Universidad de Valladolid

Escuela de Doctorado Universidad de Valladolid

PROGRAMA DE DOCTORADO EN INGENIERÍA QUÍMICA Y AMBIENTAL

TESIS DOCTORAL:

DEVELOPMENT OF AN INTEGRATED AND GREEN BIOREFINERY FROM WINERY WASTE. APPLICATION TO WINE LEES AND GRAPE STEMS

Presentada por Rut Romero Díez para optar al grado
de
Doctor/a por la Universidad de Valladolid

Dirigida por:

Soraya Rodríguez Rojo
Ana A. Matias
María José Cocero Alonso

Memoria para optar al grado de Doctora,

Con Mención Doctor Internacional,

Presentado por la Ingeniera Química:

RUT ROMERO DÍEZ

Siendo los tutores en la **Universidad de Valladolid**:

Dra. D. Soraya Rodríguez Rojo

Y

Dra. D. María José Cocero

Y

Y en el **Instituto de Biologia Experimental e Tecnológica (IBET), Lisboa (Portugal):**

Dra. D. Ana A. Matias

Valladolid, Octubre de 2018

UNIVERSIDAD DE VALLADOLID

ESCUELA DE INGENIERÍAS INDUSTRIALES

Secretaría

La presente tesis doctoral queda registrada en el folio número

_____ del correspondiente libro de registro número _____.

Valladolid, a _____ de_____ de 2018

Fdo. El encargado del registro

Soraya Rodríguez Rojo

Investigadora de BioEcoUVa, Instituto de Bioeconomía de la Universidad de Valladolid

y

María José Cocero Alonso

Catedrática (Professor)

Departamento de Ingeniería Química y Tecnología del Medio Ambiente y directora de BioEcoUVa, Instituto de Bioeconomía ambos de la Universidad de Valladolid

y

Ana A. Matias

Directora del grupo de investigación 'Nutraceuticals & Bioactives Process Technology' perteneciente a IBET (Portugal)

Certifican que:

Rut Romero Díez ha realizado bajo su dirección el trabajo "DEVELOPMENT OF AN INTEGRATED AND GREEN BIOREFINERY FROM WINERY WASTE. APPLICATION TO WINE LEES AND GRAPE STEMS", en el Departamento de Ingeniería Química y Tecnología del Medio Ambiente de la Escuela de Ingenierías Industriales de la Universidad de Valladolid. Considerando que dicho trabajo reúne los requisitos para ser presentado como Tesis Doctoral expresan su conformidad con dicha presentación.

Valladolid, a _____ de _____ de 2018

Fdo. Soraya Rodríguez Rojo

Fdo. María José Cocero Alonso

Fdo. Ana A. Matias

Reunido el tribunal que ha juzgado la Tesis Doctoral titulada "DEVELOPMENT OF AN INTEGRATED AND GREEN BIOREFINERY FROM WINERY WASTE. APPLICATION TO WINE LEES AND GRAPE STEMS" presentada por el Ingeniero Químico RUT ROMERO DÍEZ y en cumplimiento con lo establecido por el Real Decreto 99/2011 de 28 de enero de 2011 acuerda conceder por _____ la calificación de _____.

Valladolid, a _____ de _____ de 2018

PRESIDENTE SECRETARIO

VOCAL

Caminante no hay camino, se hace camino al andar.

INDEX

ABSTRACT

Abstract

Nowadays, the valorisation of residues generated in the agro-food industry for the recovery of high added values compounds, is one of the most important fields of study. A clear example of this, is the vinification grape-derived sub-products. Due to the fact that the wine sector is one of the most important activities all around the world, grapes are one of the most cultivates fruit all over the world. However, the vinification process generates huge amount of waste. Only in Spain, around 2-3 million tons of residues are generated annually. In other words, 25kg of sub-products are produced per 100L of red wine. Depending on the vinification step in which they are generated, different residues can be distinguished. The most abundant waste is the grape pomace since it represents the 62% of the total residues generated in a winery. Grape pomace is constituted by grape pulps, skins and seeds. It is obtained after the crushing of the grapes performed to get the must. The second most abundant residue are wine lees (14%). Lees are the dregs that settled at the bottom of the vessels after the wine fermentation or aging processes. Finally, grape stems and dewatered sludge are the least abundant sub-products, each of them represent a 12%. The former, are the woody skeleton of the grape bunches and they are obtained after the destemming process, just before the grape crushing. Grape stems, as grape pomace, has a lignocellulosic composition. This means that, apart from their high content of phenolic compounds, cellulose, hemicellulose and lignin are present in relative significant concentrations (up to 25%). Consequently, grape stems are also a rich source of these polymers that can be also hydrolysed into their monomeric sugars components and, posteriorly, converted in high added value products such as fuels, bulk chemicals and materials. Finally, the dewatered sludge comes from the washing and disinfection steps.

Although these wastes are considered as “non-hazardous” residues, they present a high organic load. Therefore, if they are not treated properly they can cause several environmental problems that are partially phytotoxic effects and/or soil acidification. This elevated content of organic matter, is to a large extent due to their high concentration of polyphenols. Polyphenols present more than one aromatic ring with several hydroxyl groups as substituents. They present recognized health-promoting effects due to their antioxidant, antimicrobial, anti-inflammatory and cardio protective properties. Thanks to this, they are compounds with a strong potential application in food, cosmetics, and

pharmaceutical industries. Thus, several and different processes have been developed for the recovery of polyphenols from grape sub-products, especially grape pomace. Though, few literature is available regarding the extraction of high-added-value compounds from either wine lees or grape stems. Additionally, as it was previously mentioned grape stems are a rich source of biopolymers and sugars which can be further converted into bulk materials. For this reason, this thesis is focused on the valorisation in terms of high added value compounds of both wine lees and grape stems by alternative greener methodologies. The general idea was to maximize and intensify the extraction of high added value compound from these two residues in terms of polyphenols, and also sugars and oligomers for the case of grape stems.

To do so, a total of five chapters were developed in this thesis. In Chapter I the identification of the main phenolic compounds present in aging wine lees extracts, mainly anthocyanins, was carried out. Moreover, correlations between them and different antioxidant activities were developed. In Chapter II and III, the kinetic extraction curves of anthocyanins from different types of wine lees were achieved in order to obtain the parameter values (solid-liquid ratio, solvent composition and temperature) that maximized the extraction of anthocyanins. Once these parameters were obtained, several pre-treatments were applied before the conventional solid-liquid extraction so as to intensify the extraction process. These pre-treatments involved the use of microwaves (MW), ultrasounds (US) and enzymes. Like in the previous chapter, the antioxidant activities of the different extracts was determined and main compounds were identified. The valorisation of grape stems was done in Chapters IV and V. In Chapter IV, the extraction kinetics of total polyphenols and flavonoids from grape stems were studied in the same way that for wine lees. Similarly MW were applied as a pre-treatment in order to enhance the polyphenols recovery. Regarding Chapter V, it was focus on the obtention of extracts rich in sugars and oligomers using a hydrothermal hydrolysis. Temperature and operating time were the variables studied in this chapter.

Chapter I: *Phenolic characterization of aging wine lees correlation with antioxidant activities*

In **Chapter I** aging wine lees were characterized in terms of total phenolic (TPC, as gallic acid equivalents GAE) and total flavonoid contents (TFC, as catechin equivalents, CAE). Aging wine lees come from the step in which wine is aged inside wood barrels. Once the solid phase of the wine lees were freeze-dried, they were subjected

to several solid-liquid extractions. These extractions were carried out using a solid-liquid ratio of 0.025 (0.25g of dry lees in 10mL of solvent), stirring for 5min at room temperature followed by 10 min of sonication in a ultrasonic bath. Solvents with different polarities (water, methanol, ethanol, two hydroalcoholic mixtures and acetone) were used. Total phenolic (TPC) and total flavonoid contents (TFC) were determined and expressed per gram of dried extract (DE). The mixture of 75% vol. ethanol showed the highest values with 254mg_{GAE}/g_{DE} and 146mg_{CATE}/g_{DE}, respectively. To determine the antioxidant activities (AA) of the extracts, different assays were studied. The highest HORAC (as catechin equivalents, CAE), HOSC and FRAP values (both as Trolox equivalents, TE) were obtained for the hydroalcoholic mixture with a 75% vol. ethanol (4,690$\mu mol_{CAE}/g_{DE}$, 4,527$\mu mol_{TE}/g_{DE}$ and 2,197$\mu mol_{TE}/g_{DE}$, respectively). In contrast, for ORAC method (as Trolox equivalents, TE), methanol extract showed the best value with 2,771μmol_{TE} /g_{DE}. Furthermore, correlations between TPC, TFC, individual phenolic compounds and the different AA were determined. Most relevant compounds contributing to AA were identified using data from mass spectrometry. It could be asserted that anthocyanins were the major compounds present in the wine lees extracts. Those anthocyanins which presented the 6-p-coumaroyl moiety strongly contributed ($p<0.10$) to FRAP, as well as, gallic acid and the two flavan-3-ols detected. Besides, anthocynins significantly ($p<0.10$) contributed to ORAC in a negative way. Anthocyanins also contributed negatively and positively (but not significantly) to HORAC and HOSC, respectively. Depending on the solvent used different amounts of the individual compounds were extracted which could have higher or lower activity against oxygen radicals ($ROO^{\bullet}$) or ($HO^{\bullet}$) affecting the antioxidant capacity of the extracts.

Chapter II: *Microwave and ultrasound pre-treatments to enhance anthocyanin extraction from different wine lees*

Although, there are some few works in literature regarding the recovery of phenolic compounds from wine lees, none of them have studied the extraction kinetics and the parameters that maximize their extraction through conventional solid-liquid process. Furthermore, the use of pre-treatments such as microwaves (MW) or ultrasound (US) seemed to be suitable alternatives to intensify the process and to increase the polyphenols recovery. Thus, in **Chapter II**, firstly the extraction kinetics of anthocyanins (AC, as malvidin equivalents, MALE…) from different wine lees in conventional solid-liquid extraction were studied. AC were chosen as target compounds, since they are the

main sub-family of polyphenols found in grapes. The influence of parameters such as temperature, solid-liquid ratio (R_{S-L}) and type of solvent (hydro-alcoholic mixtures) was also considered. Best parameter values chosen to get most AC out from Port win lees (2.78$mg_{MALE}/g_{DRY-LEES}$) were: a temperature of 25ºC, with a R_{S-L} of 0.10 (g/mL) and with a 50%vol. ethanol mixture. After 15min of extraction, a steady AC content was achieved. Same conditions were also applied to first fermentation and second fermentation wine lees. Final AC yields of 3.04$mg_{MALE}/g_{DRY-LEES}$ and 2.09$mg_{MALE}/g_{DRY-LEES}$ were obtained, respectively. Once the extraction kinetics were studied, the application of MW and US as pre-treatments to the conventional extraction in order to increase AC yield were assessed. With the help of a statistical surface response design, the optimum conditions which maximize the final AC content of the extracts were obtained. Evaluated parameters in this design were the R_{S-L} (g/mL), the solvent composition (% vol. ethanol) and the pre-treatment time (s). The optimal values for each parameter were: a R_{S-L} of 0.140 (g/mL), a hydro-alcoholic mixture of 40% vol. ethanol and a time pre-treatment of 90s. When MW were used at these conditions, AC extraction yield was doubled for Port wine lees (6.20$mg_{MALE}/g_{DRY-LEES}$) and the required time to achieve a constant yield was reduced from 15min to 90s, since no further increase in AC yield was observed in the subsequent conventional extraction at previously selected conditions. MW pre-treatment applied to first and second fermentation wine lees, increases the anthocyanin yield 1.50 and 1.40 times, respectively. Meanwhile, US only shortened extraction time in less proportion (from 15 to 5min). Putative identification of main extract compounds was performed by LC/MS-MS. It was interesting to notice the identification of one pyranoanthocyanin (Vitisin A) in all the different types of wine lees.

Chapter III: *Enzymatic hydrolysis to enhance the anthocyanin extraction from wine lees*

Apart from the pre-treatments studied in Chapter II, it was thought that the application of an enzymatic hydrolysis pre-treatment to wine lees will increase the release of polyphenols that may be linked or absorbed in the cell wall of yeast, one of the major components of wine lees. Therefore, in **Chapter III**, two different enzymes, Glucanex and Mannaway were tested for this purpose and, additionally, a blend of both enzymes. Incubation times for the enzymatic hydrolysis to take place, ranged from 5 to 60min. No significant differences were found in the final AC extraction yield between them. The extract obtained when the enzymatic blend was used with an incubation time of 5min, was the one which showed the highest increment (50%) in the AC extraction yield respect

the conventional extraction. On the other hand, antioxidant activity of the enzymatic extracts was evaluated via ORAC assay. The highest ORAC value was achieved for extract from first fermentation wine lees treated with Mannaway. Anthocyanins were identified via mass spectrometry and no differences in composition were detected respect to the conventional extract. The sole difference was found after pre-treatment with Mannaway with an un-identified compound detected at 280nm.

Chapter IV: *Grape stems valorization: microwave pre-treatment for polyphenol extraction*

Hitherto it was the work developed for wine lees. Concerning grape stems, a similar procedure was followed. In **Chapter IV**, an intensification of the polyphenol extraction process was also performed. Firstly, the extraction kinetics of total polyphenols (TPC) and flavonoids (TFC) content were studied following the same procedure exposed in Chapter II for wine lees. A $R_{S\text{-}L}$ of 0.10g/mL, a solvent with a 50% vol. of ethanol and 75ºC were selected as the best conditions for the recovery of bioactives. At these conditions, a TPC of 38 ± 1mg_{GAE}/g_{DS} and a TFC of 38 ± 1mg_{CATE}/g_{DS} were achieved. Since the use of MW as pre-treatment significantly increase the recovery of phenolic compounds from wine lees, this procedure was also proposed to grape stems. In this case, MW pre-treatment increased the extraction yield of TPC and TFC in a 19% and 24% respect to the conventional solid-liquid extraction, respectively. Additionally, identification and quantification of the major stilbenes (resveratrol and ε-viniferin) and flavonoids (catechin and epicatechin) was carried out with HPLC-DAD-MS/MS. According to literature, grape stems extracts have interesting anti-fungal properties. Thus, activity against *Botrytis cinarea* was determined together with the antioxidant activity, measured by ORAC assay.

Chapter V: *Conversion of grape stems into sugars rich extracts via a hydrothermal process*

Putting aside the recovery of phenolics compounds, **Chapter V** was focused in valorization of grape stems as source of cellulose, hemicellulose and lignin due to their lignocellulosic composition, as it was aforementioned. A hydrothermal process for grape stems conversion into sugars and oligomers was employed for this purpose. Studied temperatures ranged from 100ºC to 180ºC during 20-minute experiments in order to evaluate its influence on sugars recovery. Finally, a temperature of 140ºC seemed to be

the most suitable to maximize the sugars yield (264mg/g_{DS}). Besides, the effect of the operating time was also determined at this temperature. Operational times from 10 to 30min were tested. Results brought out that the higher the time, the higher the yield in terms of sugars and total carbon content. In addition, within the biorefinery concept, the effect of the MW pre-treatment carried out in Chapter IV for the extraction of polyphenols was evaluated on the conversion of grape stems into oligomers and sugars. Extractives were removed, as well as the free sugars of the raw material. On the other hand, the effect of the MW which could have an effect on the oligomers, disrupting them and improvement their extraction (10%). Finally, a kinetic model was used to fit the experimental data with an average absolute deviation around 15% for sugars and oligomers and 30% for degradation products.

INTRODUCTION

Introduction

Viticulture is one of the principal agricultural activities across the world. Thus, grapes are one of the most cultivated fruit crops, being *Vitis vinifera* variety the most abundant (Grimplet, Deluc, Cramer, & Cushman, 2007). The wine sector constitutes a sign of identity in Spain because our country was the wine greatest exporter globally in the year 2017, according to the OIV Statistical Report on World Vitiviniculture ("OIV Statistical Report on World Vitiviniculture 2017," n.d.). This wine production involves huge amounts of residues and sub-products. It is estimated that, only in Spain, around 2-3 million tons of residues are generated annually (Ruggieri et al., 2009) which means that 25kg of sub-products are produced per 100L of red wine. These sub-products have different origins and can be classified depending on the vinification process in which are formed. Figure 1 shows a schematic diagram of the wine production and the main residues generated in each step.

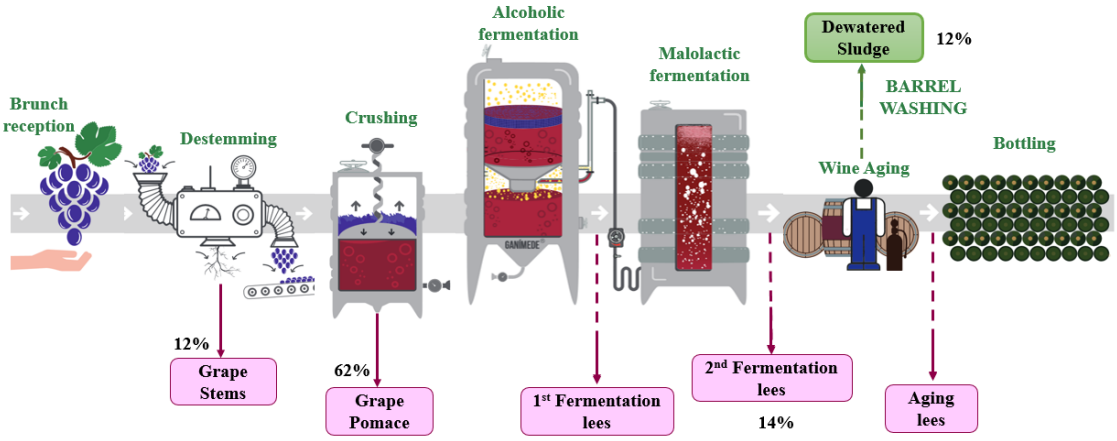


Figure 1: overall schematic diagram of the wine production, main residues and quantities of each one generated in the different stages of the vinification process.

The quantity and composition of the residues depend on the grape variety (Cruz, Domínguez, & Parajó, 2004) and the enological process (Schieber, Stintzing, & Carle, 2001). The most abundant residue is the grape pomace (mixture of grape pulps, skins and seeds), representing the 62% of the wastes generated in a winery, followed by all kinds of wine lees (14%), the stems (12%) (Devesa-Rey et al., 2011) and the dewatered sludge (12%) from the washing and disinfection steps (Flanzy, 2003). Traditionally, grape pomace and wine lees have been used as a supplement in animal feeding with a poor nutrient value, as a fermentation nutrient supplement (Dimou et al., 2015b) or to recover

chemicals, like tartaric acid (Versari, Castellari, Spinabelli, & Galassi, 2001). Sometimes they are incinerated, which entails high costs of operation and production of toxic gases potentially dangerous to human health and the environment (Mariani et al., 1992). Although these wastes are considered as "non-dangerous" residues, in many cases they are disposed in landfill constituting serious environmental problems if they are not treated properly. For instance, they may exert phytotoxic effects if applies to crops or wetlands or soil acidification associated with the high levels of polyphenols (Devesa-Rey et al., 2011; Negro, Tommasi, & Miceli, 2003). On the other hand, polyphenols are compounds with a strong potential application in food, cosmetics, and pharmaceutical industries, for their health-promoting effects due to their recognized antioxidant, antimicrobial, anti-inflammatory and cardio protective properties (Barcia et al., 2014; Landeka Jurčević et al., 2017).

1. Polyphenols

Polyphenols or phenolic compounds constitute the second group of most abundant chemical substances, after the carbohydrates in the plant kingdom (Pridham, 1965). Their biosynthesis takes place during the metabolism of the plant as a response to external pathogens, infections or ultraviolet irradiation (Beckman, 2000). From a chemical point of view, polyphenols present an extremely diverse family of molecules with a heterogeneous structure and activity. As their family name indicates, all of them present more than one aromatic ring with several hydroxyl groups as substituents. Polyphenols can be classified according to the number and distribution of the carbon atoms or by the length of the aliphatic chain linked to the aromatic core (Cheynier, Fulcrand, & Sarni, 1997). However, the most used classification criteria is based on the nature and structure of their carbon skeleton.

As result, these compounds are derived from a common carbon skeleton building block: the C_6-C_3 phenylpropanoid unit. Biosynthesis, according to this pathway, produces the two large varieties of phenols: flavonoids and non-flavonoids. The former is composed by flavonoids (C_6-C_3-C_6), proanthocyanidins [$(C_6$-C_3-$C_6)_n$], coumarins (C_6-C_3), , lignans (C_6-C_3-C_3-C_6) and lignins [$(C_6$-$C_3)_n$] (vide Figure 2). In contrast, the non-flavonoids subgroup (vide Figure 3) is formed by phenolic acids (cinnamic acids (C_6-C_3), benzoic acids (C_6-C_1)) and stilbenes (C_6-C_2-C_6) (Pereira, Valentão, Pereira, & Andrade, 2009).

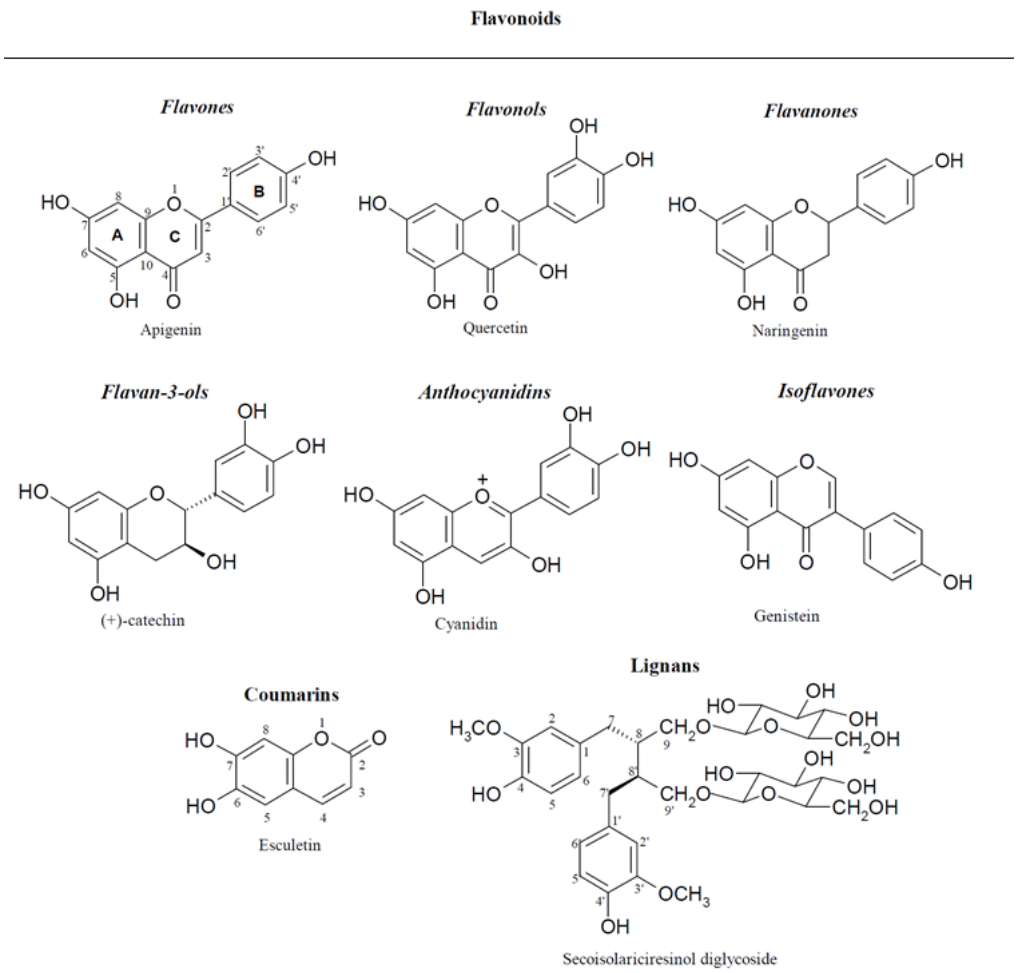


Figure 2: main structure of the flavonoid subfamily

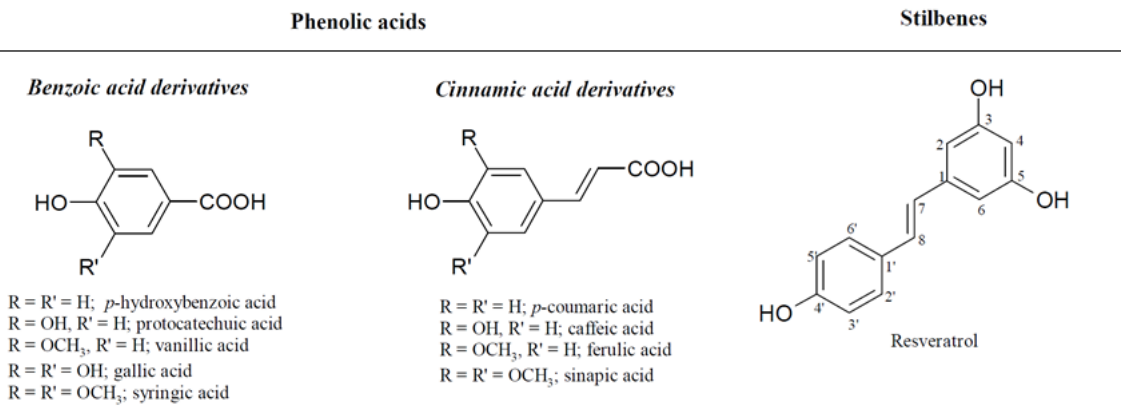


Figure 3: main structure of the non-flavonoid subfamily

The phenolic composition of the grape, and consequently of its derivates and sub-products, strongly depend on several external factors such as grape variety, climatological conditions, type of soil, the vinification process and/or the tissue take into consideration (pomace, lees, stems or seeds) (Makris, Boskou, & Andrikopoulos, 2007; Rodríguez Montealegre, Romero Peces, Chacón Vozmediano, Martínez Gascueña, & García Romero, 2006). In particular, grapes are rich in anthocyanins and phenolic acids (MONAGAS, BARTOLOMÉ, & GÓMEZ-CORDOVÉS, 2005) whereas grape stems are rich in flavanols (catechin and epicatechin) and stilbenes (resveratrol, viniferin and piceatannol). Apart from the beneficial effects that have been previously mentioned, anthocyanins are used in the agroalimentary field as natural antioxidants (instead of using synthetic antioxidants) or colorants due to the wide range of colours they can offer in function of the pH (Bordignon-Luiz, Gauche, Gris, & Falcão, 2007). Similarly, stilbenes and flavonoids have a potential interest to be used as an additives in agricultural activities due to their capacity to deter microorganisms and increasing the resistance against external pathogens thanks to the antimicrobial and anti-fungal properties (Scalbert, 1991). As an example, the stilbene resveratrol is naturally synthesized in response to several biotic and abiotic stress such as, the fungus infection derived from *Botrytis cinerea* or *Palmopara viticola*, vegetable tissue damages or frosts (Caruso et al., 2011). Its activity against *Botrytis cinarea* was firstly tested in a laboratory scale in the year 1976 (Langcake & Pryce, 1977). On the other hand, flavonoids such as catechins present good inhibitory effect against fungus growth too (Caruso et al., 2011; Weidenbörner, Hindorf, Jha, & Tsotsonos, 1990). Therefore, a revalorization of these wastes in terms of polyphenols would be interesting from the point of view of the wine market and also, it would reduce the disposal fees which are compulsory to pay to prevent contamination of the environment. The price of these taxes increase proportionally with the degree of contamination of the waste and also, depends on their nature and characteristics (Devesa-Rey et al., 2011).

A lot of information can be found in literature regarding the revalorization of grape marc (Muhlack, Potumarthi, & Jeffery, 2018). However, there is a considerable lack of information regarding the polyphenolic extraction and composition of extracts derived from wine lees and grape stems (Teixeira et al., 2014).

2. Winery residues explored in this thesis work

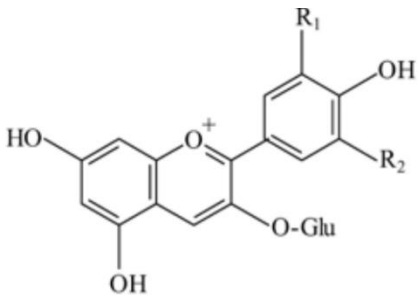
2.1 Wine lees

EEC regulation No. 337/79 states that "wine lees is the residue that forms at the bottom of recipients containing wine, after fermentation, during storage or after authorized treatments, as well as the residue obtained following the filtration or centrifugation of this product" (Pérez-Serradilla & de Castro, 2008). Different types of wine lees can be found depending on the stage of vinification they are formed. On the one hand, there are first and second fermentation lees which have been formed during the alcoholic and malolactic fermentations, respectively (Figure 1). On the other hand, in less proportion but not lees significant, aging wine lees are formed during wine aging in wood barrels. First and second fermentation wine lees are generated practically in the same proportion with around $1.4 \cdot 10^8$ kg/year of each one, only in Spain. As far as aging wine lees is concerned, a production of an order of magnitude lower (around $6.0 \cdot 10^7$ kg/year) is obtained. Commonly, wine lees are gathered by decantation from the fermentation vessels. Thus, they are presented as a mixture of dregs and wine (up to 40% in volume) and a further centrifugation steps is required to separate both phases. The composition of the solid phase includes from yeasts, metabolites and other free phenolic compounds (free flavonol aglycones and pyranoanthocyanins) (Barcia et al., 2014; Dimou et al., 2015a) and tartaric acid (100-150kg in a tone of wine lees (Yalcin, Ozcalik, Altiok, & Bayraktar, 2008)). Additionally, huge quantity of natural colorants can be found in wine lees (~12 kg/$t_{\text{red wine lees}}$) as well as β-1,3-glucans (~ 6-12 % of dry weight) (Barba, Zhu, Koubaa, Sant'Ana, & Orlien, 2016; Kopsahelis et al., 2018; Nerantzis & Tataridis, 2006).

While the liquid phase is discarded, the solid phase is dried before the extraction procedure, either with a heater or via freeze-drying (at a laboratory scale).

Main polyphenols present in red wine lees are anthocyanins (Figure 4), a subclass of the flavonoids family made of flavan-3-ol units. Most abundant anthocyanins in red wine lees are cyanidin, peonidin, delphinidin, petunidin and malvidin (and their derivatives) (Puca et al., 2013; Romero-Díez et al., 2018). Anthocyanins are water-soluble pigments responsible of providing the red colour to wines. Sometimes, these anthocyanins undergo several reactions with other low molecular weight compounds such as pyruvic acid (among others) (Marquez, Serratosa, & Merida, 2013) creating a new anthocyanin-derived pigment family, pyranoanthocyanins (Marquez, Dueñas, Serratosa, & Merida,

2012). Other minority phenolic compounds that can be found in red wines are catechins, proanthocyanidins and stilbenes.



	R_1	R_2
Malvidin	OCH_3	OCH_3
Peonidin	OCH_3	H
Cyanidin	OH	H
Delphinidin	OH	OH
Petunidin	OH	OCH_3

Figure 4: chemical structure for main anthocyanins found in red wines (DELLAGLI, Buscialà, & Bosisio, 2004).

As it was previously exposed, wine lees have been typically used for animal feeding or tartaric acid recovery. Recent studies have demonstrated that the concentration of anthocyanins is 10 times higher in lees than in grape skins (Peralbo-Molina & Luque de Castro, 2013). Besides, other bioactive compound such as squalene has been recovered from wine lees (Naziri et al., 2016). However low concentrations of squalene are presented in wine lees (0.6 g/kg dry wine lees) (Naziri, Mantzouridou, & Tsimidou, 2012). Other alternative compounds to be recovered would be the remaining yeasts, which could be revalorised or used for animal feeding (Nerantzis & Tataridis, 2006). Once the raw material is exhausted, it can be used in a pyrolysis or as a soil substrate so as to finally eliminate the residue (Arvanitoyannis, Ladas, & Mavromatis, 2006). Thus, there are several promising options to revalorise wine lees.

2.2 Grape stems

Grape stems are the skeleton of the grape bunch and consists of lignified tissues residues generated just after the destemming of the grape brunches. Likewise wine lees, they

represent a rich source of polyphenols, mainly flavonoids and stilbenes. This fact is corroborated due to the influence of destemming on the composition and quality of wine. Wines made from a non-destemmed crop generally contain higher levels of phenolic compounds (Souquet, Labarbe, Le Guernevé, Cheynier, & Moutounet, 2000).

On the other hand, since grape stems are a woody material they present a lignocellulosic structure. Thus, it is constituted by on three pseudo-components combined in a resistant structure: cellulose, hemicellulose and lignin. However, the chemical composition of grape stems differs from one study to other**.** For instance, the percentages (% on dry wet) of cellulose, hemicellulose and lignin ranging from 25-38%, 14-35% and 18-47%; respectively (Lorenzo, Moldes, Rodríguez Couto, & Sanromán, 2002; Sluiter et al., 2004). Similarly, the same differences appear for the amount of extractives initially recovered from dried grape stems which varied between 25-50% (Prozil, Evtuguin, & Lopes, 2012). Nevertheless, the content of proteins and ashes usually remains constant among the different methods (6 and 7%, respectively).

Thus, grape stems could be an alternative source for the extraction of the cellulosic fraction of the biomass (cellulose and hemicellulose, up to 50%), also known as holocellulose (Lorenzo et al., 2002). This fraction can be recovered as biopolymers and their monomeric sugars components and, posteriorly, converted in high added value products such as fuels, bulk chemicals and materials (Cherubini, 2010; Giorgia Spigno, Pizzorno, & De Faveri, 2008). For instance, cellulose is a homogeneous polymer formed by glucose monomers. Glucose can be used for further industrial applications such as the production of biofuels or hydroxymethylfurfural (Rogalinski, Ingram, & Brunner, 2008). Another well-known application of glucose is the formation of sorbitol by glucose hydrogenation (Besson, Gallezot, & Pinel, 2014; Romero, Alonso, Sastre, & Nieto-Márquez, 2016) that can be used in a wide range of applications in food, cosmetic and paper industries. Conversely, hemicellulose is a heterogeneous branched polysaccharide that mainly consists on different proportions of sugar units with different substituents (Sauter & Grafmüller, 2015). Apart from its monomeric constituents, hemicellulose polymers can be used as sustained release carriers for drug delivery (Yoo et al., 2005).

3. Extraction processes applied for the recovery of polyphenols from wine lees and grape stems.

The polyphenol extraction procedure from a natural solid material is governed by three main steps: inner transport (diffusion of the solvent into the matrix), solubility (dissolution of the solutes) and external transport (release of solutes from the solid matrix to the global solvent phase), independently of the technology used to do it. The most usual technique is the conventional solid-liquid extraction. Nonetheless, this conventional extraction procedure entails several drawbacks, such as a long operating time, use of large amount of organic solvents and further purification steps. The long operating time is usually due to mass transfer problems (external, internal and solubility). In the same way, these mass transfer limitations also explain the necessity of huge amount of solvents. Generally, these solvents are organic liquids that cannot be present in the final product in high quantities due to healthy problems. Therefore, several purification steps, usually with high energy consumption and with a severe risk of the degradation of the desired product, are required. As a result, new and greener technologies are required to the development of a more sustainable extraction process. The most common proposals are: the applications of ultrasounds (US), microwaves (MW) or supercritical fluids (SCF). Ultrasound focused their action on the external mass transfer limitation, making it negligible by cavitation effects of the solvents because of high energy pulses. In contrast, microwave affects the raw material structure, reducing the internal diffusion problems. In addition, they can locally heat the solid, also improving the solubility. Finally, SCF can promote all the limitation steps, since they have high diffusivities and the solubility can be easily tuned by little changes in pressure, temperature or by co-solvents. Each procedure is explained in detail in the following subsections together with the reported applications to the selected winery by-products.

3.1 Conventional solid-liquid extractions

Conventional solid-liquid extractions and Soxhlet extraction are the most worldwide used and studied ways of extraction of biocompounds from solid matrixes. They are also the easiest extraction process where only the material has to be in contact with a specific solvent during a determined period of time at a certain temperature. These conventional extractions are simple processes already implemented at industrial scale. However, several drawbacks are related to this process, as previously exposed.

3.1.1 Main variables related

Main variables that affect the most polyphenol recovery via solid-liquid extractions are time the solid to liquid ratio (R_{S-L}), extraction time, temperature, solvent used, particle size and pH. The extraction of polyphenols from natural matrixes is mainly affected by the R_{S-L}. If larger quantities of solvent are used during the extraction procedure, the yield also would increase. This is consistent with mass transfer principles. The concentration gradient, which is the driving force in the mass transfer, is higher when smaller R_{S-L} are used. Thus, an increase of the diffusion rate takes place (Cacace & Mazza, 2003a; Sant'Anna, Brandelli, Marczak, & Tessaro, 2012). However, it should not be forgotten that there will be a limit on the quantity of the solvent that can be used to enhance the extraction yields which is related to the exhaustion of the matrix. Additionally, the use of large quantities of solvent will involve environmental and economic problems due to concentrations steps required for the subsequent purification processes (Galvan d'Alessandro, Kriaa, Nikov, & Dimitrov, 2012). Concerning the type of solvent employed, studies of the literature revealed that ethanol-water solutions are the most suitable for the recovery polyphenols from natural matrixes, including wine less. Although it has been reported in some studies that the higher the amount of ethanol, the higher the polyphenol recovery (Wu et al., 2009). The decrease in polarity and dielectric constant values of the solvent mixture that, generally, increases the solubility of polyphenols in hydroalcoholic mixtures as the % ethanol increases (Cacace & Mazza, 2003b). However, the use of pure organic solvent, such as ethanol, may dehydrate vegetable cells which involves a lessening on the effectiveness of the alcohol to enter in the cell and diffuse polyphenols to the extract (Sant'Anna et al., 2012). Therefore, intermediate concentrations are preferable from a solubility and environmental point of view. On the other hand, acetone and methanol seem not to be profitable solvents for polyphenol recovery since acetone is much more apolar and methanol is a toxic solvent. Regarding the temperature, it is well known that the diffusivity and solubility are directly affected by the temperature (Cacace & Mazza, 2003a), increasing the polyphenol extraction rate (Sant'Anna et al., 2012). For example, gallic acid solubility in water goes from 1.5 g/g at 25ºC to 7.4 g/g at 60ºC, (Daneshfar, Ghaziaskar, & Homayoun, 2008). Nevertheless, special attention must be paid to the temperature when extracting anthocyanins as they can degrade at temperatures up to 100ºC during short exposure times of 5-10 minutes (Sólyom, Solá, Cocero, & Mato, 2014). Another parameter that may

influence the recovery of target compounds is the particle size. The smaller the particles, the faster and higher the recovery because the contact surface increases with particle size and diffusion decreasing (Amendola, De Faveri, & Spigno, 2010). Last but not least, pH does not play a major role on extraction of polyphenols. Notwithstanding it contribute to the stability and storage of the extracts. It has been proved that at low pH (between 2.5-5) anthocyanins, the main polyphenols of grapes, exhibit more stability (Wahyuningsih, Wulandari, Wartono, Munawaroh, & Ramelan, 2017). It has been also verified that the antioxidant activity of the storage extracts progressively decrease at higher pH (Amendola et al., 2010).

3.1.2 Polyphenols extraction from wine lees

Phenolic compounds from wine lees have been extracted via conventional solid-liquid extractions with different organic-water solvents and conditions by several authors. That is the case of the sequential extractions performed with an acetone:water mixture (80:20, v/v) followed by a methanol:water mixture (60:40, v/v) (Chira, Schmauch, Saucier, Fabre, & Teissedre, 2009; Dimou et al., 2015a) with a solid-liquid ratio (R_{S-L}) of 0.10 g/mL. Acidified methanol with HCl (95:5, by volume) has been also used for polyphenol recovery from a Bosnia and Herzegovina grape wine lees variety. A R_{S-L} of 0.2 g/mL was used in triplicate sequential extractions of 60 minutes and a 0.23% of polyphenols (w/w) was recovered (Landeka et al., 2017). Additionally, hydroalcoholic mixtures (varying the percentage of ethanol) have been extensively used for the recovery of polyphenols due to the expected higher yields (Alothman, Bhat, & Karim, 2009). That is the case of *Wu et al*. who recovered polyphenols from dried wine lees of a Taiwan grape variety with different hydroalcoholic mixtures varying the percentage of ethanol in a Soxhlet extraction using a R_{S-L} of 0.08 g/mL for 6 hours. The highest content of recovered PS, 24.1% (w/w), was achieved by Soxhlet 95% vol. ethanol (EtOH) extraction (Wu et al., 2009). Whereas *Dimou et al.* (Dimou et al., 2016), using a hydroalcoholic mixture with a 70% vol. EtOH were only capable of recovering 0.8% (w/w) of polyphenols from a Greek grape variety wet wine lees by performing a simulation. *Pérez-Serradilla et al.* (Pérez-Serradilla & Luque de Castro, 2011) used practically the same solvent mixture (75%vol. EtOH) from dried Syrah grape variety wine with a R_{S-L} of 0.10 g/mL during 24 hours, obtaining a recovery of 0.57% (w/w) of polyphenols. *Tao et al*., (Tao, Wu, Zhang, & Sun, 2014) after performing a statistically study in which the main variables that govern a

solid-liquid extraction, recovered a total of 0.50% (w/w) of total phenolics and 0.06% (w/w) of anthocyanins from malolactic wine lees using an ethanol (43.9% v/v) aqueous mixture at 60ºC with a R_{S-L} of 0.02 g/mL during 25 minutes. *Bosiljkov et al.* (Bosiljkov et al., 2017) proposed a conventional extraction using an acidified aqueous solution of ethanol (ethanol/water/formic acid, 50:48.5:1.5, v/v/v, pH 2.7) in a shaker for 3 hours at room temperature, achieving a final anthocyanin recovery of 0.04% (w/w). *Farías-Campomanes et al*. (Farías-Campomanes, Rostagno, Coaquira-Quispe, & Meireles, 2015) carried out some solid-liquid extractions with ethanol with a purity of 96%. They performed a 3 hour Soxhlet with a R_{S-L} of 0.04 g/mL and an extraction with an agitation of 168 rpm at 40ºC during 6 hours. Their results showed a final polyphenol recovery of 0.28 % and 0.17% (w/w), respectively.

Additionally, natural deep eutectic solvents (NADES) have been evaluated for the recovery of polyphenols from wine lees in a conventional solid-liquid extraction (Bosiljkov et al., 2017). They are liquid salts formed by a quaternary ammonium salt (e.g., choline chloride) with a metal salt or hydrogen bond donor, such as vitamins, amines, sugars, alcohols or carboxylic acids (Smith, Abbott, & Ryder, 2014). *Bosiljkov et al.* (Bosiljkov et al., 2017) extracted anthocyanins from Merlot variety grape wine lees generated during the alcoholic fermentation with different NADES prepared by them: choline chloride:citric acid (ChCit), choline chloride:oxalicacid (ChOa), choline chloride:malic acid (ChMa), choline chlo-ride:glucose (ChGlc), choline chloride:fructose (ChFru), cholinechloride:xylose (ChXyl), choline chloride:glycerol, (ChGly). A first approach to study the most favourable NADES to extract PS was performed. A RS-L of 0.02 g of dry lees per mL of the NADES were mixed in a shaker during 3 hours at room temperature. Results revealed that anthocyanin extraction was favored by the NADES choline chloride-malic acid (ChMa) with a maximum recovery of 0.06% (w/w). However, deeper investigation in this field is required.

3.1.3 Polyphenols extraction from grape stems

There are several works in literature regarding the valorization of grape steams for the recovery of polyphenols, solid-liquid extraction have been performed to grape stems. *Makris et al.* (Makris, Boskou, Andrikopoulos, & Kefalas, 2008), performed a solid-liquid extraction of dried grape stems (GS) from the Roditis and Agiorgitio cultivars (Koropi and Nemea regions, Greece) and obtained a polyphenol and flavonoid recoveries

5.8% and 5.4% (w/w), respectively. As solvent media extraction, they employed a mixture of 0.1% HCl MeOH/acetone/H_2O (60/30/10, v/v). On the contrary, *Spigno et al*. (Giorgia Spigno & De Faveri, 2007)reach much lower polyphenol extraction yields via solid-liquid extraction from GS (Barbera red grape of Piacenza, northern Italy, 2003) during 5 hours at 60ºC in a shaking incubator with a R_{S-L} of 0.25 g/mL. No significant differences were found when the extraction was performed with ethylacetate/water (9/1, v/v) (0.1% recovery of polyphenols, w/w) or ethanol (0.12% recovery of polyphenols, w/w).

3.2 Cell disruption methods

In order to intensify the recovery process of polyphenols from natural matrixes cell disruption methods (mechanical, chemical, thermal) can be applied to enhance the extraction of valuable components from the raw materials. The objective of these disruption methods is increasing the external and internal mass transfer, as it has been afore mentioned. Among them, procedures such as ultrasound assisted extraction, microwave assisted extraction are very promising. Another important methology to take into account is the enzymatic release.

3.2.1 Ultrasounds

One of the most broadly used is ultrasounds (US). US present a mechanical background as they can be considered as an energy generated by sound waves of frequencies above 18 kHz (Fijalkowska, Nowacka, Wiktor, Sledz, & Witrowa-Rajchert, 2016). US are well-known for enhancing the external mass transfer (Miano, Rojas, & Augusto, 2017). This improvement occurs due to the acoustic effects and cavitation phenomena reported. This phenomena takes place due to the high frequency sound waves generated during US application, which generates bubbles in the liquid that collapse. This collapse results in a change in temperature and pressure (Wijngaard, Hossain, Rai, & Brunton, 2012) (Figure 6). Likewise for conventional solid-liquid extractions, variables such as R_{S-L}, extraction time, temperature, solvent used, particle size and pH, influence US extractions. All of them contribute in the same way as expose for conventional solid-liquid extractions. However, special attention must be paid to the type of solvent used in a US extraction as cavitation intensity decreases as vapour pressure and surface tension are increased, which reduces US efficiency (Vilkhu, Mawson, Simons, & Bates, 2008). Another additional

variable that govern an US extraction and must be taken into account is the ultrasound power (Khan, Abert-Vian, Fabiano-Tixier, Dangles, & Chemat, 2010). It has been reported by several authors that extraction yields of PS from different natural sources improve as ultrasonic power increase (Ma, Chen, Liu, & Ye, 2009; Van Man, Anh Vu, & Chi Hai, 2017). This phenomena occurs due to the increase of the diffusion thanks to the increment of cavitation bubbles generated (Figure 5).

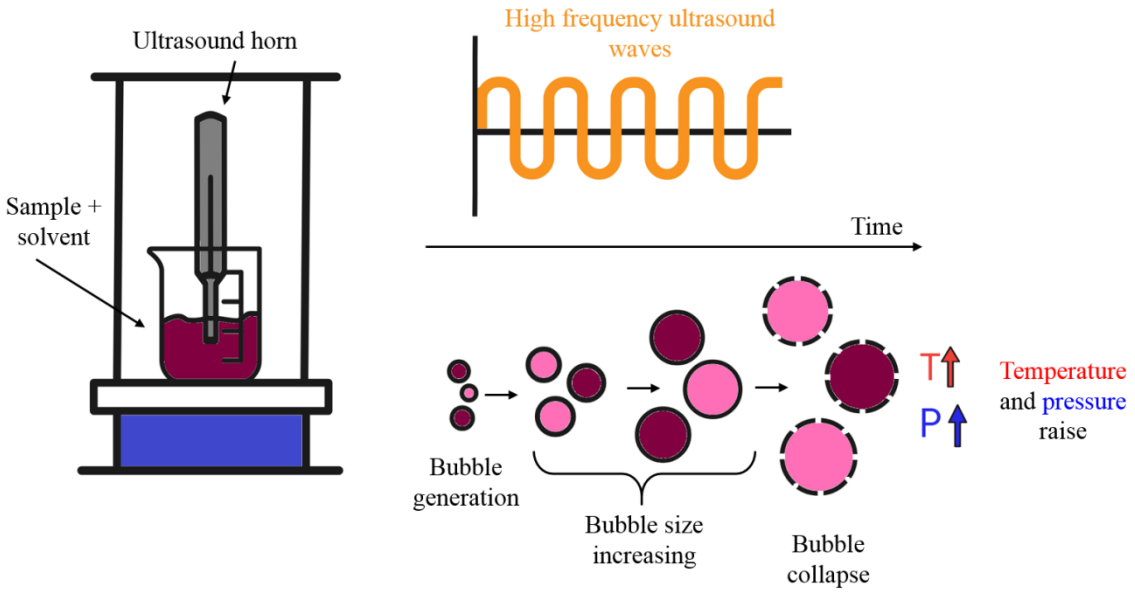


Figure 5: schematic representation of an ultrasound extraction principle and cavitation phenomenon.

However, an adequate power may be adjusted for polyphenol extraction because in some case it has been noticed extracted polyphenols compounds can undergo reactions with possible generated hydroxyl radicals at high ultrasonic powers (Van Man et al., 2017). Moreover, higher US power led to an increase of the temperature which will involve a possible degradation of the polyphenols (Tao et al., 2014). Thus, the time of sonication and temperature have to be controlled in order to get a good relationship between all the variables to avoid degradation of polyphenols.

3.2.1.1 Polyphenols extraction from wine lees using ultrasounds

Ultrasounds have been also employed as a disruption method in an attempt to maximize the recovery of polyphenols from wine lees. As an example, a US bath was employed by

Alonso et al. (Alonso, Guillén, Barroso, Puertas, & García, 2002) in order to extract polyphenols from several types or red and white wine dregs. Using methanol with a R_{S-L} of 0.10 g/mL for 15 minutes, they were able to recovery a 0.02% (w/w) of polyphenol compounds from the red Syrah grape variety wine lees. Other authors who employed a US bath were *Barcia et al*. (Barcia et al., 2014) to recover polyphenols from two *Vitis Vinifera* varieties from 2012 harvesting: Cabernet Franc and Cabernet Sauvignon. In this case, they used mixture of methanol, water, and formic acid (50:48.5:1.5 v/v) with a R_{S-L} of 0.01 g/mL for 2 minutes of treatment. They achieved recoveries around 0.04% (w/w) from the two kinds of wines. Higher yields were achieved when US assisted extraction (US-AE) was applied to wine lees which had spent one year in barrels filed with red wine (Cabernet Sauvignon 60%, Merlot 30% and Cabernet Franc 10%) (Beatriz Rivas, Ana Torrado, Ana Belén Moldes, & Domínguez*, 2006). In this work, US-AE was performed at a constant frequency of 40 kHz. A central composite design was performed in order to optimize main extraction variables (time, temperature, R_{S-L} and percent of ethanol). The optimized conditions were 25 min, 60ºC, a R_{S-L} of 0.02 g/mL and a solvent with a 43.9% of ethanol. Total recoveries of the 0.60% and 0.07% (w/w) of total PS and anthocyanins, respectively, were achieved. The enhancement of using US-AE was demonstrated since an increase of 19.8% in the polyphenol yield was obtained with respect to the conventional extraction performed at same conditions.

3.2.1.2 Polyphenols extraction from grape stems using ultrasounds

Disruption methods have been also employed for the maximization of the recovery of polyphenols from grape stems. This is the case of **US-AE (ultrasound assisted extraction)** develop by *Piñeiro et al*. (Piñeiro, Guerrero, Fernández-Marin, Cantos-Villar, & Palma, 2013). Fourteen different varieties ((Sauvignon blanc, Chardonnay, Vijiriega, Palomino fino, Tempranillo, Syrah, Garnacha, Tintilla de Rota, Vitis silvestris, Merlot, Cabernet Sauvignon, and Petit Verdo) from Rancho de la Merced Centre, in Jerez de la Frontera, Cadiz (Spain) were subjected to some optimal US-AE conditions (0.7 s cycle, 70% amplitude, 7 mm diameter probe tip, and 75 °C as extraction temperature) so as to maximize the extraction of total stilbenoids. Results revealed that the Syrah grape variety was the one which presented a higher stilbenoids recovery (0.024%, w/w), whereas Cabernet Sauvignon was the poorest (0.003%, w/w).

Barros et al. (Barros et al., 2014) used stems from different Portuguese grape varieties. Dry samples were mixture with methanol/formic acid/water (50:2:48, v/v) in a R_{S-L} of 0.10 g/mL. Then, samples were vortexed and sonicated in an ultrasonic bath for 60 min. Final recoveries of 0.17%, 0.03%, 0.15% and 0.14% (w/w) were obtained for flavonols, stilbenes, hydroxycinnamic acids and anthocyanins, respectively. Again, results showed that white (*Rabigato*) were richer in stilbenes, whereas red wines (*Sousão, Tinta Barroca*) were richer in flavonols, hydroxycinnamic acids and anthocyanins. Different concentration of stilbenes were found in the work of *Anastasiadi et al.* (Anastasiadi, Pratsinis, Kletsas, Skaltsounis, & Haroutounian, 2012) using a analogous extraction methodology. Results revealed similar averages resveratrol and viniferin concentrations for red wines (0.015% and 0.031%, w/w, respectively) and white wines (0.011% and 0.028%, w/w, respectively).

3.2.2 Microwaves

Microwaves (MW) have been also used as a cell disruption and process intensification method for more than 30 years (Ganzler, Salgó, & Valkó, 1986) in a wide range of vegetable matrixes (Rodríguez-Rojo, Visentin, Maestri, & Cocero, 2012) and even in grape residues (Álvarez et al., 2017). MW heating takes advantage of the properties of some compounds to transform the electromagnetic energy into heat. MW heating is produced by two mechanisms: the dipolar rotation and the ionic conduction. The dipolar rotation is an interaction where the polar molecules try to realign upon themselves due the electric field (Ganzler et al., 1986). This rotational movement generates an energy transfer via molecular friction. By contrast, the ionic conduction takes place when there are ions in the medium. Heat is caused thanks to the ion migration in the medium where friction loses are generated, which results in a rapidly heating up (Carballido Reboredo & Universidad de Santiago de Compostela., 2008) (Figure 6).

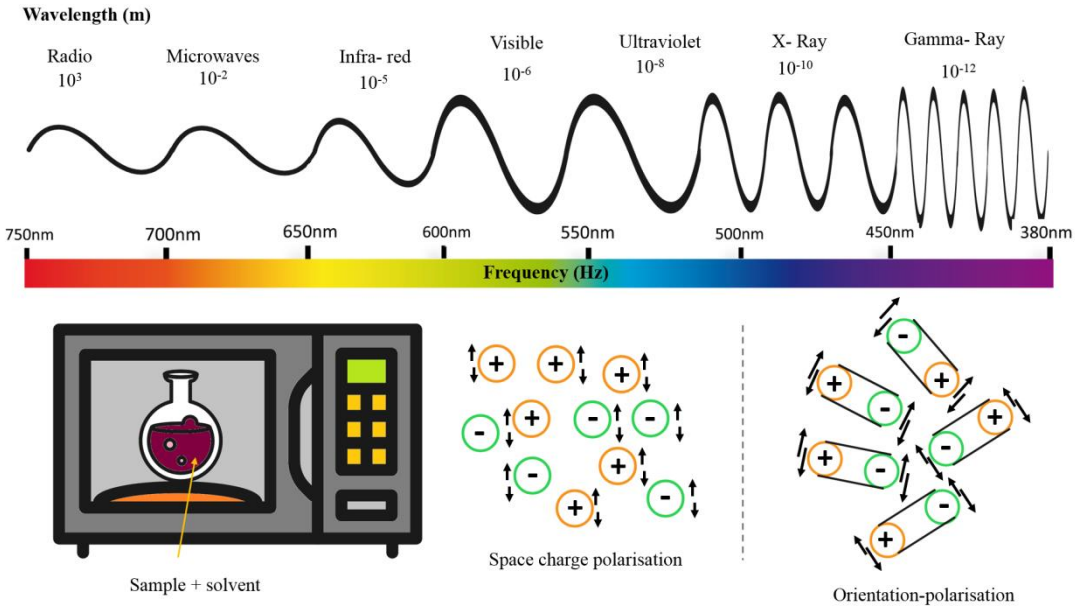


Figure 6: schematic representation of a microwave extraction principle and polarisation phenomenon.

Thus, the attractiveness of using microwaves resides in its rapidity and energy efficiency which makes that the heating process takes place in a few seconds. A double effect of the energy takes place in every MW irradiation. On one hand, the irradiation improves the cellular lysis of materials with large quantities of water due to the rapid heating and evaporation of the intracellular water. On the other hand, a non-thermal effect appears as a result of the alteration of the dielectric camps which could provide a breakdown of the hydrogen bonds of the macromolecules, breaking off their structure (Ganzler et al., 1986). An adequate selection of the solvent capable of absorbing MW irradiation is mandatory. Two main parameters affect the absorption of the irradiation by the solvent. One, the dielectric loss coefficient, which measures the efficiency by which magnetic energy is converted into heat, and the dielectric constant, which represents the ability of the material to be polarized (a measure of the energy stored) (G. Spigno & De Faveri, 2009). Due to that, solvents with high dielectric loss coefficient and dielectric constant are desired. Two, the dissipation factor, which represents the ability of the solvent to absorb microwave energy and to conduct heat to surrounding molecules (Mateus, Silva, Rivas-Gonzalo, Santos-Buelga, & De Freitas, 2003). This factor, relates the dielectric loss coefficient and dielectric constant so, the higher this factor, the faster the extraction will be. As a consequence, one of the most important parameters that affects MAE is the adequate

selection of the solvent. Table 1 shows the dielectric properties of some of the most common solvents which have been already used for this purpose.

Table 1: Dielectric properties of some solvents [74]

Solvent	Dielectric loss (ε''2.45GHz)	Dielectric constant (ε'2.45GHz)	Dissipation factor tan(ε''/ε')
Methanol	23.1	24.6	0.941
Ethanol	21.5	32.7	0.659
Water	9.9	80.4	0.123
Acetone	1.1	20.6	0.054
Ethyl Acetate	0.4	6.2	0.059

Although methanol has been found to provide better extraction yields (Casazza, Aliakbarian, Mantegna, Cravotto, & Perego, 2010), ethanol is chosen as solvent due to it is lower toxicity. Mixtures of water and ethanol are employed: the high dielectric loss of the ethanol will increase the heating rate, and the larger dielectric constant of the water enhances the polarity of the solvent. For the case of the $R_{S\text{-}L}$, as it has been previously described, low solid-liquid ratios promote the extraction of polyphenols due to a problem of solvation. However, the employment of low $R_{S\text{-}L}$ involves less energy available per millilitre of sample. Working with low $R_{S\text{-}L}$, will not allow to irradiate the material homogeneously, since most of the irradiation will be lost in heating up the solvent which is traduced in a low penetration depth (Álvarez et al., 2017). Further, it has been demonstrated the absorption of energy by the material is more relevant than the power irradiated (Alfaro, Bélanger, Padilla, & Jocelyn Paré, 2003; Sólyom, Mato, Pérez-Elvira, & Cocero, 2011). Higher levels of energy absorbed enhance the polyphenol extraction rate, but also improves polyphenols degradation as a result of an increase of the temperature (Liazid, Guerrero, Cantos, Palma, & Barroso, 2011; Pan, Niu, & Liu, 2003). Additionally, the penetration depth in a MAE of 2.45GHz represent a sever scale-up limitation, since it has been reported to be very low (Álvarez et al., 2017; Thostenson & Chou, 1999). This generates an un-uniformly irradiation in a large vessel, since only the external parts will be irradiated without affecting the matter. The degradation and the penetration depth problems can be easily overcome using higher frequencies, up to 9.15 MHz (although this will suppose a reduction of the thermal effect). As alternative, MWs

can be used as a prior step to the conventional solid-liquid extraction. With this MW pre-treatment, the material is homogenously irradiated at a low residence time. The material will be capable of absorbing an amount of energy equivalent to MAE milder radiation conditions (due to the peak of energy during the beginning of the pre-treatment) and thanks to the use of short periods of MW pre-treatment, the polyphenol degradation is avoided (Sólyom et al., 2014). Differences between a MAE process and a MW pre-treatment are visible in Figure 7.

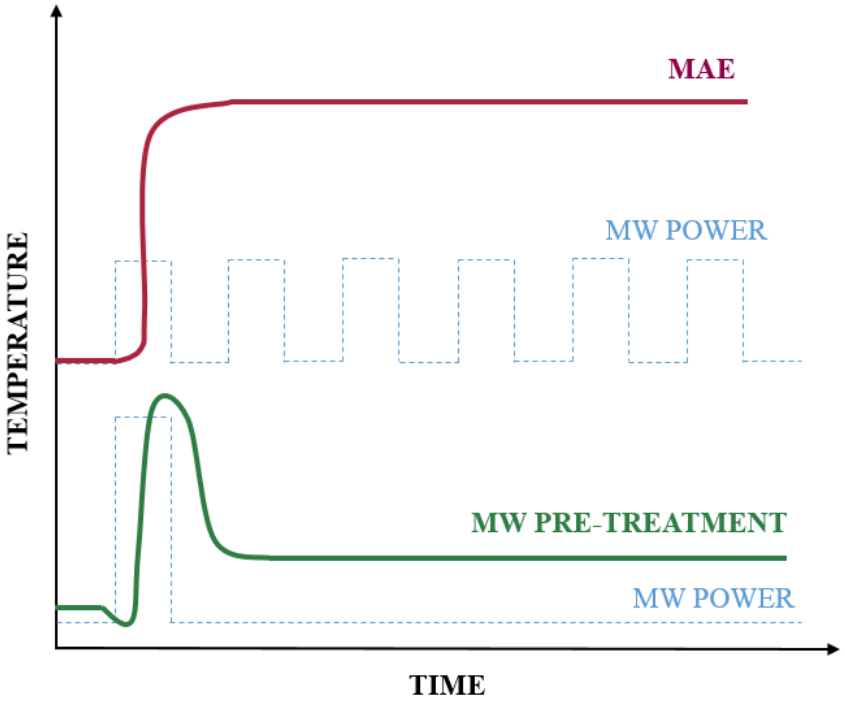


Figure 7: differences in the heating in a MAE process and a MW pre-treatment.

Parameters that govern this process are the same as those described for the for the MAE technology, such as the R_{S-L}, the solvent composition and the irradiation time. Regarding the R_{S-L}, it is worth remarking that when MW are used as a pre-treatment higher R_{S-L} in comparison to MAE processes.

3.2.2.1 Polyphenols extraction from wine lees using microwaves

Thanks to the prominent advantages of the MW irradiation aforementioned, this disruption method has been also applied to extract polyphenols from wine lees. *Pérez-Serradilla et al.* (Pérez-Serradilla & Luque de Castro, 2011) proposed an optimization of

a MW assisted extraction (MAE) varying the main parameters that govern this type of processes: irradiation power (100-200W), irradiation time (5-25min), percentage of ethanol (50-100%), and percentage of HCl (0.2-1% for pH adjustment). The optimal working variables were an irradiation power of 200W for 17 min using a hydro-alcoholic mixture with 75% vol. EtOH acidified with 1% of HCl. A final PS recovery of 0.7% (w/w) was achieved, which represent a 17% higher than those obtained from the reference conventional extraction (0.57%, w/w).

3.2.2.2 Polyphenols extraction from grape stems using microwaves

Similarly to wine lees, MAE procedures have been tested for grape stems. *Piñeiro et al.* (Piñeiro, Marrufo-Curtido, Vela, & Palma, 2017) applied a MAE in grape stems from white and red cultivars. Four white cultivars (Palomino fino, Chardonnay, Moscatel de Alejandria and Vijiriega) and sixteen red cultivars (Tempranillo, Marselan, Syrah, Petit verdot, Malbec, Carmenere, Merlot, Zinfandel, Muscat Julius, Tintilla de Rota, Cabernet franc, Vitis sylvestris-1, Vitis sylvestris-3, Regent, Tannat and Melonera) were analysed. MAE conditions were the following: a RS-L of 0.01 g/mL, a hydroalcoholic mixture with 80% vol. of EtOH, 125ºC and 5 minutes of extraction. In this case, the *Vijiriega* grape variety was the richest in stilbenes with recoveries up to 0.31% (w/w). In contrast, from *Petit verdot* variety only recoveries of 0.014% (w/w).

3.2.3 Supercritical Fluid Extraction (SFE)

Supercritical fluids represent a greener alternative to other extraction procedures. Carbon dioxide (CO_2) is the most common solvent use in supercritical fluid extraction (SFE) due to its low critical point (31ºC and 7.4 MPa) and to the fact that it is cheap, non-toxic and environmentally friendly. Thanks to this, it is recognized as a GRAS (Generally Recognized As Safe) solvent. One of the most outstanding advantages of working with SFE is the enhancement of the transport properties thanks to the low viscosity and high diffusivity (da Silva, Rocha-Santos, & Duarte, 2016) of supercritical fluids (SCF). Another important parameter to take into account is the density of a SCF, which can be easily tuned with small changes in pressure and/or temperature, which is directly related to its solvent power: the higher the density the higher solubility of solute (Khaw, Parat, Shaw, & Falconer, 2017). SFE has been already tested in other wine by-products, such as

grape marc and seeds (Fiori, De Faveri, Casazza, & Perego, n.d.), but there are only few works in which supercritical fluid extraction have been used for the recovery of polyphenols from wine lees (Farías-Campomanes et al., 2015). Another main advantage of the SFE is that the separation process of the target compounds from the SC-CO_2 is made by the expansion of the gas in a vessel in which the SC-CO_2 becomes gas and the extract is separated from it easily. However, SC-CO_2 is not the most suitable solvent for polyphenol extraction due to its low polarity in comparison to most phenols (Garcia-Salas, Morales-Soto, Segura-Carretero, & Fernández-Gutiérrez, 2010). This drawback can be overcome using small percentages (1-10%) of polar co-solvents to change SC-CO_2 polarity, thus increasing its solvating power (Herrero, Mendiola, Cifuentes, & Ibáñez, 2010). Ethanol is the most common used modifier and it has been employed in several studies for polyphenol recovery, as it produced a higher increase in the solvent polarity if compared with acetone (de Campos, Leimann, Pedrosa, & Ferreira, 2008; Farías-Campomanes et al., 2015; Prado et al., 2012). Methanol also increases notably the polarity, but it is not commonly used because it is not food grade. (Figure 8).

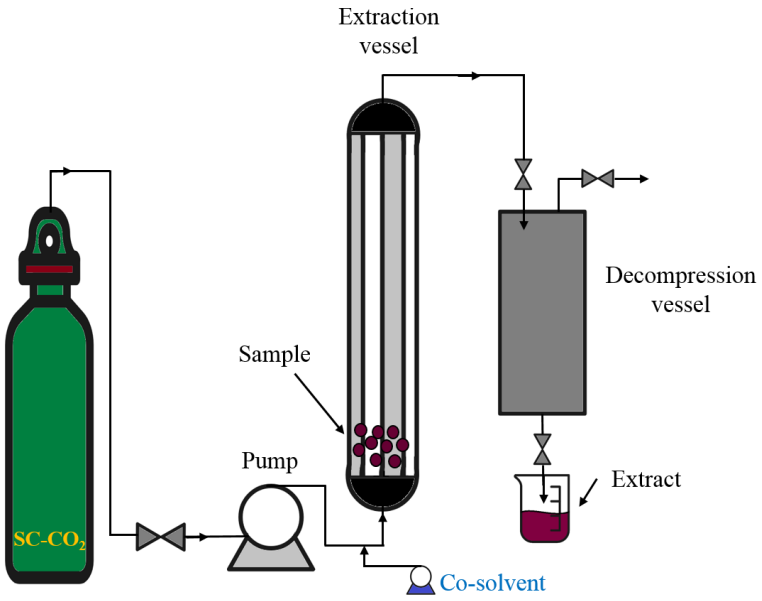


Figure 8: schematic representation of supercritical fluid extraction using CO_2 and a co-solvent for the recovery of polyphenols from wine lees.

The key of an effectively SFE lies on optimizing the different variables that govern the SFE: mainly extraction temperature and pressure due to its effect in the density of SC-CO_2, as indicated previously. Working with high densities involves higher diffusion

coefficients, hence the solvation power increase (da Silva et al., 2016). For a constant temperature, if pressure increases, density increases too. On the contrary, if temperature is raised, the density of SC-CO_2 decreases and thus the solvating power decreases too but, on the other hand, the extraction rate and extraction efficiency increase through increased diffusion and desorption (Le Floch, Tena, Ríos, & Valcárcel, 1998). So, there must be a compromise between the pressure and the temperature chosen for the extractions. On the other hand, the volumetric flow is directly related to the operational time since the higher the flow is, the less saturated the SC- CO_2 is and lower extraction times are required.

3.2.3.1 Polyphenols extraction from wine lees using supercritical fluids

Less information is available concerning the extraction of polyphenols via **s**upercritical fluid extraction (SFE). Only two works can be found in literature related to the extraction of PS from wine lees with supercritical fluids, both of them using CO_2 with ethanol as co-solvent. *Wu et al*. (Wu et al., 2009) made several SFEs with different percentages of ethanol as a co-solvent from a Taiwan grape wine lees variety at 35 MPa. Results showed that the higher the amount of co-solvent in the supercritical phase, the higher the polyphenol recovery. The parameter that influenced the most the yield and the content of PS in the extracts was the amount of co-solvent added. The highest yield (0.06%) when 0.041 kg $_{CO2+EtOH}$/g $_{DRY\ LEES}$ were used (2.5% of ethanol, w/w). Similar recovery values were obtain by *Farías-Campomanes et al*. (Farías-Campomanes et al., 2015). SC-CO_2 with a 10% ethanol as co-solvent (w/w) was employed at two different pressures, 20 and 35 MPa. Final results revealed that a direct proportional relation was obtained between the PS recovery and the ratio of kg of SCF/ kg of lees at the same temperature. A polyphenol recovery of around 0.10% (w/w) was achieved when 0.242 kg $_{CO2+EtOH}$/g $_{DRY\ LEES}$. When half of the solvent was used (0.137 kg $_{CO2+EtOH}$/g $_{DRY\ LEES}$) a recovery near 0.06% (w/w) was achieved.

3.2.4 Enzymatic release

Some polyphenols remains linked or absorbed within the cellular wall of the constituents of the winery wastes (Pérez-Serradilla & de Castro, 2008). This is the case of the wine lees that are mainly composed by yeast which have been in contact with the wine during the fermentation stages. For this reason, a breakage of the cell walls could be interesting from the recovery of polyphenols linked to their cell wall. One methodology that can be

used for this purpose is the use of enzymatic hydrolysis (Martins, Roberto, Blumberg, Chen, & Macedo, 2016).

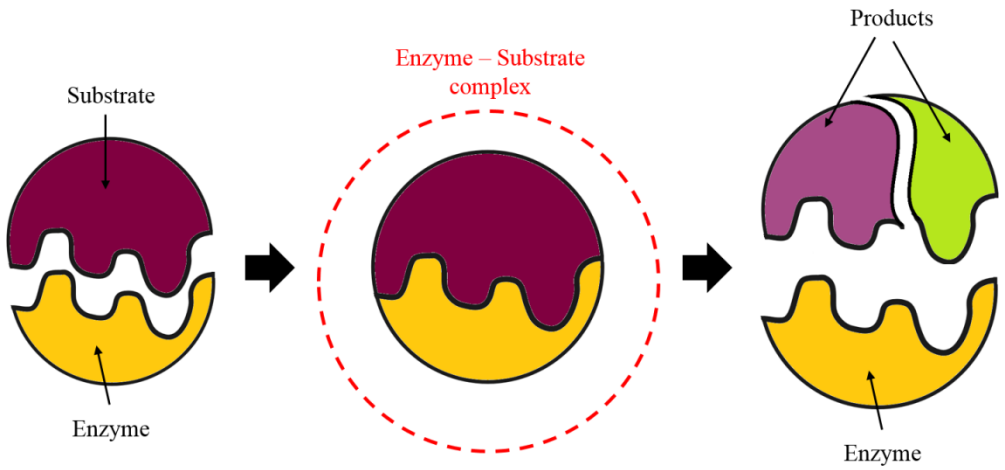


Figure 9: schematic representation of an enzymatic process for the release of polyphenols link to the yeast cell wall from wine lees.

For this kind of extraction the most important parameter to take into account is the type of enzyme. It is necessary to recognize the type of cell wall and the molecules that conform it in order to select the appropriate enzyme (Figure 9). It should be borne in mind that most of the enzymes inhibit their activity with organic solvents, thus the incubation time is normally performed in water. The incubation time is the required time to both substrate and enzyme settled together and the enzymatic hydrolysis takes place. After that, ethanol can be added to the medium (if necessary), since anthocyanin extraction yields increase with hydroalcoholic mixtures (Martins et al., 2016). Another important parameter is the incubation time, which is also dependent on the cell wall structure. Other parameters such as solid-liquid ratios, the $R_{S\text{-}L}$ and enzyme-substrate ratios, pH or temperature (Peterson, Daniel, Danson, & Eisenthal, 2007) are normally given in enzyme specification data sheets.

This technique has been applied for the recovery of polyphenols from grape pomace (Ferri et al., 2016; Martins et al., 2016) or grape seeds (Fernández, Vega, & Aspé, 2015) but never used in wine lees despite its high yeast content.

Literature

Alfaro, M. J., Bélanger, J. M. ., Padilla, F. C., & Jocelyn Paré, J. . (2003). Influence of solvent, matrix dielectric properties, and applied power on the liquid-phase microwave-assisted processes (MAP^{TM}) extraction of ginger (Zingiber officinale). *Food Research International*, *36*(5), 499–504.

Alonso, A. M., Guillén, D. A., Barroso, C. G., Puertas, B., & García, A. (2002). Determination of antioxidant activity of wine byproducts and its correlation with polyphenolic content. *Journal of Agricultural and Food Chemistry*, *50*(21), 5832–6.

Alothman, M., Bhat, R., & Karim, A. A. (2009). Antioxidant capacity and phenolic content of selected tropical fruits from Malaysia, extracted with different solvents. *Food Chemistry*, *115*(3), 785–788.

Álvarez, A., Poejo, J., Matias, A. A., Duarte, C. M. M., Cocero, M. J., & Mato, R. B. (2017). Microwave pretreatment to improve extraction efficiency and polyphenol extract richness from grape pomace. Effect on antioxidant bioactivity. *Food and Bioproducts Processing*, *106*, 162–170.

Amendola, D., De Faveri, D. M., & Spigno, G. (2010). Grape marc phenolics: Extraction kinetics, quality and stability of extracts. *Journal of Food Engineering*, *97*(3), 384–392.

Anastasiadi, M., Pratsinis, H., Kletsas, D., Skaltsounis, A.-L., & Haroutounian, S. A. (2012). Grape stem extracts: Polyphenolic content and assessment of their in vitro antioxidant properties. *LWT - Food Science and Technology*, *48*(2), 316–322.

Arvanitoyannis, I. S., Ladas, D., & Mavromatis, A. (2006). Wine waste treatment methodology. *International Journal of Food Science and Technology*, *41*(10), 1117–1151.

Barba, F. J., Zhu, Z., Koubaa, M., Sant'Ana, A. S., & Orlien, V. (2016). Green alternative methods for the extraction of antioxidant bioactive compounds from winery wastes and by-products: A review. *Trends in Food Science & Technology*, *49*, 96–109.

Barcia, M. T., Pertuzatti, P. B., Rodrigues, D., Gómez-Alonso, S., Hermosín-Gutiérrez, I., & Godoy, H. T. (2014). Occurrence of low molecular weight phenolics in Vitis vinifera red grape cultivars and their winemaking by-products from São Paulo

(Brazil). *Food Research International*, *62*, 500–513.

Barros, A., Gironés-Vilaplana, A., Teixeira, A., Collado-González, J., Moreno, D. A., Gil-Izquierdo, A., Domínguez-Perles, R. (2014). Evaluation of grape (Vitis vinifera L.) stems from Portuguese varieties as a resource of (poly)phenolic compounds: A comparative study. *Food Research International*, *65*, 375–384.

Beatriz Rivas, Ana Torrado, Ana Belén Moldes, and Domínguez, J. M. (2006). Tartaric Acid Recovery from Distilled Lees and Use of the Residual Solid as an Economic Nutrient for Lactobacillus. *Journal of Agricultural and Food Chemstry*, *54*(20), 7904–11.

Beckman, C. H. (2000). Phenolic-storing cells: keys to programmed cell death and periderm formation in wilt disease resistance and in general defence responses in plants? *Physiological and Molecular Plant Pathology*, *57*(3), 101–110.

Besson, M., Gallezot, P., & Pinel, C. (2014). Conversion of Biomass into Chemicals over Metal Catalysts. *Chemical Reviews*, *114*(3), 1827–1870.

Bordignon-Luiz, M. T., Gauche, C., Gris, E. F., & Falcão, L. D. (2007). Colour stability of anthocyanins from Isabel grapes (Vitis labrusca L.) in model systems. *LWT - Food Science and Technology*, *40*(4), 594–599.

Bosiljkov, T., Dujmić, F., Cvjetko Bubalo, M., Hribar, J., Vidrih, R., Brnčić, M., Jokić, S. (2017). Natural deep eutectic solvents and ultrasound-assisted extraction: Green approaches for extraction of wine lees anthocyanins. *Food and Bioproducts Processing*, *102*, 195–203.

Cacace, J. E., & Mazza, G. (2003a). Mass transfer process during extraction of phenolic compounds from milled berries. *Journal of Food Engineering*, *59*(4), 379–389.

Cacace, J. E., & Mazza, G. (2003b). Optimization of Extraction of Anthocyanins from Black Currants with Aqueous Ethanol. *Journal of Food Science*, *68*(1), 240–248.

Carballido Reboredo, M. R. (2008). Síntesis asistida por microondas de compuestos de interés biológico e industrial haloarenos, fulvenos, tricloropirrolidonas, azoles y flavonoides : modelado de espectros de absorción electrónica de flavonoides.*Universidad de Santiago de Compostela.*

Caruso, F., Mendoza, L., Castro, P., Cotoras, M., Aguirre, M., Matsuhiro, B.,

Antonioletti, R. (2011). Antifungal Activity of Resveratrol against Botrytis cinerea Is Improved Using 2-Furyl Derivatives. *PLoS ONE*, *6*(10), e25421.

Casazza, A. A., Aliakbarian, B., Mantegna, S., Cravotto, G., & Perego, P. (2010). Extraction of phenolics from Vitis vinifera wastes using non-conventional techniques. *Journal of Food Engineering*, *100*(1), 50–55.

Cherubini, F. (2010). The biorefinery concept: Using biomass instead of oil for producing energy and chemicals. *Energy Conversion and Management*, *51*(7), 1412–1421.

Cheynier, M., Fulcrand, V., & Sarni, H. (1997). Application des techniques analytiques à l'étude des composés phénoliques et de leurs réactions au cours de la vinification. *Analusis*, *25*, 14–21.

Chira, K., Schmauch, G., Saucier, C., Fabre, S., & Teissedre, P.-L. (2009). Grape Variety Effect on Proanthocyanidin Composition and Sensory Perception of Skin and Seed Tannin Extracts from Bordeaux Wine Grapes (Cabernet Sauvignon and Merlot) for Two Consecutive Vintages (2006 and 2007). *Journal of Agricultural and Food Chemistry*, *57*(2), 545–553.

Cruz, J. M., Domínguez, H., & Parajó, J. C. (2004). Assessment of the Production of Antioxidants from Winemaking Waste Solids. *Journal of Agricultural and Food Chemistry*, *52*(18), 5612–5620.

da Silva, R. P. F. F., Rocha-Santos, T. A. P., & Duarte, A. C. (2016). Supercritical fluid extraction of bioactive compounds. *TrAC Trends in Analytical Chemistry*, *76*, 40–51.

Daneshfar, A., Ghaziaskar, H. S., & Homayoun, N. (2008). Solubility of Gallic Acid in Methanol, Ethanol, Water, and Ethyl Acetate. *Journal of Chemical & Engineering Data*, *53*(3), 776–778.

De Campos, L. M. A. S., Leimann, F. V., Pedrosa, R. C., & Ferreira, S. R. S. (2008). Free radical scavenging of grape pomace extracts from Cabernet sauvingnon (Vitis vinifera). *Bioresource Technology*, *99*(17), 8413–8420.

Dellagli, M., Buscialà, A., & Bosisio, E. (2004). Vascular effects of wine polyphenols. *Cardiovascular Research*, *63*(4), 593–602.

Devesa-Rey, R., Vecino, X., Varela-Alende, J. L., Barral, M. T., Cruz, J. M., & Moldes,

A. B. (2011). Valorization of winery waste vs. the costs of not recycling. *Waste Management*, *31*(11), 2327–2335.

Dimou, C., Kopsahelis, N., Papadaki, A., Papanikolaou, S., Kookos, I. K., Mandala, I., & Koutinas, A. A. (2015a). Wine lees valorisation: Biorefinery development including production of a generic fermentation feedstock employed for poly(hydroxybutyrate) synthesis. *Food Research International*, *73*(50), 81–87.

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

Dimou, C., Vlysidis, A., Kopsahelis, N., Papanikolaou, S., Koutinas, A. A., & Kookos, I. K. (2016). Techno-economic evaluation of wine lees refining for the production of value-added products. *Biochemical Engineering Journal*, *116*, 157–165.

Farías-Campomanes, A. M., Rostagno, M. A., Coaquira-Quispe, J. J., & Meireles, M. A. A. (2015). Supercritical fluid extraction of polyphenols from lees: overall extraction curve, kinetic data and composition of the extracts. *Bioresources and Bioprocessing*, *2*(1), 45.

Fernández, K., Vega, M., & Aspé, E. (2015). An enzymatic extraction of proanthocyanidins from País grape seeds and skins. *Food Chemistry*, *168*, 7–13.

Ferri, M., Bin, S., Vallini, V., Fava, F., Michelini, E., Roda, A., Tassoni, A. (2016). Recovery of polyphenols from red grape pomace and assessment of their antioxidant and anti-cholesterol activities. *New Biotechnology*, *33*(3), 338–344.

Fijalkowska, A., Nowacka, M., Wiktor, A., Sledz, M., & Witrowa-Rajchert, D. (2016). Ultrasound as a Pretreatment Method to Improve Drying Kinetics and Sensory Properties of Dried Apple. *Journal of Food Process Engineering*, *39*(3), 256–265.

Fiori, L., De Faveri, D., Casazza, A. A., & Perego, P. (2009). Grape by-products: extraction of polyphenolic compounds using supercritical CO_2 and liquid organic solvent – a preliminary investigation. *CyTA-Journal of Food, 7*(3). 163-171.

Flanzy, C. (2003). *Enología : fundamentos científicos y tecnológicos*. AMV Edicione.

Galvan d'Alessandro, L., Kriaa, K., Nikov, I., & Dimitrov, K. (2012). Ultrasound assisted

extraction of polyphenols from black chokeberry. *Separation and Purification Technology*, *93*, 42–47.

Ganzler, K., Salgó, A., & Valkó, K. (1986). Microwave extraction : A novel sample preparation method for chromatography. *Journal of Chromatography A*, *371*, 299–306.

Garcia-Salas, P., Morales-Soto, A., Segura-Carretero, A., & Fernández-Gutiérrez, A. (2010). Phenolic-Compound-Extraction Systems for Fruit and Vegetable Samples. *Molecules*, *15*(12), 8813–8826.

Grimplet, J., Deluc, L. G., Cramer, G. R., & Cushman, J. C. (2007). Integrating Functional Genomics With Salinity and Water Deficit Stress Responses in Wine Grape - Vitis Vinifera. In *Advances in Molecular Breeding Toward Drought and Salt Tolerant Crops* (pp. 643–668). Dordrecht: Springer Netherlands.

Herrero, M., Mendiola, J. A., Cifuentes, A., & Ibáñez, E. (2010). Supercritical fluid extraction: Recent advances and applications. *Journal of Chromatography A*, *1217*(16), 2495–2511.

Khan, M. K., Abert-Vian, M., Fabiano-Tixier, A.-S., Dangles, O., & Chemat, F. (2010). Ultrasound-assisted extraction of polyphenols (flavanone glycosides) from orange (Citrus sinensis L.) peel. *Food Chemistry*, *119*(2), 851–858.

Khaw, K.-Y., Parat, M.-O., Shaw, P. N., & Falconer, J. R. (2017). Solvent Supercritical Fluid Technologies to Extract Bioactive Compounds from Natural Sources: A Review. *Molecules*, *22*(12), 1186.

Kopsahelis, N., Dimou, C., Papadaki, A., Xenopoulos, E., Kyraleou, M., Kallithraka, S., … Koutinas, A. A. (2018). Refining of wine lees and cheese whey for the production of microbial oil, polyphenol-rich extracts and value-added co-products. *Journal of Chemical Technology & Biotechnology*, *93*(1), 257–268.

Landeka, I., Jurčević, Dora, M., Guberović, I., Petras, M., Rimac, S., Đikić, D. (2017). Polyphenols from Wine Lees as a Novel Functional Bioactive Compound in the Protection Against Oxidative Stress and Hyperlipidaemia. *Food Technology and Biotechnology*, *55*(1), 109–116.

Landeka Jurčević, I., Dora, M., Guberović, I., Petras, M., Rimac Brnčić, S., Đikić, D.

(2017). Wine Lees Polyphenols as a Novel Functional Bioactive Compound in the Protection against Oxidative Stress and Hyperlipidemia. *Food Technology and Biotechnology*, *55*(1), 109–116.

Langcake, P., & Pryce, R. J. (1977). A new class of phytoalexins from grapevines. *Experientia*, *33*(2), 151–2.

Le Floch, F., Tena, M. ., Ríos, A., & Valcárcel, M. (1998). Supercritical fluid extraction of phenol compounds from olive leaves. *Talanta*, *46*(5), 1123–1130.

Liazid, A., Guerrero, R. F., Cantos, E., Palma, M., & Barroso, C. G. (2011). Microwave assisted extraction of anthocyanins from grape skins. *Food Chemistry*, *124*(3), 1238–1243.

Lorenzo, M., Moldes, D., Rodríguez Couto, S., & Sanromán, A. (2002). Improving laccase production by employing different lignocellulosic wastes in submerged cultures of Trametes versicolor. *Bioresource Technology*, *82*(2), 109–113.

Ma, Y.-Q., Chen, J.-C., Liu, D.-H., & Ye, X.-Q. (2009). Simultaneous extraction of phenolic compounds of citrus peel extracts: Effect of ultrasound. *Ultrasonics Sonochemistry*, *16*(1), 57–62.

Makris, D. P., Boskou, G., & Andrikopoulos, N. K. (2007). Polyphenolic content and in vitro antioxidant characteristics of wine industry and other agri-food solid waste extracts. *Journal of Food Composition and Analysis*, *20*(2), 125–132.

Makris, D. P., Boskou, G., Andrikopoulos, N. K., & Kefalas, P. (2008). Characterisation of certain major polyphenolic antioxidants in grape (Vitis vinifera cv. Roditis) stems by liquid chromatography-mass spectrometry. *European Food Research and Technology*, *226*(5), 1075–1079.

Mariani, G., Benfenati, E., Fanelli, R., Nicoli, A., Bonfitto, E., & Jacopone, S. (1992). Incineration of agro-industrial wastes and macro- and micropollutants emission. *Chemosphere*, *24*(11), 1545–1551.

Marquez, A., Dueñas, M., Serratosa, M. P., & Merida, J. (2012). Formation of vitisins and anthocyanin-flavanol adducts during red grape drying. *Journal of Agricultural and Food Chemistry*, *60*(27), 6866–74.

Marquez, A., Serratosa, M. P., & Merida, J. (2013). Pyranoanthocyanin derived pigments

in wine: Structure and formation during winemaking. *Journal of Chemistry, (2013)*.

Martins, I. M., Roberto, B. S., Blumberg, J. B., Chen, C.-Y. O., & Macedo, G. A. (2016). Enzymatic biotransformation of polyphenolics increases antioxidant activity of red and white grape pomace. *Food Research International*, *89*, 533–539.

Mateus, N., Silva, A. M. S., Rivas-Gonzalo, J. C., Santos-Buelga, C., & De Freitas, V. (2003). A new class of blue anthocyanin-derived pigments isolated from red wines. *Journal of Agricultural and Food Chemistry*, *51*, 1919–1923.

Miano, A. C., Rojas, M. L., & Augusto, P. E. D. (2017). Other Mass Transfer Unit Operations Enhanced by Ultrasound. *Ultrasound: Advances for Food Processing and Preservation*, 369–389.

MONAGAS, M., BARTOLOMÉ, B., & GÓMEZ-CORDOVÉS, C. (2005). Updated Knowledge About the Presence of Phenolic Compounds in Wine. *Critical Reviews in Food Science and Nutrition*, *45*(2), 85–118.

Muhlack, R. A., Potumarthi, R., & Jeffery, D. W. (2018). Sustainable wineries through waste valorisation: A review of grape marc utilisation for value-added products. *Waste Management*, *72*, 99–118.

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

Naziri, E., Mantzouridou, F., & Tsimidou, M. Z. (2012). Recovery of Squalene from Wine Lees Using Ultrasound Assisted Extraction—A Feasibility Study. *Journal of Agricultural and Food Chemistry*, *60*(36), 9195–9201.

Negro, C., Tommasi, L., & Miceli, A. (2003). Phenolic compounds and antioxidant activity from red grape marc extracts. *Bioresource Technology*, *87*(1), 41–44.

Nerantzis, E. T., & Tataridis, P. (2006). Integrated enology – utilization of winery by-products into high added value products. *Journal of Food Science Technology*, *3*, 1–12.

OIV Statistical Report on World Vitiviniculture 2017. Retrieved from http://www.oiv.int/en/oiv-life/oiv-2017-report-on-the-world-vitivinicultural-situation

Pan, X., Niu, G., & Liu, H. (2003). Microwave-assisted extraction of tea polyphenols and tea caffeine from green tea leaves. *Chemical Engineering and Processing: Process Intensification*, *42*(2), 129–133.

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

Pereira, D., Valentão, P., Pereira, J., & Andrade, P. (2009). Phenolics: From Chemistry to Biology. *Molecules*, *14*(6), 2202–2211. 2

Pérez-Serradilla, J. A., & de Castro, M. D. L. (2008). Role of lees in wine production: A review. *Food Chemistry*, *111*(2), 447–456.

Pérez-Serradilla, J. A., & Luque de Castro, M. D. (2011). Microwave-assisted extraction of phenolic compounds from wine lees and spray-drying of the extract. *Food Chemistry*, *124*(4), 1652–1659.

Peterson, M. E., Daniel, R. M., Danson, M. J., & Eisenthal, R. (2007). The dependence of enzyme activity on temperature: determination and vPeterson, M. E., Daniel, R. M., Danson, M. J., & Eisenthal, R. (2007). The dependence of enzyme activity on temperature: determination and validation of parameters. The Biochemical Journ. *The Biochemical Journal*, *402*(2), 331–7.

Piñeiro, Z., Guerrero, R. F., Fernández-Marin, M. I., Cantos-Villar, E., & Palma, M. (2013). Ultrasound-Assisted Extraction of Stilbenoids from Grape Stems. *Journal of Agricultural and Food Chemistry*, *61*(51), 12549–12556.

Piñeiro, Z., Marrufo-Curtido, A., Vela, C., & Palma, M. (2017). Microwave-assisted extraction of stilbenes from woody vine material. *Food and Bioproducts Processing*, *103*, 18–26.

Prado, J. M., Dalmolin, I., Carareto, N. D. D., Basso, R. C., Meirelles, A. J. A., Vladimir Oliveira, J., … Meireles, M. A. A. (2012). Supercritical fluid extraction of grape seed: Process scale-up, extract chemical composition and economic evaluation. *Journal of Food Engineering*, *109*(2), 249–257.

Pridham, J. B. (1965). Low Molecular Weight Phenols in Higher Plants. *Annual Review of Plant Physiology*, *16*(1), 13–36.

Prozil, S. O., Evtuguin, D. V., & Lopes, L. P. C. (2012). Chemical composition of grape stalks of Vitis vinifera L. from red grape pomaces. *Industrial Crops and Products*, *35*(1), 178–184.

Puca, A. A., Carrizzo, A., Villa, F., Ferrario, A., Casaburo, M., Maciąg, A., & Vecchione, C. (2013). Vascular ageing: the role of oxidative stress. *The International Journal of Biochemistry & Cell Biology*, *45*(3), 556–9.

Rodríguez-Rojo, S., Visentin, A., Maestri, D., & Cocero, M. J. (2012). Assisted extraction of rosemary antioxidants with green solvents. *Journal of Food Engineering*, *109*(1), 98–103.

Rodríguez Montealegre, R., Romero Peces, R., Chacón Vozmediano, J. L., Martínez Gascueña, J., & García Romero, E. (2006). Phenolic compounds in skins and seeds of ten grape Vitis vinifera varieties grown in a warm climate. *Journal of Food Composition and Analysis*, *19*(6–7), 687–693.

Rogalinski, T., Ingram, T., & Brunner, G. (2008). Hydrolysis of lignocellulosic biomass in water under elevated temperatures and pressures. *The Journal of Supercritical Fluids*, *47*(1), 54–63.

Romero-Díez, R., Rodríguez-Rojo, S., Cocero, M. J., Duarte, C. M. M., Matias, A. A., & Bronze, M. R. (2018). Phenolic characterization of aging wine lees: correlation with antioxidant activities. *Food Chemistry*.

Romero, A., Alonso, E., Sastre, Á., & Nieto-Márquez, A. (2016). Conversion of biomass into sorbitol: Cellulose hydrolysis on MCM-48 and d-Glucose hydrogenation on Ru/MCM-48. *Microporous and Mesoporous Materials*, *224*, 1–8.

Ruggieri, L., Cadena, E., Martínez-Blanco, J., Gasol, C. M., Rieradevall, J., Gabarrell, X., Sánchez, A. (2009). Recovery of organic wastes in the Spanish wine industry. Technical, economic and environmental analyses of the composting process. *Journal of Cleaner Production*, *17*(9), 830–838.

Sant'Anna, V., Brandelli, A., Marczak, L. D. F., & Tessaro, I. C. (2012). Kinetic modeling of total polyphenol extraction from grape marc and characterization of the extracts. *Separation and Purification Technology*, *100*, 82–87.

Sauter, J., & Grafmüller, A. (2015). Solution Properties of Hemicellulose Polysaccharides

with Four Common Carbohydrate Force Fields. *Journal of Chemical Theory and Computation*, *11*(4), 1765–1774.

Scalbert, A. (1991). Antimicrobial properties of tannins. *Phytochemistry*, *30*(12), 3875–3883.

Schieber, A., Stintzing, F. ., & Carle, R. (2001). By-products of plant food processing as a source of functional compounds - recent developments. *Trends in Food Science & Technology*, *12*(11), 401–413.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., & Templeton, D. (2004). *Determination of structural carbohydrates and lignin in biomass. NREL Biomass analysis Technology Team, Laboratory Analytical Procedure (LAP).*

Smith, E. L., Abbott, A. P., & Ryder, K. S. (2014). Deep Eutectic Solvents (DESs) and Their Applications. *Chemical Reviews*, *114*(21), 11060–11082.

Sólyom, K., Mato, R. B., Pérez-Elvira, S. I., & Cocero, M. J. (2011). The influence of the energy absorbed from microwave pretreatment on biogas production from secondary wastewater sludge. *Bioresource Technology*, *102*(23), 10849–10854.

Sólyom, K., Solá, R., Cocero, M. J., & Mato, R. B. (2014). Thermal degradation of grape marc polyphenols. *Food Chemistry*, *159*, 361–6.

Souquet, J. M., Labarbe, B., Le Guernevé, C., Cheynier, V., & Moutounet, M. (2000). Phenolic composition of grape stems. *Journal of Agricultural and Food Chemistry*, *48*(4), 1076–80.

Spigno, G., & De Faveri, D. M. (2007). Antioxidants from grape stalks and marc: Influence of extraction procedure on yield, purity and antioxidant power of the extracts. *Journal of Food Engineering*, *78*(3), 793–801.

Spigno, G., & De Faveri, D. M. (2009). Microwave-assisted extraction of tea phenols: A phenomenological study. *Journal of Food Engineering*, *93*(2), 210–217.

Spigno, G., Pizzorno, T., & De Faveri, D. M. (2008). Cellulose and hemicelluloses recovery from grape stalks. *Bioresource Technology*, *99*(10), 4329–4337.

Tao, Y., Wu, D., Zhang, Q.-A., & Sun, D.-W. (2014). Ultrasound-assisted extraction of phenolics from wine lees: modeling, optimization and stability of extracts during

storage. *Ultrasonics Sonochemistry*, *21*(2), 706–15.

Teixeira, A., Baenas, N., Dominguez-Perles, R., Barros, A., Rosa, E., Moreno, D. A., & Garcia-Viguera, C. (2014). Natural bioactive compounds from winery by-products as health promoters: a review. *International Journal of Molecular Sciences*, *15*(9), 15638–78.

Thostenson, E. T., & Chou, T.-W. (1999). Microwave processing: fundamentals and applications. *Composites Part A: Applied Science and Manufacturing*, *30*(9), 1055–1071.

Van Man, P., Anh Vu, T., & Chi Hai, T. (2017). Effect of ultrasound on extraction of polyphenols from the old tea leaves. *Annals. Food Science and Technology*, *18*(1), 44–50.

Versari, A., Castellari, M., Spinabelli, U., & Galassi, S. (2001). Recovery of tartaric acid from industrial enological wastes. *Journal of Chemical Technology and Biotechnology*, *76*(5), 485–488.

Vilkhu, K., Mawson, R., Simons, L., & Bates, D. (2008). Applications and opportunities for ultrasound assisted extraction in the food industry — A review. *Innovative Food Science & Emerging Technologies*, *9*(2), 161–169.

Wahyuningsih, S., Wulandari, L., Wartono, M. W., Munawaroh, H., & Ramelan, A. H. (2017). The Effect of pH and Color Stability of Anthocyanin on Food Colorant. *IOP Conference Series: Materials Science and Engineering*, *193*(1), 012047.

Weidenbörner, M., Hindorf, H., Jha, H. C., & Tsotsonos, P. (1990). Antifungal activity of flavonoids against storage fungi of the genus Aspergillus. *Phytochemistry*, *29*(4), 1103–1105.

Wijngaard, H., Hossain, M. B., Rai, D. K., & Brunton, N. (2012). Techniques to extract bioactive compounds from food by-products of plant origin. *Food Research International*, *46*(2), 505–513.

Wu, J.-J., Lin, J.-C., Wang, C.-H., Jong, T.-T., Yang, H.-L., Hsu, S.-L., & Chang, C. J. (2009). Extraction of antioxidative compounds from wine lees using supercritical fluids and associated anti-tyrosinase activity. *The Journal of Supercritical Fluids*, *50*(1), 33–41.

Yalcin, D., Ozcalik, O., Altiok, E., & Bayraktar, O. (2008). Characterization and recovery of tartaric acid from wastes of wine and grape juice industries. *Journal of Thermal Analysis and Calorimetry*, *94*(3), 767–771.

Yoo, M. K., Choi, H. K., Kim, T. H., Choi, Y. J., Akaike, T., Shirakawa, M., & Cho, C. S. (2005). Drug release from xyloglucan beads coated with Eudragit for oral drug delivery. *Archives of Pharmacal Research*, *28*(6), 736–42.

THESIS OBJECTIVES

Thesis objectives

The general aim of this thesis is the **valorisation** of two vinification residues, mainly in terms of polyphenols. These residues are **wine lees and grape stems** and they have been barely studied compared to other winery sub-products such as grape pomace.

Wine lees

Wine lees from different vinification steps and enological procedures will be studied. Anthocyanins will be the target compounds of study. In order to maximize and develop a suitable process for their recovery the following specific aims will be addressed.

- **Evaluation of wine lees** as a rich source of compounds with great antioxidant activity against different types of free radicals. Establish correlations between the different phenolic compounds, mainly anthocyanins, and each antioxidant activity tested.
- Study of the **kinetic curves** for conventional **extraction** for anthocyanins. Optimisation of the main parameters: the solid-liquid ratio (R_{S-L}), the solvent composition, temperature, pH and operating time. Obtention of the **set of values that promote** the extraction of anthocyanins.
- Application of a disruption method as a pre-treatment to the conventional solid-liquid extraction: **microwave (MW) and ultrasound (US).** Study of the important parameters (R_{S-L}, solvent composition and pre-treatment time) according to statistical surface area response designs. Obtention of the **optimum MW and US** variables that maximize the extraction of anthocyanins.
- Performance of the optimum MW and US conditions followed by a conventional solid-liquid extraction (at the best selected conditions).
- Application of **enzymatic hydrolysis** to wine lees. Evaluation of different enzymes and different incubation times on the release of polyphenols.
- **Extracts characterization**
    - Total polyphenol content and/or total anthocyanin content.
    - Identification and quantification of main compounds (HPLC)
    - Antioxidant activity (*in vitro*): ORAC, HORAC, HOSC and /or FRAP.

Grape stems

The recovery of polyphenols from grape stems, flavonoids will be of high relevance. Similarly to wine lees, a sequence of specific objectives will be addressed.

- Study of the **kinetic curves** for conventional **extraction** for both flavonoids and total polyphenols. Tested parameters will be: the solid-liquid ratio (R_{S-L}), the solvent composition, temperature, pH and operating time. Obtention of the **set of values that promote** the extraction of both flavonoids and total polyphenols.
- Application of a **microwaves** as a pre-treatment to the conventional solid-liquid extraction**.** Study of the important parameters (R_{S-L}, solvent composition and pre-treatment time) according to statistical surface area response designs. Obtention of the **optimum MW** variables that maximize the extraction of both flavonoids and total polyphenols
- Performance of the optimum MW conditions followed by a conventional solid-liquid extraction (at the best selected conditions).
- **Extracts characterization**
    - Total polyphenol content and/or total flavonoid content.
    - Identification and quantification of main compounds, special focus on stilbenes (HPLC).
    - Antioxidant activity (*in vitro*): ORAC.
    - Anti-fungal activity: *Botrytis cinarea*.

Regarding the extraction of oligomers and sugars from grape stems, the described process will be followed:

- **A hydrothermal conversion procedure** will be used for the conversion of the biomass into sugars and oligomers. The operating temperature and time will be the variables of study.
- **The effect of a MW pre-treatment** to recover the polyphenols on the sugars extraction yield will be also evaluated.
- A **kinetic model** will be proposed to fit the experimental data minimizing the average absolute deviation.
- **Extracts characterization**
    - Determination of pH.

- Determination of oligomers and sugars by HPLC.
- Determination of degradation products.
- Extract purity.
- Liquid product characterization: hexoses/pentoses ratio

CHAPTER 1

Phenolic characterization of aging wine lees correlation with antioxidant activities

(https://doi.org/10.1016/j.foodchem.2018.03.119)

R. Romero-Díez, S. Rodríguez-Rojo, M.J Cocero, C.M.M Duarte, A.A. Matias, M.R. Bronze. *'Phenolic characterization of aging wine lees: Correlation with antioxidant activities'*. Food Chemistry. 259, pp. 188-195. Elsevier, 27/03/2018.

Abstract

Aging wine lees are water-wastes produced during the wine aging inside wood barrels that can be considered as alternative sources of bioactive compounds. Phenolic characterization and antioxidant activity (AA) measurements of wines lees solid-liquid extracts have been undertaken on a dry extract (DE) basis. Solvents with different polarities (water, methanol, ethanol, two hydroalcoholic mixtures and acetone) were used. Total phenolic (TPC) and total flavonoid contents (TFC) were determined. The mixture of 75:25(v/v) EtOH:H_2O showed the highest values with 254mg_{GAE}/g_{DE} and 146mg_{CATE}/g_{DE} respectively. HORAC, HOSC and FRAP were used to determine the AA of the extracts being also highest for the mixture of 75:25(v/v) EtOH:H_2O (4,690 $\mu mol_{CAE}/g_{DE}$, 4,527 $\mu mol_{TE}/g_{DE}$ and 2,197 $\mu mol_{TE}/g_{DE}$, respectively). For ORAC method, methanol extract showed the best value with 2,771μmol_{TE} /g_{DE}. Correlations between TPC, TFC, phenolic compounds and AA were determined. Most relevant compounds contributing to AA were identified using data from mass spectrometry, being mainly anthocyanins.

1. Introduction

The wine industry is an important sector of the EU economy, with an approximate worldwide production of 280 million hectoliters per year (Dimou et al., 2015). This agricultural activity generates huge amounts of wastes and by-products. In Spain alone, 2-3 million tons of wastes are generated per year (Ruggieri et al., 2009), including grape pomace (62%), lees (14%), stalk (12%) and dewatered sludge (12%). Traditionally, these wastes have been used as a supplement in animal feed with a poor nutrient value, as fermentation nutrient supplement (Dimou et al., 2015) or to recover tartaric acid (Versari, Castellari, Spinabelli, & Galassi, 2001). However, in many cases, they are disposed in landfill contributing to an environmental problem due to their low pH and high content in organic matter (Bustamante et al., 2008). Sometimes they are incinerated, which entails high costs of operation and production of toxic gases potentially dangerous to human health. As an alternative, some environmental friendly technologies have emerged to revalorize and take advantage of these winemaking residues with high contents of natural bioactive compounds (Teixeira et al., 2014).

Wine lees are the least exploited waste from the wine industry. Wine lees are a water-waste residue created during the vinification process of red and white wines and

they result from the combination of the yeasts, metabolites and other free phenolic compounds such as released free flavonol aglycones and pyranoanthocyanins (Barcia et al., 2014; Dimou et al., 2015). Depending on the stage of vinification, wine lees can be classified into different groups: first and second fermentation lees (formed during the alcoholic and malolactic fermentations, respectively) and aging wine lees (formed during wine aging in wood barrels). The main factors that may influence the composition of the lees are environmental conditions, the land type, grape variety and the time of aging in the wood barrels (Rankine, Fornachon, Boehm, & Cellier, 1971)

Wine lees could be used as rich sources of anthocyanins and other (poly)phenols with a strong potential application in food, cosmetics, and pharmaceutical industries, for their health-promoting effects due to their recognized antioxidant, antimicrobial, anti-inflammatory and cardio protective properties (Barcia et al., 2014; Landeka Jurčević et al., 2017). Furthermore, the exploitation of these dregs would contribute to an environmental equilibrium and lead to extracts of great interest with important bioactive properties that can be used as antioxidant additives. For instance, grape seeds extracts have potential antioxidant properties by inhibiting lipid oxidation and antimicrobial activities against major food borne pathogens (Perumalla & Hettiarachchy, 2011). However, there is a considerable lack of information regarding the polyphenolic composition of extracts derived from wine lees in comparison to other residues, such as grape pomace, seeds and other wine by-products (Teixeira et al., 2014).

Different methodologies can be used for determination of antioxidant activity (AA). Among them, the most common assays are Ferric Reducing Antioxidant Power (FRAP) and Oxygen Radical Absorption Capacity (ORAC) assays. They have been already used to measure AA of wine and polyphenolic extracts of winery by-products (Kondrashov, Ševčík, Benáková, Koštířová, & Štípek, 2009). Hydroxyl Radical Averting Capacity (HORAC) and Hydroxyl Radical Scavenging Capacity (HOSC) assays are gaining importance in the measurement of AA of extracts from berries, also rich in anthocyanins (Matias et al., 2016). Furthermore, it is important to correlate and understand which family of polyphenols and/or compounds contribute to the different antioxidant assays, showing specific antioxidant potential for the different radicals (like Fe^{+3}, $OH^{\bullet}$ or $ROO^{\bullet}$) depending on their chemical structure (Kallithraka, Mohdaly, Makris, & Kefalas, 2005).

As far as we know, there are only a few studies published studying the antioxidant activities response to extracts of wine lees and their correlations with phenolic

composition. Some authors used ORAC assays to measure the antioxidant activity of wine lees extracts prepared using a Soxhlet extraction and a microwave assisted extraction (Pérez-Serradilla & Luque de Castro, 2011) or with ultrasounds (Alonso, Guillén, Barroso, Puertas, & García, 2002). DPPH$^{\bullet}$ assay (Wu et al., 2009) and FRAP radical scavenging activity have been also employed to measure the antioxidant ability of wine lees extracts (Landeka et al., 2017) .

The work here presented is aimed at contributing to the phenolic characterization of aging wine lees obtained from *Vitis vinifera* grape variety. The total phenolic and flavonoid contents of the extracts prepared were measured as well as the chromatographic peak areas and were correlated with results from antioxidant activity assays to find out which families and specific compounds were contributing to the antioxidant activity. Putative identification of compounds with the major contribution to the antioxidant activity of the extract was carried out.

2. Materials

2.1 Wine Lees

Aging wine lees were provided by the winery *Grupo Matarromera* (41° 38′ 33″ N, 4° 17′ 28″ W) after a 12 months aging step of a red wine in American oak barrels. The wine lees were recovered from the bottom of the barrels during the decanting process. The grapes used in the vinification process (*Vitis vinifera*, variety *Tempranillo*) were cultivated in a clay soil in *Valbuena de Duero, Ribera de Duero* Designation of Origin (*Castilla y León*), in 2013. The average ambient temperature during this year in the vineyard was 11ºC, the average precipitations were 11 mm and the middling humidity was 32%. Wine lees were centrifuged, (Avanti J-26 XPI with a rotor type *JA-10*) for 90 minutes at 10,000 rpm. The moisture content of the solid phase was 75%. Afterwards, it was freeze-dried for 48 hours (Micro Modulo EDWARDS) and kept isolated from light at ambient conditions. These lyophilized lees were used for further extractions and characterization.

2.2 Reagents

Chemicals used for extractions methodologies were: bidistilled water (Milli-Q® Integral), EtOH absolute grade anhydrous >99.9% was purchased from CARLO ERBA Reagents, methanol absolute 99.99% was from Fisher Scientific (Waltham, MA, USA), acetone with a purity of ≥ 99.5% was from Sigma-Aldrich and citric acid from Sigma-Aldrich (St Quentin Fallavier, France).

For phytochemical total phenolic content: sodium carbonate (Na_2CO_3) was from Sigma-Aldrich (St Quentin Fallavier, France), Folin-Ciocalteau reagent was from Panreac (Barcelona, Spain) and gallic acid was from Fluka (Germany).

Chemicals used for antioxidant activity assays were: 2`,2`- Azobis (2-amidinopropane) dihydrochloride (AAPH), 6- hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), caffeic acid ($C_9H_8O_4$), cobalt fluoride tetrahydrate (CoF_2), hydrogen peroxide (H_2O_2) and picolinic acid ($C_6H_5NO_2$) from Sigma-Aldrich (St Quentin Fallavier, France) and iron chloride ($FeCl_3$) from Riedel-de-Haën (Seelze, Germany). Disodium fluorescein (FS) was from TCI Europe (Antwerp, Belgium). Sodium nitrite ($NaNO_2$ >99%) was purchased from Riedel-de Haen, aluminum chloride ($AlCl_3$ >97) and sodium acetate trihydrate ($C_2H_3NaO_2$ x $3H_2O$ >99%) were acquired from Sigma-Aldrich.

HPLC analysis were performed using formic acid 98% PA-ACS, Panreac®(Barcelona, Spain), acetonitrile for HPLC Plus Gradient-ACS+Reag. Ph. Eur.-Reag. USP. Carlo Erba (Val de Reuil, France) and Milli-Q® water (Milli-Q® Integral).

3 Experimental procedure and analytical methods

3.1 Solid-liquid extractions

Different solvents were selected to perform the extraction experiments: distilled water, ethanol, acetone, methanol and two mixtures of ethanol:water (50:50 and 75:25 v/v). These extractions were carried out using the same solid:liquid ratio of 1:40 (0.25 g of dry lees in 10 mL of solvent), stirring for 5 min at room temperature followed by 10 min of sonication in a ELMA Transsonic 700/H bath. Afterwards, sample extracts were centrifuged in a Hettich MiKro 220R at 6,000 rpm during 5 min. Supernatants were separated, filtered with PVDF (Polyvinylidene difluoride) filters with a pore size of 0.22 μm and kept at 4 ºC until analysis. In order to express the analytical results in "grams per dry extract" (g_{DE}), sample extracts were evaporated until dryness, using a vacuum centrifuge (Centrivap concentrator, Labconco, Kansas City, MO, USA) with a MD 4C NT vacuum pump (Vacuubrand, Wertheim, Germany).

3.2 Extracts characterization

3.2.1 Total Phenolic Content (TPC)

The total polyphenol content was measured by the Folin-Ciocalteu colorimetric method according to the procedure described by *T.Serra et al.* (Serra et al., 2008), which

was adapted for the microplate Spectrophotometer (Genesys™ 10UV, ThermoFischer Scientific). The results of TPCs were calculated using a calibration curve for gallic acid (between the range of 50-800 $ppm_{GALLIC\ ACID}$) *(Equation 1)*:

$$y = 0.0009x - 0.0133; R^2 = 0.997 \qquad (Eq.1)$$

where *'y'* is absorbance at 765 nm and *'x'* concentration of gallic acid in mg/L. TPCs were expressed in mg of gallic acid equivalents (GAE) per gram of dry extract (mg_{GAE}/g_{DE}) ± SD.

3.2.2 Total Flavonoid Content (TFC)

The flavonoid content of the different extracts was also measured as described by *Michalska et al.* (Michalska, Ceglińska, & Zieliński, 2007) with a modification, concerning the volume of the reagents used in order to work with a 96 microplate for the microplate Spectrophotometer (Genesys™ 10UV, ThermoFischer Scientific). Absorbance was read at 510 nm. The results of TFCs were calculated using a calibration curve for catechin (between the range of 0-1000 $ppm_{CATECHIN}$ *(Equation 2)*:

$$y = 2.0421x - 0.0229; R^2 = 0.999 \qquad (Eq.2)$$

where *'y'* is absorbance at 510 nm and *'x'* concentration of catechin in mg/L. TFCs were expressed in mg of catechin equivalents (CATE) per gram of dry extract (mg_{CATE}/g_{DE}) ± SD.

3.2.3 HPLC-DAD (High Performance Liquid Chromatography)

The High Performance Liquid Chromatography (HPLC) system used was a Thermo Finnigan (Surveyor model) equipped with an autosampler, a pump and a photodiode-array detector (PDA). A pre-column (100RP-18, 5µm) and a reversed phase C18 column (LiCrospher® 100 RP-18, 250x4mm; 5µm) in a thermostated oven at 35 ºC were used for separation using a gradient elution, adapted from (Csiktusnádi Kiss et al., 2000), using water acidified with formic acid at 0.5% (v/v) as solvent A and 90% acetonitrile as solvent B. The flow rate was 0.3 mL/min with an injection volume of 20 µL. The linear solvent gradient was as follows: 0 min, 94.4% A; 15 min, 83.3% A; 20 min, 77.8% A maintained for 10 min; 55 min, 66.7% A; 80 min, 44.4% A; 120 min, 0% A maintained for 15min; 140 min; 94.4 % A constant for 10 min. The data acquisition systems was the Chromquest version 4.0 (ThermoFinnigan—Surveyor, San Jose, CA,

USA). Absorption spectra were acquired from 210 to 600 nm by a photodiode array detector. Semi-quantitative evaluation of detected compounds was expressed as the area percentage of each peak respect to the total area of the chromatogram at 280nm and 520 nm, which are the general wavelength for polyphenols and the specific wavelength for anthocyanins, respectively.

3.2.4 HPLC-MS/MS (High Performance Liquid chromatography–mass spectrometry)

The system used was a liquid chromatography Waters Alliance 2695 Separation Module (Waters®, Ireland) consisting on a system of quaternary pumps, degasificator, autosampler and a column furnace. The mass spectrometer (MS/MS) used was a MicroMass Quattromicro® API (Waters®, Ireland). For the data acquisition and processing MassLynx® 4.1 software was employed. Chromatographic separation of compounds was carried out on a LiChrospher® 100 RP-18 (250 x 4.0mm) column in an oven at 35 ºC. Chromatographic separation of compounds was carried out in a reversed-phase LiChrospher® 100 RP-18 5μm LiChroCART® 250-4 column inside a thermostated oven at 35ºC. The mobile phase consisted of formic acid (0.5% v/v in ultrapure water) (eluent A) and acetonitrile (eluent B). The gradient program used was 99:1 A:B for 5 min, from 99:1 A:B to 40:60 A:B in 40 min, from 40:60 A:B to 10:90 A:B in 45 min, held isocratically (90% B) for 10 min, from 10:90 A:B to 99:1 A:B in 10 min, and finally held isocratically (99:1 A:B) for 10 min, at a flowrate of 0.3 mL/min, with an injection volume of 20 μL. Total run time was 120 min. Absorption spectra were acquired from 210 to 600 nm by a photodiode array detector. AC were monitored at 520 nm, flavonols at 360 nm, phenolic acids at 320 nm, and phenolic compounds in general at 280 nm. Mass spectrometry was performed using an electrospray ion source in negative ion mode (ESI-). The ion source temperature was 120°C, the capillary voltage was 2.5 kV, and the source voltage was 30 V. Compounds separated by HPLC were ionized and the mass spectra were recorded in a full scan mode, between m/*z* 100 and 1500. High purity nitrogen was used as drying and nebulizing gas, and ultrahigh purity argon was used as collision gas. Different collision energy values were used in fragmentation experiments.

3.3 Evaluation of the Antioxidant activity (AA)

3.3.1 ORAC (Oxygen Radical Absorbance Capacity)

Oxygen Radical Absorbance Capacity (ORAC) is a method for the evaluation of antioxidative ability of a specific substance based on the fluorescence quenching of fluorescein sodium (FS) salt after exposure to AAPH (2,2-azobis(2-amidino-propane) dihydrochloride), which generates oxygen radicals ($ROO^{\bullet}$) at a constant rate. ORAC assay was carried out by the method described by *Feliciano et al.* (Feliciano et al., 2009) who included some modifications for the FL800 microplate fluorescence reader (Bio-Tek Instruments, Winooski, VT, USA). ORAC values were calculated using a regression equation between the Trolox concentration and the area under the decay of the FS curve (AUC) according to the calibration curve for Trolox (between the range of 5-40 μmol/L_{TROLOX}) *(Equation 3)*:

$$y = 0.4328x - 0.7811; R^2 = 0.9931 \qquad (Eq.3)$$

where *'y'* is the net AUC and *'x'* concentration of Trolox in μmol/L. The results are given in μmol of Trolox equivalents (TE) per g of dry extract ($\mu mol_{TE}/g_{DE}$) ± SD.

3.3.2 HORAC (Hydroxyl Radical Averting Capacity)

Hydroxyl radical averting capacity (HORAC) is an antioxidant method able to measure the capability of a substance to neutralize the hydroxyl radical ($HO^{\bullet}$) generated by Fenton-like reactions employing a Co(II) complex using FS as a probe. HORAC assays were performed by the method developed by *Ou et al.* (Ou et al., 2002) modified for the FL800 microplate reader and tested successfully in more publications (Serra, Duarte, Bronze, & Duarte, 2011). HORAC values were calculated using a regression equation between the caffeic acid concentration and the area under the decay of the FS curve (AUC) according to the calibration curve for caffeic acid (between the range of 0-250 μmol/$L_{CAFEIC\ ACID}$) *(Equation 4)*:

$$y = 0.0685x - 2.9112; R^2 = 0.983 \qquad (Eq.4)$$

where *'y'* is the net AUC and *'x'* concentration of cafeic acid in μmol/L. The results are expressed in μmol of equivalents of caffeic acid (CAE) per g of dry extract ($\mu mol_{CAE}/g_{DE}$) ± SD.

3.3.3 HOSC (Hydroxyl Radical Scavenging Capacity)

Hydroxyl Radical Scavenging Capacity (HOSC) is another method which also uses FS as a probe in order to evaluate the hydroxyl radical scavenging ability of a substance in a classic Fenton reaction with Fe^{+3} and H_2O_2 as a source of hydroxyl radicals. The assay was carried out by the model described by *Moore et al*. (Moore, Yin, & Yu, 2006). HORAC values were calculated using a regression equation between the Trolox concentration and the area under the decay of the FS curve (AUC) according to the calibration curve for Trolox (between the range of 0-30 μmol/L_{TROLOX}) *(Equation 5)*:

$$y = 0.7896x - 0.0158; R^2 = 0.997 \qquad (Eq.5)$$

where *'y'* is the net AUC and *'x'* concentration of Trolox in μmol/L. The results are given in μmol of Trolox equivalents (TE) per g of dry extract ($\mu mol_{TE}/g_{DE}$) ± SD.

3.3.4 FRAP (Ferric Reducing Antioxidant Power)

The FRAP assays has been compared with other antioxidant capacity methods as it is capable to reveal substances that can reduce Fe^{+3} to Fe^{+2}. FRAP assays were carried out by the protocol suggested by *Bolanos de la Torre et al*. (Bolanos de la Torre, Henderson, Nigam, & Owusu-Apenten, 2015). Absorbance was measured at 593 nm in a spectrophotometer (ThermoSpectronic Genesys 10 μV). The FRAP results were calculated according to the calibration curve for Trolox (between the range of 0-600 μmol/L_{TROLOX}) *(Equation 6)*:

$$y = 0.0015x + 0.5585; R^2 = 0.998 \qquad (Eq.6)$$

where y is absorbance at 593 nm and *'x'* concentration of Trolox in μmol/L. Results are shown in μmol of Trolox equivalents (TE) g of dry extract ($\mu mol_{TE}/g_{DE}$) ± SD.

3.4 Correlation data treatment

A correlation study using Excel 2013 was performed. Pearson´s regression coefficient '*r'* with P-value was selected. The correlation coefficient '*r*' is employed to assess if two different variable are associated and the p-value is used to quantify the idea of statistical significance of evidence in the context of null hypothesis. A 95% confidence interval for the correlation coefficient was chosen, which means that if the probability is lower than 5% ($p<0.05$), the correlation coefficient is statistically significant, according to the t-Student distribution. This correlations was performed

between the areas of all detected peaks in the chromatograms at 280 nm with TPC, TFC and the different AA tests for each solvents. Among all peaks, only 11 compounds were selected since they had a '*r*' higher than |0.90|.

3.5 Statistical Analysis

All data were expressed as means ± standard deviations (SD). Assays for TPC, TFC and AA measurements were performed, at least, in triplicate. A statistical analysis was done using SigmaStat 3.0® software. These analyses were performed to study if each individual solvent had a statistically significant effect on the measured variables that characterize the extracts (TPC, TFC, ORAC, HORAC, HOSC and FRAP). All values were tested for normal distribution and equal variance. When homogeneous variances were confirmed, data were analyzed by One Way Analysis of Variance (ANOVA) coupled with the post-hoc Holm–Sidak test ($p<0.05$ was accepted as statistically significant in all cases).

4. Results and discussion

For years, the phenolic composition of samples has been determined using spectrophotometric methodologies that are useful for a rapid screening of a large number of samples, and are not particularly expensive. However, they are not able to obtain selective information since results may be influenced by other components present in the samples. Chromatography and mass spectrometry have become important tools for characterization purposes. In the present work wine lees extracts were prepared using different solvents and were analyzed using the methodologies described, in order to characterize their phenolic content.

4.1 TPC (Total Phenolic Content) and TFC (Total Flavonoid Content)

The solubility of the phenolic compounds into different solvents, which is related with the solvent polarity used (Rocío Teruel, Garrido, Espinosa, & Linares, 2015), plays a major role in the recovery of polyphenols from different sources. Results from the total phenolic content (TPC) for the different extracts prepared in this work are presented in Table 1 and they range from 26±1 mg_{GAE}/g_{DE} to 254±24 mg_{GAE}/g_{DE} (3.6 $mg_{GAE}/g_{DRY\ LEES}$) depending on the solvent used. Water, ethanol and acetone barely extracted the phenolic compounds present in the wine lees, compared with methanol and the mixtures of ethanol:water. Usually mixtures of ethanol:water present better extracting power for

these type of compounds and in our case, the mixture corresponding to the ratio 75:25, was the best one with a value of 254±24 mg_{GAE}/g_{DE}. This value was similar to those obtained by *Jia-Jiuan Wu et al.*, who reported a 21% (w/w) recovery of the initial dried wine lees from a Taiwan grape variety with a Soxhlet extraction using 70% (vol.%) aqueous ethanol solution for 6 hours (Wu et al., 2009). In our case, we were able to extract 25% (w/w) of the initial wine lees with a dramatic reduction of time (345 min *vs* 15 min). On the contrary, much higher results were obtained by *Pérez-Serradilla et al*. (Pérez-Serradilla & Luque de Castro, 2011). They performed a Soxhlet extraction with a 75:25 EtOH:H_2O (%v/v) from dried Syrah grape variety wine with a solid-liquid ratio of 1/10 lees, during 24 hours, and obtained an extract with 547 mg_{GAE}/g_{DE}. Also *Landeka et al.* (Landeka et al., 2017) described an acidified methanolic wine lees extract from a Bosnia and Herzegovina variety, with a TPC of 23.16 $mg_{GAE}/g_{DRY\ LEES.}$ All these extracts were obtained for dry wine lees. The expected recovery using wet wine lees is lower, according to Dimou *et al*. (Dimou et al., 2016). They carried out a simulation of a global valorization process of wet wine lees, from *Merlot* variety grape, and proposed a recovery of antioxidants of only 0.8 % (w/w) by conventional solid liquid extraction with a 70:30 EtOH:H_2O (%vol.), based on lab-scale experiments.

The total flavonoid content (TFC) presented a similar behavior as the TPC and values ranged from 16±1 to 146±5 mg_{CATE}/g_{DE}. Higher flavonoid content was obtained with methanol and the mixtures of ethanol:water as shown in Table 1. Acetone, ethanol and water were the solvents with less capacity to extract all the phenolics and flavonoid family.

Table 1: Total phenolic and flavonoid content and antioxidant activity of aging wine lees extracts obtained with different solvents. The highest values (per g of dry residue) are presented in bold and the lowest values in italics. Values with different lowercase letters in the same column are significantly different ($p<0.05$).

	TPC **mg GAE /g$_{DE}$**	**TFC** **mg CAT /g DE**	**ORAC** **µmol TE /g DE**	**HORAC** **µmol CAT /g DE**	**HOSC** **µmol TE /g DE**	**FRAP** **µmol TE /g DE**	**TA** **280nm**	**TA** **520nm**
H_2O	38 ± 3^{a}	16 ± 1^{a}	471 ± 86^{a}	348 ± 35^{a}	592 ± 39^{a}	461 ± 3^{a}	$8.68\cdot10^{7}$	$2.04\cdot10^{7}$
EtOH	94 ± 8^{b}	51 ± 18^{b}	1,603 ± 227^{b}	1,245 ± 103^{b}	2,107 ± 134^{b}	1,034 ± 26^{b}	$6.95\cdot10^{7}$	$1.90\cdot10^{7}$
Acetone	26 ± 1^{a}	30 ± 3^{a}	217 ± 68^{a}	543 ± 59^{a}	281 ± 26^{a}	362 ± 6^{a}	$2.30\cdot10^{7}$ *	$2.40\cdot10^{6}$ *
MeOH	149 ± 7^{c}	112 ± 12^{c}	**2,771 ± 289^{c}**	3,963 ± 367^{c}	2,732 ± 257^{c}	1,542 ± 38^{c}	$2.72\cdot10^{8}$	$1.12\cdot10^{8}$
EtOH:H_2O (50:50)	206 ± 28^{d}	145 ± 6^{d}	1,003 ± 90^{d}	2,985 ± 389^{d}	3,912 ± 310^{d}	2,112 ± 65^{d}	**$3.13\cdot10^{8}$**	$1.99\cdot10^{8}$
EtOH:H_2O (75:25)	**254 ± 24^{e}**	**146 ± 5^{d}**	2,323 ± 289^{e}	**4,690 ± 463^{e}**	**4,527 ± 413^{e}**	**2,197 ± 84 ^{d}**	$2.75\cdot10^{8}$	**$1.77\cdot10^{8}$**

**The total areas for the acetone extract was calculated without taking into account the area of the acetone detected in the chromatogram (t_R ~ 15min) (vide Figure 2S from the supplementary material for more information)*

4.2 Antioxidant activities (AA)

The values obtained for the different antioxidant activities (AA) of the extracts are shown in Table 1. The ethanol:water extracts had higher antioxidant capacities than the rest of the extracts, especially the 75:25 EtOH:H_2O (v/v) mixture. This behavior agrees with the total phenolic concentration: the higher the TPC values, the higher the antioxidant activities (Orak, 2007). However, this tendency was not shown for the ORAC assay where the highest antioxidant activity was found for the methanol extract with 2,771±289 μmol $_{TE}$/g $_{DE}$. These ORAC values were lower compared to 6,100 μmol$_{TE}$/g $_{DE}$ obtained by *Pérez-Serradilla et al*. (Pérez-Serradilla & Luque de Castro, 2011) for a wine lees extract prepared from *Syrah* red grapes using a Soxlhet and a 75% ethanol (%, v/v.) aqueous solution with a solid-liquid ratio of 1:10. These differences between extracts may be explained by the different TPC values that was much higher (547 mg$_{GAE}$/g$_{DE}$) than ours (254±24 mgGAE/g DE), as previously mentioned). Our ORAC values are also comparable with the one obtained with an extract of grape marc with an ORAC value of 2,644 μmol$_{TE}$/g $_{DE}$ and a TPC of 222 mg$_{GAE}$/g$_{DE}$. This extract was prepared by traditional solid-liquid extraction of grape marc with a solid-liquid ratio 1:2 (g/mL) at a temperature of 60ºC for a period of 3 hours, using a mixture 50:50 EtOH:H_2O (%vol.) (Moro Gonzaléz, 2009).

Concerning FRAP assays, values for aging wine lees extracts ranged from 362± 6 to 2,197± 84 μmol$_{TE}$/g$_{DE}$ (44 ± 1 to 583 ± 18 μmol$_{TE}$/g$_{DRY\ LEES}$). These values were similar to those found in the literature for other wine lees waste extracts. For example, *Landeka et al*. (Landeka Jurčević et al., 2017) who obtained a wine lees extract from a Bosnia and Herzegovina winery with a TPC value of FRAP values of 457 μmol$_{TE}$/g$_{DRY\ LEES}$.

With the HORAC and HOSC assays, the highest values were obtained for the 75:25 EtOH:H_2O (%vol.): 4,690 ± 463 μmol $_{CATE}$/g $_{DE}$ and 4,527 ± 413 μmol $_{TE}$/g $_{DE}$ respectively. These values cannot be compared due to the absence of literature concerning these type of assays for wine lees, wines or grape extracts. However, some works used HORAC assay to measure the capacity against hydrophilic chain-breaking hydroxyl radicals of other red berries. That was the case of *Matias et al*. (Matias et al., 2016) whose cherry extracts presented a higher HORAC value (6874 ± 584 μmol $_{CATE}$/g $_{DE}$) than ours. These differences may result from the different types and concentrations of anthocyanins and phenolic acids found in cherry extracts.

Correlations between TPC and TFC values from the six extracts and the AA values are shown in Table 2. A graphic example of these correlations is shown in Figure 2S in *Supplementary Material.*

*Table 2: Correlation (r values) between TPC, TFC and the antioxidant activity results. Values of r >|-0.90| are in bold. Values with a * are statistically significant (p <0.05).*

	TPC	**TFC**
TPC	1.000*	
TFC	**0.970***	1.000*
ORAC	0.665	0.646
HORAC	**0.924***	**0.930***
HOSC	**0.992***	**0.960***
FRAP	**0.990***	**0.983***

High positive statistically significant correlations (r > 0.90) were found for HORAC, HOSC and FRAP assays. Strong correlations between FRAP and total phenolics and flavonoids have been reported in the literature (Arnous, Makris, & Kefalas, 2002; Doshi, Adsule, Banerjee, & Oulkar, 2015). However, ORAC values did not correlate with TPC and TFC ($r \leq 0.665$) since the highest value was achieved for methanolic extract and not for the hydroalcoholic mixture (75:25). This different trend may be explained by the ability of methanol to extract other molecular entities than polyphenols or even by synergetic effects between the main compounds extracted which may potentiate the scavenging of peroxyl free radicals.

4.3 Analysis by HPLC –DAD and HPLC-MS/MS

The chromatographic profiles of the extracts were compared using reverse phase chromatography and detection at 280 nm. The employed method has a good repeatability with a variation between 2-5% in peak areas and lower for retention time. Peak area of detected compounds was measured, as well as the total area (TA) of the chromatogram at 280 nm (maximum absorption for phenolic compounds) and 520 nm (maximum absorption for anthocyanins) to carry out the correlation with antioxidant activity values. TA values for each extract are shown in Table 1. These values were important to consider, as it might represent the real value of phenolic content since the interferences that occur in the spectrophotometric TPC measurement, were avoided in the chromatographic analysis. The chromatographic profiles from the different extracts analyzed were according to data obtained for TPC and TFC: water, ethanol and acetone extracts

chromatograms showed that these solvents were less efficient in the extraction of phenolic compounds from aging wine lees, comparing to MeOH, 50:50 and 75:25 EtOH:H_2O (%vol.) mixtures, being these mixtures diluted twofold. Figure 1 illustrates the chromatographic profiles obtained for aging wine lees extracts with methanol and the two hydro-alcoholic mixtures at 280 nm. Chromatographic profiles for acetone, ethanol and water extracts can be seen in *Supplementary Material*.

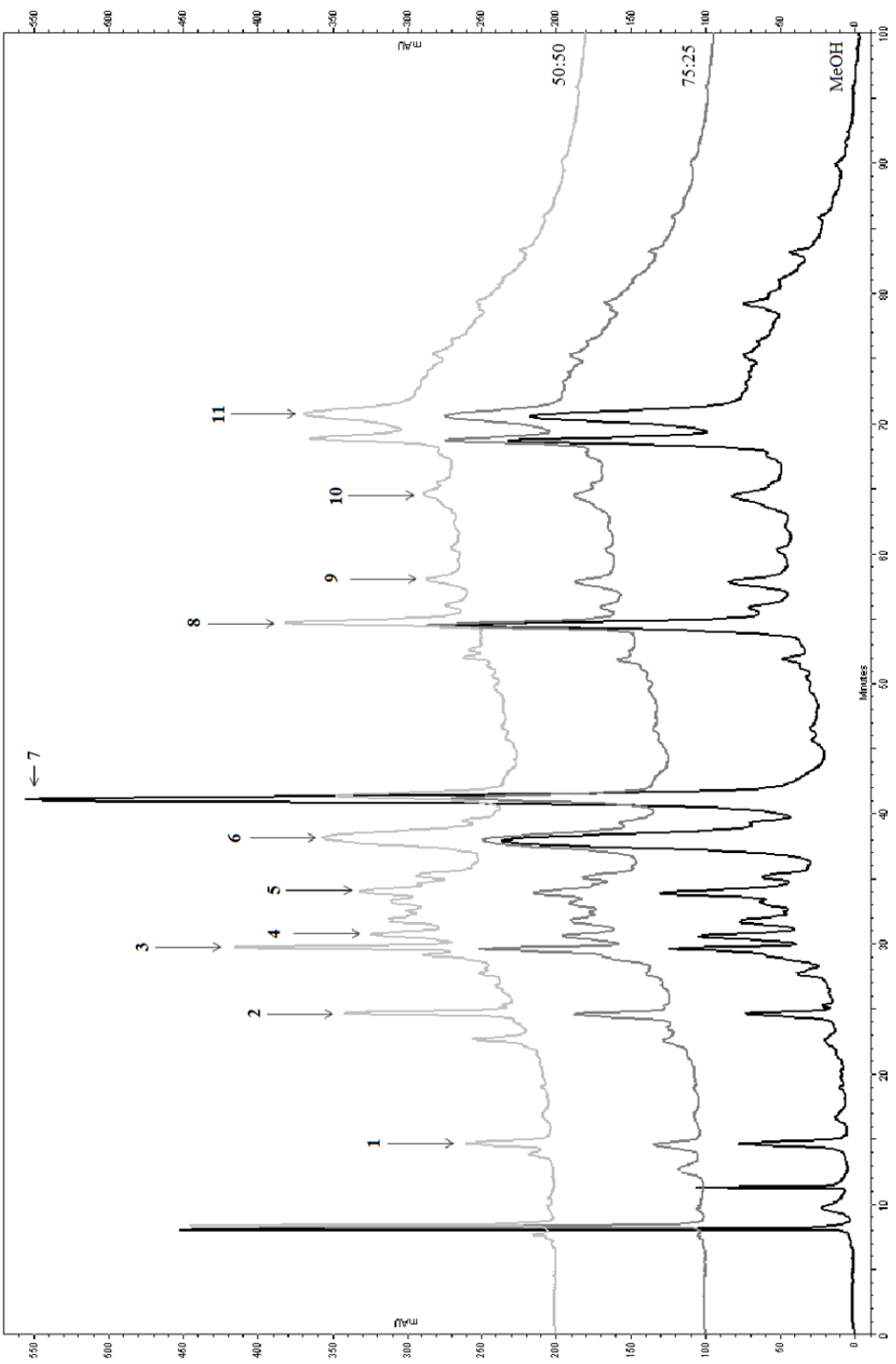


Figure 1: Chromatograms at 280 nm obtained for methanolic and hydro-alcoholic extracts of aging wine lees.

4.3.1 Compounds contributing to antioxidant activity

Compounds from methanol and hydroalcoholic mixtures were putatively identified by HPLC-MS/MS (Figure 1 and Table 3). All the peaks where present in the three extracts. The main compounds found were anthocyanins, and all the compounds were already reported for red wines (Arnous et al., 2002; Bravo, Silva, Coelho, Boas, & Bronze, 2006; Cantos, Espín, & Tomás-Barberán, 2002) and in wine lees residues (Delgado de la Torre, Priego-Capote, & Luque de Castro, 2015).

Table 3: Putative identification of main compounds in the extracts. Retention time (min), maximum absorbance (nm) (λmáx), MS and MS/MS values (m/z), putative identification, phenolic family and the percentage of each peak area in the different extracts. Numbers in brackets represent the main m/z values.

Peak nº	Retention time (min)	λmax (nm)	m/z (positive and negative mode)	[M-H] fragments (m/z)	Putative Identification	Phenolic family	Percentage (%) of each peak			Reference(s)
							MeOH	50%v.	75%v.	
1	14.7	270	169 (M^-)	[169], 125	Gallic Acid	Phenolic acid	0.59	1.29	1.16	(Delgado de la Torre et al., 2015; Hernández, Estrella, Carlavilla, Martín-Álvarez, & Moreno-Arribas, 2006)
2	24.7	328	289 (M^-)	[289] 229, 153, 137	Catechin	Flavan-3-ol	1.10	2.67	2.57	(Delgado de la Torre et al., 2015; Hernández et al., 2006), (Cantos et al., 2002)
3	29.7	529	465 (M^+)	[465] 349, 303, 147	Delphinidin-3-O-glucoside	Anthocyanin	1.46	3.62	3.96	(Delgado de la Torre et al., 2015)
4	30.6	283	289 (M^-)	[289] 271, 227, 203, 188	Epicatechin	Flavan-3-ol	1.71	4.43	3.51	(Cantos et al., 2002; Hernández et al., 2006)
5	33.9	529	479 (M^+)	[479] 317	Petunidin-3-O-glucoside	Anthocyanin	2.42	4.27	3.71	(Delgado de la Torre et al., 2015)
6	37.9	527	493 (M^+)	[493] 331	Malvidin 3-*O*-glucoside	Anthocyanin	8.20	9.93	11.35	(Delgado de la Torre et al., 2015), (Cantos et al., 2002)
7	41.4	366	477 (M^-)	[477] 301, 151	Quercetin -3-*O*-glucuronide	Flavonol	11.72	5.75	10.64	(Oszmiański et al., 2015)
8	54.5	368	317 (M^-)	[317] 179, 151	Myricetin	Flavonol	6.25	6.23	7.20	(Delgado de la Torre et al., 2015; Hernández et al., 2006)
9	57.8	529	611 (M^+)	[611] 303	Delphinidin 3-(6-p-coumaroylglucoside)	Anthocyanin	2.94	8.07	6.84	(Hernández et al., 2006; Hokkanen, Mattila, Jaakola, Pirttilä, & Tolonen, 2009)
10	64.4	529	625 (M^+)	[625] 317	Petunidin-3-(6-p-coumaroylglucoside)	Anthocyanin	3.20	8.50	7.55	(Hernández et al., 2006)
11	70.6	530	639 (M^+)	[639] 331	Malvidin 3-(6-p-coumaroylglucoside)	Anthocyanin	7.45	13.29	15.48	(Delgado de la Torre et al., 2015)

Even though a large number of peaks were detected in the HPLC chromatograms, only those peaks which showed r values ≥|-0.900| between antioxidant activity and peak areas were considered for discussion, as shown in Table 4.

*Table 4: Correlation (r values) between the peak areas at 280 nm (see Table 2 for identification) and the antioxidant activity results. Values of r >|-0.90| are in bold. Values with a * are statistically significant (p<0.05).*

	ORAC	HORAC	HOSC	FRAP
1	-0.896*	-0.414	0.774	0.896*
2	-0.840*	-0.308	0.841*	**0.940***
3	-0.734	-0.139	**0.921***	**0.985***
4	**-0.947***	-0.532	0.682	0.828*
5	**-0.951***	-0.542	0.673	0.821*
6	-0.687	-0.072	**0.946***	**0.994***
7	**0.998***	0.815*	-0.351	-0.551
8	-0.619	0.018	**0.971***	**0.999***
9	**-0.912***	-0.447	0.750	0.879*
10	-0.889*	-0.399	0.784	**0.903***
11	-0.667	-0.046	**0.954***	**0.997***

Anthocyanins were the majority of the identified compounds, being malvidin 3-O-glucoside (*7*) and malvidin 3-(6-p-coumaroylglucoside) (*12*) the most concentrated in all extracts as it is shown in Table 2. Most of the compounds were in higher concentration in the hydroalcoholic mixtures, as expected. For example, anthocyanins such as *(5)* petunidin-3-O-glucoside, *(9)* delphinidin 3-(6-p-coumaroylglucoside) and *(10)* petunidin-3-(6-p-coumaroylglucoside) were present in a higher concentration in the 50% vol. ethanol mixture with a percentage of 4.3, 8.0 and 8.5, respectively. Additionally, the 75% vol. ethanol mixture was richer in *(3)* delphinidin-3-O-glucoside (4%), *(6)* malvidin 3-O-glucoside (11.3%) and *(11)* malvidin 3-(6-p-coumaroylglucoside) (15.5%) anthocyanins. In contrast, a different tendency was observed for quercetin-3-glucuronide *(7)*, a flavonol that was at higher levels in the MeOH extract (11.7%) than in the 50:50 hydro-alcoholic mixture (5.8%) and similar to the 75:25 hydroalcoholic mixture (10.6%). The other flavonol, *(8)* myrecitin was present in smaller amounts in each extract, being higher for the 75% vol. ethanol mixture. Both flavan-3-ols, *(2)* catechin and *(4)* epicatechin, were found in higher quantities in 50%vol. ethanol extract with 2.7% and 4.4%, respectively.

Furthermore, it was possible to establish which compounds or family of compounds contributed to each AA assay and which type of oxygen radicals are affected.

Gallic acid *(1)* was the only benzoic acid identified. It presented a statistically significant negative correlation with ORAC and a statistically significant positive correlation with FRAP (*-0.896, p<0.050; 896, p<0.05* respectively*)*. This tendency is explained by the high scavenging power of gallic acid, making it capable of rapidly deactivating a wide variety of radicals via electron transfer (Marino, Galano, & Russo, 2014).

Flavan-3-ol presented statistically significant negative correlations with ORAC *(-0.840 and -0.947, p<0.05* for catechin *(2)* and epicatechin *(4)*, respectively). Moreover, both showed statistically significant positive correlations with FRAP *(0.940* for *(2)* catechin *and 0.828, p<0.05 for* epicatechin *(4)*. However, only catechin *(2)* had a statistically significant positive correlation with HOSC *(0.841, p<0.05).* These observations are consistent with published data. It has been strongly substantiated that flavanols, namely catechins and proanthocyanidins, are powerful radical quenchers in various systems (Arnous et al., 2002; Kallithraka, Mohdaly, Makris, & Kefalas, 2005)**.**

For the case of flavonols, both identified compounds showed positive correlations but for different assays. Quercetin -3-O-glucuronide *(7)* showed a statistically significant correlation with ORAC *(0.998, p<0.05*) and HORAC *(0.815, p<0.05*). On the contrary, myricetin *(8)* registered an *r* value of *0.971 and 0.999 (p<0.05)* for HOSC and FRAP, respectively.

These differences observed between assays are related to the individual molecular structure of each compound. It must be borne in mind that each assay is a measure of the antioxidant activity but using different radicals. Thus, stereoisomerism, functional groups distribution and any other structural parameters such as the oxidation state of the C-ring, the hydroxylation and methylation pattern also are expected to affect the final value (Frankel, Waterhouse, & Teissedre, 1995; Kallithraka et al., 2005). Furthermore, it has also been demonstrated that the substitution of a 3-hydroxyl for a sugar group influences the antioxidant ability of flavonols, decreasing it in a 10-15% (Gardner, McPhail, Crozier, & Duthie, 1999). Thus, the same behavior is expected for the rest of polyphenol families if this substitution takes place.

Anthocyanins' contribution seemed to have a completely different effect depending on the method used to measure the AA. For instance, for ORAC and HORAC, the effect was negative while for HOSC and FRAP was positive. However, not all of them were statistically significant. Just petunidin-3-O-glucoside *(5)*, delphinidin 3-(6-p-coumaroylglucoside) *(9)* and petunidin-3-(6-p-coumaroylglucoside) *(10)* displayed statistically significant *r* values for ORAC (*-0.951, -0.912* and *-0.889, p<0.05,*

respectively*)*. For FRAP, the result was always statistically significant *(r ≥ 0.821, p<0.05)*. Nonetheless, for HOSC, the significance was only ensured for delphinidin-3-O-glucoside *(3)*, malvidin 3-O-glucoside *(6)* and malvidin 3-(6-p-coumaroylglucoside) *(11) (r ≥ 0.921, p<0.05)*.

Since anthocyanins are the main polyphenols found in wine lees residues, correlations between the peak areas of the identified anthocyanins at 520 nm (maximum absorbance of anthocyanins) and each AA were performed. This wavelength was used to isolate anthocyanins from other possible compounds that can co-elute and can be detected at 280 nm. The *'r'* values are listed in Table 5 and, in this case, they showed the same behavior as described in the previous paragraph: negative correlations for ORAC and HORAC, positive correlations for HOSC and FRAP. These individual analyses provided a more accurate pattern regarding significance. All anthocyanins became statistically significant for ORAC *(r ≥ |0.824|, p<0.05)*. Delphinidin 3-(6-p-coumaroylglucoside) *(9)*, petunidin-3-(6-p-coumaroylglucoside) *(10)* and malvidin 3-(6-p-coumaroylglucoside) *(11)* were also statistically significant for FRAP *(r ≥ |0.888|, p<0.05)*. Furthermore, malvidin 3-(6-p-coumaroylglucoside) *(11)* had a statistically significant behavior for HOSC *(0.856, p<0.05)* too. For other anthocyanin/assay, results were not statistically significant.

*Table 5: Correlation (r values) between the peak areas at 520nm for anthocyanins (see Table 2 for identification) and the antioxidant activity results. Values of r >|-0.90| are in bold. Values with a * are statistically significant (p<0.05).*

	ORAC	HORAC	HOSC	FRAP
3	**-0.989***	-0.673	0.542	0.716
5	**-0.983***	-0.645	0.574	0.742
6	**-0.968***	-0.590	0.629	0.786
9	**-0.903***	-0.428	0.764	0.888*
10	-0.897*	-0.415	0.773	0.895*
11	-0.824*	-0.280	0.856*	**0.950***

With this new pattern is possible to establish a relation between the significance and individual molecular structure of anthocyanins. Those anthocyanins which have a -3-O-glucoside moiety, negatively contribute to ORAC. Thus, these compounds unsuccessfully scavenge $ROO^{\bullet}$. Also anthocyanins with the 6-p-coumaroyl moiety negatively contribute to ORAC. Nevertheless, they displayed positive statistically significant correlations with

FRAP, corroborating they are capable of quenching Fe^{+3} and $HO^{\bullet}$ radicals generated from a Fenton reaction by hydrogen transfer atom (HAT) mechanism with Fe^{+3} (Li et al., 2017).

Moreover, it is worth mentioning that correlations, either positive or negative, between anthocyanins and AA were found. Even though most of the researchers concurred that the different antioxidant potential is strongly dependent from total phenolic and flavanol contents, a lot of controversy appears when talking about anthocyanins. Some previous works established there is no relation between ORAC and anthocyanins (Sólyom, Solá, Cocero, & Mato, 2014) or poor correlations (Arnous et al., 2002), but others found strong correlations between AC content and AAs like in our case. As an example, *Moyer et al.*, (Richard A. Moyer, Kim E. Hummer, Chad E. Finn, Balz Frei, & Ronald E. Wrolstad, 2001) whose work reported statistically significant correlations between AC content and ORAC $(r \geq |0.460|, p<0.005)$ and FRAP $(r \geq |0.440|, p<0.005)$.

5. Conclusions

In this work aging wine lees, an underexploited waste stream from the winemaking process, is proposed as an alternative source of phenolic compounds as its extracts could be used as antioxidant additives. An extraction procedure with six solvents with different polarities (water, acetone, methanol, ethanol and two hydro-alcoholic mixtures) was established in order to characterize this raw material in terms of phenolic composition and antioxidant activity, thus providing an important contribution for the valorization of this biomass. It was found that the recovery of phenolic compounds from this raw material is higher (254±24 $mg_{GAE}/_{DE}$) when a mixture of 75:25 (v/v) of EtOH:H_2O is used. Also promising results were obtained for the different antioxidant activities assays. This hydro-alcoholic mixture was also the most advantageous solvent to provide positive antioxidant capacities for HORAC, HOSC and FRAP (4,690 μmol $_{CATE}$/g $_{DE}$, 4,527 μmol $_{TE}$/g $_{DE}$, 2,197 μmol $_{TE}$/g $_{DE}$, respectively), meanwhile the methanol extracts showed the highest ORAC value (2,771±289 μmol $_{TE}$/ g $_{DE}$). In addition, a correlation between different antioxidant activities, total phenols and identified compounds was demonstrated. It could be asserted that anthocyanins were the major compounds present in the wine lees extracts. They significantly contribute to ORAC in a negative way. Those of them which presented the 6-p-coumaroyl moiety strongly contribute to FRAP, as well as for gallic acid and both flavan-3-ols detected. Depending on the solvent used different amounts of the individual compounds are extracted which

could have higher or lower activity against oxygen radicals ($ROO^{\bullet}$) or ($HO^{\bullet}$) affecting the antioxidant capacity estimation.

References

Alonso, A. M., Guillén, D. A., Barroso, C. G., Puertas, B., & García, A. (2002). Determination of antioxidant activity of wine byproducts and its correlation with polyphenolic content. *Journal of Agricultural and Food Chemistry*, *50*(21), 5832–6.

Arnous, A., Makris, D. P., & Kefalas, P. (2002). Correlation of Pigment and Flavanol Content with Antioxidant Properties in Selected Aged Regional Wines from Greece. *Journal of Food Composition and Analysis*, *15*(6), 655–665.

Barcia, M. T., Pertuzatti, P. B., Rodrigues, D., Gómez-Alonso, S., Hermosín-Gutiérrez, I., & Godoy, H. T. (2014). Occurrence of low molecular weight phenolics in Vitis vinifera red grape cultivars and their winemaking by-products from São Paulo (Brazil). *Food Research International*, *62*, 500–513.

Bolanos de la Torre, A. A. S., Henderson, T., Nigam, P. S., & Owusu-Apenten, R. K. (2015). A universally calibrated microplate ferric reducing antioxidant power (FRAP) assay for foods and applications to Manuka honey. *Food Chemistry*, *174*, 119–23.

Bravo, M. N., Silva, S., Coelho, A. V., Boas, L. V., & Bronze, M. R. (2006). Analysis of phenolic compounds in Muscatel wines produced in Portugal. *Analytica Chimica Acta*, *563*(1), 84–92.

Bustamante, M. A., Moral, R., Paredes, C., Pérez-Espinosa, A., Moreno-Caselles, J., & Pérez-Murcia, M. D. (2008). Agrochemical characterisation of the solid by-products and residues from the winery and distillery industry. *Waste Management*, *28*(2), 372–380.

Cantos, E., Espín, J. C., & Tomás-Barberán, F. A. (2002). Varietal differences among the polyphenol profiles of seven table grape cultivars studied by LC-DAD-MS-MS. *Journal of Agricultural and Food Chemistry*, *50*(20), 5691–6.

Csiktusnádi Kiss, G. A., Forgács, E., Cserháti, T., Candeias, M., Vilas-Boas, L., Bronze, R., & Spranger, I. (2000). Solid-phase extraction and high-performance liquid chromatographic separation of pigments of red wines. *Journal of Chromatography*, *889*(1), 51–57.

Delgado de la Torre, M. P., Priego-Capote, F., & Luque de Castro, M. D. (2015). Characterization and Comparison of Wine Lees by Liquid Chromatography-Mass

Spectrometry in High-Resolution Mode. *Journal of Agricultural and Food Chemistry*, *63*(4), 116-1125.

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

Dimou, C., Vlysidis, A., Kopsahelis, N., Papanikolaou, S., Koutinas, A. A., & Kookos, I. K. (2016). Techno-economic evaluation of wine lees refining for the production of value-added products. *Biochemical Engineering Journal*, *116*, 157–165.

Doshi, P., Adsule, P., Banerjee, K., & Oulkar, D. (2015). Phenolic compounds, antioxidant activity and insulinotropic effect of extracts prepared from grape (Vitis vinifera L) byproducts. *Journal of Food Science and Technology*, *52*(1), 181–90.

Feliciano, R. P., Bravo, M. N., Pires, M. M., Serra, A. T., Duarte, C. M., Boas, L. V., & Bronze, M. R. (2009). Phenolic Content and Antioxidant Activity of Moscatel Dessert Wines from the Setúbal Region in Portugal. *Food Analytical Methods*, *2*(2), 149–161.

Frankel, E. N., Waterhouse, A. L., & Teissedre, P. L. (1995). Principal Phenolic Phytochemicals in Selected California Wines and Their Antioxidant Activity in Inhibiting Oxidation of Human Low-Density Lipoproteins. *Journal of Agricultural and Food Chemistry*, *43*(4), 890–894.

Gardner, P. T., McPhail, D. B., Crozier, A., & Duthie, G. G. (1999). Electron spin resonance (ESR) spectroscopic assessment of the contribution of quercetin and other flavonols to the antioxidant capacity of red wines. *Journal of the Science of Food and Agriculture*, *79*(7), 1011–1014.

Hernández, T., Estrella, I., Carlavilla, D., Martín-Álvarez, P. J., & Moreno-Arribas, M. V. (2006). Phenolic compounds in red wine subjected to industrial malolactic fermentation and ageing on lees. *Analytica Chimica Acta*, *563*(1), 116–125.

Hokkanen, J., Mattila, S., Jaakola, L., Pirttilä, A. M., & Tolonen, A. (2009). Identification of Phenolic Compounds from Lingonberry (*Vaccinium vitis-idaea* L.), Bilberry (*Vaccinium myrtillus* L.) and Hybrid Bilberry (*Vaccinium x intermedium* Ruthe L.) Leaves. *Journal of Agricultural and Food Chemistry*, *57*(20), 9437–9447.

Kallithraka, S., Mohdaly, A. A.-A., Makris, D. P., & Kefalas, P. (2005). Determination of major anthocyanin pigments in Hellenic native grape varieties (Vitis vinifera sp.): association with antiradical activity. *Journal of Food Composition and Analysis*, *18*(5), 375–386.

Kondrashov, A., Ševčík, R., Benáková, H., Koštířová, M., & Štípek, S. (2009). The key role of grape variety for antioxidant capacity of red wines. *E-SPEN, the European e-Journal of Clinical Nutrition and Metabolism*, *4*(1), 41–46.

Landeka, I., Jurčević, Dora, M., Guberović, I., Petras, M., Rimac, S., Đikić, D. (2017). Polyphenols from Wine Lees as a Novel Functional Bioactive Compound in the Protection Against Oxidative Stress and Hyperlipidaemia. *Food Technology and Biotechnology*, *55*(1), 109–116.

Landeka Jurčević, I., Dora, M., Guberović, I., Petras, M., Rimac Brnčić, S., Đikić, D. (2017). Wine Lees Polyphenols as a Novel Functional Bioactive Compound in the Protection against Oxidative Stress and Hyperlipidemia. *Food Technology and Biotechnology*, *55*(1), 109–116.

Li, X., Tian, Y., Wang, T., Lin, Q., Feng, X., Jiang, Q., Chen, D. (2017). Role of the p-Coumaroyl Moiety in the Antioxidant and Cytoprotective Effects of Flavonoid Glycosides: Comparison of Astragalin and Tiliroside. *Molecules*, *22*(12), 1165.

Marino, T., Galano, A., & Russo, N. (2014). Radical Scavenging Ability of Gallic Acid toward OH and OOH Radicals. Reaction Mechanism and Rate Constants from the Density Functional Theory. *The Journal of Physical Chemistry B*, *118*(35), 10380–10389.

Matias, A., Rosado-Ramos, R., Nunes, S., Figueira, I., Serra, A., Bronze, M., Duarte, C. (2016). Protective Effect of a (Poly)phenol-Rich Extract Derived from Sweet Cherries Culls against Oxidative Cell Damage. *Molecules*, *21*(4), 406.

Michalska, A., Ceglińska, A., & Zieliński, H. (2007). Bioactive compounds in rye flours with different extraction rates. *European Food Research and Technology*, *225*(3-4), 545–551.

Moore, J., Yin, J.-J., & Yu, L. L. (2006). Novel fluorometric assay for hydroxyl radical scavenging capacity (HOSC) estimation. *Journal of Agricultural and Food Chemistry*, *54*(3), 617–26.

Moro Gonzaléz, C. (2009). ES 2319032 A1 - Procedimiento De Extraccion De Polifenoles A Partir De Orujo De Uva Procedente De Destilacion. - The Lens.

Orak, H. H. (2007). Total antioxidant activities, phenolics, anthocyanins, polyphenoloxidase activities of selected red grape cultivars and their correlations. *Scientia Horticulturae*, *111*(3), 235–241.

Oszmiański, J., Nowicka, P., Teleszko, M., Wojdyło, A., Cebulak, T., & Oklejewicz, K. (2015). Analysis of Phenolic Compounds and Antioxidant Activity in Wild Blackberry Fruits. *International Journal of Molecular Sciences*, *16*(7), 14540–53.

Ou, B., Hampsch-Woodill, M., Flanagan, J., Deemer, E. K., Prior, R. L., & Huang, D. (2002). Novel fluorometric assay for hydroxyl radical prevention capacity using fluorescein as the probe. *Journal of Agricultural and Food Chemistry*, *50*(10), 2772–2777.

Pérez-Serradilla, J. A., & Luque de Castro, M. D. (2011). Microwave-assisted extraction of phenolic compounds from wine lees and spray-drying of the extract. *Food Chemistry*, *124*(4), 1652–1659.

Perumalla, A. V. S., & Hettiarachchy, N. S. (2011). Green tea and grape seed extracts. Potential applications in food safety and quality. *Food Research International*, *44*(4), 827–839.

Rankine, B. C., Fornachon, J. C. ., Boehm, E. W., & Cellier, K. M. (1971). lnfluence of grape variety, climate and soil on grape composition and on the composition and quality of table wines. *Vitis*, *10*, 33–50.

Richard A. Moyer, Kim E. Hummer, Chad E. Finn, Balz Frei, and Ronald E. Wrolstad. (2002). Anthocyanins, Phenolics, and Antioxidant Capacity in Diverse Small Fruits: Vaccinium, Rubus, and Ribes. *Journal of Agricultural and Food Chemistry, 50*(3), 519-25

Rocío Teruel, M., Garrido, M. D., Espinosa, M. C., & Linares, M. B. (2015). Effect of different format-solvent rosemary extracts (Rosmarinus officinalis) on frozen chicken nuggets quality. *Food Chemistry*, *172*, 40–6.

Ruggieri, L., Cadena, E., Martínez-Blanco, J., Gasol, C. M., Rieradevall, J., Gabarrell, X., Sánchez, A. (2009). Recovery of organic wastes in the Spanish wine industry. Technical, economic and environmental analyses of the composting process.

Journal of Cleaner Production, *17*(9), 830–838.

Serra, A. T., Duarte, R. O., Bronze, M. R., & Duarte, C. M. M. (2011). Identification of bioactive response in traditional cherries from Portugal. *Food Chemistry*, *125*(2), 318–325.

Serra, A. T., Matias, A. A., Nunes, A. V. M., Leitão, M. C., Brito, D., Bronze, R., Duarte, C. M. (2008). In vitro evaluation of olive- and grape-based natural extracts as potential preservatives for food. *Innovative Food Science & Emerging Technologies*, *9*(3), 311–319.

Sólyom, K., Solá, R., Cocero, M. J., & Mato, R. B. (2014). Thermal degradation of grape marc polyphenols. *Food Chemistry*, *159*, 361–366.

Teixeira, A., Baenas, N., Dominguez-Perles, R., Barros, A., Rosa, E., Moreno, D. A., & Garcia-Viguera, C. (2014). Natural bioactive compounds from winery by-products as health promoters: a review. *International Journal of Molecular Sciences*, *15*(9), 15638–78.

Versari, A., Castellari, M., Spinabelli, U., & Galassi, S. (2001). Recovery of tartaric acid from industrial enological wastes. *Journal of Chemical Technology and Biotechnology*, *76*(5), 485–488.

Wu, J.-J., Lin, J.-C., Wang, C.-H., Jong, T.-T., Yang, H.-L., Hsu, S.-L., & Chang, C. J. (2009). Extraction of antioxidative compounds from wine lees using supercritical fluids and associated anti-tyrosinase activity. *The Journal of Supercritical Fluids*, *50*(1), 33–41.

Supplementary Material

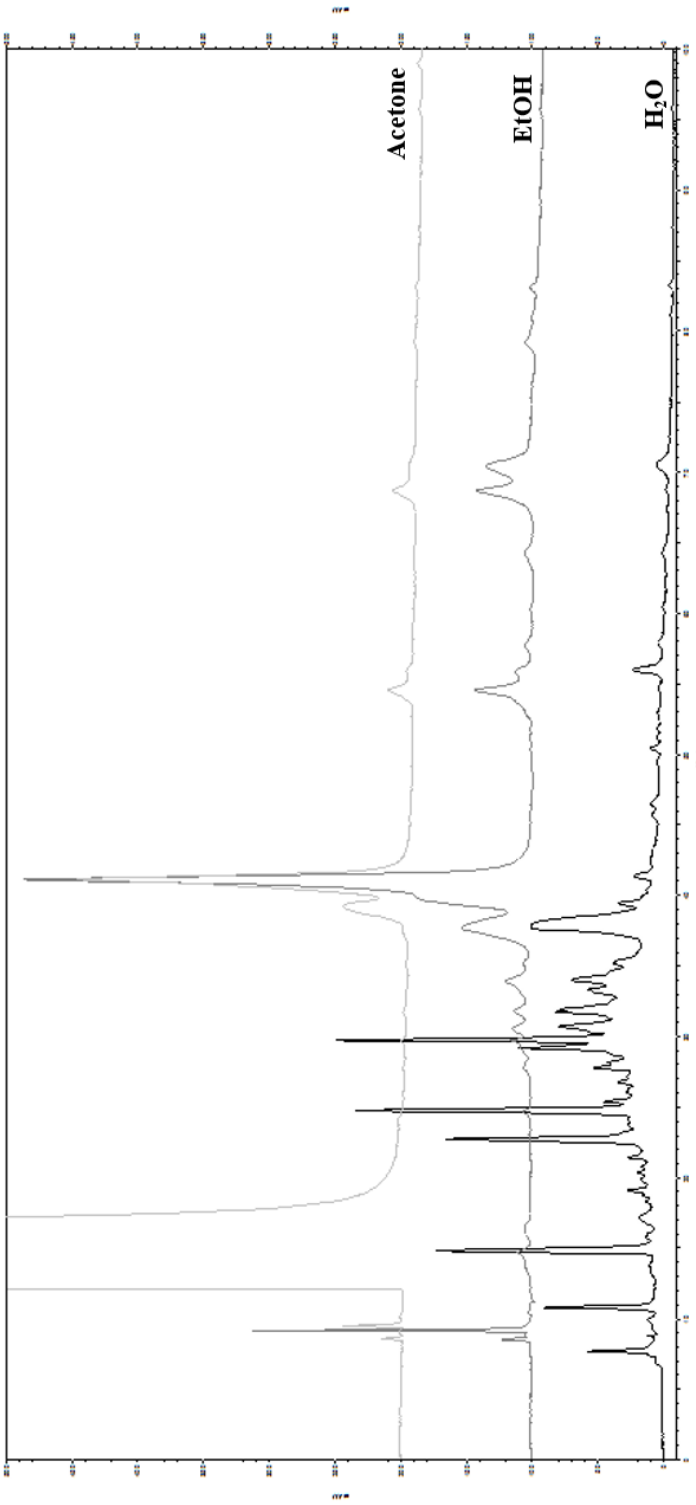
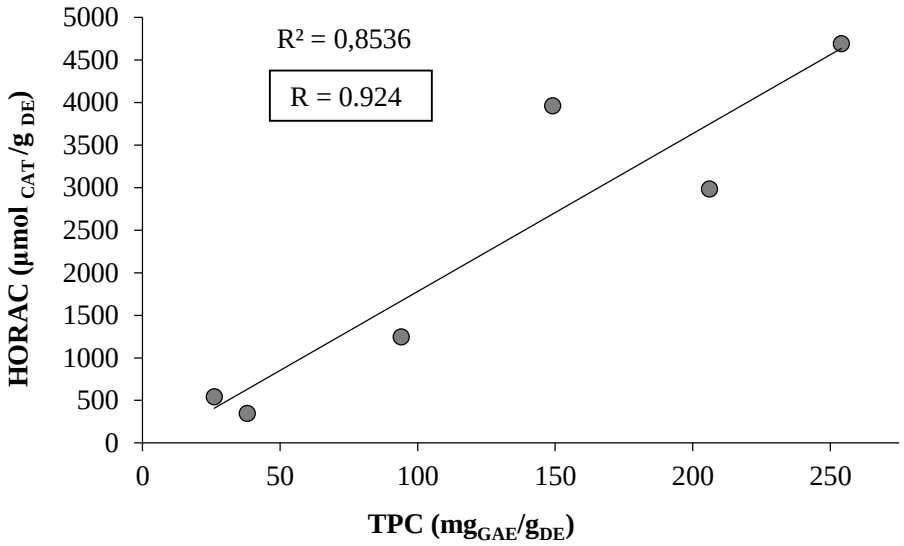


Figure 1S: Chromatograms obtained for acetone, ethanol and water of aging wine lees extracts at a wavelength of 280 nm

a)



b)

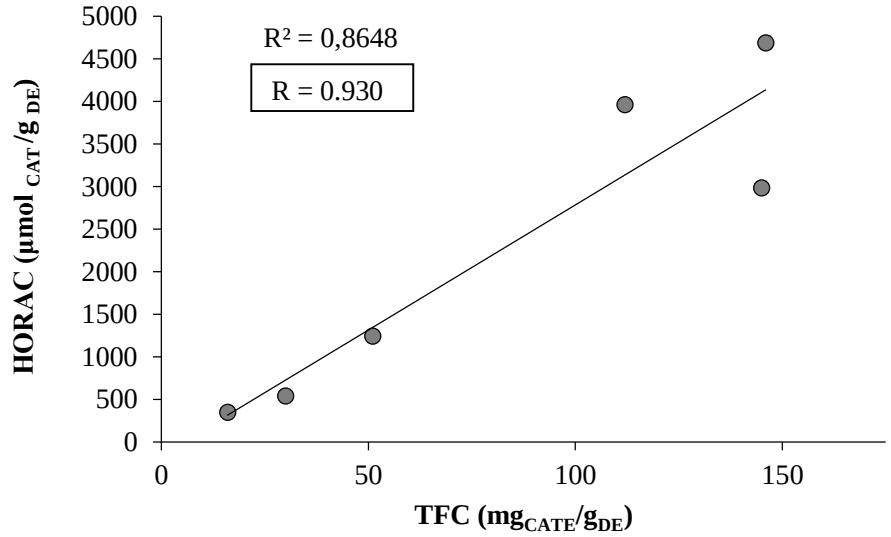


Figure 2S: example of correlation values (R^2) between HORAC antioxidant activity and a) TPC and b) TFC.

CHAPTER 2

Microwave and ultrasound pre-treatments to enhance anthocyanin extraction from different wine lees

(https://doi.org/10.1016/j.foodchem.2018.08.016)

R. Romero-Díez, M. Matos, L. Rodrigues, M.R. Bronze, S. Rodríguez-Rojo, M.J Cocero, A.A. Matias. *'Microwave and ultrasound pre-treatments to enhance anthocynins extraction from different wine lees*'. Food Chemistry. 272, pp. 258-266. Elsevier, 04/08/2018.

Abstract

Wine lees are rich in anthocyanins (AC), natural colorants with health promoting properties. The extraction kinetics of AC from different wine lees in conventional solid-liquid extraction were studied for the first time. The influence of parameters such as temperature, solid-liquid ratio (R_{S-L}) and type of solvent (hydro-alcoholic mixtures) was also studied. Furthermore, microwaves (MW) and ultrasounds (US) were used as pre-treatments (a prior step to the conventional extraction) in order to increase AC yield. Maximum extraction yield (2.78 $mg_{MALVIDIN\text{-}EQUIVALENTS}/g_{DRY\text{-}LEES}$) was achieved after 15 minutes at 25ºC, with a R_{S-L} of 1/10 (g/mL) and with a 50%vol. ethanol mixture. When MW were used AC extraction yield was doubled (6.20 $mg_{MALVIDIN\text{-}EQUIVALENTS}/g_{DRY\text{-}LEES}$) and the required time to achieve a constant yield was reduced (from 15 min to 90s). Meanwhile, US only shortened extraction time in less proportion (from 15 to 5 min). Putative identification of main extract compounds was performed by LC/MS-MS.

1. Introduction

Wine industry generates huge amounts of wastes and by-products, which are sources of high value compounds, including vine pruning, grape stalks, grape pomace and wine lees (WL) (Drosou, Kyriakopoulou, Bimpilas, Tsimogiannis, & Krokida, 2015). WL are defined by EEC regulation no.337/79 as 'the residue formed at the bottom of recipients containing wine, after fermentation, during storage or after authorized treatments, as well as the residue obtained following filtration or centrifugation'. Huge amounts of WL are produced per year; they constitute the 14% of the 2-3 million tons of wastes generated in vinification processes, only in Spain. Main components of the solid phase of WL are yeast and bacteria, responsible for the vinification process, tartaric acid salts, precipitated tannins, inorganic matter and free phenolic compounds (Pérez-Serradilla & Luque de Castro, 2011; Dimou et al., 2015). Different types of WL can be found depending on the vinification process. In the case of red wine, it is possible to find first fermentation WL (generated in the alcoholic fermentation) and second fermentation WL, (generated in the malolactic fermentation). Nevertheless, in the case of a Port wine, first fermentation is stopped by adding extra ethanol (Perestrelo, Silva, Pereira, & Câmara, 2016), and only one type of WL are generated.

Historically, WL have been used for the recovery of tartaric acid (Kontogiannopoulos, Patsios, Mitrouli, & Karabelas, 2017) or as fermentation nutrient

supplement (Dimou et al., 2015). However, in recent years the recovery of anthocyanins (AC) from WL has attracted much attention since recent studies showed that the concentration of these colorants is 10 times higher than in grape skins (Peralbo-Molina & Luque de Castro, 2013). Moreover, AC present beneficial effects on human health: its anti-inflammatory, antimicrobial and antioxidant are world-wide known (He & Giusti, 2010). Thereby, the exploitation of these dregs would led to a sustainable growth of the wine industry and would contribute to reduce winery wastes hazards, as they have been classified as pollutants by the European Union (Karpe, Beale, Harding, & Palombo, 2015).

The easiest and the most implemented way to extract compounds from a solid matrix are solid-liquid (S-L) extractions. The most used solvents to recovery polyphenols are methanol, ethanol, ethyl acetate and acetone (Muhlack, Potumarthi, & Jeffery, 2017). For example, *Pérez-Serradilla et al*. (Pérez-Serradilla & Luque de Castro, 2011) recovered bioactive compounds from WL using mixtures of ethanol and water. Acetone and methanol have been also used for the recovery of polyphenols from WL (Dimou et al., 2015). Nonetheless, substances of interest usually have an intracellular localisation which may represent a problem for the extraction procedure. The movement of those substances of interest from the inside of the cell to the solvent is usually hindered by the mass transfer processes. This is owing to all the mass transfer stages occurring in these type of extractions. In a first step, the solvent should enter the matrix (internal transport), later the dissolution of the compounds in the solvent (solubility) and the release of the solutes to the global phase (external transport). For this reason, cell disruption methods (mechanical, chemical, thermal) can be applied to promote the extraction of valuable intracellular components from diverse raw materials (Kim et al., 2016) by enhancing the mass transfer steps. Within this context, microwave (MW) assisted extraction has been broadly used to enhance the extraction of active compounds from many vegetable matrixes (Rodríguez-Rojo, Visentin, Maestri, & Cocero, 2012; Spigno & De Faveri, 2009), including grape residues such as seeds (Dang, Zhang, & Xiu, 2014) and WL (Pérez-Serradilla & Luque de Castro, 2011) as it has been proved that MW improve the extraction of intracellular compounds as it enhances the internal mass transfer (Rodríguez-Rojo, Visentin, Maestri, & Cocero, 2012) . Prominent among the advantages offered by MW is the double effect of the MW energy. On one hand, the irradiation improves the cellular lysis of materials with large quantities of water due to the rapid heating and evaporation of the intracellular water. On the other hand, a non-thermal effect

appears as a result of the alteration of the dielectric camps which could provide a breakdown of the hydrogen bonds of the macromolecules, breaking off their structure (Ganzler, Salgó, & Valkó, 1986). Thanks to the efficiency of the microwave, the heating process takes place in a few seconds. Recently, some authors (Álvarez et al., 2017) suggested using MW pre-treatment as a previous step to the conventional extraction, in which low residence time pre-treatments (below 120s) are employed.

Other way to improve the recovery of bioactive compounds from natural matrixes is the use of ultrasounds (US). US assisted extraction has already been applied to an extensive variety of raw materials from vegetable matrixes (Rodríguez-Rojo et al., 2012) and from WL (Barcia et al., 2014; Tao, Wu, Zhang, & Sun, 2014), showing an increasing of the recovery yield. US increase the external mass transfer due to their mechanical background and 'cavitation effect'. This phenomena takes place due to the high frequency sound waves generated during US application, which generates bubbles in the liquid that collapse. This collapse results in a change in temperature and pressure (Wijngaard, Hossain, Rai, & Brunton, 2012) and the release of cell contents into the medium is enhanced (Rodríguez-Rojo et al.,2012). Similarly, as MW irradiation, US could be used as pre-treatment step before a conventional sold-liquid extraction as an alternative to US assisted extraction in order to reduce the requiring extraction time.

The work presented here is a study of the extraction kinetics of AC from different types of WL. The study was focused in the maximization of AC extraction since they are the most abundant polyphenols family present in red grapes and their concentration in wine lees is even higher, as previously, indicated (Peralbo-Molina & Luque de Castro, 2013) . Parameters such as solid-liquid ratio (g/mL), type of solvent (hydro-alcoholic mixtures) and temperature were tested in conventional solid-liquid extractions. Once all the parameters were studied, the best operating conditions were selected. MW and US pre-treatments followed by solid-liquid extraction at selected conditions were also studied for intensifying AC extraction. Furthermore, process parameters for each type of pre-treatment were also investigated such as time, type of solvent (hydro-alcoholic mixtures) and solid:liquid ratio. Additionally, amplitude was studied for US. Optimum extracts were characterized in terms of total polyphenol content, total anthocyanin content and antioxidant activity. Further, putative identification of main component of the selected extracts were identified by LC-MS/MS.

2. Materials and methods

2.1 Raw material

Port WL were kindly provided by Sogrape Vinhos S.A. (Port, Portugal) in 2015 and immediately stored at 4ºC in the absence of light. The lees were centrifuged (Avanti J-26 XPI with a rotor type JA-10) for 90 minutes at 10,000 rpm. The solid phase obtained was freeze-dried (Micro Modulo EDWARDS) at -40ºC for 72 hours, in order to preserve the material and avoid the growth of bacteria. Particle size of dry lees is mainly determined by the nature of solid part of the wine lees, composed mainly by yeast and bacteria, as already mentioned; The freeze dry solid is easily crumbled by hand and it was homogenised by a soft milling step using a chopper (A320R1, Moulinex). Particle size distribution in volume was determined by laser diffraction (Malvern Mastersizer 2000) using a dry disperser accessory (Scirocco 2000); A surface weighted mean particle size value of 13 µm was obtained. Wine lees were stored at room temperature, protected from light.

First (1F) and second fermentation (2F) red WL resulting from the fermentation of *Tempranillo* grapes, from Ribera del Duero Denomination of Origin were kindly provided by Matarromera winery (Valladolid, Spain) in 2015. WL were firstly stored at 4ºC, in the absence of light, and then processed as reported above for Port WL.

2.2 Chemicals

Solvents used for extractions were absolute ethanol (99.9% Carlo Erba Reagents, France), bidistilled water (Milli-Q® Integral) and hydrochloric acid (≥37%, puriss. p.a., Riedel-de Haën, France). Chemicals used on the determination of total phenolic content were sodium carbonate (Sigma-Aldrich, France), Folin Ciocalteau reagent (Panreac, Spain) and gallic acid (Fluka, Germany). To determine total anthocyanin content potassium chloride (Riedel-de Haën, France), sodium acetate trihydrate (≥99.0%, Sigma-Aldrich, France) and hydrochloric acid (≥37%, puriss. p.a., Riedel-de Haën, France) were used to prepare the buffer solutions in bidistilled water. Chemicals used for antioxidant activity assays were: 2‘,2‘-azobis(2-amidinopropane) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and disodium fluorescein (FS) from Sigma-Aldrich (France). Sodium chloride (Sigma-Aldrich, France), potassium chloride (Riedel-de Haën, France), sodium phosphate dibasic dihydrate (Sigma-Aldrich, France) and potassium phosphate monobasic anhydrous (Amresco, USA) were used for phosphate buffer solution (PBS) preparation in bidistilled

water (Milli-Q® Integral). HPLC analyses were performed using acetonitrile (99.9%, Sigma-Aldrich, France), ultrapure water purified with a Milli-Q water purification system (Merck Millipore, USA), formic acid (99-100%, VWR-CHEM, Spain) and malvidin-3-O-glucoside chloride (Extrasynthese, France) as standard.

2.3 Methods

2.3.1 Anthocyanin Extraction Kinetics

2.3.1.1 Conventional solid-liquid extraction

Conventional S-L extractions were performed by putting in contact the desired solvent with a known amount of dry WL. Parameters such as the R_{S-L} (0.1, 0.05, 0.033 and 0.025 g/mL), type of solvent (ethanol and hydroalcoholic mixtures varying the percentage of ethanol in 25, 50 and 75%) and temperature (25, 35 and 45ºC) were studied in order to select the best conditions for AC extraction. All the S-L extractions were performed with an agitation of 300 rpm. The pH was adjusted to 2.5 with HCl when the solvent was different from pure ethanol. Samples of 1.5 mL were collected every 5 minutes during a total extraction time of 90 minutes. Total AC concentrations of each sample was measured in order to build the anthocyanin kinetic extraction curve at different conditions. Conventional S-L extractions were performed in triplicate and data were analysed by t-Student´s test (unpaired samples, unequal variances) with a significance p-value of 0.05.

2.3.1.2 Microwave pre-treatments

MW pre-treatments were carried out in a CEM Discovery One Microwave (CEM Corp.). Power was fixed at 300W since it has been found that energy levels do not have a significant effect on the anthocyanin extraction (Sólyom, Mato, Pérez-Elvira, & Cocero, 2011). A 100 mL QianCap (QLabtech) safe glass pressure reactor was employed to maintain the solvent in a liquid phase. An exact mass of 7.5g of WL was poured inside with a specified amount of solvent in order to reach the desired R_{S-L}. The mixture was homogenised before MW irradiation. Three main parameters were studied in these pre-treatment: R_{S-L} (0.2, 0.15 and 0.1 g/mL), solvent mixture (hydroalcoholic mixtures varying the percentage of water in 100, 50 and 10%) and time of microwaves applied (30, 60 and 90s). Mixtures of ethanol and water were chosen as solvent due to their environmentally friendly and low toxicity properties. Preliminary analysis of the R_{S-L} revealed that higher values of R_{S-L} were needed in comparison with those of literature for

grape marc (0.5g/mL) (Álvarez et al., 2017) since freeze dried WL absorbs high amount of solvent due to their powdery nature.

When MW pre-treatment was completed, the vessel was cooled down in an ice batch followed by the conventional S-L extraction.

A statistical surface response design was performed using Statgraphics® Centurion XVII software in order to obtain the optimum conditions which maximize the final AC content of the extracts. A central potential composite design 2^3 + stars (CCD), which establish new extremes for the low and high settings for all factors, was applied. To check the reproducibility, a triplicate of the central point was done. Three variables with three levels of response (-1, 0, 1) were employed. These three levels correspond to the minimum (-1), medium (0) and maximum (1) values of each variable. A total of 17 experiences were obtained.

Responses obtained from the statistical analysis were fitted to a second degree model (Equation 1) that took into account, not only individual interactions, but also quadratic relations between the variables:

$$Y = \beta_0 + \sum_{j=1}^{k} \beta_j X_j + \sum_{j=1}^{k} \beta_{jj} X_j^2 + \sum_{i=1}^{k} \sum_{j=1}^{k} \beta_{ij} X_i X_j \quad (1)$$

where Y corresponds to the response variable (AC content in this study), β_0, β_j, β_{jj} and β_{ij} are regression coefficients; X stands for each operating variable. The statistical evaluation was performed by analysis of variance (ANOVA) in order to identify which factors contribute the most to the response. Effects with a p-value< 0.05 are statistically significant with a level of confidence of 95%.

2.3.1.3 Sonication pre-treatments

Preliminary experiments were performed with a BRASON (101-147-035) Sonifier®Cell Disruptor Model 450 with a high gain horn of ¾" of diameter. Time of sonication and amplitude were varied in a first attempt between 30s and 90s and between 10 and 100% that correspond to an amplitude value of the sound wave of 19 and 130 μm, respectively. After sonication, samples were submitted to S-L extractions at the best conditions previously studied and the kinetic curves were built by gathering samples along time.

2.3.2 Extract Characterization

2.3.2.1 Total Phenolic Content (TPC)

The total phenolic content was determined by Folin-Ciocalteou method which consists on the reduction of the Folin-Ciocalteu reagent to produce a bluish mixture of metal oxides which intensity is proportional to the phenolic content. Protocol was followed as described elsewhere (Waterhouse, Waterhouse, & L., 2003) by putting in contact the sample with the Folin-Ciocalteou reagent and the Na_2CO_3. Absorbance of each sample was measured at 765nm against the blank in a UV 2550 Shimadzu spectrophotometer. TPC values were expressed as milligrams of gallic acid equivalents per gram of dry lees (mg_{GAE}/g_{DL}) and milligrams of gallic acid equivalents per gram of dry extract (mg_{GAE}/g_{DE}).

2.3.2.2 Anthocyanin Content

Monomeric anthocyanin pigments content was evaluated following the AOAC official method 2005.02. This pH differential method is based in the change of color of AC with pH: at pH 1.0 colored oxonium ions are formed, whereas at pH 4.5 predominates the colorless hemiketal form. The difference in the absorbance of the pigments at 520 nm is proportional to the pigment concentration. Briefly, each sample was properly diluted in pH 1.0 buffer (potassium chloride, 0.025M) and pH 4.5 buffer (sodium acetate, 0.4M) and absorbance was determined at both 520 and 700 nm (Tecan Spark 10M).

$$C_A = \frac{[(A_{520}-A_{700})_{pH1}-(A_{520}-A_{700})]\cdot M_W \cdot DF}{\varepsilon \cdot l} \cdot \frac{1}{R_{S-L}} \qquad (2)$$

where 'C_A' is the anthocyanin content expressed in mg_{MLVE}/g_{DL}; 'A' the absorbance measurements; 'M_w' the molecular weight of malvidin (493.4 $g \cdot mol^{-1}$); 'DF' is the dilution factor; 'ε' represents the molar extinction coefficient (28,000$L \cdot mol^{-1} \cdot cm^{-1}$); 'l' is the path length in cm and 'R_{S-L}' is the solid-liquid ratio ($g \cdot mL^{-1}$) used in the extraction. Anthocyanin (AC) concentration was expressed as milligrams of malvidin-3-o-glucoside equivalents per gram of dry lees (mg_{MLVE}/g_{DL}) and milligrams of malvidin-3-o-glucoside equivalents per gram of dry extract (mg_{MLVE}/g_{DE}).

2.3.2.3 Antioxidant Activity: ORAC

Oxygen Radical Absorbance Capacity (ORAC) is a method for the evaluation of antioxidant ability of a specific substance based on the fluorescence quenching of

disodium fluorescein (FS) salt after exposure to AAPH (2,2-azobis(2-amidino-propane) dihydrochloride), which generates oxygen radicals ($ROO^{\bullet}$) at a constant rate (Garrett et al., 2014). ORAC assay was carried out by the method developed by *Huang et al*. (Huang, Ou, Hampsch-Woodill, Flanagan, & Prior, 2002) and modified for the FL800 microplate fluorescence reader (Bio-Tek Instruments, Winooski, VT, USA), as described by *Feliciano et al.* (Feliciano et al., 2009). ORAC results were given in μmol of Trolox equivalents (TE) per gram of dry lees ($\mu mol_{TE}/g_{DL}$) and μmol of TE per gram of dry extract ($\mu mol_{TE}/g_{DE}$) as mean of three replicates.

2.3.2.4 Results basis: yield and richness

AC extraction yield was expressed in terms of milligrams of malvidin equivalents per gram of dry lees (mg_{MLVE}/g_{DL}) in order to maximize AC extraction of from dry WL. In addition, extracts were characterized in terms of richness to have an idea of the purity of the extracts regarding AC. Richness was expresses in milligrams of malvidin equivalents per gram of dry extract (mg_{MLVE}/g_{DE}). ORAC and TPC values were also expressed either in mg_{MLVE}/g_{DL} or mg_{MLVE}/g_{DE}.

2.3.2.5 Solid residue

Sample extracts were evaporated until dryness using a vacuum centrifuge (Centrivap concentrator, Labconco, Kansas City, MO, USA) with a MD 4C NT vacuum pump (Vacuubrand, Wertheim, Germany) for result expressions per gram of dry extract (g_{DE}).

2.3.2.6 HPLC-DAD-MS/MS (High Performance Liquid chromatography–mass spectrometry)

The mass spectrometer (MS/MS) used was a MicroMass Quattromicro® API (Waters®, Ireland). Chromatographic separation of compounds was carried out on a LiChrospher® 100 RP-18 (250 x 4.0mm) column in an oven at 35 ºC. Chromatographic separation of compounds was carried out in a reversed-phase LiChrospher® 100 RP-18 5μm LiChroCART® 250-4 column inside a thermostated oven at 35ºC. The mobile phases gradients, the ion source temperature, the capillary voltage and the source voltage employed were the same used in previous work. For the data acquisition and processing MassLynx® 4.1 software was employed.

Main compounds in the WL extracts were identified by LC-MS/MS with a method previously reported (Romero-Díez et al., 2018). The system used was a liquid chromatography Waters Alliance 2695 Separation Module (Waters®, Ireland). The mass spectrometer (MS/MS) used was a MicroMass Quattromicro® API (Waters®, Ireland). Chromatographic separation of compounds was carried out in a reversed phase LiChrospher® 100 RP-18 5µm LiChroCART® (250 x 4.0mm) column inside a thermostated oven at 35ºC. A binary mobile phase was used: eluent A consisted of solution formic acid (0.5% v/v) and eluent B was acetonitrile. It was used at a constant flowrate of 0.3 mL/min with the following gradient program: 99:1 A:B for 5 min, from 99:1 A:B to 40:60 A:B in 40 min, from 40:60 A:B to 10:90 A:B in 45 min, held isocratically (90% B) for 10 min, from 10:90 A:B to 99:1 A:B in 10 min, and finally held isocratically (99:1 A:B) for 10 min. The sample injection volume was 20 µL. Absorption spectra were acquired from 210 to 600 nm by a photodiode array detector. AC were monitored at 520 nm, flavonols at 360 nm, phenolic acids at 320 nm, and phenolic compounds in general at 280 nm. Mass spectrometry was performed using an electrospray ion source in negative and positive ion mode (ESI- and ESI+). The ion source temperature was 120°C, the capillary voltage was 2.5 kV, and the source voltage was 30 V. Compounds separated by HPLC were ionized and the mass spectra were recorded in a full scan mode, between m/z 100 and 1500. High purity nitrogen was used as drying and nebulizing gas, and ultrahigh purity argon was used as collision gas. Different collision energy values were used in fragmentation experiments. For the data acquisition and processing MassLynx® 4.1 software was employed.

2.3.3 Statistical Analysis

All data were expressed as means ± standard deviations (SD). Assays for TPC, AC content and ORAC measurements were performed, at least, in triplicate. A statistical analysis was done using SigmaStat 3.0® software. When homogeneous variances were confirmed, data were analyzed by One Way Analysis of Variance (ANOVA) coupled with the post-hoc Holm–Sidak test ($p<0.05$ was accepted as statistically significant in all cases).

3. Results and Discussion

3.1 Best extraction conditions for AC

3.1.1 Conventional S-L extractions. Extraction kinetics of AC

The selection of the best conditions that influence AC extraction was firstly carried out for Port WL, and later applied for 1F and 2F *Ribera del Duero* WL. Extractions were performed during 90 minutes, but after 15 minutes a steady AC concentration was achieved (Figure 1.A). Firstly, the effect of R_{S-L} (0.100, 0.050, 0.033 and 0.025 g/mL) was studied. The rest of parameters were kept constant: ethanol was used and a temperature was set at 25ºC. Results revealed that, AC extraction yield slightly increased as R_{S-L} decreased (Figure 1.A). A R_{S-L} of 0.100 yielded 0.61 ± 0.04 mg_{MLVE}/g_{DL}. When R_{S-L} decreased to 0.050 and 0.033, AC content increased to 0.96 ± 0.01 and 0.94 ± 0.03 mg_{MLVE}/g_{DL}, respectively. However no significant differences were found between them. For the case of a R_{S-L} of 0.025, a minor increase in the final AC concentration was observed (1.05 ± 0.10 mg_{MLVE}/g_{DL}). However, this AC extraction yield increase implied the use of four times more of solvent, which clearly involves economic and environmental issues (Drosou et al., 2015). Thus, it was decided to fix the R_{S-L} in 0.100. Additionally, this ratio has been also used by other authors for recovering of polyphenols from WL with conventional extraction (Pérez-Serradilla & Luque de Castro, 2011) .

Once the R_{S-L} was selected, four different hydro-alcoholic mixtures were studied. The content of ethanol 100%, 75%, 50% and 25% (%vol. ethanol) was varied. In this case parameters which were kept constant were the R_{S-L} (0.100) and temperature (25ºC). As shown in Figure 1.B, AC extraction was significantly enhanced as the amount of ethanol increased from 25% to 75% in the mixture (0.79 ± 0.01 to 3.04 ± 0.38 mg_{MLVE}/g_{DL}, respectively), as it was expected due to the decrease in polarity and dielectric constant values of the solvent mixture that, generally, increases the solubility of polyphenols in hydroalcoholic mixtures as the % ethanol increases (Cacace & Mazza, 2003b; Dimou et al., 2016). However, the use of 100% ethanol did not improve AC extraction (0.51 ± 0.04 mg_{MLVE}/g_{DL}) and the difference between using a 50% (2.78 ± 0.18 mg_{MLVE}/g_{DL}) or a 75% (3.04 ± 0.38 mg_{MLVE}/g_{DL}) aqueous ethanol mixture is not significant. This is mainly due to the fact that at acidic pH, AC remain as ionic molecules (flavilium cation form, AH^+) and maximum AC extraction yield is achieved at approximately 50% ethanol (Cacace & Mazza, 2003b). Therefore, the hydroalcoholic 50% vol. ethanol mixture was selected,

also from an economical point of view, since it requires a lower amount of organic solvent.

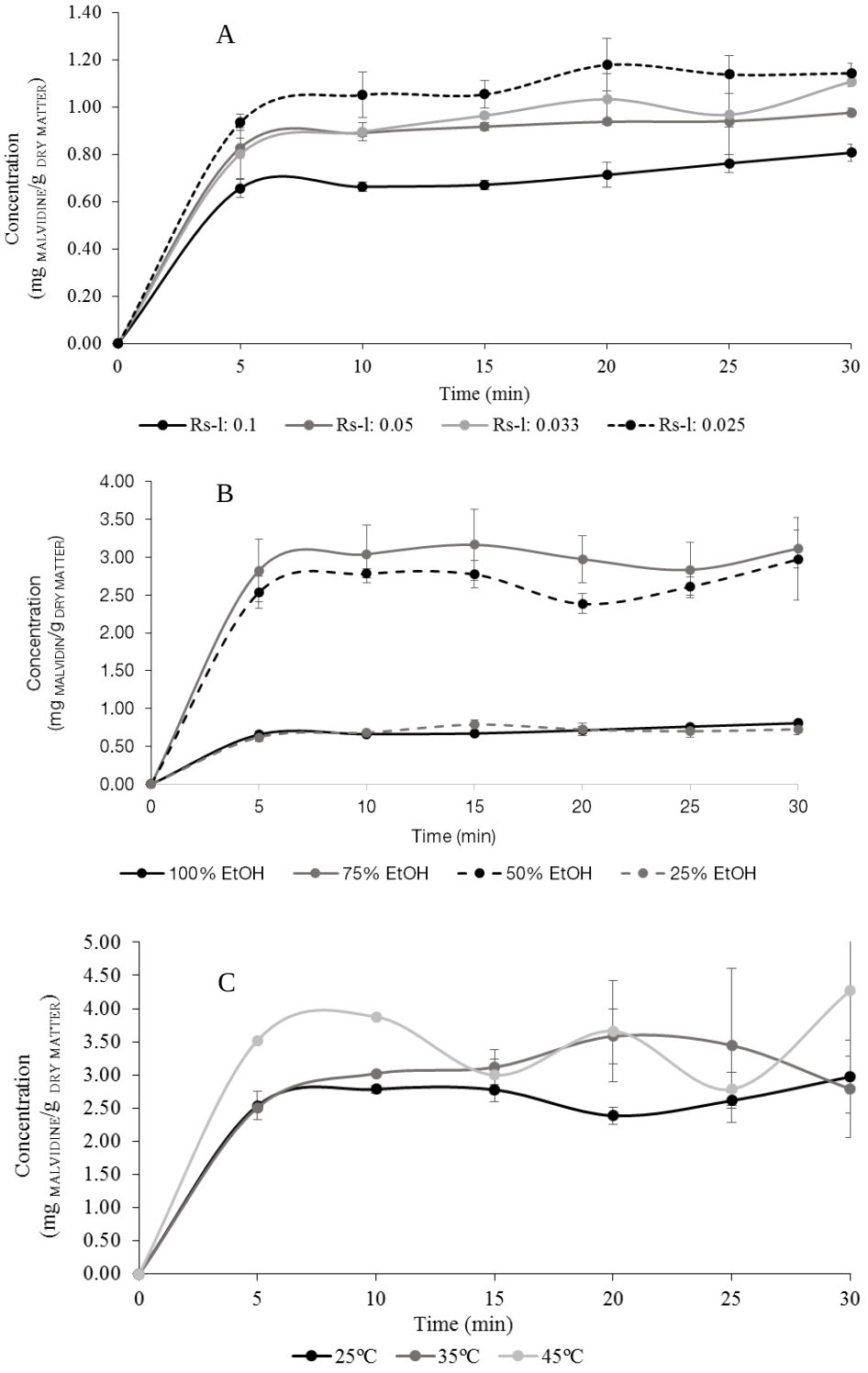


Figure 1: influence of the solid-liquid ratio (g/mL) (1.A), type of solvent (%vol. ethanol) (1.B) and temperature (ºC) (1.C) on the AC extraction from Port wine lees in conventional extraction.

At the end, the influence of the temperature on the AC yield was investigated. In this context, three temperatures were tested (25, 35 and 45ºC) maintaining, the other parameters constant (50% vol. ethanol mixture, $R_{S\text{-}L}$ of 0.100). After 15 minutes of extraction, an AC extraction yield of 2.78 ± 0.18 mg_{MLVE}/g_{DL}, 3.12 ± 0.27 mg_{MLVE}/g_{DL} and 3.00 ± 0.24 mg_{MLVE}/g_{DL} were achieved for 25, 35 and 45ºC, respectively. Within the studied range, higher temperature led to a slight increase of AC extraction rate, (Figure 1.C). Although it was expected that temperature increases the AC content by increasing the extraction coefficient (Pinelo, Fabbro, Manzocco, Nuñez, & Nicoli, 2005), no significant differences were observed in terms of AC extraction yield. So, to reduce the use of resources and energy, temperature was fixed in 25ºC. Though the use of 45ºC reduces slightly the extraction time (Figure 1.C), it has been demonstrated that low temperatures contribute to prevent anthocyanin degradation, since AC stability is compromised even at 45ºC along time (Cacace & Mazza, 2003a; Sólyom, Solá, Cocero, & Mato, 2014). AC extraction values for the study of each variable are shown in Table S.1 of the *Supplementary Material*.

As a conclusion of the influence of each parameter, best conditions for AC extraction were a $R_{S\text{-}L}$ 0.100, a mixture with 50%vol. ethanol and a temperature of 25ºC. At these conditions, a final AC extraction yield of 2.78 ± 0.18 mg_{MLVE}/g_{DL} was obtained for Port WL. These conditions were also applied to the *Ribera del Duero* WL. Final AC extraction yield of 3.04 ± 0.03 mg_{MLVE}/g_{DL} was obtained for 1F WL, while for 2F WL, lower AC content was achieved, 2.09 ± 0.38 mg_{MLVE}/g_{DL}.

Although there is few available literature regarding AC extraction from WL, the AC recovery from the different WL reached in this work are in accordance to those found in literature. *Tao et al*. (Tao et al., 2014) recovered 5.55 ± 0.19 mg_{MLVE}/g_{DL} of AC from light lees via maceration in an aqueous ethanol solution (51% vol. EtOH) at 60ºC during 36 minutes using a low $R_{S\text{-}L}$ (0.0167).

In addition, it has been proved that WL represent a richer source of AC compared to other vinification residues. For example, *Álvarez et al*. (Álvarez et al., 2017) studied the extraction kinetics of AC extraction from grape pomace from the same variety of grapes and provided by the same winery (Bodegas Matarromera) in year 2014. They performed several S-L extractions, and AC content was also measured along time. For their best conditions, only 1.20 mg_{MLVE}/g_{DL} were extracted and more than 60 minutes were required to attain a steady AC concentration. This can be due to the much lower particle size of

WL, which reduces internal mass transfer limitation and, therefore, AC become more accessible.

3.1.2 Microwave pre-treatment

Table 1 collects all MW pre-treatments performed from the statistical analysis, AC concentration just after the pre-treatment and the temperature achieved in each experiment.

Table 1: CCD design set of experiments for application of MW pretreatment in Port wine lees. AC is the anthocyanin content just after the pre-treatment, T represents the achieved temperature in the MW pre-treatment. Rows in bold represent the triplicate of the central point and 'Average CP' is an average of the central points. Runs 18 and 19 are the experiments performed with the optimized variables and 'Average OP' is the average of the optimum. AC values with an asterisk are significantly different ($p<0.05$) from the central point.

	% H_2O (v/v)	**R_{S-L} (g/mL)**	**t (s)**	**AC (mg_{MLVE}/g_{DL})**	**T (ºC)**
1	10	0.20	90	4.33*	106
2	100	0.20	90	0.14*	117
3	**50**	**0.15**	**60**	**5.64***	**73**
4	10	0.10	30	2.68*	53
5	100	0.20	30	0.03*	105
6	50	0.15	30	4.38*	60
7	100	0.10	90	0.07*	68
8	**50**	**0.15**	**60**	**5.25***	**72**
9	10	0.20	30	2.71*	65
10	**50**	**0.15**	**60**	**5.18***	**73**
11	50	0.10	60	3.62*	68
12	50	0.20	60	3.95*	80
13	10	0.10	90	4.78*	77
14	10	0.15	60	3.32*	75
15	50	0.15	90	6.78*	98
16	100	0.10	30	0.04*	38
17	100	0.15	60	0.06*	88
Average CP	**50**	**0.15**	**60**	**5.36 ± 0.25**	**72**
18	40	0.14	90	6.15	115
19	40	0.14	90	6.26	114
Average OP	40	0.14	90	6.20 ± 0.36	115

As the objective was to optimize the MW pre-treatment, AC concentrations were measured just after the pre-treatment for Port WL and, later, applied for the rest of WL. At first glance, it can be observed that AC extraction yield varied a lot depending on the

pre-treatment parameters, namely the H_2O percentage (v/v). AC concentration in the extract after pre-treatment was really low (experiment 1 as example) when only water was used. However, higher AC extraction yields were achieved when more ethanol was employed. For example in the experiment 15, AC extraction yield was twice higher (~6 mg_{MLVE}/g_{DL}) than compared with those obtained in the conventional S-L extraction (~3 mg_{MLVE}/g_{DL}).

Figure 2 shows the main effect diagram for each variable from the statistical study. There were values for the type of solvent and S-L ratios that maximize the AC extraction yield, which correspond to the optimum point. A different behaviour was observed for the time: the greater the time, the higher the extraction yield of AC. However, if time was increased, temperature would increase during the pre-treatment and the degradation of AC would take place. It is known that AC degrade at temperatures above 100ºC during exposure times of 5-10 minutes (Sólyom et al., 2014). For this reason, no experiments at higher temperature were proposed. In this work, the highest temperature achieved was 117ºC, but only during a short period of time (90s) avoiding AC degradation. Thus, the optimal values for each parameter were: a hydro-alcoholic mixture of 40% vol. ethanol, a R_{S-L} of 0.140 (g/mL) and a time pre-treatment of 90s.

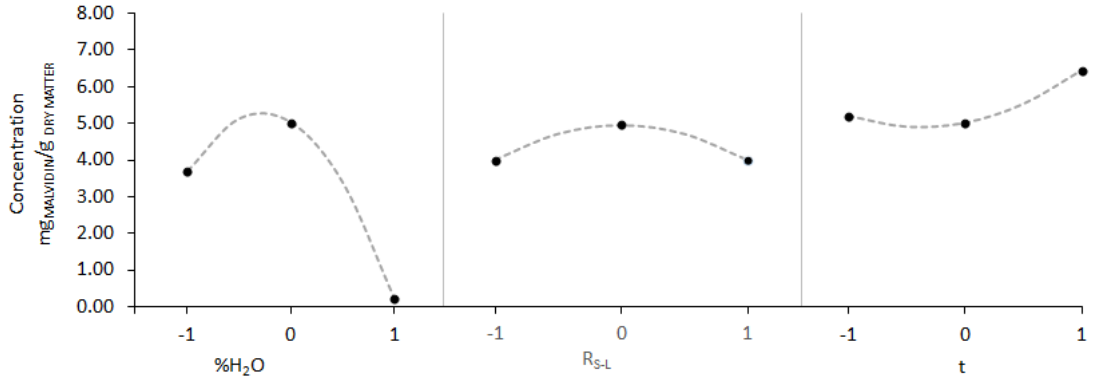


Figure 2: main effect diagram of each variable for AC content from the statistical study.

From the analysis of variance (Table S.2 in *Supplementary Material*), it could be seen that the percentage of water was the parameter which influences the most in anthocyanin extraction. Furthermore, the pre-treatment time and the interaction between the water percentage and time were also crucial for the extraction.

The regression coefficients of second-order polynomial equation (*Equation 1*) were obtained by fitting experimental results and extraction variables. The final expression for the *Equation 1* is shown below. Some parameters were negligible assuming the p-values from the ANOVA table (Table S.2 in *Supplementary Material*).

$$AC = 5.0166 + 0.624926 \cdot t - 0.00311123 \cdot R_{SL} - 1.74735 \cdot \%H_2O + 0.818187 \cdot t^2 - 0.0485444 \cdot t \cdot R_{SL} - 0.448549 \cdot t \cdot \%H_2O - 0.980728 * R_{SL}^2 + 0.060261 \cdot R_{SL} \cdot \%H_2O - 3.07077 * \%H_2O^2$$

'*AC*' corresponds to the anthocyanin extraction yield, '*t*' is the time of pre-treatment in seconds, '$R_{S\text{-}L}$' is the solid-liquid ratio in g/mL and '$\%H_2O$' is the %vol. of water of the hydroalcoholic mixture.

With the optimal pre-treatment conditions, a MW pre-treatment was performed and, in this case, followed by S-L extraction at the best studied conditions (exact amount of solvent was added in order to obtain a $R_{S\text{-}L}$ of 0.100 and hydro-alcoholic mixture of 50% vol. ethanol) for each type of WL during 30 minutes. AC extraction yields for the optimal conditions obtained for MW pre-treatments after 15 minutes are shown in Table 2.

Port WL showed the highest AC content (6.20 ± 0.36 mg_{MLVE}/g_{DL}), followed by first fermentation lees (4.45 ± 0.30 mg_{MLVE}/g_{DL}) and second fermentation lees (2.88 ± 0.22 mg_{MLVE}/g_{DL}) when MW are applied. The use of MW pre-treatment provided an increment in the AC extraction yield of 2.7, 1.5 and 1.4 times compared with the S-L extraction from Port, 1F and 2F WL, respectively (*Supplementary material)*. The effect of MW was also confirmed by the reached temperature in the optimum conditions (115ºC). High temperatures made possible the breakage of cell walls of the yeast of WL, leading to an improvement on the anthocyanin yield extraction since AC linked to cell walls became more accessible and the internal mass transfer is enhanced (Pérez-Serradilla & de Castro, 2008).

Furthermore, the implementation of MW reduces considerably the required extraction time from 15 min to 90s, as the maximum AC extraction yield was achieved just after the pretreatment (Figure 3).

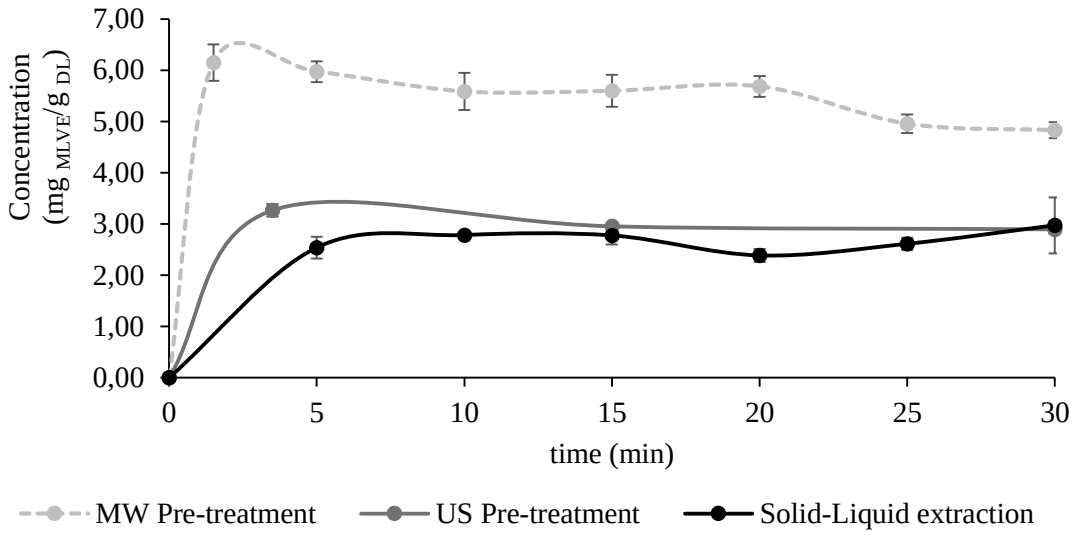


Figure 3: anthocyanin extraction kinetics comparison for Port wine between the best conditions for conventional solid-liquid extraction, the optimal conditions for MW and US pre-treatments.

No comparison about AC extraction could be done due to the absence of information in literature since previous works measured the TPC instead of AC content when MW are used to recover polyphenols from WL. However, some authors (Pérez-Serradilla & Luque de Castro, 2011) proved the efficiency of MW to enhance polyphenols extraction from WL in a 10% compared to the yields obtained with a Soxhlet extraction. Furthermore, AC yields from WL were higher if comparing to those achieved from grape pomace by *Álvarez et al*. (Álvarez et al., 2017), who achieved AC concentration values up to 1.75 $mg_{MLVE}/g_{\text{DRY POMACE}}$ applying their optimized parameters for MW pre-treatments.

Table 2: total phenolic content, anthocyanin concentration and ORAC values for conventional solid-liquid extracts and MW and US pre-treatments after 15 minutes of extraction. Values with an asterisk in the same row are significantly different (P<0.05) for each type of wine lees.

		TPC (mg_{GAE}/g_{DL})	**TPC (mg_{GAE}/g_{DE})**	**AC (mg_{MLVE}/g_{DL})**	**AC (mg_{MLVE}/g_{DE})**	**ORAC ($\mu mol_{TE}/g_{DL}$)**	**ORAC ($\mu mol_{TE}/g_{DE}$)**
Port wine lees	E_{S-L} 15'	27.70 ± 0.18	68 ± 7	2.78 ± 0.18	3.57 ± 0.23	195 ± 20	453± 45
	MW 15'	***42.04 ± 0.22*** *	106 ± 3 *	***6.20 ± 0.36*** *	7.99 ± 0.44 *	402 ± 42 *	1041 ± 107 *
	US 15'	-	-	3.17 ± 0.08	2.91 ± 0.13	312 ± 34 *	574 ± 64
1st Fermentation	E_{S-L} 15'	28.12 ± 0.08	232 ± 5	3.04 ± 0.03	17.07 ± 0.32	392 ± 42	3201 ± 347
	MW 15'	37.03 ± 0.15	***295 ± 13***	4.45 ± 0.30 *	***18.56 ± 1.26***	***655 ± 63*** *	***4952 ± 480****
2nd Fermentation	E_{S-L} 15'	23.42 ± 0.11	196 ± 10	2.09 ± 0.38	10.95 ± 0.77	304 ± 20	2484 ± 159
	MW 15'	23.44 ± 0.17	269 ± 11 *	2.88 ± 0.22	12.03 ± 1.14	512 ± 54 *	3867± 406 *

3.1.3 Sonication pre-treatments

First sonication experiments were performed with the lowest (30s and 10% amplitude) and the highest (90s and 100% amplitude) conditions, followed by a conventional S-L extraction for Port WL. No significant differences were observed in the final AC concentration between the use of ultrasounds (3.02 ± 0.13 mg_{MLVE}/g_{DL} and 3.17 ± 0.08 mg_{MLVE}/g_{DL}) and the conventional S-L extraction (2.78 ± 0.18 mg_{MLVE}/g_{DL}). It was thought that maybe the time of sonication pre-treatment was very short, so a trial with a longer time of processing was carried out. The conditions of 5 minutes and amplitude of 55% were defined for the ultrasounds assisted extraction (USAE) of polyphenols based on a previous work for a different raw material (Rodríguez-Rojo et al., 2012). These conditions were applied to Porto WL and an AC extraction yield of 2.94 ± 0.10 mg_{MLVE}/g_{DL} was achieved. From these results it could be concluded that the AC extraction yield was not enhanced with the use of US. US enhanced the external mass transfer and not the internal mass transfer, which is the limiting step for the AC extraction from WL, so similar yields were achieved. However, US produced a reduction of the required time to achieve a steady AC extraction yield from 15min to 5min as can be seen in Figure 3.

3.2 Extracts characterization: yield and richness

3.2.1 Total Phenolic Content (TPC)

TPC quantification was performed only for those extracts obtained at best conditions for the S-L extractions and the optimized MW pre-treatments. Results for the TPC are shown in Table 2. Extracts obtained when MW were used as pre-treatment showed higher yields, being the Port WL extract the one with the highest and significant TPC yield (42.04 ± 0.22 mg_{GAE}/g_{DL}). For the conventional S-L extractions, similar TPC yields were achieved for each type of WL, ranking from 23.42 ± 0.11 to 27.70 ± 0.18 mg_{GAE}/g_{DL}. Nevertheless, these TPC recoveries changed a lot if they were expressed in terms of richness: milligrams of gallic acid equivalents per gram of dry extract (mg_{GAE}/g_{DE}). The richest extract in terms of TPC were *Ribera del Duero* WL extracts, either for a conventional S-L extraction or with MW pre-treatment. TPC richness for conventional extraction ranging from nearly 196 to 232 mg_{GAE}/g_{DE}. Port WL appeared to be the poorest extract with only 67.94 ± 6.55 $mg_{GAE}/g_{DE.}$ Same tendency was observed when MW were applied: 1F WL were the richest extract (294.17 ± 12.81 mg_{GAE}/g_{DE}) followed by 2F WL extract (268.53 ± 10.92 mg_{GAE}/g_{DE}). The explanation of these differences can be related

to the vinification process. Port WL presented a higher sugar concentration because first fermentation is stopped by adding extra ethanol and all sugars were not processed (Perestrelo et al., 2016). These sugars remained linked to WL and were also extracted together with AC reducing the richness of the extract in terms of TPC and AC. Sugars and their degradation compounds concentrations in WL extracts after 15 minutes of extraction are shown in Table S.3 of the *Supplementary material.*

Although TPC values in this work were lower than those achieved when a MAE was applied to 1F WL of syrah grapes (532 mg_{GAE}/g_{DL}), mainly due the use of different grape varieties (Pérez-Serradilla & Luque de Castro, 2011), WL can be considered as a suitable source of polyphenols if compared with the results from others grape residues. *Casazza et al*. (Casazza, Aliakbarian, Mantegna, Cravotto, & Perego, 2010) extracted polyphenols from differences types of *Vitis Vinifera* wastes, in particular grape seeds and skins, using non-conventional techniques such as HPTE (high pressure and temperature extraction), UAE (ultrasound-assisted extraction) and MAE (microwave-assisted extraction). The TPC in grape seeds was far higher (110 to 60 $mg_{GAE}/g_{DRY\ MATTER}$) than in grape skin (20 to 35 $mg_{GAE}/g_{DRY\ MATTER}$) for every type of extraction. Similar TPC values were achieved in literature from grape pomace (261.5 ± 2.5 mg_{GAE}/g_{DE}) (Álvarez et al., 2017) when MW were used as pre-treatment.

3.2.2 Antioxidant Activity

ORAC assay was performed to evaluate the antioxidant ability of the extracts, obtained with or without pre-treatments, as peroxyl radical scavengers. Table 2 shows ORAC values in $\mu mol_{TE}/g_{DL}$ and $\mu mol_{TE}/g_{DE}$ for conventional S-L extracts and MW pre-treatments after 15 minutes of extraction. A direct relation between the AC concentrations and the ORAC values was found: the larger the concentration of AC, the greater the ORAC value. When pre-treatments were used, TPC and AC content increased and also ORAC values enhanced proportionally. For example, AC richness from Port WL increased when MW pre-treatment was applied in 2.2 times (6.2 ± 0.4 mg_{GAE}/g_{DL}) compared with the S-L extraction (2.78 ± 0.18 mg_{GAE}/g_{DL}) and the ORAC values did also improved: 2.1 times (1040 ± 107 $\mu mol_{TE}/g_{DE}$) with MW than without them (453 ± 45 $\mu mol_{TE}/g_{DE}$). 1F WL showed the highest ORAC values for both yield and richness as it was expected due to it was the richest extract in terms of TPC and AC. Moreover, the highest increment in ORAC activity between S-L extraction (3201 ± 347) and when MW were applied (4952 ± 480 $\mu mol_{TE}/g_{DE}$) was found for 1F WL extract. Port WL presented

almost five times lower antioxidant capacitiy, with ORAC values up to 1040 ± 107 µmol $_{TE}/g_{DE}$, when MW pre-treatment was applied. These ORAC values were smaller than those obtained by *Pérez-Serradilla et al*. (Pérez-Serradilla & Luque de Castro, 2011), who achieved similar ORAC values when a conventional extraction was performed and a MAE was applied to 1F WL from *Syrah* grape variety, 6100 and 6250 $\mu mol_{TE}/g_{DE}$, respectively, due to the higher content on TPC as previously mentioned. In contrast, if ORAC values obtained from WL were compared with those obtained from grape pomace extracts, it could be said that WL extracts presented higher antioxidant activities. *Álvarez et al.* (Álvarez et al., 2017) reported ORAC values between 1200- 2750 µmol $_{TE}/g_{DE}$ for different MW pre-treatments.

3.2.3 HPLC analysis

HPLC analyses were performed in order to determine the main compounds present in WL extracts after 15 minutes of extraction. Figure S.2 (of the *Supplementary Material*) shows, as an example, the chromatographic profile at 280 nm of a conventional extraction (A) and after the MW pre-treatment (B) from Port WL. From this figure it is possible to corroborate the effect of the MW pre-treatment on the amount of extracted polyphenols.

As this work was focused on the maximization of the extraction of AC, an exhaustive study for their determination was performed. Same AC were found in extracts from 1F and 2F WL, meanwhile for Port WL different compounds appeared. These discrepancies can be seen in Figure 4 where a comparison between chromatograms at 520 nm of the WL 1F extract (A) and the Port WL extract (B) after the MW pre-treatment is displayed.

For the determination of the anthocyanins present in the extracts, the LC-MS/MS was used for the qualitative determination of the main compounds. Putative identification of AC was also tested with other studies already reported in literature (Cantos, Espín, & Tomás-Barberán, 2002; Delgado de la Torre, Priego-Capote, & Luque de Castro, 2015; Sanz et al., 2012; Schwarz, Quast, von Baer, & Winterhalter, 2003; Vallverdú-Queralt et al., 2015; Wu & Prior, 2005) with comparable matrices and databanks ("Database on Polyphenol Content in Foods - Phenol-Explorer," n.d.; "PhytoHub," n.d.).

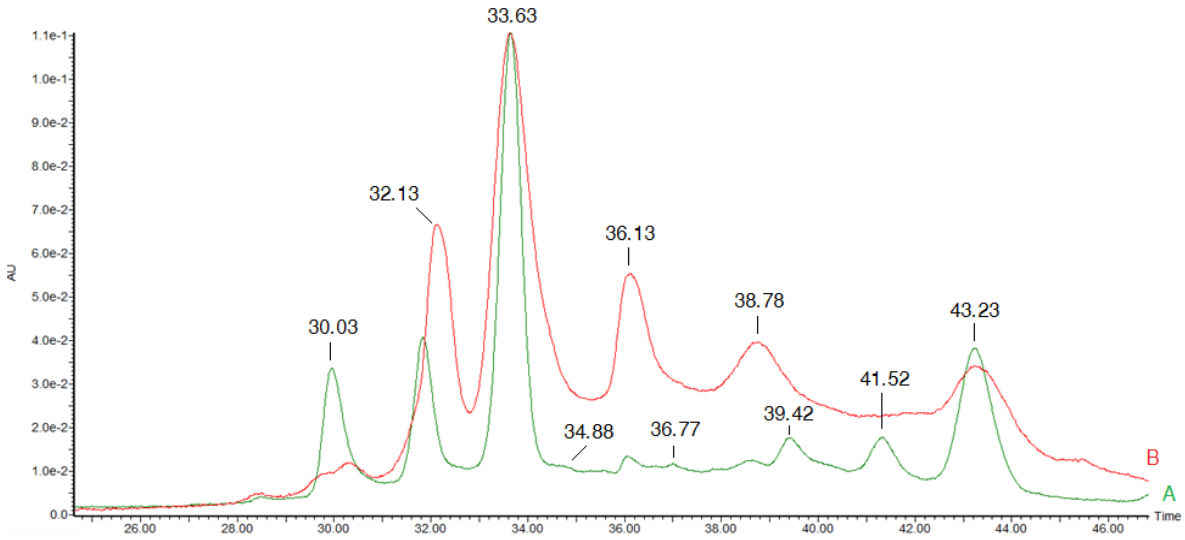


Figure 4: comparison of the chromatographic profiles at 520 nm for the extracts obtained after MW pre-treatment of first fermentation wine lees (A) and Port wine lees (B)

A total of twelve anthocyanins were identify as Table 3 shows. The respective m/z values, the fragmentations, the putative identification, the phenolic subclass and the extract in which each anthocyanin appeared is also shown in Table 3. The most interesting finding in this study was the presence of a pyranoanthocyanin, Vitisin A, in both types of extracts at a retention time of 32.13 min. These A-type vitisins are adducts resulting from the cycloaddition of pyruvic acid, a metabolite of the alcoholic fermentation (Marquez, Serratosa, & Merida, 2013) to anthocyanin molecules, usually formed during the maturation of wine. Until now, these types of pyranoanthocyanins had been only identified in the dregs of an old Port wine bottle (Marquez et al., 2013; Oliveira et al., 2010) and in aged red Chilean wine (Schwarz et al., 2003). Additionally, another anthocyanin, petunidin-3-O-glucoside, appeared to be co-eluted at the same retention time (32.13 min) in both Port and Ribera del Duero extracts. However, in Port wine lees, Vitisin A was present in higher amounts than petunidin-3-O-glucoside, whereas in Ribera del Duero wine lees the opposite was observed. This tendency can be seen in Figure S.3 in the *Supplementary Material*, where the signal for the Vitisin A (m/z 561) is much more pronounced than the one for the petunidin-3-O-glucoside (m/z 479), which suggests that Vitisin A is present in higher amounts than petunidin-3-O-glucoside in Port wine lees. In the same way, for Ribera del Duero extracts, the opposite was observed from Figure S.4. Nonetheless, to the best of our knowledge, these types of pyranoanthocyanins have never

been identified neither in 1F nor 2F Ribera del Duero extracts. Thus, with the help of the novel extraction technique that has been introduced in this study, it was possible to extract Vitisin-A from Ribera del Duero wine lees.

Table 3: Putative identification of main anthocyanins (520nm $\lambda_{máx}$), in 1F wine lees and Port wine lees extracts, retention time (t_R: min) of each compound, M-H^+ values (m/z), MS/MS values and wine lees extracts where each compound appeared.

Retention time (min)	$[M-H]^+$ (m/z)	MS/MS	Putative identification	Phenolic subclass	Ribera del Duero wine lees	Port wine lees
30.03	463	303	Delphinidin-3-O-glucoside	Anthocyanin	✓	✓
32.13	561	399	Vitisin A	Pyranoanthocyanin	✓	✓
	479	317	Petunidin-3-O-glucoside	Anthocyanin	✓	✓
33.63	493	331	Malvidin-3-O-glucoside	Anthocyanin	✓	✓
34.88	507	-	Delphinidin 3-O-(6"-p-acetylglucoside)	Anthocyanin	✓	χ
36.13	707	399	10-carboxypyranomalvidin-3-6”-p-coumaroyl-glucoside	Anthocyanin	✓	✓
36.77	491	-	Cyanidin 3-O-(6"-p-acetylglucoside)	Anthocyanin	✓	χ
38.78	535	331	Malvidin-3-O-6”-acetyl-glucoside	Anthocyanin	χ	✓
39.42	611	303	Delphinidin 3-O-(6"-p-coumaroyl-glucoside)	Anthocyanin	✓	χ
41.52	625	317	Petunidin 3-O-(6"-p-coumaroyl-glucoside)	Anthocyanin	✓	✓
43.23	639	331	Malvidin 3-O-(6"-p-coumaroyl-glucoside)	Anthocyanin	✓	✓

Apart from these AC, there were anthocyanins identified only in one type of extract, which may be directly correlated to the vinification process. That was the case of compounds such as delphinidin 3-O-(6"-p-acetylglucoside), cyanidin 3-O-(6"-p-acetylglucoside) and delphinidin 3-O-(6"-p-coumaroyl-glucoside) which only appeared in *Ribera del Duero* WL extracts. Additionally, a derivate from malvidin (malvidin-3-O-6”-acetyl-glucoside) was found only in Porto WL extracts.

Although, the study here presented was focused on the optimization of AC extraction and their identification, chromatograms of major polyphenol families present in the extracts such as flavonoids or hydroxycinnamic acids are displayed in *Supplementary material* in Figure S.5, Figure S.5 and Figure S.7 at 280 nm, 320 nm and 360 nm, respectively. The putative identification of those compounds with higher concentrations is shown in Table S.4 in the *Supplementary material.*

Some of the components were also identified in aging wine lees and correlated with the antioxidant activity of the extract (Romero-Díez et al., 2018) such as myricetin (flavonol) and several antocyanins (3-O-glucoside of delphinidin, petunidin and malvidin, and 3-O-(6"-p-coumaroyl-glucoside) of delphinidin, petunidin and malvidin).

4. Conclusions

WL have been pointed out as an important source of phenolic compounds, namely anthocyanins. AC extraction kinetics from WL were studied. Parameters selected as the best for AC recovery in S-L extractions were: $R_{S\text{-}L}$ of 0.100 g/mL, a hydro-alcoholic mixture with 50% EtOH (% vol.) and at 25ºC. Furthermore, two different pre-treatments have been tested for AC recovery from WL. On the one hand, the use of MW pre-treatment enhanced internal mass transfer, increasing AC extraction yield and reducing the processing time, as well. On the other hand, US only influenced the processing time having no effect on the AC extraction yield. Different origin WL were processed, being red WL (*Ribera del Duero*) those that presented richer extracts and higher antioxidant activity respect to Port WL. Main compounds were identified finding distinctive anthocyanins for both types of lees. Furthermore, a pyranoanthocyanin, Vitisin A, was identified in both types of lees being predominantly present in Port WL.

References

Álvarez, A., Poejo, J., Matias, A. A., Duarte, C. M. M., Cocero, M. J., & Mato, R. B. (2017). Microwave pretreatment to improve extraction efficiency and polyphenol extract richness from grape pomace. Effect on antioxidant bioactivity. *Food and Bioproducts Processing*, *106*, 162–170.

Barcia, M. T., Pertuzatti, P. B., Rodrigues, D., Gómez-Alonso, S., Hermosín-Gutiérrez, I., & Godoy, H. T. (2014). Occurrence of low molecular weight phenolics in Vitis vinifera red grape cultivars and their winemaking by-products from São Paulo (Brazil). *Food Research International*, *62*, 500–513.

Cacace, J. E., & Mazza, G. (2003a). Mass transfer process during extraction of phenolic compounds from milled berries. *Journal of Food Engineering*, *59*(4), 379–389.

Cacace, J. E., & Mazza, G. (2003b). Optimization of Extraction of Anthocyanins from Black Currants with Aqueous Ethanol. *Journal of Food Science*, *68*(1), 240–248.

Cantos, E., Espín, J. C., & Tomás-Barberán, F. A. (2002). Varietal differences among the polyphenol profiles of seven table grape cultivars studied by LC-DAD-MS-MS. *Journal of Agricultural and Food Chemistry*, *50*(20), 5691–6.

Casazza, A. A., Aliakbarian, B., Mantegna, S., Cravotto, G., & Perego, P. (2010). Extraction of phenolics from Vitis vinifera wastes using non-conventional techniques. *Journal of Food Engineering*, *100*(1), 50–55.

Dang, Y.-Y., Zhang, H., & Xiu, Z.-L. (2014). Microwave-assisted aqueous two-phase extraction of phenolics from grape (*Vitis vinifera*) seed. *Journal of Chemical Technology & Biotechnology*, *89*(10), 1576–1581.

Database on Polyphenol Content in Foods - Phenol-Explorer. (n.d.). Retrieved October 18, 2017, from http://phenol-explorer.eu/

Delgado de la Torre, M. P., Priego-Capote, F., & Luque de Castro, M. D. (2015). Characterization and Comparison of Wine Lees by Liquid Chromatography-Mass Spectrometry in High-Resolution Mode. *Journal of Agricultural and Food Chemistry*, *63*(4), 1116-1125.

Dimou, C., Kopsahelis, N., Papadaki, A., Papanikolaou, S., Kookos, I. K., Mandala, I., & Koutinas, A. A. (2015). Wine lees valorisation: Biorefinery development including

production of a generic fermentation feedstock employed for poly(hydroxybutyrate) synthesis. *Food Research International*, *73*(50), 81–87.

Dimou, C., Vlysidis, A., Kopsahelis, N., Papanikolaou, S., Koutinas, A. A., & Kookos, I. K. (2016). Techno-economic evaluation of wine lees refining for the production of value-added products. *Biochemical Engineering Journal*, *116*, 157–165.

Drosou, C., Kyriakopoulou, K., Bimpilas, A., Tsimogiannis, D., & Krokida, M. (2015). A comparative study on different extraction techniques to recover red grape pomace polyphenols from vinification byproducts. *Industrial Crops and Products*, *75*, 141–149.

Feliciano, R. P., Bravo, M. N., Pires, M. M., Serra, A. T., Duarte, C. M., Boas, L. V., & Bronze, M. R. (2009). Phenolic Content and Antioxidant Activity of Moscatel Dessert Wines from the Setúbal Region in Portugal. *Food Analytical Methods*, *2*(2), 149–161.

Ganzler, K., Salgó, A., & Valkó, K. (1986). Microwave extraction : A novel sample preparation method for chromatography. *Journal of Chromatography A*, *371*, 299–306.

Garrett, A. R., Weagel, E. G., Martinez, A. D., Heaton, M., Robison, R. A., & O'Neill, K. L. (2014). A novel method for predicting antioxidant activity based on amino acid structure. *Food Chemistry*, *158*, 490–6.

He, J., & Giusti, M. M. (2010). Anthocyanins: Natural Colorants with Health-Promoting Properties. *Annual Review of Food Science and Technology*, *1*(1), 163–187.

Huang, D., Ou, B., Hampsch-Woodill, M., Flanagan, J. A., & Prior, R. L. (2002). High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. *Journal of Agricultural and Food Chemistry*, *50*(16), 4437–44.

Karpe, A. V., Beale, D. J., Harding, I. H., & Palombo, E. A. (2015). Optimization of degradation of winery-derived biomass waste by Ascomycetes. *Journal of Chemical Technology & Biotechnology*, *90*(10), 1793–1801.

Kontogiannopoulos, K. N., Patsios, S. I., Mitrouli, S. T., & Karabelas, A. J. (2017). Tartaric acid and polyphenols recovery from winery waste lees using membrane separation processes. *Journal of Chemical Technology & Biotechnology*, *92*(12),

2934–2943.

Marquez, A., Serratosa, M. P., & Merida, J. (2013). Pyranoanthocyanin derived pigments in wine: Structure and formation during winemaking. *Journal of Chemistry*, *2013*.

Muhlack, R. A., Potumarthi, R., & Jeffery, D. W. (2017). Sustainable wineries through waste valorisation: A review of grape marc utilisation for value-added products. *Waste Management*, *72*, 99–118.

Oliveira, J., Azevedo, J., Silva, A. M. S., Teixeira, N., Cruz, L., Mateus, N., & de Freitas, V. (2010). Pyranoanthocyanin dimers: a new family of turquoise blue anthocyanin-derived pigments found in Port wine. *Journal of Agricultural and Food Chemistry*, *58*(8), 5154–9.

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

Perestrelo, R., Silva, C., Pereira, J., & Câmara, J. S. (2016). Wines: Madeira, Port and Sherry Fortified Wines – The Sui Generis and Notable Peculiarities. Major Differences and Chemical Patterns. *Encyclopedia of Food and Health*, 534–555.

Pérez-Serradilla, J. A., & de Castro, M. D. L. (2008). Role of lees in wine production: A review. *Food Chemistry*, *111*(2), 447–456.

Pérez-Serradilla, J. A., & Luque de Castro, M. D. (2011). Microwave-assisted extraction of phenolic compounds from wine lees and spray-drying of the extract. *Food Chemistry*, *124*(4), 1652–1659.

PhytoHub. (n.d.). Retrieved October 18, 2017, from http://phytohub.eu/

Pinelo, M., Fabbro, P. Del, Manzocco, L., Nuñez, M. J., & Nicoli, M. C. (2005). Optimization of continuous phenol extraction from Vitis vinifera byproducts. *Food Chemistry*, *92*(1), 109–117.

Rodríguez-Rojo, S., Visentin, A., Maestri, D., & Cocero, M. J. (2012). Assisted extraction of rosemary antioxidants with green solvents. *Journal of Food Engineering*, *109*(1), 98–103.

Romero-Díez, R., Rodríguez-Rojo, S., Cocero, M. J., Duarte, C. M. M., Matias, A. A., &

Bronze, M. R. (2018). Phenolic characterization of aging wine lees: correlation with antioxidant activities. *Food Chemistry*, *259*, 188-195.

Sanz, M., Fernández de Simón, B., Esteruelas, E., Muñoz, Á. M., Cadahía, E., Hernández, M. T., Martinez, J. (2012). Polyphenols in red wine aged in acacia (Robinia pseudoacacia) and oak (Quercus petraea) wood barrels. *Analytica Chimica Acta*, *732*, 83–90.

Schwarz, M., Quast, P., von Baer, D., & Winterhalter, P. (2003). Vitisin A Content in Chilean Wines from *Vitis vinifera* Cv. Cabernet Sauvignon and Contribution to the Color of Aged Red Wines. *Journal of Agricultural and Food Chemistry*, *51*(21), 6261–6267.

Sólyom, K., Mato, R. B., Pérez-Elvira, S. I., & Cocero, M. J. (2011). The influence of the energy absorbed from microwave pretreatment on biogas production from secondary wastewater sludge. *Bioresource Technology*, *102*(23), 10849–10854.

Sólyom, K., Solá, R., Cocero, M. J., & Mato, R. B. (2014). Thermal degradation of grape marc polyphenols. *Food Chemistry*, *159*, 361–6.

Spigno, G., & De Faveri, D. M. (2009). Microwave-assisted extraction of tea phenols: A phenomenological study. *Journal of Food Engineering*, *93*(2), 210–217.

Tao, Y., Wu, D., Zhang, Q.-A., & Sun, D.-W. (2014). Ultrasound-assisted extraction of phenolics from wine lees: Modeling, optimization and stability of extracts during storage. *Ultrasonics Sonochemistry*, *21*(2), 706–715.

Vallverdú-Queralt, A., Boix, N., Piqué, E., Gómez-Catalan, J., Medina-Remon, A., Sasot, G., Lamuela-Raventos, R. M. (2015). Identification of phenolic compounds in red wine extract samples and zebrafish embryos by HPLC-ESI-LTQ-Orbitrap-MS. *Food Chemistry*, *181*, 146–151.

Waterhouse, A. L., Waterhouse, & L., A. (2003). Determination of Total Phenolics. *Current Protocols in Food Analytical Chemistry*. Hoboken, NJ, USA: John Wiley & Sons, Inc.

Wijngaard, H., Hossain, M. B., Rai, D. K., & Brunton, N. (2012). Techniques to extract bioactive compounds from food by-products of plant origin. *Food Research International*, *46*(2), 505–513.

Wu, X., & Prior, R. L. (2005). Systematic Identification and Characterization of Anthocyanins by HPLC-ESI-MS/MS in Common Foods in the United States: Fruits and Berries. *Journal of Agricultural and Food Chemistry*, *53*(7), 2589–2599.

Nomenclature

Abbreviations

1F: first fermentation

2F: second fermentation

AA: antioxidant activity

AAPH: 2, 2-azobis(2-amidino-propane) dihydrochloride

AC: anthocyanins

CCD: central composite design

CP: central point

DAD: diode array detection

DE: dry extract

DL: dry lees

FS: disodium fluorescein

GAE: gallic acid equivalents

GRAS: general recognize as safe

HPLC: high performance liquid chromatography

HPTE: high pressure and temperature extraction

MAE: microwave assisted extraction

MLVE: malvidin-3-o-glucoside equivalents

MS/MS: mass spectrometer

MW: microwave

OP: optimum point

ORAC: oxygen radical absorbance capacity

SD: standard deviation

S-L: solid-liquid

TE: trolox equivalents

TPC: total phenolic compounds

UAE: ultrasound-assisted extraction

US: ultrasounds

USAE: ultrasound assisted extraction

Greek letters

Y: response variable (anthocyanin concentration, mg_{MLVE}/g_{DL})

β_0: independent coefficient

β_j, β_{jj}, β_{ij} : interaction coefficients for variables "i" and "j"

X: stands for each operating variable

Symbols

A: absorbance, nm

DF: dilution factor

l: path length, cm

M_w: molecular weight of malvidine, m/mol

$ROO^{\bullet}$: oxygen radicals

$R_{S\text{-}L}$: solid-liquid ratio, g/mL

Supplementary Material

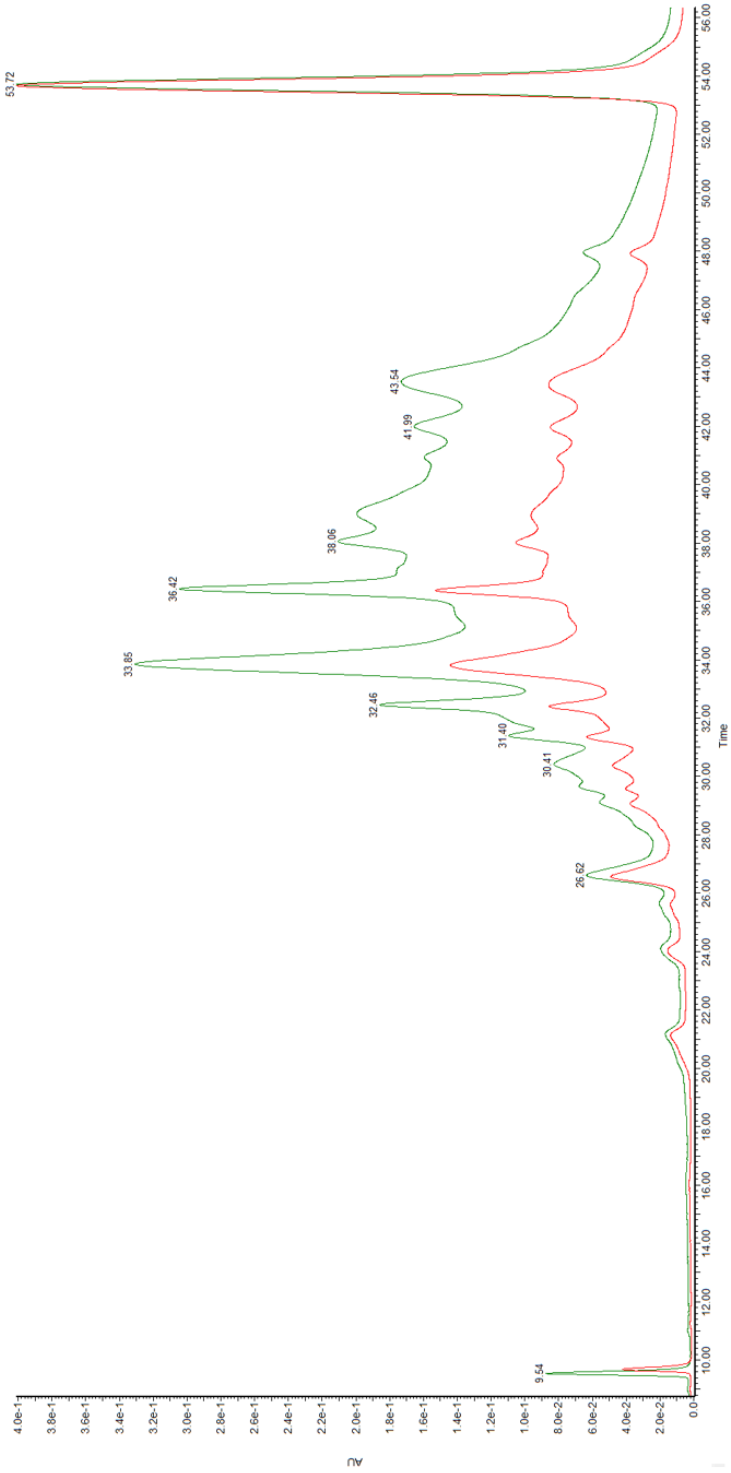


Figure S.1: comparison of the chromatographic profiles at 280nm for the extracts obtained from Port wine lees with a conventional solid-liquid ratio (red) and applying MW pre-treatment (green).

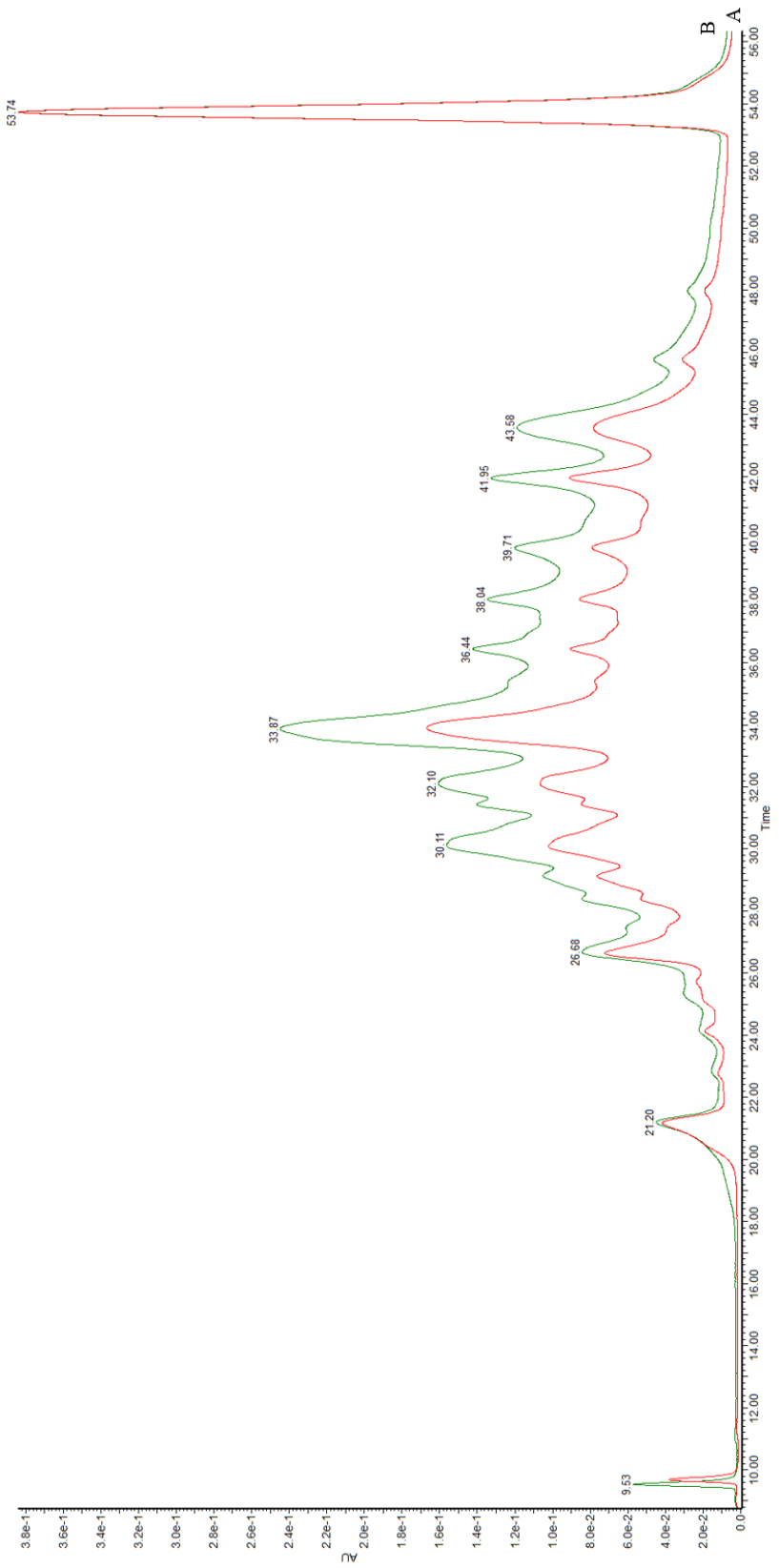


Figure S.2: comparison of the chromatographic profiles at 280nm for the extracts obtained from first fermentation wine lees with a conventional solid-liquid ratio (A) and applying MW pre-treatment (B)

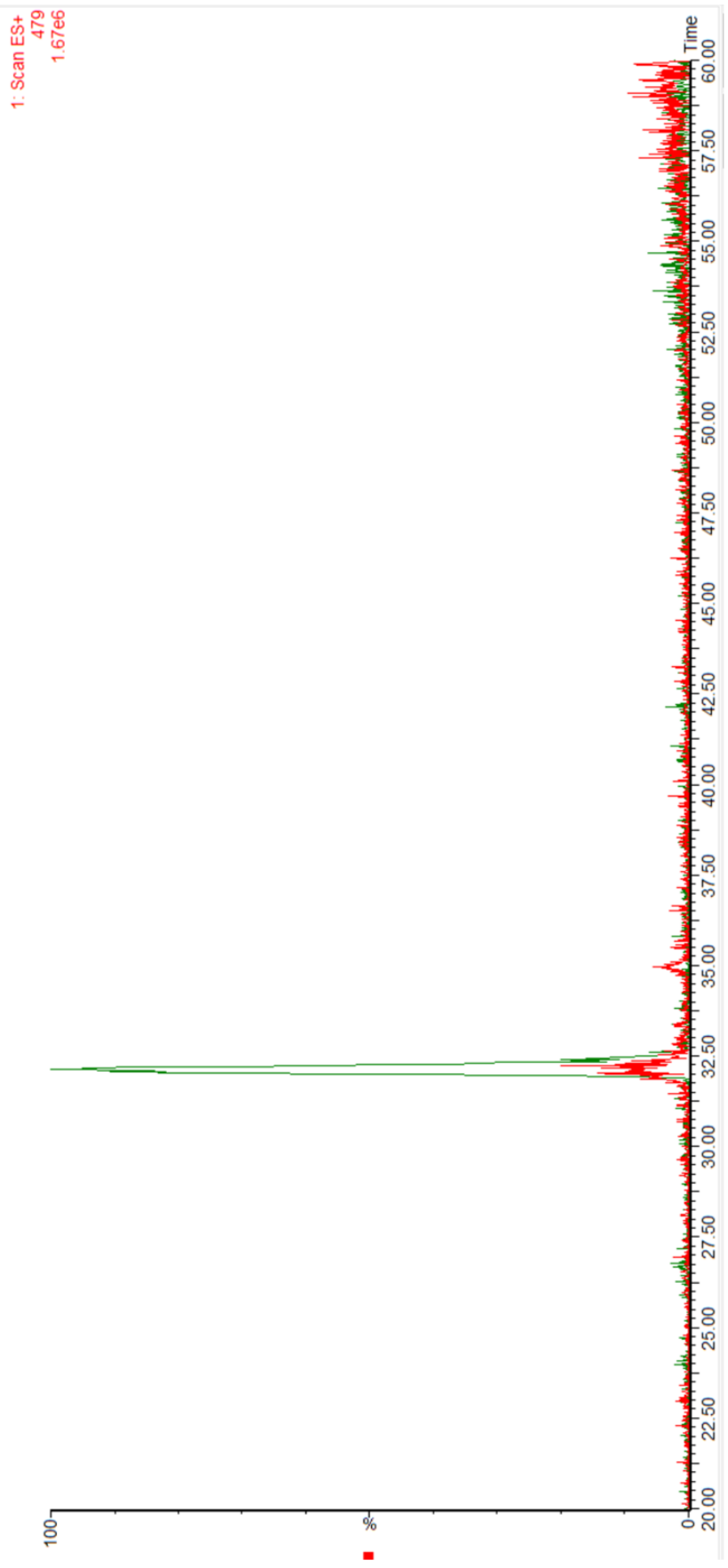


Figure S.3: comparison of the m/z intensities of Visitin A (green line) (m/z =561) and petunidin-3-O-glucoside (red line) (m/z=479) for the Port extract obtained MW pre-treatment.

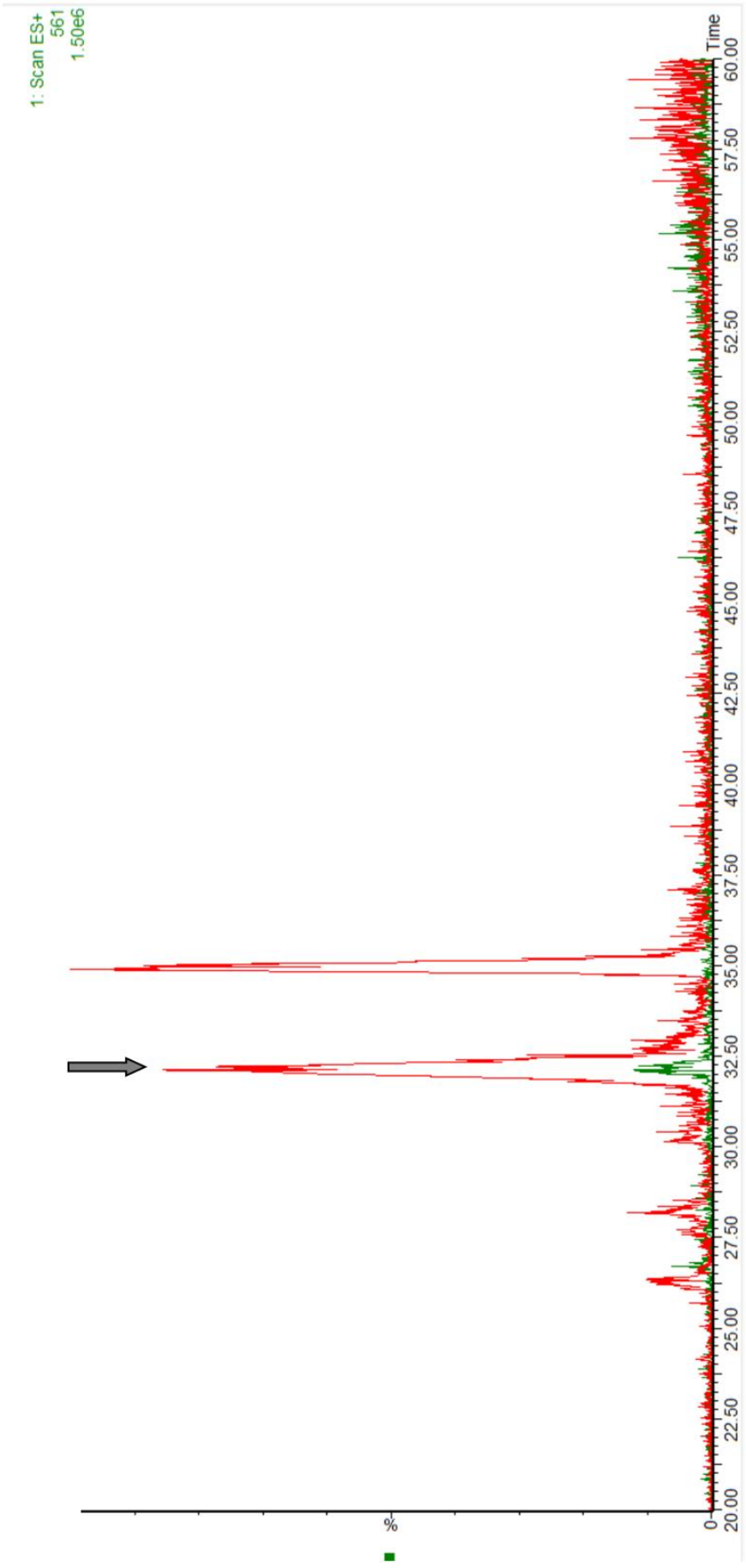


Figure S.4: comparison of the m/z intensities of Visitin A (green line) (m/z =561) and petunidin-3-O-glucoside (red line) (m/z=479) for the Ribera del Duero extract obtained MW pre-treatment.

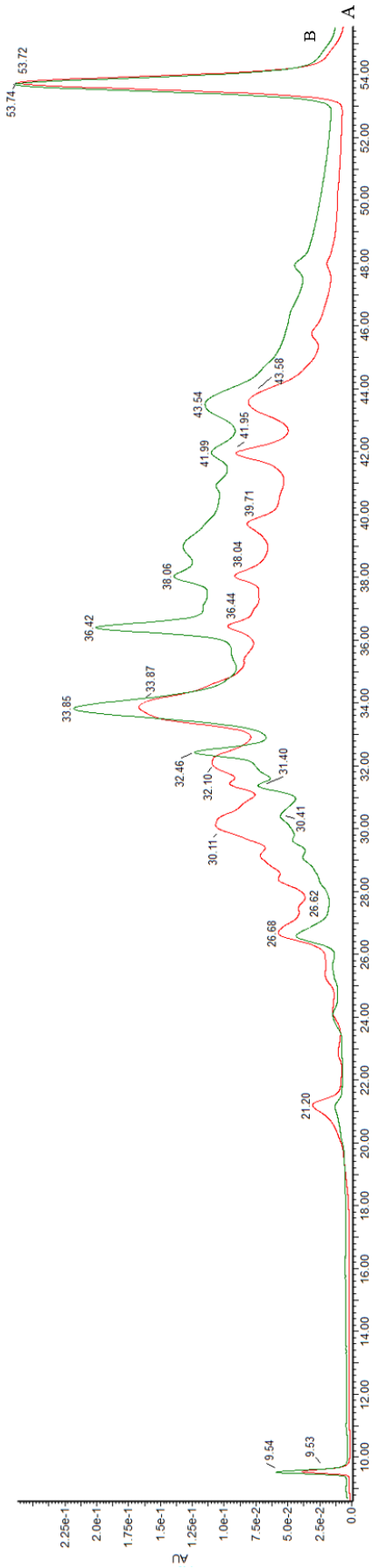


Figure S.5: comparison of the chromatographic profiles at 280nm for the extracts obtained after MW pre-treatment of first fermentation wine lees (A) and Porto wine lees (B).

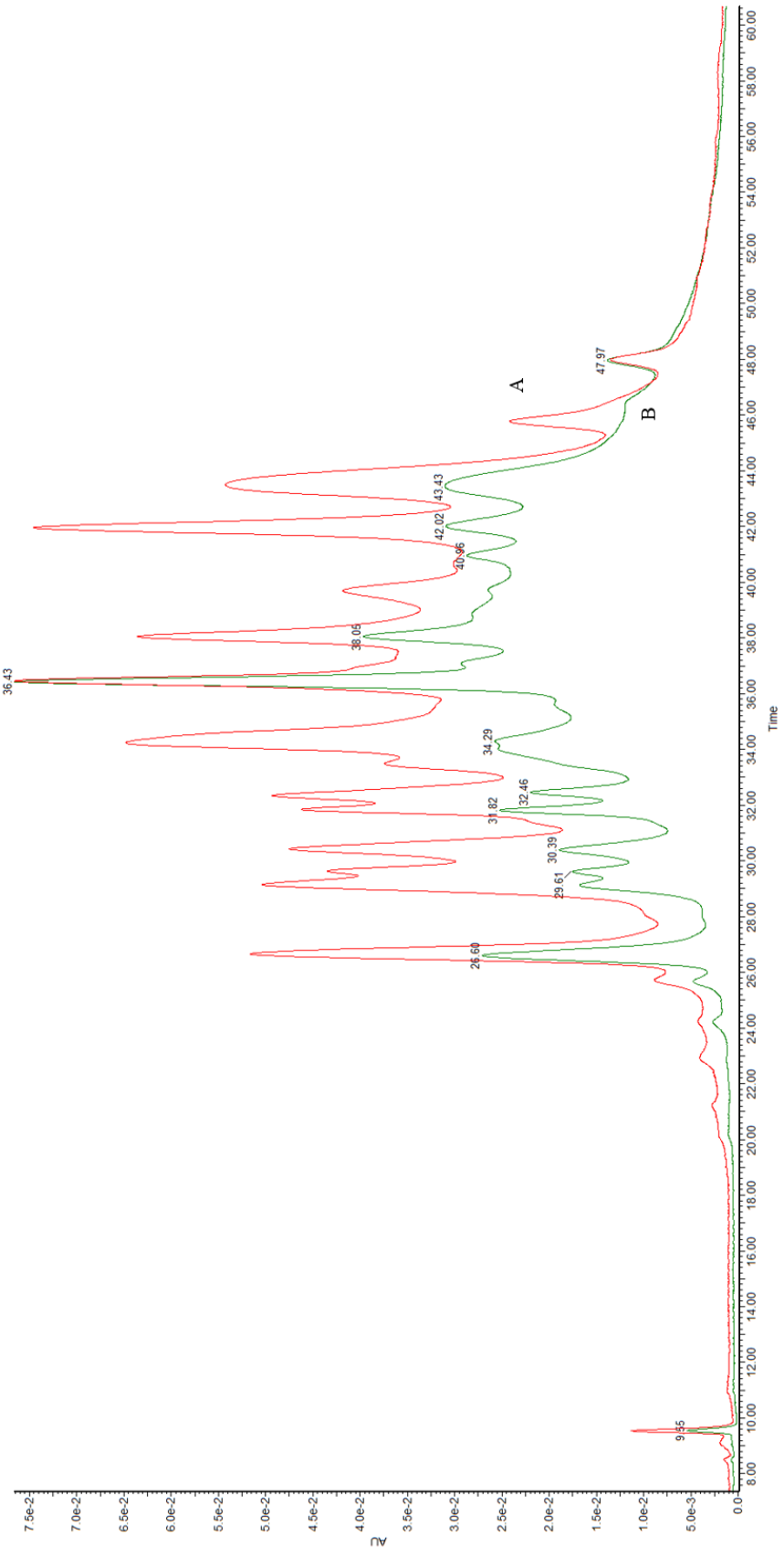


Figure S.6: comparison of the chromatographic profiles at 320nm for the extracts obtained after MW pre-treatment of first fermentation wine lees (A) and Porto wine lees (B).

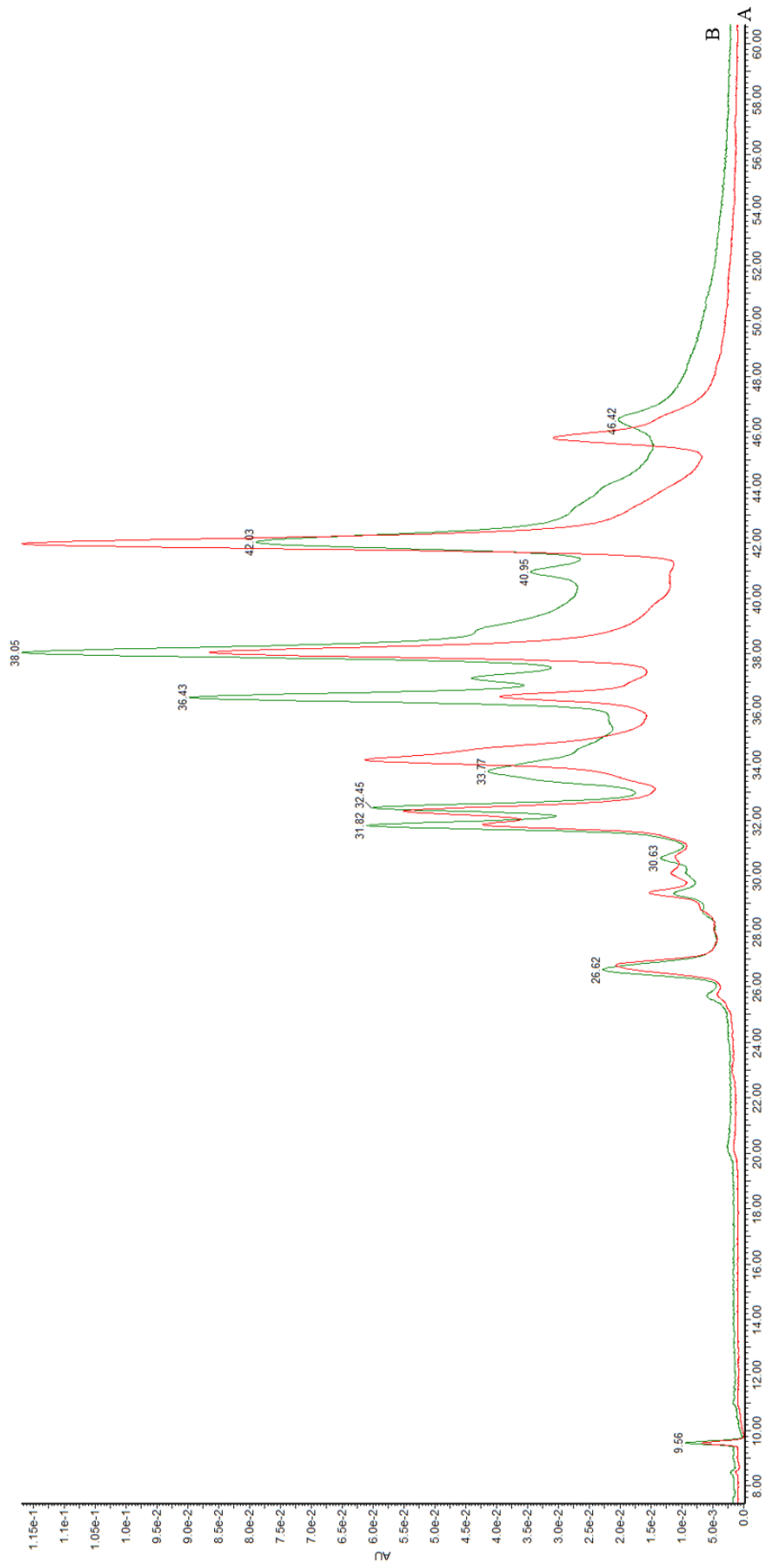


Figure S.7: comparison of the chromatographic profiles at 360nm for the extracts obtained after MW pre-treatment of first fermentation wine lees (A) and Porto wine lees (B).

Table S.1: anthocyanin extraction yields (mg_{MLVE}/g_{DL}) for the study of 'a': solid-liquid ratio (g/mL), 'b': percentage of ethanol (%vol.) and 'c': temperature (ºC). Values with different lowercase letters in each row are significantly different ($p<0.05$)

a)

R_{S-L} (g/mL)	AC (mg_{MLVE}/g_{DL})
0.1	0.81 ± 0.04^a
0.05	0.96 ± 0.01^a
0.033	0.94 ± 0.03^a
0.025	1.05 ± 0.10^a

b)

Ethanol (%vol.)	AC (mg_{MLVE}/g_{DL})
25	0.79 ± 0.01^a
50	2.78 ± 0.18^b
75	3.04 ± 0.38^b
100	0.66 ± 0.04^a

c)

T (ºC)	AC (mg_{MLVE}/g_{DL})
25	2.78 ± 0.18^a
35	3.12 ± 0.27^a
45	3.00 ± 0.24^a

Table S.2: ANOVA for total anthocyanin response. It is consider statically significant for p-values < 0.05.

Source	DF	Sum of squares	Mean square	F-Value	p-Value
A:%H_2O	**1**	**30.532**	**30.532**	**114.78**	**0.0000**
B:R_{S-L}	1	9.679E-05	9.679E-05	0.00	0.9853
C:t	**1**	**3.905**	**3.905**	**14.68**	**0.0064**
AA	**1**	**25.264**	**25.264**	**94.98**	**0.0000**
AB	1	0.029	0.029	0.11	0.7507
AC	**1**	**1.609**	**1.609**	**6.05**	**0.0435**
BB	**1**	**2.576**	**2.576**	**9.69**	**0.0170**
BC	1	0.018	0.018	0.07	0.7977
CC	**1**	**1.793**	**1.793**	**6.74**	**0.0356**
Total Error	7	1.862	0.266007		
Total (corr.)	16	82.217			

Table S.3: Sugars and their degradation compounds concentrations (ppm) in wine lees extracts after 15 minutes of extraction analyzed by HPLC according to the method described in (Cantero et al., 2015)*

	Sugar and derivates compounds concentrations (ppm)					
Compound	**MW-1F**	**S-L 1F**	**MW-2F**	**S-L 2F**	**MW-Porto**	**S-L Porto**
Celobiose	1345	160	497	144	1503	716
Glucose	26	463	796	552	14637	8654
Xylose	626	205	155	62	-	-
Fructose	-	-	-	-	23589	14050
Arabinose	479	289	196	101	1314	1138
Piruvaldehide	942	459	610	425	-	-
Lactic Acid	5207	353	718	191	-	-
Formic Acid	3840	495	4578	1576	4713	3076
Acetic Acid	403	131	175	91	-	-
Levulinic Acid	750	12	135	29	-	-
Acrilic Acid	106	22	306	90	-	-

**Cantero, D.A., Vaquerizo, L., Martinez, C., Bermejo, M.D., Cocero, M.J., 2015. Selective transformation of fructose and high fructose content biomass into lactic acid in supercritical water. Catal. Today 255, 80–86. doi:10.1016/J.CATTOD.2014.11.013*

Table S.4: Putative identification of main phenolics (λ_{max} 360nm, 320nm, 280nm), in 1F wine lees and Porto wine lees extracts. Wine lees extracts where each compound appeared, M-H$^-$ values (m/z), putative identification.

Retention time (min)	Mass [M-H]$^-$ (m/z)	Putative identification	Mass (g/mol)	Phenolic subclass (λ_{max})	Ribera del Duero wine lees	Porto wine lees
31.82	479 (317)	Myricetin-3-O-glucoside	480.38	Flavonol (360nm)	✓	χ
33.77	463 (301)	Quercetin-3-O-glucoside	464.37	Flavonol (360nm)	✓	χ
36.46	507 (345)	Syringetin-3-O-glucoside	508.43	Flavonol (360nm)	✓	✓
38.05	317	Myricetin	318.24	Flavonol (360nm)	✓	✓
42.03	301	Quercetin	302.24	Flavonol (360nm)	✓	✓
45.78	285	Kaempferol	286.23	Flavonol (360nm)	✓	χ
46.42	315	Rhamnetin	316.26	Flavonol (360nm)	χ	✓
26.60	311 (179,149)	Caftaric acid	312.23	Hydroxycinnamic acid (320nm)	✓	✓
29.61	865/577/289; 295 (163,149)	Procyanidin trimer/dimer/catechin/epicatechin; Coutaric acid	866.77/578.52/290.26; 296.23	Flavanols; Hydroxycinnamic acid (320nm)	✓	Catechin; Coumaric acid
21.20	169	Gallic acid	170.12	Hydroxybenzoic acid (280nm)	✓	✓

CHAPTER 3

Enzymatic hydrolysis to enhance the anthocyanin extraction from wine lees

Abstract

Wine lees constitute an important percentage (14%) of the residues generated in a winery. Due to their natural composition, they present an important concentration of yeast derived from the fermentation processes. Thus, the application of an enzymatic hydrolysis to this residue would liberate the phenolic compounds linked to the yeast cell walls. Two enzymes, Glucanex and Mannaway were tested for this purpose and, additionally, a blend of both enzymes. Incubation times ranging from 5 to 60min were studied without significant differences respect to anthocyanin extraction. The highest increment (22%) in the anthocyanin yield was obtained for the enzymatic blend with an incubation time of 5 min. On the other hand, the highest ORAC value was achieved for extract from first fermentation wine lees treated with Mannaway. Anthocyanins were identified by mass spectrometry.

1. Introduction

In the last decades there has been a great increase in the valorisation of agricultural wastes in order to recover high added value compounds. A clear example of this is the case of winery grape-residues which constitute a rich source of polyphenols (Garcia-Alonso, Minihane, Rimbach, Rivas-Gonzalo, & de Pascual-Teresa, 2009), commonly used in industries such as alimentary, pharmaceutic or cosmetic because of their high bioactivity and beneficial effects for human health (Barcia et al., 2014; Moreno-Montoro, Olalla-Herrera, Gimenez-Martinez, Navarro-Alarcon, & Rufián-Henares, 2015). Wine production has always represent an important sector within the agricultural activities in the EU. Huge amounts of wastes are generated during the vinification process, from the grape harvesting to the wine bottling. Among these residues, vine pruning, grape stalks, grape pomace and wine lees can be found (Drosou, Kyriakopoulou, Bimpilas, Tsimogiannis, & Krokida, 2015). The latter are solid-liquid wastes generated during the fermentation steps that sediment on the bottom of the barrels or as the solid by-products obtained following filtration or centrifugation. Different types of wine lees can be found depending on the vinification process. In the case of red table wine, it is possible to find first fermentation wine lees (generated in the alcoholic fermentation) and second fermentation wine lees (generated in the malolactic fermentation). Nevertheless, in the case of a Port wine, first fermentation is stopped by adding extra ethanol

(Perestrelo, Silva, Pereira, & Câmara, 2016), and only one type of wine lees are generated.

Only in Spain, around 60kg of lees are generated per ton of grapes (Pérez-Bibbins, Torrado-Agrasar, Salgado, Oliveira, & Domínguez, 2015). They are mainly formed by yeasts, polyphenolic compounds, mainly anthocyanins, and inorganic material. Although these dregs were used for the production of tartaric acid (Versari, Castellari, Spinabelli, & Galassi, 2001; Yalcin, Ozcalik, Altiok, & Bayraktar, 2008), few works are available regarding the extraction of polyphenols from them. However, recent studies showed the potential of these residues as an alternative sources of natural colorants, since anthocyanins and their derivates concentrations in wine lees are ten times higher than the concentrations found in the grape skins (Peralbo-Molina & Luque de Castro, 2013).

Additionally, there are some studies that revealed the presence of anthocyanins linked and/or absorbed within the cellular wall of yeasts during fermentations steps (Pérez-Serradilla & de Castro, 2008). It is world-wide known, that wine lees can absorb phenolic compounds since they have been commonly used for the decolouring of sparkling wines, such as cava or champagne (Ibern-Gómez et al., 2000; Vasserot, Caillet, & Maujean, 1997). Thus, the same absorption process takes place during the fermentation steps, where yeast lees and the wine are together during months. This is corroborated due to the fact that anthocyanins and their derivates concentrations decreased in wines after being in contact with wine lees (Vasserot et al., 1997). Nonetheless, the bonds of polyphenols to the yeast cell walls led to controversy among the researches. There is not a general assumption about how the phenolic compounds are linked to the yeast. On the one hand, there are those hypothesis in which polyphenols are said to be linked by weak and reversible adsorption interactions between anthocyanins and yeast walls such as hydrophobic interactions or hydrogen bonds (A. Morata et al., 2003). On the other hand, other authors suggested the idea that stronger covalent bonds appear between the polyphenols and the yeast walls (Xu et al., 2014).

In this sense, it is necessary to get an accurate methodology to a break or degrade the cell wall. Thus, the use of enzymes which can act on the specific compounds that conform the cellular wall, could provide its disruption and consequently the release of polyphenols linked to the yeasts cell wall (Martins, Roberto, Blumberg, Chen, & Macedo, 2016). Generally, the strain *Saccharomyces*

Cerevisiae is the responsible of the fermentation steps in the winemaking process (Jolly, Augustyn, & Pretorius, 2033). Though they are present naturally in grapes, extra quantities of theses yeast are added to the wine at the cellar, due to its low concentration. The mainly constituents of the yeasts cell walls are polysaccharides and glycoproteins and they create two differentiated regions: an external protein layer and an internal polysaccharide wall (Borchani et al., 2014). Its structural composition is mostly made of β-(1→3) glucans (50-55%), β-(1→6) glucan that appears in a lower concentration (10-15%), and mannoproteins (Aimanianda et al., 2009). Polysaccharides have a structural function whereas the mannoprotein layer acts as a filler and it is important for the permeability of the cell wall (Kollár et al., 1997). Therefore, the degradation of the cell wall polysaccharides is a fundamental step to improve the release of polyphenols.

Enzymatic pre-treatment has been already carried out by cell wall hydrolyse enzymes, to grape fruit and grape pomace. For instance, *Fernández et al*. (Fernández, Vega, & Aspé, 2015) used cellulase, tannase and pectinase to obtain enzymatic extracts from grape skins and seed. Enzymatic hydrolysis was carried out in the appropriate buffer and once the incubation time was finished, ethanol was added to the medium (50%vol. ethanol). Results showed that the most favourable results for grape skins were obtained when pectinase was employed, increasing the total polyphenol concentration by 2.5 times respect to the control. Cellulase and tannase only increased the liberation of polyphenols by 1.35 and 1.29 times. For the case of grape seed, the three enzymes were able to increase the total phenol release by 1.26, 1.32 and 1.34 times respect to the control ($p<0.05$) for pectinase, cellulase and tannase, respectively. The addition of ethanol to the media, once the enzymatic hydrolysis has taken place, increases the release of polyphenols. This fact was also corroborated by other authors to both red (Ferri et al., 2016) and white grape pomace (Ferri et al., 2017), increasing the phenolic concentration when ethanol is added respect to the water extracts. This behaviour was expected due to the fact that hydroalcoholic mixtures have been reported to increase the solubility of polyphenols (Cacace & Mazza, 2003; Dimou et al., 2016). On the other hand, other authors found that tannase was most effective in the hydrolysis of polymeric polyphenolics as it increased total phenol content by 1.61times in red grape pomace and 1.22 times in white grape pomace compared to the respective untreated grape pomaces. However, pectinase was not particularly effective in the hydrolysis (Martins et al., 2016). This

might be to grape pomace is a mixture of grape skins and seeds, in a variable proportion.

From the best of our knowledge, enzymatic pre-treatment has never been applied to wine lees and also, it has to be taken in mind the composition if the yeasts cell wall, so enzymes able to degrade the yeast cell walls, and consequently liberate the linked polyphenols and the yeasts cell wall compounds are needed, such as β-glucanases and/or mannosidases. For example Varelas et al. (Varelas, Tataridis, Liouni, & Nerantzis, 2016) employed Glucanex 200 G (G) as a source of β-glucanases in order to liberate the β-glucans of the cell walls of *Saccharomyces Cerevisiae* from wine residues. Mannaway (KHN01057) (M) is an enzyme with an endo-1,4- β -mannosidase enzyme activity which has not been already used in yeast. This enzyme is used in order to evaluate its activity in the mannoprotein layer that conform the yeast (Mateos-Aparicio, Molina, & Redondo-Cuenca, 2015).

This work is aimed to the study of an enzymatic pre-treatment as a mechanism to increase the anthocyanin content in the final extract, compared to the traditional solid-liquid extract, by breaking the established links between anthocyanins and the cellular wall of yeasts during fermentations steps. Two different types of enzymes were tested, Glucanex (G) and Mannaway (M), and a combination of both (GM) in different types of wine lees. Enzymatic reaction time was evaluated. Anthocyanin content (AC) was measured in each experiment and identification of extracted compounds were performed by HPLC.

2. Materials and methods

2.1 Materials

Two types of *Tempranillo* grape variety wine lees were provided by the winery Matarromera from a *Ribera del Duero* Denomination of Origin wine (Valladolid, Spain) of 2015. These lees were first (1F) and second fermentation (2F) wine lees. Moreover, studies with other variety of grape and vinification process were performed. Porto wine lees were gathered in this case by the winery Sogrape located in Porto (Portugal) in 2015. All wine lees were centrifuged (Avanti J-26 XPI with a rotor type JA-10) for 90 minutes at 10,000 rpm and lyophilized for 48 hours (Micro Modulo EDWARDS) in order to preserve the material and avoid the growth of bacteria.

Lees were powdered using a using a Braun MR 6550 CA mincer in order to obtain a homogenous matrix and then, storage in dark place at room temperature.

Chemicals used for extractions methodologies were ethanol (96% v/v, Panreac) and distilled water. To determine anthocyanin content, two salt were used to prepare the buffers in distilled acidified water: potassium chloride (KCl) and sodium acetate ($CH_3CO_2Na \cdot 3H_2O$).

Enzymes used for the enzymatic pre-treatment were Mannaway (KHN01057) and Glucanex (KM24400201) kindly provided from the Nutrition and Bromatology Department II, Pharmacy Faculty, University Complutense de Madrid (Madrid, Spain).

2.2Methods

2.2.1 Conventional extraction

In order to compare the anthocyanin extraction yields obtained with an enzymatic hydrolysis, results were compared to the yields obtained in the conventional solid-liquid extractions carried out in our previous work (Romero-Díez et al., 2018).

2.2.2 Enzymatic assisted extraction

Firstly the enzymatic reaction has to take place, enzymes need the required time to break the linking bonds the cell yeast walls of wine lees, which acts as the substrate. This time of enzymatic reaction is a main variable to study in the process, so different incubation times were tested (5, 15, 30 and 60 minutes). This step was performed by adding a determine amount of specific enzyme to a lees solution. Solid-liquid ratios and enzyme-substrate ratios are also main parameters of the enzymatic reaction and they were kept constant. A $R_{S\text{-}L}$ of 1/10 (g/mL) was chosen for the incubation time from (Romero-Díez et al., 2018). The incubation step was only performed in water in order to preserve the activity of the enzymes which can be halted by the ethanol (Fernández et al., 2015). A temperature of 37ºC and a pH of 5 were selected as according to the enzyme specification sheets ("Novozymes | Enzymes & Microorganisms," n.d.). Extractions were performed in an erlenmeyer protected from the light with an agitation of 300 rpm. The enzyme-substrate ratio is different for each of the two enzymes used. On one hand, Mannaway is presented in a yellow liquid form and the enzyme-substrate ratio is expresses as

volume of enzyme per mass of substrate. For our experiments, an initial volume 100 µL of Mannaway was added per gram of lees according to literature (Mateos-Aparicio et al., 2015). On the other hand, Glucanex is a white powder and the ratio here must be expressed as mass of enzyme per volume of solvent. Following the procedures of previous works to extract β-glucans from *Saccharomyces cerevisiae* yeasts a ratio of 0.015 mg $_{enzyme}$/mL$_{solvent}$ was chosen (Varelas et al., 2016).

Once the incubation time passed, ethanol was added to the mixture in the same rate as water in order to get a final mixture solvent of 50% vol. ethanol. Hydroalcoholic mixtures have been established as the best solvent type to maximize anthocyanin extraction from wine lees due to an increment of their solubility (Romero-Díez et al., 2018). Extraction continued for 20 minutes more and volume samples of 1.5 mL were collected every 5 minutes to measure anthocyanin content (AC) along the time towards build the kinetic extraction curve for the different conditions.

2.2.3 Anthocyanin Content (AC)

A pH differential method was used for the anthocyanin content (Lee et al., n.d.). It is based in the change of colour of anthocyanins with pH. At pH=1 coloured oxonium ions are formed whereas at pH=4.5 not. Absorbance was measures at 520nm. Additional measure at 700nm was made to correct for hazel. The difference between thisabsorbance is proportional to the anthocyanins concentration. It is expressed as equivalents of malvidin per gram of dried lees (mg$_{MLVE}$/g$_{DL}$) ± standard deviation (SD).

Two buffers were used: a 0.025M solution of potassium chloride with pH=1 and other of 0.4M, pH=4.5 of sodium acetate. Samples were diluted in these buffers and then, absorbance is measured at 520 and 700nm for each buffer. The equation used to calculate the concentration of anthocyanins was:

$$C_A = \frac{[(A_{520}-A_{700})_{pH1}-(A_{520}-A_{700})]\cdot M_W \cdot DF}{\varepsilon \cdot l} \cdot \frac{1}{R} \qquad \textit{(Equation 1)}$$

Where C_A is the anthocyanin content expressed in mg$_{MLVE}$/g$_{DM}$; A the absorbance measurements; M_w the molecular weight of malvidin (493.4 g/mol); DF is the dilution factor; ε represents the molar extinction coefficient (28,000L/mol·cm); l is the path length in cm and R is the solid-liquid ratio (g$_{DL}$/mL) used in the extraction.

2.2.4 High Performance Liquid chromatography–mass spectrometry (HPLC-DAD-MS/MS)

The spectrometer (LC) system used was a liquid chromatography Waters Alliance 2695 Separation Module (Waters®, Ireland). The mass spectrometer (MS/MS) used was a MicroMass Quattromicro® API (Waters®, Ireland). Chromatographic separation of compounds was carried out in a reversed phase LiChrospher® 100 RP-18 5μm LiChroCART® (250 x 4.0mm) column inside a thermostated oven at 35ºC. Mobile phase and other characteristic features of the analysis procedure, such as the volume injection or data acquirement have been previously reported in the work of *Romero-Díez et al*. (Romero-Díez et al., 2018).

2.2.5 Antioxidant activity: ORAC

In order to measure the antioxidant ability of the enzymatically prepared extracts, the Oxygen Radical Absorbance Capacity (ORAC) was measured. This assays is based on the fluorescence quenching of disodium fluorescein (FS) salt after exposure to AAPH (2,2-azobis(2-amidino-propane) dihydrochloride), which generates oxygen radicals ($ROO^{\bullet}$) at a constant rate (Garrett et al., 2014). ORAC assay was carried out as described as *Huang et al*. (Huang, Ou, Hampsch-Woodill, Flanagan, & Prior, 2002). ORAC results were given in μmol of Trolox equivalents (TE) per gram of dry lees ($\mu mol_{TE}/g_{DL}$) ± SD.

2.2.6 Statistical analysis

All data are expressed as means ± standard deviations (SD) and individual experiments were performed at least in triplicate.

3. Results

3.1 Conventional extraction

Anthocyanin contents for the solid-liquid extraction for all types of lees are shown in ¡Error! No se encuentra el origen de la referencia.. Ribera del Duero wine lees from the first fermentation showed the highest AC after 15 minutes (3.04±0.10 mg_{MLVE}/g_{DL}) followed by the Port wine lees 2.78±0.18 mg_{MLVE}/g_{DL}) and the second

fermentation win lees 2.09±0.38 mg_{MLVE}/g_{DL}). These values were those reported in Chapter 2. Results of AC are gathered in Table 1.

Table 1: anthocyanins extraction yields after the conventional solid-liquid extraction for first fermentation (1F), second fermentation (2F) and Port wine lees.

	AC (mg_{MLVE}/g_{DL})		
t (min)	**1F wine lees**	**2F wine lees**	**Porto wine lees**
0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
5	2.84 ± 0.11	1.93 ± 0.07	2.54 ± 0.21
10	3.00 ± 0.06	2.01 ± 0.07	2.78 ± 0.06
15	3.04 ± 0.03	2.09 ± 0.10	2.78 ± 0.18
20	3.02 ± 0.05	2.08 ± 0.06	2.39 ± 0.13

3.2 Enzymatic assisted extraction to first fermentation wine lees

Time of the enzymatic pre-treatment was firstly evaluated for first fermentation wine lees and then best conditions were applied for the rest of the lees. Once the lees mass was weighted (7.5g) in an erlenmeyer, the exact volume of distilled water to have a R_{S-L} of 0.10 (g/mL) was poured inside. Regarding the Mannaway enzyme, the chosen quantity of enzyme employed (100 µL $_{enzyme}/g_{substrate}$) did not improve the recovery yield (data no shown). Thus, a three times more concentrated enzyme-substrate ratio was employed (300 µL $_{enzyme}/g_{substrate}$).

Hereafter, the enzyme is added to the mixture, whether G, M, or a combination of both of them (GM) and it is let to act over the yeast cell walls. Four incubation times were studied: 5, 15, 30 and 60 minutes. When the enzymatic pre-treatment time has expired, a sample of the mixture is taken and then, ethanol is added in the same volume as water was. Extraction results can be seen in Table 2.

Table 2: anthocyanin content along the time for different incubations times in first fermentation wine less for each enzyme, Mannaway (a)-Glucanex (b) and the combination of both (M+G) (c) . t =0 represents the moment after the incubation takes places. Following times represent AC when ethanol was added. Values with a superscript asterisk are significantly different ($p<0.05$) for each time.

AC (mg_{MLVE}/g_{DL})

a)	**Pre-treatment time $_{Mannaway}$ (min)**			
t (min)	**5**	**15**	**30**	**60**
0	0.41 ± 0.02*	0.47 ± 0.05*	0.39 ± 0.06*	0.49 ± 0.06*
5	3.34 ± 0.98	4.14 ± 0.13	3.92 ± 0.02	4.23 ± 0.43
10	3.81 ± 0.36	4.17 ± 0.14	4.19 ± 0.06	4.02 ± 0.08
15	3.75 ± 0.18	4.50 ± 0.29	4.17 ± 0.26	4.26 ± 0.05
20	3.81 ± 0.02	4.17 ± 0.43	4.14 ± 0.05	4.20 ± 0.05
b)	**Pre-treatment time $_{Glucanex}$ (min)**			
t (min)	**5**	**15**	**30**	**60**
0	0.27 ± 0.02*	0.18 ± 0.11*	0.11 ± 0.03*	0.42 ± 0.10*
5	3.81 ± 0.02	3.76 ± 0.25	3.83 ± 0.02	3.93 ± 0.22
10	3.71 ± 0.31	3.85 ± 0.30	3.99 ± 0.03	3.67 ± 0.43
15	3.84 ± 0.25	4.04 ± 0.08	3.90 ± 0.18	3.90 ± 0.24
20	3.82 ± 0.37	4.02 ± 0.05	4.01 ± 0.03	4.01 ± 0.35
c)	**Pre-treatment time $_{M+G}$ (min)**			
t (min)	**5**	**15**	**30**	**60**
0	0.53 ± 0.06*	0.49 ± 0.04*	0.86 ± 0.37*	0.61 ± 0.00*
5	4.37 ± 0.07	4.08 ± 0.27	4.38 ± 0.08	3.93 ± 0.25
10	4.40 ± 0.40	4.27 ± 0.05	4.36 ± 0.23	4.10 ± 0.07
15	4.18 ± 0.33	4.55 ± 0.12	4.39 ± 0.10	4.09 ± 0.06
20	4.65 ± 0.23	4.32 ± 0.04	4.21 ± 0.13	3.97 ± 0.05

As it can be seen in Table 1, no significant differences ($p<0.05$) were achieved from the different incubation times. Only significant differences were achieved when ethanol was added to the medium (t>5min), as expected (Fernández et al., 2015; Ferri et al., 2016, 2017). Without the addition of ethanol, anthocyanin extraction yields obtained

after enzymatic pre-treatments during 60min of incubation followed by a 20-min conventional extraction were very low (around 0.45 mg_{MLVE}/ g_{DL}). AC results showed that, when Glucanex or Mannaway are used individually, slightly (but not significant) increments were observed with the pre-treatment time. For the case of Mannaway an increment on the AC of the 10% is achieved when 60 minutes of pre-treatment (4.20 mg_{MLVE}/ g_{DL}) are used instead 5 minutes (3.81 mg_{MLVE}/ g_{DL}). Meanwhile, when Glucanex is used in this conditions only an increment of 5% on the AC is attained (3.82 – 4.01 mg_{MLVE}/ g_{DL}, respectively). Although there is no data available about the extraction of phenolics from wine lees using an enzymatic hydrolysis, other authors have evaluated its efficiency in other vinification residues. Additionally, another challenge for the comparison between works in which an enzymatic hydrolysis is performed, is that they are usually focused on the total polyphenol content. Whereas in our work, the targeted compounds were the anthocyanins. For example, *Fernández et al*. (Fernández et al., 2015) treated enzymatically a dried grape skin and obtain similar enhancements. Cellulase and tannase increased the liberation of phenols by 1.35 and 1.29 times, respectively. Higher extraction yields were achieved (1.61-hold higher) when tannase was employed in a enzymatic hydrolysis to red grape pomace (Martins et al., 2016). *Ferri et al*. (Ferri et al., 2016), who worked with dried red grape pomace performed enzymatic hydrolysis with Fungamyl (α-amylase) and Celluclast (cellulase) followed by the addition of ethanol. The total phenolic content, after the addition of ethanol when Celluclast was used, increased significantly by 1.22 times, respect to the control performed at the same conditions (37ºC, 2h). On the other hand, Fungamyl showed a significant negative effect on the total phenolic content. It decreased a 22% in comparison with the control performed at the same conditions (24ºC, 2h). For the case of anthocyanins, Fungamyl showed the same negative effect.

A different trend was observed when a combination of both enzymes (G+M) is used, conversely a decrease of 15% on the AC is perceived from applying 5 (4.65 mg_{MLVE}/ g_{DL}) or 60 (3.97 mg_{MLVE}/ g_{DL}) minutes of pre-treatment. On the other hand, the AC release from *Ribera del Duero* first fermentation wine lees for an incubation of 5 min increases 1.50 times when using the enzymatic blend respect to the individual enzymes for 5min incubation time. Same behaviour was obtained by *Fernández et al.* (Fernández et al., 2015). They used a enzymatic blend to increase the total phenol release of grape seeds and skin, showing an increment on the final phenol content of 1.28 and 1.27 times higher,

respectively, compared to the obtained by individual enzymes. This phenomena can be explained to the presence of fibre in the yeast cell wall (Aimanianda et al., 2009) which challenges enzyme access. The synergistic activity of the enzymatic blend could have led to the digestibility of the cell wall increasing the anthocyanin release. Taking this in mind, the mixture of G+M would be the best choice to enhance the extraction of anthocyanins with an increase around 1.5 times for an incubation time of 5min and a conventional extraction of 15min, in comparison with the conventional extraction.

In addition to all aforementioned, the yield increments on the final AC exposed in our work varied and are influenced by several factors. For instance, the initial raw materials differ a lot from each other and the enzyme variety greatly influence the liberation of linked polyphenols (Martins et al., 2016). Similarly, it is important to take into consideration that other works from literature only measured the total polyphenols content, whereas we were only measuring the AC. Moreover, longer incubation times were used by other authors (Fernández et al., 2015; Martins et al., 2016) (from 2 to 24h) than ours (5-60min) which may have enhance the release of polyphenols. However, it does not mean that the longer the time, the higher final extraction yield. Long-time enzyme treatments have revealed to have a significantly negative effect on phenol recovery, probably due to metabolite degradation during extended incubation (Meyer, Jepsen, & Sørensen, 1998). A clear example of this was found by *Ferri et al.* (Ferri et al., 2016) who found that after 6h of incubation time a 2.3-fold decrease was detected the final polyphenol recovery respect to a 2h incubation time.

3.3 Comparison of the enzymatic assisted extraction to all wine lees types

Enzymatic pre-treatments were also applied to the other types of lees, second fermentation *Ribera de Duero* wine lees and *Porto* wine lees. For this case, pre-treatments with individual enzymes, G and M, and 5 min of incubation was chosen since there were no significant differences among the different evaluated incubation times. From results showed in Figure 1, it is possible to remark the differences between all types of lees. As it was supposed, first fermentation wine lees provides the best results of AC (~3.80 mg_{MLVE}/ g_{DL}) after enzymatic pre-treatment than second fermentation lees (~2.65 mg_{MLVE}/ g_{DL}) because their lower concentration of yeasts. Apart from the natural yeasts present in grapes, wine cellars add yeasts to the must to ensure the fermentation step takes place (Romano, Fiore, Paraggio, Caruso, & Capece, 2003). Following the first

fermentation, the must is pressed to separate the liquid and the grape pomace, where yeasts are dragged together with grape skins and seeds. After this, the second fermentation continues very slowly (Chambers & Pretorius, 2010) due to the less concentration of yeasts which will lead to settle. Furthermore, lower AC were obtained for Porto wine lees (~2.70 mg_{MLVE}/ g_{DL}). This was because the vinification process for Porto wine differs from the rest of wines and the must may not be in contact with yeasts enough time for anthocyanins to be linked to the yeast cell walls. In Porto wine, first fermentation is stopped in the middle of the process by adding extra ethanol (Perestrelo et al., 2016), and the fermentation step is not completed, reason why Porto wine is sweeter than other wines.

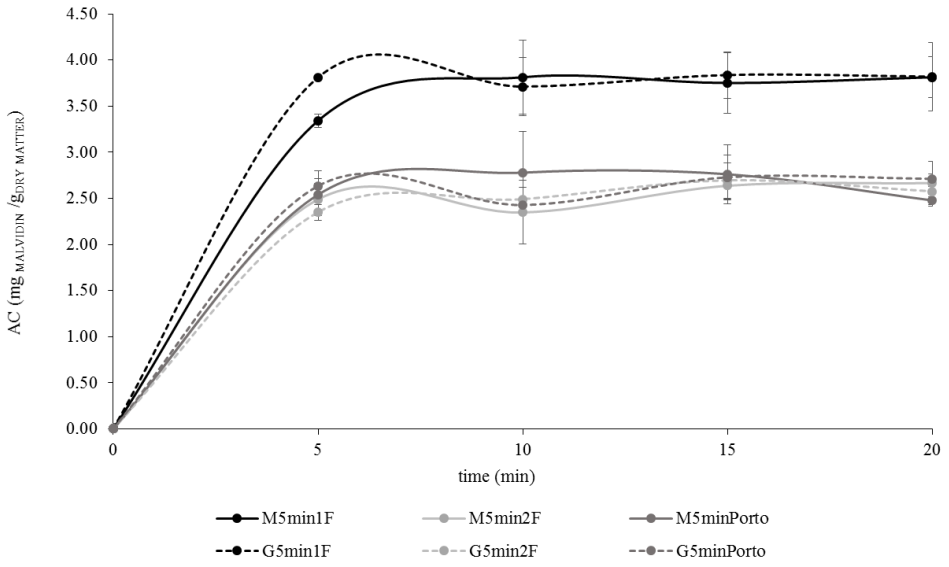


Figure 1: comparison of AC after the enzymatic pre-treatments at 5min of incubation time with G and M enzymes for each type of lees.

3.4 Comparison of the different pre-treatments for the recovery of polyphenols form wine lees: microwaves, ultrasounds and enzymatic hydrolysis.

Similar results were achieved for AC from first fermentation wine lees when a microwave pre-treatment was used (4.30 ± 0.06 mg_{MLVE}/ g_{DL}) (Romero-Díez et al., 2018). However, the required time to achieve a steady concentration in much lower (90s) than the needed for the best enzymatic conditions (60 min). A comparison of microwave and the enzymatic pre-treatment can be seen in Figure 2. Moreover, it should be not forgotten that the price of enzymes is really high (Fernández et al., 2015), and more if we are using

a mixture of both, which may make this pre-treatment more expensive and less profitable than the microwave pre-treatment which would have a negative economic impact on the polyphenol recovery process. Further thermo-economic analysis are required in order to corroborate which technique will be the most suitable for polyphenol recovery.

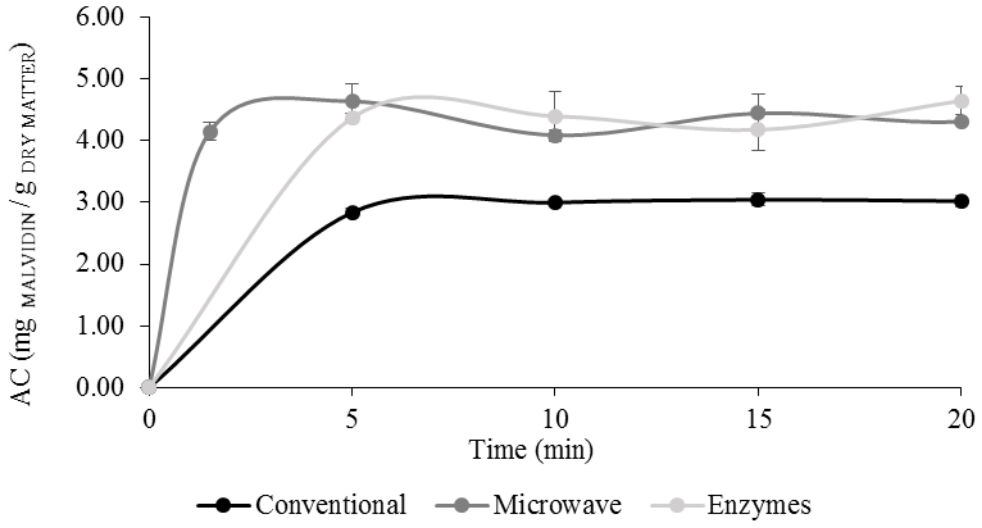


Figure 2: comparison of AC for microwave and enzymatic pre-treatment (G+M) for a 60 minutes of incubation and the traditional solid-liquid extraction for Ribera del Duero first fermentation wine lees.

3.5 HPLC analysis

HPLC analysis were only performed to the selected extracts. These extracts were those obtain after 5min of incubation for each type of enzyme. These 5-minutes extracts were chosen since no significant differences were observed for higher incubation times (Table 1).

Anthocyanins present in all the extracts from the different types of wine lees were the same for all the enzymatic extracts as it can be seen in Figure 4.

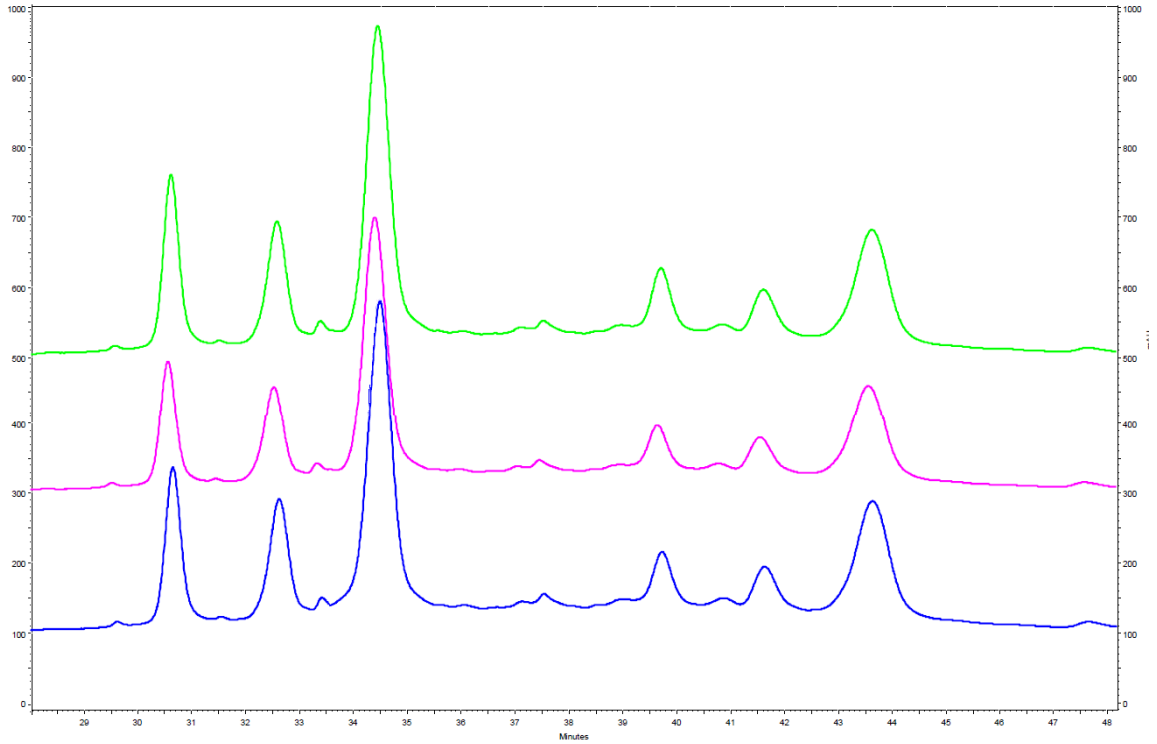


Figure 4: chromatographic profile at 520nm of the first fermentation wine lees extracts obtained enzymatically with an incubation time of 5 min for Mannaway (blue), Glucanex (pink) and the enzymatic blend (green)

As well, anthocyanins putative identifications via MS/MS is shown in Table 3. Identified anthocyanins were the same as those found in other works with the same wine lees (Chapter 2 o (Romero-Díez et al., 2018), except for the case of the pyranoanthocyanins Vitisin A. it seemed that Vitisin A was only extracted when a microwave pre-treatment was employed (Romero-Díez et al., 2018).

Table 3: putative identification of the anthocyanins found in wine lees extracts treated enzymatically.

R_t (min)	**$[M-H]^+$ (m/z)**	**MS/MS (m/z)**	**Putative identification**
29.75	465	303	Delphinidin-3-O-glucoside
31.72	479	317	Petunidin-3-O-glucoside
33.70	493	331	Malvidin-3-O-glucoside
39.48	611	303	Delphinidin 3-O-(6"-p-coumaroyl-glucoside)
41.80	625	317	Petunidin 3-O-(6"-p-coumaroyl-glucoside)
43.88	639	331	Malvidin 3-O-(6"-p-coumaroyl-glucoside)

Apart from the anthocyanins, the most interesting finding was the detection of a compound at around the minute 41, which is only released when the Mannaway enzyme was used, as it is shown in Figure 5. Nonetheless, this compound has not been already identified and it is pending of clarify if it is or not a polyphenol. The most defined $[M-H]^+$ values for this compound were 375, 263 and 219 (m/z). Further work is required in order to determine the unknown compound.

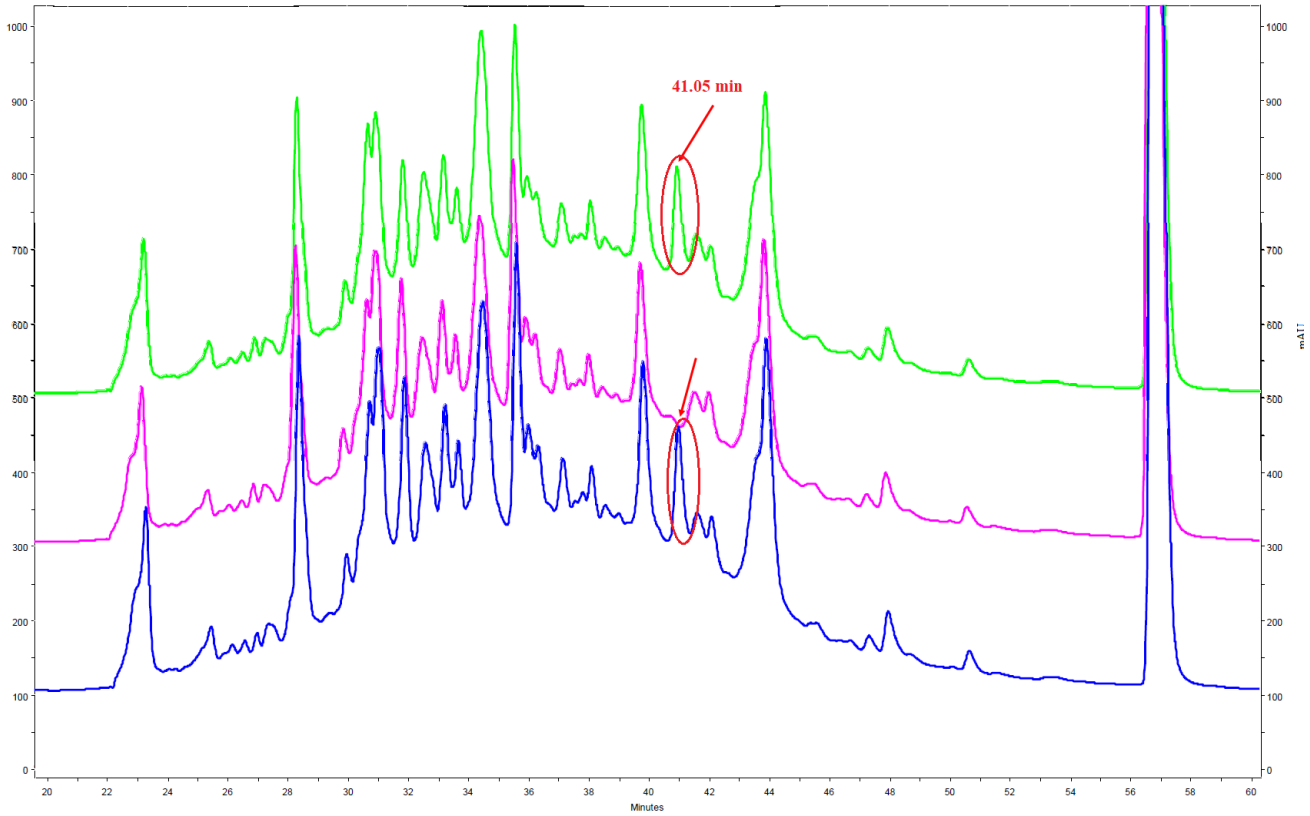


Figure 5: chromatographic profile at 280nm of the first fermentation wine lees extracts obtained enzymatically with an incubation time of 5 min for Mannaway (blue), Glucanex (pink) and the enzymatic blend (green)

3.6 Antioxidant activity: ORAC

The antioxidant activity of the selected extracts was evaluated via ORAC assay. These extracts were those for the different wine lees types performed with an enzymatic incubation of 5 min. ORAC results are shown in Table 3.

Table 3: comparison between the antioxidant activities for the different wine lees extracts treated enzymatically and the conventional solid-liquid extract. Values with different letters in the same row are significantly different ($P<0.05$) for each type of wine lees.

	ORAC ($\mu mol_{TE}/g_{DL}$)			
	Conventional extraction	**Enzymatic hydrolysis**		
		Mannaway	**Glucanex**	**G+M**
1st Fermentation	392 ± 42^{a}	418 ± 18^{b}	413 ± 9^{b}	334 ± 2^{a}
2nd Fermentation	304 ± 20^{a}	325 ± 11^{a}	352 ± 30^{b}	-
Port wine lees	195 ± 20^{a}	276 ± 21^{b}	344 ± 20^{c}	-

As it can be seen, enzymatic extracts from first fermentation wine lees showed the highest ORAC values for each enzymatic treatment. Since the AC increased after the enzymatic hydrolysis, the antioxidant capacities of the extracts increased too. The same trend was observed for second fermentation and Port wine lees. Furthermore, for these two types of lees, the highest ORAC value was obtained when the enzymatic hydrolysis was carried out with Glucanex (352 and 344 $\mu mol_{TE}/g_{DL}$, respectively). On the contrary, for first fermentation wine lees, Mannaway-treated extracts was the one which exhibited a higher (but not significant, $p<0.05$) ORAC value than the Glucanex-treated extracts. For the case of the enzymatic blend (G+M) in first fermentation wine lees, it displayed the lowest values. Nevertheless, this value was expected to be the highest since it gave the most concentrate extract in anthocyanins (vide Table 1).

No works can be found in literature about the antioxidant activity of wine lees extracts produced via enzymatic hydrolysis. Thus the comparison of our values respect to other can be only perform for other winery residues, such as the red grape pomace. This is the case of *Martins et al.* (Martins et al., 2016) evaluated the ORAC value of their grape pomace extracts enzymatically treated. Values up to 1100, 700 and 850 $\mu mol_{TE}/g_{DRY\ POMACE}$ when tannase, pectinase plus cellulase and a blend of the three enzymes were used, respectively. Besides, these increments in the ORAC values were almost in accordance with the increment of the total phenolic content. For the extract enzymatically treated with tannase, and increment on the total phenolic content of 1.60 times respect to the control was achieved. For the same extract, the ORAC value exhibited an increment of 1.80 times. These values are higher than ours. Furthermore, in our case the proportionality between the AC and the antioxidant activity was not maintained. For example, for first fermentation wine lees, increments of 1.25 and 1.50 times for TPC were achieved when enzymes were used individually or in a blend, respectively, but the ORAC values only increased 1.10 times with the individual enzymes and further, a decrese in ORAC was observed after the pre-treatment with the blend. Several factor can prove these discrepancies. The most important were the differences between the two different raw materials used and the types of enzymes employed in each case. Probably, the fact that our incubation time was far less (5-60min) compared to the one they used (5h), would also influence the phenolic release. Least but not less, is the fact that we were only measuring the AC whereas other works took into account all the polyphenols subgroups.

4. Conclusions

In this work the performance of an enzymatic hydrolysis has been proposed to different types of wine lees pre-treatment to enhance anthocyanins extraction. Results showed slight increments of around 25% when Glucanex and Mannaway were employed individually for a 5min incubation time respect to the conventional AC extraction yield. Higher increments (up to 1.50 times) were obtained when the enzymatic blend G+M was employed for the same incubation time. However, no significant differences ($p<0.05$) were obtained for longer incubations times, up to 60min. Antioxidant activity of the extracts was also evaluated via ORAC. Antioxidant activity was in accordance with the AC yields only when Glucanex and Mannaway were used individually. Wine lees enzymatically pre-treated led to higher anthocyanins concentrations, which led to higher ORAC values. On the contrary, when the enzymatic blend G+M was used, an increment on the AC yield was obtained, although this increment was not shown in the ORAC results. Data revealed that the extracts with the highest antioxidant activity was the first fermentation wine lees extract, treated with Mannaway (418 $\mu mol_{TE}/g_{DL}$). Main anthocyanins have been identified by HPLC-DAD-MS/MS. Further, an un-identified compound was detected at 280nm only after the incubation with Mannaway.

References

A. Morata, M. C. Gómez-Cordovés, J. Suberviola, B. Bartolomé, B. Colomo, and Suárez, J. A. (2003). Adsorption of Anthocyanins by Yeast Cell Walls during the Fermentation of Red Wines. *The Journal of Agricultural and Food Chemistry*, *51*(14), 4084-4088.

Aimanianda, V., Clavaud, C., Simenel, C., Fontaine, T., Delepierre, M., & Latgé, J.-P. (2009). Cell wall beta-(1,6)-glucan of Saccharomyces cerevisiae: structural characterization and in situ synthesis. *The Journal of Biological Chemistry*, *284*(20), 13401–12.

Barcia, M. T., Pertuzatti, P. B., Rodrigues, D., Gómez-Alonso, S., Hermosín-Gutiérrez, I., & Godoy, H. T. (2014). Occurrence of low molecular weight phenolics in Vitis vinifera red grape cultivars and their winemaking by-products from São Paulo (Brazil). *Food Research International*, *62*, 500–513.

Borchani, C., Fonteyn, F., Jamin, G., Paquot, M., Blecker, C., & Thonart, P. (2014). Enzymatic process for the fractionation of baker's yeast cell wall (Saccharomyces cerevisiae). *Food Chemistry*, *163*, 108–113.

Cacace, J. E., & Mazza, G. (2003). Optimization of Extraction of Anthocyanins from Black Currants with Aqueous Ethanol. *Journal of Food Science*, *68*(1), 240–248.

Chambers, P. J., & Pretorius, I. S. (2010). Fermenting knowledge: the history of winemaking, science and yeast research. *EMBO Reports*, *11*(12), 914–20.

Dimou, C., Vlysidis, A., Kopsahelis, N., Papanikolaou, S., Koutinas, A. A., & Kookos, I. K. (2016). Techno-economic evaluation of wine lees refining for the production of value-added products. *Biochemical Engineering Journal*, *116*, 157–165.

Drosou, C., Kyriakopoulou, K., Bimpilas, A., Tsimogiannis, D., & Krokida, M. (2015). A comparative study on different extraction techniques to recover red grape pomace polyphenols from vinification byproducts. *Industrial Crops and Products*, *75*, 141–149.

Fernández, K., Vega, M., & Aspé, E. (2015). An enzymatic extraction of proanthocyanidins from País grape seeds and skins. *Food Chemistry*, *168*, 7–13.

Ferri, M., Bin, S., Vallini, V., Fava, F., Michelini, E., Roda, A., Tassoni, A. (2016).

Recovery of polyphenols from red grape pomace and assessment of their antioxidant and anti-cholesterol activities. *New Biotechnology*, *33*(3), 338–344.

Ferri, M., Rondini, G., Calabretta, M. M., Michelini, E., Vallini, V., Fava, F., Tassoni, A. (2017). White grape pomace extracts, obtained by a sequential enzymatic plus ethanol-based extraction, exert antioxidant, anti-tyrosinase and anti-inflammatory activities. *New Biotechnology*, *39*(Pt A), 51–58.

Garcia-Alonso, M., Minihane, A.-M., Rimbach, G., Rivas-Gonzalo, J. C., & de Pascual-Teresa, S. (2009). Red wine anthocyanins are rapidly absorbed in humans and affect monocyte chemoattractant protein 1 levels and antioxidant capacity of plasma. *The Journal of Nutritional Biochemistry*, *20*(7), 521–529.

Garrett, A. R., Weagel, E. G., Martinez, A. D., Heaton, M., Robison, R. A., & O'Neill, K. L. (2014). A novel method for predicting antioxidant activity based on amino acid structure. *Food Chemistry*, *158*, 490–6.

Huang, D., Ou, B., Hampsch-Woodill, M., Flanagan, J. A., & Prior, R. L. (2002). High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. *Journal of Agricultural and Food Chemistry*, *50*(16), 4437–44.

Ibern-Gómez, M., Andrés-Lacueva, C., Lamuela-Raventós, R. M., Buxaderas, S., Singleton, V. L., & Torre-Boronat, M. C. de la. (2000). Browning of Cava (Sparkling Wine) During Aging in Contact with Lees Due to the Phenolic Composition. *American Journal of Enology and Viticulture*, *51*.

Jolly, N., Augustyn, O. P. H., & Pretorius, I. S. (2033). The Effect of Non-Saccharomyces Yeasts on Fermentation and Wine Quality. *South African Journal for Enology and Viticulture*, *24*(2).

Kollár, R., Reinhold, B. B., Petráková, E., Yeh, H. J., Ashwell, G., Drgonová, J., Cabib, E. (1997). Architecture of the yeast cell wall. Beta(1→6)-glucan interconnects mannoprotein, beta(1-->)3-glucan, and chitin. *The Journal of Biological Chemistry*, *272*(28), 17762–75.

Lee, J., Durst, R. W., Wrolstad, R. E., Barnes, K. W., Eisele, T., Giusti, M. M., Wightman, J. D. (2005). Determination of Total Monomeric Anthocyanin Pigment Content of Fruit Juices, Beverages, Natural Colorants, and Wines by the pH Differential

Method: Collaborative Study. *Journal of AOAC International*, *88*(5), 1269–78.

Martins, I. M., Roberto, B. S., Blumberg, J. B., Chen, C.-Y. O., & Macedo, G. A. (2016). Enzymatic biotransformation of polyphenolics increases antioxidant activity of red and white grape pomace. *Food Research International*, *89*, 533–539.

Mateos-Aparicio, I., Molina, M., & Redondo-Cuenca, A. (2015). Scavenging activity of dpph radical of the enzymatically obtained oligosaccharides from himanthalia elongata. *Current Nutrition & Food Science*, *11*, 60–64.

Meyer, A. S., Jepsen, S. M., & Sørensen, N. S. (1998). Enzymatic Release of Antioxidants for Human Low-Density Lipoprotein from Grape Pomace. *Journal of Agricultural and Food Chemistry*, *46*(7), 2439–2446.

Moreno-Montoro, M., Olalla-Herrera, M., Gimenez-Martinez, R., Navarro-Alarcon, M., & Rufián-Henares, J. A. (2015). Phenolic compounds and antioxidant activity of Spanish commercial grape juices. *Journal of Food Composition and Analysis*, *38*, 19–26.

Novozymes | Enzymes & Microorganisms. Retrieved May 22, 2017, from http://www.novozymes.com/en

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

Perestrelo, R., Silva, C., Pereira, J., & Câmara, J. S. (2016). Wines: Madeira, Port and Sherry Fortified Wines – The Sui Generis and Notable Peculiarities. Major Differences and Chemical Patterns. In *Encyclopedia of Food and Health* (pp. 534–555).

Pérez-Bibbins, B., Torrado-Agrasar, A., Salgado, J. M., Oliveira, R. P. de S., & Domínguez, J. M. (2015). Potential of lees from wine, beer and cider manufacturing as a source of economic nutrients: An overview. *Waste Management*, *40*, 72–81.

Pérez-Serradilla, J. A., & de Castro, M. D. L. (2008). Role of lees in wine production: A review. *Food Chemistry*, *111*(2), 447–456.

Romano, P., Fiore, C., Paraggio, M., Caruso, M., & Capece, A. (2003). Function of yeast species and strains in wine flavour. *International Journal of Food Microbiology*,

86(1), 169–180.

Romero-Díez, R., Matos, M., Rodrigues, L., Bronze, M. R., Rodríguez-Rojo, S., Cocero, M. J., & Matias, A. A. (2018). Microwave and ultrasound pre-treatments to enhance anthocynins extraction from different wine lees. *Food Chemistry, 272*, 258-266.

Varelas, V., Tataridis, P., Liouni, M., & Nerantzis, E. T. (2016). Application of different methods for the extraction of yeast β-glucan. *E-Journal of Science & Technology (e-JST)*, *11*(1), 75–89.

Vasserot, Y., Caillet, S., & Maujean, A. (1997). Study of Anthocyanin Adsorption by Yeast Lees. Effect of Some Physicochemical Parameters. *American Journal of Enology and Viticulture*, *48*, 433–437.

Versari, A., Castellari, M., Spinabelli, U., & Galassi, S. (2001). Recovery of tartaric acid from industrial enological wastes. *Journal of Chemical Technology and Biotechnology*, *76*(5), 485–488.

Xu, C., Yagiz, Y., Borejsza-Wysocki, W., Lu, J., Gu, L., Ramírez-Rodrigues, M. M., & Marshall, M. R. (2014). Enzyme release of phenolics from muscadine grape (Vitis rotundifolia Michx.) skins and seeds. *Food Chemistry*, *157*, 20–29.

Yalcin, D., Ozcalik, O., Altiok, E., & Bayraktar, O. (2008). Characterization and recovery of tartaric acid from wastes of wine and grape juice industries. *Journal of Thermal Analysis and Calorimetry*, *94*(3), 767–771.

CHAPTER 4

Grape stems valorization: microwave pre-treatment for polyphenol extraction

Abstract

Only in Spain, around 0.24-0.36 million tons of grape stems (GS) are produced annually. Although, GS have been usually employed for compost or burned for disposal, they can constitute an alternative source of polyphenols, mainly flavonoids and stilbenes. Thus, the extraction kinetics of total polyphenols (TPC) and flavonoids (TFC) content have been studied via solid-liquid extraction varying the solid-liquid ratio (R_{S-L}), solvent composition (% of ethanol in the hydroalcoholic mixture) and the temperature. A R_{S-L} of 0.10 g/mL, a solvent with a 50% of ethanol (v/v) and 75ºC were selected as the best conditions for the recovery of bioactives from GS given a TPC of 38 ± 1 mg_{GAE}/g_{DS} and a TFC of 38 ± 1 mg_{CATE}/g_{DS}. In order to enhance these recoveries, MW irradiation was proposed as a prior step to the conventional solid-liquid extraction. With the help of a statistical surface response study the R_{S-L}, the solvent composition and the irradiation time that maximize the TPC on the one hand, and the TFC on the other, were obtained. MW pre-treatment increased the extraction yield of TPC and TFC in a 19% and 24%, respectively. The antioxidant activity of the extracts was measured by ORAC assay and the anti-fungal activity against *Botrytis cinarea* was determined. Additionally, identification of the major stilbenes and flavonoids was carried out with HPLC-DAD-MS/MS.

1. Introduction

Grape stems (GS) are the woody skeleton from the grape bunches. Only in Spain, they represent around a 12% of the 2-3 million tons of wastes generated per year in a cellar (Ruggieri et al., 2009). Usually, GS are composted or burned for disposal, constituting an environmental problem (Spatafora, Barbagallo, Amico, & Tringali, 2013). However, recent studies have revealed the potential of these residues as a natural source of polyphenols. Polyphenols are natural organic compounds characterized by the presence of more than one phenol molecule with one or more hydroxyl radicals in their structure. They are worldwide known for their beneficial properties to human health (He & Giusti, 2010; Pandey & Rizvi, 2009) such as anti-inflammatory, antibacterial, antioxidant activity and anti-fungal activity (Ismail, Marjan, & Foong, 2004). The latter attracts a lot of attention in the research field since fungal and bacterial infections in agriculture produce great losses (Spadaro & Gullino, 2004) and many crops have to be

discarded if they are affected (Salgado, Rodríguez-Rojo, & Cocero, 2017). Thus, it is important to develop specific products to fight against these external pathogens.

In this sense, the interest in the production of natural products with anti-fungal and antibacterial activities has increased. One alternative may be grape stems extracts which are rich in stilbenes and flavonoids, two families of polyphenols which present great anti-fungal properties (Zachová et al., 2018). For the case of stilbenes, they are non-flavonoid compounds with a characteristic C_6–C_2–C_6 structure (Zachová et al., 2018). Whereas flavonoids are frequently found in their glycosylated or esterified forms consisting of C_6-C_3-C_6 rings, namely rings A and B linked by three-carbon-ring C (Wang, Li, & Bi, 2018). For example, the stilbene resveratrol is naturally synthesized in response to several biotic and abiotic stress such as, the fungus infection derived from *Botrytis cinarea* (Adrian, Jeandet, Veneau, Weston, & Bessis, 1997) or *Palmopara viticola*. On the other hand, flavonoids such as catechins present good antioxidant and antimicrobial activities against a wide range of pathogenic bacteria (Scalbert, 1991; Vázquez-Armenta et al., 2017). Furthermore, the inhibitory effect of flavonoids against fungus growth have been reported (Cotoras, García, Lagos, Folch, & Mendoza, 2001; Weidenbörner, Hindorf, Jha, & Tsotsonos, 1990)

Within this context, grape stems represent a good source of bioactive compounds with potential interest to be used as an additives in agricultural activities (Scalbert, 1991). Therefore, a valorisation of GS in terms of polyphenols seems to be interesting from the searching of natural alternatives as anti-fungal products. Several technologies have been used for this purpose. Among them, the conventional solid-liquid extraction have been broadly used with different solvents (Dias et al., 2015; Piñeiro, Guerrero, Fernández-Marin, Cantos-Villar, & Palma, 2013; Piñeiro, Marrufo-Curtido, Vela, & Palma, 2017). However, greener technologies have recently emerged, such as microwave assisted extraction (MAE) and ultrasound assisted extraction (US-AE). Among their prominent advantages respect conventional extractions, it is remarkable to mention the decrease of the organic solvent consumption and the process intensification they could offer, increasing the yield of the process and decreasing the operating time. *Piñeiro et al.* (Piñeiro et al., 2013) proved that at optimized conditions US-AE increased 4 fold the recovery of resveratrol in comparison with a conventional solid-liquid extraction carried out with an ethanol-water mixture. Moreover, grape stems has been also submitted to a MAE process (Piñeiro et al., 2017) varying parameters such as temperature, time and

solid-liquid ratio. Considering all the results, they concluded that MAE under optimum conditions revealed a great extraction of polyphenols from GS.

Although MAE is a powerful tool for extraction of polyphenols from natural matrixes, it presents a main drawback that may be overcome: the sever scale-up limitations. The penetration depth in the MAE of 2.45GHz has been reported to be very low (Álvarez et al., 2017; Thostenson & Chou, 1999), which generates an uniformly irradiation in a large vessel since only the external parts will be irradiated without affecting the matter. Using higher frequencies, up to 9.15 MHz, this problem can be easily overcome confronted although this will suppose a reduction of the thermal effect. In this way, MWs can be used as a pre-treatment to the conventional solid-liquid extraction, where the material is homogenously irradiated for a low residence time. The material will be capable of absorbing an amount of energy equivalent to MAE milder radiation conditions due to the peak of energy during the beginning of the pre-treatment. It has been demonstrated the absorption of energy by the material was more relevant than the power irradiated (Y. Li et al., 2012). Another benefit in using MWs in short periods of time at high temperature is the avoidance of polyphenol degradation (Sólyom, Solá, Cocero, & Mato, 2014). This technique has been already employed successfully by other authors (Álvarez et al., 2017; Romero-Díez et al., 2018) for the recovery of polyphenols from grape marc and wine lees, respectively.

In view of the above, this work is aimed at studying the valorisation of GS in terms of polyphenols recovery, focusing on two polyphenols families: stilbenes and flavonoids. To carry out this work, firstly the extraction kinetics of total polyphenols and total flavonoids were investigated. Variables such as the solid-liquid ratio, temperature, solvent composition and particle size were studied. After selecting the parameters which maximize the extraction of polyphenols and flavonoids. Secondly, MW pre-treatments were optimized from an operational point of view with a statistical surface response study. Tested parameters were the solid-liquid ratio, solvent composition and time of pre-treatment. Characterization of the optimized extracts were performed with the analysis of total polyphenol content, total flavonoid content, HPLC-DAD and HPLC-MS/MS. Antioxidant activity and antifungal activity were measured via ORAC and against the growth of *Botritys cinerea*, respectively.

2. Materials and methods

2.1 Grape Stems

Grape stems were kindly provided by the winery *Grupo Matarromera* (41° 38′ 33″ N, 4° 17′ 28″ W) in October 2017. These vines belong to the grape variety *Vitis vinifera, Tempranillo* and were cultivated in a clay soil in *Valbuena de Duero, Ribera de Duero* Designation of Origin (*Castilla y León*). The average ambient temperature during that year in the vineyard was 13.9ºC and the average precipitations were 7.5 mm. Grape bunches were harvested in September 2017 and stems were mechanically removed before grape pressing. They were washed with water in order to remove any residual wine traces. Traces of grapes linked to grape stems were manually removed. Stems were washed with tap water twice in order to remove the rests of grape juice. Stems were sun dried for 48 hours and afterwards, stems were placed in an oven at the temperature of 65ºC for other 24 hours. Later, they were grounded using a chopper (A320R1, Moulinex) and sieved. A fraction with an average length between 4-6 mm was collected and stored at room temperature.

2.2 Chemicals

Solvents used for extractions were absolute ethanol denaturalized (99.9%, Sigma-Aldrich), bidistilled water (Milli-Q® Integral) and hydrochloric acid (≥37%, Sigma-Aldrich). Chemicals used on the determination of total phenolic content were sodium carbonate (Sigma-Aldrich, St. Quentin Fallavier, France), Folin Ciocalteau reagent (Panreac, Barcelona, Spain) and gallic acid (Fluka, Germany). To determine total anthocyanin content potassium chloride (Riedel-de Haën, France), sodium acetate trihydrate (≥99.0%, Sigma-Aldrich, France) and hydrochloric acid (≥37%, Sigma-Aldrich) were used to prepare the buffer solutions in bidistilled water.

The determination of the antioxidant activities was carried out with: 2‘,2‘-azobis(2-amidinopropane) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and disodium fluorescein (FS) from Sigma-Aldrich (St. Quentin Fallavier, France). Sodium chloride (Sigma-Aldrich, St. Quentin Fallavier, France), potassium chloride (Riedel-de Haën), sodium phosphate dibasic dihydrate (Sigma-Aldrich, St. Quentin Fallavier, France) and potassium phosphate monobasic anhydrous (Amresco, Solon, OH, USA) were used for phosphate buffer solution (PBS) preparation in bidistilled water (Milli-Q® Integral). HPLC analyses were performed using acetonitrile (99.9%, Sigma-Aldrich), ultrapure water purified with

a Milli-Q water purification system (Merck Millipore, Billerica, MA, USA), formic acid (99-100%, VWR-CHEM, Spain) and malvidin-3-O-glucoside chloride (Extrasynthese, France) as standard.

For the anti-fungal activity, malt extract agar was purchased from Panreac (Spain). *Botrytis cinarea* was cultivated and isolated in Campus La Yutera, (Universidad de Valladolid, Palencia, Spain).

2.3 Methods

2.3.1 Extraction Kinetics

2.3.1.1 Conventional solid-liquid extraction

Extraction kinetic curves for both polyphenols and flavonoids were studied via solid-liquid extractions. Main parameters that govern these type of extractions were analyzed so as to promote the extraction of bioactive compounds. These parameters were the R_{S-L} (0.1, 0.07 and 0.04 g/mL), composition of the solvent (varying the percentage of ethanol in the hydroalcoholic mixture, 20, 50 and 80% vol.) and temperature (25, 50 and 75ºC). A grape stem mass of 7.5 g was measured and mixed with the specific amount of solvent so as to obtain the specific R_{S-L} value. The extractions were carried out at 500 rpm during 60 minutes. Solvent pH was adjusted with HCl to a final value of 3 since polyphenol stability is higher in acidic media (Brianceau, Turk, Vitrac, & Vorobiev, 2016). Extraction kinetics curves for polyphenols and flavonoids were built by measuring the total polyphenol and flavonoid content, respectively, for the collected samples every 5 minutes. At the end of this study, the conditions that promote the extraction of bioactive compounds from grape stems were obtained.

2.3.1.2 Microwave pre-treatments

For the MW pre-treatments, a CEM Discovery One Microwave (CEM Corp.) was employed with a constant power of 300W. Safe closed glass pressure reactors of 100 mL QianCap (QLabtech) were used for the pre-treatments to maintain the solvent in liquid state in all the pre-treatment. Like in the conventional solid-liquid extractions a mass of 7.5 g of grape stems was poured inside with the solvent and the mixture was manually homogenised before MW irradiation. Some parameters such as the R_{S-L} (0.10, 0.30 and 0.50 g/mL), solvent mixture (hydroalcoholic mixtures varying the percentage of ethanol in 20, 50 and 80% vol.) and time of microwaves irradiation (30, 60 and 90s), were varied so as to study their influence on the extraction of both, polyphenols and flavonoids. R_{S-L}

up to 0.50 g/mL were employed so that MW energy would be better transfer directly to the grape stems and less energy is lost in heating up the whole liquid. Additionally, these values of R_{S-L} have already been employed by other authors for the recovery of polyphenols from grape marc (Álvarez et al., 2017).

In order to analyse the influence of these parameters in the purposed range and to obtain the optimum conditions which maximize the final polyphenol and flavonoids contents of the extracts, a statistical surface response design (Box-Behnken) was performed using Statgraphics® Centurion XVII. A total of 15 experiments were get with a triplicate of the central point. Three levels of response (-1, 0, 1) were employed that correspond to the minimum (-1), medium (0) and maximum (1) proposed values of each variable. Temperature of each experiment was calculated by the Peng-Robinson equation of state taking into account the final pressure achieved. The conventional mixture rules for vapour-liquid equilibrium, assuming ideal liquids were used (Michelsen & Mollerup, 2007).

A second degree model (Equation 1) was employed to fit the response achieved from the statistical analysis:

$$Y = \beta_0 + \sum_{j=1}^{k} \beta_j X_j + \sum_{j=1}^{k} \beta_{jj} X_j^2 + \sum_{i=1}^{k} \sum_{j=1}^{k} \beta_{ij} X_i X_j \quad (1)$$

where Y corresponds to the response variable (TPC or TFC content), β_0, β_j, β_{jj} and β_{ij} are the regression coefficients for intercept, linearity, square and interaction, respectively; X stands for each operating variable.

The statistical evaluation was performed by analysis of variance (ANOVA) in order to identify which factors significantly contribute the most to the response. Effects with a P-value< 0.10 were chosen as a statistically significant with a level of confidence of 90%.

Once the Equation 1 was fitted with the corresponding parameters, an optimization was performed and the optimal conditions that maximize either the polyphenol content or the flavonoid content were determined. The optimum MW pre-treatments for TPC and TFC were applied to grape stems and followed by the solid-liquid extraction at the conditions selected as best (R_{S-L} of 0.10 g/mL, 50% vol. ethanol and 75ºC). This procedure was carried out in triplicate in order to test the reproducibility of the process.

2.3.2 Extract Characterization

2.3.2.1 Total Phenolic Content (TPC)

The total polyphenol content was measured by the Folin-Ciocalteou colorimetric method (Waterhouse, Waterhouse, & L., 2003). As a resume, 40µL of the diluted sample were mixed with 3 mL of distillate water and 200 µL of Folin-Ciocalteou reagent. The mixture was shaken for 5 minutes with the help of a vortex. After, 600 µL of 20% sodium carbonate were added and incubated at 40 °C during 30 min. The results of TPCs were calculated using a calibration curve for gallic acid (between the range of 50-800 $ppm_{GALLIC\ ACID}$) (Equation 2):

$$y = 0.0009x - 0.0133; R^2 = 0.997 \qquad (Eq.2)$$

where 'y' is absorbance at 765 nm and 'x' concentration of gallic acid in mg/L. TPCs were expressed in mg of gallic acid equivalents (GAE) per gram of dry stem (mg_{GAE}/g_{DS}) ± standard deviation (SD).

2.3.2.2 Total Flavonoid Content (TFC)

The flavonoid content (TFC) of the different extracts was measured as described by *Michalska et al.* (Michalska, Ceglińska, & Zieliński, 2007). Briefly, 1 mL of the extract were diluted in 10 mL of distilled water. Then, 300 µL of $NaNO_2$ (5% w/w) were added and the mixture was left to react during 5 minutes. Afterwards, 500 µL of $AlCl_3$ (2% w/w) and 500 µL of NaOH (1M) were poured in the mixture and 6 minutes of incubation at room temperature were needed. Absorbance was read at 510 nm (Shimadzu UV/vis Spectrophotometer). The results of TFCs were calculated using a calibration curve for catechin (between the range of 0-1000 ppm $_{CATECHIN}$ (Equation 3) :

$$y = 2.0421x - 0.0229; R^2 = 0.999 \qquad (Eq.3)$$

where *'y'* is absorbance at 510 nm and *'x'* concentration of catechin in mg/L. TFCs were expressed in mg of catechin equivalents (CATE) per gram of dry stem (mg_{CATE}/g_{DS}) ± SD.

2.3.2.3 Antioxidant Activity: ORAC

Oxygen Radical Absorbance Capacity (ORAC) was the method employed for the evaluation of antioxidant ability of the extracts. The fundamentals of this assays relays

on the fluorescence quenching of disodium fluorescein (FS) salt after exposure to AAPH (2,2-azobis(2-amidino-propane) dihydrochloride), which generates oxygen radicals ($ROO^{\bullet}$) at a constant rate (*Garrett et al., 2014*). ORAC assay was carried out by the method developed by *Huang et al*. (Huang, Ou, Hampsch-Woodill, Flanagan, & Prior, 2002). ORAC Results in µmol Trolox equivalents (TE) per gram of dry stem ($\mu mol_{TE}/g_{DS}$) ± SD.

2.3.2.4 Anti-fungal activity: *Botrytis cinerea* culture

Botryti cinarea, the fungus, was isolated from vines in our university (Campus La Yutera, Universidad de Valladolid, Palencia, Spain). The followed protocol was the same as described by *Salgado et al.* (Salgado et al., 2017) with a minor modification. Ethanol was eliminated from the extract by rotary evaporation (Heidolph) at 40ºC under vacuum (0.08 MPa) to avoid the inhibition of fungal growth. In the end, an extract with 4% vol. ethanol was obtained. Therefore, the blank was prepared with distilled water and the same amount of ethanol. Briefly, the agar medium was autoclaved. When cooled until around 65-75ºC, 20 mL of agar were mixed with 5 mL of the corresponding sample or blank. Later, the mixture was poured into Petri dishes and left to solidify overnight. The following day, the fungus was placed in the centre of the surface and let for incubation at 22ºC during one week. The growth area was measured with a ruler by measuring the diameter in two perpendicular directions. Averages and standard deviations were calculated for each sample.

2.3.2.5 HPLC- PDA-MS/MS (High Performance Liquid chromatography–mass spectrometry)

Samples were analyzed by HPLC-DAD-MS, using a Waters Alliance 2695 Separation Module (Waters, Ireland) system equipped with a quaternary pump, a degasser, an autosampler and a column oven. The liquid chromatography system was coupled to a photodiode array detector 996 PDA (Waters, Ireland), and to a mass spectrometer MicroMass Quattromicro® API (Waters, Ireland). All data were acquired and processed by MassLynx® 4.1 software. Chromatographic separation of compounds was carried out in a reversed-phase LiChrospher® 100 RP-18 5µm LiChroCART® 250-4 column inside a thermostated oven at 35ºC. The mobile phase consisted of formic acid (0.5% V/V in ultrapure water) (eluent A) and 0.5% v/v formic acid in acetonitrile (eluent B). The gradient program used was 99:1 A:B for 5 min, from 99:1 A:B to 40:60 A:B in

40 min, from 40:60 A:B to 10:90 A:B in 45 min, held isocratically (90% B) for 10 min, from 10:90 A:B to 99:1 A:B in 10 min, and finally held isocratically (99:1 A:B) for 10 min, at a flowrate of 0.3 mL/min, with an injection volume of 20 μL. Total run time was 120 min. Absorption spectra were acquired from 210 to 700 nm by a photodiode array detector.

Tandem mass spectrometry (MS/MS) detection was performed on a Micromass® Quattro Micro triple quadrupole (Waters®, Ireland) using an electrospray ionization (ESI) source operating at 120ºC and applying a capillary voltage of 2.5 kV and a source voltage of 30 V. The compounds were ionized in positive or negative ion mode, and the spectra of the column eluate were recorded in the range m/z 60 – 1500. High purity nitrogen (N_2) was used both as drying gas and as a nebulizing gas. Ultra-high purity argon (Ar) was used as collision gas. Collision energies were optimized for each compound. The analysis was performed in multiple reaction monitoring (MRM) mode in order to achieve a higher selectivity and sensitivity. Whenever possible, two transitions were used in order to identify and quantify the compounds in samples, with a maximum deviation of 15% between MRM1/MRM2 ratios.

2.3.3 Statistical Analysis

All data were expressed as means ± standard deviations (SD). Assays for TPC, TFC, ORAC and antifungal activities were performed, at least, in triplicate. The data were analysed by t-Student's test (unpaired samples, unequal variances) with a significance p-value of 0.05 ($p<0.05$ was accepted as statistically significant in all cases).

3. Results and Discussion

3.1 Best extraction conditions for TPC and TFC

3.1.1 Conventional S-L extractions. Extraction kinetics of TPC and TFC

Temperature and solvent composition were kept constant at 25ºC and 20% of ethanol, respectively, to study the influence of the R_{S-L} variation among 0.10, 0.07 and 0.04 g/mL. Results revealed that no significant differences (see Table S.1) were found among the three R_{S-L} values neither for TPC nor TFC as shown in Figure 1 (A1 and A2). Since 0.10 g/mL is the R_{S-L} that requires lower amount of solvent, it was chosen as the best value to extract both polyphenols and flavonoids, which is also favourable from an economic and environmental point of view (Drosou, Kyriakopoulou, Bimpilas, Tsimogiannis, & Krokida, 2015).

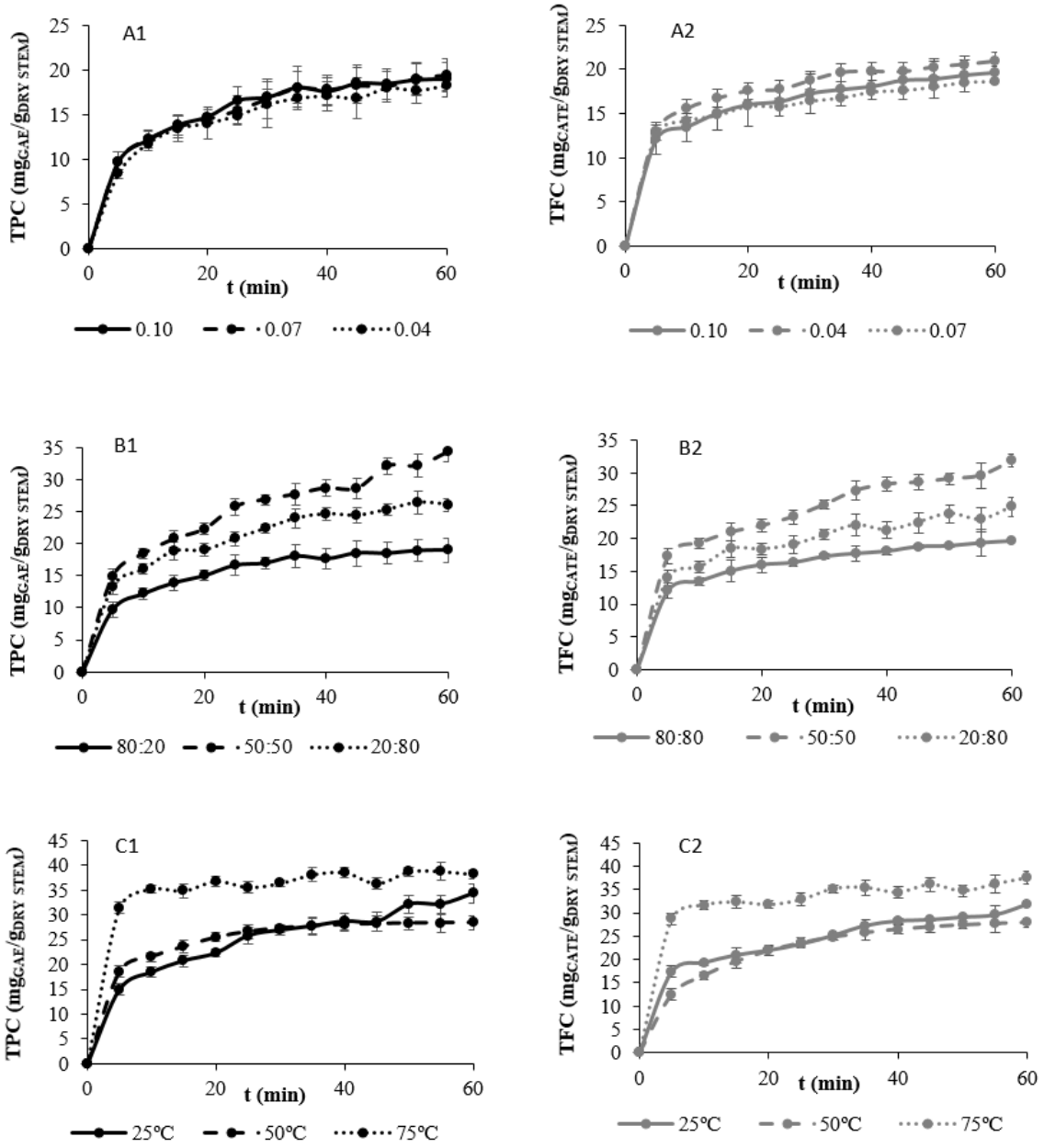


Figure 1: TPC and TFC values in the study of extraction kinetic curves. Figures A1 and A2 shows the variation in TPC and TFC, respectively, for the tested R_{S-L} values at 25ºC and with a hydroalcoholic mixture with a 20% (v/v) of ethanol. Figures B1 and B2 shows the variation in TPC and TFC, respectively, for the tested solvent composition at 25ºC and with a R_{S-L} of 0.10. Figures C1 and C2 shows the variation in TPC and TFC, respectively, for the tested temperatures with a R_{S-L} of 0.10 and a hydroalcoholic mixture with a 50% (v/v) of ethanol.

The final values of TPC and TFC working at a R_{S-L} of 0.10 g/mL at 25ºC and 20% of ethanol were 19.0 ± 1.9 mg_{GAE}/g_{DS} and 19.6 ± 0.7 mg_{CATE}/g_{DS}, respectively. To check out the influence of the solvent composition in the final concentrations of

polyphenols and flavonoids, extractions were performed at three different volume percentages of ethanol (20, 50 and 80% vol. ethanol) in the hydroalcoholic mixture solvent maintaining a $R_{S\text{-}L}$ of 0.10 g/mL and a temperature of 25ºC. From Figure 1 (B1 and B2) it is possible to appreciate the differences achieved as the percentage of ethanol is varied. If the concentration of ethanol is increased from 20 to 50% vol. ethanol, there is a huge increment on both TPC and TFC values from 19.0 ± 1.9 mg_{GAE}/g_{DS} and 19.6 ± 0.7 mg_{CATE}/g_{DS} to 34.3 ± 1.9 mg_{GAE}/g_{DS} and 31.8 ± 0.1 mg_{CATE}/g_{DS} respectively. Nevertheless, if the percentage of ethanol is increased until 80%, TPC and TFC values decrease (26.1 ± 1.9 mg_{GAE}/g_{DS} and 24.8 ± 1.2 mg_{CATE}/g_{DS}, respectively). Thus, the solvent composition selected as the best for extracting bioactive compounds from grape stems was a mixture with a 50% vol. ethanol. As it is broadly known, hydroalcoholic mixtures represent the best solvent for the recovery of bioactive compounds from natural matrixes (Brianceau et al., 2016; Spigno & De Faveri, 2007). It has been proved that a combination of ethanol water facilitates the recovery of polyphenols with an intracellular localisation by altering the cell membranes in the phospholipid bilayer (Goldstein & Chin, 1981). Additionally, ethanol has been proved to decrease the hydrophobic interactions and hydrogen bounding between procyanindins and the cell-wall structure (Brianceau et al., 2016). Nonetheless, large quantities or even pure organic solvent could lead to a dehydration of the vegetable cell which complicates the entrance of alcohol into the cell and, consequently, the bioactive compounds leakage to the bulk extract (Sant'Anna, Brandelli, Marczak, & Tessaro, 2012).

Once the best $R_{S\text{-}L}$ and solvent composition parameters were selected, the last variable tested was the temperature. Extractions were performed at 25, 50 and 75ºC for a $R_{S\text{-}L}$ of 0.10 g/mL and with a solvent with a 50% vol. of ethanol. Although it was supposed that temperature would increase the solubility and consequently the extraction of both polyphenols and flavonoids, it was not until 75ºC when a significant difference (Table S.1) was observed as displayed in Figure 1 (C1 and C2). Carrying out the extractions at 75 ºC, a TPC of 38.2 ± 1.0 mg_{GAE}/g_{DS} and a TFC of 37.6 ± 1.5 mg_{CATE}/g_{DS} were achieved, which correspond to a significant increments of a 12% and 18% compared with extractions performed at 25ºC, respectively. Furthermore, from the plotted kinetic extraction curves it is possible to corroborate that the temperature raised the extraction rate, achieving a stable concentration faster than when 25ºC and 50ºC were used. For this reason, 75ºC was chosen as the temperature that promoted the extraction of polyphenols

present in grape stems. Furthermore, at this temperature the thermal degradation of both stilbenes (Lee et al., 2014; Piñeiro, Palma, & Barroso, 2006) and flavonoids (N. Li, Taylor, Ferruzzi, & Mauer, 2012; Lončarić, Pablo Lamas, Guerra, Kopjar, & Lores, 2018) does not take place. As expected, temperature favours the extraction of both polyphenols and flavonoids from GS by increasing solubility and diffusion coefficient (Spigno & De Faveri, 2007).

Therefore, the conditions that favour the extraction of both polyphenols and flavonoids from grape stems were a R_{S-L} of 0.10 g/mL and with a solvent with a 50% vol. of ethanol at a temperature of 75ºC.

The comparison of these data is challenging since in the works reporting the bioactives extraction from GS the attention is usually paid to the identification and quantification of stilbenes and main flavonoids and no to a global quantification. *Makris et al.* (Makris, Boskou, & Andrikopoulos, 2007), performed a solid-liquid extraction of dried GS from the Roditis and Agiorgitio cultivars (Koropi and Nemea regions, Greece) and obtained a TPC and TFC values of 58 mg_{GAE}/g_{DS} and TFC of 54 mg_{CATE}/g_{DS}, respectively. Those content values were higher than ours, but smaller was their R_{S-L} (0.03 g/mL) after performing three successive extractions. As solvent media extraction, they employed a mixture of 0.1% HCl MeOH/acetone/H_2O (60/30/10, v/v). On the contrary, *Spigno et al.* (Spigno & De Faveri, 2007) reach much lower polyphenol extraction yields via solid-liquid extraction from GS (Barbera red grape of Piacenza, northern Italy, 2003) during 24 hours at 60ºC in a shaking incubator with a R_{S-L} of 0.25 g/mL. No significant differences were found when the extraction was performed with ethylacetate/water (1.6 mg_{GAE}/g_{DS}) (9/1, v/v) or ethanol (1.2 mg_{GAE}/g_{DS}).
Nonetheless, discrepancies could be found due to the fact that several factors affect the composition of the GS (grape variety, climatological conditions, extraction and analytical methods) (Ruiz-Moreno et al., 2015).

3.1.2 Microwave pre-treatment

The results of TPC and TFC obtained just after the MW-pretreatment according to the statistical set up of experiments are gathered in Table 1.

Table 1: CCD design set of experiments for application of MW pre-treatment in grape stems. TPC and TFC are the total polyphenol and flavonoid contents just after the pre-treatment, 'T'

represents the achieved temperature in the MW pre-treatment. Rows in bold represent the triplicate of the central point and 'Average CP' is an average of the central points. TPC and TFC values with an asterisk are significantly different ($p<0.05$) from the central point.

Exp.	R_{S-L} (g/mL)	Solvent %EtOH	Time (s)	T (ºC)	TPC ($mg_{GAE}/g_{DRY\ STEM}$)	TFC ($mg_{CAT}/g_{DRY\ STEM}$)
1	0.50	50	30	93	51	35
2	0.30	20	30	61	10*	9*
3	0.10	20	60	83	42	36
4	0.50	20	60	93	30*	30
5	0.10	80	60	90	28*	20*
6	0.50	80	60	109	30*	30
7	0.50	50	90	117	28*	28*
8	**0.30**	**50**	**60**	**109**	**46**	**32**
9	0.30	20	90	106	9*	8*
10	0.10	50	90	103	38	52*
11	**0.30**	**50**	**60**	**108**	**47**	**36**
12	0.30	80	90	118	27*	21*
13	0.30	80	30	88	18*	14*
14	0.10	50	30	103	25*	25*
15	**0.30**	**50**	**60**	**109**	**43**	**31**
Average CP	0.30	50	60	109	45 ± 2	33 ± 3

At first glance, it can be observed that a solvent with a 50% vol. ethanol have higher TPC and TFC values (up to ~50 and ~35 mg_{GAE}/g_{DS} and mg_{CATE}/g_{DS}). The trend of the effect of the three variables in extraction of TPC and TFC is quite similar as it can be shown in the main effects diagrams (vide Figure 2.A and Figure 2.B) with major differences in the effect of the R_{S-L}. Concerning the latter parameter, the higher the R_{S-L}, the higher the TPC (Figure 2.A). On the contrary, for the case of TFC a clear minimum was achieved at the medium value (0.30 g/mL). Regarding the percentage of ethanol and the irradiation time, the maximum recoveries were obtained at parameters closed to the medium values (50% vol. ethanol and 60s, respectively). The effect of the ethanol was also observed for the solid-liquid extraction (see *Section 3.1.1*).

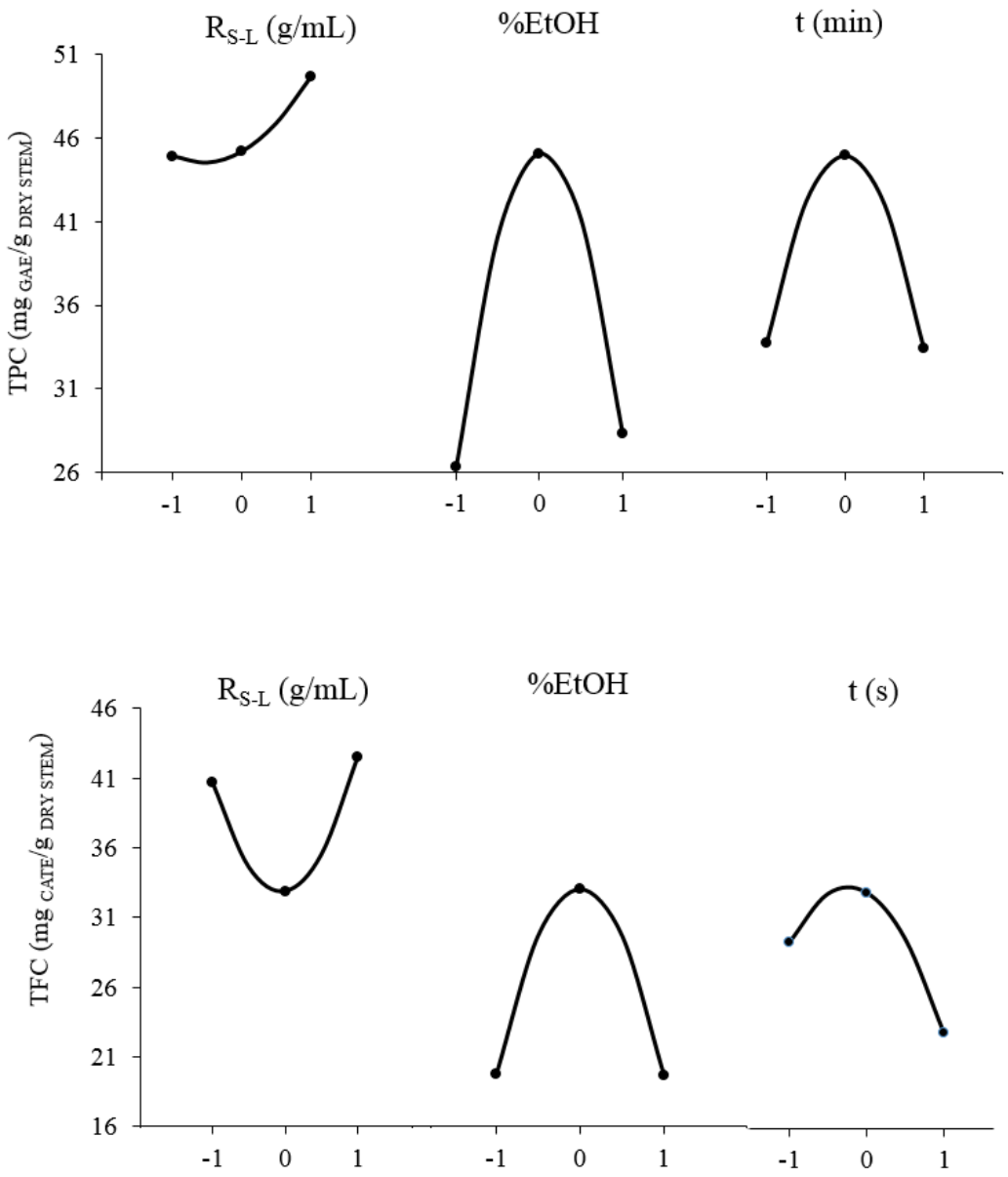


Figure 2: main effect diagrams for each variable (R_{S-L} (g/mL), %EtOH and time (s)) on the final total polyphenol content (A) (TPC) and total flavonoid content (B) (TFC).

According to the analysis of variance (vide *Supplementary Material* Table S.2 and Table S.3), it could be seen that the quadratic effect of the percentage of ethanol in the mixture was the most significant parameter in the extraction of polyphenols and flavonoids (p-value of 0.0135 and 0.0147, respectively). Additionally, in the case of TPC, the irradiation time was also a significant variable (p-value of 0.0609) whereas for the recovery of flavonoids, the R_{S-L} (p-value of 0.0601) presented a higher role than the irradiation time (p-value of 0.1190). However, the cross effect between the R_{S-L} and the

irradiation time was significant for the extraction of flavonoids (p-value of 0.0601). As it is shown in Figure 3 small R_{S-L}, it is better to have long irradiation times since there is more solvent in the media to be heated before the irradiation reaches the grape stems. On the contrary, for high R_{S-L} is preferable to have short irradiation times due to there is less solvent to be heated until the irradiation reaches the solid raw material. Longer irradiation time may cause an excessive increment of the temperature and the toasting of the grape stems. This cross effect between the R_{S-L} and the irradiation time also applies for the extraction of polyphenols, but in this case the significance level was slightly lower (p-value of 0.1066).

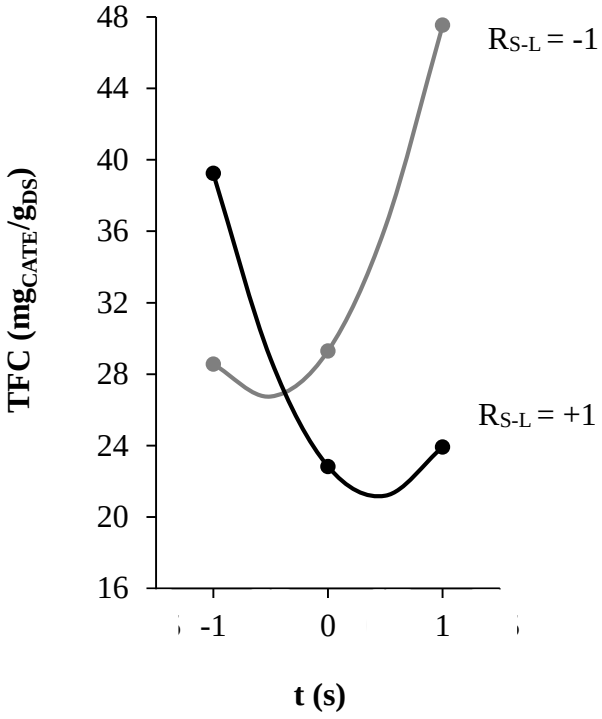


Figure 3: interaction diagram between the R_{S-L} (g/mL) and the time (s) for the final total flavonoid content (TFC).

The regression coefficients of second-order polynomial equation were obtained by fitting experimental results and extraction variables. The final expression for the maximization of the extraction of polyphenols and flavonoids are shown in Equation 5 and Equation 6, respectively.

$$\boldsymbol{TPC} = 45.2333 + 2.375 \cdot R_{S-L} - 0.0625 \cdot \%EtOH - 0.1625 \cdot t + 2.10822 \cdot R_{S-L}^2 + 6.925 \cdot R_{S-L} \cdot \%EtOH - 9.025 \cdot R_{S-L} \cdot t - 17.8667 \cdot \%EtOH^2 + 2.55 \cdot \%EtOH \cdot t - 11.5167 \cdot t^2 \quad \textit{(Eq. 5)}$$

$$\boldsymbol{TFC} = 32.8667 + 0.9125 \cdot R_{S-L} - 0.025 \cdot \%EtOH - 3.2375 \cdot t + 8.75417 \cdot R_{S-L}^2 + 4.4 \cdot R_{S-L} \cdot \%EtOH - 8.575 \cdot R_{S-L} \cdot t - 13.2208 \cdot \%EtOH^2 + 1.9 \cdot \%EtOH \cdot t - 6.79583 \cdot t^2 \quad \textit{(Eq. 6)}$$

'*TPC*' and *'TFC'* corresponds to the polyphenol and flavonoid extraction yield, respectively, '*t*' is the time of pre-treatment in seconds, 'R_{S-L}' is the solid-liquid ratio in g/mL and '$\%H_2O$' is the %vol. of water of the hydroalcoholic mixture.

With these two equations, parameter values that maximize the extraction of both polyphenols and flavonoids are gathered in Table 2 together with the predicted TPC and TFC values.

Table 2: optimal microwave conditions for TPC and TFC, predicted values by the model and TPC and TFC values for experiments performed at the optimized microwave variables for TPC (runs 16 to 18) and TFC (runs 19 to 21). 'Average TPC' and 'Average TFC' is the average of the triplicates with ± SD, respectively.

		R_{S-L}	Solvent	Time	T	TPC	TFC
	Exp.	**(g/mL)**	**%EtOH**	**(s)**	**(ºC)**	**($mg_{GAE}/g_{DRY\ STEM}$)**	**($mg_{CAT}/g_{DRY\ STEM}$)**
Opt. TPC (predicted TPC: 52 mg_{GAE}/g_{DS})	**16**	0.50	55	36	98	46	41
	17				97	44	38
	18				98	46	39
Average TPC		0.50	55	36	98	45 ± 1	39 ± 1
Opt. TFC (predicted TFC: 48 mg_{CAT}/g_{DS})	**19**	0.10	47	86	103	43	44
	20				104	43	48
	21				104	42	48
Average TFC		0.10	47	86	104	43 ± 3	47 ± 2

MW pre-treatments at the optimum conditions were carried out and later followed by the conventional solid-liquid extraction at the conditions selected as the best for TPC and TFC in *Section 3.1*.1. The optimum TPC and TFC values obtained after the MW pre-treatment and the solid-liquid extraction are shown in Table 2. MW pre-

treatment increased the extraction yield of TPC and TFC in a 19% and 24%, respectively, compared to the conventional solid-liquid extractions at the best conditions as Figure 4 shows (TPC = 38.2 ± 1.0 mg_{GAE}/g_{DS}, TFC = 37.6 ± 1.5 mg_{CATE}/g_{DS}). Nevertheless, the experimental data for the TPC maximization did not coincide with the predicted value whereas the optimum TFC fitted perfectly (vide Table 2).

The enhancement of the polyphenol and flavonoid extraction yields using MW irradiation to GS has been previously tested by *Piñeiro et al.* (Piñeiro et al., 2017). They performed a MW assisted extraction (MAE) at 125ºC for 20min.Whereas, in this work, MW irradiation was only used as a pre-treatment which involve small energetic consumptions (from an economic point of view). Furthermore, polyphenols are less time (< 2 min) exposed to high temperatures (T>100ºC), something to be borne in mind since polyphenols are thermolabile (Spigno & De Faveri, 2007). Apart from the increasing solubility and diffusion coefficient achieved with the MW pre-treatment which facilitates the extraction of polyphenols from GS, probably the irradiation degrades the vacuole compartments where a huge number of flavonoids are located releasing this compounds to the media and making them more removable (Agati, Azzarello, Pollastri, & Tattini, 2012).

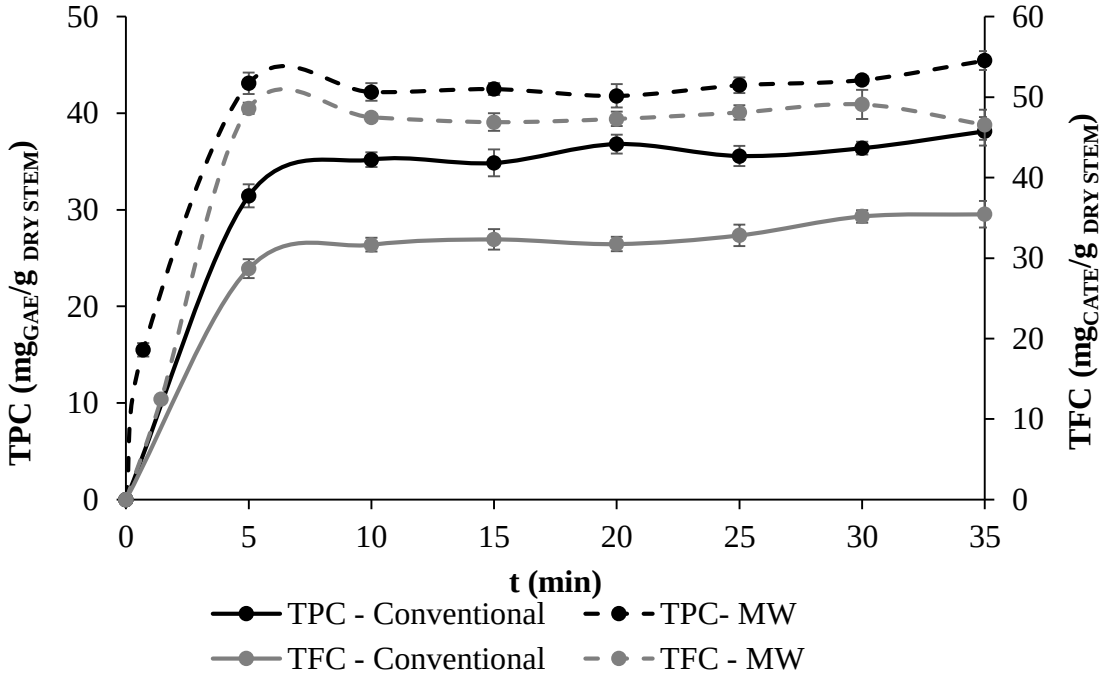


Figure 4: comparison of the TPC and TFC between the conventional solid-liquid extraction at (RS-L of 0.10 g/mL, a solvent with a 50% (vol.) of ethanol and a temperature of 75ºC) and the optimums points of the MW pre-treatment.

3.2 Extracts characterization

3.2.1 Antioxidant Activity: ORAC

ORAC was measured for the best conventional solid-liquid ratio and the two extracts obtained from the optimum points of the MW pre-treatment for TPC and TFC. For the conventional solid-liquid extraction an ORAC value of 791 ± 90 $\mu mol_{TE}/g_{DS}$ was achieved. For the optimum points for TPC and TFC higher antioxidant capacities were achieved (848 ± 110 $\mu mol_{TE}/g_{DS}$ and 1056 ± 56 $\mu mol_{TE}/g_{DS}$, respectively). However, only the ORAC value for the maximization of the TFC was significantly different ($p>0.05$). Few data have been found in literature regarding the antioxidant activity of GS extracts via ORAC. Similar values were obtain by *Gonzalez-Centeno et al.* (González-Centeno et al., 2012) who measured the antioxidant potential of stems by ORAC for 10 different grape varieties. Highest ORAC value, 1128 $\mu mol_{TE}/g_{DS}$, was found for the most concentrated extract in polyphenols (115 mg_{GAE}/g_{DS}) derived from a *Callet* grape variety. The lowest ORAC, 404 $\mu mol_{TE}/g_{DS}$, was achieved for the least rich polyphenol extract (*Merlot variety*) (47 mg_{GAE}/g_{DS}), as expected.

3.2.2 HPLC analysis

The identification of the compounds present in the different extracts was carried out by comparison of the retention time of pure standards and the spectra at 280 nm. In every extract, the same compounds were obtained. Notwithstanding, different concentrations were found among the extracts, being these higher in those performed with the MW pre-treatment in accordance to the intensity of the peaks. Henceforth, only the extract performed with the MW conditions that maximized TFC, was used for the analysis. This is due to the fact that it presented a higher ORAC activity. Furthermore, its TPC value was very close to the one obtained at the MW conditions that maximized the extraction of polyphenols (vide Table 2) .Identified compounds were catechin, epicatechin, procyanidin B2, trans-resveratrol and ε-viniferin.

The effect of the extraction temperature is well visible in the Figure 5. The higher the temperature, the higher the concentration of each identified compound.

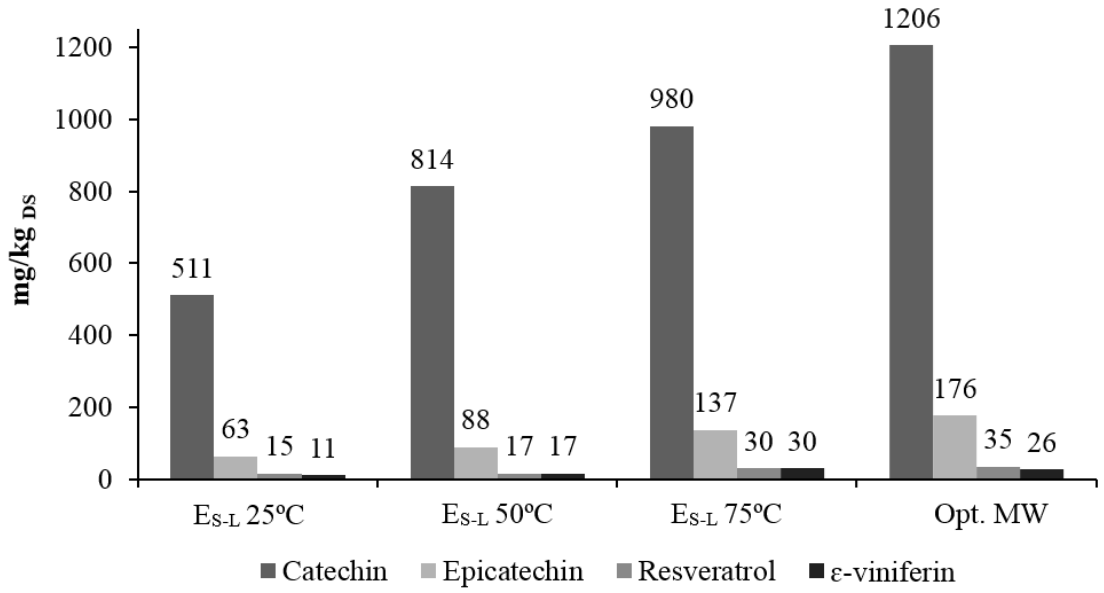


Figure 5: concentration of the main compounds presented in the different grape stems extracts.

Furthermore, the effect of the MW irradiation was corroborated for the optimum TFC pre-treatment. Especially for the catechin concentration (1206 mg/kg$_{DS}$), which increased a 23% respect to the solid-liquid extraction performed at the best studied conditions (980 mg/kg$_{DS}$). Same compounds have been already identified in more GS extracts from other grape varieties and with other extraction procedures. Most of the studies were focused on the revalorization of GS as an alternative source of stilbenes. That is the case of *Piñeiro et al.* (Piñeiro et al., 2017), who obtained different concentrations of resveratrol and ε-viniferin from 20 different varieties of grape stems, apart from piceatannol. In this case, an optimized MAE hydroalcoholic mixture with 80% (v/v) EtOH with a R_{S-L} of 0.01 during 5 min) was used to obtain the extracts a wide range of lyophilized stems. Concentrations of 73 mg/kg$_{DS}$ of ε-viniferin and 229 mg/kg$_{DS}$ of resveratrol were obtained for the *Tempranillo* variety. This fact also corroborates what was previously indicated, the comparisons and similitudes among the different extracts strongly depend on several external factors, such the geographical localization (Ruiz-Moreno et al., 2015). On the other hand, when an ultrasound assisted extraction was performed (single extraction cycle with 75 °C as extracting temperature, a R_{S-L} of 0.033, 80% ethanol in water as extracting liquid, 7 mm diameter probe tip, 70% amplitude, 0.7 s cycle time, and 15 min extraction time) to 22 GS varieties, lower stilbenes

concentrations were achieved (Piñeiro et al., 2013). Although, grape clusters were grown in the same place, concentrations values of resveratrol and ε-viniferin varied a lot for the same grape variety depending on the year the samples were harvested in the 2010, 2011, and 2012. For the case of *Tempranillo* variety, in the year 2012, no resveratrol were detected and only 28 mg/kg_{DS} of ε-viniferin were obtained. However, in the year 2010, the extracts from the same grape variety concentrations showed concentrations around 88 mg/kg_{DS} and 81 mg/kg_{DS} of resveratrol and ε-viniferin, respectively. Nonetheless, these values were lower if compared with other grape variety like *Tintilla de Rota* in the year 2010. For this grape variety a resveratrol concentration of 119 mg/kg_{DS} was obtained, whereas the concentration of ε-viniferin was around 92 mg/kg_{DS}. In this case, significant differences were achieved between the control extraction and the US-AE applied to all the grape varieties. Thus, the concentrations achieved in the present work using MW as a pre-treatment were in the ranged to those obtained with a MAE or with US-AE. Other authors also focused their attention on the concentration of flavonoids such as catechin and epicatechin. Extractions from stems of three red grape cultivars (Mandilaria, Mavrotragano and Voidomatis) were performed at a R_{S-L} of 0.20g/mL with a solvent mixture of MeOH/H_2O/HCl (90:9.5:0.5 v/v) sonicated for 10min in an ultrasound bath. The solid was re-extracted three additional times. Averages concentrations of catechin and epicatechin of 1094 and 100 mg/kg_{DS} were found, respectively. (Anastasiadi, Pratsinis, Kletsas, Skaltsounis, & Haroutounian, 2012). These values were lower than the concentrations obtained for these compounds in the present work, which means that the MW pre-treatment enhance the extraction of catechin and epicatechin.

3.2.3 Anti-fungal activity (*Botrytis cinerea* culture)

The growth area of the *Botrytis cinerea* culture also indicated discrepancies among the concentrations of bioactives in the different extracts. From Figure 6, the growth area for the blank (distilled water) was around 6204 mm^2. In contrast, the optimum flavonoid extract performed with the MW pre-treatment inhibited the growth of the fungus in a 46% (with only a growth area of 2837 mm^2), which demonstrate the potential application of the extract as antifungal.

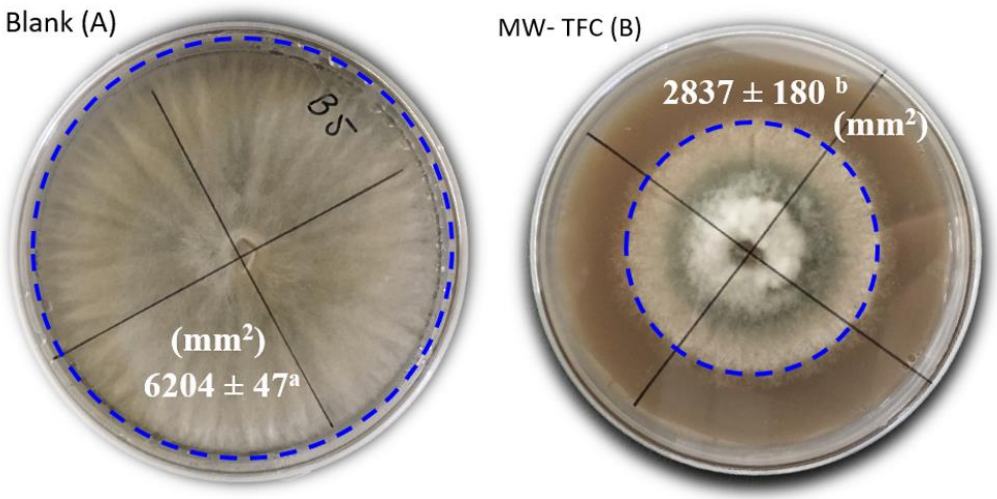


Figure 6: comparison of the growth areas for the blank (distilled water) (A) and the MW optimum point for TFC (B).

The anti-fungal properties of GS extracts have been also validated in other works. *Ruiz-Moreno et al.* (Ruiz-Moreno et al., 2015) tested the effectiveness of GS extracts as preservatives in the wine industry to inhibit the microbial and fungal spoilage. Particularly, for the *Botrytinia fuckeliana* (also known as its anamorphic *Botrytis cinarea*) GS extracts were able to significantly delay the fungus growth.

4. Conclusions

In this work, the kinetic extraction curves for both total polyphenols and flavonoids conventional solid-liquid extractions from GS have been studied. The use of a $R_{S\text{-}L}$ of 0.10 g/mL with a solvent with a 50% vol. ethanol at 75ºC seemed to promote the extraction of bioactive compounds. In the same sense, the optimum MW pre-treatments (<90s) were capable of increasing the final TPC and TFC in a 19% and 24%, respectively and the antioxidant activity compared the conventional procedure. For the case or the *Botrytis cinarea* culture, the optimum MW extract showed a great anti-fungal activity. The effect of the MW was also visible in the concentration of individual components as HPLC shown. For instance, a concentration of 30 and 980 mg/kg_{DS} of resveratrol and catechin, respectively, were obtained with the conventional solid-liquid extraction. Whereas when MW was employed these concentrations increased to 35 and 1206 mg/kg_{DS}, respectively.

References

Adrian, M., Jeandet, P., Veneau, J., Weston, L. A., & Bessis, R. (1997). Biological Activity of Resveratrol, a Stilbenic Compound from Grapevines, Against Botrytis cinerea, the Causal Agent for Gray Mold. *Journal of Chemical Ecology*, *23*(7), 1689–1702.

Agati, G., Azzarello, E., Pollastri, S., & Tattini, M. (2012). Flavonoids as antioxidants in plants: Location and functional significance. *Plant Science*, *196*, 67–76.

Álvarez, A., Poejo, J., Matias, A. A., Duarte, C. M. M., Cocero, M. J., & Mato, R. B. (2017). Microwave pretreatment to improve extraction efficiency and polyphenol extract richness from grape pomace. Effect on antioxidant bioactivity. *Food and Bioproducts Processing*, *106*, 162–170.

Anastasiadi, M., Pratsinis, H., Kletsas, D., Skaltsounis, A.-L., & Haroutounian, S. A. (2012). Grape stem extracts: Polyphenolic content and assessment of their in vitro antioxidant properties. *LWT - Food Science and Technology*, *48*(2), 316–322.

Brianceau, S., Turk, M., Vitrac, X., & Vorobiev, E. (2016). High voltage electric discharges assisted extraction of phenolic compounds from grape stems: Effect of processing parameters on flavan-3-ols, flavonols and stilbenes recovery. *Innovative Food Science & Emerging Technologies*, *35*, 67–74.

Cotoras, M., García, C., Lagos, C., Folch, C., & Mendoza, L. (2001). Antifungal activity on Botrytis cinerea of flavonoids and diterpenoids isolated from the surface of Pseudognaphalium Spp. *Boletín de La Sociedad Chilena de Química*, *46*(4), 433–440.

Dias, C., Domínguez-Perles, R., Aires, A., Teixeira, A., Rosa, E., Barros, A., & Saavedra, M. J. (2015). Phytochemistry and activity against digestive pathogens of grape (Vitis vinifera L.) stem's (poly)phenolic extracts. *LWT - Food Science and Technology*, *61*(1), 25–32.

Drosou, C., Kyriakopoulou, K., Bimpilas, A., Tsimogiannis, D., & Krokida, M. (2015). A comparative study on different extraction techniques to recover red grape pomace polyphenols from vinification byproducts. *Industrial Crops and Products*, *75*, 141–149.

Garrett, A. R., Weagel, E. G., Martinez, A. D., Heaton, M., Robison, R. A., & O'Neill,

K. L. (2014). A novel method for predicting antioxidant activity based on amino acid structure. *Food Chemistry*, *158*, 490–6.

Goldstein, D. B., & Chin, J. H. (1981). Interaction of ethanol with biological membranes. *Federation Proceedings*, *40*(7), 2073–6.

González-Centeno, M. R., Jourdes, M., Femenia, A., Simal, S., Rosselló, C., & Teissedre, P.-L. (2012). Proanthocyanidin Composition and Antioxidant Potential of the Stem Winemaking Byproducts from 10 Different Grape Varieties (Vitis vinifera L.). *Journal of Agricultural and Food Chemistry*, *60*(48), 11850–11858.

He, J., & Giusti, M. M. (2010). Anthocyanins: Natural Colorants with Health-Promoting Properties. *Annual Review of Food Science and Technology*, *1*(1), 163–187.

Huang, D., Ou, B., Hampsch-Woodill, M., Flanagan, J. A., & Prior, R. L. (2002). High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. *Journal of Agricultural and Food Chemistry*, *50*(16), 4437–44.

Ismail, A., Marjan, Z. M., & Foong, C. W. (2004). Total antioxidant activity and phenolic content in selected vegetables. *Food Chemistry*, *87*(4), 581–586.

Lee, D.-U., Kim, H. M., Lee, G. D. and Jeon, S.-H(2014). Effects of thermal treatments on the stability of trans-resveratrol and yeast inactivation in trans-resveratrol-amplified grape juice. *Archives of Biological Sciences*, *66*(1), 323-329.

Li, N., Taylor, L. S., Ferruzzi, M. G., & Mauer, L. J. (2012). Kinetic Study of Catechin Stability: Effects of pH, Concentration, and Temperature. *Journal of Agricultural and Food Chemistry*, *60*(51), 12531–12539.

Li, Y., Han, L., Ma, R., Xu, X., Zhao, C., Wang, Z., Hu, X. (2012). Effect of energy density and citric acid concentration on anthocyanins yield and solution temperature of grape peel in microwave-assisted extraction process. *Journal of Food Engineering*, *109*(2), 274–280.

Lončarić, A., Pablo Lamas, J., Guerra, E., Kopjar, M., & Lores, M. (2018). Thermal stability of catechin and epicatechin upon disaccharides addition. *International Journal of Food Science & Technology*, *53*(5), 1195–1202.

Makris, D. P., Boskou, G., & Andrikopoulos, N. K. (2007). Polyphenolic content and in

vitro antioxidant characteristics of wine industry and other agri-food solid waste extracts. *Journal of Food Composition and Analysis*, *20*(2), 125–132.

Michalska, A., Ceglińska, A., & Zieliński, H. (2007). Bioactive compounds in rye flours with different extraction rates. *European Food Research and Technology*, *225*, 545–551.

Michelsen, M. L., & Mollerup, J. M. (2007). *Thermodynamic models : fundamentals and computational aspects*. Tie-Line Publications.

Pandey, K. B., & Rizvi, S. I. (2009). Plant polyphenols as dietary antioxidants in human health and disease. *Oxidative Medicine and Cellular Longevity*, *2*(5), 270–8.

Piñeiro, Z., Guerrero, R. F., Fernández-Marin, M. I., Cantos-Villar, E., & Palma, M. (2013). Ultrasound-Assisted Extraction of Stilbenoids from Grape Stems. *Journal of Agricultural and Food Chemistry*, *61*(51), 12549–12556.

Piñeiro, Z., Marrufo-Curtido, A., Vela, C., & Palma, M. (2017). Microwave-assisted extraction of stilbenes from woody vine material. *Food and Bioproducts Processing*, *103*, 18–26.

Piñeiro, Z., Palma, M., & Barroso, C. G. (2006). Determination of trans-resveratrol in grapes by pressurised liquid extraction and fast high-performance liquid chromatography. *Journal of Chromatography A*, *1110*(1–2), 61-65.

Romero-Díez, R., Matos, M., Rodrigues, L., Bronze, M. R., Rodríguez-Rojo, S., Cocero, M. J., & Matias, A. A. (2018). Microwave and ultrasound pre-treatments to enhance anthocynins extraction from different wine lees. *Food Chemistry, 272*, 258-266 .

Ruggieri, L., Cadena, E., Martínez-Blanco, J., Gasol, C. M., Rieradevall, J., Gabarrell, X., Sánchez, A. (2009). Recovery of organic wastes in the Spanish wine industry. Technical, economic and environmental analyses of the composting process. *Journal of Cleaner Production*, *17*(9), 830–838.

Ruiz-Moreno, M. J., Raposo, R., Cayuela, J. M., Zafrilla, P., Piñeiro, Z., Moreno-Rojas, J. M., … Cantos-Villar, E. (2015). Valorization of grape stems. *Industrial Crops and Products*, *63*, 152–157.

Salgado, M., Rodríguez-Rojo, S., & Cocero, M. J. (2017). Barley and yeast β-glucans as new emulsifier agents for the development of aqueous natural antifungal

formulations. *Carbohydrate Polymers*, *174*, 1114–1120.

Sant'Anna, V., Brandelli, A., Marczak, L. D. F., & Tessaro, I. C. (2012). Kinetic modeling of total polyphenol extraction from grape marc and characterization of the extracts. *Separation and Purification Technology*, *100*, 82–87.

Scalbert, A. (1991). Antimicrobial properties of tannins. *Phytochemistry*, *30*(12), 3875–3883.

Sólyom, K., Solá, R., Cocero, M. J., & Mato, R. B. (2014). Thermal degradation of grape marc polyphenols. *Food Chemistry*, *159*, 361–6.

Spadaro, D., & Gullino, M. L. (2004). State of the art and future prospects of the biological control of postharvest fruit diseases. *International Journal of Food Microbiology*, *91*(2), 185–194.

Spatafora, C., Barbagallo, E., Amico, V., & Tringali, C. (2013). Grape stems from Sicilian Vitis vinifera cultivars as a source of polyphenol-enriched fractions with enhanced antioxidant activity. *LWT - Food Science and Technology*, *54*(2), 542–548.

Spigno, G., & De Faveri, D. M. (2007). Antioxidants from grape stalks and marc: Influence of extraction procedure on yield, purity and antioxidant power of the extracts. *Journal of Food Engineering*, *78*(3), 793–801.

Thostenson, E. T., & Chou, T.-W. (1999). Microwave processing: fundamentals and applications. *Composites Part A: Applied Science and Manufacturing*, *30*(9), 1055–1071.

Vázquez-Armenta, F. J., Silva-Espinoza, B. A., Cruz-Valenzuela, M. R., González-Aguilar, G. A., Nazzaro, F., Fratianni, F., & Ayala-Zavala, J. F. (2017). Antibacterial and antioxidant properties of grape stem extract applied as disinfectant in fresh leafy vegetables. *Journal of Food Science and Technology*, *54*(10), 3192–3200.

Wang, T., Li, Q., & Bi, K. (2018). Bioactive flavonoids in medicinal plants: Structure, activity and biological fate. *Asian Journal of Pharmaceutical Sciences*, *13*(1), 12–23.

Waterhouse, A. L., Waterhouse, & L., A. (2003). Determination of Total Phenolics. In *Current Protocols in Food Analytical Chemistry*. Hoboken, NJ, USA: John Wiley & Sons, Inc.

Weidenbörner, M., Hindorf, H., Jha, H. C., & Tsotsonos, P. (1990). Antifungal activity of flavonoids against storage fungi of the genus Aspergillus. *Phytochemistry*, *29*(4), 1103–1105.

Zachová, Z., Tříska, J., Vrchotová, N., Balík, J., Sajfrtová, M., & Sovová, H. (2018). Combining high-pressure methods for extraction of stilbenes from grape cane. *The Journal of Supercritical Fluids*.

CHAPTER 5

Conversion of grape stems into sugars rich extracts via a hydrothermal process

Abstract

Grape stems, a waste generated during the destemming of the vinification process, represents around the 12% of the total residues generated in a winery. They represent a source of sugar based compounds (monomers and oligomers) due to their lignocellulosic structure and composition. A hydrothermal process for grape stems conversion into sugars and oligomers was employed. A range of temperatures from 100ºC to 180ºC was tested for 20-minute experiments in order to evaluate its influence on sugars recovery. A temperature of 140ºC seemed to be the most suitable to maximize the sugars yield (264mg/$g_{DRY\ STEM}$). The effect of the operating time, from 10 to 30 min, was also determined at this temperature. Results brought out that the higher the time, the higher the yield in terms of sugars and total carbon content. Finally, a kinetic model was used to fit the experimental data with an average absolute deviation around 15% for sugars and oligomers and 30% for degradation products.

1. Introduction

Estimations indicate that, only in Spain, around 2-3 million tons of residues are generated annually during the vinification process (Ruggieri et al., 2009). This means that around 25kg of sub-products are produced per 100L of red wine. These residues comprises grape pomace, wine lees and grape stems (Teixeira et al., 2014). This work focused the attention to the latter, the grape stems, which constitutes around 12 to 16% of these residues (Devesa-Rey et al., 2011). In general, all these sub-products are not considered hazardous, but their high content of organic (together with their seasonal production) could generate potential pollution problems (Spigno, Pizzorno, & De Faveri, 2008). In this context, the so called bioeconomy, economy based on the conversion of biomass processing residues into valuable products, is gaining strength in the last decades. Furthermore, this interest is also motivated by the legislation, which has increased significantly the residue disposal fees (Devesa-Rey et al., 2011). In order to reduce the problems associated to grape stems disposals, several alternatives have emerged as a solution such their use as fertilizer after composting (Bertran, Sort, Soliva, & Trillas, 2004), as absorbent material for the removal of heavy metals from aqueous solutions (Martínez et al., 2006; Villaescusa et al., 2004) or as source of natural antioxidants

(Chupin et al., 2015; Piñeiro, Guerrero, Fernández-Marin, Cantos-Villar, & Palma, 2013; Ruiz-Moreno et al., 2015).

Grape stems are the woody skeleton of the grape bunch. As many other agricultural wastes, stems present a lignocellulosic nature. Thus, it is constituted by three pseudo-components combined in a resistant structure: cellulose, hemicellulose and lignin. However, the composition of grape stems differs a lot from the different species and environmental conditions as well as the method employed for the raw material characterization. For instance, the percentages (% on dry weight) of cellulose, hemicellulose and lignin range from 25-38%, 14-35% and 18-47%, respectively. Similarly, the same differences appear for the amount of extractives initially recovered from dried grape stems which varied between 25-50%. Nevertheless, the content of proteins and ashes usually remains constant among the different methods, 6 and 7%, respectively (Lorenzo, Moldes, Rodríguez Couto, & Sanromán, 2002; A. Sluiter et al., 2004).

According to their composition, grape stems could be an alternative source for the extraction of the cellulosic fraction of the biomass (cellulose and hemicellulose, up to 50%), also known as holocellulose. This fraction can be recovered as biopolymers and sugars. Moreover, these polymers can be also hydrolysed into their monomeric sugars components and, posteriorly, converted in high added value products such as fuels, bulk chemicals and materials (Cherubini, 2010; Spigno et al., 2008). For instance, cellulose is a homogeneous polymer formed by glucose monomers. Glucose can be used for further industrial applications such as the production of biofuels or hydroxymethylfurfural (Rogalinski, Ingram, & Brunner, 2008). Another well-known application of glucose is the formation of sorbitol by glucose hydrogenation (Besson, Gallezot, & Pinel, 2014; Romero, Alonso, Sastre, & Nieto-Márquez, 2016) that can be used in a wide range of applications in food, cosmetic and paper industries Conversely, hemicellulose is a heterogeneous branched polysaccharide that mainly consists on different proportions of sugar units with different substituents (Sauter & Grafmüller, 2015). Its composition and structure differ a lot from the different types of wood and from the part they belong to -stem, branches, roots or bark- (Eero Sjöström, 1993). For the case of softwoods, mannose is the most common hemicellulosic monomer followed by xylose, glucose, galactose and arabinose. In contrasts, in hardwood materials xylose is the most important monomer followed by mannose, glucose and galactose (Bobleter, 1994). A differentiating feature

of hemicellulose respect to the cellulose is their low molecular weight and low degree of polymerization which make hemicellulose solubility higher and easily to be fragmented into their constitutional sugar units (Farhat et al., 2017). Hemicellulose polysaccharides can be used such as emulsifiers, drug carriers, plastic films for the protection of foods or superabsorbent hydrogels (Gallina, Alfageme, Biasi, & García-Serna, 2018). Whereas their monomeric constituents can be also employed for the production of second generation ethanol and fuels (Aristidou & Penttilä, 2000).

Different procedures have been employed for lignocellulosic biomass conversion (Carvalheiro, Duarte, & Gírio, 2008). For example, concentrated acid or diluted acid pre-treatments, in which an acid is used as a catalyst, are suitable for sugar extraction processes. Nonetheless, these processes entail several drawbacks since it is an expensive methodology regarding the separation processes at the end of the extraction. Furthermore, the use of acids leads to corrosion problems which increase operating costs (Hamelinck, Hooijdonk, & Faaij, 2005). On the other hand, it is worth mentioning the worldwide use of alkaline treatments. Alkaline treatments can be divided into two major groups, those which employ sodium, potassium or calcium hydroxide and those in which ammonia is used. The main advantage of the processes is the use of low temperature and pressure. However, a restriction arises when some alkali is converted into irrecoverable salts or incorporated as salts into biomass (Mosier et al., 2005). Similarly, alkaline peroxide extraction takes place when H_2O_2 is added to the alkaline pre-treatment in order to enhance kinetic extraction at lower temperatures by favouring the lignin removal. Furthermore, microwave treatments (Azhar, Henriksson, Theliander, & Lindström, 2015) and ionic liquid extraction (Froschauer et al., 2013) have been used. Another group of extraction methodologies with a great potential for the conversion of sugars are the hydrothermal pre-treatments. These processes are characterized by using liquid hot water between 150ºC and 230ºC (G. Garrote, Domínguez, & Parajó, 1999) able to extract holocellulose.

Temperatures below 100ºC do not make a hydrolytic effect on the material (Abatzoglou, Chornet, Belkacemi, & Overend, 1992), meanwhile cellulose degradation occurs when working above 250ºC (Rubio Torres, Heitz, Chauvette, Chornet, & Overend, 1986). Apart from being green processes, relatively high holocellulose extraction yields can be obtained with them. That is the case of *Pedras et al*. (Pedras et al., 2017) who used sub-critical water (170-210ºC and 100bar) to obtain a rich carbohydrate extract from

white grape pomace. Results showed that assays at 210 °C led to the highest recovery of carbohydrates (85% of total amount of initial carbohydrates). Additionally, *Sánchez-Bastardo et al*. (Sánchez-Bastardo, Romero, & Alonso, 2017) were able to recover high amount of arabinoxylans (58-71%) from wheat bran using hydrothermal processes assisted by heterogeneous catalysts. Similarly, the combination of carbon dioxide and water has been also demonstrated to be a suitable option for biomass conversion into sugars (Relvas, Morais, & Bogel-Lukasik, 2015). The effect of the acidify media is due to the presence of carbonic acid derived from the carbon dioxide. This, promote the kinetic constants for the production of either intermediated products or final compounds in comparison to pure water reactions to conversion yields up to 70.3% (Magalhães da Silva, Morais, & Bogel-Łukasik, 2014).

With the appropriate procedure, oligomers and monomeric sugars can be recovered from grape stems. It is also important to bear in mind that the operating conditions have to be selected appropriately in order to avoid sugar degradation or solubilized oligomers cleavage. Therefore, this work is aimed at extracting oligomers and their monomeric sugars from grape stems, considered as a hardwood, by a hydrothermal method. Due to the major problem related with second generation fuels are associated to the expensive required pre-treatments in order to make cellulose more accessible, the effect of a MW treatment was analyse in the extraction of oligomers and sugars. Oligomers, sugars and degradation products were analysed via HPLC. The purity of the extracts was measured by the total organic carbon (TOC). Furthermore, a kinetic model was used to fit the experimental data.

2. Materials and methods

2.1 Materials

2.1.1 Raw material: grape stems

Grape stems (GS) belonging to the grape variety *Vitis vinifera, Tempranillo,* were provided by the winery *Grupo Matarromera* (41° 38′ 33″ N, 4° 17′ 28″ W). These vines were cultivated in a clay soil in *Valbuena de Duero, Ribera de Duero* Designation of Origin (*Castilla y León*). The average ambient temperature during this year in the vineyard was 13.9ºC, the average precipitations were 7.5mm. Grapes were harvested in September 2017 and stems were mechanically separated before grape pressing. They were washed with water in order to remove any residual wine traces and the traces of grapes linked to grape stems were manually removed. Stems were let dry at the sun during

48h. In order to ensure the total dryness, GS were placed in an oven at the temperature of 65ºC for 24h. Later they were ground using a chopper (A320R1, Moulinex) into small pieces with an average length between 4-*6mm*.

2.1.2 Chemicals

Distilled water was used as an extraction solvent. The standards employed for the analysis HPLC were: glucose (99%), xylose (99%), rhamnose (99%), galactose (99%), arabinose (99%), mannose (99%), fructose (99%), glyceraldehyde (95%), glycolaldehyde (99%), lactic acid (85%), formic acid (98%), acetic acid (99%), levulinic acid (98%), acrylic acid (99%), pyruvaldehyde (40%), 5-hydroxymethilfurfural (99%), furfural (99%) and erythrose (75%). All these chemicals were purchased from Sigma Aldrich (Spain) and used as received.

2.2 Methods

2.2.1 Chemical characterization of the raw material

The composition of GS was determined according to the Laboratory Analytical Procedure (LAP) for biomass analysis provided by the National Renewable Energy Laboratory (NREL) (a Sluiter et al., 2008). This procedure has been previously described in prior works of our group (Gallina, Cabeza, et al., 2018; Sánchez-Bastardo et al., 2017).

2.2.2 Extraction procedure

Extractions were carried out in an AISI 304 stainless steel vessel with a volume of 170 mL. An electric resistance heater (275 W) around the vessel was used to heat up the extractor until the desired operational temperature, controlled by a PID. Pressure generated inside the extractor (2-10 barg) was measured with a pressure gauge (0 - 25barg). To carry out the experimental procedure 13g of dried stems were introduced in the extractor vessel with 130mL of distilled water. A relative high suspension concentration ($100 g \cdot L^{-1}$) was chosen in comparison to previous works of the group (Sánchez-Bastardo et al., 2017) due to the lower initial content of the holocellulose in the raw material used in this work.. Then the extractor vessel was closed and put in a stirring plate at 1,200rpm. Once the desired temperature was achieved the operating time started to run. The operating time was fixed at 20 min to maximize both, the oligomers length and the sugars extraction yield (Gallina, Cabeza, et al., 2018). At the end of the experiments, the extractor vessel was cooled in an ice bath. The liquid extracts were

centrifuged for 7,800rpm during 10min (SIGMA 2–16P; SIGMA) and later, vacuum filtered and stored at 4ºC until further analysis. Analysis were performed in triplicate for each experience.

Regarding the operational temperature, a range from 100 to 180ºC was considered. These temperature were used to study the whole range in which the cellulosic fraction of biomass can be hydrothermally recovered without a huge degradation (Carvalheiro et al., 2008).

On the other hand, for the best yield obtained at 20min, the role of a different operational time was also assed performed experiment at 10 and 30min. Additionally, the effect of a pre-treatment to recover the extractives (mainly polyphenols) on the sugars extraction yield was evaluated. This recovery was performed by a microwave (MW) pre-treatment using a CEM Discovery One Microwave (CEM Corp.). For the pre-treatment a solid-liquid ratio of 0.10g/mL, a 47% of EtOH in the hydroalcoholic mixture and an operating time of 86s. More details about the experimental procedure and set up can be found in a previous work (Romero-Díez et al., 2018). After the recovery of polyphenols, the remaining solid was dried in an oven at 65ºC for 48h. Once this solid was dried, it was treated following the same hydrothermal procedure as the used for the other samples for the obtention of sugars.

All the experiments and the operational conditions are listed in Table 1.

*Table 1: list of experiments and main concentration values (sugars, degradation products, oligomers and TOC). *Aleatory experiments were performed in duplicate to verify reproducibility of the process.*

	T	t	P	pH	Sugars	Degradation Compounds	Total Oligomers	Pentose Olig. (HC)	Hexose Olig. (C)	TOC	Total extracted yield
Exp.	**(ºC)**	**(min)**	**(barg)**				**(mg/g_{DS})**			**(mg/L)**	**%**
1	100	20	2	4.28	109 ± 7	1.0 ± 0.3	496 ± 11	33 ± 1	463 ± 9	17063 ± 624	61
2	120	20	2	4.16	121 ± 8	1.0 ± 0.4	505 ± 9	40 ± 2	465 ± 7	15348 ± 1178	63
3*	140	20	3	4.02	264 ± 5	2.0 ± 0.0	316 ± 14	74 ± 5	242 ± 10	13952 ± 315	58
4*	160	20	7	4.06	249 ± 7	42.8 ± 5.0	357 ± 7	90 ± 1	267 ± 7	13120 ± 485	65
5*	180	20	10	4.04	171 ± 1	71.3 ± 8.0	361 ± 10	161 ± 3	200 ± 7	13247 ± 958	60
6	140	10	3	3.94	280 ± 8	3.2 ± 0.1[a]	256 ± 15	50 ± 4	206 ± 11	18603 ± 1683	54
7	140	30	4	4.06	302 ± 9	9.4 ± 0.6	357 ± 23	86 ± 5	270 ± 18	20840 ± 454	67
8	160	20	5	4.33	109 ± 7	35 ± 1.0	393 ± 14	201 ± 6	191 ± 8	8603 ± 84	54

2.2.3 Extract Characterization

2.2.3.1 Determination of pH

A Phenomenal pH meter using a refillable glass electrode model 221 with a built-in PT 1000 temperature sensor was employed for the pH determination of the liquid extracts after the extractions.

2.2.3.2 Determination of oligomers and sugars

A High Performance Liquid Chromatography (HPLC) apparatus from Waters Corporation, consisting on an isocratic pump (Waters 1515), an automatic injector (Waters 717) and an IR detector (Waters 2414), was employed for the determination of sugars (monosaccharides and oligomers). A Supelcogel Pb (Supelco) column (milli-Q water as mobile phase, 0.5mL·min^{-1} as flow rate and 85°C as temperature) was used. Total oligomers were quantified as the difference between the sugars obtained after a hydrolysis step, and the initial content of sugars in the extract.

This hydrolysis of the samples was performed according to the Laboratory Analytical Procedure (LAP) of the NREL (a Sluiter et al., 2008). Briefly, 0.8 mL of sulfuric acid (72%) were added to 20mL extract aliquot and autoclaved at 121 °C for 1 h. Later, samples were filtered (pore size 0.22μm, Diameter 25mm, Nylon; FILTER-LAB).

2.2.3.3 Determination of the degradation products

Degradation products in the extracts were directly analyse after the extraction process using the same HPLC equipment. In this case, Sugar SH-1011 (Shodex) column was employed (sulfuric acid 0.01 N as mobile phase, 0.8mL·min^{-1} as flow rate and 50°C as temperature). Furthermore, apart from the Waters IR detector 2414 employed for the determination of acids and aldehydes, a UV–vis detector (Waters 2487; Waters Corporation) at a wavelength of 254nm was used for the determination of 5-hidroxymethylfurfural (5-HMF) and furfural. Samples were also filtered (pore size 0.22μm, Diameter 25mm, Nylon; FILTER-LAB) prior to their analysis in the HPLC.

2.2.3.4 Overall yield

Total Organic Carbon (TOC) analysis was employed in order to determine the purity of the liquid extract in a Shimadzu TOC-VCSH equipment. Prior to analysis, samples were diluted in accordance with the operating range of the equipment (0-500ppm$_{CARBON}$).

3. Results and Discussion

3.1 Chemical characterization of the raw material

The characterization of the raw material was done following the method described in *Section 2.2.1*. The result was an 11.5% of insoluble lignin, 3.7% of soluble lignin, 15.3% of cellulose, 9.2% of hemicellulose, 50.9% of extractives, 9.3% of proteins and 0.1% of ash. Thus, it can be checked that the amount of available sugars to be recovered is around 25%, which is a low value compared to those data reported in bibliography for grape stems (around 50%) (Lorenzo et al., 2002; Prozil et al., 2012 Spigno et al., 2008). However, the extractives represent almost a half of the initial composition. Therefore, once they have been recovered by a pre-treatment, the solid residue obtained would have a relatively higher holocellulose content.

3.2 Extraction yield of the raw material

3.2.1 Oligomers, sugars and degradation products

The effect of the temperature on the oligomer concentration in the liquid phase can be seen in Figure 1.A. In this plot, it can be observed that the oligomer yield had an almost constant value until 120 ºC, when a decrease was obtained. This decrease would be related with the fact that, at temperatures above 120ºC, the operational conditions were severe enough to degrade the oligomers into sugars. Additionally, this behavior agreed with the literature, where it was demonstrated that temperatures around 100-120ºC had a low hydrolytic effect (Abatzoglou et al., 1992). This statement is in line with the experimental profiles obtained for sugar and degradation products concentrations (Figure 1.B) too. Regarding sugars, a maximum can be observed at 140ºC and, for degradation products, they started to be different from zero also at 140ºC. At higher temperatures, a slight increment in the oligomers was the result, whereas sugars started to decrease and degradation product linearly increased. These behaviors could be explained by the fact that the increase in temperature would not be severe enough to degrade the oligomers, since they would be shorter than the obtained at lower temperatures (Chen, Lawoko, & Heiningen, 2010; Gallina, Cabeza, et al., 2018). Unlike oligomers, sugars degradation would be promoted by higher temperatures. This difference could be related with the pH, which was around 4, since it would be too high to promote oligomers breaking but low enough to ensure the sugars degradation. A result that agreed with previous works (A.

Cabeza, Piqueras, Sobrón, & García-Serna, 2016; Li, Jiang, Jia, & Xu, 2014), where a higher role of the pH on sugar degradation was confirmed.

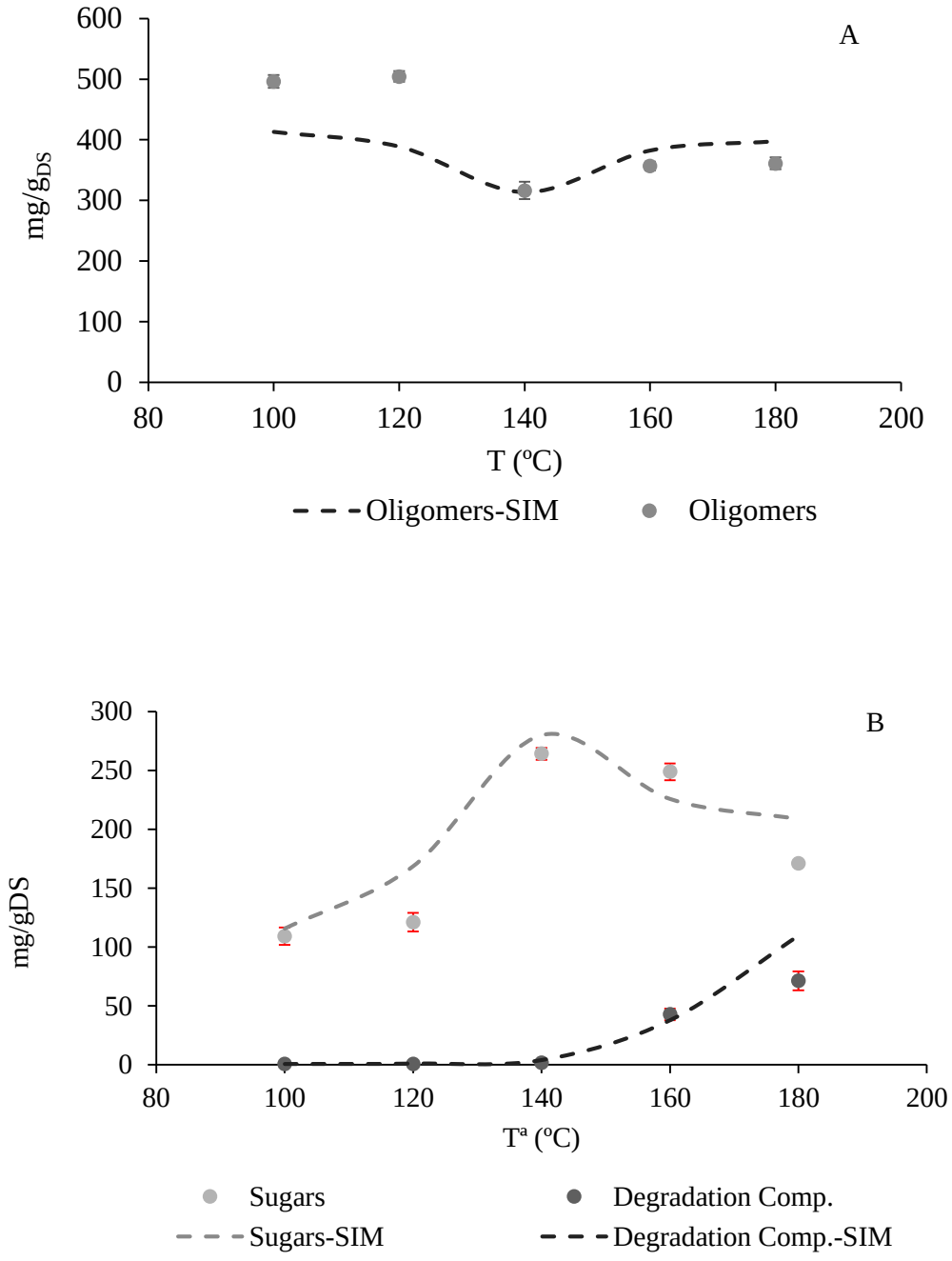
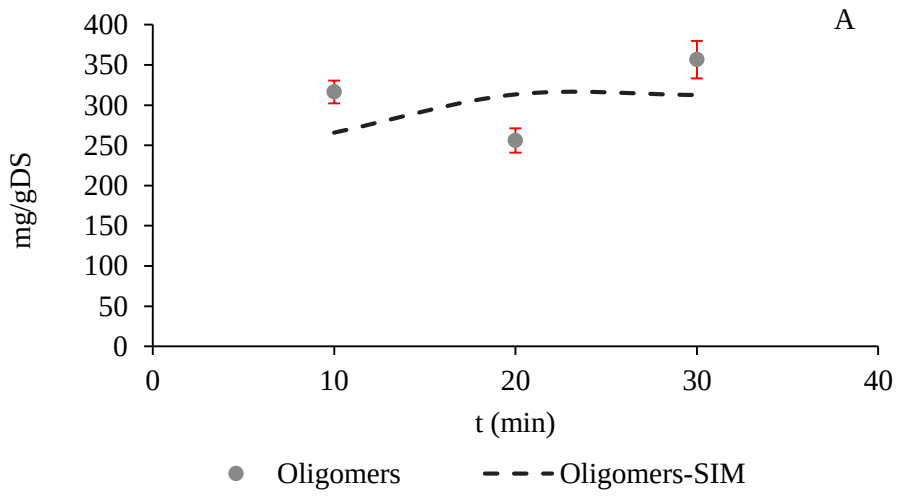


Figure 1: Oligomers (A), sugars and degradation products (B) concentration with temperature at 20 min of reaction time.

On the other hand, it can be seen in Figure 2 the effect of the time. The graph shows the evolution of the oligomers, sugars and degradation products along time when

the operational temperature was fixed at 140ºC. This temperature was selected since it implied the highest yield in sugars (Figure 2.B). From the results displayed in Figure 2.A, it can be concluded that a time lower or higher than 20min implied a higher amount of oligomers. Nevertheless, the sugar concentration just increased when the operation was done for 30min. With regards degradation products, they were always far lower than the other species due to the low operational temperature. Thus, it seems that the optimal operational conditions would be 140ºC and an operational time longer than 20min. However, if the sugar yield is the objective, the improvement at 30min is just around a 5%, which could not be high enough to justify an increase in the operational time of 50%. On the contrary, if the aim is to maximize the oligomer production, the yield was enhanced up to 40%, which would support the use of the longest operational time studied. Regarding the degradation products the increment was quite high (260%). Nevertheless, the quantitative value was still 2 orders of magnitude below the sugars (only around 6 mg/g_{DS}). Thus, it is negligible.

As a whole, it can be observed that grape stems seemed to have a completely different behaviour if compared with other woody biomasses since higher sugars yield were expected at a temperature of 180ºC (Cocero et al., 2018a). Nevertheless, the extraction yield respect to the biomass able to be recovered was similar to the reported by other authors in literature (67 vs 60%, respectively) (Gallina, Cabeza, Biasi, & García-Serna, 2016; Yedro et al., 2017).



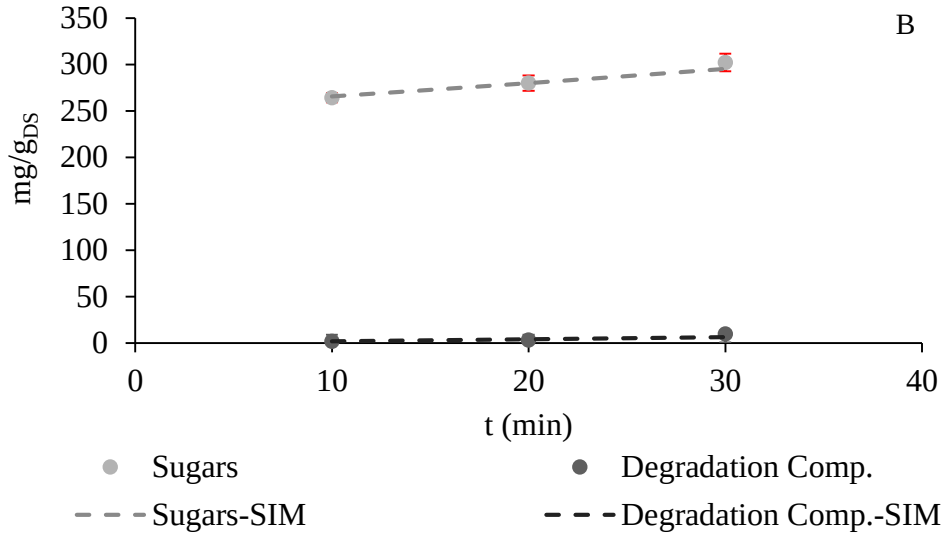


Figure 2: Oligomers (A), sugars and degradation products (B) concentration with time at 140ºC.

3.2.2 Hexoses/pentoses ratio

Hexoses and pentoses oligomers were also measured. Glucose and fructose were the constituents of the former. Meanwhile, pentoses oligomers were mainly formed by xylose, galactose and arabinose. In Figure 5.A a completely unexpected result was observed: hexoses oligomers (C) (usually assumed to come from cellulose) were the main compound recovered during the whole extraction. Whereas, pentoses' oligomers (HC) (hemicellulose's oligomers) were not started to be really extracted until 140ºC, which was a more conventional behaviour (Cocero et al., 2018b). This result could be explained by two different arguments. The first one would be that some residual oligomers from the grapes have remained in the stem after the washing due to the high amount of extractives (Spigno et al., 2008). The second one could be that the amount of amorphous cellulose in this kind of biomass were far higher, and it is more easily to extract. There are no general values about the percentages of amorphous cellulose in the initial raw materials. However, some authors reported that it can varied between 50-75% for the case of Brazilian Ginseng (Alvaro Cabeza et al., 2017). Additionally, there content of amorphous cellulose in grape stems has been barely studied. However, there is a work in which the degree of crystallinity of the cellulose that conforms grape stems has been reported to be 75.4% (Prozil, Evtuguin, & Lopes, 2012). This value is much higher than the found in

woods (55-65%) and close to values reported for cotton or bacteria cellulose (Hon, 1994). Similarly, when the effect of the time was assessed (Figure 3), the same result was obtained: hexoses' oligomers were the main compound in the bulk for each time. The variations between them could be related to two opposite events: the releasing of new oligomers form the solid and the degradation of the oligomers in the bulk.

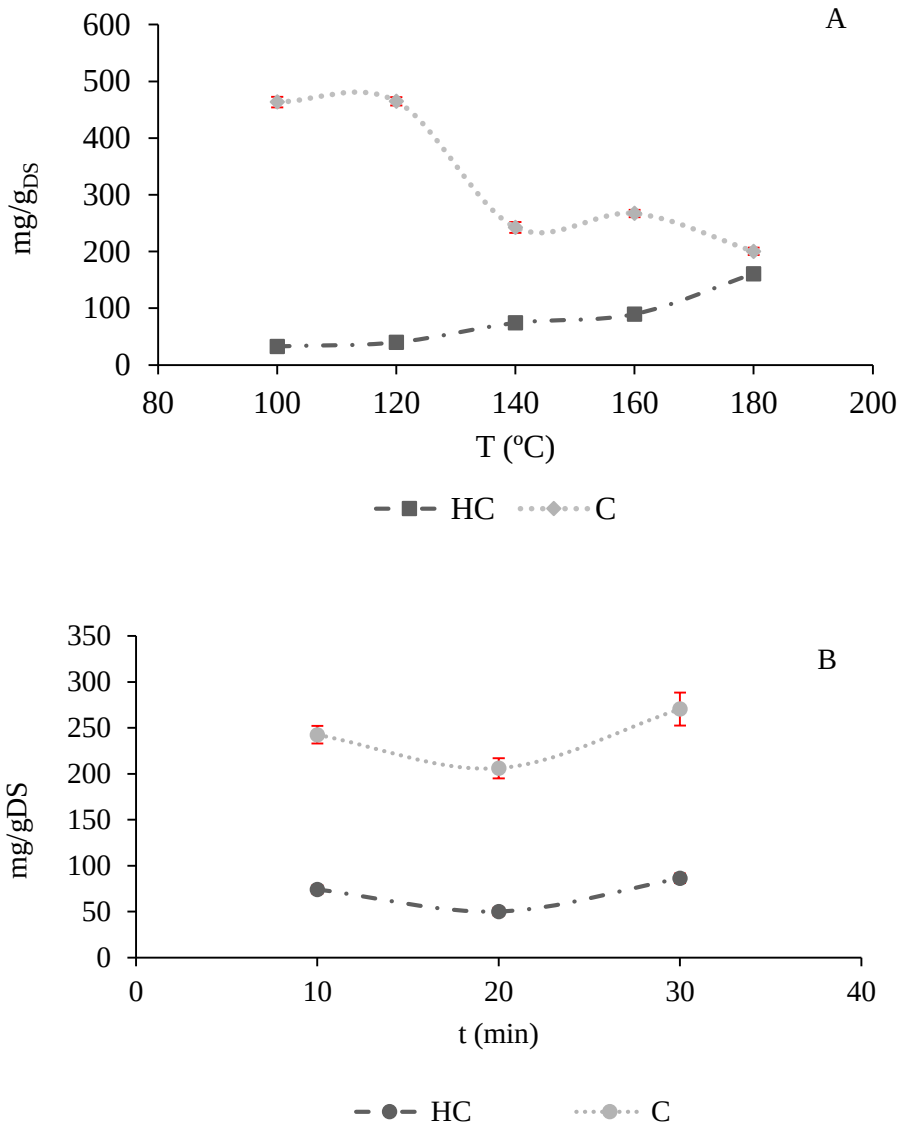
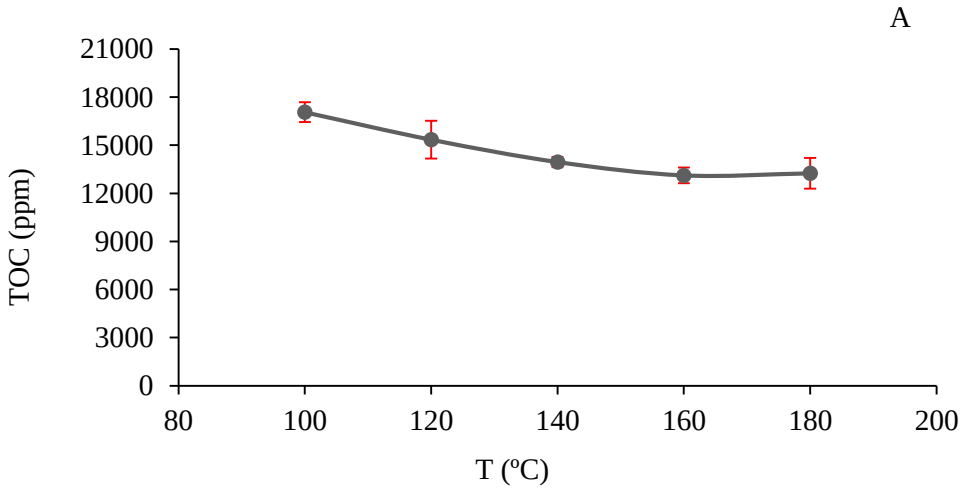


Figure 3: hexoses (HC) and pentoses (C) oligomers concentration evolution with temperature at 20 min of reaction time (A) and with time at 140ºC (B). (Lines to guide the eye)

3.2.3 Overall yield

Total organic Carbon (TOC) was used to measure the purity of the extracts. The role of the operational temperature on the TOC of the extract can be seen in Figure 4.A. The trend displayed in this graph showed a decreasing in the value of the TOC when temperature was raised. This result would not be in line with the expected behaviour since, higher temperatures should promote the extraction. However, considering the high amount of extractives of the raw material, some light can be shed on the results. At the operational conditions, these compounds could degrade into volatiles (like in a pyrolysis process), explaining the decrease of the organic content. This assumption, would be also promoted by the fact that the TOC had a plateau at the highest temperatures (160 and 180ºC), when just the less thermolabile compounds would be present in the extract. Additionally, these results also agreed with the value for the TOC obtained when the preliminary pre-treatment to recover the polyphenols was done (experiment 8) (*see Section 3.2.3).*



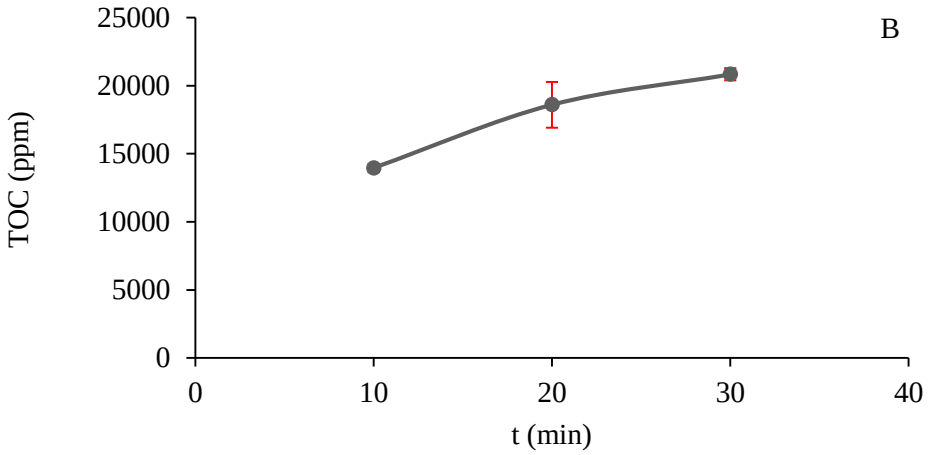


Figure 4: Temperature effect at 20 min and (A) and operating time effect at 140ºC (B) on the Total Organic Carbon content (TOC) of the extract obtained. (Lines to guide the eye)

Concerning the effect of operational time on the TOC, a proportional relation between both was obtained (Figure 4.B). The effect of the time was again considered at 140ºC since this temperature implies the maximum sugars releasing. This result is in line with the explanation performed in *Section 3.2.1*, where it was also obtained that the longer the operational time the higher the yield. Thus, it seems that a higher time would be the best result to maximize the extraction yield (although the increase was just around 12%). However, analysing the trend depicted in Figure 4.B, it is clear that for a time much higher than 30min the extraction is not going to be far more improved.

3.2.4 Effect of the polyphenol recovery on the sugars extraction yield

Since the amount of extractives initially present in the raw material constitutes around 50% of the whole sample, a preliminary recovery of this fraction could make sense. This recovery was done as described in *Section 2.2.2*. After the MW pre-treatment, an extract rich in flavonoids was obtained (~ 45$mg_{CAT}/g_{DRY\ STEM}$).

The effect of this MW pre-treatment can be analysed by the comparison of the values obtained for the experiments 4 and 8, since they were carried out at the same operational conditions (160ºC and 20min). It can be checked that the main effect of the MW treatment was a reduction in the TOC (34%), which agreed with the fact that the

extractives would have been removed, as previously mentioned. Additionally, this would also confirm the statement exposed in *Section 3.2.2* for the decrease of the TOC along temperature due to the extractives thermal degradation (Deng, Yang, Capanoglu, Cao, & Xiao, 2018; Zupančič, Lavrič, & Kristl, 2015). Furthermore, the free sugars of the raw material would have been recovered too, explaining the decrease in the sugar content after the MW pre-treatment (56%). Surprisingly, the oligomer content followed an opposite trend, being higher in the sample treated with the MW (10%). This result could be due to the effect of the MW which could have an effect on the oligomers, disrupting them and improvement their extraction. A similar effect was observed for the sugar hydrolysis due to the athermal effect of MW (Budarin, Clark, Lanigan, Shuttleworth, & Macquarrie, 2010; Fan et al., 2013). This non-thermal effect relies on the alteration of the dielectric camps which could provide a breakdown of the hydrogen bonds of the macromolecules, disrupting the structure of the raw material (Ganzler, Salgó, & Valkó, 1986).

3.2.5 Modelling and fitting

The data showed in section 3.2.1 was fitted by a simplification of a kinetic model previously developed by our research group (A. Cabeza, Piqueras, Sobrón, & García-Serna, 2016; A. Cabeza, Sobrón, Yedro, & García-Serna, 2015). The model used in the present work assumes: (1) a constant porosity, (2) isothermal conditions, (3) a perfect mixture regime and (4) first order kinetics (Pronyk & Mazza, 2010; Rivas, González-Muñoz, Santos, & Parajó, 2014). The reaction path way showed in Figure 5 was also used. This mechanism assumed biomass to decompose into two different families of oligomers and free sugars (reaction 2). The first family would represent the oligomers with a high molecular weight but soluble in water (Olig-SOL) (reaction 1), whereas the second family would be shorter oligomers that can break to produce sugars (Olig-SUG) (reaction 3). The mechanism was completed considering the sugars degradation (reaction 6) and with the glimmers hydrolysis in the liquid phase (reactions 4 and 5). On the other hand, it must be remarked that the pH was not included in the model since the reactor is a batch system, which meant a globalized and constant value for this variable during the process. Otherwise, it should be an independent term included in the kinetics.

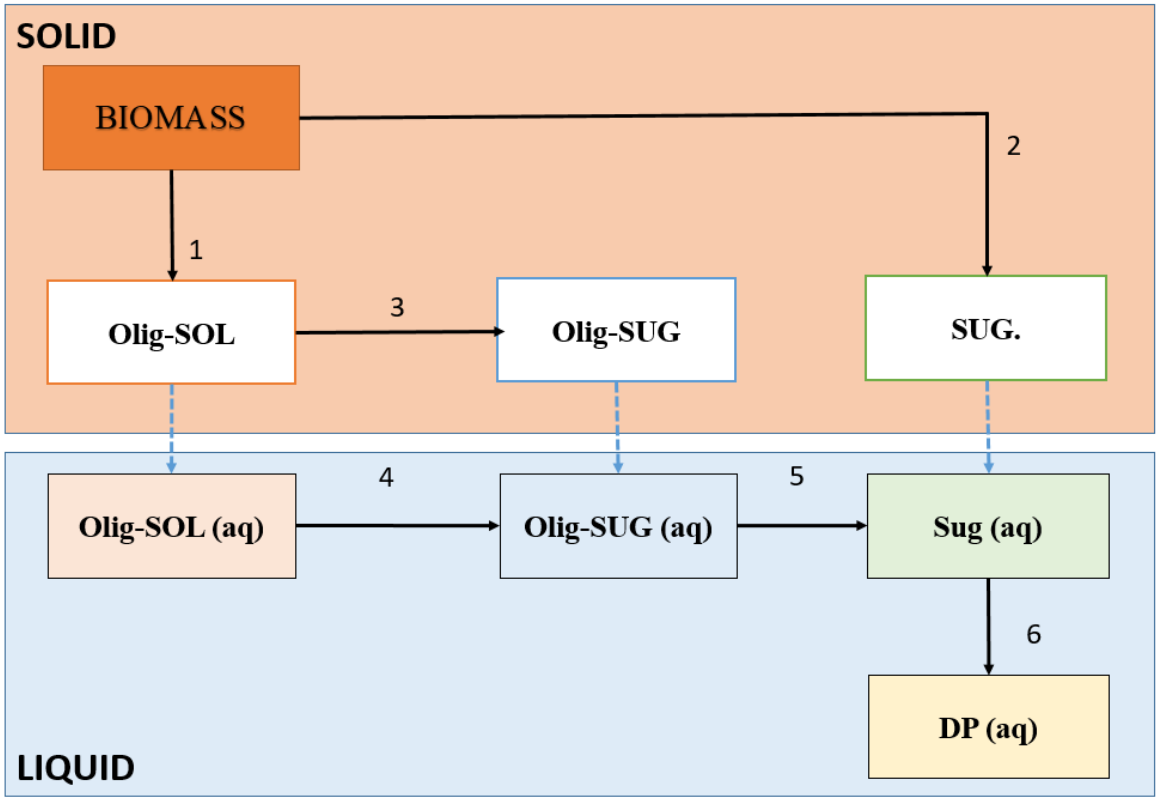


Figure 5: proposed reaction mechanism.

The mathematical model was obtained applying a transient mass balance to each compound in both phases, liquid (Eq. 1) and solid (Eq. 2). It can be checked that both equation are quite similar and they only have three different terms: accumulation $\left(\frac{d_{Cj}}{d_t}\right)$, reaction $\left(r_j\right)$, and mass transfer $\left(k_j \cdot a \cdot (C_j^* - C_j)\right)$. The reaction term was assumed to be a first order kinetic with an Arrhenius' temperature dependence and the mass transfer was defined by an external expression with a linear driving force. This driving force was calculated as the difference between a simulated equilibrium concentration $\left(C_j^*\right)$, in the last layer of liquid in contact with the solid and the globalized concentration in the bulk $\left(C_j\right)$. Finally, this equilibrium concentration was computed using a linear relation between the solid (C_j^s) and the liquid concentrations $\left(C_j^* = H_j \cdot C_j^s\right)$. Thus, H_j was the equilibrium constant between the solid and liquid phases.

$$\frac{dC_j}{dt} = \frac{1}{\varepsilon}\left(r_j + k_j \cdot a \cdot \left(C_j^* - C_j\right)\right) \quad \textbf{(1)}$$

$$\frac{dC_j^s}{dt} = \frac{1}{1-\varepsilon}\left(r_j - k_j \cdot a \cdot \left(C_j^* - C_j\right)\right) \quad \textbf{(2)}$$

Once the model was put forward, it was validated by the minimization of the Absolute Average Deviation (A.A.D.) between the simulated values and the experimental data using as objective function the Eq. 3. The model was solved using the Euler's method (explicit) and the minimization was done using the Solver tool of Excel. The average error for the validation was 14, 15 and 30% for oligomers, sugars and degradation products, respectively. Furthermore, it is visually displayed in Figure 1. It must be remarked that the error for the degradation products was higher due to the low values measured (near zero) at the lowest temperatures.

$$\boldsymbol{A.A.D.} = \sum_{\boldsymbol{i=1}}^{\boldsymbol{n}} \frac{|\boldsymbol{X_{EXP} - X_{SIM}}|}{\boldsymbol{X_{EXP}}} \cdot \mathbf{100}\ [=]\% \qquad \textbf{(3)}$$

On the other hand, it can be checked that the simulated trend for the oligomers along the time does not follow the experimental trend (Figure 2.A). In contrast, it increased up to reach a plateau, which would agree with the expected behavior (Piqueras et al., 2017).

3.2.5.1 Model parameters: mass transfer and kinetics

The mass transfer parameters (equilibrium constant and mass transfer coefficient) are listed in Table 2. Regarding the equilibrium constant (H), it can be observed that it always increased with temperature (as it was expected), following a linear trend (regression coefficients higher than 0.84). Moreover, it can be also checked that the values for the first soluble oligomers (Olig-SOL) and the oligomers before sugar formation (Olig-SUG) were the same at low temperatures. This would mean that both would have a similar length and structure (Kruse & Dinjus, 2007; Miller-Chou & Koenig, 2003; Teo, Tan, Yong, Hew, & Ong, 2010), which also explain the constant value in the oligomer yield for low temperatures displayed in Figure 1.A. On the other hand, the mass transfer coefficient had the same value for all the experiments since the mixing rate was the same in all the cases.

Table 2: mass transfer parameters.

H	Olig-SOL	Olig-SUG	SUG
100	0.079	0.079	0.19
120	0.08	0.081	0.228
140	0.08	0.081	0.235
160	0.08	0.097	0.344
180	0.081	0.105	0.419
R^2	0.93	0.84	0.91
k_a	**Olig-SOL**	**Olig-SUG**	**SUG**
	0.076	0.076	0.256

Concerning the kinetics, they are arrayed in Table 3. For the reactions that took place in the solid (reactions from 1 to 3), it can be checked that the cleavage of the biomass into oligomers was faster than the releasing of sugars (A. Cabeza et al., 2016), as it was expected. Astonishingly, the activation energy for the degradation of the Olig-SOL was lower than the values for the initial biomass, which would not make sense since the Olig-SOL should be shorter than the biomass. However, the pre-exponential factor for these oligomers was 2 orders of magnitude lower too. Which finally led to a lower oligomers' kinetics.

Table 3: kinetic parameters.

k	**Reaction 1** *(BIOMASS to Olig-SOL)*	**Reaction 3** *(Olig-SOL to Olig-SUG)*	**Reaction 2** *(BIOMASS to Sugars)*
E_a/R	1,577	1,157	2,910
$\ln(k_o)$	1.18	0.06	3.2
k_{aq}	**Reaction 4** *(Olig-Sol to Olig-SUG)*	**Reaction 5** *(Olig-SUG to Sugars)*	**Reaction 6** *(Sugars to degradation products)*
E_a/R	1,015	3,981	9,641
$\ln(k_o)$	0.09	4.32	16.98

Finally, for the aqueous reaction, it can be perceived that the cleavage of the Olig-SOL was easier, which was awaited since the hydrolysis would be promoted by the protons released into the water due to the hemicellulose deacetylation (Gil Garrote, Domínguez, & Parajó, 2002; Parajó, Garrote, Cruz, & Dominguez, 2004). With regards

to degradation of the Olig-SUG and the sugars, their kinetics had a far lower value than those related to the break of the Olig-SOL. This result could be explained by the lower length of the Olig-SUG and the sugars, which would make them more sensitive to the proton concentration. Since the length of the Olig-SUG and the sugars would be lower, a higher activation energy would be required to degrade them. Explaining why the catalytic effect of the protons would have a higher effect on these two compounds. And, as it was explained in Section 3.2.1, it was not high enough to promote sugar production and their degradation (Figure 2).

4. Conclusions

This work assessed the extraction of the holocellulosic fraction of grape stems by a hydrothermal treatment with a yield of 67%.Temperature and time were studied as the key operational variables, obtaining that 140ºC was the most suitable condition if sugars are the target compounds. Regarding the operating time, the longer it was, the higher extraction yields were achieved (oligomers, sugars and carbon content). Additionally, an unexpected behaviour was observed, since the extract was mainly composed of hexoses based compounds. Which was not awaited at the tested condition since, in principle, they were too mild to fractionate cellulose.

On the other hand, a kinetic model for this biomass conversion was proposed and validated, being able to reproduce the experimental data with deviations lower than 30% for all the cases.

References

Abatzoglou, N., Chornet, E., Belkacemi, K., & Overend, R. P. (1992). Phenomenological kinetics of complex systems: the development of a generalized severity parameter and its application to lignocellulosics fractionation. *Chemical Engineering Science*, *47*(5), 1109–1122.

Aristidou, A., & Penttilä, M. (2000). Metabolic engineering applications to renewable resource utilization. *Current Opinion in Biotechnology*, *11*(2), 187–198.

Azhar, S., Henriksson, G., Theliander, H., & Lindström, M. E. (2015). Extraction of hemicelluloses from fiberized spruce wood. *Carbohydrate Polymers*, *117*, 19–24.

Bertran, E., Sort, X., Soliva, M., & Trillas, I. (2004). Composting winery waste: sludges and grape stalks*1. *Bioresource Technology*, *95*(2), 203–208.

Besson, M., Gallezot, P., & Pinel, C. (2014). Conversion of Biomass into Chemicals over Metal Catalysts. *Chemical Reviews*, *114*(3), 1827–1870.

Bobleter, O. (1994). Hydrothermal degradation of polymers derived from plants. *Progress in Polymer Science*, *19*(5), 797–841.

Budarin, V. L., Clark, J. H., Lanigan, B. A., Shuttleworth, P., & Macquarrie, D. J. (2010). Microwave assisted decomposition of cellulose: A new thermochemical route for biomass exploitation. *Bioresource Technology*, *101*(10), 3776–3779.

Cabeza, A., Piqueras, C. M., Sobrón, F., & García-Serna, J. (2016). Modeling of biomass fractionation in a lab-scale biorefinery: Solubilization of hemicellulose and cellulose from holm oak wood using subcritical water. *Bioresource Technology*, *200*, 90–102.

Cabeza, A., Santos, D. T., Meireles, M. A. A., Sobrón, F., Cocero Alonso, M. J., & García-Serna, J. (2017). Pretreatment Effect on the Thermal Degradation of a Feedstock with Low Hemicellulose Content: Brazilian Ginseng. *Energy & Fuels*, *31*(7), 7123–7131.

Cabeza, A., Sobrón, F., Yedro, F. M., & García-Serna, J. (2015). Two-phase modelling and simulation of the hydrothermal fractionation of holm oak in a packed bed reactor with hot pressurized water. *Chemical Engineering Science*, *138*, 59–70.

Carvalheiro, F., Duarte, L. C., & Gírio, F. M. (2008). Hemicellulose biorefineries: a

review on biomass pretreatments. *Journal of Scientific & Industrial Research*, 849–864.

Chen, X., Lawoko, M., & Heiningen, A. van. (2010). Kinetics and mechanism of autohydrolysis of hardwoods. *Bioresource Technology*, *101*(20), 7812–7819.

Cherubini, F. (2010). The biorefinery concept: Using biomass instead of oil for producing energy and chemicals. *Energy Conversion and Management*, *51*(7), 1412–1421.

Chupin, L., Maunu, S. L., Reynaud, S., Pizzi, A., Charrier, B., & Charrier-EL Bouhtoury, F. (2015). Microwave assisted extraction of maritime pine (Pinus pinaster) bark: Impact of particle size and characterization. *Industrial Crops and Products*, *65*, 142–149.

Cocero, M. J., Cabeza, Á., Abad, N., Adamovic, T., Vaquerizo, L., Martínez, C. M., & Pazo-Cepeda, M. V. (2018a). Understanding biomass fractionation in subcritical & supercritical water. *The Journal of Supercritical Fluids*, *133*, 550–565.

Cocero, M. J., Cabeza, Á., Abad, N., Adamovic, T., Vaquerizo, L., Martínez, C. M., & Pazo-Cepeda, M. V. (2018b). Understanding biomass fractionation in subcritical & supercritical water. *The Journal of Supercritical Fluids*, *133*, 550–565.

Deng, J., Yang, H., Capanoglu, E., Cao, H., & Xiao, J. (2018). Technological aspects and stability of polyphenols. *Polyphenols: Properties, Recovery, and Applications*, 295–323.

Devesa-Rey, R., Vecino, X., Varela-Alende, J. L., Barral, M. T., Cruz, J. M., & Moldes, A. B. (2011). Valorization of winery waste vs. the costs of not recycling. *Waste Management*, *31*, 2327–2335.

Eero Sjöström. (1993). *Wood Chemistry, Fundamental and Applications*. Elsevier.

Fan, J., De bruyn, M., Zhu, Z., Budarin, V., Gronnow, M., Gomez, L. D., Clark, J. (2013). Microwave-enhanced formation of glucose from cellulosic waste. *Chemical Engineering and Processing: Process Intensification*, *71*, 37–42.

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

Froschauer, C., Hummel, M., Iakovlev, M., Roselli, A., Schottenberger, H., & Sixta, H. (2013). Separation of Hemicellulose and Cellulose from Wood Pulp by Means of Ionic Liquid/Cosolvent Systems. *Biomacromolecules*, *14*(6), 1741–1750.

Gallina, G., Alfageme, E. R., Biasi, P., & García-Serna, J. (2018). Hydrothermal extraction of hemicellulose: from lab to pilot scale. *Bioresource Technology*, *247*, 980–991.

Gallina, G., Cabeza, Á., Biasi, P., & García-Serna, J. (2016). Optimal conditions for hemicelluloses extraction from Eucalyptus globulus wood: hydrothermal treatment in a semi-continuous reactor. *Fuel Processing Technology*, *148*, 350–360.

Gallina, G., Cabeza, Á., Grénman, H., Biasi, P., García-Serna, J., & Salmi, T. (2018). Hemicellulose extraction by hot pressurized water pretreatment at 160 ºC for 10 different woods: Yield and molecular weight. *The Journal of Supercritical Fluids*, *133*, 716–725.

Ganzler, K., Salgó, A., & Valkó, K. (1986). Microwave extraction : A novel sample preparation method for chromatography. *Journal of Chromatography A*, *371*, 299–306.

Garrote, G., Domínguez, H., & Parajó, J. C. (1999). Hydrothermal processing of lignocellulosic materials. *Holz Als Roh- Und Werkstoff*, *57*(3), 191–202.

Garrote, G., Domínguez, H., & Parajó, J. C. (2002). Interpretation of deacetylation and hemicellulose hydrolysis during hydrothermal treatments on the basis of the severity factor. *Process Biochemistry*, *37*(10), 1067–1073.

Hamelinck, C. N., Hooijdonk, G. van, & Faaij, A. P. (2005). Ethanol from lignocellulosic biomass: techno-economic performance in short-, middle- and long-term. *Biomass and Bioenergy*, *28*(4), 384–410.

Hon, D. N.-S. (1994). Cellulose: a random walk along its historical path. *Cellulose*, *1*(1), 1-25.

Kruse, A., & Dinjus, E. (2007). Hot compressed water as reaction medium and reactant: Properties and synthesis reactions. *The Journal of Supercritical Fluids*, *39*(3), 362–380.

Li, H.-Q., Jiang, W., Jia, J.-X., & Xu, J. (2014). pH pre-corrected liquid hot water

pretreatment on corn stover with high hemicellulose recovery and low inhibitors formation. *Bioresource Technology*, *153*, 292–299.

Lorenzo, M., Moldes, D., Rodríguez Couto, S., & Sanromán, A. (2002). Improving laccase production by employing different lignocellulosic wastes in submerged cultures of Trametes versicolor. *Bioresource Technology*, *82*(2), 109–113.

Magalhães da Silva, S. P., Morais, A. R. C., & Bogel-Łukasik, R. (2014). The CO_2-assisted autohydrolysis of wheat straw. *Green Chem.*, *16*(1), 238–246. G

Martínez, M., Miralles, N., Hidalgo, S., Fiol, N., Villaescusa, I., & Poch, J. (2006). Removal of lead(II) and cadmium(II) from aqueous solutions using grape stalk waste. *Journal of Hazardous Materials*, *133*(1–3), 203–211.

Miller-Chou, B. A., & Koenig, J. L. (2003). A review of polymer dissolution. *Progress in Polymer Science*, *28*(8), 1223–1270.

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

Parajó,J., Garrote,G., Cruz,J., and Dominguez,H. (2004). Production of xylo-oligosaccharides by autohydrolysis of lignocellulosic materials. *Trends in Food Science & Technology*, *15*(3–4), 115–120.

Pedras, B., Salema-Oom, M., Sá-Nogueira, I., Simões, P., Paiva, A., & Barreiros, S. (2017). Valorization of white wine grape pomace through application of subcritical water: Analysis of extraction, hydrolysis, and biological activity of the extracts obtained. *The Journal of Supercritical Fluids*, *128*, 138–144.

Piñeiro, Z., Guerrero, R. F., Fernández-Marin, M. I., Cantos-Villar, E., & Palma, M. (2013). Ultrasound-Assisted Extraction of Stilbenoids from Grape Stems. *Journal of Agricultural and Food Chemistry*, *61*(51), 12549–12556.

Piqueras, C. M., Cabeza, Á., Gallina, G., Cantero, D. A., García-Serna, J., & Cocero, M. J. (2017). Online integrated fractionation-hydrolysis of lignocellulosic biomass using sub- and supercritical water. *Chemical Engineering Journal*, *308*, 110–125.

Pronyk, C., & Mazza, G. (2010). Kinetic Modeling of Hemicellulose Hydrolysis from Triticale Straw in a Pressurized Low Polarity Water Flow-Through Reactor.

Industrial & Engineering Chemistry Research, *49*(14), 6367–6375.

Prozil, S. O., Evtuguin, D. V., & Lopes, L. P. C. (2012). Chemical composition of grape stalks of Vitis vinifera L. from red grape pomaces. *Industrial Crops and Products*, *35*(1), 178–184.

Relvas, F. M., Morais, A. R. C., & Bogel-Lukasik, R. (2015). Kinetic modeling of hemicellulose-derived biomass hydrolysis under high pressure CO2–H2O mixture technology. *The Journal of Supercritical Fluids*, *99*, 95–102.

Rivas, S., González-Muñoz, M. J., Santos, V., & Parajó, J. C. (2014). Acidic processing of hemicellulosic saccharides from pine wood: Product distribution and kinetic modeling. *Bioresource Technology*, *162*, 192–199.

Rogalinski, T., Ingram, T., & Brunner, G. (2008). Hydrolysis of lignocellulosic biomass in water under elevated temperatures and pressures. *The Journal of Supercritical Fluids*, *47*(1), 54–63.

Romero-Díez, R., Matos, M., Rodrigues, L., Bronze, M. R., Rodríguez-Rojo, S., Cocero, M. J., & Matias, A. A. (2018). Microwave and ultrasound pre-treatments to enhance anthocynins extraction from different wine lees. *Food Chemistry*, *272*, 258–266.

Romero, A., Alonso, E., Sastre, Á., & Nieto-Márquez, A. (2016). Conversion of biomass into sorbitol: Cellulose hydrolysis on MCM-48 and d-Glucose hydrogenation on Ru/MCM-48. *Microporous and Mesoporous Materials*, *224*, 1–8.

Rubio Torres, M., Heitz, M., Chauvette, G., Chornet, E., & Overend, R. P. (1986). Conversion and solubilization profiles of a prototype hardwood (Populus tremuloides) following aqueous thermomechanical pre-treatment. *Biomass*, *10*(2), 85–96.

Ruggieri, L., Cadena, E., Martínez-Blanco, J., Gasol, C. M., Rieradevall, J., Gabarrell, X., Sánchez, A. (2009). Recovery of organic wastes in the Spanish wine industry. Technical, economic and environmental analyses of the composting process. *Journal of Cleaner Production*, *17*(9), 830–838.

Ruiz-Moreno, M. J., Raposo, R., Cayuela, J. M., Zafrilla, P., Piñeiro, Z., Moreno-Rojas, J. M., … Cantos-Villar, E. (2015). Valorization of grape stems. *Industrial Crops and Products*, *63*, 152–157. h

Sánchez-Bastardo, N., Romero, A., & Alonso, E. (2017). Extraction of arabinoxylans from wheat bran using hydrothermal processes assisted by heterogeneous catalysts. *Carbohydrate Polymers*, *160*, 143–152.

Sauter, J., & Grafmüller, A. (2015). Solution Properties of Hemicellulose Polysaccharides with Four Common Carbohydrate Force Fields. *Journal of Chemical Theory and Computation*, *11*(4), 1765–1774.

Sluiter, a, Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., & Templeton, D. (2008). *Determination of Sugars , Byproducts , and Degradation Products in Liquid Fraction Process Samples Laboratory Analytical Procedure (LAP).*

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., & Templeton, D. (2004). *Determination of structural carbohydrates and lignin in biomass. NREL Biomass analysis Technology Team, Laboratory Analytical Procedure (LAP).*

Spigno, G., Pizzorno, T., & De Faveri, D. M. (2008). Cellulose and hemicelluloses recovery from grape stalks. *Bioresource Technology*, *99*(10), 4329–4337.

Teixeira, A., Baenas, N., Dominguez-Perles, R., Barros, A., Rosa, E., Moreno, D. A., & Garcia-Viguera, C. (2014). Natural bioactive compounds from winery by-products as health promoters: a review. *International Journal of Molecular Sciences*, *15*(9), 15638–78.

Teo, C. C., Tan, S. N., Yong, J. W. H., Hew, C. S., & Ong, E. S. (2010). Pressurized hot water extraction (PHWE). *Journal of Chromatography A*, *1217*(16), 2484–2494.

Villaescusa, I., Fiol, N., Martínez, M., Miralles, N., Poch, J., & Serarols, J. (2004). Removal of copper and nickel ions from aqueous solutions by grape stalks wastes. *Water Research*, *38*(4), 992–1002.

Yedro, F. M., Grénman, H., Rissanen, J. V., Salmi, T., García-Serna, J., & Cocero, M. J. (2017). Chemical composition and extraction kinetics of Holm oak (Quercus ilex) hemicelluloses using subcritical water. *The Journal of Supercritical Fluids*, *129*, 56–62.

Zupančič, Š., Lavrič, Z., & Kristl, J. (2015). Stability and solubility of trans-resveratrol are strongly influenced by pH and temperature. *European Journal of Pharmaceutics and Biopharmaceutics*, *93*, 196–204.

CONCLUSIONS

Conclusions

This thesis is a contribution to the valorization of winery waste (or by-products) within the framework of the biorefinery concept. The work is focused in the polyphenolic fraction recovery of two underexploited residues the wine lees and grape stems. Additionally, the use of the latter as lignocellulosic feedstock for the production of a sugar rich extract was also addressed. Alternative and greener intensification processes, some of them which have never been applied to these residues, were proposed and evaluated. The main conclusions of this work according to the obtained results, taking into consideration the objectives proposed at the beginning, are exposed below.

Wine lees

Wine lees characterization

- Extracts rich in polyphenols were obtained from aging wine lees via solid-liquid extractions with solvents of different polarity. The hydroalcoholic mixture with a 75% vol. ethanol seemed to be the most suitable solvent for the recovery of phenolic compounds and consequently, the one which maximizes the antioxidant activities.

- Relative quantification of the polyphenols which significantly contributed ($p<0.10$) to the different antioxidant activities was performed via HPLC peak areas. A total of 12 compounds were identified, among them 6 were anthocyanins, 2 flavonols, 2 Flavan-3-ol and 1 phenolic compound.

- Correlations between extracted phenolic compounds and the different antioxidant activities were obtained. Significant correlations were found for those anthocyanins with 6-p-coumaroyl moiety and FRAP assay, as well as, the phenolic acid and the two flavan-3-ols detected. Anthocyanins also contributed positively (but not significantly) to HOSC.

Maximization of the extraction of polyphenols

- From the study of the kinetic curves for solid-liquid extraction for anthocyanins, the parameters selected as the best in order to enhance the extraction of

anthocyanins were a temperature of 25ºC, with a R_{S-L} of 0.10 (g/mL) and with a 50%vol. ethanol mixture. At these conditions a final steady anthocyanin concentration of 2.78mg_{MALE}/g_{DL} was achieved for Port wine lees after 15min of extraction. First fermentation and second fermentation wine lees gave final anthocyanin yields of 3.04mg_{MALE}/g_{DL} and 2.09mg_{MALE}/g_{DL}, respectively.

- The effect of several pre-treatments were evaluated on the final anthocyanin extraction yields:

    - Microwave (MW) pre-treatment significantly increased ($p<0.05$) the recovery of anthocyanins from all wine lees types. The optimum MW conditions that maximize the anthocyanin yield were: a hydro-alcoholic mixture of 40% vol. ethanol, a R_{S-L} of 0.140 (g/mL) and a time pre-treatment of 90s. At these conditions, AC extraction yield was doubled for Port wine lees (6.20mg_{MALE}/g_{DL}) and operational was reduced 10 fold (from 15min to 90s). At the same conditions, anthocyanin yield of 4.45mg_{MALE}/g_{DL} and 2.88mg_{MALE}/g_{DL} were achieved for first fermentation and second fermentation wine lees, respectively.

    - Ultrasound (US) did not seemed to be a suitable pre-treatment for the recovery of anthocyanins. Results revealed that the increment on the final anthocyanin yield obtained by US pre-treatment were not significant different respect to the conventional extraction yield ($p<0.05$).

    - Enzymatic hydrolysis was tested with Glucanex and Mannaway enzymes, as well as with a blend of both enzymes. Data showed that an incubation time of 5min was enough since no significant differences were obtained ($p<0.05$) among the other times. The addition of ethanol was crucial for the anthocyanin recovery. Best anthocyanin extraction yield was achieved when the enzymatic blend was employed (4.65mg_{MALE}/g_{DL}).

- The antioxidant activities of the extracts derived from these pre-treatments revealed higher ORAC values respect to the conventional extracts. When MW

pre-treatment was applied, a pyranoanthocyanin was identified in all wine lees types.

Grape stems

Maximization of the extraction of polyphenols

- From the study of the kinetic curves for solid-liquid extraction for anthocyanins, the parameters selected as the best in order to boost the extraction of total polyphenol and flavonoid contents were: a temperature of 75ºC, a R_{S-L} of 1/10 (g/mL) and with a 50%vol. ethanol mixture. At these conditions final polyphenol and flavonoid concentrations of 38.2 ± 1.0 mg_{GAE}/g_{DS} and 37.6 ± 1.5 mg_{CATE}/g_{DS} were obtained, respectively, after a 60min extraction.

- MW pre-treatment was also studied for grape stems. In this case variables that maximize the extraction of total polyphenols were a R_{S-L} of 0.50g/mL, a solvent mixture with a 55% vol. ethanol and a pre-treatment time of 36s. At this conditions, an extract with a 45 mg_{GAE}/g_{DS} was obtained. On the other hand for the maximization of flavonoids a R_{S-L} of 0.10g/mL, a solvent mixture with a 47% vol. ethanol and a pre-treatment time of 86s were required (47mg_{CATE}/g_{DS}).

- Grape stem extract rich in flavonoids obtained when the MW pre-treatment was applied, showed a great significant ($p<0.05$) antioxidant (1056 ± 56 $\mu mol_{TE}/g_{DS}$) and anti-fungal activity (inhibition of the fungus in a 46% respect to the blank).

Conversion of grape stems into oligomers and sugars

- The assessment of the extraction of the holocellulosic fraction of grape stems by a hydrothermal treatment was evaluated. A temperature of 140ºC was the most suitable for the recovery of sugars. Regarding the operating time, the longer (from 10 to 30min) the higher extraction yields were achieved (oligomers, sugars and total carbon content). An unpredicted trend was achieved since the extract was mainly composed of hexoses despite the mild processing conditions.

- Apart from removing the polyphenols compounds, as well as the free sugars of the raw material, the MW treatment could have affected the oligomers, disrupting them and improvement their extraction (10%).

- The kinetic model proposed for this biomass conversion was validated with an average absolute deviation around 15% for sugars and oligomers and 30% for degradation products.

FUTURE WORK

Future work

Supplementary studies could be performed following the work developed in this thesis.

- The extractions for the recovery of polyphenols from both residues could be carried out without previously dried the raw material. This study will give an idea about the recovery extraction yields depending on the drying of the material and from an economic point of view. In this case, apart from the pre-treatments already studied in this thesis, solvent-free MW pre-treatment could be tested in order to compare the polyphenols extraction yields.
- Regarding the enzymatic hydrolysis, further work should be done to have more insight in the action of the enzymes. Different enzymes could be assessed, as well as different enzymes concentrations per substrate mass. Complementary analysis such as total phenolic (TPC) and flavonoid (TFC) content and possible β-glucans and mannoproteins released. Besides the evaluation of the performance of the enzymatic hydrolysis to wet wine lees would be also significant.
- Application of another extraction procedure which has never been applied to wine lees, in order to enhance the internal mass transfer. High hydrostatic pressure (HHP) is a promising alternative of which potential relies on the enhancement of the mass transfer increasing cell permeability. It could improve the recovery of bioactive from wine lees, since it has been successfully implemented for the cell disruption of *Saccharomyces cerevisae* for the release of internal substances.
- Concerning the characterization of the extracts, deeper identification of compounds could be perform (not only anthocyanins). For the case of grape stems, identification of other compounds which seemed to appear in higher concentrations, apart from the flavonoids and stilbenes, would we necessary. Similarly, more fungus can be tested in order to compare the anti-fungal activity of grape stem extracts in comparison with commercialised products from natural origin with well-established anti-fungal properties.
- Since it has been proved the great antioxidant capacity of both wine lees and grape stems extracts, different encapsulation processes could be assessed in order to protect these bioactive compounds along time. Furthermore, this encapsulation or coating would provide the bioactive compounds higher absorption or release in the a huge range of applications (cosmetic, pharmaceutical, alimentary, etc)

- For the case of the conversion of grape stems into oligomers and sugars, the hydrothermal process could be done together with some catalysts which would improve and accelerate the conversion. Moreover, it would be of high relevance measured the molecular weight of the released oligomers due to wide range of applications.
- Techno-economic evaluation in order to explore the viability of the optimized parameters and the proposed bio-refinery concept

RESUMEN

INTRODUCCIÓN

La industria vinícola es uno de los sectores más importantes de la economía de la Unión Europea. Esto hace que la uva uno de los frutos más cultivados en todo el mundo. Se calcula que alrededor de 60 millones de toneladas son producidas anualmente, de las cuales el 80% se usan para la elaboración de vino. Uno de los problemas asociados con esta actividad es la gran cantidad de residuos que se generan, principalmente durante los procesos de prensado y fermentación del vino. Sólo en España, se generan anualmente alrededor de unos 2-3 millones de toneladas de residuos. En otras palabras, se producen 25kg de subproductos por cada 100L de vino tinto. Dependiendo de la etapa en que la se forman, pueden distinguirse diferentes residuos. Entre los residuos más abundantes se encuentra el orujo de uva ya que representa el 62% de los residuos totales generados en una bodega. El orujo de uva está constituido por las semillas, pieles y pulpa de uva y se obtiene después de la trituración de las uvas para obtener el mosto. El segundo residuo más abundante son lías, que representan un 14%. Las lías son los posos que se forman por decantación en la parte inferior de los recipientes durante las etapas de fermentación o procesos del envejecimiento del vino. Por último, y en menor cantidad, se encuentran los tallos o raspón de uva y las aguas residuales. Cada uno de estos residuos representa un 12%. El raspón de uva, constituye el esqueleto leñoso del racimo de uva y se obtienen tras el proceso de despalillado, justo antes del prensado de la uva. El raspón de uva, al igual que el orujo de uva, tiene una composición lignocelulósica. Esto significa que, aparte de su alto contenido de compuestos fenólicos; celulosa, hemicelulosa y lignina están presentes en concentraciones relativamente altas (hasta 25%). En consecuencia, el raspón de uva también constituye una fuente rica de estos polímeros que pueden ser, a su vez, hidrolizados en sus componentes monoméricos. Y, posteriormente, convertirlos en productos de alto valor añadido tales como combustibles, productos químicos y materiales. Finalmente, las aguas residuales provienen de los procesos de lavado y desinfección llevados a cabo en la bodega.

Aunque estos residuos se consideran como residuos "no peligrosos", presentan una alta carga orgánica. Por lo tanto, si no se tratan adecuadamente pueden causar varios problemas ambientales que son efectos fito-tóxicos y/o acidificación del suelo. Este elevado contenido de materia orgánica, aparte de otros compuestos, se debe en gran parte a su alta concentración de polifenoles. Los polifenoles son compuestos

caracterizados por poseer más de un anillo aromático con varios grupos hidroxilo como sustituyentes. Los polifenoles presentan efectos beneficiosos para la salud debido a sus propiedades antioxidantes, antimicrobianas, antiinflamatorias y cardioprotectivas. Gracias a estas propiedades, estos compuestos tienen un alto valor añadido y pueden ser utilizados en industrias como la alimenticia y farmacéutica, así como aditivos cosméticos. Debido a esto, son muchos los procesos han sido desarrollados para la recuperación de polifenoles a partir de los subproductos de uva, especialmente del orujo de uva. Sin embargo, son pocos los trabajos en los que se estudie la extracción de estos compuestos de alto valor añadido a partir de lías o del raspón. Además, como se ha mencionado anteriormente, el raspón de uva es una fuente rica de los biopolímeros y azúcares, los cuales que pueden ser utilizados para producir otros químicos y productos derivados. Por todo ello, esta tesis se centra en la revalorización de lías de vino y raspón de uva. Para llevarlo a cabo, se han empleado tecnologías alternativas cuya finalidad es promover la sostenibilidad. La idea general era maximizar e intensificar la extracción de estos compuestos de alto valor añadido a partir de estos dos residuos en términos de polifenoles. También se ha propuesto una revalorización del raspón de uva en términos de azúcares y oligómeros.

OBJETIVOS

El objetivo general de esta tesis es la **revalorización** de dos residuos procedentes del proceso de vinificación, principalmente en cuanto a polifenoles se refiere. Estos residuos son **las lías y el raspón de uva,** los cuales han sido parcamente estudiados en comparación con otros subproductos como lo es el orujo de uva.

Lías de vino

Se va a trabajar con lías de las diferentes etapas de vinificación y diferentes procedimientos enológicos. Las antocianinas, serán los compuestos de interés ya que son los compuestos mayoritarios. Con el fin de maximizar y desarrollar un proceso adecuado para su recuperación se abordarán los siguientes objetivos específicos.

- **Evaluación de lías de vino** como una fuente rica de compuestos con gran actividad antioxidante contra diferentes tipos de radicales libres. Establecer correlaciones entre el diferente compuesto fenólicos, principalmente antocianinas, y cada actividad antioxidante evaluada.
- Estudio de las **cinéticas de extracción** de antocianinas. Optimización de los parámetros principales: la relación sólido-líquido (R_{S-L}), la composición del

solvente y temperatura. Obtención del **conjunto de variables que favorecen** la extracción de antocianinas.

- Aplicación de tratamientos previo a la extracción sólido-líquido convencional para favorecer la recuperación de antocianinas: **microondas (MW) y ultrasonido (US).** Estudio de los parámetros importantes que rigen estos procesos ($R_{S\,L}$, composición solvente y tiempo de tratamiento previo) acorde a un estudio estadístico (diseño superficie respuesta). Obtención de las variables **óptima MW y US** que maximizan el proceso.
- Aplicación de las condiciones óptimas de MW y US, seguidas de una extracción sólido-líquido convencional (en las mejores condiciones seleccionadas previamente).
- Aplicación de **hidrólisis enzimática** a las lías del vino. Evaluación de enzimas diferentes y tiempos de incubación en la liberación de polifenoles.
- **Caracterización de extractos**
  - Contenido de antocianinas o polifenoles totales.
  - Identificación y cuantificación de los principales compuestos (HPLC).
  - Actividad antioxidante (*en vitro*): ORAC, HORAC, HOSC y/o FRAP.

Raspón de uva

Recuperación de polifenoles de uva, especialmente flavonoides y estilbenos.

- Estudio de las **cinéticas de extracción** de polifenoles y flavonoides mediante extracciones convencionales. Optimización de los parámetros principales: la relación sólido-líquido (R_{S-L}), la composición del solvente y temperatura. Obtención del **conjunto de variables que favorecen** la extracción de antocianinas.
- Aplicación de microondas como un tratamiento previo a la extracción sólido-líquido convencional**.** Estudio de los parámetros importantes que rigen estos procesos ($R_{S\,L}$, composición solvente y tiempo de tratamiento previo) acorde a un estudio estadístico (diseño superficie respuesta). Obtención de las variables de **MW óptima** que maximiza la extracción de flavonoides y polifenoles totales.
- Aplicación de las condiciones óptimas de MW, seguido de una extracción sólido-líquido convencional (en las mejores condiciones seleccionadas previamente).

- **Caracterización de extractos**
    - Contenido de antocianinas o polifenoles totales.
    - Identificación y cuantificación de los principales compuestos (HPLC).
    - Actividad antioxidante (*en vitro*): ORAC.
    - Actividad anti-hongos: *Botrytis cinarea*.

Con respecto a la extracción de azúcares y oligómeros, se seguirá el proceso descrito:

- Se utilizará **un procedimiento de conversión hidrotermal** para la conversión de la biomasa en azúcares y oligómeros. La temperatura y tiempo de operación serán las variables de estudio.
- Se evaluará e**l efecto de un tratamiento previo de MW** para recuperar los polifenoles en rendimiento de extracción de los azúcares.
- Se propondrá un **modelo cinético** para ajustar los datos experimentales.
- **Caracterización de extractos**
    - Determinación de pH.
    - Determinación de azúcares por HPLC y oligómeros.
    - Determinación de productos de degradación de.
    - Pureza del extracto.
    - Caracterización de producto líquido: relación/pentosas hexosas

PLANTEAMIENTO DE LA TESIS

Para llevar a cabo esta tesis, se han desarrollado un total de cinco capítulos. En el **Capítulo I** se ha procedido a la identificación de los principales compuestos fenólicos presentes en extractos de lías de envejecimiento. Los compuestos identificados fueron mayoritariamente antocianinas. En este mismo capítulo, se establecieron correlaciones entre las diferentes actividades antioxidantes estudiadas y los diferentes compuestos identificados. En los **Capítulos II y III**, se han estudiado las cinéticas de extracción de antocianinas en los diferentes tipos de lías de vino. Tras varias extracciones convencionales, las condiciones (relación sólido-líquido, composición del solvente y temperatura) que favorecían la extracción de antocianinas fueron obtenidas. Una vez obtenidos estos parámetros, se aplicaron varios tratamientos previos a la extracción convencional sólido-líquido convencional con el fin de intensificar dicho proceso. Para llevar a cabo estos pretratamientos, se emplearon irradiación de microondas (MW),

ultrasonidos (US) y enzimas. Como en el capítulo anterior, se determinó la actividad antioxidante de los diferentes extractos obtenidos. De igual modo, los principales compuestos presentes en los extractos fueron identificados. En cuanto a la revalorización del raspón de uva, se desarrolló en los capítulos IV y V. En el **Capítulo IV**, se estudiaron las cinéticas de extracción de polifenoles totales y flavonoides de la misma manera que se hizo en las lías del vino. Del mismo modo, la radiación por MW fue aplicada como un pretratamiento con el fin de mejorar la extracción de polifenoles. Por otro lado, en el **Capítulo V** se llevó a cabo la conversión del raspón de uva en extractos ricos en azúcares y oligómeros mediante una hidrólisis hidrotermal. La temperatura y tiempo de operación fueron las variables estudiadas en este capítulo.

Capítulo I. Caracterización fenólica de lías de envejecimiento: correlación con actividades antioxidantes

En el **Capítulo I** se centró en la caracterización de lías de envejecimiento en términos de contenido total de polifenoles (TPC, como equivalentes de ácido gálico GAE) y contenido total de flavonoides (TFC, como equivalentes de catequina, CAE). Las lías de envejecimiento se obtienen del fondo de las barricas de madera en las que tiene lugar el envejecimiento del vino. Las lías fueron centrifugadas y una vez que se obtuvo la fase sólida, ésta fue liofilizada. Tras la liofilización, el sólido se sometió a varias extracciones sólido-líquido. Para realizar estas extracciones, primero se llevaron a un vortex durante 5 min a temperatura ambiente, seguido de 10 minutos en un baño de ultrasonidos. La relación sólido-líquido empleada fue de 0.025 (0,25 g de lías liofilizadas en 10 mL de disolvente). Varios solventes con diferentes polaridades (agua, metanol, etanol, dos mezclas de agua-etanol y acetona) fueron empleados. EL contenido total de polifenoles (TPC) y el contenido total de flavonoides (TFC) fueron determinados y expresado por gramo de extracto seco (DE). Para la determinación de la actividad antioxidante (AA) de los extractos producidos, se emplearon diferentes ensayos *in-vitro* (ORAC, HORAC, HOSC y FRAP). Los datos de TPC, TFC y las diferentes AAs se pueden ver en la Tabla 1.

Tabla 1: contendidos totales de polifenoles (TPC) y flavonoides (TFC) y las diferentes actividades antioxidantes para los extractos de lías de envejecimiento de vino obtenidos con diferentes solventes. Los valores más altos de cada caracterización se presentan en negrita, mientras que los menores están en cursiva. Valores con diferentes letras superíndices en la misma columna, son significativamente diferentes ($p<0.05$).

	TPC **mg GAE /gDE**	**TFC** **mg CAT /g DE**	**ORAC** **µmol TE /g DE**	**HORAC** **µmol CAT /g DE**	**HOSC** **µmol TE /g DE**	**FRAP** **µmol TE /g DE**	**TA** **280nm**	**TA** **520nm**
H_2O	38 ± 3^{a}	16 ± 1^{a}	471 ± 86^{a}	348 ± 35^{a}	592 ± 39^{a}	461 ± 3^{a}	$8.68 \cdot 10^{7}$	$2.04 \cdot 10^{7}$
EtOH	94 ± 8^{b}	51 ± 18^{b}	$1{,}603 \pm 227^{b}$	$1{,}245 \pm 103^{b}$	$2{,}107 \pm 134^{b}$	$1{,}034 \pm 26^{b}$	$6.95 \cdot 10^{7}$	$1.90 \cdot 10^{7}$
Acetone	26 ± 1^{a}	30 ± 3^{a}	217 ± 68^{a}	543 ± 59^{a}	281 ± 26^{a}	362 ± 6^{a}	$2.30 \cdot 10^{7}$ *	$2.40 \cdot 10^{6}$ *
MeOH	149 ± 7^{c}	112 ± 12^{c}	**$2{,}771 \pm 289^{c}$**	$3{,}963 \pm 367^{c}$	$2{,}732 \pm 257^{c}$	$1{,}542 \pm 38^{c}$	$2.72 \cdot 10^{8}$	$1.12 \cdot 10^{8}$
EtOH:H_2O (50:50)	206 ± 28^{d}	145 ± 6^{d}	$1{,}003 \pm 90^{d}$	$2{,}985 \pm 389^{d}$	$3{,}912 \pm 310^{d}$	$2{,}112 \pm 65^{d}$	**$3.13 \cdot 10^{8}$**	$1.99 \cdot 10^{8}$
EtOH:H_2O (75:25)	**254 ± 24^{e}**	**146 ± 5^{d}**	$2{,}323 \pm 289^{e}$	**$4{,}690 \pm 463^{e}$**	**$4{,}527 \pm 413^{e}$**	**$2{,}197 \pm 84^{d}$**	$2.75 \cdot 10^{8}$	**$1.77 \cdot 10^{8}$**

De los resultados obtenidos, la mezcla de etanol al 75% vol. Fue la que mostró valores más altos de TPC y TFC con 254mgGAE/g_{DE} y 146 mg_{CATE}/g_{DE}, respectivamente. Para el caso de las diferentes AAs, tanto el valor más alto de HORAC (como equivalentes de catequina, CAE) como de HOSC y FRAP (ambos como equivalentes Trolox, TE) se obtuvieron para la mezcla hidroalcohólica con 75% vol. de etanol (690µmolCAE/g_{DE}, 527µmolTE/g_{DE} y 197µmolTE/g_{DE}, respectivamente). En cambio, para el método ORAC (en equivalentes de Trolox, TE), el extracto de metanol fue el que mostró el mejor valor con 771$\mu mol_{TE}/g_{DE}$.

Compuestos más relevantes que contribuyen a la AA fueron identificados utilizando los datos de espectrometría de masas. Podría afirmarse que las antocianinas son los principales compuestos presentes en los extractos de lías de vino como se muestra en la Tabla 2. Además, se determinaron correlaciones entre TPC, TFC, compuestos fenólicos identificados y las diferentes AAs. Estas correlaciones se muestran en las Tablas 3 y 4, respectivamente.

Tabla 2: identificación de los principales compuestos presentes en los extractos. Tiempo de retención (min), absorbancia máxima (nm) (λmáx), valores MS y MS/MS (m/z), familia fenólica y porcentaje del área de cada pico en los diferentes extractos. Los números entre corchetes representan el valor más grande de m/z.

Pico nº	Tiempo de retención (min)	λmax (nm)	m/z (modo positivo y negativo)	[M-H] fragmentos (m/z)	Identificación	Familia fenólica	Porcentaje (%) de cada pico		
							MeOH	50%v.	75%v.
1	14.7	270	169 (M^-)	[169], 125	Gallic Acid	Phenolic acid	0.59	1.29	1.16
2	24.7	328	289 (M^-)	[289] 229, 153, 137	Catechin	Flavan-3-ol	1.10	2.67	2.57
3	29.7	529	465 (M^+)	[465] 349, 303, 147	Delphinidin-3-O-glucoside	Anthocyanin	1.46	3.62	3.96
4	30.6	283	289 (M^-)	[289] 271, 227, 203, 188	Epicatechin	Flavan-3-ol	1.71	4.43	3.51
5	33.9	529	479 (M^+)	[479] 317	Petunidin-3-O-glucoside	Anthocyanin	2.42	4.27	3.71
6	37.9	527	493 (M^+)	[493] 331	Malvidin 3-*O*-glucoside	Anthocyanin	8.20	9.93	11.35
7	41.4	366	477 (M^-)	[477] 301, 151	Quercetin -3-*O*-glucuronide	Flavonol	11.72	5.75	10.64
8	54.5	368	317 (M^-)	[317] 179, 151	Myricetin	Flavonol	6.25	6.23	7.20
9	57.8	529	611 (M^+)	[611] 303	Delphinidin 3-(6-p-coumaroylglucoside)	Anthocyanin	2.94	8.07	6.84
10	64.4	529	625 (M^+)	[625] 317	Petunidin-3-(6-p-coumaroylglucoside)	Anthocyanin	3.20	8.50	7.55
11	70.6	530	639 (M^+)	[639] 331	Malvidin 3-(6-p-coumaroylglucoside)	Anthocyanin	7.45	13.29	15.48

Las antocianinas que presenta el radical 6-p-coumaroyl contribuyen significativamente (*p < 0,10*) al ensayo FRAP, así como, el ácido gálico y dos flavan-3-ols detectados. Además, las antocianinas contribuyen significativamente de forma negativa (*p < 0,10*) al ensayo ORAC. Las antocianinas también contribuyen de forma negativa y positiva (pero no significativamente) para HORAC y HOSC, respectivamente.

*Tabla 3: Correlaciones (valores r) entre los valores de TPC, TFC y las diferentes AAs. Valores con un r >|0.90| se presentan en negrita. Valores con un * son estadísticamente significativos (p <0.05).*

	TPC	**TFC**
TPC	1.000*	
TFC	**0.970***	1.000*
ORAC	0.665	0.646
HORAC	**0.924***	**0.930***
HOSC	**0.992***	**0.960***
FRAP	**0.990***	**0.983***

*Tabla 4: Correlaciones (valores r) entre las áreas de los picos a 280nm y las diferentes AAs. Valores con un r >|0.90| se presentan en negrita. Valores con un * son estadísticamente significativos (p <0.05).*

	ORAC	**HORAC**	**HOSC**	**FRAP**
1	-0.896*	-0.414	0.774	0.896*
2	-0.840*	-0.308	0.841*	**0.940***
3	-0.734	-0.139	**0.921***	**0.985***
4	**-0.947***	-0.532	0.682	0.828*
5	**-0.951***	-0.542	0.673	0.821*
6	-0.687	-0.072	**0.946***	**0.994***
7	**0.998***	0.815*	-0.351	-0.551
8	-0.619	0.018	**0.971***	**0.999***
9	**-0.912***	-0.447	0.750	0.879*
10	-0.889*	-0.399	0.784	**0.903***
11	-0.667	-0.046	**0.954***	**0.997***

Dependiendo del solvente utilizado, se obtuvieron diferentes cantidades de los compuestos identificados, los cuales pueden tener una mayor o menor actividad contra los radicales de oxígeno (ROO•) o (HO•) que afectan a la capacidad antioxidante de los extractos.

Capítulo II: pretratamientos con microondas y ultrasonidos para mejorar la extracción de antocianinas de diferentes tipos de lías.

Aunque existen algunos trabajos en bibliografía con respecto a la recuperación de compuestos fenólicos de lías de vino, en ninguno de ellos se han estudiado las cinéticas de extracción ni los parámetros que maximizan su extracción en extracciones convencionales sólido-líquido. Además, el uso de tratamientos previos como microondas (MW) o ultrasonido (US) podrían intensificar el proceso y aumentar la recuperación de polifenoles. Así, en el **Capítulo II**, se obtuvieron en primer lugar las cinéticas de extracción de antocianinas (CA, como equivalentes de malvidina, MLVE por gramo de lías) de las diferentes lías de vino a través de varias extracciones convencionales sólido-líquido. Las antocianinas fueron elegidas como los compuestos de interés, ya que son la subfamilia mayoritaria de polifenoles en uvas. La influencia de parámetros tales como la temperatura, la relación sólido-líquido (R_{S-L}) y el tipo de solvente (mezclas de hidro-alcohólico) fueron estudiadas. Las variables que mejoran la extracción de antocianinas de las lías de vino Oporto (2,78 mg_{MLVE}/g_{DL}) fueron: una temperatura de 25 º c, con un R_{S-L} de 0.10 (g/mL) y con una mezcla con un 50% vol. de etanol. Después de 15min de extracción, se alcanzó un régimen estacionario para la concentración de antocianinas extraídas. Estas mismas condiciones se aplicaron también a lías de primera y segunda fermentación de vino se. Rendimientos finales de 3,04 mg_{MLVE}/g_{DL} y 2,09 mg_{MLVE}/g_{DL} se obtuvieron, respectivamente. Una vez que se estudió la cinética de extracción, se evaluó la aplicación de MW y US como tratamientos previos a la extracción convencional sólido-líquido con el fin de aumentar la extracción de AC. Con la ayuda de un diseño estadístico de superficie de respuesta, se obtuvieron las condiciones óptimas que maximizan el contenido final de AC. Los parámetros evaluados en este diseño fueron la R_{S-L} (g/mL), la composición del disolvente (etanol del vol. %) y el tiempo de tratamiento (s). Los valores óptimos para cada parámetro fueron: un R_{S-L} de 0.140 (g/mL), una mezcla hidroalcohólica de etanol al 40% vol. y un tratamiento de 90 segundos. Cuando este óptimo de MW se aplicó a las lías, el rendimiento de extracción de AC de lías de vino de Oporto se duplicó (6,20 mg_{MLVE}/g_{DL}). El tiempo requerido para alcanzar dicho rendimiento se redujo de 15 min a 90s. El tratamiento previo de MW aplicado a las lías de la primera y segunda fermentación, aumenta los rendimientos de extracción de antocianinas 1.50 y 1.40

veces, respectivamente. Mientras tanto, los US no mejoran la extracción de antocianinas de las lías e vino, sino que sólo acorta tiempo de extracción en menor proporción que el MW (de 15 a 5min). Los principales resultados de la caracterización de los extractos de las diferentes lías de vino están recogidos en la Tabla 5.

*Tabla 1: contenido total de polifenoles (TPC) y antocianinas (AC) y valores de ORAC par5los extractos obtenidos mediante la extracción convencional y con los pre-tratamientos de MW y US después de 15min de extracción. Valores con un * son significativamente diferentes (P<0.05) para cada tipo de lías.*

		TPC (mg_{GAE}/g_{DL})	TPC (mg_{GAE}/g_{DE})	AC (mg_{MLVE}/g_{DL})	AC (mg_{MLVE}/g_{DE})	ORAC ($\mu mol_{TE}/g_{DL}$)	ORAC ($\mu mol_{TE}/g_{DE}$)
Lías de Oporto	E_{S-L} 15'	27.70 ± 0.18	68 ± 7	2.78 ± 0.18	3.57 ± 0.23	195 ± 20	453± 45
	MW 15'	***42.04 ± 0.22*** *	106 ± 3 *	***6.20 ± 0.36*** *	7.99 ± 0.44 *	402 ± 42 *	1041 ± 107 *
	US 15'	-	-	3.17 ± 0.08	2.91 ± 0.13	312 ± 34 *	574 ± 64
1ª Fermentación	E_{S-L} 15'	28.12 ± 0.08	232 ± 5	3.04 ± 0.03	17.07 ± 0.32	392 ± 42	3201 ± 347
	MW 15'	37.03 ± 0.15	***295 ± 13***	4.45 ± 0.30 *	***18.56 ± 1.26***	***655 ± 63*** *	***4952 ± 480****
2ª Fermentación	E_{S-L} 15'	23.42 ± 0.11	196 ± 10	2.09 ± 0.38	10.95 ± 0.77	304 ± 20	2484 ± 159
	MW 15'	23.44 ± 0.17	269 ± 11 *	2.88 ± 0.22	12.03 ± 1.14	512 ± 54 *	3867± 406 *

La identificación de los principales compuestos de los extractos se realizó por LC/MS-MS (Tabla 6). Cabe destacar la identificación de un pyranoanthocyanin (Vitisin A) en

todos los tipos de lías de vino cando se aplica el pre-tratamiento óptimo de microondas.

Tabla 6: identificación de las principales antocianinas en los diferentes tipos de lías de vino (520nm, $\lambda_{máx}$), tiempo de retención (t_R, min), valores de M-H$^+$ y MS/MS (m/z) y extractos en los que aparecen.

Retention time (min)	[M-H]$^+$ (m/z)	MS/MS	Putative identification	Phenolic subclass	Ribera del Duero wine lees	Port wine lees
30.03	463	303	Delphinidin-3-O-glucoside	Anthocyanin	✓	✓
32.13	561	399	Vitisin A	Pyranoanthocyanin	✓	✓
	479	317	Petunidin-3-O-glucoside	Anthocyanin	✓	✓
33.63	493	331	Malvidin-3-O-glucoside	Anthocyanin	✓	✓
34.88	507	-	Delphinidin 3-O-(6"-p-acetylglucoside)	Anthocyanin	✓	χ
36.13	707	399	10-carboxypyranomalvidin-3-6"-p-coumaroyl-glucoside	Anthocyanin	✓	✓
36.77	491	-	Cyanidin 3-O-(6"-p-acetylglucoside)	Anthocyanin	✓	χ
38.78	535	331	Malvidin-3-O-6"-acetyl-glucoside	Anthocyanin	χ	✓
39.42	611	303	Delphinidin 3-O-(6"-p-coumaroyl-glucoside)	Anthocyanin	✓	χ
41.52	625	317	Petunidin 3-O-(6"-p-coumaroyl-glucoside)	Anthocyanin	✓	✓
43.23	639	331	Malvidin 3-O-(6"-p-coumaroyl-glucoside)	Anthocyanin	✓	✓

Capítulo III: aplicación de hidrólisis enzimática a diferentes tipos de lías para favorecer la extracción de antocianinas.

Aparte de los tratamientos estudiados en el Capítulo II, la aplicación de un pretratamiento de hidrólisis enzimática a las lías de vino puede provocar la liberación de los polifenoles que están ligados o absorbidos en la pared celular de las levaduras, las cuales son uno de los principales componentes de lías. En el **Capítulo III**, se probaron dos enzimas diferentes, Glucanex y Mannaway y, además, una mezcla de ambas enzimas con este propósito. La hidrólisis enzimática se llevó a cabo para las mejores condiciones de extracción sólido-líquido obtenidas en el Capítulo II. Una vez pasado el tiempo de incubación, se añade etanol (en la cantidad necesaria para obtener un solvente con un 50% vol. de etanol) al medio para maximizar la extracción de antocianinas. Se probaron diferentes tiempos de incubación para la hidrólisis enzimática, los cuales variaban entre 5 y 60 minutos. Sin embargo, no se encontraron diferencias significativas en el rendimiento de extracción de antocianinas para los diferentes tiempos de incubación estudiados. Estos valores de rendimiento para cada tipo de enzima y tiempo de incubación pueden verse en la Tabla 7.

*Tabla 7: concentración de antocianinas a lo largo del tiempo para las diferentes enzimas Mannaway (a)-Glucanex (b) y la combinación de ambas (M+G) (c). t =0 representa el momento justo antes de que la incubación haya tenido lugar. Los tiempos siguientes representan la concentración de antocianinas con el tiempo una vez añadido el etanol. Valores con un * son significativamente diferentes (p<0.05) para cada tiempo.*

AC (mg_{MLVE}/g_{DL})				
a)	**Tiempo incubación $_{Mannaway}$ (min)**			
t (min)	**5**	**15**	**30**	**60**
0	0.41 ± 0.02*	0.47 ± 0.05*	0.39 ± 0.06*	0.49 ± 0.06*
5	3.34 ± 0.98	4.14 ± 0.13	3.92 ± 0.02	4.23 ± 0.43
10	3.81 ± 0.36	4.17 ± 0.14	4.19 ± 0.06	4.02 ± 0.08
15	3.75 ± 0.18	4.50 ± 0.29	4.17 ± 0.26	4.26 ± 0.05
20	3.81 ± 0.02	4.17 ± 0.43	4.14 ± 0.05	4.20 ± 0.05
b)	**Tiempo incubación $_{Glucanex}$ (min)**			
t (min)	**5**	**15**	**30**	**60**
0	0.27 ± 0.02*	0.18 ± 0.11*	0.11 ± 0.03*	0.42 ± 0.10*
5	3.81 ± 0.02	3.76 ± 0.25	3.83 ± 0.02	3.93 ± 0.22
10	3.71 ± 0.31	3.85 ± 0.30	3.99 ± 0.03	3.67 ± 0.43
15	3.84 ± 0.25	4.04 ± 0.08	3.90 ± 0.18	3.90 ± 0.24
20	3.82 ± 0.37	4.02 ± 0.05	4.01 ± 0.03	4.01 ± 0.35
c)	**Tiempo incubación $_{M+G}$ (min)**			
t (min)	**5**	**15**	**30**	**60**
0	0.53 ± 0.06*	0.49 ± 0.04*	0.86 ± 0.37*	0.61 ± 0.00*
5	4.37 ± 0.07	4.08 ± 0.27	4.38 ± 0.08	3.93 ± 0.25
10	4.40 ± 0.40	4.27 ± 0.05	4.36 ± 0.23	4.10 ± 0.07
15	4.18 ± 0.33	4.55 ± 0.12	4.39 ± 0.10	4.09 ± 0.06
20	4.65 ± 0.23	4.32 ± 0.04	4.21 ± 0.13	3.97 ± 0.05

El extracto obtenido cuando se utilizó la mezcla enzimática con un tiempo de incubación de 5 minutos, fue el que mostró el incremento más alto (50%) respecto a la extracción convencional. Por otro lado, la actividad antioxidante de los extractos enzimáticos se evaluó mediante ensayo ORAC. El valor más alto de ORAC fue alcanzado para extracto de lías de primera fermentación vino tratadas con Mannaway. Se identificaron las antocianinas presentes en los extractos mediante espectrometría de masas (Tabla 8).

Tabla 8: identificación de las antocianas presentes en los extractos obtenidos mediante hidrólisis enzimática

T_R (min)	$[M-H]^+$ (m/z)	MS/MS (m/z)	Identificación
29.75	465	303	Delphinidin-3-O-glucoside
31.72	479	317	Petunidin-3-O-glucoside
33.70	493	331	Malvidin-3-O-glucoside
39.48	611	303	Delphinidin 3-O-(6"-p-coumaroyl-glucoside)
41.80	625	317	Petunidin 3-O-(6"-p-coumaroyl-glucoside)
43.88	639	331	Malvidin 3-O-(6"-p-coumaroyl-glucoside)

La única diferencia fue encontrada después del tratamiento previo con Mannaway con un compuesto no identificado detectado en 280nm (Figura 1).

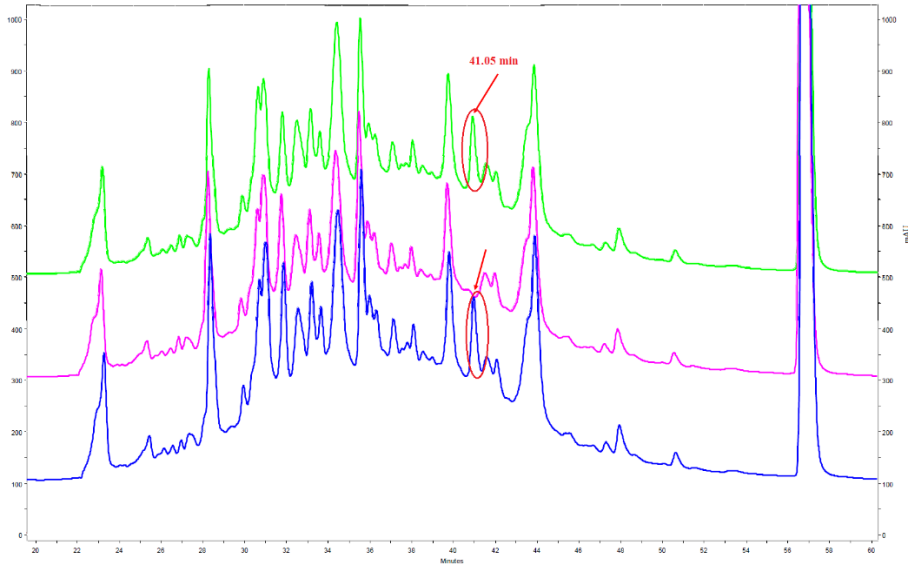


Figura 1: cromatrograma a 280nm del extracto de primera fermentación obtenido para una incubación de 5 min para Mannaway (azul), Glucanex (rosa) y la mezcla enzimática (verde)

A modo de resumen la Figura 2, recoge la comparativa de los pre-tratamientos aplicados a las lías de primera fermentación.

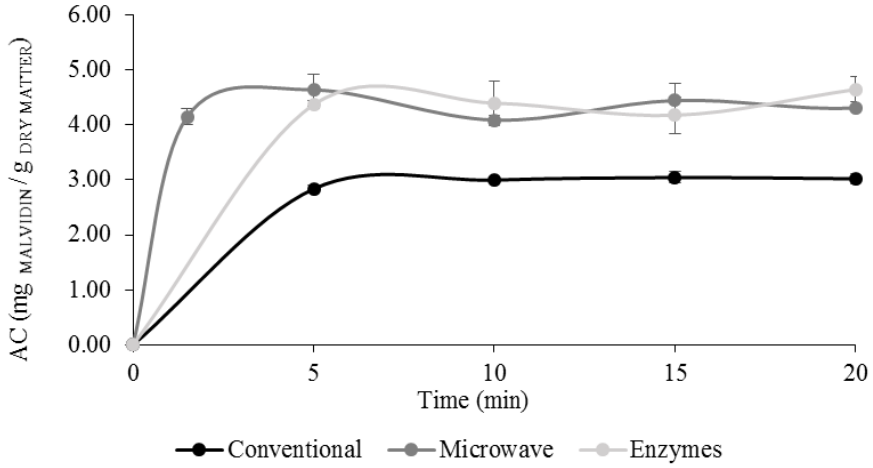


Figura 2: comparación de la concentración de antocianinas de los extractos obtenidos mediante la extracción convencional y aplicando los tratamientos con MW o enzimas (mezcla enzimática con 60min de incubación),

Capítulo IV. Revalorización del raspón de uva: pretratamiento con MW para la extracción de polifenoles.

En el **Capítulo IV**, se llevó a cabo una intensificación del proceso de extracción de polifenoles del raspón de uva. En primer lugar, se estudiaron las cinéticas de extracción de polifenoles totales (TPC) y flavonoides totales (TFC) siguiendo el mismo procedimiento expuesto en el Capítulo II para las lías de vino. El estudio de las diferentes variables puede verse en la Figura 3.Después de este estudio, los valores de las variables que mejoran la recuperación de compuestos bioactivos a partir del raspón de uva fueron una $R_{S\text{-}L}$ de 0,10 g/mL, un solvente con un 50% vol de etanol y una temperatura de 75 ºC. En estas condiciones, un valor de TPC de 38 ± 1mg_{GAE}/g_{DS} y de TFC de 38 ± 1 mg_{CATE}/g_{DS} fueron obtenidos.

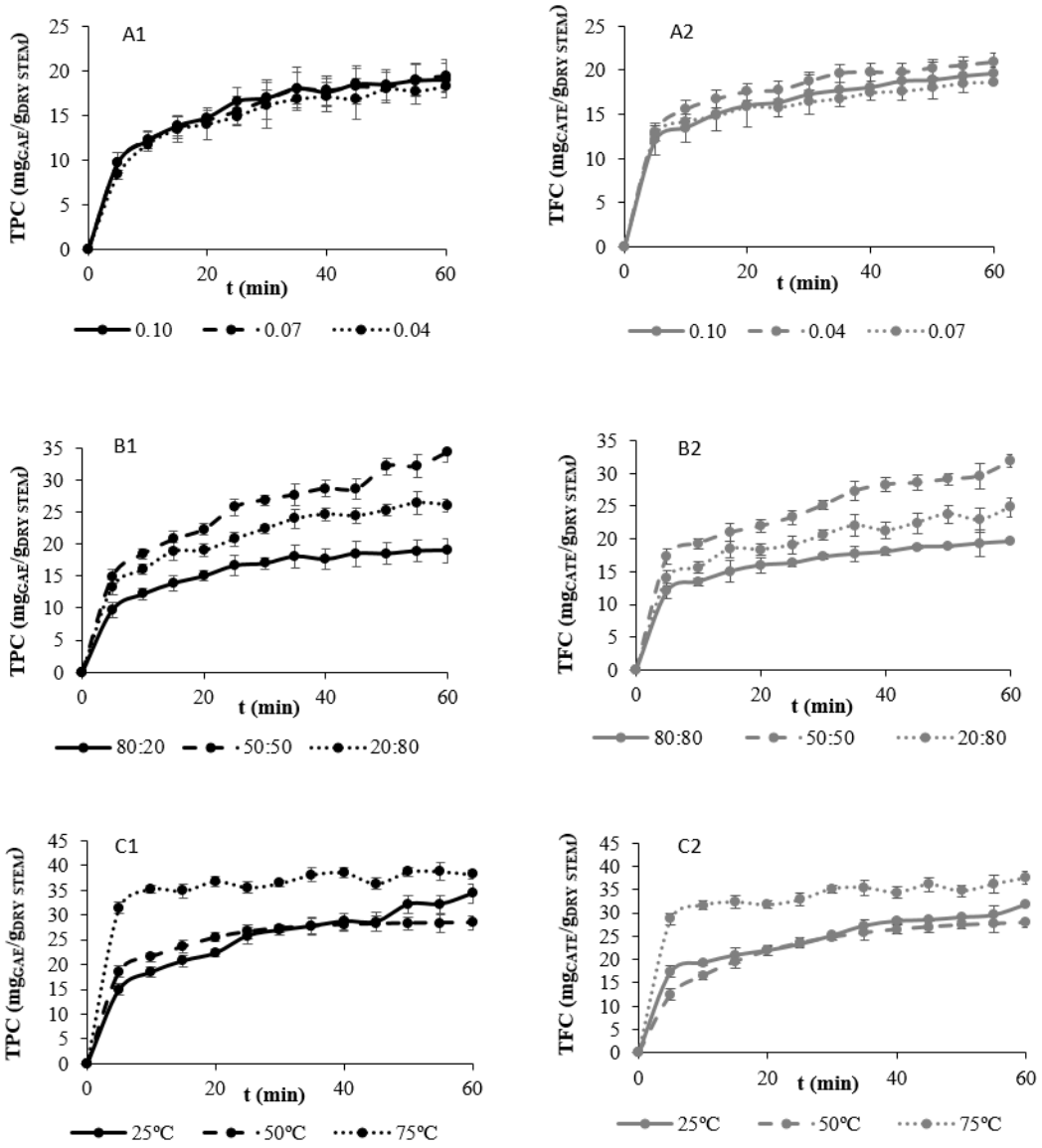


Figura 3: valores de TPC y TFC para el estudio de las cinéticas de extracción. En las figuras A.1 y A.2 se muestra la variación de concentración de TPC y TFC, respectivamente, con la R_{S-L} para una temperatura de 25ºC y un solvente con un 20% vol. de etanol. En las figuras B.1 y B2 se muestra la variación de concentración de TPC y TFC, respectivamente, con la composición del solvente para una temperatura de 25ºC y un R_{S-L} de 0,10 (g/mL). En las Figuras C.1 y C.2 se muestra la variación de concentración de TPC y TFC, respectivamente, con la temperatura para un solvente con un 50% vol. de etanol y un R_{S-L} de 0,10 (g/mL).

Dado que el pretratamiento de MW aumentó significativamente la recuperación de los compuestos fenólicos de lías de vino, este procedimiento también se aplicó al raspón de uva. En este caso, también se llevó a cabo un diseño de experimentos estadístico de superficie de respuesta. Tras la optimización las variables óptimas para el pretratamiento con microondas que maximizaban la extracción de polifenoles fueron:

una R_{S-L} 0.50 de g/mL, un solvente con un 55%vol de etanol y un tiempo de tratamiento de 36s. Para el caso de los flavonoides, las condiciones óptimas fueron: una R_{S-L} 0.10 de g/mL, un solvente con un 47%vol de etanol y un tiempo de tratamiento de 86s. En estas condiciones, el tratamiento con MW aumentó el rendimiento de extracción de TPC y TFC en un 19% y 24% respecto a la convencional extracción sólido-líquido, respectivamente como se muestra en la Figura 4.

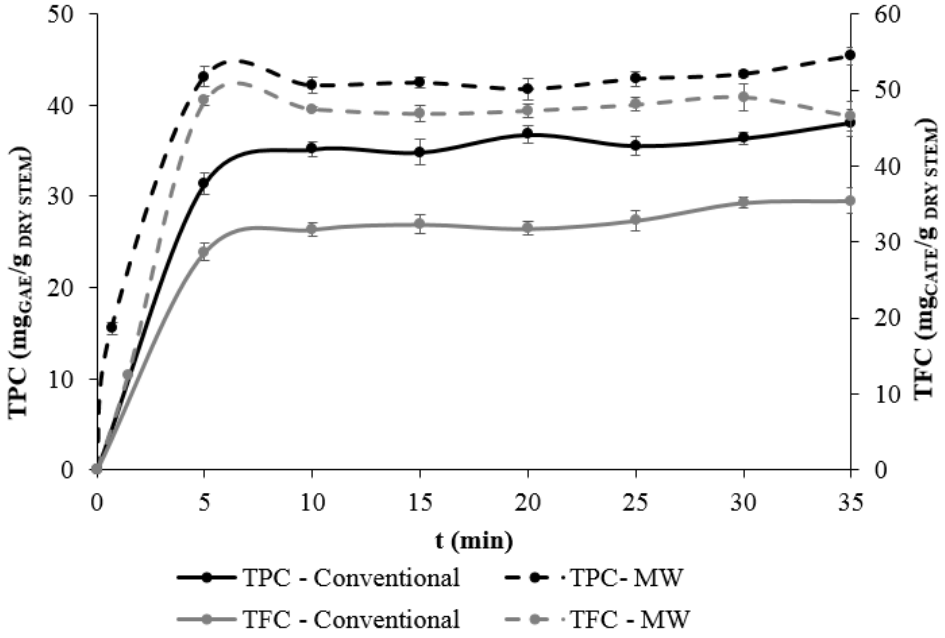


Figura 4: comparación de los valores de TPC y TFC alcanzados con la extracción convencional sólido-líquido (R_{S-L} 0.10 de g/mL, un solvente con un 50%vol de etanol y una temperatura de 75ºC) y a las condiciones óptimas de cada tratamiento con MW.

De manera adicional, se identificaron y cuantificaron por cromatografía los principales estilbenos (resveratrol y ε-viniferin) y flavonoides (catequina y epicatequina) presentes en los extractos (Figura 5).

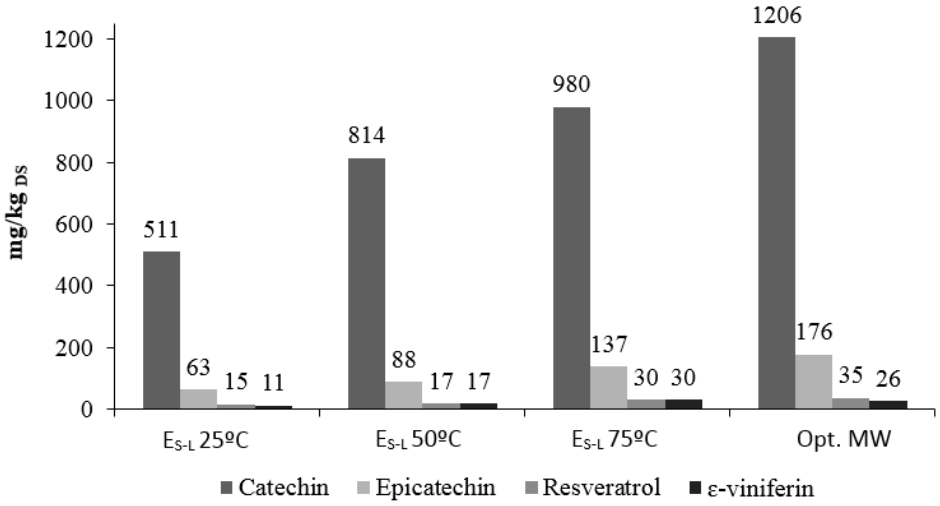


Figura 5: rendimiento de extracción de los principales estilbenos y flavonoides presentes en los diferentes extractos de raspón de uva.

Según la bibliografía, estos extractos exhiben interesantes propiedades anti fúngicas. Así, se determinó dicha actividad contra el hongo *Botrytis cinarea* junto con la actividad antioxidante, medida por el ensayo ORAC (Figura 6).

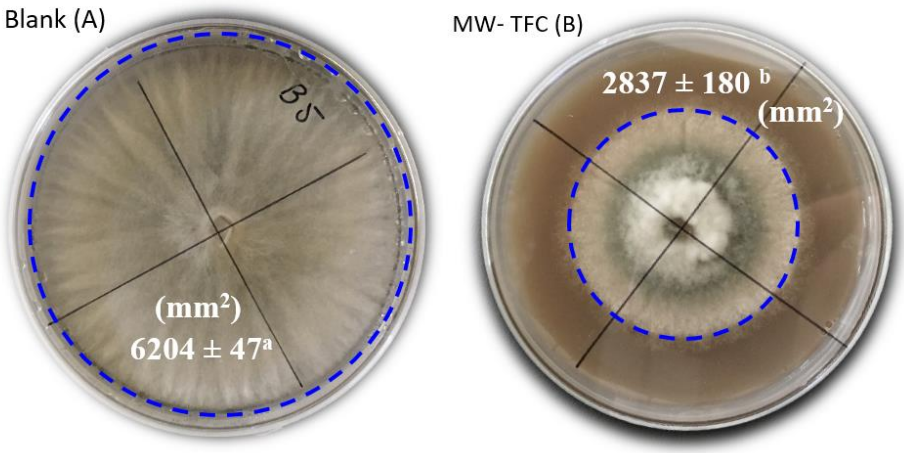


Figura 6: comparación de las áreas de crecimiento para el blanco (agua destilada) (A) y el extracto óptimo de MW para TFC (B).

Capítulo V. Conversión del raspón de uva en extractos ricos en azúcares a través de un proceso hidrotermal.

El **Capítulo V** se centró en valorización del raspón de como una fuente de celulosa, hemicelulosa y lignina y por ello, como fuente rica en azúcares y oligómeros, los cuales tiene un amplio abanico de aplicaciones. Para la obtención de extractos ricos en azúcares y oligómeros se empleó un proceso hidrotermal. Para evaluar la influencia de la temperatura en el proceso de recuperación de azúcares, temperaturas entre 100 y 180ºC durante experimentos con una duración de 20 minutos. Según los resultados, una temperatura de 140ºC parece ser la más adecuada maximizar el rendimiento de azúcares (264mg/g_{DS}) (Figura 7).

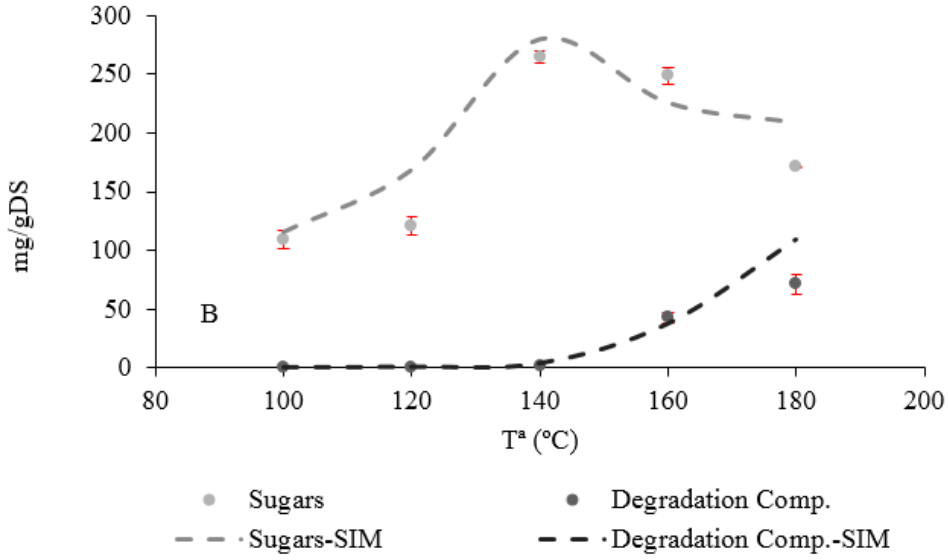


Figura 7: variación de los azúcares y compuestos de degradación con la temperatura para un tiempo de extracción de 20min.

Una vez seleccionada la mejor temperatura, se evaluó el efecto del tiempo de operación a 140ºC. El tiempo de operación fue variado entre 10 y 30min. Los resultados muestras que a mayor tiempo, mayor será el rendimiento en términos de azúcares y contenido de carbono total (Figure 8). Todos los experimentos realizados para este capítulo se encuentran recogido en la Tabla 9.

Tabla 9: listado de experimentos y valores principales de concentraciones (azúcares, productos de degradación, oligómeros y TOC).

	T	t	P	pH	Azúcares	Compuestos degradación	Oligómeros Totales	Pentosas Olig. (HC)	Hexosas Olig. (C)	TOC	Rendimiento total extracción
Exp.	**(ºC)**	**(min)**	**(barg)**				**(mg/g_{DS})**			**(mg/L)**	**%**
1	100	20	2	4.28	109 ± 7	1.0 ± 0.3	496 ± 11	33 ± 1	463 ± 9	17063 ± 624	61
2	120	20	2	4.16	121 ± 8	1.0 ± 0.4	505 ± 9	40 ± 2	465 ± 7	15348 ± 1178	63
3	140	20	3	4.02	264 ± 5	2.0 ± 0.0	316 ± 14	74 ± 5	242 ± 10	13952 ± 315	58
4	160	20	7	4.06	249 ± 7	42.8 ± 5.0	357 ± 7	90 ± 1	267 ± 7	13120 ± 485	65
5	180	20	10	4.04	171 ± 1	71.3 ± 8.0	361 ± 10	161 ± 3	200 ± 7	13247 ± 958	60
6	140	10	3	3.94	280 ± 8	3.2 ± 0.1[a]	256 ± 15	50 ± 4	206 ± 11	18603 ± 1683	54
7	140	30	4	4.06	302 ± 9	9.4 ± 0.6	357 ± 23	86 ± 5	270 ± 18	20840 ± 454	67
8	160	20	5	4.33	109 ± 7	35 ± 1.0	393 ± 14	201 ± 6	191 ± 8	8603 ± 84	54

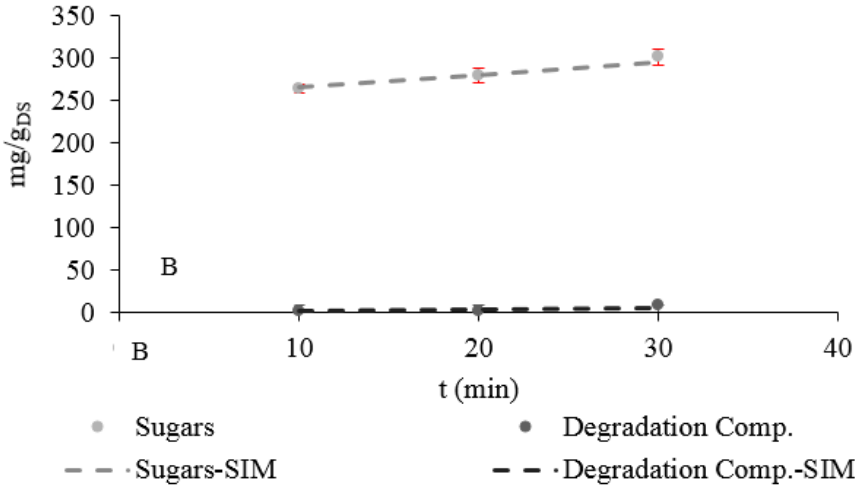


Figura 8: variación de los azúcares y compuestos de degradación con el tiempo de operación a una temperatura de 140ºC.

Además, dentro del concepto de bio-refinería, se evaluó el efecto del tratamiento previo MW llevado a cabo en el Capítulo IV para la extracción de polifenoles en la conversión del raspón de uva en oligómeros y azúcares. El efecto que el MW podría tener sobre los oligómeros alterándolos y mejorando su extracción (10%). Finalmente, se ajustaron los datos experimentales gracias a un modelo cinético con una desviación absoluta promedio alrededor del 15% para los azúcares y oligómeros y del 30% para los productos de degradación.

CONCLUSIONES

Esta tesis contribuye a la valorización de residuos vinícolas. El trabajo se centra en la recuperación de polifenoles de dos residuos poco explotados, como son las lías y el raspón de uva. Además, el raspón de uva se puede utilizar como fuente natural de obtención de azúcares y de oligómeros por su estructura lignocelulósica. Las principales conclusiones de este trabajo según los resultados obtenidos, teniendo en cuenta los objetivos propuestos al principio, se exponen a continuación.

Lías de vino

Caracterización de lías de vino

- Se han obtenido extractos ricos en polifenoles a partir de lías de envejecimiento mediante extracciones sólido-líquido convencionales con solventes de diferente polaridad. La mezcla hidroalcohólica con un 75% vol. de etanol resultó ser el disolvente más adecuado para dicha, y en consecuencia, el que maximiza las actividades antioxidantes.

- La cuantificación de los polifenoles que contribuían significativamente ($p < 0,10$) con las diferentes actividades antioxidantes fue realizada a través de las áreas de los picos de HPLC. Un total de 12 compuestos fueron identificados, entre ellos 6 antocianinas, 2 flavonoles, 2 Flavan-3-ol y 1 ácido fenólico.

- Se obtuvieron correlaciones entre los compuestos fenólicos extraídos y las diferentes actividades de antioxidantes. Se encontraron correlaciones significativas para las antocianinas que tienen en su molécula el radical *6-p-coumaroyl* y para el ensayo FRAP. Las antocianinas también contribuyeron positivamente (pero no significativamente) a HOSC.

Maximización de la extracción de polifenoles

- A partir del estudio de las cinéticas de extracción de antocianinas de las lías, los parámetros que mejoraban la extracción de antocianinas fueron una temperatura de 25 ºC, con un R_{S-L} de 0,10 (g/mL) y con un solvente con un 50% vol. de etanol. En estas condiciones se logró una concentración de antocianinas final de 2,78 mg /g_{DL} de lías de vino de Oporto después de 15 min de extracción. Lías de primera y segunda fermentación dieron rendimientos finales de 3,04 mg/g_{DL} y 2,09 mg /g_{DL}, respectivamente.

- Se evaluó l efecto de varios pre-tratamientos en los rendimientos de extracción de antocianinas:

    - El tratamiento previo de microondas (MW) aumentó significativamente ($p < 0.05$) la recuperación de las antocianinas de todos los tipos de lías de vino. Las condiciones óptimas que maximizan la producción de antocianinas fueron: una mezcla hidroalcohólica con un 40% vol. de etanol, un R_{S-L} de 0,140 (g/mL) y tratamiento de 90s. En estas condiciones, el rendimiento de extracción de AC se duplicó para las lías de vino de Oporto (6,20 mg/g/$_{DL}$) y el tiempo de extracción se redujo 10 veces (de 15 minutos a 90s). En las mismas condiciones, rendimiento de antocianina de 4,45 mg /g_{DL} y 2,88 mg /g_{DL} se obtuvieron para las lías de primera y segunda fermentación, respectivamente.

    - Los ultrasonidos (US) no resultaron ser un tratamiento previo apropiado para la recuperación de las antocianinas. El incremento en el rendimiento final de antocianinas no fue significativamente diferentes respecto al rendimiento de extracción convencionales ($p < 0.05$).

- La hidrólisis enzimática fue llevado a cabo con enzimas Glucanex y Mannaway, así como con una mezcla de ambas enzimas. Mejor rendimiento de extracción de antocianinas se logró cuando se empleó la mezcla enzimática de ambas enzimas con 5min de incubación (4,65 mg/g_{DL}).

➢ Las actividades el antioxidante de los extractos derivados de estos tratamientos previos revelaron valores de ORAC más alto respecto a los extractos convencionales. Cuando se aplicó el pre-tratamiento de MW, una piranoantocianina fue identificada en todos los tipos de lías de vino.

Raspón de uva

Maximización de la extracción de polifenoles

➢ A partir del estudio de las cinéticas de extracción de flavonoides y polifenoles a partir del raspón de uva, los parámetros que mejoraban la extracción de ambos flavonoides y polifenoles fueron: una temperatura de 75 ºC, un R_{S-L} de 1/10 (g/mL) y con una mezcla 50% vol. de etanol. En estas condiciones, concentraciones finales de polifenoles y flavonoides de 38,2 ± 1,0 mg_{GAE}/g_{DS} y 37,6 ± 1.5 mg_{CATE} /g_{DS} se obtuvieron, respectivamente, después de un tiempo de extracción de 60 min.

➢ También se estudió la aplicación del pre-tratamiento con MW para el raspón de uva. En este caso variables que maximizan la extracción de polifenoles totales fueron un R_{S-L} de 0.50 g/mL, un solvente con 55% vol. de etanol y un tiempo de tratamiento de 36s. En estas condiciones, se obtuvo un extracto con una 45 mg_{GAE}/g_{DS}. Por otro lado para la maximización de flavonoides un R_{S-L} de 0,10 g/mL, un solvente con un 47% vol. de etanol y un tiempo de tratamiento de 86s fueron requeridos (47mg_{CATE} /g_{DS}).

➢ El extracto rico en flavonoides obtenidos al aplicar el pre-tratameinto de MW mostró una gran capacidad antioxidante significativa ($p < 0.05$) (1056 ± 56$\mu mol_{TE}/g_{DS}$) y actividad anti-fúngica (inhibición del crecimiento del hongo en un 46% respecto al blanco).

Conversión de los tallos de uva en oligómeros y azúcares

➢ Se evaluó la extracción de la parte holocelulósica del raspón de uva mediante un tratamiento hidrotermal. Una temperatura de 140ºC resultó ser la más adecuada para la recuperación de los azúcares. A tiempos de operación más largos (de 10 a 30min), mayor es el rendimiento de extracción alcanzado (oligómeros, azúcares y contenido de carbono total). Se logró una tendencia poco esperada,

ya que el extracto estaba principalmente compuesto de hexosas a pesar de la condiciones de procesamiento fueron suave.

- Aparte de recuperar los compuestos polifenólicos, así como los azúcares libres de la materia prima, el pre-tratamiento de MW podría haber afectado los oligómeros, alterándolos y mejora su extracción (10%).

- El modelo cinético propuesto para la conversión de la biomasa se validó a través de una desviación absoluta promedio alrededor del 15% para los azúcares y oligómeros y del 30% para los productos de degradación.

AGRADECIMIENTOS

Agradecimientos

En primer lugar quiero agradecer a María José Cocero, jefa del grupo de investigación de Procesos a Alta Presión, por haberme dado la oportunidad de realizar la tesis en su grupo y por ser tutora de este trabajo. También me gustaría agradecer de forma especial a mi directora Soraya Rodríguez Rojo, un apoyo fundamental, que se ha dedicado a guiarme y a ayudarme día a día durante todos estos años.

Agradecer também a minha cotutora portuguesa Ana Matias pela proveitosa estada no grupo dos 'Nutraceuticals & Bioactives Process Technology' do IBET durante qausi dois anos.

Me gustaría agradecer a todos mis compañeros del Departamento de Ingeniería Química que han hecho estos 4 años más llevaderos, aunque necesito hacer menciones especiales. En primer lugar y más importante, Álvaro Cabeza, que más que un amigo es un hermano. Un apoyo incondicional, siempre dispuesto a ayudarme, y sobre todo, a escucharme. MUCHAS GRACIAS por todo. A Ana, por su apoyo incondicional, amistad y la gran cantidad de momentos vividos juntas. A mis chicas Celia, Yoana, Nerea y Nuria. En especial a las dos primeras, gracias por aportarme tanto y por todo lo compartido. Sé que siempre podré contar con vosotras (y viceversa!). A mis divertidos amigos Álvaro Sastre y Víctor. Al resto de los componentes de "marichochos", Marta y Alberto. À linda da Inês, quem em pouco tempo passou a convertirse numa das melhores amigas que uma pessoa pode imaginar. He compartido muchos buenos momentos con todos vosotros, tanto dentro como fuera del trabajo. Me llevo amistades para toda la vida.

Á Vanessa e o Daniel, dois dos meus colegas portugueses. Á jeitosa da Vanessa, ela sempre estava lá para ajudarme desde o inicío. Ao Daniel, muito obrigada. Graças a ti o tempo que passé lá foi muito mais agradável e divertido.

Por último, quiero dedicar la tesis a las personas más importantes para mí. A mis padres y mi hermano; por vuestro apoyo, vuestra compañía, vuestras visitas a Portugal, por estar siempre ahí y por todos vuestros consejos; gracias a vosotros soy lo que soy y no puedo estar más orgullosa de ello. A Alberto, por aparecer en el momento justo. Siempre apoyándome y aguantándome, hasta en los momentos más difíciles. Las palabras se quedarían cortas aquí para decir lo que os quiero.

ABOUT THE AUTHOR

About the author

Rut Romero Díez (Palencia, 23 July 1989) studied Chemical Engineering in the University of Valladolid from 2007 to 2012. During her studies, he started in research with the work: "*Solubility of Paracetamol and Polyvinylpyrrolidone in mixtures of CO_2 and solvents. Study of the influence of the solvent*" in 2012 with a Scholarship Erasmus (EuRopean Community Action Scheme for the Mobility of University Students) conceded by the European Union (EU) during 7 months at the *Friederich-Alexander Universität, Erlangen-Nürnberg* (Germany).

In 2014, the author graduated in a Master Degree in Engineering Thermodynamics of Fluids. During these time she was involved in the researching work of "*Titanium dioxide nanoparticle coating in fluidized bed via supercritical anti-solvent process (SAS)*". She also began her PhD thesis in 2014 in High Pressure Processes group in the department of Chemical Engineering and environmental technology in the University of Valladolid under the supervision of Doctor Soraya Rodríguez Rojo and Professor María José Cocero Alonso. The same year Rut achieved a MARIE SKLODOWSKA-CURIE ACTIONS fellowship within the European project WineSense (Research on extraction and formulation intensification processes for natural actives of wine, part of the Marie Curie Industry-Academia Partnerships and Pathways) in iBET (Instituto de Biologia Experimental e Tecnológica) Oeiras, (Lisbon). This mobility was for a period of 20 months (from January 2015 to September 2016), in the Nutracêuticos e Libertação Controlada group headed by Doctor Ana Matias.

PUBLICATIONS

Víctor Martín, Rut Romero-Díez, Soraya Rodríguez-Rojo, María José Cocero. '*Titanium dioxide nanoparticle coating in fluidized bed via supercritical anti-solvent process (SAS)'.* Chemical Engineering Journal. 279, pp. 425 - 432. Elsevier, 01/11/2015.

(https://doi.org/10.1016/j.cej.2015.05.014)

R. Romero-Díez, S. Rodríguez-Rojo, M.J Cocero, C.M.M Duarte, A.A. Matias, M.R. Bronze. *'Phenolic characterization of aging wine lees: Correlation with antioxidant activities'*. Food Chemistry. 259, pp. 188-195. Elsevier, 27/03/2018.

(https://doi.org/10.1016/j.foodchem.2018.03.119)

R. Romero-Díez, M. Matos, L. Rodrigues, M.R. Bronze, S. Rodríguez-Rojo, M.J Cocero, A.A. Matias. *'Microwave and ultrasound pre-treatments to enhance anthocynins extraction from different wine lees'*. Food Chemistry. 272, pp. 258-266. Elsevier, 04/08/2018.

(https://doi.org/10.1016/j.foodchem.2018.08.016)

CONFERENCES

- Víctor Martín Velasco, Rut Romero Díez, Soraya Rodríguez Rojo and María José Cocero Alonso. '*Coating of nanoparticle agglomerates via SAS in fluidized bed'*. 14th European Meeting on Supercritical Fluids (Marseille). 2014. Poster.

- Víctor Martín Velasco, Rut Romero Díez, Soraya Rodríguez Rojo and María José Cocero Alonso. '*Nanoparticle aggregates coating in supercritical fluidized bed by supercritical antisolvent Technology'*. VII Reunión de expertos en fluidos comprimidos (Barcelona). 2014. Poster communication.

- Rut Romero-Díez, Ana A. Matias, Soraya Rodríguez Rojo and María José Cocero Alonso. '*Wine lees as an alternative source of polyphenol-derived compounds'*. 9th World Congress on Polyphenols (Malta). 2015. Poster communication.

- Rut Romero-Díez, Liliana Rodrigues, Rosario Bronze, Soraya Rodríguez Rojo, María José Cocero Alonso and Ana A. Matias. *'Extraction kinetics of anthocyanins from wine lees: impact of two distinct pre-treatments'*. (Holand). 2016. Poster communication.

- Rut Romero-Díez, Liliana Rodrigues, Rosario Bronze, Soraya Rodríguez Rojo, María José Cocero Alonso and Ana A. Matias. *'Pretreatment effect on anthocyanin Extraction kinetics from different wine lees'.* (Poland). 2017. Oral communication.

- Rut Romero-Díez, J. Rubio, Ana A. Matias, Rosario Bronze, María José Cocero Alonso and Soraya Rodríguez Rojo. *'Using microwaves as a pre-treatment for enhancing the extraction of polyphenols from grape stems'.* (France). 2018. Oral communication.

- Rut Romero-Díez, J. Rubio, M. Matos, Ana A. Matias, Rosario, María José Cocero Alonso and Soraya Rodríguez Rojo. '*Green grape stems biorefinery: polyphenols and hemicellulose extraction*'. (Lisbon). 2018. Poster communication.

PROJECTS

WineSense Project, *"Research on extraction and formulation intensification processes for natural actives of wine".* Industry-Academia Partnerships and Pathways. FP7-PEOPLE-2013-IAPP. Grant agreement no.: 612208**.** Project start date: 01/10/2013. Duration of the project: 48 months.

DIRECTED WORKS

Internship co-direction (150h). Edwin Hoyos Duran. 2017. University of Valladolid.

Final Project degree co-direction. Joana García Rubio. 'Extracción de compuestos bioactivos mediante pre-tratamiento con microondas de subproductos vinícolas. Valorización del raspón de la uva'. 2018. University of Valladolid.