Vasco José Costa de Lima

Licenciado em Ciências da Nutrição

Valorisation of phenolic compounds
from grape pomace

Dissertação para obtenção do Grau de Mestre em Tecnologia
e Segurança Alimentar

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ABSTRACT

Every year millions of tonnes of waste are generated by the food industry. Producing wine is major cultural and economical activity but is also responsible for a large amount of waste in a short period of time. Grape pomace is the main by-product, a cheap material known for being rich in phenolic and other valuable compounds. This work aimed at optimizing the conditions (duration, temperature, solid:liquid ratio and concentration of the enzyme) for sequential extractions (water and acetone-based) process from red and white grape pomace (*Vitis vinifera* cultivars Merlot and Garganega, respectively) with the addition of five enzymes (Pectinex Ultra-SPL, Pentopan Mono BG, Celluclast, Driselase and Viscozyme). Several classes of extracted compounds (total phenolic content, protein, reducing sugars, tannins, flavonoids, anthocyanins and flavanols) were then quantified by spectrophotometric assays and specific phenols identified and quantified through a chromatographic technique.

The optimum determined conditions for the extraction apllied to the assays were 2 hours, 50 ⁰C, 1:10 solid:liquid ratio and 2% enzyme concentration.

Results obtained reveal that doing sequential extractions allowed for the recovery of more content for the extracts. It also showed some differences between both grape pomaces. Also, using enzymes was beneficial for achieving higher extracted phenolic content.

The findings show that grape pomace is a good source for phenolic compounds that can be exploited as ingredients for application on the food, pharmaceutical or cosmetic industries.

KEY WORDS: Grape pomace; phenolic compounds; extraction; enzymatic digestion; organic solvents; RP-HPLC-DAD

x

RESUMO

Anualmente milhões de toneladas de resíduos são gerados pela indústria alimentar. A produção de vinho é um atividade com grande importância cultural e económica, mas também é responsável por uma grande quantidade de resíduos que são produzidos num curto período de tempo. O bagaço de uva é o principal subproduto, um material barato conhecido pela sua riqueza em compostos fenólicos e outras substâncias valiosas.

Este trabalho pretendeu otimizar as condições de extração (duração, temperatura, razão sólido:líquido, concentração da enzima) para um processo de extrações sequenciais (com água e depois com acetona) a partir de bagaço de uvas tintas e brancas (*Vitis vinífera* cultivares Merlot e Garganega, respetivamente) com adição de enzimas (Pectinex Ultra-SPL, Pentopan Mono BG, Celluclast, Driselase e Viscozyme). Várias classes de compostos extraídos (compostos fenólicos totais, proteínas, açúcares redutores, taninos, flavonoides, antocianinas e flavanóis) foram quantificadas com ensaios espectrofotométricos e compostos fenólicos específicos foram identificados e quantificados recorrendo a uma técnica cromatográfica.

As condições ótimas determinadas para extração e que foram aplicadas aos ensaios foram 2 horas, 50 ºC, razão sólido:líquido 1:10 e 2% de concentração de enzima.

Os resultados obtidos revelam que fazer extrações sequenciais permite recuperar mais compostos fenólicos para os extractos. Também mostraram algumas diferenças entre os dois bagaços de uva. O uso de enzimas foi benéfico para atingir maiores recuperações destas substâncias.

Estas descobertas demonstram que o bagaço de uva é uma boa fonte de compostos fenólicos que podem ser explorados como ingredientes com aplicação para a indústria alimentar, farmacêutica ou cosmética.

PALAVRAS-CHAVE: Bagaço de uva; compostos fenólicos; extração; digestão enzimática; solventes orgánicos; RP-HPLC-DAD

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ABBREVIATIONS, SYMBOLS AND ACRONYMS

ABS	ABSORBANCE
AE	ACETONE EXTRACTION
ANOVA	ANALYSIS OF VARIANCE
BOD	BIOCHEMICAL OXYGEN DEMAND
BSA	BOVINE SERUM ALBUMINE
BSA EQ	BOVINE SERUM ALBUMINE EQUIVALENTS
CAT	CATECHIN
CAT EQ	CATECHIN EQUIVALENTS
COD	CHEMICAL OXYGEN DEMAND
C-PIC	CIS-PICEID
C-RESV	CIS-RESVERATROL
EC	EPICATECHIN
EGC	EPIGALLOCATECHIN
GA	GALLIC ACID
GAE	GALLIC ACID EQUIVALENTS
GLU	GLUCOSE
GLU EQ	GLUCOSE EQUIVALENTS
HPLC	HIGH PERFORMANCE LIQUID CHROMATOGRAPHY
HPLC-DAD	HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH DIODE-ARRAY DETECTION
HSD	HONESTLY SIGNIFICANT DIFFERENCE
PHA	POLYHYDROXYALKANOATE
PHB	POLY-3-HYDROXYBUTYRATE
PROT	PROTOCATECHUIC ACID
QUERC	QUERCETIN
RP-HPLC	REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY
RUT	RUTIN
SPE	SOLID PHASE EXTRACTION
SPSS	STATISTICAL PACKAGE FOR THE SOCIAL SCIENCES
SYR.A	SYRINGIC ACID
TF	TOURIGA FRANCA
TN	TOURIGA NACIONAL
TPC	TOTAL PHENOLIC CONTENT
TR	TINTA RORIZ
USA	UNITED STATES OF AMERICA
UV-VIS	ULTRAVIOLET-VISIBLE
V/V	VOLUME/VOLUME

Van.A	Vanillic acid
w/v	weight/volume
WE	Water extraction
w/w	weight/weight

1 INTRODUCTION

1.1 THE IMPORTANCE OF FOOD WASTE MANAGEMENT

To suppress the world needs, the food industry is always working to produce food and drinks and this leads to large amounts of waste being constantly generated, which in addition to represent a great loss of valuable materials, also raises serious economic and environmental management problems (Mateo and Maicas, 2015), regarding the storage, transformation, and/or deposition of the wastes (González-Centeno et al., 2013). Many of these residues, however, have the potential to be reused into other production systems (Mateo and Maicas, 2015), because they contain a great variety of valuable compounds and biological active species (Torres et al., 2002). They are considered affordable sources of valuable components since current technologies allow the extraction of target compounds and their introduction as functional additives (Galanakis, 2012) and the recovery of bioactive food constituents, which could be used in other industries, such as pharmaceutical or food industry, is a high value option (de Torres et al., 2015).

Almost 90 million tonnes of food waste are expelled from the food manufacturing industry every year. This nutrient and water rich waste putrefies on accumulation, providing breeding grounds for microbiological spoilage (Ravindran and Jaiswal, 2016).

Food wastes are composed of complex ingredients, which have been released from the original material. Fruits and vegetables processing wastes are widely investigated substrates due to the possibility of extracting several types of antioxidants and dietary fibres (Galanakis, 2012).

1.1.1 RESIDUES GENERATED

.Agricultural by-product stream is an abundant and promising feedstock for industrial production of energy and materials since it pursues two major goals: environment protection and economic profit (Ping et al., 2011).

Food industries produce large amount of vegetable and fruit waste, which affects municipal landfills because of its high biodegradability, leachate and methane emissions (Mirabella et al., 2014).

Tomato pomace (4 000 000 tonnes/year in Europe), apple pomace (3 000 000-4 200 000 tonnes/year worldwide) and olive pomace (2 881 500 tonnes/year worldwide) are some of the biggest waste estimate examples, but the list of agro residues is wide. Wheat bran and rice bran, rice and sesame husk, wheat straw, brewer's spent grain, oat mill waste, sugarcane bagasse, waste vegetable oil, potato and orange peel, grape pomace, chicken and slaughterhouse by-products, fish leftovers, shrimp and crab shells and cheese whey are being generated, reused and studied for alternative valorisation (Ravindran and Jaiswal, 2016).

The wastes from fruit and vegetables processing generally contain large amounts of suspended solids, and present high biochemical (BOD) and chemical oxygen demand (COD), which influence possible recovery solutions and treatment costs. According to the United Nations

Industrial Development Organization, BOD range from 3.2 g/l for bakery products to 0.53 g/l for meat specialties, while COD range from 7 g/l to 0.9 g/l. Waste organic composition includes about 75% sugars and hemicellulose, 9% cellulose and 5% lignin. Wastes mainly consist of carbohydrates and relatively small amounts of proteins and fat, with moisture content of 80-90%. Finally, the wastewaters contain dissolved compounds, pesticides, herbicides and cleaning chemicals (Mirabella et al., 2014).

Different types of high-added-value components have been recovered from agro-food by-products, such as antioxidant components, carbohydrates, sugars, pectins, proteins and phenolic compounds (Castro-Muñoz et al., 2016). Besides these recovery examples, there are several applications for industrial waste that are being studied: the production of biofuels (like bioethanol); industrial enzymes (oxidative enzymes such as cellulase, laccase, amylase, xylanase, phytase and lipase); bioactive/nutraceutical substances; nanoparticles (silica extracted from rice husk or xylan obtained from wheat bran were tested in different processes); biodegradable plastics (food waste and agriculture residue have been used as substrate for the production of polyhydroxyalkanoates (PHAs) and poly-3-hydroxybutyrate (PHBs), which are replacements for petroleum-derived plastics; chitosan (can be produced from shrimp shells); or collagen (using fish waste as raw material) (Ravindran and Jaiswal, 2016).

1.2 GRAPE POMACE

Winemaking is a seasonal activity that requires considerable amounts of resources like water, fertilizers and organic amendments (Mateo and Maicas, 2015) and produces large quantities of waste (over 16 million tons in 2010) (González-Centeno et al., 2013) during a short period every year causing an accumulation of waste that represents a serious environmental problem (Jara-Palacios et al., 2014b), being important to minimize its impact (Zhang et al., 2017).

Considering that winery by-products account for more than 30% of the grapes used for winemaking (González-Centeno et al., 2013), there is a large amount of wastewater and organic wastes being generated (Mateo and Maicas, 2015). The biggest by-product is grape pomace, which consists mainly of pressed skins, seeds and stems (Ferri et al., 2016) and it is estimated that for each 6 L of wine, 1 kg of grape pomace is generated, which is usually destined to animal feed or composting (Tournour et al., 2015). Other waste products are grape leaves, wine lees, wastewater, among others (Mateo and Maicas, 2015). Concerning wastewater production, every litre of wine produces 7 litres of winery wastewater, hence, wastewater recycling represents a sustainable operation to lower the environmental impact of winemaking. Its volume and composition are dependent on the time of the year, the size of the winery and the type of wine produced and contains water and cleaning chemicals (like sodium hydroxide (NaOH) or potassium hydroxye (KOH)) (Hirzel et al., 2017).

Grape pomace originates from both white wine production, where the juice is separated from the pomace prior to fermentation, and red wine production, where the pomace is separated after fermentation (Achmon et al., 2016). Once the juice has been extracted, the skin, stalks and seeds

are all redundant and if not treated effectively, can constitute several environmental hazards. Stacking grape pomace produces methane gas, attracts flies and pests and releases foul odours and leachates (solutions of tannins with other compounds of the pomace) can cause oxygen depletion, contamination of soil, surface and ground waters (Arvanitoyannis et al., 2006, Iora et al., 2015). Therefore, disposal of grape pomace has long been a problem for wineries (Arvanitoyannis et al., 2006) not only for the amounts considered but also because winery and distillery waste has a low pH (mean range values of 3.8-5.5) and electrical conductivity (1.62– 6.15 Ds/m), high organic matter content (669–920 g/kg), high concentrations of polyphenols (1.2– 19.0 g/kg) and low concentrations of micronutrients and heavy metals contents. These properties are incompatible with agricultural requirements (e.g., the high concentration of polyphenols has an inhibitory effect on plant seed germination (Vergara-Salinas et al., 2013) as certain polyphenols exert phytotoxic and antimicrobial effects (González-Centeno et al., 2014)). Therefore the waste must be pre-treated before use in the fields for example by means of a microbiological approach (Mateo and Maicas, 2015).

Reuse of the grape pomace depends on its composition and characteristics. Because grape pomace is a highly perishable product (due to the high moisture content) and given the high volumes generated during harvest season, the utilization of fresh grape pomace is unfeasible and requires an appropriate method of preservation or appropriate use (de Torres et al., 2015, Goula et al., 2016). Also, processing grape pomace is challenging due to its high bioactivity (fermentability), susceptibility to enzymatic degradation (pectinases), and sensitivity to thermal degradation (Monrad et al., 2014).

Grape pomace can be reused through oil extraction, antioxidant and antibacterial agents preparation (Zhu et al., 2015) and, from a nutritional perspective, polyphenols are the most important constituents (Kammerer et al., 2005). Large amounts of the residual quantities of bioactive substances remain in the vegetable tissues: phenolic acids, several flavonoids, flavanols (e.g. catechin, epicatechin and epigallocatechin) and other phenolic compounds (proanthocyanidins or condensed tannins) (Tournour et al., 2015).

Grape pomace composition, water contents and physicochemical properties may vary, depending on grape variety and the vinification procedures used (Kammerer et al., 2004, Mateo and Maicas, 2015). Those differences are explored in the next sub-chapters.

1.2.1 GRAPE

Grape is one of the crops with largest production, globally, with reports of more than 74.5 million tons produced in 2014 (Food and Agriculture Organization of the United Nations, 2017) or 75.7 million tons in 2015 (Organisation of Vine and Wine, 2016). Grapes and products obtained therefrom, like wine, grape juice, jams and raisins are of economic importance (Fontana et al., 2013) and countries like China, Italy, the United States of America (USA), France and Spain lead the world in grape production (Organisation of Vine and Wine, 2016).

There are about 60 species of grape (Zhu et al., 2015) and the species most commonly cultivated for wine production is *Vitis vinifera* (Devesa-Rey et al., 2011). Its chemical composition is dependent on the variety of the grape and growth environmental factors (Rondeau et al., 2013), which may explain some differences between varieties in polyphenols and essential fatty acids profiles (Ribeiro et al., 2015). Environmental conditions like vine water status have been associated with differences in grape polyphenols as it is clear that it affects fruit growth (Kennedy et al., 2000).

Nutritionally, grapes are rich in water (80,6% for white grapes and 78,9% for red grapes) and sugars (17,3% for white grapes and 18,6% for red grapes) with very small amounts of fat, fibre and protein (Instituto Nacional de Saúde Doutor Ricardo Jorge, 2015). The majority of the dietary fibre accumulate in the skins, seed and pulp, which remain as pomace (Zhu et al., 2015). The content of vitamins and minerals, as in other fruits or vegetables, make grapes very interesting from the nutritional point of view. Grapes are also rich in a large amount of different phenolic compounds distributed in pulp (10%), seeds (60–70%) and skin (28–35%) (Ribeiro et al., 2015), amounting to total phenolic compounds concentrations of ca. 2180, 3745, 234, and 350 mg gallic acid equivalent (GAE)/100g in seed, skin, flesh, and leaf, respectively (Xia et al., 2010). These compounds are the main responsible for colour, taste, mouth feel and oxidation (Ribeiro et al., 2015) and may show different biological and antioxidant properties (Jara-Palacios et al., 2014a). Differences in the phenolic profile can be due to pruning and preparation systems, phytosanitary conditions and maturity of the grapes, as well as soil composition, geographic origin, cultivation practices, exposure to diseases and weather conditions (Kammerer et al., 2014, Xia et al., 2010). Climate is the most important factor for viticulture, especially temperature, and vines prefer moderate conditions. Increased growing temperatures accelerate the metabolic processes and metabolite accumulation, however there is a limit after that metabolic processes are stopped or reduced significantly (Conde et al., 2007). Light is also a necessary factor and it was hypothesized that reduced light decreased anthocyanins and other flavonoids accumulation, while increased light had a positive effect on the flavonoid content of grapes. However, this hypothesis is difficult to prove due to the differences in other factors, like cultivar and site, and also because it's hard to separate the effects of light and temperature (Downey et al., 2006).

When looking at the grape pomace constituents, there are qualitative and quantitative differences in phenolic composition (Jara-Palacios et al., 2014a). Skins are rich in anthocyanins and flavonols (Xia et al., 2010, Rodríguez Montealegre et al., 2006), seeds are rich in procyanidins (Drosou et al., 2015) and flavanols like catechin and epicatechin (Xia et al., 2010, Jara-Palacios et al., 2014a) and those two compounds can represent up to 60% of the phenolics present in the seeds (López-Miranda et al., 2016). Stems are rich in tannins (Beres et al., 2017) and flavanols (Jara-Palacios et al., 2014a).

1.2.2 WINE

Wine has been produced since the dawn of agriculture during the Neolithic period over 8000 years ago and it has become an integral part of culture, society, and religion around the world (Zhang et al., 2017). Wine production is one of the most important agriculture activities throughout the world. According to a report by the International Organisation of Vine and Wine, in 2016, 259 million hl were produced globally, tough this represented a decrease from 2015 and one of lowest production over the last 20 years, and Italy, France, Spain and the U.S.A. were the countries with biggest wine production (Organisation of Vine and Wine, 2016). In 2015, 239 million hl were consumed, mostly in USA, France, Italy, Germany and China (Organisation of Vine and Wine, 2016).

Wine can be defined as the product obtained exclusively from the total or partial alcoholic fermentation of fresh grapes, whether or not crushed, or of grape must (Council of the European Union, 2008), and there are several different ways of making it. From industrial production to craft wine, there are variations on the processes, but, more than that, the kind of wine desired (and the grapes used) influences the stages of winemaking and formulations of the product. The process will also influence the composition and characteristics of the grape pomace. There are some common basic steps for white and red wine production: the grapes are harvested during a specific time of the year and transported to the winery. There, the grapes suffer the separation of the stalks and are crushed, which breaks the skin and allows the juice to flow. From this point on, the process is differentiated: for white wine production, after the pressing and decantation stages, the must is physically separated from the white grape pomace. The must enters a fermentation process, where the sugars are converted into alcohol, carbon dioxide and heat. Filtration and clarification are next, before the wine is bottled (Klapa, 2015, Vorobieva, 2013).

In the case of red wine production, after the crushing of the grapes, the fermentation starts. The difference for the white wine fermentation stage is the presence and contact of the must with the solid parts like seeds, skins and sometimes even stems. The alcohol produced during the fermentation extracts the pigments and other bioactive compounds from the skins so, this contact is important to influence the colour and other attributes of the wine. The wine is then pressed and red grape pomace is generated. It is stored and then, in the case of some red wines, malolactic fermentation occurs, where, the naturally present, malic acid is converted to lactic acid by existing bacteria. The stabilization and filtration stages are next, which precede the bottling of the wine (Klapa, 2015, Vorobieva, 2013).

Residues generated from the vinification consist of plant remains derived from the de-stemmed grapes, the sediments obtained during clarification, bagasse from pressing, and lees, which are obtained after different decanting steps. The wastewater generated from vinification lees contains grape pulp, skins, seeds and dead yeasts used in the alcoholic fermentation (Devesa-Rey et al., 2011).

The differences in the production process result, typically, in the red grape pomace having a higher alcohol content, but lower sugar content than the white grape pomace (Zhang et al., 2017). There are also differences in the phenolic profile with anthocyanins being known as the main

polyphenolic in red grapes and flavanols being more abundant in white grape varieties (Xia et al., 2010).

1.2.3 GRAPE POMACE VALORISATION

Considering that eighty percent of the worldwide grape production is used for winemaking, the volume of residues produced represents serious management issues (Fontana et al., 2013). On Figure 1.1, the main residues of wine production are presented, along with their contribution to the waste generated. It's clear that grape pomace is the main residue. Besides the large amount of phenolic compounds, grape pomace is rich in dietary fibre (carbohydrates that cannot be digested by the bodies' enzymes) and is composed by protein, soluble sugars, lipids and inorganic matter (Table 1.1).

Grape pomace

62% of the waste generated

Wine lees

14%

Grape Stalk

12%

Wastewater sludge

12%

Figure 1.1 - Organic wastes produced in the wine industry. Values for both grape pomaces, adapted from (Ruggieri et al., 2009). image of grape pomace own photograph;other images were taken from www.wineaustralia.com (wine lees), https://www.shutterstock.com (grape stalk) and The University of Adelaide, https://www.adelaide.edu.au/ (wastewater sludge).

Table 1.1 - Composition information for red and white grape pomace. Results are expressed in g/100 g of fresh weight. The values for white grape pomace are the average for the Chardonnay, Macabeu, Parallada and Premsal Blanc cultivars. For the red grape pomace are only shown the values for the cultivar used in this work. Adapted from (González-Centeno et al., 2010).

	Red grape pomace (Merlot cultivar)	White grape pomace
Moisture	53.9	67.1
Dietary fibre	37.4	23.9
Lipids	0.5	0.8
Soluble sugars	2.4	3.2
Protein	3.8	3.1
Ash	2.1	1.9

According to the European Council Regulation (EC) 479/2008 on the common organization of the wine market, grape marc/pomace (solid remains of grape after pressing for juice) and lees must be sent to alcohol distilleries, to produce exhausted grape marc and a liquid waste (vinasse). However, small wine-producers usually do not obey with this law, and generate grape marc and wine lees together with grape stalk as organic waste. Aerobic depuration of the winery effluents, vinasse and winery wastewater, generates another solid waste, known as winery-sludge (Devesa-Rey et al., 2011).

Historically, grape pomace was used to make grape spirit, but a surplus of grape spirit led to a global drop in prices, which meant that the producers were no longer able to recover costs from their waste and had to pay freight to dispose of it. With increased wine production, it became imperative to relieve an oversupply of grape pomace (Arvanitoyannis et al., 2006).

Using vegetable waste as animal feed brings some problems that affect its feasibility, like its high water content (often exceeds 80%) (San Martin et al., 2016), or high levels of phenolic compounds (Kammerer et al., 2004) makes handling more difficult. Also, the analytical composition of such waste can vary significantly throughout the year and, consequently, the animal feed manufacturers must alter their feed formulations depending on the composition (San Martin et al., 2016). The presence of polymeric polyphenols, like lignin, reduces digestibility due to the inhibition of cellulolytic and proteolytic enzymes and the growth of rumen bacteria (Fontana et al., 2013), but other compounds like tannins have been associated as well to the animal intolerance (González-Centeno et al., 2014). Those problems are amplified when considering that the large amounts of waste being generated are stocked in a short period of a few weeks of the year (Kammerer et al., 2004).

Composting is also a possibility to treat winery waste, It's a cheap and convenient method that can produce worthwhile fertilizer, with the benefit of carbon sequestration. However, despite this positive points, there are always the possibilities, mentioned before, of heavy metal accumulation, inhibition of root growth and nitrogen leaching. Composting requires great control of temperature, moisture and aeration, to avoid anaerobic digestion (Zhang et al., 2017).

Besides animal feed or composting, nowadays grape pomace has been used for the recovery of ethanol, organic acids like tartrates, malates and citric acid, but is also a rich source of grape seed oil, hydrocolloids, anthocyanins and dietary fibre (Kammerer et al., 2004, Rondeau et al., 2013). It can also be used for the production of bioethanol, a eco-friendly oxygenated fuel, due to its richness in soluble sugars that can be easily fermented by yeast. This potentially allows for a decreased consumption of fossil fuels (Zabed et al., 2017).

The extraction of bioactive substances from skins and seeds (Kammerer et al., 2014) opens the possibility for the recovery of valuable products like high quality culinary oil from the seeds or the recovery of hydrocolloids and dietary fibres from the skins (Kammerer et al., 2005). The recovered oil is rich in linolenic acid (~12–20%, w/w), protein (11%, w/w) and non digestible carbohydrates (60–70%, w/w), phenolic and non-phenolic antioxidants and can also be used in cosmetic formulations (Naziri et al., 2014).

The recovery of dietary fibre can be channelled through its use in natural texturizers and functional ingredients in food, pharmaceutical, and cosmetic industries (Zhu et al., 2015).

Wine lees are mainly composed by yeast cells (*Saccharomyces cerevisiae*) and tartaric acid and there's evidence they can be used to recover phenolic compounds and β-glucans (Naziri et al., 2014).

The extraction of polyphenols from waste material represents an attractive, sustainable and cost effective source of these high-value biological bioactives, which could be incorporated into foods. Due to the increasing demand for nutraceutical and antioxidant compounds, the study of grape pomace polyphenols exploitation may be useful for industrial purposes (Jara-Palacios et al., 2015). The following chapter presents a literature review of the studies already made addressing this issue.

1.3 POLYPHENOLS EXTRACTION FROM GRAPE POMACE

The extraction procedure is an important step in the recovery, isolation, and identification of bioactive compounds (Fontana et al., 2013) and must be adapted to the targeted compound and to the type of matrix (Puértolas and Barba, 2016) and there is no standard extraction methodology.

When considering grape pomace, it's important to remember that vinification plays a key role on the extraction yields of phenolic compounds and processes like the maceration technique, such as skin maceration vs. thermovinification, fermentation temperature, the application of pectinolytic enzymes, the maceration time, yeast type and pressing parameters are known to have an impact (Kammerer et al., 2014).

However, before getting into the extraction, its methods or goals, it's important to consider the necessity of a pre-treatment because of the large volumes of grape pomace produced and the ease of deterioration. Drying is a commonly used method due to its action inhibiting the growth of microorganisms and delaying chemical reactions. However, the temperatures used should be

lower than 60 ºC because phenolic compounds are heat sensitive. Hot-air or solar drying are preferred methods because of the low investment and operating cost (Drosou et al., 2015). Contrarily, freeze-drying is considered a gentle drying technique because the thermal degradation is minimized (Barcia et al., 2014). But there are other reasons for having pre-treatments: to improve the amount of the recovered compound and/or to reduce extraction time. To facilitate the extraction, processes such as reducing the particle size by mechanical procedures or enzymatic maceration are used (Puértolas and Barba, 2016).

When optimising the methods of extraction it's important to consider what the ultimate goals are: maximizing yields, suiting the demands of industrial processing, clarifying the high added-value ingredients from impurities and toxic compounds, avoiding deterioration and loss of functionality during processing and ensuring the food grade nature of the final product (Galanakis, 2012), which is essential for the food or cosmetic indrustries, but not for bioplastics applications. In addition, the technological and economical feasibility must be assured, to make it viable as an alternative valorisation (Puértolas and Barba, 2016).

The enclosment of those compounds on plant cell vacuoles and cell walls and in lipoproteins bilayers complicates their recovery. Thus, the need to achieve higher extraction yields leads to deeper studies on conventional or non-conventional processes (Barba et al., 2015).

In conventional processes, the industrial extraction of polyphenols can be a batch or continuous process combining water with other solvents, using moderate temperatures (50–60 °C) and having rather long duration (3–20 h) (El Darra et al., 2013). This kind of solid-liquid extraction is very common and there are several combinations of solvents used, extraction times and temperatures being reported (Fontana et al., 2013), because these are important parameters to be optimized (Ghafoor et al., 2009). Solid-liquid extraction is characterized by the mass transport where the analyte contained in a solid matrix migrates into a solvent phase that is in contact with the matrix and it is affected by concentration gradients and diffusion coefficients which are influenced by the parameters described before, as well as the method and the solvent used, the particle size and the presence of interfering substances (Fontana et al., 2013).

The particle size of the pomace is an important variable because it has been noted that the lower the particle size the higher is the yield extracted (Sánchez et al., 2009, Spigno et al., 2007) and this is explained by the increment of the superficial area available for mass transfer (Spigno et al., 2007) or enzyme accessibility (Puri et al., 2012). This was shown on a study when crushing pomace prior to the extraction resulted on a >10 fold increase of total phenolics extracted (Meyer et al., 1998).

Methanol, ethanol, acetone, ethyl acetate are some of the organic solvents applied, either alone or in mixtures (López-Miranda et al., 2016). The use of enzymes is also a big resource because through an enzymatic treatment it is possible to enhance the release of the polyphenols trapped inside the cell structures of the pomace (Ferri et al., 2016), which accounts for an increased

extraction yield (Franco et al., 2008) and can be achieved by single enzyme or the combination of different enzymes, aiming at a bigger effect.

This method, however, displays some complications or disadvantages, such as the toxicity of some solvents to human health (like methanol) or the environment, the necessity to use low/moderate temperatures, the difficulty of recovering the compounds without damaging the structure of the source material (which would contaminate the extract) or even the potential loss of compounds due to ionization, hydrolysis and oxidation during extraction (Puértolas and Barba, 2016, Fontana et al., 2013). Other possible disadvantage is that the recovery of compounds is often limited by the mass transfer resistances of both phases (González-Centeno et al., 2014). Those situations might translate in longer extraction times or low final yields (Puértolas and Barba, 2016).

To be more environmental friendly would mean to reduce solvent consumption, extraction times and to increase the yields and the quality of the extracts (Ghafoor et al., 2009) but other methods and technologies have been studied and developed to try to achieve those goals and they are known as non-conventional processes. Examples are supercritical fluid extraction, ultrasound-assisted extraction, microwave-assisted extraction, accelerated solvent extraction, high voltage electric discharges (Barba et al., 2015, González-Centeno et al., 2014), pulsed ohmic heating (El Darra et al., 2013) or polymeric adsorber resins (López-Miranda et al., 2016).

Of the methods listed before, some may still require the use of organic solvents or the technology and equipment needed are still not ready to scale up to an industrial level, due to price or complexity (López-Miranda et al., 2016). Others, like ultrasounds, pulsed electric fields and high voltage electric discharges, by physically affecting the permeability of cell, can enhance mass transfer processes in an environmental friendly way (Barba et al., 2015).

1.4   OBJECTIVES

When considering all these technologies and the studies already done, the desire to obtain higher extraction yields is a common target of the process. However, reducing or replacing organic extraction solvents without affecting the extraction yield is also a challenge (López-Miranda et al., 2016), due to its hazards to human health and the environment, but also to its costs.

Therefore, the objective of this work was to optimize a combined phenol extraction protocol (use of enzymes and solvents) to apply in white and red grape pomace, aiming for higher extraction yields while considering the hazards of organic solvents to the human health (on food or cosmetic applications) and the environment, but also the costs of the solvents and the enzymes.

To accomplish that, several different classes of extracted compounds were quantified by spectrophotometric assays and specific phenols were identified and quantified by High Performance Liquid Chromatography (HPLC) diode array technique.

2 MATERIALS AND METHODS

2.1 GRAPE POMACE

In this work frozen red grape pomace (at -20 ºC) (Figure 2.1), derived from *Vitis vinifera* L. cultivar Merlot, and frozen white grape pomace (at -20 ºC) (Figure 2.2), from *Vitis vinifera* L. cultivar. Garganega, were ground in a kitchen blender. The pomace was composed by skins, seeds and stalks. The pomaces were delivered in October of 2016 by a wine producer.

Figure 2.1 - Merlot cultivar (after fermentation and pressing)

Figure 2.2 - Garganega cultivar (after pressing)

2.2 CHARACTERIZATION OF THE GRAPE POMACE

2.2.1 DRY WEIGHT

To calculate the dry weight of the red and white grape pomaces, 6 samples of approximately 5 grams for each kind of pomace were weighed and placed 24 hours at 80ºC in an oven. After taking the samples out, and after temperature dropped to room temperature in a desiccator, they were weighed again and their dry weight was determined using Equation 2.1.

$$dry\ weight\ (\%) = \left(\frac{dry\ weight}{fresh\ weight} \right) * 100 \qquad \text{(Equation 2.1)}$$

This determination was made on raw grape pomace prior to any extraction. All the other assays were performed on the supernatants collected after each extraction procedure.

2.2.2 TOTAL PHENOLS QUANTIFICATION

The protocol used was adapted from (Ferri et al., 2013, Singleton et al., 1999). The following solutions were used: gallic acid (GA) stock solution (5 mg/mL): 50 mg of GA dissolved in 1 mL of methanol added to 9 mL of deionized water; GA working solution (50 µg/mL): 50 µl GA stock solution added to 4.950 mL of deionized water; 20% sodium carbonate (Na_2CO_3) in water: 2 g of Na_2CO_3 in 10 mL of deionized water.

After putting the appropriate volume of sample or standard in a 2 mL Eppendorf tube, deionized water was added until it reached 1.6 mL of volume. 100 μL of Folin-Ciocalteu reagent was added, the tube was stirred and then, a 5 min incubation period at room temperature and in the dark, was followed. Then, 300 μL of the 20% Na_2CO_3 solution was added and the tube was stirred again. After a 30 min incubation period at 40ºC in the dark, the absorbance was read at 765 nm in the spectrophotometer (Jasco V-530 UV/VIS). Protein was not removed before adding Folin reagent, although this reagent is also useful to quantify protein. Therefore, in this determination, protein may interfere with the results especially if some aminoacids with aromatic side chains are present. The value provided by the spectrophotometer (in μg of GA equivalents per 2 mL) was then divided by the volume of sample used (μL) to obtain the concentration (c) expressed in μg of GA equivalents/μL of sample or mg/mL. The following equation (Equation 2.2) was used to determine the concentration (C) of GA equivalents per g of pomace:

$$C \left({}^{mg\ GAequivalents}/_g \right) = (c \times dilution\ factor)$$ (Equation 2.2)

The conversion from mg GA equivalents/ g of pomace to mg GA equivalents/ g of dry pomace was made by dividing C by the value for dry weight: 0.3775 (for red grape pomace) or 0.318 (for white grape pomace).

For the construction of the calibration curve the following μg of GA were used: 0 μg, 1 μg, 2 μg, 3 μg, 5 μg and 10 μg and it was followed the same procedure as for the samples.

This protocol was used for the optimization of the extraction conditions and for the quantification after extraction.

When characterizing the extracted content: for each enzyme or control there were two biological replicates and for each one, two technical replicates were made (the two 1.8 mL aliquots of supernatant described in 2.3.1). The same applies to the other protocols from 2.2.3 to 2.2.8.

2.2.3 PROTEIN QUANTIFICATION

Adapted from (Lowry et al., 1951), this protocol used the next reagents: Solution A: 2% Na_2CO_3 in 0.1 N NaOH; Solution B: 0.5% $CuSO_4$ (copper sulfate) in 0.1% potassium sodium tartrate (NaK); Solution C: 50 mL of solution A + 1 mL of solution B; 1N NaOH; 50% Folin-Ciocalteu reagent (dilution in deionized water); Bovine Serum Albumine (BSA) standard solution (200 μg/100 μL in deionized water).

The volume of sample or standard was put in a glass test tube (maximum of 800 μl) and 200 μL of 1N NaOH was added and deionized water was inserted until 1 mL of total volume was reached. Then, 5 mL of freshly prepared solution C was added and the glass tubes were stirred and incubated 10 min at room temperature. 500 μL of 50% Folin-Ciocalteu reagent was added. After 30 minutes incubation at room temperature, the absorbance could be read at 750 nm in the spectrophotometer.

For the construction of the calibration curve the following µg of BSA were used: 0 µg, 10 µg, 20 µg, 40 µg, 80 µg, 100 µg, 150 µg and 200 µg.

Results were obtained following the same rationale described in 2.2.2 and expressed in mg BSA equivalents per gram of dry pomace (mg BSA eq/g dry pomace)

This assay and the following assays in this sub chapter were only used for the characterization of the extracted products (supernatants).

2.2.4 REDUCING SUGARS QUANTIFICATION

The protocol used was adapted from (Bailey et al., 1992). It used: D(+)-glucose (0.8 mg/mL) in deionized water; and 3,5-Dinitrosalicylic acid (DNS) reagent: 75 g of NaK were dissolved in 50 mL of 2M NaOH and 100-150 mL were reached with deionized water; 0.25g of DNS are dissolved and the final volume of 250 mL is completed with deionized water.

The volume of sample or standard is placed in a 1.5 mL Eppendorf tube and the volume of 400 µL was completed with deionized water. Then 600 µL of DNS reagent was added and the tubes were stirred and spent 7 minutes incubating at 100 ºC. After cooling down, the samples were read at 550 nm in the spectrophotometer.

To create the calibration curve the following concentrations were used: 0 mg of glucose (GLU)/mL of water, 0.2 mg/mL, 0.4 mg/mL, 0.5 mg/mL, 0.6 mg/mL and 0.8 mg/mL.

The calculations were made in the way described at 2.2.2 and the final results will be expressed in mg of glucose equivalents per g of dry pomace (mg GLU eq/g of dry pomace)

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2.2.5 TANNINS QUANTIFICATION

The quantification was done through an adapted protocol from (Porter et al., 1986). The reagent used was 15 mg of iron trichloride ($FeCl_3$) dissolved per 100 mL of a 50% of 1-butanol, 50% 12N (hydrochloric acid) HCl solution.

For each sample two 2 mL Eppendorf tubes with 0.3 mL of sample and 0.9 mL of the reagent solution were used. The first tube was incubated in boiling water for 30 minutes, while the second tube was incubated at room temperature, in the dark, for 30 minutes. The absorbance (Abs) for the second tube could be read immediately at 550 nm, using deionized water as blank, in the spectrophotometer while the boiled tube must be cool before reading the absorbance.

If necessary the samples were diluted with deionized water.

To calculate the concentration of tannins, Equation 2.3 was applied. The conversion to mg tannins per g of dry pomace was made like in 2.2.2.

$$Tannins\left(\frac{g}{L}\right) = (first\ tube\ Abs - second\ tube\ Abs) * 0.1736 * dilution\ factor \qquad \text{(Equation 2.3)}$$

2.2.6 Flavonoids quantification

This quantification followed a protocol adapted from (Zhishen et al., 1999, Ferri et al., 2013). The next solutions were used: 5% (w/v) sodium nitrite ($NaNO_2$) in deionized water; 10% (w/v) aluminium trichloride ($AlCl_3$) in deionized water; 1M NaOH; catechin stock solution (50 mg/mL), in methanol; catechin working solution (100 µg/mL).

After placing the sample or standard in a 1.5 mL Eppendorf tube, 400 µL of deionized water and 30 µL of the 5% (w/v) $NaNO_2$ solution were added and the tube was stirred and incubated at room temperature for 5 minutes. 30 µL of the 10% (w/v) $AlCl_3$ was inserted in the tube. The tube was stirred and a 6 minute incubation period followed before the addition of 200 µL of 1M NaOH. Deionized water completed the volume until 1 mL and the absorbance could be read at 510 nm in the spectrophotometer, using deionized water as blank.

To create the calibration curve the following concentrations were used: 0 mg of catechin/mL of water, 2 mg/mL, 4 mg/mL, 6 mg/mL, 8 mg/mL, 10 mg/mL, 12 mg/mL and 14 mg/mL.

Results for flavonoids were obtained by following the rationale in 2.2.2 and will be expressed in mg of catechin equivalents per gram of dry pomace (mg CAT eq/g of dry pomace).

2.2.7 Anthocyanins quantification

This protocol was adapted from (Serafini-Fracassini et al., 2002). For each sample the absorbance was read in the spectrophotometer at 530 nm and 657 nm and Equation 2.4 was used to calculate the ΔAbs for each sample. The result was then expressed per g dry weight using the rationale described in 2.2.2.

$$\Delta\,Abs = Abs\,(530\,\text{nm}) - (0.25 * Abs\,(657\,\text{nm}))$$ (Equation 2.4)

2.2.8 Flavanols quantification

The protocol applied was adapted from (McMurrough and McDowell, 1978) and uses the following reagents: 0.1% 4-(Dimethylamino)cinnamaldehyde (DMAC) in a 75% methanol, 25% 12N HCl solution; catechin stock solution (50 g/L), in methanol; and catechin working solution (0.5 g/L), diluted in methanol from the stock solution.

The volume of standard or samples was placed in 1.5 mL Eppendorf tubes and deionized water was used to complete 100 µL and then 500 µL of 0.1% DMAC solution was added and a 2 minute incubation period followed before the addition of 500 µL of deionized water. The absorbance was read at 640 nm in the spectrophotometer, using deionized water as blank.

For the construction of the calibration curve the following µL of the catechin working solution were used: 2 µL, 4 µL, 10 µL, 20 µL, 40 µL, 60 µL, 80 µL and 100 µL. A logarithmic curve was used and Equation 2.5 was applied to calculate the concentration (x, g/L).

$$y\,(abs\,640\,nm) = a * \ln(x) + b \hspace{4cm} \text{(Equation 2.5)}$$

The rest of the calculations were made like it's explained for the total phenolic compounds assay (2.2.2) The results will be expressed in mg of catechin equivalents per gram of dry pomace (mg CAT eq/g of dry pomace).

2.3 OPTIMIZATION OF THE EXTRACTION PROCESS

Several conditions for the solid:liquid extraction of phenolic compounds were tested in order to optimize the process.

2.3.1 TESTING DIFFERENT SOLID:LIQUID RATIOS AND TEMPERATURES

To discover the optimal extraction conditions, two different solid: liquid ratios (1:5 and 1:10) (wet matter) and three different temperatures (30 ºC, 50 ºC and 70 ºC) were tested. The ratios in a dry basis would be represented as 1:13.3 and 1:26.5 for red grape pomace and 1:15.7 and 1:31.5 for white grape pomace. For the 1:5 ratio 5 g of wet pomace and 25 mL of deionized water were used and for the 1:10 it was 3 g + 30 mL of deionized water. Each sample + water was placed inside of a plastic tube and its pH was measured in a Beckman 340 pH/Temp meter.

Two samples of red grape pomace and two samples of white grape pomace were subjected to each ratio/temperature combination. After 2 hours of incubation, at each temperature tested, with agitation (150 rpm), the tubes were taken out and allowed to cool down before the pH was measured again.

The samples were then centrifuged for 5 minutes at 5000 rpm and at 20 ºC (Eppendorf centrifuge 5804) and the supernatant was extracted and measured. Two 1,8 mL supernatant aliquots of each tube were then placed in 2 mL Eppendorf tubes and frozen at -20 ºC for future assays. The solid content and liquid obtained after the centrifugation were stored at -20 ºC.

The different extraction conditions were compared by determining the total polyphenol content (Folin-Ciocalteu assay) present in each Eppendorf tube.

2.3.2 TESTING DIFFERENT INCUBATION TIMES AND ENZYME CONCENTRATIONS

After selecting the best conditions for extraction (1:10 solid:liquid ratio and 50 ºC) the next step was to introduce the enzymatic digestion in order to evaluate its effect on the extraction process. The five enzymes tested were Pentopan Mono BG (xylanase from *Aspergillus oryza*, 2500 U/mL), Pectinex Ultra SPL (pectinase from *Aspergillus aculeatus*, 3800 U/mL), Celluclast (cellulase from *Trichoderma reesei*, 700 U/g), Driselase (from *Basidiomycetes* sp., protein ≥10 % by biuret) and Viscozyme (cellulolytic enzyme mixture from *Aspergillus* sp.), all from Sigma-Aldrich, Milan, Italy, and they were added to the tubes after the first pH determination. Two different concentrations were used: 1% (w/w) and 2% (w/w) of the dry weight of the grape pomace present in each tube. Equation 2.6 was used to calculate the amount of enzyme necessary, in grams, to use. The percentages of dry weight determined in the beginning were used for the calculations. As some

of the enzymes (Pectinex, Celluclast and Viscozyme) were in an aqueous solution their density was used in Equation 2.7.

$$Enzyme\ added\ (g) = enzime\ concentration\ (\%) \frac{(weight\ of\ the\ pomace\ (g)*dry\ weight\ (\%))}{100} /100$$

(Equation 2.6)

$$Enzyme\ added\ (mL) = \frac{enzyme\ added\ (g)}{density\ (g/mL)}$$

(Equation 2.7)

The optimal temperature and pH working conditions for the enzymes were also considered (Table 2.1) and this factor also had influence in the choice of the temperature (2.3.1). When measuring the pH for the tubes with the pomace and 30 mL of deionized water, the red grape pomace showed lower pH values, below the optimal range for pH for the enzymes so it was necessary to adjust the pH values with NaOH (with a low concentration, e.g.0.01M) for the red grape pomace tubes to 4.5 prior to add the enzymes.

Table 2.1 - Data on the pH and temperature conditions for the enzyme preparations used

Enzyme	pH		Temperature (ºC)		Reference
	stable at	optimum	active	optimum	
Pectinex Ultra SPL	4-9	4.5	15-50	50	(Novozymes, 2015c, National Centre for Biotecnology Education, 2016c, Novozymes, 2015a)
Celluclast	4-9	4.5-6		50-60	(Novozymes, 2015b, National Centre for Biotecnology Education, 2016b)
Pentopan Mono BG	4-6		up to 75		(Joint FAO/WHO Expert Committee on Food Additives, 2003) (data for xylanases)
Driselase	4-9	4.5-6		50-60	(National Centre for Biotecnology Education, 2016b, Novozymes, 2015b) (driselase is used as crude powder containing laminarinase, xylanase and cellulose - as cellulase activity is superior data from cellulase of Celluclast was used)
Viscozyme		3.3-5.5		25-55	(National Centre for Biotecnology Education, 2016a)

Along with controls for the red and white grape pomaces and using 50 ºC as the temperature, 2 hours and 4 hours incubation were performed. After this period, the tubes were taken out and boiled for 10 minutes to stop the enzymatic digestion. Then, after a cool down period, pH was measured again, the tubes were centrifuged at 5000 rpm and 20ºC for 5 minutes, the supernatant

was extracted and the aliquots from the supernatant (1.8 mL) were prepared and frozen, along with the solid remains and the remaining liquid.

The total phenolic content assay was performed to compare the different extraction combinations applied.

2.3.3 TESTING THE USE OF SEQUENTIAL EXTRACTION WITH ACETONE

The final step to optimize the extraction process was the introduction of the solvent acetone in a sequential batch. Based on the total polyphenol content extracted data provided by unpublished past works of the project where this work was placed in, 15 mL of a 75% (v/v) acetone/water solution was selected. Doing both extractions sequentially allows for testing the enzymes, but also to test if a solvent is able to recover more compounds that might had been still been on the sample.

This acetone solution was added to the solid content obtained after the first extraction (enzymatic digestion in aqueous solution), and a second extraction was performed incubating for 2 hours at 50 °C.

The tubes were centrifuged for 5 minutes at 5000 rpm and 20 °C and the liquid was extracted and measured and aliquots were prepared and stored at -20 °C for future assays.

2.4 HPLC ANALYSIS

The final step of the work was the qualitative analysis of the phenolic compounds extracted from the pomaces on the prior steps. To achieve this, a chromatography process was used.

With a Reverse-Phase High Performance Liquid Chromatography (RP HPLC) equipped with an on-line diode array detector (HPLC-DAD), two samples for each enzymatic treatment (and controls), for each extraction (water/enzymes or acetone) and grape pomace were tested.

The process started with a 1 mL aliquot for each digestion being used to extract its phenols content. The water extracts could be used directly, while the samples extracted with the acetone solution needed first to pass through a centrifugal evaporator (Savant Speed Vac PD1) at 45 °C to evaporate the solvent. These samples were then diluted in 5 mL of deionized water.

Then, using a Millipore apparatus, the phenols content was extracted by SPE (Solid Phase Extraction) technique, by passing the samples through a Strata-X column (Phenomenex srl) with polymeric reversed phase resin that trap the phenolic compounds and using 100 % (v/v) methanol to elute them after to 2 mL Eppendorf tubes. A pump is connected to the apparatus. The process starts with column equilibration, by passing 2 mL of methanol through the column, followed by 2 mL of HPLC grade water. After this, all the sample volume is added and the resin was washed with another 2 mL of HPLC grade water and then it was dried. When this is done, 1,8 mL of methanol is added (until the resin and column are dry) and a 2 mL Eppendorf tube is placed inside the apparatus to collect the phenolic compounds eluted in the methanol. Finally, the tube is removed and the resin was washed with 2 mL of a 70:30 acetonitrile:methanol solution.

The tubes were then placed in the centrifugal evaporator at 45 ⁰C until the methanol was completely evaporated.

The tube content was resuspended using 20 µL of acetonitrile (ACN) and 180 µL of a 0.2 % (v/v) acetic acid solution and 20 µL and was injected into the RP-HPLC equipment (Jasco, Großumstad, Germany; detector MD-2010, Plus, Jasco Instruments, Großumstad, Germany; column Gemini® 5 µm C18 110 Å, LC Column 150 x 4.6 mm, Ea, Phenomenex; precolumn SecurityGuard Ea, Phenomenex). The solvent gradient used was as follows: 0 min ACN/0.2% v/v acetic acid pH 3.0 (9:91 v/v); 3 min ACN/0.2% acetic acid (9:91 v/v); 8 min ACN/0.2% acetic acid (14:86 v/v); 10 min ACN/0.2% acetic acid (16:84 v/v); 13 min ACN/0.2% acetic acid (20:80 v/v); 17 min ACN/0.2% acetic acid (37:63 v/v); 24 min ACN/0.2% acetic acid (37:63 v/v); 27 min ACN/0.2% acetic acid (100:0 v/v); 29 min ACN/0.2% acetic acid (100:0 v/v); 33 min ACN/ 0.2% acetic acid (9:91 v/v); 37 min ACN/0.2% acetic acid (9:91 v/v) (Ferri et al., 2009).

The chromatograms were analysed at five different wavelengths: 270 nm to determine gallic acid (GA), protocatechuic acid, epigallocatechin (EGC), catechin, vanillic acid, syringic acid, epicatechin (EC), epigallocatechin gallate, vanillin, epicatechin gallate and *trans*-cinnamic acid; 285 nm to determine *cis*-piceid, *cis*-resveratrol, *cis*-resveratroloside, and naringenin; 305 nm for *trans*-piceid, *p*-coumaric acid, *trans*-resveratroloside and *trans*-resveratrol; 323 nm for chlorogenic, caffeic, sinapic, ferulic and piceatannol acids; and 365 nm to quantify rutin, myricetin, quercetin and kaempferol. The retention times used for identification are presented on Table 2.2.

Table 2.2 – Retention times used for identification of selected compounds

Wavelength	Compound	Retention time (min)	Wavelength	Compound	Retention time (min)
270 nm	Gallic acid	5.0	**305 nm**	trans-Piceid	20.6
	Protocatechuic acid	8.2		p-Coumaric acid	21.3
	Epigallocatechin	11.0		trans-Resveratroloside	22.2
	Catechin	13.4		trans-Resveratrol	26.6-26.8
	Vanillic acid	15.3	**323 nm**	Chlorogenic acid	14.9
	Syringic acid	16.2		Caffeic acid	15.6
	Epicatechin	16.9		Sinapic acid/Ferulic acid	22.7
	Epigallocatechin gallate	18.3-18.8		Piceatannol	23.7
	Vanillin	19.9	**365 nm**	Rutin	22.2
	Epicatechin gallate	22.6		Myricetin	25.5
	trans-Cinnamic acid	29.6-30		Quercetin	28.4
285 nm	cis-Piceid	23.5		Kaempferol	33.7
	cis-Resveratrol	24.2			
	cis-Resveratroloside	28.8			
	Naringenin	32			

Peaks were identified by comparison with known standards. To identify the compounds, the known spectra and retention times were considered when looking at the peaks and spectra presented by the analysis. After the identification and using the area of the peak in relation to the area and concentration of the standards it was possible to quantify the presence of such compounds on the samples through a series of calculations involving the molecular weight of the sample, the dilution used, the amount of grape pomace used and the initial extraction volume (of water or of 75% (v/v) acetone). Data were finally expressed as mg of compound/g of dry pomace.

2.5 STATISTICAL ANALYSIS

For the construction of the calibration curves, a coefficient of determination of at least 0.975 was required. Averages and standard deviations presented were done using Microsoft Excel 2013 (Windows) and for the analysis of variance (ANOVA) and Tukey HSD tests were performed with a significance level <0.05 using IBM SPSS Statistics for Windows version 24.0.

3 RESULTS AND DISCUSSION

3.1 DETERMINATION OF THE DRY WEIGHT OF THE GRAPE POMACE

The determination of the dry weight for red and white grape pomace was the first analysis done and the values presented on Table 3.1 were necessary for other procedures, like the amount of enzyme used later on the work. The moisture values are also presented on Table 3.1 and the red grape pomace had a higher dry weight (37.75%) than the white grape pomace (31.80%) and therefore a lower moisture percentage which is in line with the fact that white grapes having slightly more water (Instituto Nacional de Saúde Doutor Ricardo Jorge, 2015). On table 3.2 there is a summary of moisture values found on other works focusing other cultivars.

The moisture content determined in this work is well within the range of the other works and the values presented on Table 3.2 show a big range for moisture percentages (especially for red grape pomace) that can be explained by several reasons such as cultivar and used vinification procedures. The higher moisture content for the white grape pomace it's in line with the other works.

All the cultivars present high moisture content, making the pomace highly perishable and point out the need for adequate treatment (de Torres et al., 2015).

Table 3.1 - Averages and standard deviations for the dry weight and moisture contents of the grape pomaces

Grape pomace	Red	White
Dry weight (%)	37.8 ± 0.7	31.8 ± 1.1
Moisture (%)	62.3 ± 0.7	68.2 ± 1.1

Table 3.2 - Moisture results for grape pomaces from different *Vitis vinifera* cultivars

Red grape pomace			White grape pomace		
cultivar	Moisture (%)	Reference	cultivar	Moisture (%)	Reference
Merlot	62.3	*present work*	Garganega	68.2	*present work*
Merlot	53.9	(González-Centeno et al., 2010)	Chardonnay	63.9	(González-Centeno et al., 2010)
Agiorgitiko	59.5	(Drosou et al., 2015)	Macabeu	72.2	
Agiorgitiko	81.7	(Goula et al., 2016)	Parellada	62.8	
Dunkelfelder	57.8	(Barba et al., 2015)	Prensal Blanc	69.3	
Cabernet Sauvignon	61.5	(González-Centeno et al., 2010)			
Callet	55.6				
Manto Negro	63.6				
Tempranillo	55.7				
Syrah	50.2				
Mix (Sangiovenese and Montelpuciano)	54.0	(Ferri et al., 2016)			

3.2 OPTIMIZATION OF THE EXTRACTION PROCESS

3.2.1 TESTING DIFFERENT SOLID:LIQUID RATIO AND TEMPERATURES

The first step to optimize the extraction process tested three different temperatures (30ºC, 50ºC and 70ºC) and two solid:liquid ratios (1:5 and 1:10), incubating for two hours while shaking. The results are shown on Figure 3.1 and the statistical significance information on Table 3.3.

This data shows that there were no differences between the two grape pomaces. It also shows that the temperature and the ratio significantly affected the results, although there was no interaction between them and/or the grape pomace type.

For both pomace the extracted phenols content increases along with the temperature and the highest results for each pomace are at 70ºC and 1:10 ratio. Other studies also showed a higher extraction with increasing temperatures. A study testing extraction times of 5 and 30 minutes and temperatures of 50, 100, 150 and 200 ºC on red grape pomace (Cabernet Sauvignon cultivar) got the highest results for temperatures between 150 and 200 ºC, when using pressurized hot water (Vergara-Salinas et al., 2013). Another work testing conditions for polyphenolics extraction using microwave activation, discovered that applying during 8 minutes, 100 ºC, was the optimum time/temperature (ranging between 60-120 ºC) (Brahim et al., 2014). These studies are examples of the variation of time and temperature. The motive behind an increase of the working temperature is related to the enhancement of the solubility of solute and the diffusion coefficient (Spigno et al., 2007). However, using those high temperatures for longer periods would not be viable, as the integrity of polyphenols may not be achieved. Indeed, some reports done on the effect of drying as a pre-treatment reveal that when temperatures below 70 ºC are employed, the integrity of fruit polyphenols is retained (Goula et al., 2016).

When considering the ratio, its influence is visible since the extracted content was always higher when comparing for the same pomace and temperature used. Extracting more polyphenols with the 1:10 ratio (over the 1:5 ratio) is consistent with mass transfer principles, since it increases the concentration gradient (Pinelo et al., 2005) as it was observed by other authors (Goula et al., 2016, Spigno et al., 2007). Naczk and Shahidi (2004) also observed that changing the ratio from 1:5 to 1:10 led to higher extraction yields of total phenolics and condensed tannins from canola meals.
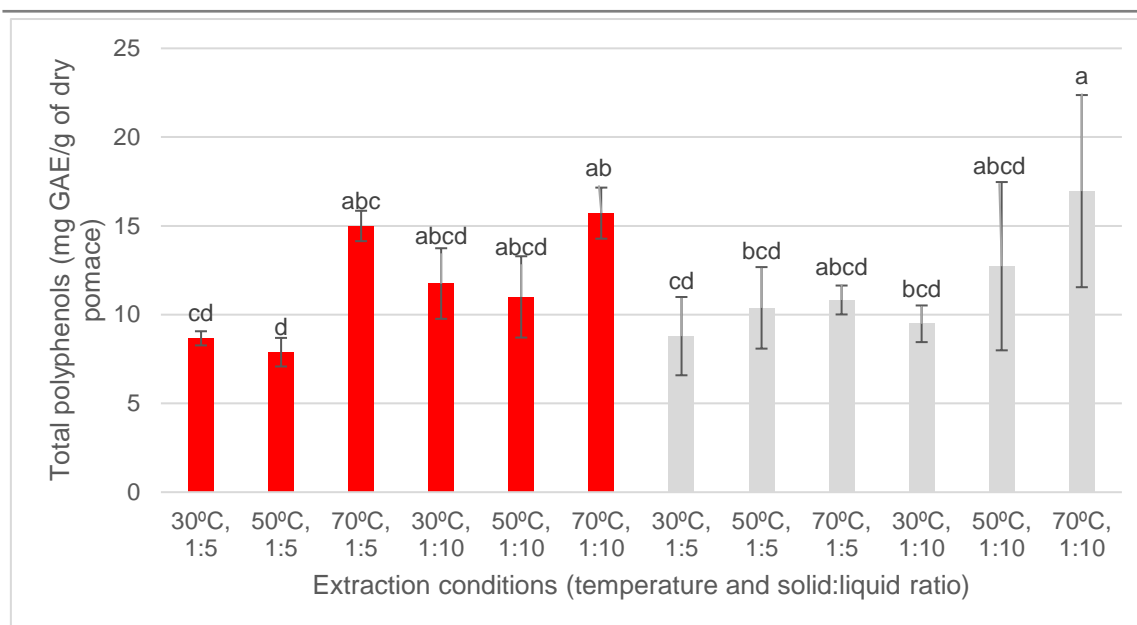
Figure 3.1 – Total phenolic content for red grape pomace and white grape pomace when applying different extraction conditions. Red bars: red grape pomace; Grey bars: white grape pomace. Different letters in each bar indicate statistical differences according to the Tukey test (p<0.05).

Table 3.3 – Statistical significance values for each factor and their interactions obtained through an ANOVA test

Factors	Significance
Grape pomace	ns
Temperature	***
Ratio	***
Grape pomace* Temperature	ns
Grape pomace* Ratio	ns
Temperature* Ratio	ns
Grape pomace* Temperature* Ratio	ns
ns – p>0.05; * p < 0.05; ** p< 0.01; *** p< 0.001	

After this step the selected conditions were 50ºC and 1:10 ratio. The ratio was selected due to the globally higher extracted content. Since there was no significant difference between the averages obtained when using 50ºC and 70º C (for red and white pomace when using 1:10 ratio), the 50 ºC temperature was selected because it fits the optimal temperature range of the enzymes used, it has a lower energy consumption and reduces the risk of the irreversible chemical changes to this compounds caused by the incubation since they are heat labile (Goula et al., 2016).

3.2.2 Testing different enzymes, incubation times and enzymes concentrations

Using the conditions selected in the prior step, different enzymes, the time of the incubation and the concentration of the enzymes were tested. So, for two or four hours of incubation, the samples were extracted with 1% or 2% (dry weight) of the selected enzyme, testing five enzymes for each pomace. A control test was also performed, where no enzyme was added. The tested enzymes were Pectinex Ultra SP-L, Pentopan Mono BG, Celluclast, Driselase and Viscozyme.

These enzymes were selected because they target the plant cell wall, which is a major factor for the release of phenolic compounds. Although the use of maceration and temperature has some effects on that, the release of these compounds can be enhanced via enzyme catalysed degradation of cell-wall polysaccharides (Pinelo and Meyer, 2008). The plant cell wall is composed of cellulose (35–50%), hemicellulose (20–30%, mostly xylan) and lignin (20–30%) (Walia et al., 2017). Pectinex is pectinase (Novozymes, 2015c), Pentopan is xylanase (Novozymes, 2016), Celluclast has cellulase activity (Novozymes, 2015b), Driselase is a mix of cellulase, laminarase and xylanse activities (Novozymes, 2017a) and Viscozyme is a multi-enzyme complex with a wide range of carbohydrases (including cellulase and xylanase) (Novozymes, 2017b). This means that these products have dfferent targets on the cell wall, but the goal is the same: to create breaches for a better release of the phenolic compounds trapped inside the cell.

The selected concentrations are within the range observed on other works since some of the enzymes had been previously tested with interesting results (Ferri et al., 2016, Ferri et al., 2017, Meyer et al., 1998, Kammerer et al., 2005) while others were tested for the first time on this material, although they had been used in other plant materials, like Viscozyme which was used in marigold flower to extract carotenoids (Puri et al., 2012).

Those times for the incubation were selected based on previous works by other authors (Ferri et al., 2016, Ferri et al., 2017, Antoniolli et al., 2015) and with the consideration of not having long periods in order to protect the phenolic compounds.

The results are shown on Figure 3.2, for red grape pomace and Figure 3.3 for white grape pomace and on Table 3.4 the statistical significance for the conditions and their interaction can be found.
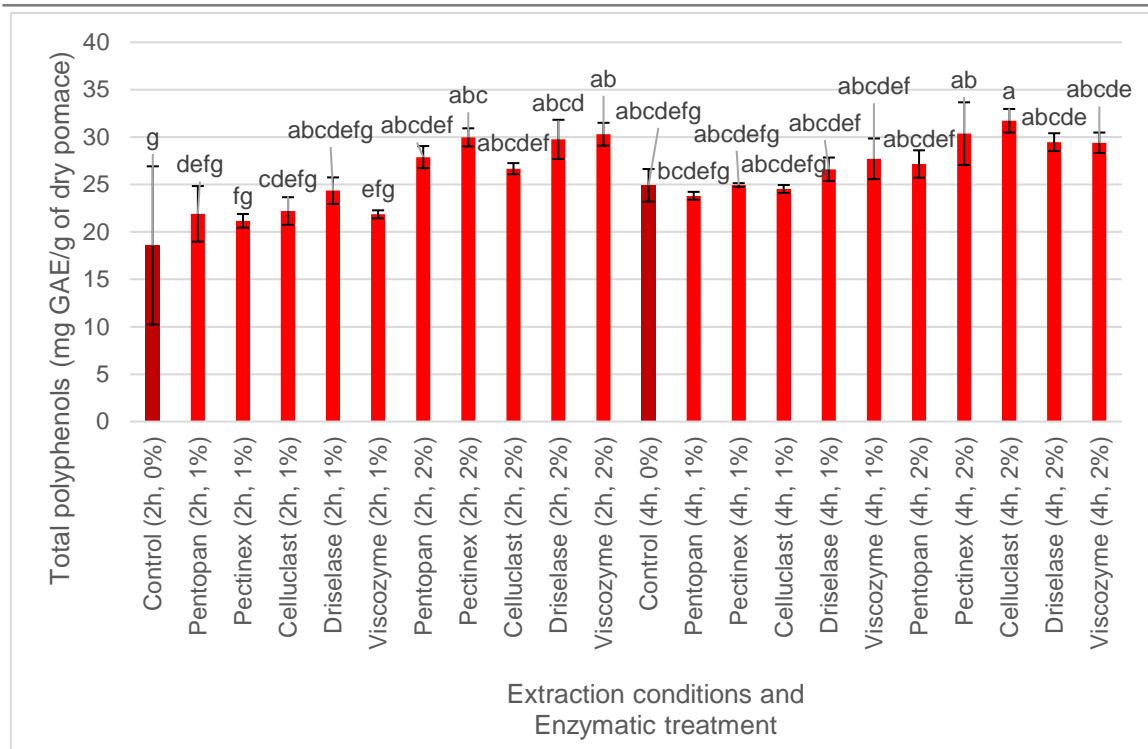
Figure 3.2 - Total phenolic content extracted from red grape pomace when applied different extraction conditions. Different letters in each bar indicate statistical differences according to the Tukey test (p<0.05).
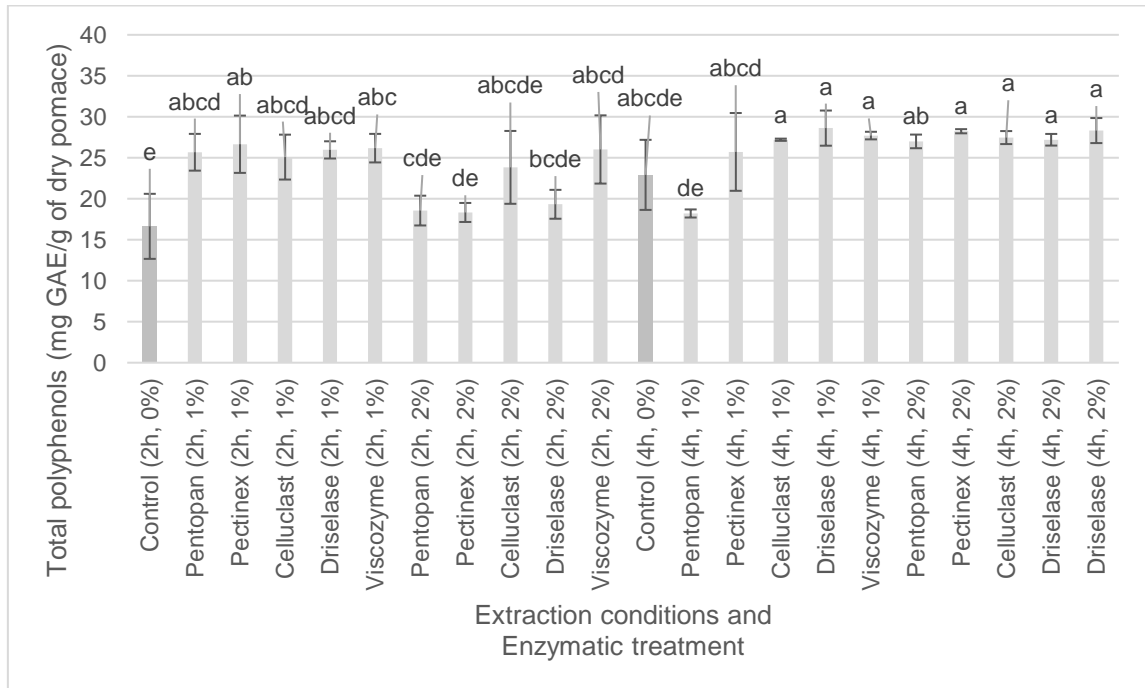
Figure 3.3 - Total phenolic content extracted from white grape pomace when applied different extraction conditions. Different letters in each bar indicate statistical differences according to the Tukey test (p<0.05).

According to the values on Table 3.4, the kind of pomace influences the extracted content and therefore the results are presented for each pomace. It is also clear that there are differences between enzymes, their concentration and the duration of incubation. There are also some significant interactions between the factors tested.

There seems to be a light tendency for extracting more from the red pomaces, especially with 2% concentration of enzymes, where for the whites there is a bigger variation between enzymes and a higher extraction can be accomplished with higher extraction time (4 h) (Figures 3.2 and 3.3). The tendency to extract more from red grape pomace is consistent with the work from Martins and colleagues (2016), where the red grape pomace values were higher (with the enzymes or without).

Table 3.4 – Statistical significance values for each factor and their interactions obtained through an ANOVA test

Factors	Significance
Grape pomace	***
Enzyme	***
Concentration	***
Time	***
Grape pomace* Enzyme	ns
Grape pomace* Concentration	***
Grape pomace* Time	ns
Enzyme* Concentration	ns
Enzyme* Time	ns
Concentration* Time	*
Grape pomace* Enzyme* Concentration	ns
Grape pomace* Enzyme* Time	ns
Grape pomace* Concentration * Time	***
Enzyme* Concentration* Time	*
Grape pomace* Enzyme* Concentration* Time	*
ns – p>0.05; * p < 0.05; ** p< 0.01; *** p< 0.001	

After these tests, the decision was to select 2 hours of incubation and 2% of enzyme as the time and enzyme concentration used (along with 50 ºC and 1:10 ratio selected before).

When considering the percentage selected it's explained by the higher TPC extracted with 2%, over the controls) than with 1%, especially for red pomace and this is shown on Figure 3.2, where 2% bars are higher than 1% bars. This was expected, since there are twice as much enzyme to act on the pomace. For white pomace (Figure 3.3), this difference is not so significant. The different behaviours between red and grape pomaces are reflected in the interaction grape pomace* concentration (Table 3.4). Similar results were obtained in the study of Ferri et al. (2017), which tested six enzymes on white grape pomace extracts, three of them (Pectinex Ultra SPL, Pentopan and Celluclast) also tested in the present work. Regarding the three concentrations used by those authors (0.5 %, 1% and 2%), the 2% concentration results were significantly higher, with the exception of the Pectinex Ultra SPL, where 1% was the best option.

Concerning extraction time, no significant differences were observed between 2 and 4 hours extraction time for red pomace, and for white pomace with 1% enzyme. Differences among 2 and 4 hours extraction time were only significant for white pomace with 2% enzymes. Therefore an extraction time of 2 h was chosen to reduce the need of time for the process to be accomplished with the resulting benefits in terms of energy and other resources being used. Moreover, selecting a shorter incubating time reduces the risk of a thermal destruction of the polyphenols (Drosou et al., 2015). Ferri and colleagues (2016) obtained a similar result. In their study that compared different incubation times, they noted that the extracted content from red grape pomace during 2 hours of incubation was significantly bigger than the content recovered in 6 or 24 h.

Differences among the different enzymes will be discussed in chapter 3.3.

3.3 CHARACTERIZATION OF THE POMACE EXTRACTS

3.3.1 TOTAL PHENOLS QUANTIFICATION

The total polyphenol quantification was the standard assay to evaluate different extracting conditions. However, on the whole chapter 3.3 the results presented refer to the analysis of the supernatant recovered after each extraction on the selected conditions for the water extraction (50 ºC, 1:10 ratio, 2 hours and 2% enzyme concentration) and for the acetone extraction (50ºC, 2 hours, 75% acetone) and they compare the effect on each enzymatic treatment for both grape pomaces.

By doing a sequential extraction with acetone it was possible to first test the use of enzymes in water and then use a solvent to discover if there was still compounds left to extract and, if so, how much it was possible to recover.

These compounds are important to the sensory and nutritional quality of fruits, vegetables and other plants but also have physiological and morphological importance. The main groups for these compounds are phenolic acids, flavonoids, tannins, stilbenes and lignans (Ignat et al., 2011).

The results for the total phenolic content (TPC) extracted from the grape pomaces by each treatment are shown in Table 3.5. The averages and standard deviations are expressed in mg of galic acid equivalents per gram of dry grape pomace.

Table 3.5 - Total phenolic content for each treatment on red and white grape pomaces

TPC (mg GAE/g dry pomace)	Red grape pomace			White grape pomace		
	Water extraction	Acetone extraction	Total	Water extraction	Acetone extraction	Total
Control	22 ± 3 [b]	32 ± 4 [a]	54 ±3 [ab]	18 ± 6 [c]	23 ± 0.9 [a]	41 ± 0.3 [b]
Pentopan	22 ± 4 [b]	32 ± 3 [a]	55 ± 3 [ab]	19 ± 2 [bc]	25 ± 0.8 [a]	42 ± 1 [ab]
Pectinex	30 ± 1 [a]	27 ± 1 [a]	57 ± 1 [ab]	18 ± 1 [bc]	25 ± 4 [a]	44 ± 4 [ab]
Celluclast	22 ± 3 [b]	31 ± 3 [a]	53 ± 3 [b]	24 ± 4 [ab]	31 ± 5 [a]	55 ± 9 [ab]
Driselase	30 ± 2 [a]	28 ± 1 [a]	58 ± 2 [ab]	19 ± 2 [bc]	27 ± 2 [a]	45 ± 1 [ab]
Viscozyme	30 ± 1 [a]	29 ± 1 [a]	59 ± 2 [a]	26 ± 4 [a]	31 ± 4 [a]	57 ± 8 [a]

Different letters in each column indicate statistical differences according to the Tukey test ($p<0.05$)

Looking at the data presented on Table 3.5 it seems that it was possible to extract a higher phenolic content from red grape pomace, with the exception of the Celluclast treatment which exhibited a value for the white pomace similar to those presented for red pomace. In both pomaces, the Viscozyme treatment got significant highest results (59 mg GAE/g for red grape pomace and 57 mg GAE/g for white). Results presented in Table 3.5 and Figures 3.4 and 3.5 are slightly different from results presented in Figures 3.2 and 3.3 because with some enzymatic treatments additional tests were executed, and those results were added to the data presented in Figures 3.2 and 3.3. Table 3.5 present the initial data obtained.

Globally, with acetone, it was possible to extract a higher amount of compounds (41-45% more for some treatments for red and 20-40% for all the white treatments), with the exceptions of Pectinex, Driselase and Viscozyme treatments for red grape pomace that could be related to the fact that in those treatments, the content recovered with water was significantly higher (1.4-fold) than in the other treatments. That can also be confirmed looking at Figure 3.4, where it's shown that the percentage of content extracted with water by those three treatments was higher and over 50%. Figure 3.5, for white pomace, confirms the influence of the acetone extraction, with percentages of extraction between 55-59%.

Red grape pomace acetone extraction following enzymatic digestion, did not show any differences between enzymes. Viscozyme was able to extract a higher content of total phenols than Celluclast, but without statistical significance to the other treatments.
Regarding white pomace, some differences between treatments were pointed out. Viscozyme presented significantly higher numbers (1.4-fold) for the water extraction than the others enzymes (except Celluclast) but Celluclast was the only other enzyme better than the control. For the acetone extraction no differences were found and when water and acetone data were summed up Viscozyme was significantly better than the control (1.4-fold) and Celluclast was the second best enzyme.
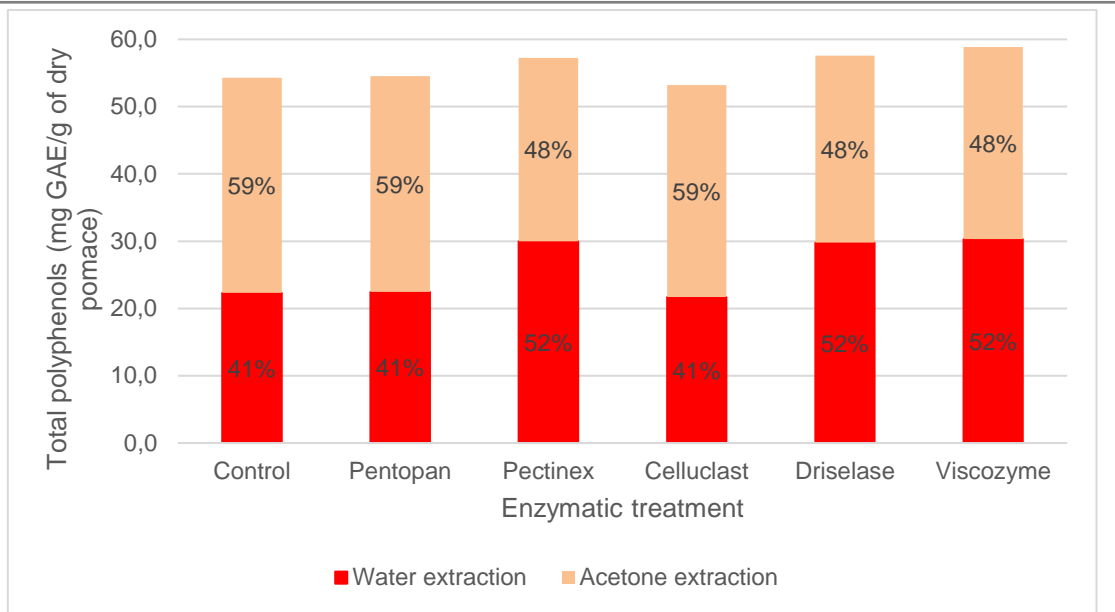
Figure 3.4 - Cumulative averages for extracted phenolic compounds by each enzymatic treatment followed by 75% acetone extraction from red grape pomace. The percentages indicate the recovered phenols amount related to each type of extraction over the total.
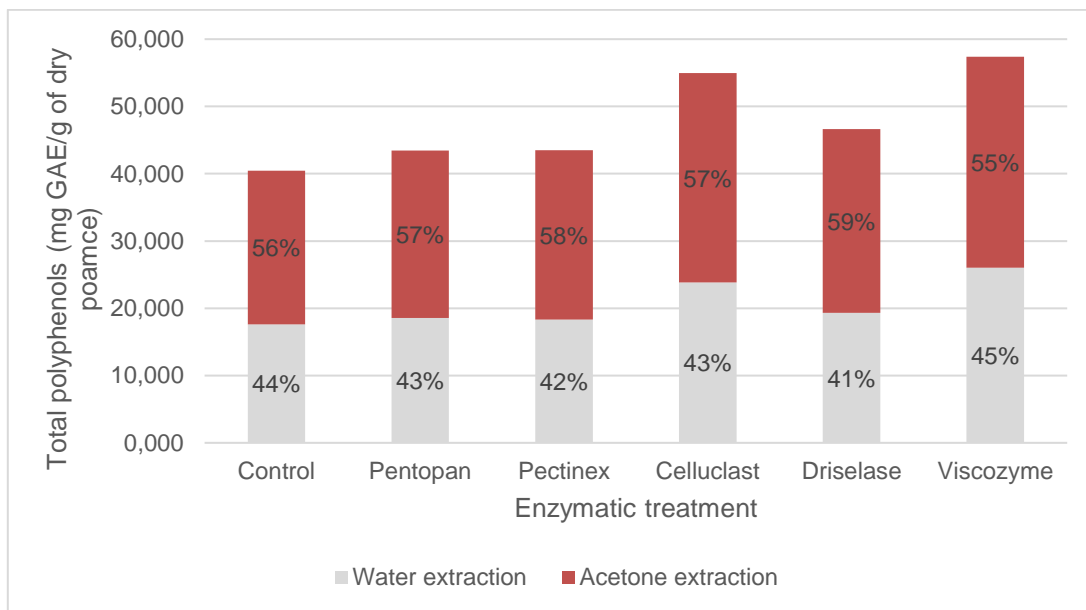
Figure 3.5 - Cumulative averages for extracted phenolic compounds by each enzymatic treatment followed by 75% acetone extraction from white grape pomace. The percentages indicate the recovered phenols amount related to each type of extraction over the total.

The determination of the phenolic compounds present in grape pomace samples is a common procedure since it's the most used assay to analyse, optimize and compare extraction methodologies. Table 3.6 and Table 3.7 show some selected works for red and white grape pomace for *Vitis vinifera* cultivars, which determined the total phenolic compound contents spectrophotometrically.

Table 3.6 – Comparison of literature works for total phenolic content extracted from red grape pomace (*V. vinifera* cultivars)

Cultivar	Solvent information	Additional information	TPC (mg GAE/g dry pomace)	Reference
Malbec	50% ethanol		32	(Antoniolli et al., 2015)
Pinot noir	50% acetone		0.01	(Cheng et al., 2012)
Pinot meunier			4	
Pinot noir	50% ethanol		0.8	
Pinot meunier			2	
Pinot noir	50% methanol		1	
Pinot meunier			3	
Ruby Cabernet	methanol	maceration	431	(García-Becerra et al., 2016)
Ruby Cabernet		Soxhlet	224	
Monastrel	50% ethanol	stirring	21	(López-Miranda et al., 2016)
Monastrel		ultrasound	24	
Monastrel	water	stirring	4	
Monastrel		ultrasound	4	
Maximo IAC 138-22	methanol	Control	51	(Martins et al., 2016)
Maximo IAC 138-22		5% (w/w) tannase	82	
Maximo IAC 138-22		2.5 % (w/w) pectinase, cellulose	52	
Maximo IAC 138-22		1.66 % (w/w) tannase, pectinase, cellulose	60	
Negro amaro	80% ethanol, 1:30 ratio (w/v)		42	(Negro et al., 2003)
Pinot noir	Ethanol:water:formic acid 50:48.5:1.5, (v/v)		90	(Reis et al., 2016)
Cabernet Sauvignon	40% ethanol, 1:50 ratio (w/v)		25-30	(Ribeiro et al., 2015)
Merlot			~30	
Barbera	10 % ethanol		388	(Spigno et al., 2007)
Barbera	20 % ethanol		393	
Barbera	30 % ethanol		471	
Barbera	40 % ethanol		474	
Barbera	50 % ethanol		451	
Barbera	60 % ethanol		338	
Tinta Roriz (TR)	1st extraction: ethanol		69	(Tournour et al., 2015)
Tinta Roriz	2nd extraction: water		76	
Touriga Franca (TF)	1st extraction: ethanol		100	
Touriga Franca	2nd extraction: water		106	
Touriga Nacional (TN)	1st extraction: ethanol		132	
Touriga Nacional	2nd extraction: water		142	
(TR+TF+TN)	1st extraction: ethanol		104	
(TR+TF+TN)	2nd extraction: water		103	
Refošk	50% acetone		17	(Vatai et al., 2009)
Merlot			20	
Cabernet			20	

Table 3.7 - Comparison of literature works for total phenolic content extracted from white grape pomace (*V. vinifera* cultivars)

Cultivar	Solvent information	Additional information	TPC (mg GAE/g dry pomace)	Reference
Chardonnay	Consecutive extractions of 80% acetone and 60% methanol		39	(González-Centeno et al., 2013)
Macabeu			31	
Parellada			47	
Prensal Blanc			36	
Zalema	75% methanol		25	(Jara-Palacios et al., 2014b)
Pedro Ximénez			7	
Moscatel			22	
Baladí			5	
Parellada			31	
Sauvignon blanc			23	
Montepila			5	
Airén			12	
Verdejo			13	
Prensal Blanc	Sequentially, 50% methanol and 70% acetone		35	(Llobera and Cañellas, 2008)
Moscato	methanol	Control	37	(Martins et al., 2016)
Moscato		5% (w/w) tannase	45	
Moscato		2.5 % (w/w) pectinase, cellulose	43	
Moscato		1.66 % (w/w) tannase, pectinase, cellulose	41	

When looking to Table 3.6 and Table 3.7 the first noticeable thing is that there is a huge range of values for total phenolic content, especially for red grape pomace and values are globally higher for red grape pomace, in accordance with the present work. Also, following the literature overview, more researches were published on red respect to white grape pomace. Interesting is the fact that our results fit the range of results presented in those two tables.

The results on Tables 3.6 and 3.7 reveal that several different extraction procedures have been experimented, while trying to have higher yields, but with lower costs and lower environmental prejudice. Changes to the pre-treatment of the pomace like stirring or ultrasound methods (which provided similar results) (López-Miranda et al., 2016) or employing various solvents and enzymes have been studied. There is also a sequential extraction study where samples were first subjected to ethanol and then to water (Tournour et al., 2015) having similar or slightly bigger results on the second extraction, similarly to what happened on the present work. This demonstrates that there is still an important phenols content in pomace to recover after the first extraction and adding additional steps with the same or other solvents could be a great advantage to improve the amount extracted from the grapes.

The study of the enzyme influence on extraction is also important because results show that, with the right combination of enzyme and concentration, they might be beneficial for the process,

especially if they can replace the use of organic solvents, which is interesting for some of the applications of the recovered phenolic compounds (e.g. food or cosmetics). Their use was studied (Martins et al., 2016) and some results on red and white grape pomace can be seen on Tables 3.6 and 3.7.

The results obtained in the present work for red grape pomace (53-59 mg GAE/ g dry pomace) fit well within such a big range of values. Differences in cultivar, climatic conditions, winemaking process or extraction conditions could explain some differences in values. Specifically, when looking to Merlot results (Ribeiro et al., 2015, Vatai et al., 2009), both values presented on Table 3.6 are similar to the values for each extraction made (water or acetone) on this work, even if there were differences on the solvent used or its percentage. When considering the summed up content of the water/enzyme and acetone extractions, a higher phenol amount (2 to 3-fold) was recovered in the present work respect to what presented previous cited papers.

For white grape pomace the results on Table 3.5 match those found on Table 3.7. For this pomace the range of values presented was much smaller and that might be explained with the lower number of studies found.

However, it's important to note that if enzymes were being used and only one extraction had to be selected, it would be possible to have good results with water for both pomaces, even if there are differences between enzymes.

3.3.2 PROTEIN CONTENT OF THE EXTRACTS

Using a modified Lowry assay the protein content was determined for each extraction and the results (including the total of the two extractions) are shown on Table 3.8 (averages and standard deviation). On Figures 3.6 and 3.7 the cumulative averages for each extraction are shown. Results represent the amount that was extracted from each g of dry pomace.

Table 3.8 shows that from white grape pomace it was possible to extract more proteins, with the exception of the control and Pentopan. Also water extractions recovered more content than acetone extractions, for both pomaces. This can be confirmed in Figures 3.6 and 3.7, although that for white pomace the difference between extractions was bigger, reaching 68% extracted in water against 32% from acetone, in the control and the Pectinex treatments.

When looking at red grape pomace alone, there weren't any significant differences between treatments in both water and acetone extractions or in the total, with the exception of Pentopan and Pectinex which recovered slightly more proteins.

During white pomace water extraction, all the enzymatic treatments worked better (5-19% more protein recovered) than the control, and Pectinex extracted the highest content (with significance over the control and Pentopan). With acetone, Driselase was the best option, but Viscozyme and Celluclast were also collecting more (70, 56 and 48%, respectively) than the control, Pentopan

and Pectinex. The sum of both extractions revealed that Driselase and Viscozyme were better options, with significance (1.3-fold) over the control and Pentopan.

So, for protein, some enzymatic treatments were successful on white grape pomace, but not with the red pomace.

Table 3.8 - Protein content for each treatment extracted from red and white grape pomaces

Protein (mg BSA eq/g dry pomace)	Red grape pomace			White grape pomace		
	Water extraction	Acetone extraction	Total	Water extraction	Acetone extraction	Total
Control	100 ± 8 a	84 ± 5 a	184 ± 12 a	118 ± 9 c	54 ± 1 d	172 ± 9 c
Pentopan	105 ± 8 a	87 ± 5 a	192 ± 12 a	124 ± 6 bc	62 ± 3 cd	182 ± 2 bc
Pectinex	107 ± 3 a	83 ± 4 a	190 ± 3 a	140 ± 3 a	66 ± 12 bcd	207 ± 14 ab
Celluclast	102 ± 4 a	84 ± 5 a	185 ± 4 a	135 ± 5 ab	80 ± 10 abc	214 ±14 ab
Driselase	102 ± 7 a	85 ± 1 a	186 ± 7 a	133 ± 5 ab	92 ± 1 a	226 ± 4 a
Viscozyme	102 ± 4 a	85 ± 7 a	187 ± 9 a	136 ± 11 ab	84 ± 8 ab	221 ± 17 a

Different letters in each column indicate statistical differences according to the Tukey test (p<0.05)

Figure 3.6 – Cumulative averages for extracted proteins by each enzymatic treatment followed by 75% acetone extraction from red grape pomace. The percentages indicate the recovered protein amount related to each type of extraction over the total.
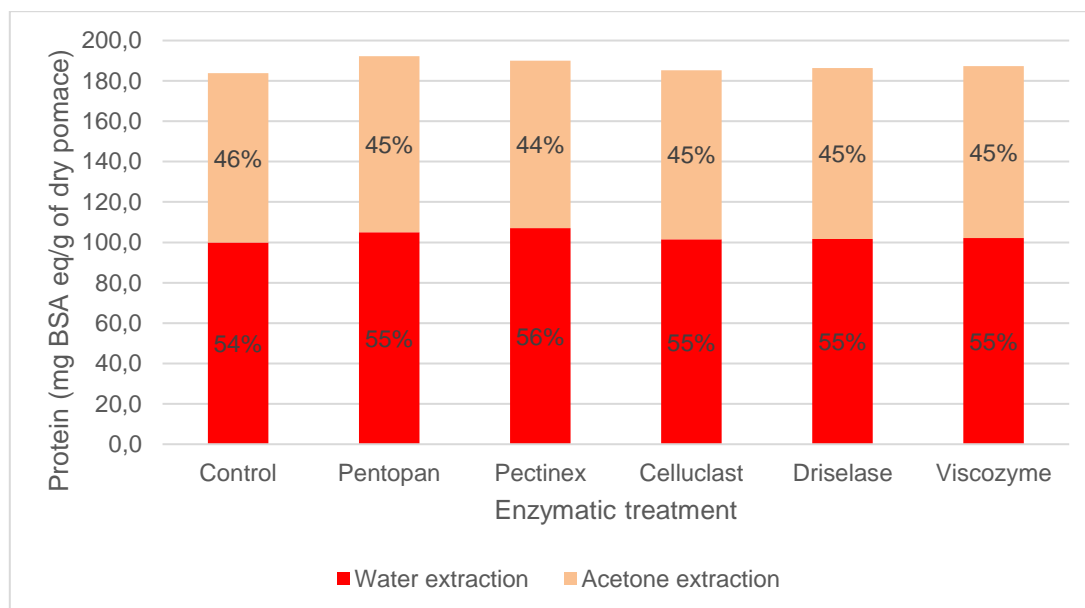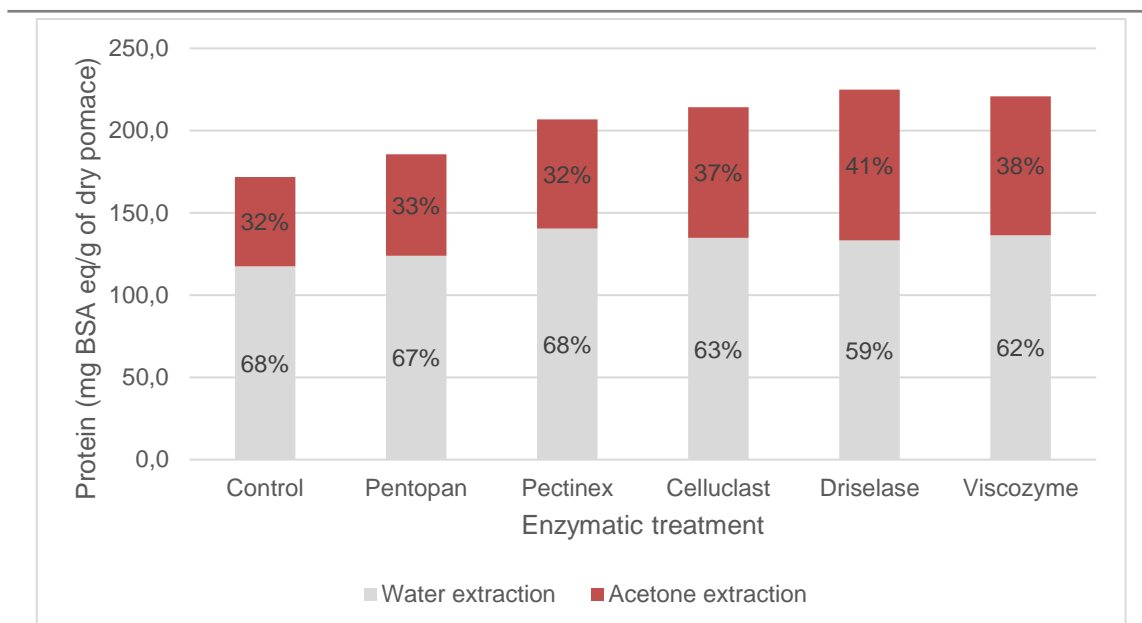
Figure 3.7 - Cumulative averages for extracted proteins by each enzymatic treatment followed by 75% acetone extraction from white grape pomace. The percentages indicate the recovered protein amount related to each type of extraction over the total.

The results obtained for the protein quantification are superior to those found by other authors. This was attributed to the fact that the protein content of the enzymes was not taken into account. This may result also from interferences associated with the method, e.g. the presence of phospholipids. A work done with red grape pomace, using the Kjeldahl method, extracted 132.3 mg/g of dry Merlot pomace and 53.2 mg/g from Cabernet Sauvignon (Ribeiro et al., 2015). The values for red grape pomace presented on Table 3.8 are 1.4 and 3.8-fold, respectively, higher than these obtained by this work. A second study, using white grape pomaces, applied the micro-Kjeldahl method for nitrogen quantification and then calculated the protein content of 131 mg/g of dry pomace from Prensal Blanc (Llobera and Cañellas, 2008). This result is 1.3-1.7 times lower than all the total values for white grape pomace on Table 3.8, but can be compared to the results for the water extraction. There were methodological differences between the present work and the previous two studies, besides different cultivars being tested. The present work recovered more protein than the two previously reported works which accounted for all the protein present in the sample of pomace given the use of Kjeldahl methods. As pointed out, since protein content of the enzymes was not taken into account and the method has interferences, results obtained are useful only to clarify differences among enzymatic treatments and control, and a comparison with results presented on literature obtained by other methodologies should be done with precaution.

Analysing the protein content of these pomaces was important in order to have a better understanding of the material. Proteins have roles on the structure of cell walls or can be connected, as lipoproteins, to polyphenols (Barba et al., 2015).

3.3.3 REDUCING SUGARS CONTENT OF THE EXTRACTS

The analysis proceeded with the quantification of the reducing sugars and Table 3.9 exhibits the result of the determination (averages and standard deviations). Figures 3.8 and 3.9 display the cumulative averages for each grape and extraction. Results represent the amount that was extracted from each g of dry pomace.

The first notable thing is that the values for white were far bigger (4 to 10-fold) and than those in red pomace and this could be explained by the differences in the white and red winemaking processes. For red wine, the pomace is collected after a fermentation step (Achmon et al., 2016), which consumes sugars, while for white wine pomace are collected before fermentation. The biggest difference between both pomaces is seen on the results for the water extraction. The contribution of each extraction is also different, since for white pomace over 80% was extracted through the water extraction, whereas for red pomace the content decreases to 45-65%. This can be explained by the fact that reducing sugars are water soluble and therefore, after this first extraction with water, there might not be a lot more to extract. The higher amount (3.4 to 6.3-fold) extracted with water in the white pomace, compared with red pomace, is linked with the wine making process, as explained before.

Looking at the red grape pomace values, the enzymatic treatments had a significant influence because with the exception of Viscozyme, all the treatments had smaller results than the control on the water extraction. For the acetone extraction, Viscozyme was the best treatment with slightly more content removed. The total reflects the influence of the water extraction, with the control and Viscozyme treatments exhibiting significantly higher extracted content (1.5 to 1.6-fold).

On the water extraction from white pomace Celluclast got a better result, significantly higher than the control, Pentopan and Driselase and, while there were no differences found on the acetone extraction, the total content extracted revealed a significant difference between Celluclast and Driselase.

Table 3.9 – Reducing sugars content for each treatment extracted from red and white grape pomaces

Reducing sugars (mg GLU eq/g dry pomace)	Red grape pomace			White grape pomace		
	Water extraction	Acetone extraction	Total	Water extraction	Acetone extraction	Total
Control	0.25 ± 0.03 a	0.13 ± 0.02 b	0.38 ± 0.03 a	1.0 ± 0.1 b	0.25 ± 0.03 a	1.3 ± 0.1 ab
Pentopan	0.11 ± 0.0 b	0.13 ± 0.01 b	0.24 ± 0.01 b	1.0 ± 0.3 b	0.23 ± 0.03 a	1.3 ± 0.1 ab
Pectinex	0.13 ± 0.0 b	0.13 ± 0.004 b	0.26 ± 0.004 b	1.1 ± 0.1 ab	0.26 ± 0.04 a	1.4 ± 0.2 ab
Celluclast	0.12 ± 0.01 b	0.12 ± 0.06 b	0.24 ± 0.01 b	1.2 ± 0.4 a	0.23 ± 0.02 a	1.5 ± 0.04 a
Driselase	0.12 ± 0.01 b	0.13 ± 0.004 b	0.25 ± 0.01 b	0.9 ± 0.1 b	0.21 ± 0.00 a	1.2 ± 0.03 b
Viscozyme	0.23 ± 0.02 a	0.16 ± 0.01 a	0.39 ± 0.02 a	1.1 ± 0.1 ab	0.25 ± 0.03 a	1.3 ± 0.1 ab

Different letters in each column indicate statistical differences according to the Tukey test ($p < 0.05$)
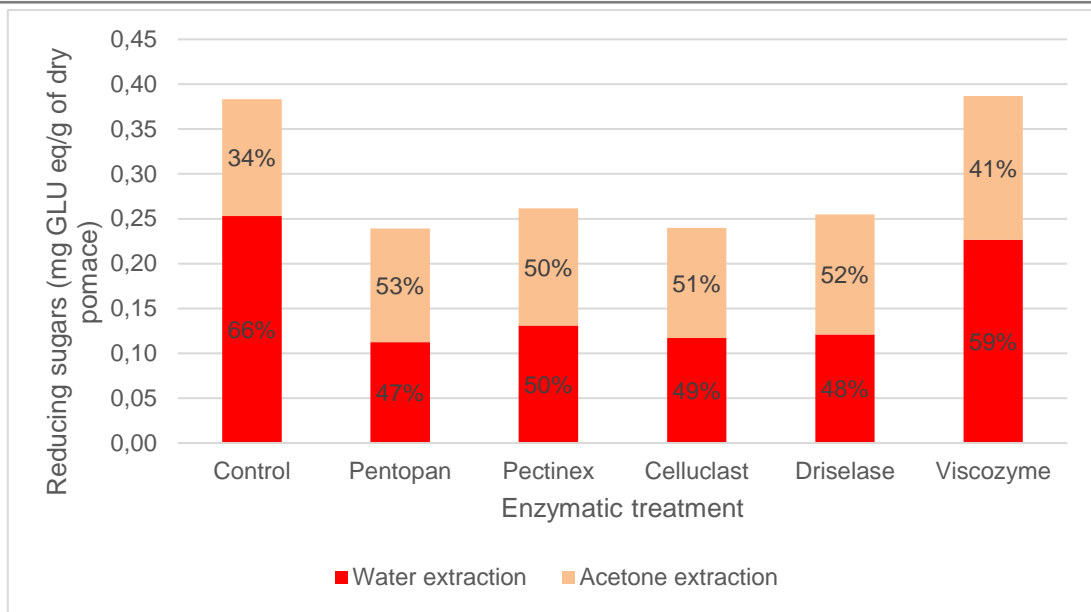
Figure 3.8 - Cumulative averages for extracted reducing sugars by each enzymatic treatment followed by 75% acetone extraction from red grape pomace. The percentages indicate the recovered reducing sugars amount related to each type of extraction over the total.
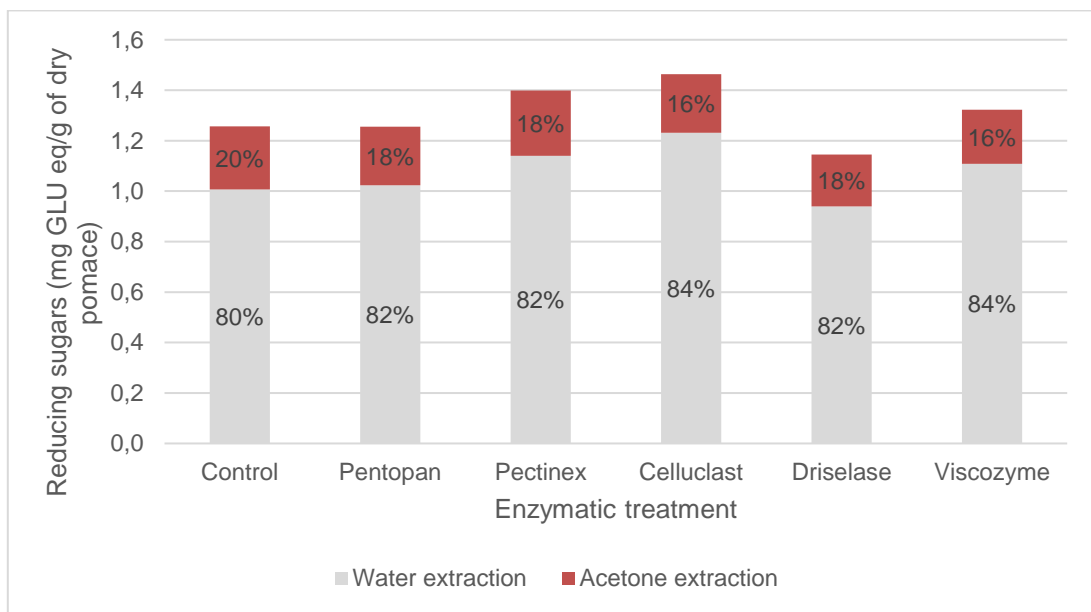
Figure 3.9 - Cumulative averages for extracted reducing sugars by each enzymatic treatment followed by 75% acetone extraction from white grape pomace. The percentages indicate the recovered reducing sugars amount related to each type of extraction over the total.

There are not a lot of works quantifying reducing sugars on grape pomace. It was only possible to find one paper where the authors determined the content from Cabernet Sauvignon and Merlot grape pomaces, obtaining 307.3 mg of glucose equivalents/g of dry pomace for the first and 19.2 mg of glucose equivalents/g dry pomace for the latter (Ribeiro et al., 2015). There is a big difference between both values (16-fold) but also between these values and those here presented (49-fold for the Merlot and the Viscozyme value presented here). That can be explained with the fact that the determination by Ribeiro and others was made with all the pomace sample, and not only in the extract, as it happened here. Further extractions on the present work might had been

able to extract more reducing sugars. The lower reducing sugars content extracted can also be due to the fact that reducing sugars can react with aminoacids through the Maillard reaction at 50ºC and therefore they were not available to react with DNS. The differences between works could also be related to variations in the winemaking steps, since longer fermentation times could be responsible for lowering reducing sugars content.

This quantification was important to know better the pomaces and try to understand if reducing sugars can be interfering in other determinations. In previous works, sugars present on grape pomace have also been used to produce bioethanol (Zabed et al., 2017).

3.3.4 TANNINS CONTENT OF THE EXTRACTS

Tannins can be divided in hydrolysable and condensed tannins and they have diverse effects on biological systems because they are potential metal ion chelators, protein precipitating agents and biological antioxidants (Ignat et al., 2011).

The results on the quantification (averages and standard deviations) of tannins are presented on Table 3.10. Generally, white grape pomace extracts were 31-40% richer in tannins than red pomace's. Although it was not on total tannin content, but separated condensed and hydrolysed content, a previous work done on red and white grape pomace found a higher condensed tannins content on red grape pomace (1.7 to 3-fold) and a higher hydrolysed tannins content on white grape pomace (2 to 100% more) (Martins et al., 2016).

The water extractions for both pomaces recovered more tannin content than the acetone extractions (Figures 3.10 and 3.11), with extracted percentages close to 60% in all the treatments for the water extracts from pomaces. This situation was not observed in a previous study on white pomace where the tannin extracted amount in the water was smaller than the ethanol-extracted content (Ferri et al., 2017).

In the present work, about the red grape pomace it can be seen that there weren't differences on the water extraction, while for the acetone extraction Pentopan yielded more tannins than Pectinex, although this was not represented on the total recovered amount where there were no differences between treatments.

For the white pomace, no differences were found among the water extractions, but with the acetone, Viscozyme got a worse result than the control. Pectinex was the best option when considering the total tannins recovered and had a significantly higher value than Pentopan.

Table 3.10 - Tannins content for each treatment on red and white grape pomaces

Tannins (mg/g dry pomace)	Red grape pomace			White grape pomace		
	Water extraction	Acetone extraction	Total	Water extraction	Acetone extraction	Total
Control	9.3 ± 1 [a]	5.7 ± 0.1 [ab]	15.0 ± 1 [a]	12.0 ± 0.1 [a]	8.4 ± 0.04 [a]	20.4 ± 0.1 [ab]
Pentopan	9.7 ± 0.1 [a]	5.8 ± 0.1 [a]	15.4 ± 0.2 [a]	12.3 ± 0.6 [a]	8.3 ± 0.1 [ab]	20.1 ± 0.04 [b]
Pectinex	10.0 ± 0.6 [a]	5.5 ± 0.1 [b]	15.6 ± 0.6 [a]	12.5 ± 0.2 [a]	8.4 ± 0.1 [ab]	20.8 ± 0.2 [a]
Celluclast	9.7 ± 0.4 [a]	5.7 ± 0.02 [ab]	15.4 ± 0.5 [a]	11.9 ± 0.3 [a]	8.3 ± 0.03 [ab]	20.2 ± 0.3 [ab]
Driselase	9.0 ± 0.2 [a]	5.7 ± 0.04 [ab]	14.7 ± 0.3 [a]	12.1 ± 0.1 [a]	8.3 ± 0.02 [ab]	20.2 ± 0.1 [ab]
Viscozyme	9.1 ± 0.5 [a]	5.6 ± 0.2 [ab]	14.7 ± 0.6 [a]	12.4 ± 0.3 [a]	8.2 ± 0.1 [b]	20.6 ± 0.4 [ab]

Different letters in each column indicate statistical differences according to the Tukey test ($p < 0.05$)

Figure 3.10 - Cumulative averages for extracted tannins by each enzymatic treatment followed by 75% acetone extraction from red grape pomace. The percentages indicate the recovered tannin amount related to each type of extraction over the total.
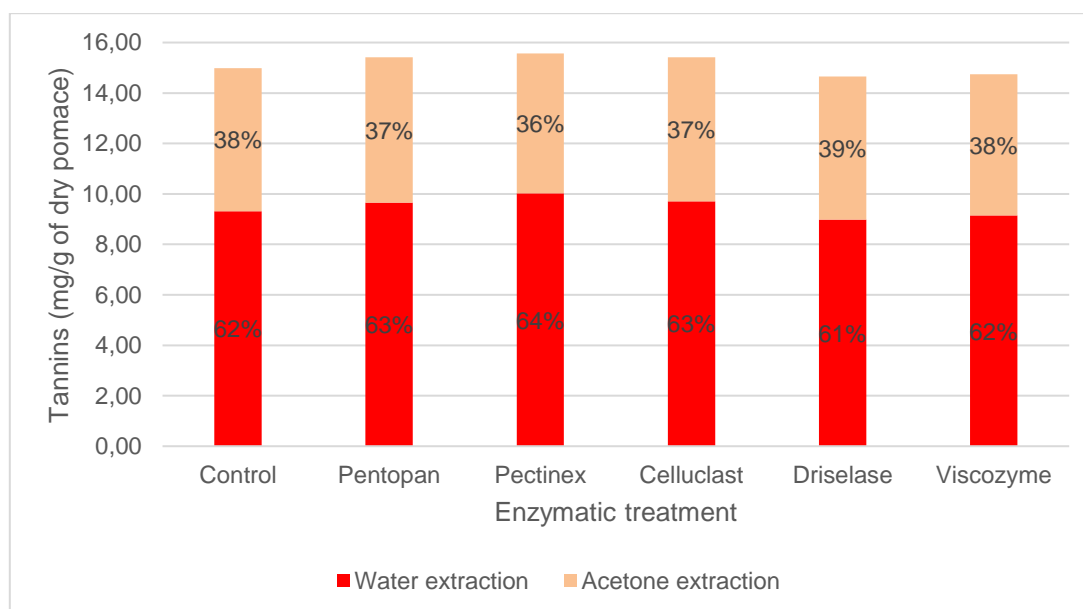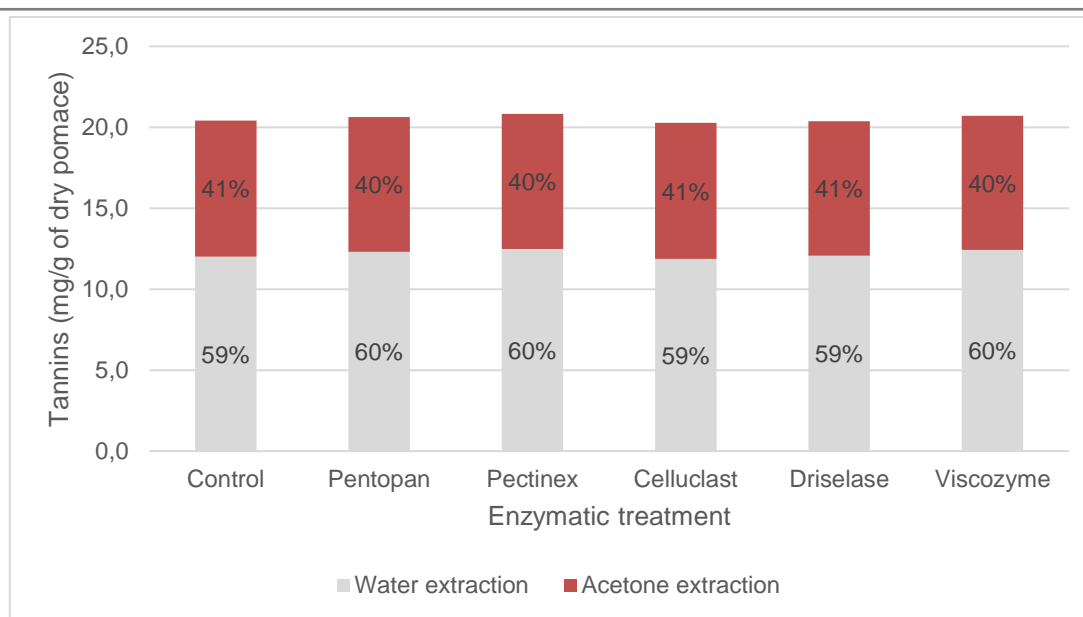
Figure 3.11 - Cumulative averages for extracted tannins by each enzymatic treatment followed by 75% acetone extraction from white grape pomace. The percentages indicate the recovered tannin amount related to each type of extraction over the total.

A study (Vergara-Salinas et al., 2013), using pressurized hot water extraction, with 1:10 solid:solvent ratio, tested different temperatures and extraction times in Cabernet Sauvignon pomace (red grapes). The results ranged between 4 to 15 mg of catechin equivalents per g of dry pomace (the highest result was at 150ºC for 5 minutes, and the lowest for 30 minutes at 50 ºC). The extraction conditions 50 ºC and 30 min of incubation are those closest to the present work, and the results are the same range to acetone data here presented (Table 3.10). However, the authors' best result is similar to the total content obtained in this study for Viscozyme or Driselase and 2-7% lower than the results for the other treatments. The increase of temperature to 200 ºC led to decrease of the extracted content to 6 mg catechin equivalents/g dry pomace (5 minutes of incubation) and 2 mg catechin equivalents/g dry pomace (30 minutes). Comparing both methodologies it seems that the use of solvents allowed for lower temperatures but, depending on the method, it might be possible to adjust the time and temperature to extract more tannins.

The tannin content was also measured in a second study on Barbera grape pomace (red grapes), where after selecting the duration and temperature of maceration (5 hours and 60 ºC), the percentage of water/ethanol solution was tested (Spigno et al., 2007). The content obtained varied from 6.5 mg/g of dry pomace (10% of water) to 20.0 (40% water) and although the cultivar, methodology and solvent used were different, most of these values are well within what was extracted in the present work for red grape pomace even if this study's best result was 28% higher than what was calculated on the Pectinex treatment, the highest total value for red pomace (Figure 3.10).

A third study (Martins et al., 2016) on Maximo IAC 138-22 (red grapes) and Moscato (white grape) pomaces calculated the condensed and hydrolysed tannins content. The use of enzymes was tested without extraction improvement, in accordance to present results. For red grape pomace the control extracted 28.6 mg catechin equivalents/g dry pomace of condensed tannins. That result is 1.4-fold higher than the yield here achieved and for white, the result was 1.8 to 1.9-fold

smaller. Their values for hydrolysed tannins were 4.8 mg/g for red and 4.9 mg/g for white (Martins et al., 2016). A fourth study (Negro et al., 2003), on Manto Negro (red) grape pomace recovered 22.3 mg/g dry pomace of condensed tannins, which is still 43-52% bigger than what was recovered here for total tannins on both extractions, but similar to the result of the third study present. Very different is a result from a fifth study (Llobera and Cañellas, 2008), where a value of 168.3 mg/g dry pomace for condensed tannins is presented for Prensal Blanc (white) grape pomace. This result is 8.1-fold higher than the Pectinex value (Table 3.10).

These results demonstrate the possibility to recover tannins which may be used as antioxidants (e.g. for feed purposes) (Ignat et al., 2011) but also allow for a better knowledge of the pomaces, which is important since, tannins are inhibitors of digestive enzymes or, for example, there is a strong interaction between tannins and protein on grape seed (Vorobieva, 2013).

3.3.5 FLAVONOIDS CONTENT OF THE EXTRACTS

Flavonoids are low molecular weight compounds whose variation in composition results in major flavonoids classes like flavonols, flavones, flavanones, flavanols, isoflavones, flavanonols and anthocyanidins. They are important antioxidants due to their actions as reducing agents, hydrogen donors and singlet oxygen quenchers and they also have a metal chelating potential. There is great interest in researching this compounds for the promotion of preventive health care through the consumption of fruits and vegetables (Ignat et al., 2011).

The results obtained by the flavonoids quantification can be seen in Table 3.11 (averages and standard deviations). Generally the difference between pomaces was small, with red grape pomaces having slightly richer extracts. Acetone extractions gave slightly better results, but in the case of white grape pomace, it was almost equal to the content extracted to the water. Those numbers can be seen in Figures 3.12 and 3.13, where the percentages of recovered flavonoids by each extraction (of the total content recovered) are presented. The results for the percentages of recovery by both extractions, for the white grape pomace, don't match what was observed on a work by Ferri and colleagues (2017) where the solvent extraction (ethanol) was responsible for most of the recovered flavonoid content.

No differences between treatments were found for the water extraction from red grape pomace and, although Celluclast recovered significantly more (1.2-fold) than the control and Pectinex with the acetone solution, on the final content no differences were found.

For the white grape pomace, when extracting with water, a significant difference was found between Viscozyme (the best treatment) and the control (1.3-fold), Pentopan (1.1-fold) e Pectinex (1.2-fold). In addition Celluclast and Driselase also showed 1.2-fold higher amounts than the control. No differences were observed when the acetone solution was used, but the total extracted content showed similar results to the water extraction: Driselase and Viscozyme were the best options, significantly better than all the other treatments.

Table 3.11 – Flavonoids content for each treatment on red and white grape pomaces

Flavonoids (mg CAT eq/g dry pomace)	Red grape pomace			White grape pomace		
	Water extraction	Acetone extraction	Total	Water extraction	Acetone extraction	Total
Control	6.8 ± 0.7 [a]	9.2 ± 0.6 [b]	16.0 ± 1 [a]	6.2 ± 0.2 [c]	7.0 ± 0.2 [a]	13.1 ± 0.4 [c]
Pentopan	6.7 ± 0.1 [a]	9.8 ± 0.4 [ab]	16.5 ± 0.5 [a]	6.8 ± 0.4 [bc]	7.4 ± 0.4 [a]	13.9 ± 0.01 [bc]
Pectinex	7.1 ± 0.2 [a]	9.0 ± 0.4 [b]	16.1 ± 0.4 [a]	6.7 ± 0.6 [bc]	7.4 ± 0.9 [a]	14.1 ± 1 [bc]
Celluclast	7.0 ± 0.5 [a]	10.9 ± 1.4 [a]	17.9 ± 2 [a]	7.2 ± 0.5 [ab]	7.7 ± 0.4 [a]	15.0 ± 0.5 [bc]
Driselase	7.2 ± 0.2 [a]	9.7 ± 0.3 [ab]	16.8 ± 0.4 [a]	7.4 ± 0.5 [ab]	8.0 ± 0.1 [a]	15.1 ± 0.1 [a]
Viscozyme	7.1 ± 0.3 [a]	10.0 ± 0.8 [ab]	17.1 ± 0.9 [a]	7.8 ± 0.1 [a]	7.7 ± 0.9 [a]	15.5 ± 0.8 [a]

Different letters in each column indicate statistical differences according to the Tukey test ($p<0.05$)

Figure 3.12 - Cumulative averages for extracted flavonoids by each enzymatic treatment followed by 75% acetone extraction from red grape pomace. The percentages indicate the recovered flavonoid amount related to each type of extraction over the total.
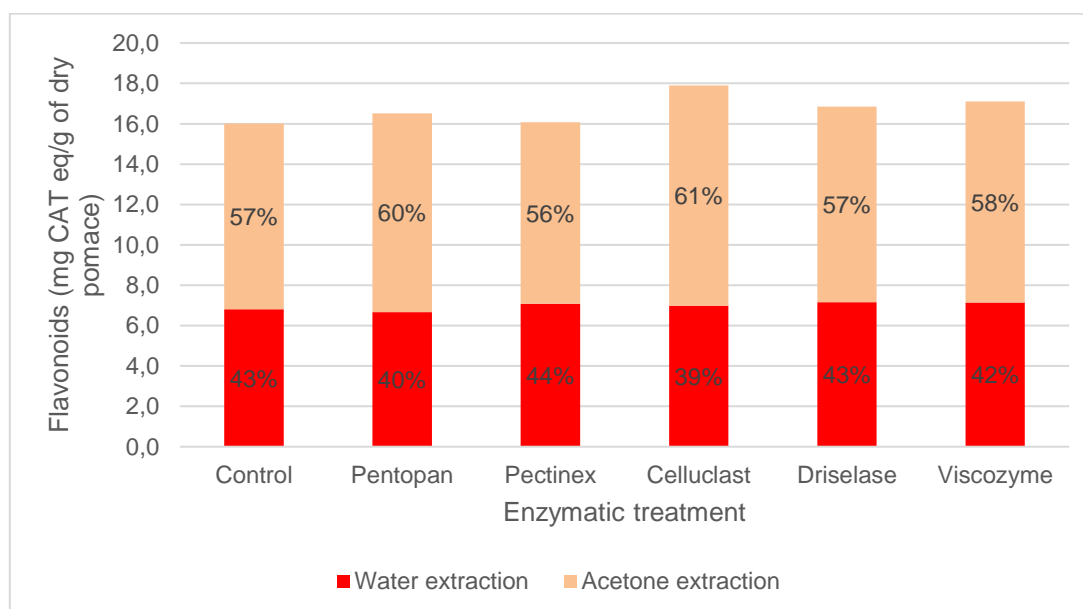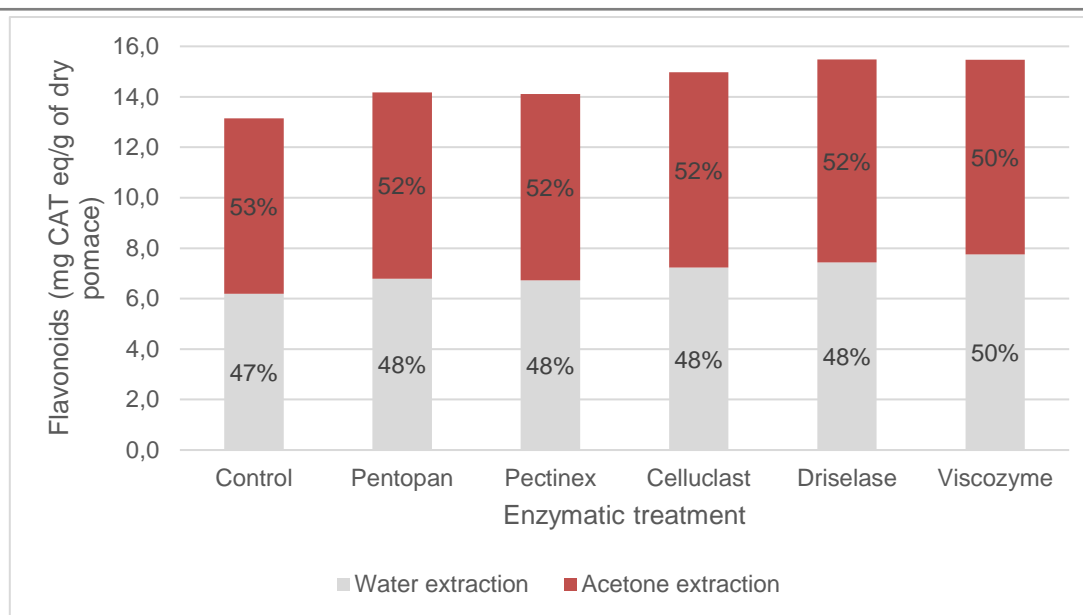
Figure 3.13 - Cumulative averages for extracted flavonoids by each enzymatic treatment followed by 75% acetone extraction from white grape pomace. The percentages indicate the recovered flavonoid amount related to each type of extraction over the total.

The content extracted seems to be in accordance with flavonoid levels reported on other studies, although 2.2 to 2.5-folds lower than the 39.4 mg Cat eq/g of dry pomace obtained from the Negroamaro pomace (red cultivar) when using 80% ethanol in the process (Negro et al., 2003).

A second study used 40% ethanol at a 1:50 solid-solvent ratio and extracted around 12,5 mg CAT eq/g of dry pomace for the Cabernet Sauvignon cultivar and 17.5 mg CAT eq/g dry pomace for Merlot cultivar (Ribeiro et al., 2015), both red grape cultivars. These values (obtained with an extraction of 24 hours at 25 ºC) are similar to what was reported in this work.

However, all of these results seem to be much lower (3 to 8-fold) than the values obtained with methanol by two different methods: maceration (147 mg CAT eq/g dry pomace) and Soxhlet (127 mg CAT eq/g dry pomace) by a third selected work, on Ruby Cabernet red grape pomace (García-Becerra et al., 2016).

3.3.6 ANTHOCYANINS CONTENT OF THE EXTRACTS

Anthocyanins are water-soluble vacuolar pigments that may appear as red, purple or blue, depending on the pH and they occur in all plant tissues (Ignat et al., 2011). Besides their use as natural food colorant, they are protective against cellular oxidants (Vergara-Salinas et al., 2013). These compounds are located on the grape skin and are the main responsible for the red colour on grapes and wines. However, a high proportion of anthocyanins remains on the pomace after their removal from the must (Lingua et al., 2016).

In this quantification it was possible to detect a bigger content of anthocyanins on red grape pomace, as it can be seen in Table 3.12, where the averages and standard deviations for this quantification are presented. Although the difference between pomace was greater for the control and Pentopan treatments. The variations between grapes was expected since anthocyanins are

mainly associated to red grapes although white grapes have been shown to synthesize anthocyanins during the final period of ripening (Kammerer et al., 2004). Also a study made on grape skins from red and white cultivars did not found anthocyanins on any of the white cultivars tested (Katalinić et al., 2010).

As expected, the majority of the recovered content was extracted with water for both pomaces and that is also visible in Figure 3.14 (for red pomace) but especially in Figure 3.15 (for white grape pomace), with at least 90% of the content extracted in the water.

On red pomace, Celluclast and Driselase enzymatic treatments worked better than Pentopan and for the acetone extraction, with 21% more content recovered, Pectinex got a better result than the control, Pentopan and Driselase. So, when the total content was quantified, Celluclast and Driselase were the best enzymes, with significantly better values (1.2-fold) than Pentopan.

For the white pomace Celluclast was better than the Viscozyme (19% more content), control (72% more) and Pentopan (79% more) for the water extraction but when using acetone, the enzymes recovered 4-22% less anthocyanins than the control. The total extracted content confirmed Celluclast was the better option, with significantly better results than all the other treatments.

Table 3.12 – Anthocyanins content for each treatment on red and white grape pomaces

Anthocyanins (Δ Abs/g dry pomace)	Red grape pomace			White grape pomace		
	Water extraction	Acetone extraction	Total	Water extraction	Acetone extraction	Total
Control	37 ± 5 [ab]	10.4 ± 0.3 [b]	47 ± 5 [ab]	25 ± 2 [c]	2.8 ± 0.05 [a]	28 ± 2 [c]
Pentopan	33 ± 0.8 [b]	10.3 ± 0.1 [b]	43 ± 0.9 [b]	24 ± 4 [c]	2.3 ± 0.01 [c]	23 ± 0.1 [d]
Pectinex	36 ± 0.7 [ab]	11.2 ± 0.5 [a]	47 ± 0.3 [ab]	38 ± 2 [ab]	2.5 ± 0.1 [bc]	40 ± 2 [b]
Celluclast	40 ± 0.5 [a]	10.7 ± 0.3 [ab]	51 ± 0.3 [a]	43 ± 1 [a]	2.6 ± 0.08 [ab]	45 ± 1 [a]
Driselase	40 ± 3 [a]	10.5 ± 0.1 [b]	50 ± 3 [a]	39 ± 3 [ab]	2.5 ± 0.1 [bc]	39 ± 0.05 [b]
Viscozyme	35 ± 2 [ab]	10.8 ± 0.4 [ab]	46 ± 1 [ab]	36 ± 2 [b]	2.7 ± 0.2 [ab]	39 ± 2 [b]

Different letters in each column indicate statistical differences according to the Tukey test (p<0.05)
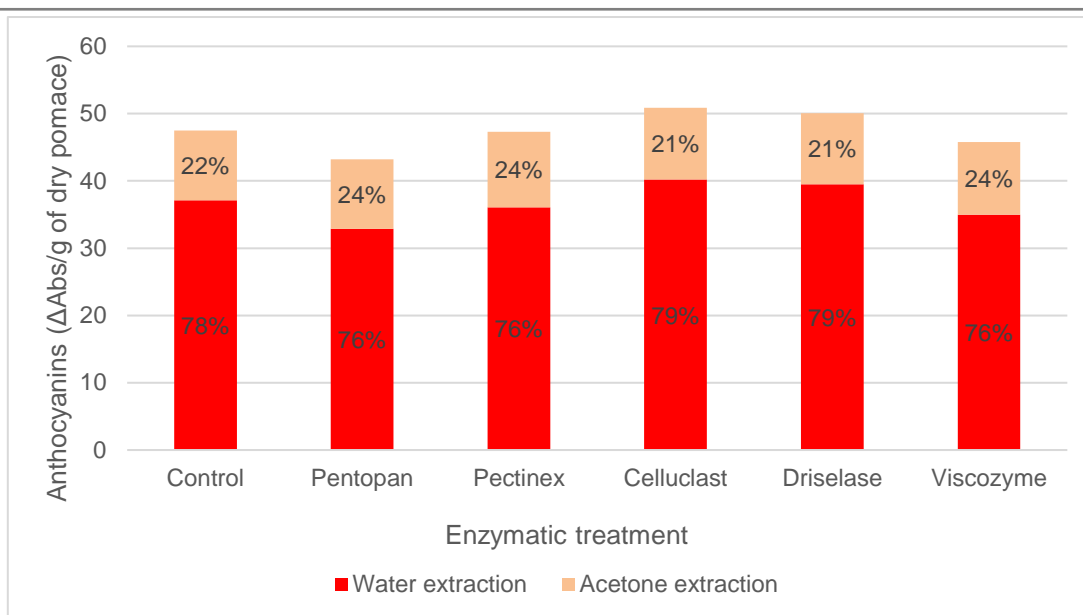
Figure 3.14 - Cumulative averages for extracted anthocyanins by each enzymatic treatment followed by 75% acetone extraction from red grape pomace. The percentages indicate the recovered anthocyanin amount related to each type of extraction over the total.

Figure 3.15 - Cumulative averages for extracted anthocyanins by each enzymatic treatment followed by 75% acetone extraction from white grape pomace. The percentages indicate the recovered anthocyanin amount related to each type of extraction over the total.

3.3.7 FLAVANOLS CONTENT OF THE EXTRACTS

Flavanols constitute the most complex sub-family of flavonoids. They can be simple monomers like (+)- catechin or (-)-epicatechin but also oligomers or polymers called proanthocyanidins (Lorrain et al., 2013).

The quantification of flavanols (averages and standard deviations) is presented on Table 3.13. In this assay, the recovered content was slightly higher for red grape pomace (from 8% more with

Driselase to 41% more with Pentopan or without enzymes) and Celluclast seems to be the best option for both pomaces.

Globally, the acetone extraction was more efficient in particular for red grape pomace, as it is seen on Figures 3.16 and 3.17.

For red grape pomace, Celluclast was the best enzyme on the extraction made with water, significantly better than all the other treatments (from 16-48% more flavanols recovered). Similar thing happened with the acetone, where, with the exceptions of the control and Pentopan, Celluclast proved to have a significant effect. On the total amount, naturally Celluclast was the best option, with a 0.50 to 1.2-fold increase respect to the average of the other samples.

Looking at the water/enzymatic extraction values for white pomace, Celluclast appears, again, to be the most efficient treatment, better than Pectinex (1.4-fold) and Viscozyme (1.3-fold). With the acetone, Celluclast was the best option, and with 1.4-fold significance over Pectinex. When the total was calculated, Celluclast repeated the highlight for best treatment (12-40% more content recovered than the other treatments) and Pectinex the worst, but there was no other difference, apart from those two treatments.

Generally, for this assay, Celluclast was the most efficient for both pomaces, unlike Pectinex, which got the worst values. These results are in agreement with those obtained for flavonoids and anthocyanins.

Table 3.13 – Flavanols content for each treatment on red and white grape pomaces

Flavanols (mg CAT eq/g dry pomace)	Red grape pomace			White grape pomace		
	Water extraction	Acetone extraction	Total	Water extraction	Acetone extraction	Total
Control	12.2 ± 0.9 [b]	18.4 ± 3 [abc]	31 ± 2 [b]	9.9 ± 0.9 [ab]	12.6 ± 1 [ab]	22 ± 2 [ab]
Pentopan	10.5 ± 0.5 [bc]	20.3 ± 0.6 [ab]	31 ± 0.8 [b]	10.4 ± 1 [ab]	12.7 ± 0.3 [ab]	22 ± 0.1 [ab]
Pectinex	9.6 ± 0.4 [c]	15.5 ± 3 [bc]	25 ± 3 [c]	8.8 ± 0.7 [b]	10.9 ± 0.7 [b]	20 ± 0.9 [b]
Celluclast	14.2 ± 1 [a]	22.2 ± 4 [a]	36 ± 4 [a]	12.2 ± 2 [a]	15.7 ± 3 [a]	28 ± 5 [a]
Driselase	11.5 ± 1 [b]	15.7 ± 0.6 [bc]	27 ± 1 [bc]	10.1 ± 1 [ab]	14.9 ± 0.05 [ab]	25 ± 0.7 [ab]
Viscozyme	9.6 ± 0.5 [c]	14.4 ± 1 [c]	24 ± 2 [c]	9.5 ± 0.05 [b]	12.3 ± 0.5 [ab]	22 ± 0.6 [ab]

Different letters in each column indicate statistical differences according to the Tukey test (p<0.05)
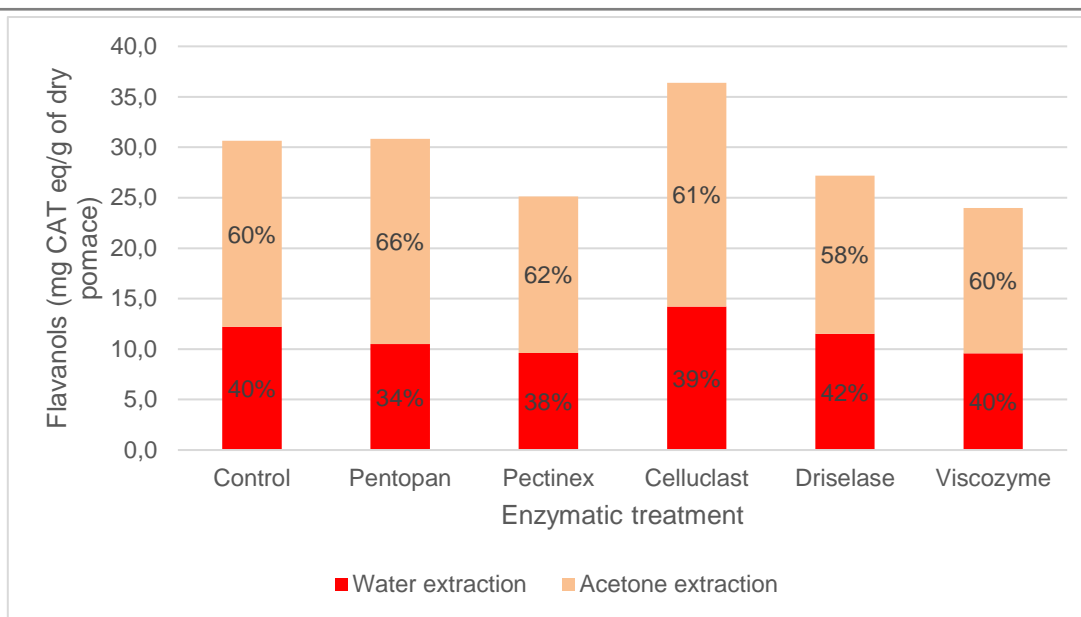
Figure 3.16- Cumulative averages for extracted flavanols by each enzymatic treatment followed by 75% acetone extraction from red grape pomace. The percentages indicate the recovered flavanol amount related to each type of extraction over the total.

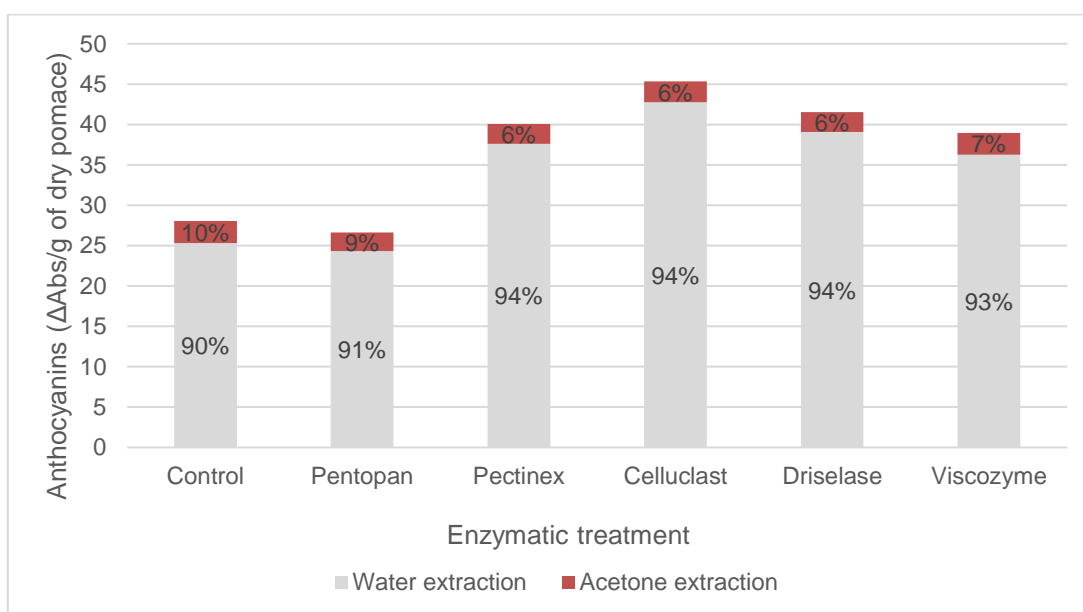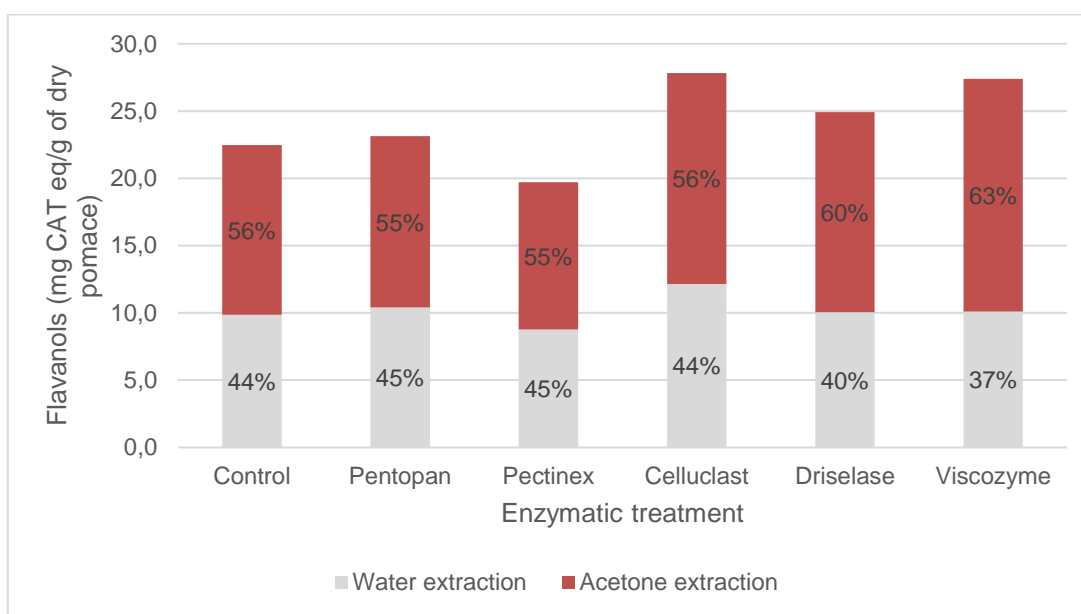

Figure 3.17 - Cumulative averages for extracted flavanols by each enzymatic treatment followed by 75% acetone extraction from white grape pomace. The percentages indicate the recovered flavanol amount related to each type of extraction over the total.

The results obtained are 7.1 to 9.9-fold higher than the 2.82 mg/g dry pomace extracted from Zalema grape pomace (white cultivar) (Jara-Palacios et al., 2014a), although this value represents the sum of the individual flavanols compounds quantified. Another study, for white grape pomaces from 9 different cultivars, presented total flavanols values, obtained by the sum of each compound identified, between 3.25 mg/g dry pomace (Baladí cultivar) and 8.74 mg/g dry pomace (Moscatel cultivar) (Jara-Palacios et al., 2014b). Those values were obtained using 75% methanol and the highest value is similar to the control value for the water extraction (9.9 mg CAT eq/g dry pomace), but 25-80% lower than the values of the acetone extraction.

On other paper analysed, nine samples of white and red grape pomace finding a big range of values 0.29 mg/g of dry pomace for Viura (white cultivar) and 1.99 mg/g dry pomace for Merlot (González-Paramás et al., 2004). In particular, the Merlot result by these authors seems to be much lower (12 to 18-fold) than the values found on this work. A third study using different temperatures, hours of incubation, solvent and enzymes (2 hours of incubation at 30 ºC) obtained more efficient extractions from 95% ethanol than water, similarly to present data, as the solvent employed was able to extract more (Ferri et al., 2017).

3.4   HPLC ANALYSIS

The final step of the work was to identify and quantify the compounds extracted from the grape pomace. Two enzyme treatments were selected for each pomace plus the control: Viscozyme and Celluclast for white pomace and Pentopan and Celluclast for red pomace. These enzymes were selected considering the best results of compound quantifications, but also looking at the prices of each enzyme. In fact, it's important to consider the costs associated to the enzymes, to assure that it's possible to scale up the optimised protocols to an industrial setting.

For this analysis the extracted content for the water/enzymes and following acetone extractions were considered. HPLC analysis was made by 45 minutes runs where several chromatograms were generated. Figures 3.18 and 3.19 refer to acetone extraction, for red and white grape pomace, at 270 nm.
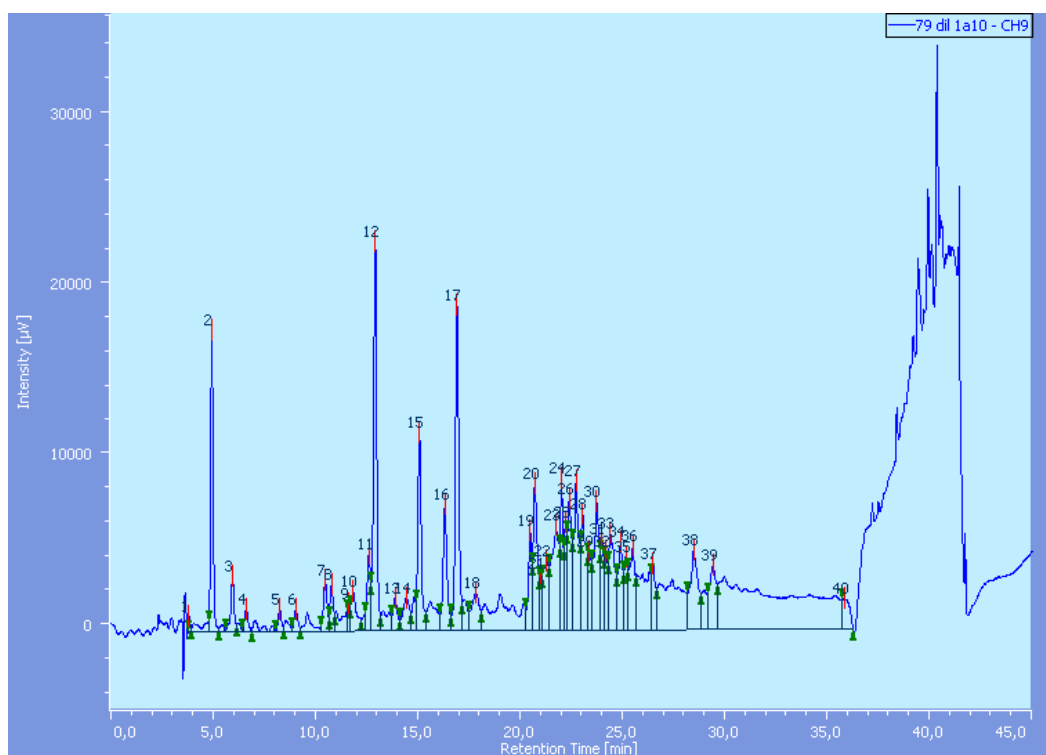
Figure 3.18 – Chromatogram obtained by RP-HPLC-DAD injection, at 270 nm, for red grape pomace acetone extracts

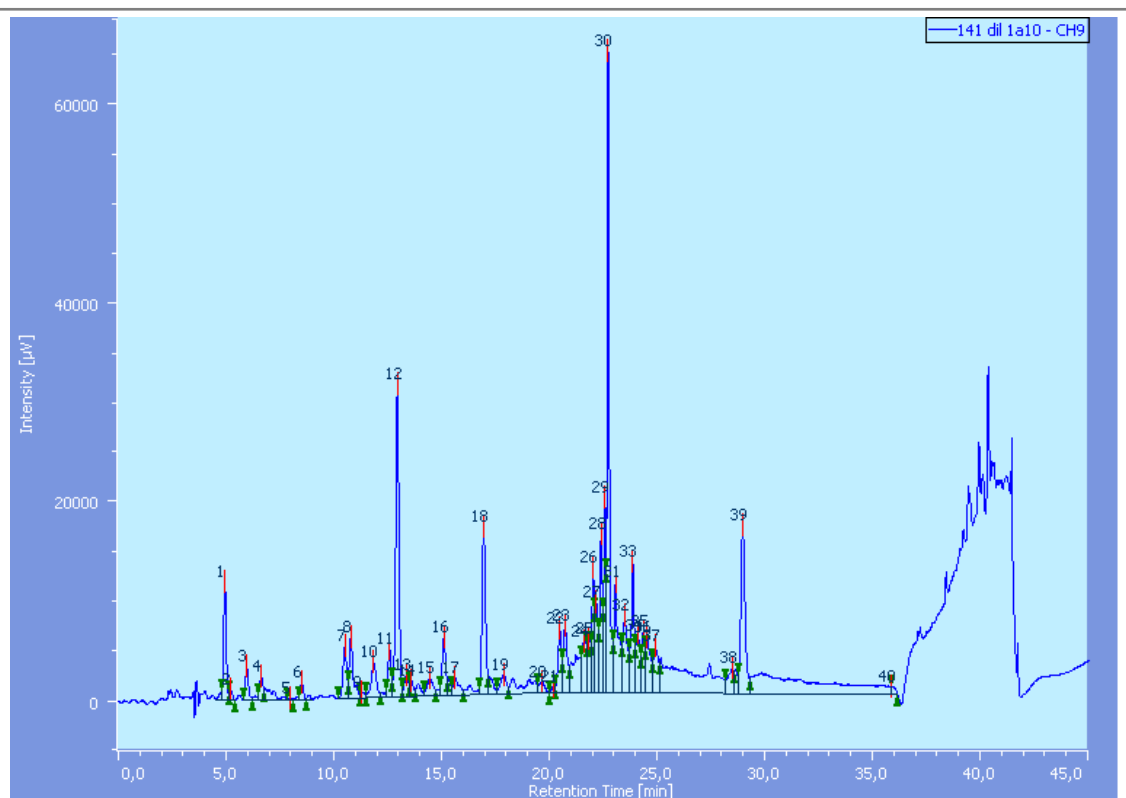**Figure 3.19 - Chromatogram obtained by RP-HPLC-DAD injection, at 270 nm, for white grape pomace acetone extracts**

The identified compounds and their concentration (means and standard deviations in mg compound/g dry pomace) extracted from both pomaces are presented on Table 3.14. Also, on Figure 3.20 the cumulative concentration of the compounds for each treatment is displayed.

Table 3.14 – Quantification of the identified compounds extracted from the pomaces (mg compound/g of dry pomace)

		Treatment	GA	PROT	EGC1	EGC2	CAT1	CAT2	VAN.A	SYR.A	EC	c-PIC	c-RESV	RUT	QUERC
Red grape pomace	Water extraction	Control	0.11 ± 0.03	0.02 ± 0.00	0.44 ± 0.03	0.52 ± 0.09	0.25 ± 0.01	0.50 ± 0.04	0.12 ± 0.00	0.06 ± 0.00	0.26 ± 0.02	0.09 ± 0.04	0.01 ± 0.01	-	0.01 ± 0.01
		Pentopan	0.09 ± 0.06	0.02 ± 0.00	0.43 ± 0.16	0.53 ± 0.09	0.18 ± 0.06	0.62 ± 0.26	0.13 ± 0.02	0.06 ± 0.00	0.26 ± 0.26	0.09 ± 0.02	-	-	-
		Celluclast	0.11 ± 0.10	0.02 ± 0.00	0.58 ± 0.13	0.59 ± 0.04	0.30 ± 0.04	0.78 ± 0.21	0.16 ± 0.05	0.08 ± 0.01	0.44 ± 0.12	0.12 ± 0.05	-	-	0.01 ± 0.01
	Acetone extraction	Control	0.01 ± 0.01	0.01 ± 0.00	0.20 ± 0.11	0.12 ± 0.01	0.11 ± 0.02	0.71 ± 0.25	0.06 ± 0.02	0.03 ± 0.01	0.43 ± 0.13	0.14 ± 0.04	-	-	0.15 ± 0.01
		Pentopan	0.03 ± 0.03	0.01 ± 0.00	0.28 ± 0.03	0.05 ± 0.05	0.08 ± 0.02	0.75 ± 0.04	0.06 ± 0.00	0.03 ± 0.00	0.45 ± 0.02	0.13	-	-	0.15 ± 0.03
		Celluclast	0,02 ± 0.01	0,01 ± 0.01	0,37 ± 0.06	-	0,10 ± 0.02	0,81 ± 0.15	0,07 ± 0.01	0,04 ± 0.00	0,50 ± 0.09	0,17 ± 0.09	-	-	0,21 ± 0.03
White grape pomace	Water extraction	Control	0.22 ± 0.04	0.01 ± 0.01	0.96 ± 0.28	1.38 ± 0.21	-	0.75 ± 0.10	-	-	0.27 ± 0.01	0.10 ± 0.14	0.01 ± 0.00	0.42 ± 0.15	-
		Viscozyme	0.25 ± 0.04	0.01 ± 0.01	0.98 ± 0.03	1.35 ± 0.16	-	0.84 ± 0.14	-	-	0.30 ± 0.07	0.18 ± 0.06	0.01 ± 0.01	0.43 ± 0.05	0.01 ± 0.01
		Celluclast	0.25 ± 0.04	0.01 ± 0.01	0.98 ± 0.13	1.57 ± 0.06	0.31 ± 0.01	0.86 ± 0.01	-	-	0.34 ± 0.03	0.19 ± 0.01	0.01 ± 0.00	0.42 ± 0.04	-
	Acetone extraction	Control	0.02 ± 0.02	0.00 ± 0.00	0.18 ± 0.09	0.23 ± 0.03	-	0.59 ± 0.08	-	-	0.27 ± 0.07	0.03 ± 0.03	-	0.20 ± 0.01	0.11 ± 0.01
		Viscozyme	0.03 ± 0.03	0.00 ± 0.00	0.31 ± 0.05	0.24 ± 0.06	0.41 ± 0.50	0.73 ± 0.04	-	-	0.33 ± 0.03	0.05 ± 0.02	-	0.21 ± 0.04	0.12 ± 0.00
		Celluclast	0.01 ± 0.01	0.00 ± 0.00	0.37 ± 0.03	0.26 ± 0.03	0.07 ± 0.01	0.90 ± 0.08	-	-	0.35 ± 0.03	0.07 ± 0.03	-	0.19 ± 0.04	0.10 ± 0.02

GA- gallic acid; PROT- protocatechuic acid; EGC- epigallocatechin; CAT- catechin; VAN.A- vanillic acid; SYR.A- syringic acid; EC- epicatechin; c-PIC- cis-piceid; c-RESV- cis-resveratrol; RUT- rutin; QUERC- quercetin
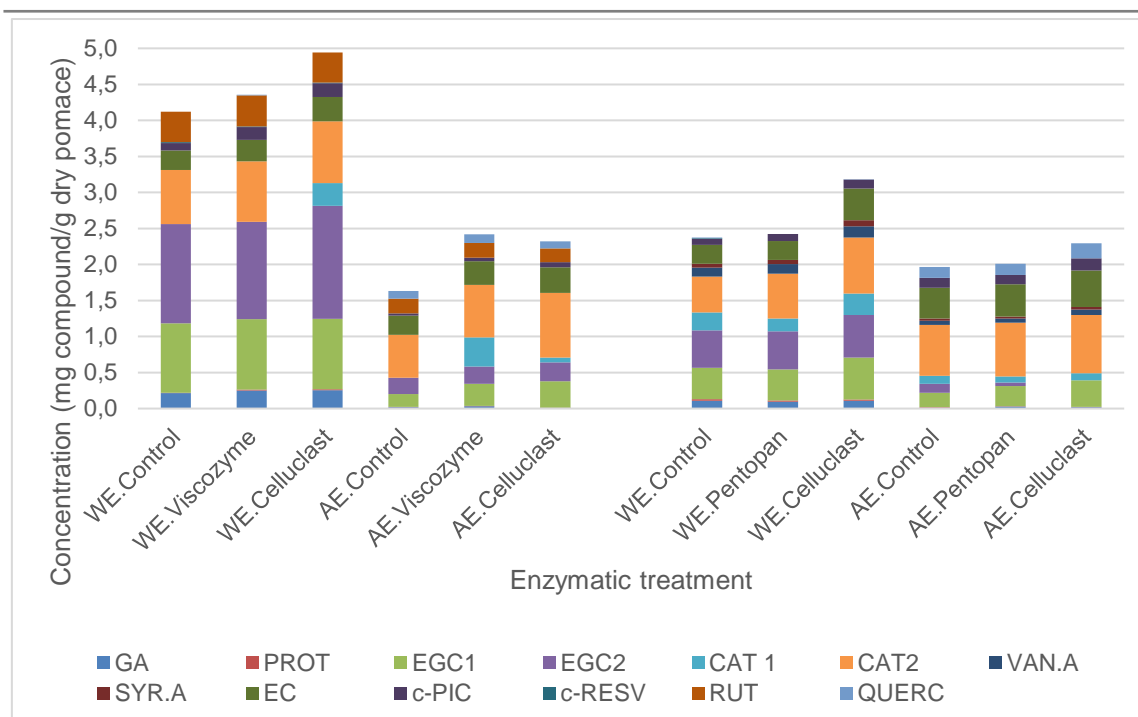
Figure 3.20 – Cumulative concentrations of quantified compounds by HPLC for each enzymatic treatment (GA- gallic acid; PROT- protocatechuic acid; EGC- epigallocatechin; CAT- catechin; VAN.A- vanillic acid; SYR.A- syringic acid; EC- epicatechin; c-PIC- cis-piceid; c-RESV- cis-resveratrol; RUT- rutin; QUERC- quercetin; WE- water extraction; AE- acetone extraction). The bars on the left represent white pomace, while the bars on the right are from red grape pomace.

This data show a wider spectrum of compounds and a higher total concentration was recovered from white grape pomace than from red pomace. In addition some compounds, like vanillic and syringic acids, were only found on red grape pomace, while rutin was only found on white grape pomace. Another important note is that, fot both pomaces, most of the content was recovered in the water extracts, especially for white grape pomace.

The present data represent an advance on what is known since there isn't an abundance of works with the identification and quantification of phenolic compounds on grape pomace and it was possible to find only another work where red and white grape pomace were studied at the same time, on the same conditions. On that work, that quantified compounds like gallic acid, catechin, quercetin and rutin, only the latter was recovered with higher concentration on white pomace (Martins et al., 2016).

In the present work, in general, for both pomaces, epigallocatechin, catechin and epicatechin were found on the biggest concentrations while some compounds like protocatechuic acid or cis-resveratrol were found only in very small amounts.

On Table 3.14 and Figure 3.20 it's possible to see catechin and epigallocatechin identified twice as EGC1 and 2 and CAT 1 and 2. This means that the same compound was identified in two peaks having two different retention times, as consequence of conformations, probably due to a different glycosylation process.

Globally, all of the identified compounds are either phenolic acids, flavonoids (flavanols and flavonols) or stilbenes. Phenolic acids can be divided in hydroxybenzoic and hydroxycinnamic

acids and several compounds from both classes were searched for but only gallic, protocatechuic, vanillic and syringic acids were found, all hydroxybenzoic acids. The results related to the four mentioned compounds were maily recovered in water extracts and in white grape pomace. While gallic acid was the main phenolic acid found in white pomace, conversely vanillic acid was that most abundant in red grape pomace. Gallic acid is a relevant compound since it acts as a precursor of hydrolysable tannins (Teixeira et al., 2014) and it has free radical scavenger activity (Xia et al., 2010). Almost all of the gallic acid content was extracted with the water, on both pomaces.

It seems that the use of enzymes was important to extract a higher phenolic yield than the controls. Globally Celluclast allowed for slightly better results on most extractions, especially with the water/enzymes (13-20% more for white pomace and 31-34% for red). The results for Pentopan (on red grape pomace) are similar to the control. In acetone extracts only Viscozyme (for white pomace) is more efficient than the control.

Looking at all the extractions, most of the content was recovered with the water/enzyme treatments, mainly on white grape pomace. On this pomace the difference in phenol content between the two extractions can be attributed to epigallocatechin and catechin, since the change of the levels of the other compounds is not significant. This could be attributed to a higher concentration of both compounds present in the pomace which were then largely recovered after the first water/enzyme extraction. Opposite results were obtained in recent works utilising a sequential (water and ethanol) extraction process for white grape pomace where epigallocatechin and catechin recovery was mainly through the ethanol (Ferri et al., 2017) or with the recovery of catechin from red grape pomace (Ferri et al., 2016).

When looking at Table 3.14, the data for gallic acid are comparable to most of the results found on other studies (Rockenbach et al., 2011, Antoniolli et al., 2015, Cheng et al., 2012, Ferri et al., 2017, Jara-Palacios et al., 2014a, Jara-Palacios et al., 2014b). Similar trend was found also for protocatechuic and vanillic acids. Conversely, for the syringic acid from red pomace, the results on this work are much smaller (from 3.5 to 230-fold for the Viscozyme water value) to those obtained by other authors (Antoniolli et al., 2015, Tournour et al., 2015, Ribeiro et al., 2015).

Flavonols represent most of the content of the identified compounds, regardless of the pomace or treatment considered. Catechin, epicatechin and epigallocatechin were found on the biggest concentrations and these compounds have been associated to important bioactivities like antibacterial functions (for catechin and epicatechin) and free radical scavenging, anticancer and anti-inflammation activity for catechin (Xia et al., 2010).

Compounds like catechin or epicatechin have been reported as the main phenolic compound extracted from pomaces of different origins by several authors (Tournour et al., 2015, Jara-Palacios et al., 2014b, Rockenbach et al., 2011, Bonilla et al., 1999, Reis et al., 2016, Martins et al., 2016, Ferri et al., 2017, Antoniolli et al., 2015, Cheng et al., 2012) with results similar to some of the values presented on Table 3.14 like 0.55 mg of catechin/g dry pomace (with Malbec red

grape pomace) (Antoniolli et al., 2015), 0.80 mg catechin/g dry pomace (with Moscato white grape pomace (Martins et al., 2016), 0.33 mg epicatechin/g dry pomace (Zalema white grape pomace) (Jara-Palacios et al., 2014b) or 0.26 mg epicatechin/g dry pomace (Merlot grape pomace) (Rockenbach et al., 2011) but it's noticeable that there is a great variability of values presented by different authors, including those on Merlot pomaces.

Two stilbenes were identified in the samples: cis-piceid and cis-resveratrol. In the case of the latter, a very small amount was extracted after water enzymatic digestion of white pomace and control of red pomace, but for cis-piceid a bigger content was extracted from all the treatments on both pomaces. On red pomace, more cis-piceid content was recovered from the water (42-55% more), while for white pomace, most of the content was extracted through the acetone (2.7 to 3.6-fold). Cis-resveratrol was detected in almost every wine regardless of the origin or winemaking process, although it is not a natural constituent of the grapes but his formation is due to isomerisation after exposure to natural UV light (Atanacković et al., 2012). This compound has not been the subject of a lot of publications since it was only recently that the presence of cis-resveratrol on white grape pomace was reported for the first time (Ferri et al., 2017).

There aren't a lot of works quantifying these two compounds since the main stilbene searched has always been only trans-resveratrol. Only two papers reported results on both compounds and presented some similar results, to the present work, of cis-piceid for white pomace (0.1-0.12 mg/g dry pomace) (Ferri et al., 2017) but for red pomace the results are similar (0.12-0.15 mg/g) for the ethanol extracts from dried grape pomace and higher (0.33-0.44 mg/g) for wet pomace ethanol extracts (Ferri et al., 2016) than those displayed on Table 3.14. Cis-resveratrol was not detected on both studies.

Rutin and quercetin were the flavonols identified. This compounds, associated with free radical scavenging activity (Xia et al., 2010), belong to the flavonoid family, where anthocyanins and flavanols like the catechins are included.

Rutin was not found on the Merlot samples of the present work, unlike other Merlot extractions where contents of 0.41 mg/g dry pomace (Rockenbach et al., 2011) or 1.7 mg/g (Ribeiro et al., 2015) were discovered. For white grape pomace, a study testing enzyme-assisted extractions obtained values of 0.15 mg/g dry pomace from Moscato grapes (Martins et al., 2016) and this value is comparable to what was extracted using the acetone here, but less than half of what was in the water.

Quercetin is associated to antibacterial functions and enhancing plasma nitric oxide level (Xia et al., 2010). In this work quercetin was detected in both pomaces, especially when the acetone was used. It was found at similar concentrations to what was presented on Merlot pomace (Lingua et al., 2016) but very different concentrations from other Merlot report (1.7 mg quercetin/g dry pomace) (Ribeiro et al., 2015). In white pomace, the quercetin levels in water extracts (0.01 mg/g dry pomace with the Viscozyme treatment) are very close to those found by other authors on Moscato (Martins et al., 2016) or Zalema (Jara-Palacios et al., 2014a) cultivars. Also it seems that most of the quercetin was recovered with the acetone. The results for quercetin on the present

work agree with the idea that the by-products of winemaking from red varieties present higher amounts of flavonols than those from white varieties (Teixeira et al., 2014).

On the present work rutin, when present on white pomace, was the main flavonol, but that was not the case in a study with red grape pomace from Syrah, Merlot and Cabernet Sauvignon cultivars, where quercetin was in the main compound of this family (Lingua et al., 2016).

Compounds absent from Table 3.14 were not found on the samples. This is the case for trans-resveratrol, whose presence was expected (Rockenbach et al., 2011). This stilbene is abundant on grape skins and present in higher concentrations in red grape varieties, rather than white varieties (Careri et al., 2003). Resveratrol is an important compound since it is a potent antimutagenic, antioxidant, anti-inflammatory and anti-proliferative agent (Rockenbach et al., 2011). This last study extracted t-resveratrol contents of 0.06 mg/g dry pomace for Merlot grapes or 0.04 mg/g for Cabernet Sauvignon but, other study, with Malbec grape pomace extracted a lower content of 0.006 mg/g dry pomace (Antoniolli et al., 2015), demonstrated that it was present in small amounts.

Antoniolli and co-workers also recovered piceatannol (not found on the present work), another stilbene, and reported that its content in grapes is about 4-times lower than that of resveratrol (Antoniolli et al., 2015).

Concentrations of stilbenes can vary a lot since they are produced as a response to physiological stressing factors like ozone and UV-C radiation. Therefore, the content can be modified by the industrial process used for winemaking (Teixeira et al., 2014).

The absence of some hydroxycinnamic acids was not expected. Compounds like caffeic, p-coumaric or ferulic acids have been previously detected in other grape cultivars, with results from Merlot or other cultivars (Antoniolli et al., 2015, Jara-Palacios et al., 2014a, Martins et al., 2016, Ramirez-Lopez and DeWitt, 2014, Reis et al., 2016, Ribeiro et al., 2015, Tournour et al., 2015).

Flavonols like kaempferol or myrecetin were found on some publications concerning Merlot pomaces (Lingua et al., 2016, Ribeiro et al., 2015), in spite of not being detected on this work. The latter has not been detected in organic residues from white grape varieties and it's been suggested that could be related to the absence of the enzyme flavonoid-3',5'-hydroxylase (Teixeira et al., 2014).

4 CONCLUSION

Millions of tonnes of food waste are produced yearly, becoming an economical and environmental problem that needs to be better addressed. The wine industry is responsible for producing large amounts of wastes in a short period of time and grape pomace is the biggest example where considerable quantities of phenolic compounds remain. These valuable compounds can be recovered through extraction protocols and applied to various purposes on the food, animal feed, packaging or cosmetic industries.

This work aimed at optimizing a combined phenol extraction protocol (using enzymes and solvents) and the first step was to select the best conditions of temperature, solid:liquid ratio, time of incubation and concentration of enzyme. After testing different possibilities, 50 ºC, 1:10 solid:liquid ratio, 2 hours and 2% enzyme concentration were the selected conditions for the next steps of the work.

With a sequential extraction method (first with water/enzymes followed by 75% acetone), five enzymes were applied to the extraction of phenolic compounds from red and white grape pomace and the extracts were characterized. The performance of the enzymes was dependent on the quantification, grape or extraction, but it seems that there was a tendency for Viscozyme to extract higher contents of total phenolics, protein or reducing sugars; while flavonoids were better extracted with Celluclast. Globally, the use of enzymes improved the extraction of the compounds. Also, it was noticed that doing a sequential extractions allowed for a greater recovery of phenolic compounds from red and white grape pomace.

Through the application of a HPLC technique, some selected compounds were detected and quantified, with epigallocatechin and catechin being the most concentrated. It also showed that it was possible to recover a higher phenolic yield from white grape pomace rather than the red grape pomace.

The results present on this work prove that the recovery of phenolic compounds from grape pomace it's one of the viable possibilities to reuse this waste as a cheap source of rich bioactive compounds that can, later, be used on other industries.

Future work is needed to continue improving the extraction conditions, but also all the process from the moment of pomace collection should be considered. Such a rich and perishable material needs to be preserved or treated right after it's collected from the production of wine. The efficiency of the extraction is also dependent on the pre-treatments used or the extraction method and new techniques have been tested and optimized to make it more appealing for use outside of the laboratory. Future work should also consider if one specific group of phenolic compounds is the intended for recovery, since there are several differences between the classes of these compounds, in regard to the extraction, that research should go deep further onto.

On the line of research used in this work perhaps would be interesting to see if a solid:liquid ratio of 1:20 would result in better recoveries. Also, a different time and temperature balance might improve the outcome of the extractions, but it must be adjusted to the target compounds.

The application of the enzymes can represent an effective way to enhance the extraction yields or even to try to reduce the solvents' usage, but more must be known about the possibilities of these or other enzymes representing a benefit for the recovery. Regarding the use of enzymes, there are lots of possibilities to study: from the type of enzyme, to the concentration or even some potential synergistic effect of some enzymes when combined. The amount of enzyme used could be defined considering the amount of protein within the enzymatic preparation, instead of considering the weight of the enzyme preparation in the mixture (as in the study done). It should be also studied the hypothesis of recovering the enzymes used through an immobilization technique would allow their reuse for further work.

Optimum conditions for the recovery of these valuable compounds would mean another revenue for the wine industry, a cheap source of phenolic compounds for other industries and a big effort diminishing environmental burdens.

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