cellulose	0,04	0,06	0,25	0,25
	Xylan	-	0,06	0,25	0,25
	L-Arabinitol	-	0,06	0,25	0,25
	Galactitol	0,05	0,08	0,25	0,25
	D-Manitol	-	0,03	0,31	0,31
	D-Sorbitol	-	0,02	0,59	0,59
	xilitol	-	0,04	0,20	0,20