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SUSTAINABLE EXPLOITATION OF WINERY BY-PRODUCTS TO RECOVERY BIOACTIVE COMPOUNDS USING MEMBRANE TECHNOLOGY

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Esame finale anno 2021

Declaration

I declare that this thesis, as well as the results reported in it, are the product of my work and that as far I know, it does not contain material previously published or written by another person, nor does it contain material that has been used to obtain a degree or diploma in any other educational Institution.

I also declare that I give fair recognition in the thesis to the people who contributed with their work and, finally, I declare that this thesis is the product of my own work with the permitted support of third parties regarding the conception of the project, in the style of the presentation, or to written expression.

Cesena. 29 September 2021

ABSTRACT

Currently, the use of waste generated in the agri-food industry for the recovery of valuable compounds is one of the most important fields of study. Among the agri-food processes, the cultivation of grapes for the production of wine generates large amounts of waste, whose elimination, can significantly reduce environmental impacts. Therefore, like in all food industries, the by-products from the winemaking process have been investigated to consider methods for their treatment, minimization, and prevention.

However, during the last few years there has been a growing interest in their recovery as valuable compounds for nutraceutical applications.

The efficient and economic recovery of these molecules represents a key point for the evaluation of the compounds derived from this process. The enormous amount of waste produced in winemaking, combined with well-designed extraction methods and existing technologies, leads to the recovery, recycling and improved sustainability of high-value ingredients added to the food chain. However, the high bioactivity of the compounds, together with their susceptibility to enzymatic and thermal degradation, make their handling difficult. In this context, this PhD thesis discusses different extraction processes, membrane operation integration and microencapsulation of bioactive compounds for their separation, protection and conservation.

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CHAPTER 1

Nomenclature:

AD: Anaerobic digestion

COD: Chemical demand for oxygen

db: Dry basis

dw: Dry weight

EU: Europe Union

ha: hectare

hL: Hecto liters

HPLC: High Performance Liquid Cromatography

mhl: millions of hectoliters

t: tons

vol: volume

1.0. Introduction

Grape cultivation is one of the most extensive crops in the world with an annual production of 92 million tons and cultivated vineyards covering an area of 7.9 million ha. China is one of the largest grape cultivators producing 13.5 million tons, followed by Italy with 8.5 million, United States with 6.9 million, Spain with 6.6 million and France with 6.2 million tons. The commercialization of the grape ranges from raw consumption to the production of juices, wines, jams, raisins, vinegar, jelly, seed extract and seed oil; however, 80% of the whole grape production is destined to winemaking (Zhu et al., 2015, FAOSTAT, 2018). Table 1 displays the major grapes and wine producers in 2009, showing that Italy leads world wine production with 54.8 million hectoliters (mhl), followed by France with 48.6 mhl and Spain with 44.4 mhl (OIV, 2019), and the grape *Vitis vinifera* is the most cultivated grape species for the production of wine (Devesa-Rey et al., 2011).

Table 1. Major grapes and wine producers (Modified from OIV, 2019)

Countries	Table grape (%)	Dried grape (%)	Wine grape (%)	Wine production (mhl)
USA	16.3	18.1	65.6	23.9
Chile	26	3.9	70.2	12.9
Argentina	0.9	5.5	93.7	14.5
Brazil	53.5	0.0	46.5	3.1
Spain	4.0	0.0	96	44.4
France	0.4	0.0	99.6	48.6
Italy	13.5	0.0	86.5	54.8
Germany	0.4	0.0	99.6	10.3
Rumania	6.9	0.0	93.1	5.1
South Africa	15.8	15.5	68.7	9.5
China	84.1	5.6	10.3	9.1
Australia	7.1	1.9	90.9	12.9

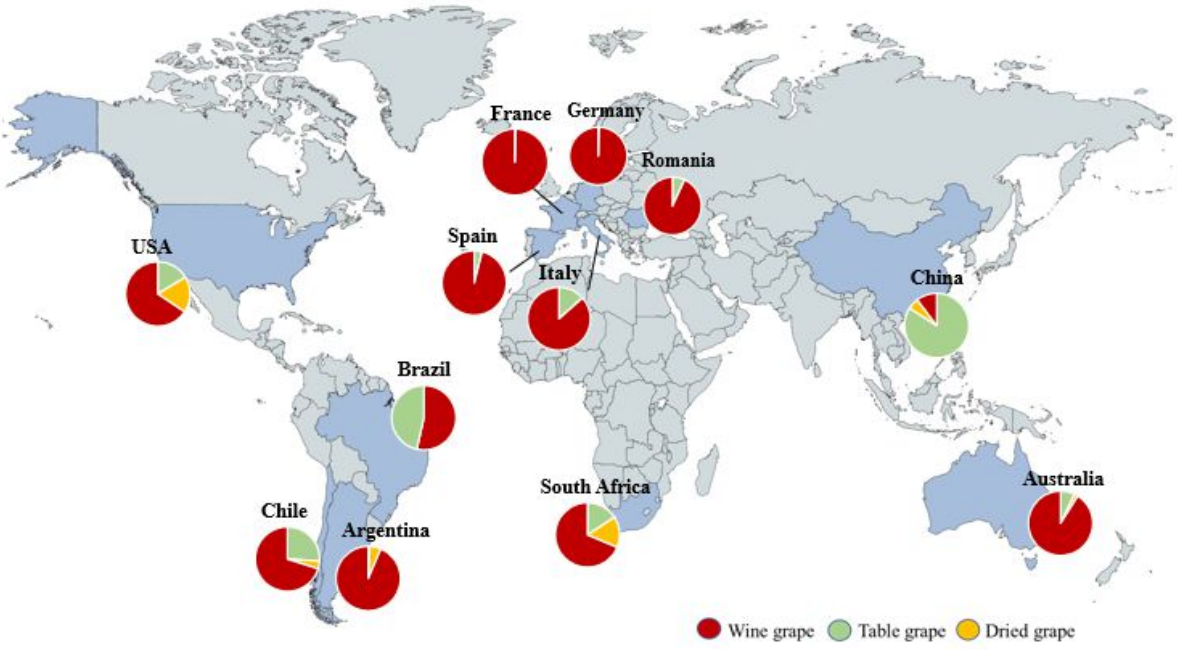


Figure 1. Schematic representation of the major grape producers (Modified from OIV, 2019)

The wine sector is responsible for the generation of a huge amount of waste (Figure 1) at the primary production level (vine shoots from pruning and cuttings); then during the first step of processing (stems, grape pomaces) (Grainger & Tattersall, 2007) and then following the fermentation stage (grape pomaces, lees). The amount of waste generated by the winemaking process can vary due to different factors such as the harvest. Even so, it is estimated that for one ton of grapes, is produced 0.13 t of grape pomace and 0.06 t of wine lees. The main by-product of the wine industry is grape pomace, which consists of skin, pulp, stalks and seeds (Galanakis, 2017; Oliveira & Duarte, 2016). Table 2 presents percentages of the compounds of these products.

Table 2. Percentage of by-products and major compounds from grapes during the winemaking process (Nerantzis & Tataridis, 2006)

By-products	Content in grape (%)	Major compounds
Grape stalks	2.5 – 7.5	-
Grape pomace (wet)	25 – 45	Sugars, phenolics, tartrate and fibers
Grape seeds	3 - 6	Grape seed oil and phenolics
Wine lees	3.5 – 8.5	Pigments, tartrate, ethanol and beta-1.3-glucans

As shown in figure 2. Through the wine process, waste is generated in different outputs. Skin and seeds comprise of approximately 20-30% of the waste generated during the winemaking process; however this depends on the grape cultivation techniques, pressing process and the different stages of fermentation (Dwyer et al., 2014; Laufenberg et al., 2003). At a European level, the wine industry produces approximately 14 million tons of waste per year (Schieber et al., 2001; Torres et al., 2001).

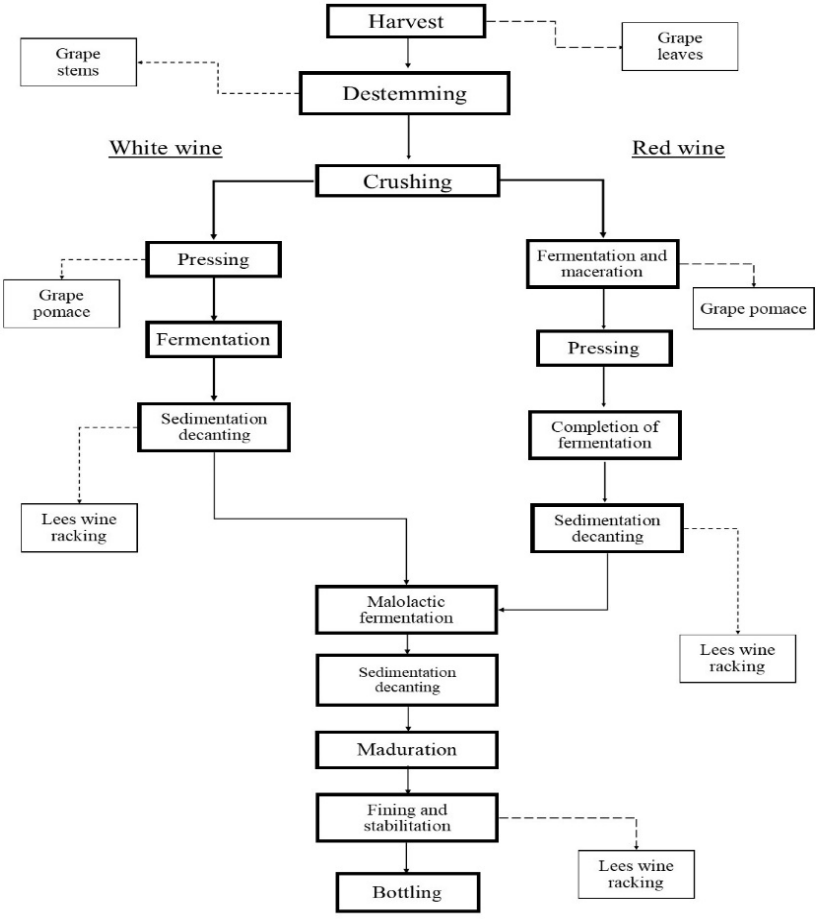


Figure 2. Diagram of the wine process and generation of by-products. (Modiefied fromDevesa-Rey et al., 2011)

In Europe, the wine sector is regulated by European legislation. In particular, the "Common Market Organization" (CMO), which was introduced for the first time in 1999 through the EU Regulation

(Reg.) No 1493/99. It was then reformed in 2008 by the EU Reg. No 479/2008 and 555/2008. Years later, in 2013 was taken other reformed under the EU Reg. No 1308/2013 of the European Parliament and of the Council where it was implemented "organization of the markets in agricultural products".

The focus of these reforms is to organize the way the EU wine market is managed in order to ensure that wine production matches demand, eliminate wasteful intervention, and reorient public spending in order to make European wine, more competitive. Regarding residues from the vinification process, producers are required to dispose of the by-products from winemaking, or any other type of grape processing, following the guidelines set out in the EU legislation. In wineries where the production of wine or must, is higher than 25 hL, the lees must not be removed from the cellar before the denaturation process, preventing their usage in winemaking. In addition, the over pressing of grape pomace is strictly prohibited (Galanakis, 2017).

The following presents details on the wastes generated in the wine sector, and possible treatment strategies for their exploitation.

Vine shoots. Classified as non-wood lignocellulosic agricultural residue, generated in large quantities on commercial vineyards. These residues are considered as novel biomass but scarcely exploited. Therefore, one of the main challenges of the wine sector is to identify strategies in order to increase its value. In this context, the interest in the scientific community in obtaining biomass from agro-industrial waste has been notorious due it can be used as an energy source or as a raw material in the wood industry, which consequently would provide additional income to fruit producer and also support a more sustainable system.

An example of these efforts is the study conducted by the authors (Velázquez-Martí et al., 2011). They reported the positive incidence of the shade of the plantation structure in the quantity of vine shoot produced. The horizontal trellis type plantation structure has produced a quantity of 4.2 t/ha

of vine shoot, which is more than double that of the plantation structure with a high trellis and more than triple with the standard trellis system.

Grape stalks. Comprise of approximately 14% of the total solid waste obtained during the vinification process.

They are mainly composed of cellulose (30.3%), hemicellulose (21.0%), lignin (17.4%), tannins (15.9%), protein (6.1%) and polysaccharides as heteroxylan (Prozil et al., 2012).

Grape skin. Approximately 50% of the grape pomace; the ratio of skin to seeds can vary greatly depending on the grape variety. Grape skins are of great interest, due to their composition: protein (5÷12%), ash (2%÷8%), soluble sugars (70%) and high fiber content. They are also considered a source of anthocyanidins and anthocyanins, which are natural pigments with antioxidant properties (Deng et al., 2011; Ivan et al., 2011; Teixeira et al., 2014)

*Grape seed. M*ostly fiber (40%), essential oil (16%), protein (11%), complex phenolic compounds such as tannins (7%), sugars and minerals. Phenolic monomers are present such as (+) - catechin, (-) - epicatechin and (-) - epicatechin-3-O-gallate, as well as dimetric, trimeric and tetrameric procyanindis (de Campos et al., 2008; Saito et al., 1998).

Grape pomace. One of the most abundant by-products, comprising of approximately 60% of the waste derived from the winemaking process. Grape pomace is composed of a mixture of skin, seeds and residual stalks, as well as a significant amount of secondary metabolites (phenolic acids, flavonols, proanthocyanidins, flavanols, anthocyanidins, stilbenes), with recognized biological properties (antioxidant, antimicrobial, anti-inflammatory, anticancer, cardiovascular protection). They are predominantly used in the pharmaceutical, food and cosmetic industries (Galanakis, 2017; Teixeira et al., 2014).

*Wine lees. R*esidue consisting of dead yeast cells, yeast residue, or particles which precipitate at the bottom of the wine tanks or barrels (Hwang et al., 2009).

Grape pomace and wine are discussed below with regard to their chemical composition, impact on the environment and possible exploitation in several economic sectors.

1.1. Chemical composition of the grape pomace and wine lees

1.1.1. Grape pomace

Grape pomace production is generated after the harvest. Red grape is stripped of its stems and placed in containers where it is crushed. The must is then left in the tank for no more than three days.

The addition of pomace, specifically the skins, provides the necessary pigments (anthocyanins) to create the red color of the wine. During this time, the spontaneous fermentation process occurs. Once fermentation is nearly complete, the must (which is described as juice and pomace together) is pressed to extract the remaining juice from the interior of the grape. The liquid is then separated from the solids, which are then crushed a second time. Grape pomace contains anthocyanin pigments which contribute to the red color of the wine. Therefore, during the red winemaking process, the juice is kept together with the pomace and is fermented at temperatures of between 28 and 30 °C. They remain together for two weeks until the yeast has converted all of the sugars into alcohol. Concerning white grapes, after the harvest they are stripped of their stems, before being crushed and deposited in containers, which allow the continuous extraction of the must from the bottom of the tank. Since a color is not desirable in white wine, the pomace is not included in the fermentation process.

The sugar content of white grape pomace is still high, however the alcohol content is low, therefore an additional step of fermentation is required before distillation (Dwyer et al., 2014; Silva et al., 2000). If the pomace has been obtained from the red winemaking process, it is a fermented grape pomace with a low sugar content and low phenolic content. If however, it is obtained from the grape juice or white winemaking processes, the grape pomace is not fermented, therefore it is potentially richer in sugar and phenolic content compared to fermented grape pomace.

This type of waste can be classified into two groups as follows:

- Seedless pomace, which contains the pulp, skin and stems
- Pomace containing seeds

The seedless pomace is a rich source of polyphenols, lipids, indigestible fibers, proteins and minerals, as well as containing a significant amount of anthocyanins. In addition, grape seeds contain proteins, indigestible carbohydrates such as cellulose and pectin and significant amounts of different types of antioxidants, such as tocopherols and B-carotenes, flavonoids, procyanidins, resveratrol, sugars and minerals. This part of the grape pomace has received a great deal of interest, due to the high content of phenolic compounds, which are not completely extracted during vinification. These compounds are well known for being responsible for their antioxidant properties.(Beres et al., 2017; Brenes et al., 2016; Sotiropoulou et al., 2017). Table 3 shows the chemical parameters of grape pomace.

Table 3. Chemical parameters of grape pomace

Parameters (dry basis)	Value composition	References
Ash (%) db	5.5	(Llobera & Cañellas, 2007)
Moisture (%)	8.0 – 9.8	(de Campos et al., 2008; Drevelegka & Goula, 2020)
Protein (%)	12	(Llobera & Cañellas, 2007)
Pectin (%)	3.9	(Sousa et al., 2014)
Total dietary fibre (%)	70.0	(Llobera & Cañellas, 2007)
Total phenols (mg GAE/ 100 g)	2.4 – 2.6	(Llobera & Cañellas, 2007; Louli et al., 2004)
Glucose	7.9	(Sousa et al., 2014)
Fructose	8.9	(Sousa et al., 2014)

Grape seeds are rich in unsaturated acids such as oleic and linoleic, and phenolic compounds. The chemical composition of grape seed is approximately 6.5% moisture, 11% protein, 5.7% ash, 46% acid insoluble lignin and 1.4% acid soluble lignin (Prado et al., 2014).

1.1.2. Wine lees composition

Another by-product generated from the winemaking process is wine lees, produced during alcoholic fermentation. Wine less is defined by the Council Regulation (n. 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 of this product (Pérez-Serradilla & Luque de Castro, 2011).

The composition of lees is variable. They are mainly composed of microorganisms (mostly yeasts and bacteria), insoluble carbohydrates from the cellulosic and hemicellulosic fractions, β-glucans, squalene (found in the lipophilic nucleus of lipid globules within cells that are surrounded by a thick and rigid cell wall) and in a lesser proportion tartaric acid and inorganic matter. Wine lees play a significant role in wine processing by interacting with polyphenolic compounds. However, this residue releases enzymes favoring the hydrolysis and transformation of polyphenolic substrates with high added value, such as gallic acid or ellagic acid (Naziri et al., 2012, 2016; Pérez-Serradilla & de Castro, 2008; Vattem & Shetty, 2003). Furthermore, wine lees is composed of lignin, proteins, metals and organic salts (mainly tartrates) and have a liquid phase rich in ethanol and organic acids, such as lactic acid or acetic acid. The high protein content and total nitrogen concentration, combined with the considerable presence of essential amino acids such as tyrosine, valine gran and aminocaproic acid, led to the proposal for the use of wine lees as a source of protein for ruminants. However, the high content of polyphenols bound with proteins, makes a large part of the lees fraction not assimilable (Delgado De La Torre et al., 2015b; Molina-Alcaide et al., 2008). However, phenolic compounds are one of the main contributors to the color and flavor of wine, and it should be noted that the type of phenolic compounds present in wine, depends on the origin of the grape and the climate where it is growing (Pérez-bibbins et al., 2015).

Regarding the amount of polyphenols, wine lees contain between 1.9 and 16.3 g of polyphenols/kg which is dependent on the type of wine and method of processing. Polyphenols in wine lees can be found both in the liquid fraction (wine) and in the solid form, as a result of their adsorption on the yeast cell walls during winemaking. This adsorption mechanism will depend on the type and quantity of phenolic compounds, the grape variety, the degree of maturity, the maceration method

and the fermentation temperature (Bustamante et al., 2008; De Iseppi et al., 2020; Zhijing et al., 2016)

1.2. The main components recovered from winemaking by-products

The recovery of biologically active compounds from the byproducts of winemaking is well documented. The most common approaches are directed towards the production of natural grape extracts. For example, grape seed has one of the highest concentrations of monomeric proanthocyanidins found in fruit. It is also noted that resveratrol is the phenolic compound with the highest antioxidant activity in grapes (Crespo & Brazinha, 2010).

The bioactive phytochemicals found in winery byproducts are mainly represented by polyphenols, which arise biogenetically from two main biosynthetic pathways:

- Shikimate and,
- Acetate

These compounds are structurally composed of one or more aromatic rings joined by different moieties. Therefore, their chemical structures range from simple molecules (phenolic acids), to complex polymeric structures (tannins) (Katalinić et al., 2010; Kris-Etherton et al., 2002). The phenolic compounds obtained from waste from the wine industry belong to different classes which include phenolic acids (hydroxybenzoic and hydroxycinnamic), flavonoids (flavanols, flavan-3-ols, proanthocyanodins, flavones and flavonols) and stilbenes. All of which have been reported to be responsible for biological effects (El Gharras, 2009; Kähkönen et al., 1999).

Polyphenolic compounds found in grape pomace have a wide range of biological properties such as antiallergenic, anti-inflammatory, anticancer, anti-aging, antimicrobial, antioxidant, antithrombotic, antilipotropic, insulinotropic, cardioprotective and vasodilatory. Through these mechanisms, polyphenolic compounds have multifunctional activities in the human body. As previously mentioned, grapes contain phenolic compounds in their skin, seeds and short stems, which

constitute around 10-11% of the dry weight of extractable phenols. These compounds found in grape pomace have been classified in grape pomace as follows:

- Phenolic acids
- Flavonoids
- Tannins

The most abundant phenolic compounds are anthocyanins that impart the red color to grapes after ripening, hydroxybenzoic and hydroxycinnamic acids, flavan-3-ols (such as catechins and proanthocyanidins), flavanols and stilbenes (Beres et al., 2017; Makris et al., 2007; Sirohi et al., 2020a)

1.2.1. Phenolic acids

Phenolic acids have a functional carboxylic group and are divided into hydroxycinnamic acids and hydroxybenzoic acids. Hydroxycinnamic acids are found in foods, unlike hydroxybenzoic acids, and they usually exist in free form except in frozen, sterilized, or fermented foods. Concerning hydroxycinnamic acids, they predominantly include gallic, ρ-coumaric, ferulic, caffeic, chlorogenic and sinapic acids (Yu & Ahmedna, 2013). The most abundant phenolic acids in winemaking by-products are the benzoic and cinnamic acids. Figure 3. presents a diagram of the classification of phenolic compounds.

1.2.2. Hydroxybenzoic acids

The derivatives of hydroxybenzoic acid are mainly ρ-hydroxybenzoic acid, protocatechic acid, tanic acid, vanillic acid, derivatives of gallic acid and syringic acid. In this context, gallic acid is presented as the most abundant hydroxybenzoic acid in grape stems, skin and seeds followed by syringic acid in grape stems and protocateuic acid in grape seeds and skins (Anastasiadi et al., 2012; Apostolou et al., 2013; Di Lecce et al., 2014).

The antimicrobial effect of grape pomace is usually attributed to different phenolic compounds, predominantly phenolic acids (Such as gallic acid followed by p-hydroxybenzoic and vanillic acids). In this context gallic acid was found to be a strong antimicrobial agent in grape seed extract, suggesting the high power of hydroxycinnamic acid, compared to its corresponding hydroxybenzoic acid, due to its lower polarity, which means that it has to cross through the membrane (Margarita Corrales et al., 2009; García-Lomillo & González-SanJosé, 2017).

1.2.3. Hydroxycinnamic acids

Hydroxycinnamic acid is present within all parts of the fruit of the grape, and a significant amount is located in the external part of the skin when the grape is ripe. However, the concentration of hydroxycinnamic acid decreases as the ripening process advances. Although, the total content of this phenolic class is proportional to the size of the fruit.

The main hydroxycinnamic acids present in grapes and wine are caftaric, ρ-coutaric and fertaric acids, in which the caftaric and fertaric are the acids that are predominantly present in the trans-form, while a small fraction of p- coutaric is in the cis-form. The phenolic profile of these organic acids derived from the winemaking process, depends on the type of residue and the type of grape (Teixeira et al., 2014).

1.2.4. Flavonoids

Flavonoids are the most abundant phenolic compound in grapes and wines and are a crucial element in determining the quality parameters of red wine. Flavonoids can be classified as anthocyanins, flavonols and flavan-3-ols in grapes (Cheng et al., 2020).

Flavonol biosynthesis occurs during the grape development and ripening phases. The highest concentration of flavonols in grapes has been found to be between 3-4 weeks post-veraison. This physiological process, together with the harvest time, contributes significantly to the final quality and quantity of flavonoids in the residues of the winemaking process. The comparison of the

composition of flavonoids in the residues of the wine process showed that flavan-3-ols are found in similar concentrations in both the skins and in the seeds of the grape (Cook & Samman, 1996).

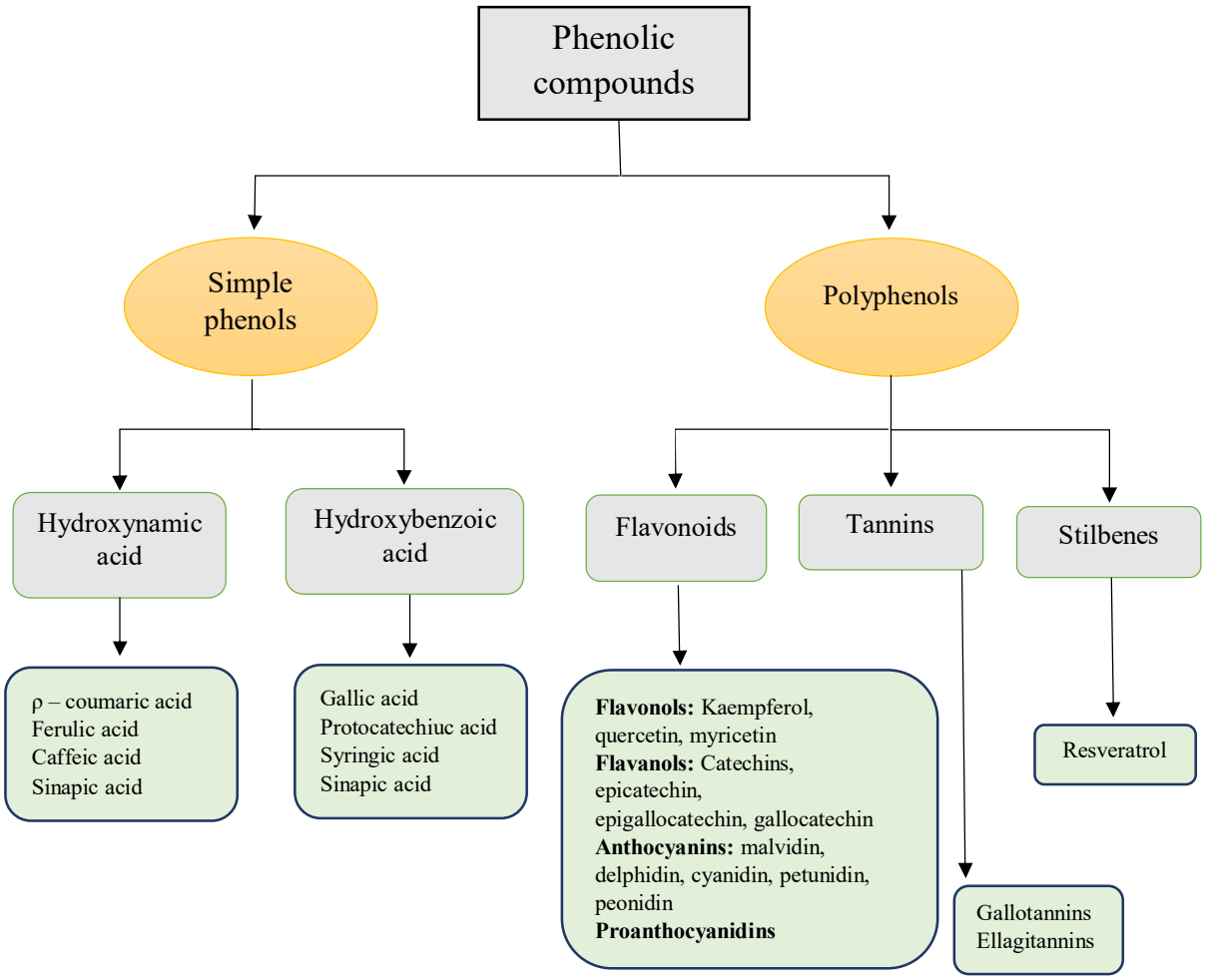


Figure 3. Classification of polyphenolic compounds

1.2.5. Flavonols

Flavonols are a type of flavonoid compound derived from the secondary metabolism of plants.

In grapes, flavonols are present within the skin and are extracted from the must during the maceration stage of red wine vinification. However, compared to other flavonoid compounds such as anthocyanins and proanthocyanidins, flavonols are present in lower concentrations (Castillo-Muñoz et al., 2007, 2009; Favre et al., 2018). The distribution of flavonols in the different types of

residues produced in the wine process (stems, seeds, skins, pomace and leaves) present significant differences concerning the individual flavonols and their relative proportions. In addition, these compounds are present in greater quantity within red winemaking waste compared to waste from the white winemaking process. Regarding the analysis of individual flavonols in red grape stems, it has been observed that there are a wide range of flavanols, although the predominant one present is quercetin 3-O-glucuronide (around 128 mg·g−1·dw). However, a lower amount of quercetin 3-O-glucuronide (1.34 mg·g−1·dw) has been detected in grape pomace originating from the frapatto grape cultivars.

(Amico et al., 2008; Negro et al., 2003; Souquet et al., 2000). The phytochemical profile of the flavonols present in grape pomace are mainly quercetin-3-O-glucuronide, which has been found to be the predominant compound.

1.2.6. Flavanols

Flavanols are the most common polyphenols in the human diet and are mostly found in fruits, cocoa, tea, wine, nuts and beans. This group of polyphenols exist in a monomeric (catechin and epicatequin) and oligomeric (proanthocyanidins (PAs)) or condensed form such as tannins, depending on the molecular weight and also aglycone form and esterified with gallic acid (Aherne & O'Brien, 2002; Aron & Kennedy, 2008).

It is noted that tannins are complex, high molecular weight phenolic compounds that can be divided into two different groups:

- Condensed tannins, also known as proanthocyanidins, are made up of subunits of flavan-3-ols monomers and their structures vary according to their subunit structure, degrees of polymerization and the linkage position. This group of compounds also represents a significant proportion of the bioactive phytochemicals in winemaking residues such as procyanin dimers B1, B2, B3 and B4, and procyanin trimers C1, C2 and C3. Condensed tannins represent a significant amount of the grape pomace, around 21-52% (dry weight matter)

- Hydrolyzable tannins are complex polyphenols that can be degraded into smaller units mainly in sugars and phenolic acids.

The flavanols in products derived from grape processing exist in monomeric, oligomeric and polymeric forms, with an estimated 40% of monomers and oligomers in the external part of the intestine. The non-substantial part of flavonoids that are not absorbed, pass into the colon where they undergo a microbiotic catabolism before being absorbed as low molecular weight phenolic acids (Monagas et al., 2010; Ou & Gu, 2014; Teixeira et al., 2014; Teng & Chen, 2019).

1.2.7. Flavones

Flavones are compounds with a double bond between carbon C2 and C3, which differs from flavanols due to the absence of the hydroxyl group at carbon 3.

These phytochemical compounds have been found in very low amounts, including luteolin, compared to the major flavonoids such as (+)- catechin and (-) – epicatechin (Çetin et al., 2011).

1.2.8. Anthocyanins

Anthocyanins are compounds belonging to the group of flavonoids which are composed of secondary metabolites found in some vegetables and fruits such as grape, blueberry, sweet potato, red cabbage, black carrot and bean husk (Ock et al., 2007).

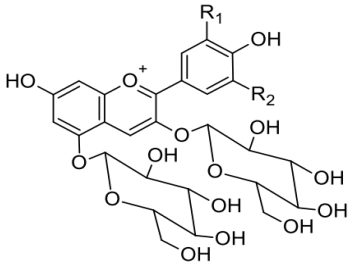
The main anthocyanins present in grapes are cyanidin, peonidin, delphinidin, petunidin, malvidin and their derivatives of glycosylation and/or acylation (Pomar et al., 2005; Trikas et al., 2017).

During the vinification and storage of wine, a biochemical transformation of flavonoids takes place. During the first steps of fermentation in the vinification process, anthocyanins biochemically transform into pigments, such as pyranoanthocyanins, which are formed by the reaction of the anthocyanidin-3-glucosin with low molecular weight compounds such as 4-vinylphenol, pyruvic acid and flavanols (Castañeda-Ovando et al., 2009; Lago-Vanzela et al., 2014). Table 4. Anthocyanins present in grape tissue and residues from the winemaking process.

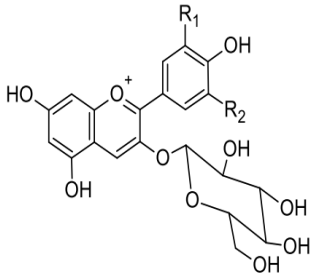
Table 4. Summary of some of the anthocyanins found in grape tissue and residue from the winemaking process

Compound	Skin	Wine lees	Pomace	Reference
Cy-3-*O*-Glc	≤0.04 (mg*g^{-1} dw, HPLC-DAD)	-	≤0.01 (mg*g^{-1} dw, HPLC-UV-DAD)	(Amico et al., 2008; Ruberto et al., 2007)
Cy-3-*O*-(6'-*O*-acetyl)-Glc	-	-	≤0.01 (mg*g^{-1} dw, HPLC-UV-DAD)	(Amico et al., 2008)
Del-3-*O*-Glc	≤4.2 (mg* g^{-1} dw, HPLC-FD)		≤1.20 (mg*g^{-1} dw, HPLC-UV-DAD)	(Amico et al., 2008; Ky et al., 2014; Ruberto et al., 2007)
Del-3-*O*-(6'-*O*-ρ-c coumaryl)-Glc	-	-	≤2.2 (mg*g^{-1} dw, HPLC-UV-DAD)	(Ruberto et al., 2007)
Mv-3- *O*-Glc	12.10-16.50 (mg*g^{-1} dw, HPLC-DAD)	0.09 (mg*g^{-1} dw, HPLC-DAD)	0.06-10.40 (mg*g^{-1} dw, HPLC-DAD)	(Amico et al., 2008; Pérez-Serradilla & Luque de Castro, 2011)
Mv-3-*O*-(6'- ρ-coumaroyl)-Glc		11.7 (mg*g^{-1} dw, HPLC-DAD)	≤27.10 (mg*g^{-1} dw, HPLC-DAD)	(Amico et al., 2008; Pérez-Serradilla & Luque de Castro, 2011; Ruberto et al., 2007)
Pn-3-*O*-Glc	1.90-7.10 (mg*g^{-1} dw, HPLC-DAD)		0.02 (mg*g^{-1} dw, HPLC-UV-DAD)	(Amico et al., 2008)

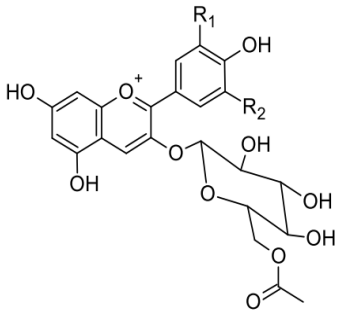
The major anthocyaninic compound is malvidin-3-O-glucoside, which is found in grape skins at a concentration of 16.30 mg*g^{-1} dw, as well as in grape pomace at a concentration of 10.40 mg*g^{-1}. The second most abundant anthocyaninic compound is peonidin-3-O-glucoside, which is present in the skin of grapes and wine at a concentration ranging from 0.70 to 11.50 mg * g-1.dw. (Amico et al., 2004, 2008; Ky et al., 2014). Figure 4. Shows the structures of anthocyanins found in twenty one grape varieties.



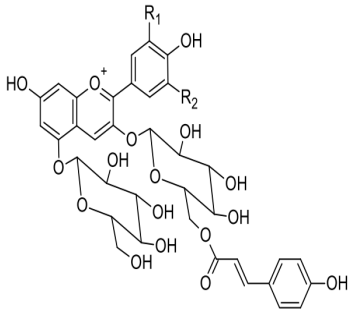
delphinidin (Dp) 3,5-*O*-diglucoside R_1 = OH; R_2 = OH
cyanidin (Cy) 3,5-*O*-diglucoside R_1 = H; R_2 = OH
petunidin (Pt) 3,5-*O*-diglucoside R_1 = OCH_3; R_2 = OH
peonidin (Pn) 3,5-*O*-diglucoside R_1= OCH_3; R_2 = H
malvidin (Mv) 3,5-*O*-diglucoside R_1 = OCH_3; R_2= OCH_3



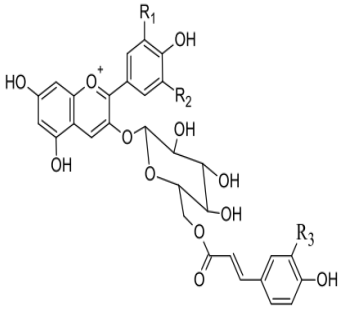
Dp-3-*O*-monoglucoside R_1 = OH; R_2 = OH
Cy-3-*O*-monoglucoside R_1 = H; R_2 = OH
Pt-3-*O*-monoglucoside R_1 = OCH_3; R_2 = OH
Pn-3-*O*-monoglucoside R_1= OCH_3; R_2 = H
Mv-3-*O*-monoglucoside R_1 = OCH_3; R_2= OCH_3



Dp-3-*O*-(6-*O*-acetyl) monoglucoside R_1 = OH; R_2 = OH
Cy-3-*O*-(6-*O*-acetyl) monoglucoside R_1 = H; R_2 = OH
Pt-3-*O*-(6-*O*-acetyl) monoglucoside R_1 = OCH_3; R_2 = OH
Pn-3-*O*-(6-*O*-acetyl) monoglucoside R_1= OCH_3; R_2 = H
Mv-3-*O*-(6-*O*-acetyl) monoglucoside R_1 = OCH_3; R_2= OCH_3



Dp-3-*O*-(6-*O*-*p*-coumaroyl)-5-*O*-diglucoside R_1 = OH; R_2 = OH
Cy-3-*O*-(6-*O*-*p*-coumaroyl)-5-*O*-diglucoside R_1 = H; R_2 = OH
Pt-3-*O*-(6-*O*-*p*-coumaroyl)-5-*O*-diglucoside R_1 = OCH_3; R_2 = OH
Pn-3-*O*-(6-*O*-*p*-coumaroyl)-5-*O*-diglucoside R_1= OCH_3; R_2 = H
Mv-3-*O*-(6-*O*-*p*-coumaroyl)-5-*O*-diglucoside R_1 = OCH_3; R_2= OCH_3



Dp-3-*O*-(6-*O*-*p*-coumaroyl)monoglucoside R_1=OH; R_2=OH; R_3=H
Cy-3-*O*-(6-*O*-*p*-coumaroyl)monoglucoside R_1=H; R_2=OH; R_3=H
Pt-3-*O*-(6-*O*-*p*-coumaroyl)monoglucoside R_1=OCH_3; R_2=OH; R_3=H
Pn-3-*O*-(6-*O*-*p*-coumaroyl)monoglucoside R_1=OCH_3; R_2=H; R_3=H
Mv-3-*O*-(6-*O*-*p*-coumaroyl)monoglucoside R_1 = OCH_3; R_2=OCH_3; R_3=H
Mv-3-*O*-(6-*O*-caffeoyl)monoglucoside R_1 = OCH_3; R_2=OCH_3; R_3=H

Figure. 4 Structures of anthocyanins identified in twenty one different hybrid red grape varieties (Flamini et al., 2013)

1.2.9. Stilbenes

Stilbenes are phytoalexins found in consumer plants, especially grapes. Grapes produce stilbenes in response to the expose to a number of physiological factors, including ozone and UV-C radiation, which can modify the stilbene content of grape residue through the process of the industry to obtain must. Resveratrol is the primary stilbene that is neutralized in grapes, wine and residues from the winemaking process, and the concentration of resveratrol found depends significantly on the state of

maturity and the variety of the grape. The most important stilbenes in grapes are resveratrol-3-O-b-d-glucopyranoside, resveratrol in both its steral forms (*cis* and *trans*) and dimers of piceatanol. Resveratrol is present in several organic residues originating from the winemaking process. The wine processing method used determines the concentration of resveratrol found in the final product (Flamini et al., 2013; González-Barrio et al., 2006; Püssa et al., 2006; Ribeiro De Lima et al., 1999).

1.3. Products achieved from winemaking by-products

Winemaking leads to the production of large amounts of waste, which increases the chemical oxygen demand (COD) and the biochemical oxygen demand (BOD_5), due to the high pollution load. In addition, the recycling and use of the grape pomace (Figure 5) and wine lees (Figure 6) bring advantages at an economic and environmental level in different areas of production.

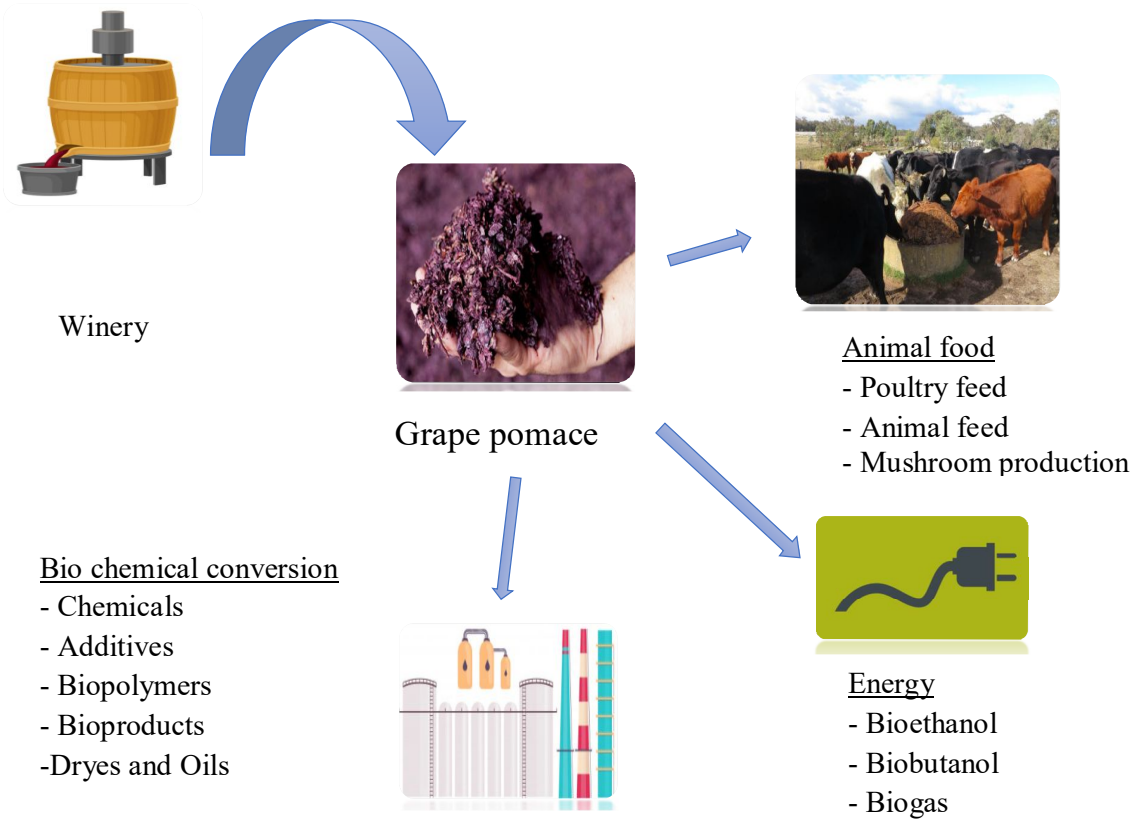


Figure 5. Applications from grape pomace. Modified (Sirohi et al., 2020a)

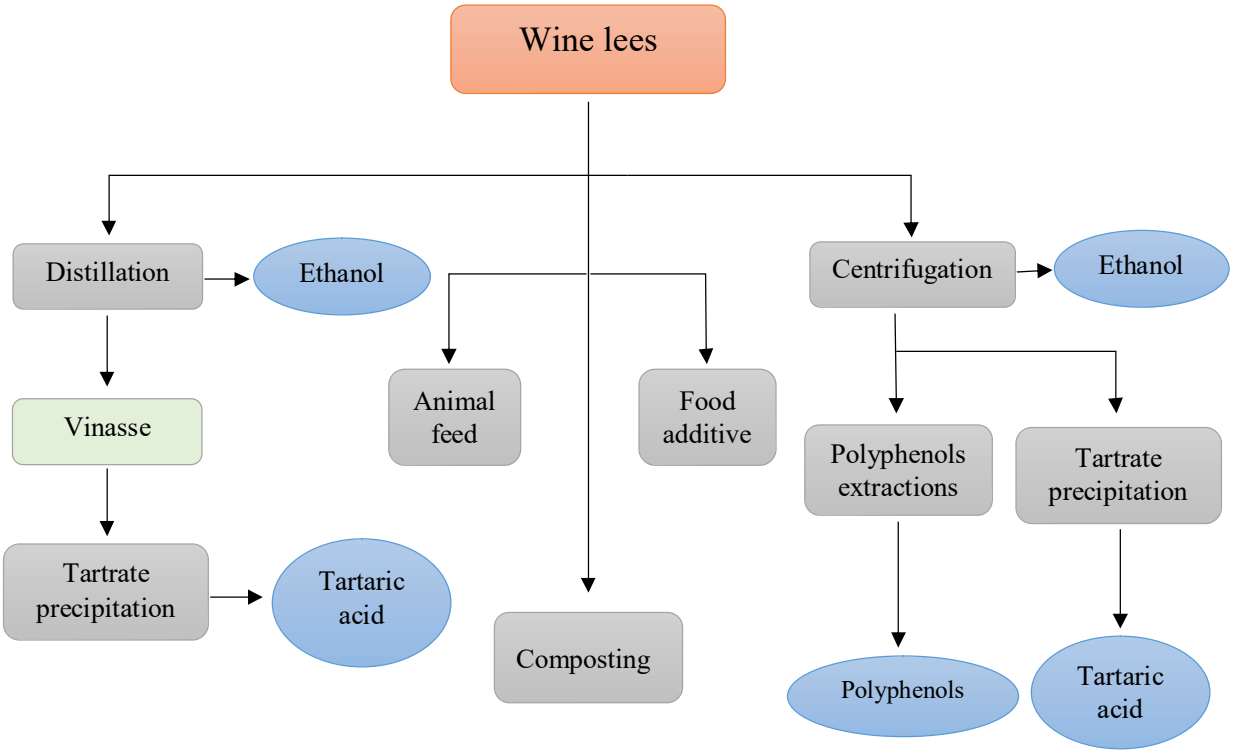


Figure 6. Applications from wine lees

The following presents a summary of various processes and useful products extracted from winemaking wastes:

1.3.1. Antioxidants

Wineries throughout the world produce large amounts of wine and consequently large amounts of by-products are generated. This leads to a need to develop alternative uses for these residues, such as in soil conditioners or fertilizers. As has been explained, the residues from the winemaking process contain significant levels of phenols, which have strong antioxidant properties. Therefore, the by-products from winemaking provide an opportunity to recover phenols and are in fact a cheap and abundant source of phenols (Pinelo et al., 2006). Figure 7 shows chemical structure of bioactive compounds found in the grape.

The antioxidant activity present in phenolic compounds are due to their free radical scavenging and metal chelating, which are influenced mainly by the number of OH groups and their position in the phenolic ring (Hogan et al., 2009).

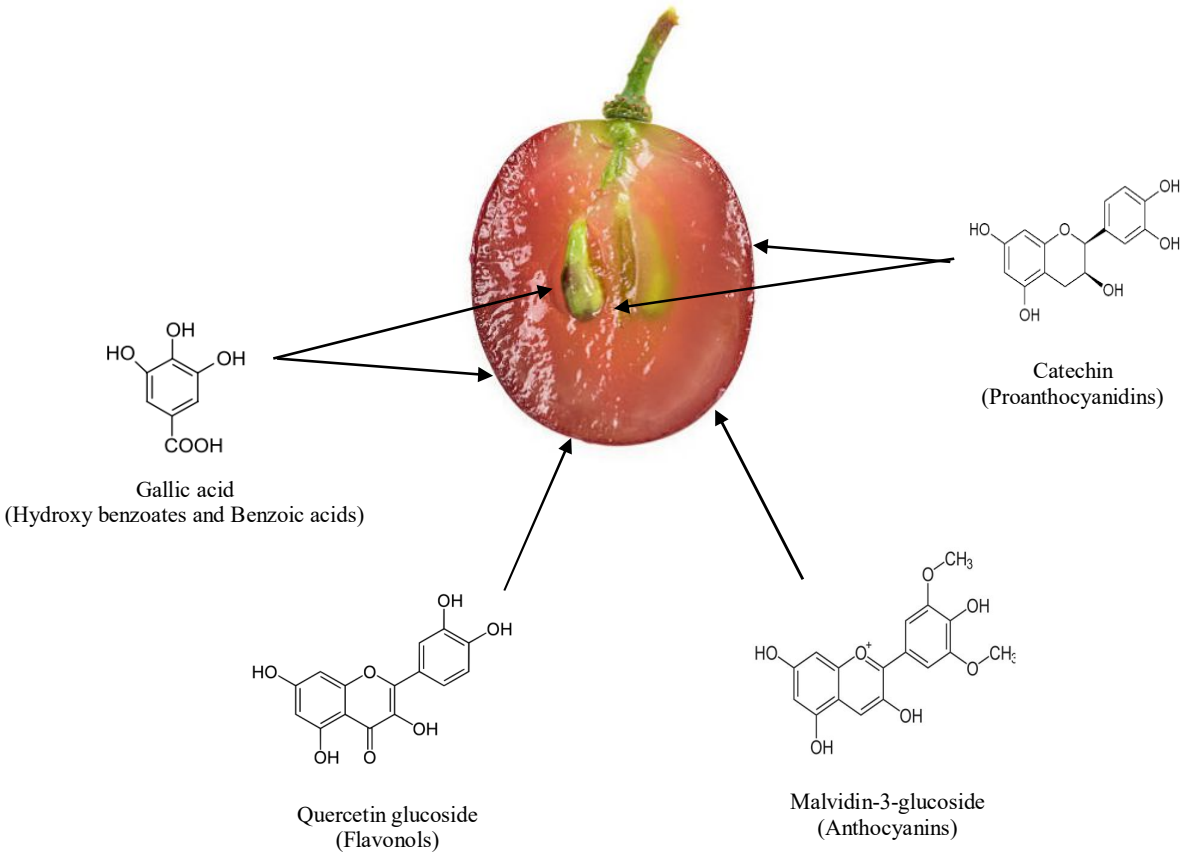


Figure 7. Structures of the bioactive compounds present in grapes

The high content of polyphenols presents in the seeds provide a high antioxidant activity. One of the major polyphenol compounds found in grape seeds are flavanols, where catechin is the predominant compound. Flavanols have significant biological and medicinal properties and are particularly known for their antioxidant activities. In addition, these compounds have antiulcer, anticarcinogenic, antimutagenic, anti-inflammatory, antiallergic and antitoxic effects.

In addition, the hydrolysis of the lignin fraction can produce aromatic phenolic compounds (for instance, low molecular mass alcohols, aldehydes, ketones or acids) making also vine shoots a significant source of phenols (Bashir et al., 2016; Delgado-torre et al., 2012).

The delignification process with sodium hydroxide (NaOH) has been used to isolate lignin in a liquid fraction, so that the lignin can be removed, increasing the accessibility of the enzymes within the solid residue, which is also enriched in cellulose. After the alkaline hydrolysis, the presence of ferulic acid, p-coumaric on the part of the hydroxyxinamic acids and gallic acid on the part of the hydroxybenzoic acids have been noted. These compounds are widely used in food, pharmaceutical and cosmetic industries due to their antioxidant properties (Bustos et al., 2005; Max et al., 2010)

1.3.2. Biofuels

The rapidly growing demand for heat, electricity and cold has caused a huge burden on nature, creating an ever-growing need for renewable alternatives. The reuse of biodegradable wastes from the agri-food sector are helping to provide sustainable biofuels and reduce reliance on non-renewable fuels. Environmental professionals have advised to use natural resources more efficiently and also to increase the use of renewable energy, such as biofuels, or the transformation and use of biomass, to be a beneficial way of reducing our reliance on non-renewable fuels. This is due to the relatively uniform distribution of biofuels and biomass throughout the world. The use of waste and raw materials to obtain biofuels helps to sustain the economy, and furthermore it reduces environmental pollution due to the reuse of waste materials.

Most winemaking residues, such as grape pomace, contain carbohydrates which can be converted into ethanol and other biofuels. The conversion of biomass from plants into a liquid form can be difficult due to its complex natural structure, which is not always easy to break down unless an enzymatic pretreatment is used. The part that remains after the conversion to biofuels is used for animal feed or fertilizers (Devesa-Rey et al., 2011; Devi & Sumathy, 2017; Sirohi et al., 2020a).

Grape pomace is also suitable to produce biochar, bio-oils or mixed gas mixtures when heated to low or high temperatures. The alcoholic fermentation of grape pomace generates bioethanol, oleanolic acid and residues which can act as insulation materials (Sirohi et al., 2020a).

Ethanol is produced by the conversion of the sugar content into alcohol with the production of carbon dioxide, under controlled environmental conditions. Grape pomace residues contain mostly water-soluble carbohydrates, such as glucose and fructose, which can be converted into ethanol through the fermentation process. In the fermentation process, a part of the sugar is assimilated by yeast and then transformed into glycerol, acrylaldehyde and lactic acid. The more complex carbohydrate structures can be broken down by the addition of the yeast *Saccharomyces cerevisiae* and converted into ethanol. One of the many uses that ethanol has is the possibility of being used as a transportation fuel and hence a renewable substitute for gasoline (Braide et al., 2016; Sirohi et al., 2020b).

Different studies have investigated and demonstrated the functionality of grape pomace biomass to convert into biofuels.

Ethanol production based on the presence of significant amounts of fermentative sugar, after the pressing of grapes, was investigated by (Korkie et al., 2002). According to the authors, the best method to release the additional monosaccharides from grape pomace could be to use the yeast isolated from grape pomace, which is capable of hydrolyzing the polysaccharides and fermenting the sugars (glucose and fructose) to release ethanol. Furthermore, the fermentation of these residual sugars can increase the economic value of grape pomace as it is a significant additional source of ethanol production from grapes.

A similar study obtained ethanol from grape pomace and sugar beet pomace during solid-state fermentation, using the yeast *Saccharomyces cerevisiae*. The fermentation showed a maximum concentration of ethanol at 48 hours and the yield of ethanol in the sugars consumed was more than 82% (Rodríguez et al., 2010).

However, in the production of biogas, waste from wineries contains a low content of nitrogen and phosphorus, which does not favor aerobic degradation. Grape pomace contains polyphenolic compounds that have phytotoxic and antimicrobial properties. In addition, it has a low pH due to the high concentration of organic acids such as citric, tartaric and malic acids, which make it difficult to eliminate waste, so a biological treatment is needed that could consequently increase the chemical demand for oxygen (COD) (Da Ros et al., 2016).

Anaerobic digestion is a viable biological treatment for the waste from wineries, as it stabilizes the waste products while producing useful gases. The anaerobic digestion process also reduces organic pollutants such as polyphenols, therefore increasing yield, as the antimicrobial potential inhibits the fermentation process.

Furthermore, the proper use of biogas for energy generation, can reduce greenhouse gases and can prevent the release of biogenic methane into the atmosphere (Appels et al., 2008; Da Ros et al., 2014; Moraes et al., 2014; Y. Zheng et al., 2012). Another important biofuel produced during the fermentation of biomass from the winemaking process is biobutanol. This fuel is produced in a similar way to ethanol, through the microbial fermentation of sugar, cellulosic feedstocks and starch. Biobutanol is able to reduce the carbon footprint by more than 85%, compared to other fuels (gasoline and other ethanol fuels). Compared to ethanol, this fuel is less corrosive and explosive, and has been found to reduce hydrocarbon, carbon monoxide, and nitrogen dioxide emissions.

Biobutanol can be mixed with other biofuels in high concentrations, while ethanol has an 85% limit to be mixed in motor engines. Using anaerobic bacteria (*Clostridium saccharobutylicum*) white grape pomace can be converted into biobutanol. It is an eco-friendly alternative to replace non-renewable fossil fuels (Sirohi et al., 2020b).

Other technological processes have also obtained biofuels derived from the residues of the winemaking process. An example of this is the production of bio-oil. Through the distributed rapid pyrolysis process, the conversion of biomass into bio-oil can be achieved, which is subsequently used in the production of motor fuels or chemicals, through the gasification of bio-oils.

Bio-oil can be produced economically on small scales that match the size of the profitable biomass collection points, and it can be stored at the nearest transportation point (or port). It is then subsequently shipped, together with the production of many other plants of pyrolysis, to a central site for the generation and use of synthesis gases such as the production of synthetic natural gas, diesel fuel, dimethyl ether, ethanol, and higher alcohols (Zheng et al., 2019).

Bio-oils generate less pollution as well as 50% less NO_x emissions, compared to diesel in a gas turbine. Grape seed has been used as a potential source for the production of bio-based petroleum derivatives and value-added products, through the transesterification and epoxidation process. The bio diesel produced through the transesterification process of the grape seed showed excellent low temperature properties. Grape seed also showed less cetane number than restricted limit presenting characteristics such as flash point, ester content, viscosity and acid value, similar to that of vegetable oils (Haro et al., 2018; Sirohi et al., 2020a).

1.3.3. Bio-energies

Bio-energies have gained attraction because they are renewable and clean and can be produced by various types of organic biomass waste. Food waste is an example of a feedstock used to produce different types of high value bio-energies, due to the large amount of organic matter. For example, processes such as anaerobic digestion (AD) are widely used for the production of bio-methane (Latha et al., 2019; Zou et al., 2020).

Anaerobic digestion is considered an important contributor of energy across Europe and the residues from the winemaking process represent a consistent source that can be used in this technology.

In order to produce bio-energy from winery by-products, it is important to consider the variability of the characteristics of the substrates, which can cause different biogas productions. This includes characteristics such as the type of grape, origin and the vinification technologies (Da Ros et al., 2016).

A study reported that the best bio-methane potential was achieved using white grape pomace with a production of 0.273 m^3CH_4/KgVS, while the red grape pomace produced a yield of just 0.101 m^3CH_4/KgVS (Fabbri et al., 2015).

Other types of waste have also been investigated for their potential in the production of viable biogas. Biogas production maximization was achieved through anaerobic co-digestion of wine lees with waste activated sludge in mesophilic and thermophilic conditions. Three organic loads (2.8, 3.3 and 4.5 KgCOD/m^3d) and different hydraulic retention times (21, 19 and 16 days) were tested in order to obtain the best operating conditions. This study achieved a biogas production yield of 0.40 NM^3_{biogas}/$KGCOD_{fed}$ (Da Ros et al., 2014).

Another study evaluated the production of biogas and methane through the anaerobic digestion of fresh grape pomace using a laboratory scale plant. The yield was 110 Nm^3/t sv of methane, which was obtained much faster than with other types of biomasses. In addition, they verified that the incorporation of grape seeds in the anaerobic digestion process has positive effects on the production of biogas and methane (Failla & Restuccia, 2014).

In another similar study, the production of biogas and methane was estimated through the batch anaerobic digestion of grape pomace, grape pulp and seeds. A cumulative methane production of 0.125, 0.165 and 0.053 Nm^3 Kg COD^{-1} was obtained for grape pomace, pulps and seeds, respectively. In this study, anaerobic biodegradability was increased by grinding grape pomace, pulp and seeds by 13.1%, 4.8% and 22.2%, respectively (El Achkar et al., 2016).

Another mode of anaerobic digestion from the residues of the wine process was tested by (Da Ros et al., 2016). Through both anaerobic digestion in batch and continuous mode, they achieved a methane production of 0.133 Nm^3CH_4/$KgVS_{fed}$ for grape stalks and 0.370 Nm^3CH_4/$KhVS_{fed}$ for wine lees. Furthermore, when they evaluated the anaerobic digestion process at 55 ° C, under mesophilic conditions, the production potential for grape pomace increased to 84%, compared to values reported in literature.

The functionality of the best performance of biogas production was calculated through the combustion of biogas generated by grape pomace, which could produce 1520 GWh/h of heat and 1245 GWh/y of electricity, and the electricity produced can be used directly by the wineries themselves.

The use of residues from the winemaking process for the production of biogas through anaerobic digestion is a highly attractive process for the winemaking industry, due to the opportunities in generating income from the waste and also significantly reducing waste disposal costs (Rebecchi et al., 2013).

1.3.4. Biofertilizers

Further technologies have been developed to optimize the reuse of waste produced in the winemaking industry and give added value to the conversion of the by-products. The remaining solid residue can be used as a biofertilizer in agriculture. In this way, sustainability in the bioeconomy (Figure 8) can be implemented in terms of biorefinery concepts applied to solid waste from winemaking process (Bharathiraja et al., 2020).

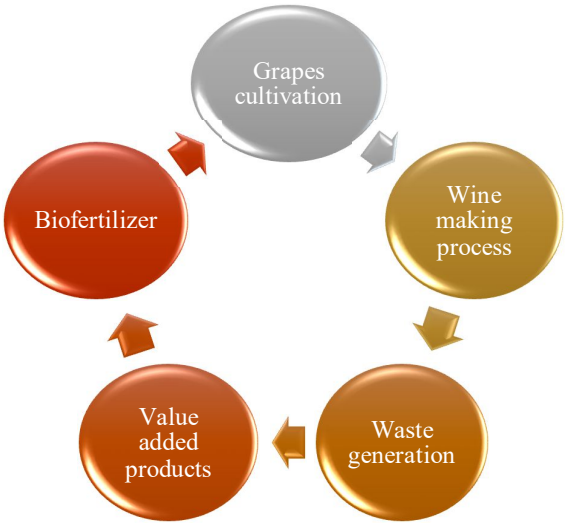


Figure 8. Circular economy model from the wineries residues

The composting of organic material is an easy and suitable method of transforming agro-industrial waste into products suitable for use, such as soil fertilizers. However, the organic matter produced by the wine industry waste can be recycled as a soil conditioner, based on its organic composition and nutritional compounds. Compost derived from winemaking residues has been described as of good quality, with beneficial physicochemical characteristics and sufficient nutrients. In addition, the compost obtained by the residues from the winemaking process provides the following three benefits (Arvanitoyannis et al., 2006):

- Increasing the humidity of the natural organic matter, therefore facilitating the incorporation of water in the soil and improving the water-holding capacity of the soil. This is an important factor for the quality and specificity of wine production.
- Enabling nitrogen to be released gradually, which is particularly important for vineyards, as the soils can suffer from having high nitrogen levels.
- Its high to moderate ratio of potassium values, therefore the addition of compost to the soil is beneficial, as the presence potassium is considered a quality factor in wines.

The potential of the residues obtained from the winemaking process to be converted into compost has been shown in a number of studies.

For instance, the authors (Ferrer et al., 2001) conducted research into co-composting grape waste with hen droppings for application on corn crops. They demonstrated that there was a significant improvement to the yield of corn dry matter when the compressed grape waste was mixed with hen droppings (10% w/w) hen droppings improved the activating effect and the aeration during the composting process.

The study showed satisfactory results when the products were applied in several doses (between 1000-4000 kg/ha) as a soil conditioner for corn seed germination in greenhouses.

The addition of hen droppings had a significant effect on corn dry matter yields (14% increase). Furthermore, it was noted that adding a dose of 3000 kg/ha was considered the optimal condition, when it was used as supplemented with triple superphosphate (TSP) in agronomic trials.

1.3.5. Biochar generation

The process of pyrolysis, which is the thermal processing of agricultural waste, municipal waste and industrial by-products in low or no oxygen conditions at a temperature of 300 and 800 ° C, produces the solid material known as Bio carbon.

The physicochemical properties of bio carbon depend on the type of raw material and the conditions of the thermal processing stage.

Biochar improves the quality of the soil in several ways including, increasing its pH, increasing its capacity to retain moisture and improving crop yields, in addition, it has been shown to stimulate the activity of fungi and microbes with beneficial properties. Furthermore, it improves the cation exchange capacity and retains nutrients in the soil. In this context, grape pomace can be converted to biochar by anaerobic pyrolysis (Sirohi et al., 2020a).

A study investigated the pyrolysis of different agricultural and biofuel production residues, including grape residues, sugarcane residues, dried distiller's grains, palm oil residues, apple pomace and forest residues. Using a pilot bubbling fluidized bed pyrolyzer operated under a range of temperature conditions between 300 and 600 ° C and two steam residence times (2 and 5 s). They were able to determine the behavior of the pyrolysis process and observed that the grape residue gave the highest yield of biochar among all the types of residues evaluated (Xu et al., 2011).

1.3.6. Generation of Biopolymers

Biopolymers are synthesized from natural sources, either chemically from a biological material, or biosynthesized by living organisms. Synthetic plastics have important properties such as the ease of forming shapes, their heat seal ability, flexibility and impact strength. They area also light weight, low cost, have a good tensile and tear strength and they have good barrier properties to oxygen,

even though they can remain in the atmosphere for a long time. To solve this problem, biopolymers are a great substitute but with a high cost on the raw material. The use of waste generated by the agri-food industry to generate biopolymers and other products can help towards reducing the environmental impact and reducing the costs of waste disposal.

One study reported the production of biopolymers using de-phenolized and fermented grape pomace.

By extracting polyphenolic compounds from grapes and undergoing anaerobic acidogenic digestion of dephenolized grape pomace, they helped to convert the organic molecular complex into simpler volatile fatty acids. In conclusion, the authors achieved the production of polyhydroxybutyrate biopolymers by incorporating a rich stream of simpler volatile fatty acid (Smith et al., 2016).

1.3.7. Enzymes production

The residues from the wine process offer a green alternative for enzymes which can be implemented in the recovery of bioactive compounds. Through solid-state fermentation it is possible to obtain hydrolytic enzymes from grape pomace.

The study conducted by (Teles et al., 2019) successfully achieved the production of hydrolytic enzymes through solid-state fermentation by means of the mutant *Aspergillus niger* 3T5B8 using a mixture of grape pomace and wheat bran as substrate. This combination with a substrate proved to be more effective for the extraction of compounds with a high content of proanthocyanidins and high antioxidant activity.

1.3.8. Biocomposite production

Another strategy which has been developed is the use of waste from winemaking (grape pomace) to produce biocomposite based formulations. A recent study on this subject by (Ferri et al., 2020) used poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), which is useful as a renewable and biodegradable matrix to obtain biocomposites. The authors performed a solvent-based and

pressurized liquid extraction to recover polyphenols. They then used the residual solid part from the extraction for the production of biocomposite materials, by directly mixing with poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV polymer), which can therefore function as a renewable and biodegradable matrix to obtain new materials with lower cost and comparable properties. The recovery of bioactive compounds and the reuse of the extraction product for the generation of biocomposites, fully represents the concept of circular economy.

1.3.9. Animal feed

The presence of grape pomace as an agri-industrial waste is of concern to the wine industry and has led to the search for potential ways to utilize it, with low-cost techniques, in animal feed. The understanding of the components of grape pomace in animal nutrition is essential to minimizing the risk of possible intoxication, or possible variation to the quality of the meat. For instance, grape pomace can act on ruminal biohydrogenation, altering the fatty acid profile of meat. Furthermore, the presence of grape pomace in animal nutrition for consumption can increase the oxidative stability of the meat, due to the presence of phenolic compounds.

A study showed that replacing 5% of the corn with grape pomace, in the daily food of piglets, produced beneficial effects in the animals. The polyphenols from the 5% grape pomace are absorbed into the blood of the piglets that receive it. These bioactive molecules have a beneficial action on the health status of animals and also increase antioxidant activity in the liver, spleen and kidneys which are key organs in the metabolism of nutrients (Chedea et al., 2019).

1.4. Recovery of bioactive compounds from winemaking by-products

The negative environmental impacts from the winemaking process are significant despite recent improvements in the waste management systems; therefore, it is necessary to further develop activities to ensure better sustainability in the wine sector. Solid residues regarding wine production correspond to approximately 1/3 of the grapes used, which is represented in millions of tons of

residues. The disposal of large amounts of this waste can lead to significant environmental impacts. Therefore, like all waste from the food industry, the by-products of grape recovery have been considered as a matter of treatment, minimization and prevention.

Consequently, during the last years there has been a growing interest not only in the safe disposal of residues from the winemaking process, but also in the recovery of valuable compounds for nutraceutical applications. The recovery of waste and by-products from wineries could represent a solution to reduce the environmental impact of the wine industry (Makris et al., 2007). Among the bioactive compounds, the recovery of polyphenols from grape processing residues and from by-products, by means of different methods, has become a subject of interest.

The recovery of these molecules in an efficient and economical way represents a key point for the valorization of the compounds derived from grapes. The enormous amount of waste produced from processed grapes, combined with well-designed extraction methods and existing technologies, lead to the recovery, recycling and sustainability of high-value ingredients added to the internal food chain. Therefore, waste management represents both an ecological and an economic issue (Barba et al., 2016; Drosou et al., 2015a; Fontana et al., 2013a).

Difficulties in the management of wine processing waste arise because wine production is seasonal, (only during harvest). Therefore, any treatment processes must be carried out in a non-continuous way. Large amounts of waste are generated in wineries over a short annual period of time, so treatment of grape pomace and other by-products is necessary shortly after the various stages of winemaking. The high bioactivity of the compounds of grape process residues, along with their susceptibility to enzymatic degradation and their sensitivity to thermal degradation, make handling them challenging. As a consequence, effective conservation and an efficient recovery process are often required (Atanacković et al., 2012; Drosou et al., 2015b; Miljić et al., 2014)

In this context, different extractions and technologies for the separation and protection of bioactive compounds, such as membrane technologies and microencapsulation by spray drying, are presented as alternatives will be discussed in the following chapters.

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CHAPTER 2

Nomenclature:

BSA: Bovine serum albumin

CCD: Central composite design

DoE: Design of Experiments

dw: Dry weight

EHD: Electro hydrodynamic

FRAP: ferric reducing/antioxidant power

HVED: High voltage electric shock

mM: Milimol

OIV: Organization of vine and wine

PHWE: Pressurized hot water extraction

RPM: Revolution per minute

SD: Standard deviation

v/v: Volume/Volume

2.0. EXTRACTION METHODS FOR THE BIOACTIVE COMPOUNDS

ABSTRACT

Grape pomace is a by-product of the winemaking process and consists of the peels, seeds and stems originating from the post-harvest to the crushing of the grapes. This organic waste comprises of 20 to 25% of the weight of the grape (Yu & Ahmedna, 2013). During the winemaking process only small amounts of phytochemicals are transferred from the grapes to the wine, however large quantities remain in the pomace. Another type of residue from the wine process is wine lees, which includes dead cells, yeast, organic compounds and bioactive compounds from the grape that settle at the bottom of the tank after the vinification process.

The chemical composition of the various compounds in both the skin and the seeds have been studied and quantified by (Martinez et al., 2016; Yu & Ahmedna, 2013). In reference to the skin of the grape, polyphenolic compounds are 2-6.5% and are abundant as anthocyanins. On the other hand, the seed of the grape represents a content of 60% of total polyphenolic compounds which are in the form of phenolic acids, flavonoids, procyanidins and resveratrol. The authors (Martinez et al., 2016) proposed the use of bioactive compounds found in grape pomace in the cosmetics and pharmaceutical industries as well as in the nutraceutical industry in certain food additives.

The largely unexplored field of the treatment and exploitation of the residues from the winemaking process has caused a detrimental environmental impact despite the economic benefits (García-Lomillo & González-SanJosé, 2017). There are several environmental factors that discourage disposal to landfills. The high phenolic content in in both of these aforementioned residues decreases the pH, having a detrimental effect on the rate of biological degradation of the organic wastes. In addition, the organic wastes have the potential to generate bad odours, attract undesirable pests, contaminate surface waters and deplete oxygen levels in groundwater (Arvanitoyannis et al., 2006; Chand et al., 2009; Dwyer et al., 2014).

As a result of the above points, the optimization of several bioactive compound extraction techniques has been studied in the present chapter with the grape pomace and wine lees as a matrix and ultrasound, ethanol and microwave and enzymatic method as possible extraction media.

2.1. Introduction

For a long time grape pomace from the winemaking process has been wasted due to the lack of investigation into the potential application and the economic benefits of the byproduct (García-Lomillo & González-SanJosé, 2017); the carefree approach to the large amount of wine industry wastes (5-9 millions tons per year) (Meyer et al., 1998; Schieber et al., 2001), and to their environmental impact and management (Fontana et al., 2013b; Louli et al., 2004). Especially in small-scale wineries, waste legislation is not always followed, further contributing to environmental problems (Devesa-Rey et al., 2011).

In the wine industry, large quantities of wastes are produced in a short period, such as during the harvest, from August to October, which if disposed in landfill, can be harmful for the environment because of the high level of phenolic compounds which decrease the pH of the waste, thus increasing their resistance to biological degradation (Dwyer et al., 2014).

Other environmental problems are attraction of undesirable pests, contamination of surface water and depletion of oxygen in the ground and groundwater (Arvanitoyannis et al., 2006; Chand et al., 2009).

The potential use of by-products from grape processing can be a promising sustainable alternative, not only driven by the idea of reducing the environmental impact, but also by the possibility of improving the quality of food and developing ingredients and high value products. (Abarghuei et al., 2010; Martinez et al., 2016). As a consequence, the recovery of phenolic compounds from grape by-products in the wine industry, has received increasing attention in recent years, and industries are finding higher values and environmentally sustainable alternatives for these residues. Moreover,

in recent years consumers have become increasingly aware of the use of food additives and that certain foods have beneficially functional properties. This has led to an increasing demand to identify natural alternatives and use safer sources of antioxidant foods (Fontana et al., 2013b).

Antioxidants are substances capable of preventing or delaying the rate oxidation, a chain reaction involving free radicals, which takes place in autoxidables materials (Louli et al., 2004). As mentioned above, the phenolic compounds, which include important secondary metabolites such as flavonoids and anthocyanins, are the main bioactive phytochemical compounds present in wine, waste and by-products (Katalinić et al., 2010; Kris-Etherton et al., 2002; Macheix et al., 1991). Despite their sensory properties, they have been demonstrated to have health related benefits (Cvejić et al., 2016; Vilkhu et al., 2008). However, these compounds are contained in vacuoles of plant cells and in lipoproteins which are insoluble structures making them difficult to extract (Corrales et al., 2008).

With this goal, several extraction methods have been investigated, such as polymeric adsorbent resins, subcritical water, ultrasound, pressurized liquid extraction and microwave (Femenia, Rosselló, & Simal, 2015; Kammerer & Gajdos, 2005; Metivier et al., 1968; Oward, 2003; Miguel A. Pedroza et al., 2015; Revilla, 1998; Zhi & Luke, 2005).

The recovery of compounds could be achieved in five different stages described in Figure 1 (Galanakis, 2012), although several of the stages could be avoided, as the most important thing is to achieve an adequate separation of the compounds. The figure shows the recovery stages for the bioactive compounds from food waste.

Figure.1. Stages for the recovery of high value compounds from food waste

There are a number of well-established technologies to recover the target compounds; in addition, there are several emerging technologies (Table 1), where the main objective is the effective isolation of the compounds of interest in the matrix to be analyzed. Within these five stages, different steps are evident, such as in the preparation of the material, in the removal of macromolecules, in the dissociation of clusters and molecular complexes, in the removal of the impurities from the co-extracts and in obtaining of the final product.

2.1.1. Conventional extraction techniques

One of the important steps for the successful recovery of bioactive compounds is to have a range of options available, which are known as the conventional extraction techniques.

The selection of the extraction technique will depend on the compound of interest. In addition, there are important points to consider such as maximizing the yield of the compound of interest, considering the demand to the industrial process, purifying the ingredient with high added value,

avoiding deterioration, oxidation or decrease of functional properties, guaranteeing the safety of the final product and ensuring the sustainability of the process in the industry (Galanakis, 2015).

Table 1. Stages of recovery of valuable compounds (Modified from Galanakis, 2012)

	Established technologies	**Emerging technologies**
Macroscopic pre-treatments	Wet milling, thermal and/or vacuum concentration, mechanical pressing, Freeze Drying, centrifugation and microfiltration.	Foam mat drying, electro osmotic dewatering, electro hydrodynamic (EHD) Drying low temperature plasma treatment
Macro and micro molecules separation	Alcohol precipitation, ultrafiltration, isoelectric solubilization, precipitation, extrusion	Colloidal gas aphrons, ultrasound-assisted crystallization, pressurized microwave-assisted extraction
Molecule extraction	Solvent, acid, alkali, microwave assisted, steam diffusion, hydro distillation, supercritical fluid	Ultrasonic, laser ablation, Pulsed Electric Field, high voltage electrical discharge.
Isolation and purification	Adsorption, chromatography, nanofiltration electrodialysis	magnetic fishing, aqueous two-phase separation, membrane ion exchange chromatography
Product formation	Spray and freeze drying, emulsions, extrusion	Nanotechnology, pulsed fluid bed agglomeration

Among the techniques that have been experimented with in conventional pre-treatments, is thermal drying the grape pomace before treatment by ultrasound, for the extraction of polyphenolic compounds. However, it is important to note that generally the use of high temperatures is avoided, due to the evidence that when thermal pre-treatment is performed at 60 °C, there is a significant decrease in the polyphenolic content (Goula et al., 2016)

Several conventional techniques have been developed to separate compounds of interest from the agro-industrial residues (grape pomace). Table 2 gives examples of some of the extraction methods, extracted compounds and the treatments used to recover the bioactive compounds.

Table 2. Conventional methods to recover bioactive compounds from grape pomace

Extraction method	Food matrix or food by-products	Compounds	Treatment	References
Microwave	Grape pomace	Polyphenols	300 W, 100 °C. mg GAE/100 g DM	(Álvarez et al., 2017)
Pulsed ohmic heating	Grape pomace	Polyphenols	Extraction of polyphenols in water (50 °C). mg GAE/100 g DM	(Darra et al., 2013)
Pressurized hot water extraction (PHWE)	Grape N.R (Not reported)	Polyphenols	Ethanol/water (70:30 vol %). mg GAE/100 g DM	(Rajha et al., 2014)
Extraction with solvent	Grape pomace (Cabernet Sauvignon)	Polyphenols	EtoAc mg GAE/100 g DM	(Pinta et al., 2018)
Pressurized hot water extraction (PHWE)	Grape N.R (Not reported)	Polyphenols	Ethanol/water (70:30 vol %) mg GAE/100 g DM	(Rajha et al., 2014)
Extraction with solvent	Grape pomace (Cabernet Sauvignon)	Polyphenols	EtoAc mg GAE/100 g DM	(Pinta et al., 2018)
Maceration	Grape pomace and stem	Polyphenols	Maceration in 60 min at room temperature with 50% Methanol and 70% acetone	(Llobera & Cañellas, 2007)
Maceration	Grape berries (Pinot Noir)	Anthocyanins and other polyphenols	- Water and acidified methanol - Methanol, 96% ethanol and distilled water	(Lee & Rennaker, 2011)
Maceration at room temperature and hot extraction	Grape pomace	Tocopherols	Soxhlet extraction for 4 h at 65 °C	(Özkan & Gokturk baydar, 2006)
Microwave	Wine lees (From Syrah grapes)	Polyphenols	17 minutes of operation time	(Pérez-Serradilla & Luque de Castro, 2011)

One of the best-known conventional extractions that has been established for decades is extraction with solvent, which requires long extraction times and relatively large amounts of solvents (Bonfigli et al., 2017). The solvent extraction technique is very versatile due to the range of solvents that can be used, making it attractive and widely used; although it should be noted that the extraction solvent applied will depend on the properties of the by-product and the compound of interest to be extracted. In the case of grape pomace, ethyl acetate represented a good candidate for the extraction of phenolic acids, flavanones, flavonones, flavonols, flavanols, stilbenes and coumarins. However, methanol was the most suitable solvent for the extraction of anthocyanins from grape pomace (Pinta et al., 2018).

A recent study (Zhijing et al., 2018) used a 50/50 (v/v) ethanol solution for the extraction of several bioactive compounds from red and white wine lees, that had been produced in the early fermentation and during aging. The results showed that winemaking affected the antioxidant activity, and the amount of total tannins and polyphenols correlated to the winemaking technique used.

Solvent extraction has been modified and updated over time with the implementation of new technologies such as solid liquid extraction. (Posadino et al., 2018) demonstrated that solid liquid extraction is capable of extracting antioxidant compounds such as anthocyanins from grape pomace, as it uses a negative gradient pressure from the inside to the outside of the solid matrix, thus transporting the extractable compounds out of the matrix. Moreover, this same study investigated the biological activity capacity of the extract and its ability to counteract the oxidative stress induced by hydrogen peroxide (Posadino et al., 2018). The research demonstrated that the adequate treatment of the by-product is an important factor in obtaining not only sufficient quantity of bioactive compounds but also their biological capacity.

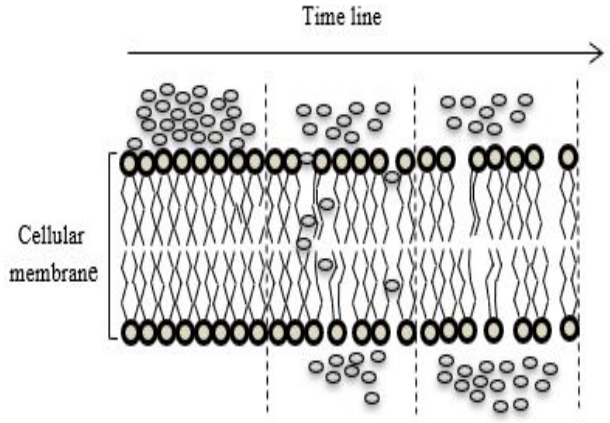


Figure. 2. Conventional solvent extraction

Figure.2 shows the schematic representation of the conventional solvent extraction. The solvent enters the solid matrix by diffusion and the solute dissolves to reach the concentration, which is limited by the characteristics of the solids (Angiolillo et al., 2015). The polarity of the solvent is an important factor in the extraction process because it influences the ability to dissolve a special group of antioxidant compounds and therefore the estimation of antioxidant activity (Singh et al., 2014).

Another technology that has been widely studied during the last decade is microwave assisted extraction. This technique combines the use of energy emanating from the microwaves (in the electric and magnetic field) with traditional extraction by solvent. Microwave energy is used to heat the solvents that are in contact with the solid or liquid sample, causing a partition of the compounds of interest from the sample into the solvent. Electromagnetic waves heat the molecules through the two mechanisms of ionic conduction and the rotation of the dipole, where they act simultaneously in the solvent and in the sample, converting the microwave energy into thermal energy (Llompart et al., 2018).

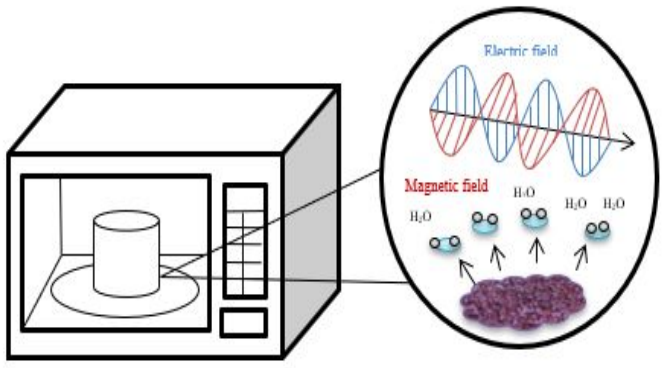


Figure. 3. Microwave Assisted Extraction

Figure.3 shows the schematic representation of microwave assisted extraction. This technique uses electromagnetic fields of 300 MHz to 300 GHz that consists of two perpendicular fields, electric field and magnetic field. (Angiolillo et al., 2015). The irradiation improves the cellular lysis of materials with large quantities of water, due to the rapid heating and evaporation of the intracellular water (Matos et al., 2018)

This extraction technique has been used in fruits such as *Morus nigra* L. maximizing the bioactive metabolites and inhibitory activity of tyrosinase (Koyu et al., 2018), in the *Gordonia axillaris* extracting flavonoid compounds and phenolics such as gallic acid, protocatechuic acid, epicatechin, ferulic acid among others, which in the future can be isolated, identified and evaluated for their antimicrobial and anticancer properties (Li et al., 2017). In plants such as *Adathoda vasica* and *Cymbopogon citratus*, recovering bioactive compounds has important pharmaceutical relevance

(Simha et al., 2016). In addition, agro-industrial waste such as grapefruit peel, where the extraction of pectin from the peel using tartaric acid as a solvent, under conditions such as a grapefruit peel ratio/solvent 1:40 (weight/volume), irradiation time of 9 minutes at 660 W and a pH of 1.5, results in an excellent pectin extraction yield of 23.83%, pure pectin of 80.88%, high methoxyl pectin of 92.75%. This pectin can be used in the food industry for the production of jams and beverages (Quoc et al., 2015).

In relation to the treatment with microwaves on winemaking by-products such as the grape pomace, wine lees, seeds and skin separately have been studied during the last ten years with positive results. In 2011 a study investigated the influence of the variables in the extraction of anthocyanins from the grape skin (Tintilla de rota) assisted by microwaves. The research found that the most significant variables for the extraction were the operating time of 5 minutes at 100 °C and 40% methanol in water as a solvent, thus extracting three acid derivatives (Maldivina 3-cumumilglucoside (cis), malvidin 3-caffeine-glucoside and petunidin 3-p-coumaroilglucoside) and significantly reducing the time of microwave operation in comparison to the classic method of maceration (from 5 hours to 5 minutes) obtaining almost the same values in anthocyanins content (Liazid et al., 2011). In the same year, the microwave assisted technique was used for the extraction of phenolic compounds in wine lees obtained from the Syrah variety. The research showed that with a short operating time of 17 minutes, with a hydroalcoholic solvent (ethanol 75%, hydrochloric acid 1% in water) in a ratio of 1:10 (w/v)

it was possible to obtain 53.2 mg of gallic acid per 100 g of dry matter, suggesting that the lees from wine, was an undervalued material and could be an alternative to the use of skins and grape seeds for the extraction of bioactive compounds (Pérez-Serradilla & Luque de Castro, 2011). Years later another study was carried out using the microwave technique on the skin of the dry white grape Chardonnay variety in the extraction of phenolic compounds. Variables such as vessel geometry, irradiation cycles and operation time were investigated. Their results were compared with a conventional solid-liquid extraction, concluding that the microwave assisted technique did not influence the vessel geometry and demonstrated that the microwave assisted technique allowed for an 83% saving in extraction time, while obtaining the same polyphenol content (Miguel A Pedroza et al., 2015).

Microwave assisted extraction has been shown to perform better than several other extraction techniques as several variables have been improved by accelerating the extraction kinetics. It has been proven that the technique accelerates the extraction kinetics of various compounds such as sugars and fibers, however its effect on phenolic compounds is much greater. The grape pomace from the Tempranillo variety was treated with the microwave assisted technique and it was found that the optimal operating conditions were treatment with microwaves for 120 seconds at 300 Watts of power. Under these conditions the yield of the polyphenols extracted improved by 57%, compared to a comparable sample without the microwave treatment (Álvarez et al., 2017). Conventional extractions can also be found in reflux, cold maceration, simple distillation techniques and Soxhlet (Bandar et al., 2013).

2.1.2. Emergent extraction techniques

In recent years, various technologies have been developed for the extraction of compounds in an effort to replace extraction methodologies with conventional solvents (Otero-Pareja et al., 2015). As with conventional extraction techniques, the type of extraction to be used will depend on the nature of the subtract. Usually, conventional treatments are restricted by various problems which are

difficult to overcome, such as overheating of the food matrix, high energy consumption, loss of functionality of the compound being extracted, poor stability and compliance with increasingly strict legal requirements on the safety of materials. These disadvantages can be overcome by being replacing various conventional techniques with emerging treatments (Galanakis, 2012; Galanakis & Schieber, 2014).

Among the emerging pre-treatments of the grape pomace is the drying foam mat (EHD). Due to the high humidity contained in the grape pomace (2.5-3.0 kg/kg dry basis), thermal treatments for drying are expensive, and it has been found that the use of EHD provides benefits in terms of energy efficiency (Artynenko & Udra, 2017). Concerning the separation of compounds, research developed in 2016 presented the possible combinations of already established techniques with emerging techniques for oenological objectives, such as the use of temperature, micro-oxygenation and mixing cycles in wine lees. After the treatment it was noted that there was an increase in color, polysaccharide content and polyphenolic content (Fia, 2016).

One of the most widely used techniques in the last years for the extraction of compounds of interest, is the ultrasound technique. The effect of density of acoustic energy on the extraction yields of the phenolic and tartaric compounds was studied. Ultrasound assisted extraction was performed in a 25 kHz bath system using 50% aqueous ethanol as the solvent. The results obtained indicated that increasing the density of the frequency, increased the yield of the phenolic compounds and the tartaric esters. Increasing the temperature (40 °C) was another variable that had a positive effect, as it caused an increase in the spread of compounds of interest in the solvent (Tao et al., 2014).

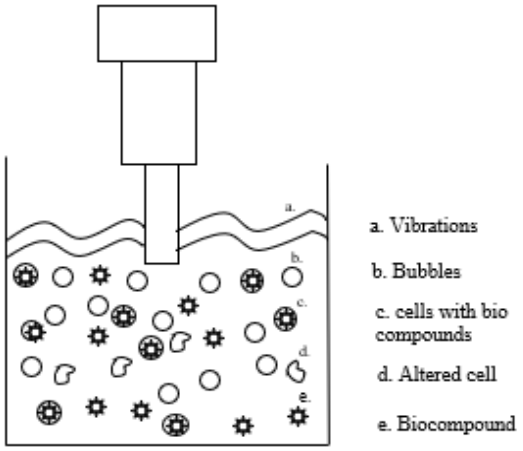


Figure.4 shows the schematic representation of ultrasound assisted extraction. Due to the high frequency sound waves, a cavitation effect is generated that produces an increase in mass transfer. High frequency waves generate bubbles that collapse producing a change in temperature and pressure in the medium releasing cellular content (Rodríguez-rojo et al., 2012; Wijngaard et al., 2012)

Figure.4. Ultrasound Assisted Extraction

Techniques involving the use of energy are increasingly being used for extraction purposes. This is the case in the application of high voltage electric shock (HVED). This technique directly introduces energy into an aqueous solution through a plasma channel, which is formed by a high current electric discharge by means of two electrodes that are submerged in the solution. Research carried out in 2011 managed to optimize the conditions of the HVED extraction system on grape pomace (*Vitis vinifera* variety) to obtain an extract rich in polyphenolic content. The optimal conditions were to apply 80 kJ/kg with a distance of 5 mm between the electrodes, a solid/liquid ratio of 5 followed by diffusion with 30% ethanol, in water at a temperature of 60 °C for 30 minutes. These conditions produced 2.8± 0.4 g GAE/100 g of dry matter. The antioxidant activity obtained was 66.8 ± 3.1 g TEAC/kg dry matter (Boussetta et al., 2011).

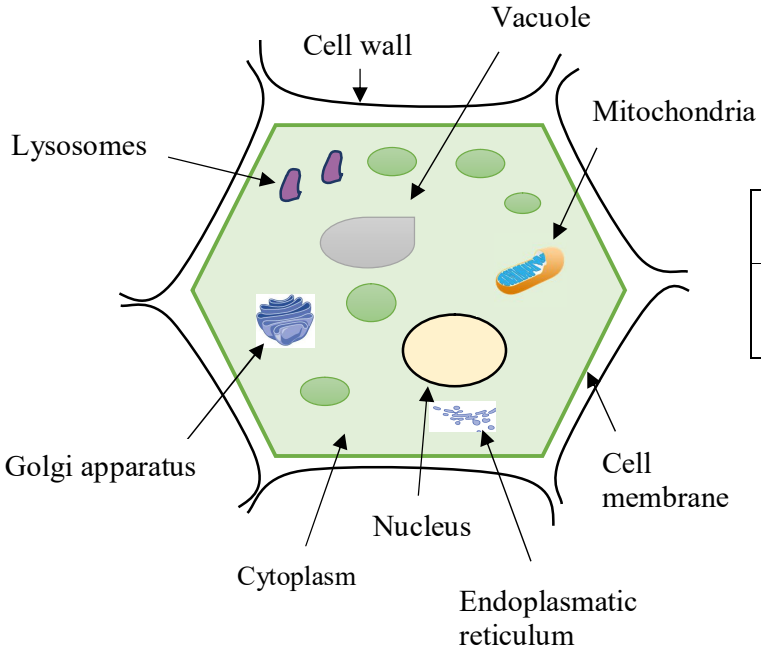
As previously mentioned, for the extraction of bioactive compounds there is a compendium of conventional and emerging techniques such as solid-liquid extraction, pressurized liquid and extraction with pressurized hot water. Supercritical fluid extraction, microwave, ultrasound-assisted extraction and pulsed electric field extraction are amongst the most sustainable alternatives to traditional methods (Kammerer et al., 2005).

2.1.3. Premises and recent advances on the usage of hydrolytic enzymes for the recovery of bioactive compounds from grape pomace

In the process of extracting bioactive compounds such as polyphenols, organic solvents are usually used mixed with or without water. The extraction can be controlled by different parameters such as the selection of extraction procedures, flow rate used for each extraction, type of solvent, temperature, pressure and time. Other techniques developed have given similar results, which implies that the use of microorganisms and enzymes in extraction techniques is a promising alternative to conventional extraction methods, for the exploitation of wine wastes to obtain bioactive compounds.

The advantages of this method are the selectivity and specificity of the action of the enzyme which has the ability to catalyze reactions in mild aqueous solutions. Furthermore, the application of enzymes is an environmentally friendly method for the extraction of polyphenols (such as flavonoids) due to a decrease in the amount of solvent required, a reduction in extraction times and an increase extraction yield (Chávez-González et al., 2020).

Different types of enzymes from fungi, bacteria, plant extracts and animal organs such as pectinases, glucanases, hemicellulases, cellulases etc. either mixed or alone, decompose the cell wall by hydrolysis of the biopolymeric components in such a way that it can increase the permeability of the cell wall and increase the yield of the bioactive compounds extracted. The hemicellulose backbone can be hydrolyzed by endoxylanases, endomannanases, β-xylosidase, and β-mannanases. The degradation of the pectin backbone requires a series of enzymes such as pectiniliases, endo and exo polygalacturonases, endo and exo rhamnogalacturonases and rhamnogalacturonliases. In addition, by-product enzymes such as arabinases, galactosidases, and feruloyl esterases are required to hydrolyze the side chains of polysaccharides (Castro-vazquez et al., 2016; Zha et al., 2019).



Organule	Bioactive and other value compounds and enzymes
Chromoplast	Carotenoids
Vacuoles	Sugar, organic acids and polyphenols
Chloroplasts	Tocopherols, chlorophylls

Figure. 5. Location of the bioactive compounds inside the plant cell. Figure modified from (Galanakis, 2015).

Grape pomace is characterized by the presence of pectin polysaccharides, which are key constituents responsible for the conformation and mechanical properties of the cell walls within plant tissues. Due to their complex conformational arrangement into main and secondary chain fragments, pectins constitute major limiting factors for the extraction of polyphenolic compounds from vegetal tissues both from fresh pomace during macerative fermentation and from fermented wine pomaces, which have been depleted of their extractable compounds during the vinification process (Pinelo et al., 2006). Pectolytic enzymes (generally referred as pectinases) play a highly specific hydrolytic activity responsible for the degradation/disruption of macromolecules of biological interest (primarily polysaccharide chains). Pectinases are naturally present in grapes, being partly responsible for the berries ripening process, and they are inactivated by pH and SO_2 levels occurring during the vinification process; on the contrary, pectinases isolated from fungi can exert their activity under oenological conditions, and they are currently used in oenology as maceration adjuvants (Revilla & Gonzalez-San Jose, 2003).

The usage of pectolytic enzymes to facilitate the extraction of bioactive compounds from grape pomaces is regulated by the International Organization of Vine and Wine (OIV), which has identified the *Aspergillus niger* and *Trichoderma* fungi as suitable sources of pectinases for oenological uses. Commercial formulations include pectinases, emicellulases, glucanases and glucosidases; since the *Aspergillus niger* enzymes exhibit reduced hydrolytic activity in intact berries, grapes are usually crushed before applying the treatment (OIV, 2012).

According to previous findings (Chamorro et al., 2012; Fernández et al., 2015; Romero-Cascales et al., 2012) the activity of pectolytic enzymes is mainly influenced by the following factors: pH, temperature and contact time. These enzymes act in the temperature range 8-55°C, with peak activities in the mean region of the interval. In general, enzymatic activity increases with increasing temperatures; when the temperature falls below 15°C higher dosages and contact times are required to elicit extraction, and they must be doubled when the temperature values fall to < 12°C (Porto et al., 2013). In relation to the parameter of time, the oenological enzymes suppliers recommend a minimum 1-hour exposure under oenological conditions for soft grape tissues (pulp), up to 2 hours for toughest tissues as grape skin and seeds (Stambuk et al., 2016), In relation to potential limiting factors, it has been proven that alcohol content up to 15% (v/v) and SO_2 concentrations falling within the legal limits guarantee the activity of commercial enzymes from fungi (Chamorro et al., 2012). Noting the above, the characteristics of oenological pectolytic enzymes give potential for being utilized in assisting the extraction of valuable bioactive compounds, i.e., polyphenols, from fermented grape pomaces made available as vinification by-products from wineries.

2.2. Materials and methods

2.2.1. Description of the samples

Red grape pomace (60% Cabernet sauvignon, 30% Sangiovese, 10% Syrah) and fresh red wine lees (from several grape varieties) were collected from a local winery (Terre Naldi, Faenza, Italy) and stored at -20 °C. For the experiments, pomace and lees were thawed and then dried at 50 °C for 24

h (Figure 6). Moreover, exhausted red grape pomace was collected from a local distillery and stored at room temperature were also used as a sample.

Figure. 6. Red grape pomace (left) and lees (right) from vinification process

2.2.2. Evaluation of extraction methods

Several experiments were set up to evaluate the efficiency of different extraction methods in the three samples. In the first experiment the treated samples were:

1) Fresh red grape pomace

2) Red wine lees

3) Red grape pomace exhausted, which comprises the pomace after being distilled

The compared extraction methods were: 1) microwave assisted; 2) organic solvent (ethanol); 3) ultrasonic; 4) enzymatic coupled with ultrasonic irradiation; 5) enzymatic extraction. The performance of each extraction method was evaluated in terms of the total polyphenols extracted. In particular, for microwave-assisted extraction, organic solvent extraction, ultrasonic and enzymatic coupled with ultrasonic irradiation the performance was evaluated by means of Response Surfaces using experimental Central Composite Design (CCD) using the statistical software Design Expert 11.0. (Statease, MN, USA). For the ezymatic extraction the performance was evaluated by means of Response Surface using experimental Box-Behnken design with the help of the statistical software JMP 14.0 (SAS Institute Srl, Milan, Italy).

The following section describes the methodology of the extraction processes and the related experimental designs, which have driven the optimization of process conditions.

2.2.3. Microwave-assisted extraction

Microwave assisted extraction was carried out according to the methodology described elsewhere (Pérez-Serradilla & Luque de Castro, 2011) by using a domestic microwave oven (Model, JT 356 WH, Whirlpool, USA) with settable power levels and irradiation time (Figure 7). In order to optimize the extraction conditions, a three-level was set up, using the factors time (0.5, 2, 3 min) and power (90, 160, 350 Watts) (Table 3). Twelve experimental conditions were obtained (Table 4). Microwave-assisted extraction was performed in duplicate, and the results were reported as mean value ± standard deviation between two measurements. In all experiments, 1 g of grape pomace was mixed with 10 milliliters of extracting solution (75% ethanol, 1% HCl, 24% H_2O).

Table. 3. Independent factors selected for DoE in the microwave extraction method

Microwave operation conditions	**Level**		
	Low	**Medium**	**High**
Time (min)(min)	0.5	2	3
Power (Watts)(Watts)	90	160	350

Figure. 7. External (left) and internal (right) view of microwave device

Table.4. Twelve combinations of DoE for the microwave assisted method

Run	Microwave power (Watts)	Microwave time (min)
1	90	2
2	160	2
3	160	3
4	90	3
5	350	0.5
6	350	2
7	90	0.5
8	350	3
9	90	1
10	160	1
11	160	0.5
12	350	1

2.2.4. Conventional Solvent Extraction

The ethanol based extraction was conducted in accordance with previous studies (Zhijing et al., 2018). A three level DoE was set up, and the variables ethanol concentration (20, 35, 50%) and time of treatment (5, 42.5, 80 min) were evaluated, as reported in Table 5. Conventional solvent extraction was performed in duplicate, and the results were reported as mean value ± standard deviation between two measurements.

Table 5. Independent factors of the DoE used in the ethanol extraction method

Ethanol operation conditions	Level		
	Low	Medium	High
Agitation time (min)	5	42.5	80
Ethanol concentration (%)	20	35	50

In each experiment, 5 milliliters of ethanol solution were added to 0.5 g of sample. After which, the sample was stirred for the time under investigation and centrifuged at 3000 RPM for 5 minutes, to obtain the supernatant (Figure 8).

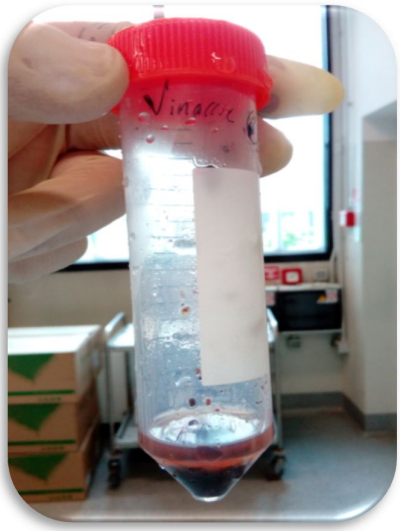


Figure. 8. Sample of red wine lees during ethanol extraction

Table 6 shows the 9 experimental combinations obtained by the statistical software based on a CCD model for ethanol extraction method.

Table. 6. The nine combinations of DoE for solvent/ethanol method

Run	Exposure time (min)	Ethanol concentration (%)
1	80	20
2	42.5	50
3	42.5	35
4	80	35
5	5	35
6	80	50
7	5	50
8	5	20
9	42.5	20

2.2.5. Ultrasound Assisted Extraction

Two different solvents (acidified ethanol and not-acidified ethanol) were tested according to previous study (Jensen et al., 2007). Grape pomace (250 mg) was mixed with either 5 or 10 mL of solvent as reported in Table 7. Ultrasound assisted extraction was performed in duplicate and the results were reported as mean value ± standard deviation between two measurements.

The treatment conditions regarding the solid/extraction solution ratio are obtained based on preliminary analyzes and differ in the volume of the extraction solution (5 and 10 mL). In order to define the composition of the solvent for best extraction performance and the best extraction condition in terms of solvent volume, a CCD-type experimental design was applied with three independent variables: 1) ethanol concentration in solvent (5 and 10%); 2) acidified water for ethanol solvent preparation (Presence: YES/Absence: NO); 3) solvent volume (mL). The solutions were the following:

- *Acidified water:* 4.14 mL of 37% HCl were added to H_2O in a final volume of 500 mL.
- *Acidified ethanol (5%):* 475 mL of acidified water (0.1 M with HCl) were added with 25 mL of absolute ethanol.
- *Non-acidified ethanol (5%):* 475 mL of water were added with 25 mL of absolute ethanol.
- *Non-acidified ethanol (10%):* 450 mL of water were added with 50 mL of absolute ethanol.
- *Acidified ethanol (10%):* 450 mL of acidified water (0.1 M with HCl) were added with 50 mL of absolute ethanol.

The ultrasound (Figure 9) set up was as follows: frequency of sonication (20 KHz), temperature (24 °C) and time (5 cycles of 30 seconds each) as previously reported (Palma, Piñeiro, Rostagno & Barroso, 2006). After treatment the sample was centrifuged at 4000 rpm for 15 minutes at room temperature to separate the superntant.

Figure 9. Ultrasound equipment

Table 7. Independent factors for the ultrasound method

Operation conditions	Level	
	Low	High
Ethanol (%)	5	10
Acidified water	No	Yes
Solvent volume (mL)	5	10

The experimental combinations for the evaluation of ultrasound assisted extraction are reported in Table 8.

Table.8. Experimental design for the ultrasound assisted method

Run	Ethanol concentration (%)	Acidified water*	Solvent (mL)
1	10	Yes	5
2	5	No	5
3	5	Yes	10
4	10	Yes	10
5	5	Yes	5
6	10	No	10
7	5	No	10
8	10	No	5

*Indicates use of acidified water for ethanol standard preparation

2.2.6. Ultrasonic-Assisted Enzymatic Extraction

Polyphenols extraction by means of hydrolytic/pectolytic enzymes (Lafase® He Grand Cru from Laffort, Bordeaux, France) coupled with ultrasonic assistance, was carried out based on the previous study (Palma et al., 2006). Dried grape pomace (250 mg) was mixed with 20 mL of buffer (50 mM succinic acid, 50 mM borax, pH 4.0) and three enzymatic dosages (10, 20 or 30 mg/g of dried pomace) at 40 °C for 2 h. The irradiation was applied by means of a high intensity ultrasonic liquid processor (VCX 50s-VC 75, Sonics, Newtown, CT, USA) at 16 W, for different cycles of treatment (4 to 10), each of 1-minute duration. The CCD experimental design was applied to two independent factors: enzyme dosage (mg/g) and cycles (No.) Each factor was evaluated at three levels (low, medium and high), as reported in Table 9. Ultrasonic assisted-enzymatic extraction was

performed in duplicate, and the results were reported as mean value ± standard deviation between two measurements.

Table.9. Independent factors for the enzymatic coupled to ultrasound extraction

Operation conditions	**Level**		
	Low	**Medium**	**High**
Enzymatic dosage (mg/g)	10	20	30
Cycles (No.)	4	7	10

Table 10 reports the experimental combinations which were tested during the experiment.

Table. 10. Design experiment for the ultrasonic-assisted enzymatic extraction

Run	**Enzimatic dosage (mg/g)**	**Cycles (No.)**
1	10	10
2	20	7
3	10	7
4	20	10
5	30	4
6	20	7
7	20	7
8	20	7
9	10	4
10	30	10
11	30	7
12	20	7
13	20	4

2.2.7. Enzymatic extraction from grape pomace

A Box-Behnken experimental design was applied to 4 independent factors: 1) enzyme dosage; 2) pH of the extraction buffer; 3) extraction temperature; 4) extraction time. Each factor was tested at three distinctive levels, as reported in Table 11.

Table 11. Factors defined as independent variables of the experimental design and relative levels.

Factors	Levels		
	-1	*0*	*1*
Time of extraction (h)	2	3	4
Enzyme dosage (mg/g)	10	20	30
pH of the extraction buffer	2	3	4
Extraction temperature (°C)	40	45	50

Total polyphenols and tannins concentration were labelled as the iron reactive polyphenols and the optimal number of experiments in the DoE was assessed by the Box-Behnken algorithm. The algorithm was forced to operate in a limited range of experiments (minimum 12 – maximum 24). Results of the computation are reported in the Table 12, where "*n"* represents the number of experiments, "*I*" represents the computational matrix including the whole information about factors and levels and "*p*" is the number of unknown variables. A maximum of 10 iterations/s has been applied to the algorithm, which showed convergence after 8 iterations. This resulted in a design composed by 19 experiments (Figure 10).

Table 12. Computations by the Box-Behnken algorithm

No. experiments	**Log(|1/n*I|^1/p)**	**Log(|I|)**	**Log(|I|^1/p)**
12	-0.235	7.595	6.981
13	-0.232	7.935	7.615
14	-0.234	8.207	8.165
15	-0.231	8.510	8.821
16	-0.225	8.810	9.524
17	-0.221	9.088	10.228
18	-0.218	9.338	10.903
19	-0.216	9.565	11.555
20	-0.219	9.741	12.088
21	-0.220	9.923	12.665
22	-0.220	10.102	13.256
23	-0.219	10.282	13.882
24	-0.219	10.454	14.506

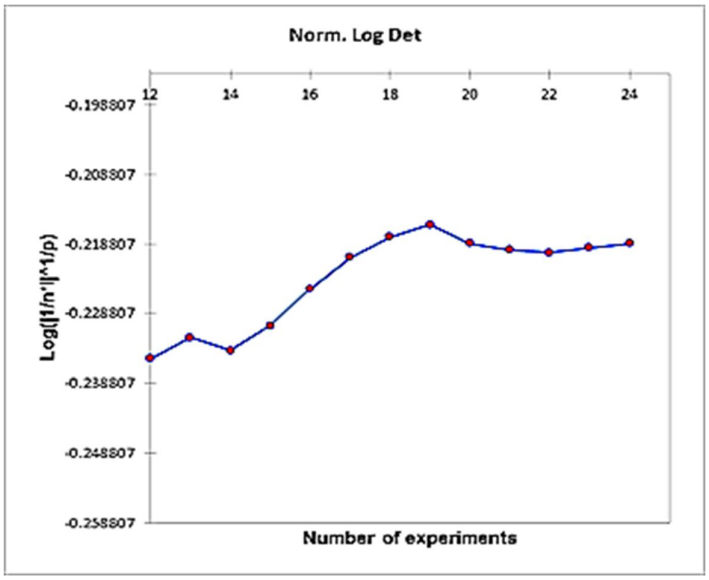


Figure 10. Graphical representation of the iteration algorithm suggesting the maximum number of experiments (19)

The objective of the experiment was *a priori* defined to obtain the optimal factors and levels combination to maximize the iron-reactive polyphenols and the tannins extractions. Under these premises, optimal conditions will be identified as the maximum values of the response surfaces.

Table 13 summarizes the combinations of factors and levels as suggested by the experimental design.

Table 13. The Box-Behnken experimental design

Run	Enzyme dosage (mg/g)	pH extraction buffer	Temperature (°C)	Time (h)
1	20	3	40	2
2	10	4	40	4
3	20	3	50	3
4	10	3	50	4
5	10	2	50	2
6	10	2	45	2
7	10	3	45	3
8	10	4	45	3
9	30	4	50	2
10	30	2	40	3
11	20	2	50	4
12	30	3	40	2
13	20	4	45	2
14	20	2	40	3
15	30	2	45	4
16	10	4	40	4
17	30	4	50	3
18	30	3	45	4
19	20	4	45	4

Pomaces were provided by local companies and composed of a mixture of different grape varieties (both red and white); the raw material included skins, seeds and traces of vine stems.

The pectolytic enzyme used in this experiment, Lafase HE Grand Cru, is a mixture of pectolytic enzymes (pectinases, cinnamil esterases) which is commercially available as a red grapes maceration coadjutant. It was purchased from Laffort (Laffort Italia S.R.L. Alessandria, Italia) as a lyophilized powder and dissolved according to the buffer solutions and concentrations suggested by the experimental design (Table 13).

Buffer solutions were prepared according to the literature (Buffer Solutions Other Than Standard, 1999) as detailed in Table 33, and pH values were adjusted by HCl 1M and NaOH 1M standardized solutions. The enzyme was fully solubilized in all buffers under the experimental conditions provided in this DoE (Table 14).

Table 14. Composition of buffer solutions according to (Buffer Solutions Other Than Standard, 1999)

pH value	Buffer composition
2	490.1 mL HCl 0.1 M and 500.9 mL glycine 0.1 M
3	170.8 mL HCl 0.1 M and 820.2 mL di glycine 0.1 M
4	820.2 mL succinic acid 0.05 M and 170.8 mL sodium borate solution (19.404 g $Na_2B_4O_7.10H_2O$/L)

The enzyme dosages were expressed as mg lyophilized powder/g raw material (pomaces) and modified according to the concentration suggested by the experimental design. The grape pomaces were dried under controlled temperature (40 ± 1°C) for 3 days and ground down to obtain a uniform, micrometric mesh and maximize the interface of contact with enzymes. The ratio (w/v, g/mL) between raw pomace treated and enzymatic solution volume was kept constant at the 1:80 g mL^{-1} level; in more detail, 0.25 grams of the raw dried pomace were treated by adding 20 mL of buffer containing variable concentrations of the enzyme.

To account for the different temperature levels provided by the experimental design samples were kept under controlled temperature (values from DoE ± 1°C) and under continuous stirring (100 rpm) in an 810 Climatic Hood thermostat (Enrico Bruno S.r.l., Turin, Italy).

Control samples were arranged to account for the effect of temperature, buffer pH and time in the absence of the enzyme and run apart from the experiments provided by DoE.

At the end of the extraction time enzymes were deactivated by heating the working solution at 90°C for 1 minute. Extracts (Figure 11) were centrifuged at 4500 rpm for 20 minutes at room temperature using an Allegra™ 2IR centrifuge (Beckman Coulter, Inc., Brea, US). Then the surnatant was filtered using 0.45 μm cellulose acetate membranes and stored in glass vials under inert atmosphere (nitrogen).

Optimal extraction parameters obtained from preliminary DoE were tested in a different grape pomace batch; a control sample (extraction under optimal conditions excluding the enzyme) was provided.

Reagents and standards used for the analysis of the extract were purchased from Sigma-Aldrich (St. Louis, US).

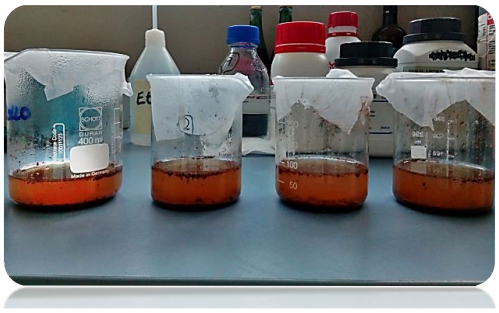


Figure 11. Typical extract obtained following the enzymatic assisted procedure

2.3. Analysis of the extracts

Polyphenolic compounds (including total polyphenols and tannins) were determined in all extracts from DoE according to the colorimetric method described by (Harbertson et al., 2002).

A volume of the extract is mixed with the BSA protein (Bovine serum albumin). In this way, the protein is bequeathed to the tannin in the extract, forming a complex of tannins. Subsequently, the protein tannin complex is precipitated and washed with a ferric chloride solution that forms a colored complex that can be read at 510 nm.

The amount of color is proportional to the amount of tannins in the extract. Results were expressed as mg (+)-catechin (CE)/100 g dw of the sample.

The extract obtained under optimized conditions (and related control extract) was fully characterized according to the total polyphenols and tannins content (Harbertson et al., 2002) total proteins (Vincenzi et al., 2015), alcohol content and reducing sugars (OIV, 2014), 2,2-diphenyl-1-picrylhydrazyl (DPPH•) antiradical assay (Brand-Williams et al., 1995) and ferric reducing/ antioxidant power (FRAP) assay (Benzie & Strain, 1996).

2.4. Analytical evaluations

2.4.1. Determination of total polyphenols

In particular, for the extraction methods were: 1) microwave assisted; 2) organic solvent (ethanol); 3) ultrasonic; 4) enzymatic coupled with ultrasonic irradiation, the quantification of total polyhenols compounds was carried out by means of the Folin-Ciocalteu method (Singleton & Rossi, 1965). One milliliter of sample (diluted and centrifuged at the occurrence) was placed in a 100 milliliters flask, then an aliquot of distilled water and 5 milliliters of the Folin Ciocalteu reagent were added. The solution was stirred and in the time-lapse 30 sec- 8 min, 20 milliliters of sodium bicarbonate at 15% were added. The final volume (100 mL) was reached by distilled water. After two hours, the absorbance at 750 nm was registered. Results were expressed as mg of gallic acid/100 g of dry weight for grape pomace and as mg of gallic acid/L for wine lees. Determination of total polyphenols in the different extractions were performed in duplicate and the results were reported as mean value ± standard deviation between two measurements.

For the 5) enzimatic extraction method, the polyphenolic compounds (including total polyphenols and tannins) were determined in all extracts from DoE according to the colorimetric method described by (Harbertson et al., 2002). Results were expressed as mg (+)-catechin (CE)/100 g dw of the sample.

Determination of total polyphenols and tannins in the enzimatic extraction were performed in duplicate and the results were reported as mean value ± standard deviation between two measurements.

2.5. Statistical processing

All extractions were performed in duplicate, and the results were reported as mean value ± standard deviation between two measurements. In particular, for 1) microwave assisted; 2) organic solvent (ethanol); 3) ultrasonic; 4) enzymatic coupled with ultrasonic irradiation, the one-way analysis of variance (ANOVA; significance $\rho \leq 0.05$) was performed using Design Expert 11.0 (Design Expert,

Stat-Ese, Inc. MN, USA). Tukey's HSD post-hoc test was performed using Minitab®17.1.0 (Minitab, Ltd. UK) statistical software. For the 5) enzymatic extraction, the results of the total polyphenols and tannins determinations were added as the iron reactive polyphenols and tannins for the experimental design results. Correlations between different combinations of factors and optimal extraction conditions were determined by means of a non-linear, multiple regression, to fit a second-order equation with respect to the dependent variables. Results were visualized in the three-dimensional domain by means of response surfaces. The experimental design analysis, the calculation of the provisional model and the evaluation of fitting parameters (mean, standard deviation, regression, F-test, ANOVA) together with the response surfaces were obtained using the JMP 14.0 statistical software (SAS Institute Srl, Milan, Italy).

2.6. Results and Discussion

2.6.1. Microwave-assisted extraction

2.6.1.1. Red grape pomace

The results of the experimental design for the extraction of phenolic compounds from fresh red grape pomace by means of the microwave method are shown in Table 15.

Table 15. Concentrations of total polyphenols extracted (mean ±SD) from fresh red grape pomace by different combinations of microwave extraction method.

Run	Microwave power (Watts)	Microwave time (min)	Total polyphenols (mg GAE/100 g)
1	90	2	759.6 ± 97.9^{CD}
2	160	2	690.4 ± 85.1^{CD}
3	160	3	1548.8 ± 12.7^{B}
4	90	3	804.8 ± 59.6^{C}
5	350	0.5	334.9 ± 85.1^{EF}
6	350	2	2283.7 ± 115^{A}
7	90	0.5	169.3 ± 72.4^{F}
8	350	3	2512.7 ± 12.7^{A}
9	90	1	280.7 ± 127.7^{EF}
10	160	1	208.4 ± 25.5^{EF}
11	160	0.5	69.9 ± 34^{F}
12	350	1	467.5 ± 42.5^{DE}

Values followed by different letters are significantly different according to the Tukey's HSD test ($p \leq 0.05$).

2.6.1.1.1. Effect of variables on total polyphenols

Quantitative significant differences were identified for total polyphenolic compounds extracted under different experimental conditions (Table 15). The concentration ranged from 69.9 to 2512.7 mg of gallic acid/100 g of dry weight (average value of 844.2 ± 64.25); with the highest value reached at 350 Watts with 3 minutes microwave treatment. These results were confirmed by response surface analysis (Figure 12), where curvature (representing total polyphenols) progressively increases up to the previously mentioned microwave conditions. Some authors report (Wang et al., 2010) that the improvements seen in the recovery of the product of interest when using microwave extraction, is generally attributed to its heating effect, which occurs due to the dipole rotation of the solvent in the microwave field. This temperature rise increases the solubility of the compound of interest.

In accordance with our results, some authors (Krishnaswamy et al., 2013) reported that by increasing the microwave power (from 100 to 200 Watts) and the operating time (from 2 to 6 minutes) a significant improvement from 913 to 1524.1 mg GAE/100 g of phenolic compounds extraction can be achieved. In the above-mentioned study, authors used ethanol at 60% (v/v) as a solvent.

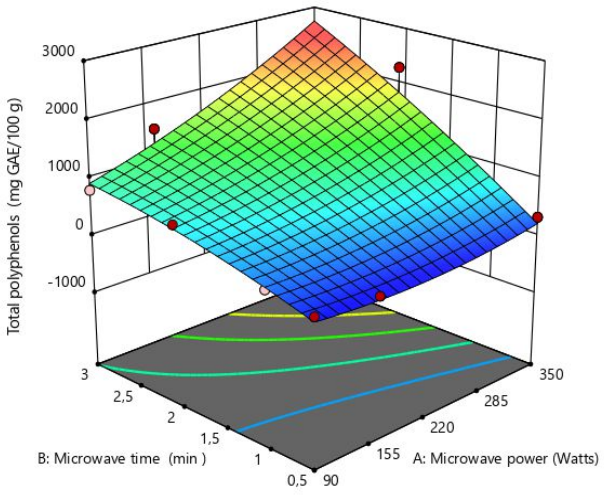


Figure.12. Response surface of total polyphenol extracted from red grape pomace in relation to the effects variables: A: Microwave power (Watts); B: Microwave time (min)

In our study, the high amount of extracted total polyphenols is likely related to both the microwave condition (350 Watts for 3 minutes) and the solvent (ethanol 75% v/v) addition.

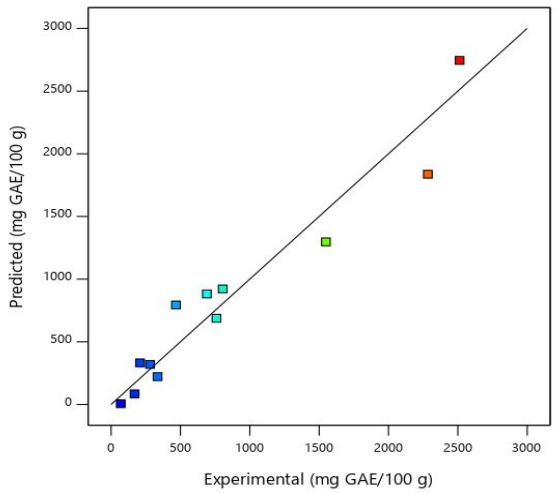
2.6.1.1.2. Statistical analysis

The statistic of the experiment and the results of the analysis of variance (ANOVA) for the quadratic model are reported in Table 16. The main information is given by the values of *F* and *p* (Amini et al., 2008; Kalavathy M. et al., 2009), if *p* is less than 0.05, the model is considered statistically significant (Kim et al., 2003).

Table. 16. Effect of factors (single or their interactions) on the extraction of total polyphenols by microwave method

Source	Sum of squares	df	Mean Square	F-value	p-value	
Model	7.043E+06	5	1.409E+06	16.26	0.0020	significant
A – Microwave power	1.892E+06	1	1.892E+06	21.85	0.0034	
B – Microwave time	4.833E+06	1	4.833E+06	55.79	0.0003	
AB	8.986E+05	1	8.986E+05	10.37	0.0181	
A^2	33215.77	1	33215.77	0.3834	0.5585	
B^2	23062.67	1	23062.67	0.2662	0.6243	
Residual	5.198E+05	6	86625.56			
Cor Total	7.563E+06	11	1.409E+06			

In the present experiment A and B were significant factors, which indicates the importance of the effects of microwave power and operating time on the extraction of polyphenols. Their interaction (AB) was significant as well. As regard R^2 values (Figure 13), the "predicted R^2" of 0.5848 was in reasonable agreement with the "adjusted R^2" of 0.8740. The "accuracy of adequacy or adequate precision" which is defined as the signal/noise ratio measure, must be greater than 4 to be considered desirable (Muthukumar et al., 2003). The ratio of 13.16 suggested an adequate signal. According to the statistical analysis presented above, this model can be used to navigate the designed space.



Std. Dev.	294.32	**R^2**	0.9313
Mean	844.23	**Adjusted R^2**	0.8740
C.V. %	34.86	**Predicted R^2**	0.5848
		Adeq Precision	13.1610

Figure.13. Correlation chart of observed values/predicted values (total polyphenols (mg GAE/100 g dry weight) obtained from exhausted red grape pomace, and analysis of Variance (ANOVA) for the linear correlation test

The predictive model of the extraction of polyphenols from the dry red grape pomace by means of the microwave method is shown in Figure 14. The high predictive capacity of the linear regression model was demonstrated by a coefficient R^2 of 0.9313. Moreover, a *p*-value of 16.26 suggested that the model is significant. The response surface analysis provides information on the effect of each factor on the obtained model. In this view, is possible to identify the value of each factor above which the extractability of the compounds of interest is optimized. For the present experiment, the conditions which have optimized the polyphenol extraction are shown in Table 17.

Table.17. Optimization of the extraction parameters by the experimental model

Factors	Optimal condition
Microwave power (Watts)	333.4
Microwave operation time (min)	2.93

2.6.1.2. Red wine lees

The results of the experimental design for the extraction of phenolic compounds from red wine lees by means of the microwave method are summarized in Table 18.

Table 18. Concentrations of total polyphenols extracted (mean ±SD) from red wine lees at different experimental combinations for microwave extraction method

Run	Microwave power (Watts)	Microwave time (min)	Total polyphenols (mg GAE/100 g)
1	90	2	293 ± 122.6^{DEF}
2	160	2	436.3 ± 56.2^{CD}
3	160	3	538.8 ± 10.2^{BC}
4	90	3	368.9 ± 52.8^{CDE}
5	350	0.5	466.5 ± 13.6^{BCD}
6	350	2	933.9 ± 6.8^{A}
7	90	0.5	162.8 ± 6.8^{F}
8	350	3	970.1 ± 34^{A}
9	90	1	190.6 ± 11.9^{F}
10	160	1	170.1 ± 0.0^{F}
11	160	0.5	242.4 ± 27.2^{EF}
12	350	1	617.1 ± 1.7^{B}

Values followed by different letters are significantly different according to the Tukey's HSD test ($p \leq 0.05$).

2.6.1.2.1. Effect of variables on total polyphenols

Statistically significant differences were identified between the amounts of polyphenolic compounds extracted in the different combinations of experiments. The concentration of phenolic compounds extracted from the red wine lees ranged from 162.8 to 970.1 mg of gallic acid/100 g of dry weight (average value 449.2 ± 28.68).

Similar to the grape pomace experiment (Section 2.6.1.1 of this present chapter), the highest polyphenols extraction (970.1 ± 34 mg gallic acid/100 g of dry weight) was obtained with microwave power set at 350 Watts for 3 minutes treatment. The result was confirmed by the analysis of the response surface (Figure 19). Recent studies (Álvarez et al., 2017) demonstrated that an increase of microwave power from 60 to 100 Watts, coupled with 40 minutes treatment, can improve the extraction of polyphenols from less by up to 48%.

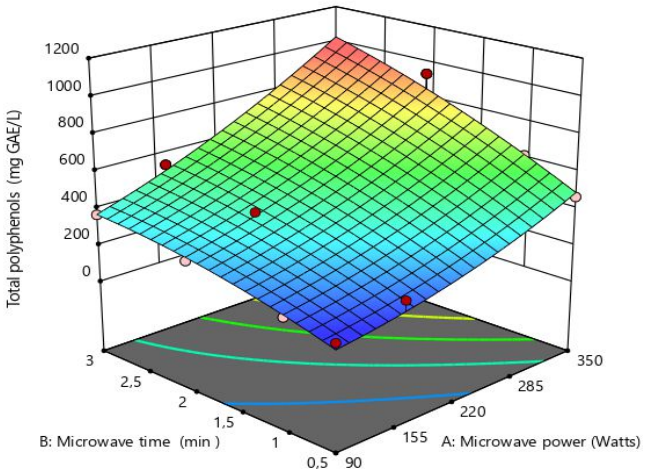


Figure.19. Response surface of concentration of total polyphenol versus the effects variables: A: Microwave power (Watts); B: microwave time (min)

The use of maximum power and maximum operation time, in addition to the use of solvent (75% ethanol (v/v)) in this experiment, confirmed the behavior obtained in the previous extraction experiment with grape pomace (In the section 2.6.1.1).

2.6.1.2.2. Statistical analysis

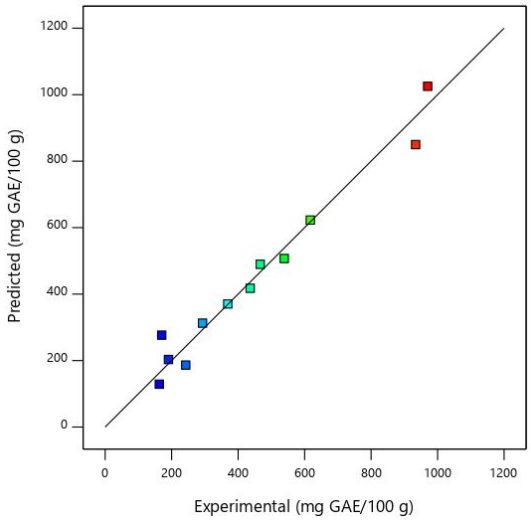
The significance of the effects the variables have in the experiments are analysed using ANOVA for the quadratic model are tabulated in the Table 19.

Table. 19. Effect of factors (single or their interactions) on the extraction of total polyphenols by microwave extraction

Source	Sum of squares	Df	Mean Square	F-value	p-value	
Model	8.152E+05	5	1.630E+05	34.72	0.0002	significant
A – Microwave power	5.0706E+05	1	5.076E+05	108.10	< 0.0001	
B – Microwave time	2.583E+05	1	2.583E+05	55.01	0.0003	
AB	27334.23	1	27334.23	5.82	0.0524	
A^2	3919.90	1	3919.90	0.8347	0.3961	
B^2	3390.30	1	3390.30	0.7219	0.4281	
Residual	28177.32	6	4696.22			
Cor Total	8.434E+05	11	1.630E+05			

The A and B factors, as well as their interaction (AB), significantly affected the polyphenols extraction.

Regarding the results of the statistical analysis in this experiment, the model obtained in the extraction of polyphenols from red wine lees by means of microwaves is shown in figure 15.



Std. Dev.	68.53	**R^2**	0.9666
Mean	449.24	**Adjusted R^2**	0.9387
C.V. %	15.25	**Predicted R^2**	0.8098
		Adeq Precision	18.4949

Figure.15. Correlation chart of observed values/predicted values (total polyphenols (mg GAE/100 g dry weight) obtained from red wine lees and analysis of Variance (ANOVA) for the linear correlation test.

A high predictive capacity was verified, which is reflected in the linear regression analysis with an R^2 coefficient equivalent to 0.9666, which means an optimal linear response. The model F value of 18.4949 suggested that the model is significant. There is only a 0.02% chance that such a large F-value will occur due to noise. Furthermore, the probability $\rho < 0.0002$ also validated that the model is significant.

As observed at the R^2 values and the predicted R^2 of 0.8089 was in reasonable agreement with the "adjusted R2" of 0.9387. The "accuracy of adequacy or adequate precision" which is defined as the mean of the signal/noise ratio must be greater than 4 to be considered desirable. The ratio of 18.4949 suggested a suitable signal. According to the statistical analysis presented above, this model can be used to navigate the design space. The response surface provides a deep analysis of the effect of the factors according to the corresponding model. However, the combination of the estimated effects makes it possible to identify the values above which the extractability of the compounds of interest is optimized, in this case polyphenolic compounds. For the present experiment, the design model provided an optimal combination of factors that is shown in table 20.

Table. 20. Optimization of the extraction parameters by the experimental model

Factors	Optimal condition
Microwave power (Watts)	349.8
Microwave operation time (min)	2.99

2.6.2. Conventional Solvent Extraction

2.6.2.1. Red grape pomace

Results of the experimental design for the extraction of phenolic compounds from fresh red grape pomace by the conventional solvent method are shown in Table 21.

Table 21. Concentrations of total polyphenols extracted (mean ±SD) from fresh red grape pomace by different combinations of conventional solvent extraction

Run	Exposure time (min)	Ethanol concentration (%)	Total polyphenol (mg GAE/100 g)
1	80	20	309.9 ± 23.9^{A}
2	42.5	50	229.2 ± 141.4^{A}
3	42.5	35	355.7 ± 27.3^{A}
4	80	35	377.3 ± 201.1^{A}
5	5	35	519.5 ± 75^{A}
6	80	50	403.9 ± 187.4^{A}
7	5	50	424.3 ± 1.7^{A}
8	5	20	355.7 ± 27.3^{A}
9	42.5	20	234.0 ± 114.2^{A}

Values followed by different letters are significantly different according to the Tukey's HSD test ($p \leq 0.05$).

No statistically significant differences were identified between the amounts of polyphenolic compounds extracted in the different combinations of experiments. The concentration of phenolic compounds extracted from the red grape pomace is in the range of 234.0 ± 114.2 to 519.5 ± 75 mg GAE/100 g dry weight with a mean value equivalent to 356.6 ± 88.8 mg GAE/100 g dry weight.

A uniform behavior was evidenced in terms of ethanol concentration factors (%), exposure time (min) and the response to polyphenolic content. As observed in the response surface (Figure 16), the curvature increases at the opposite sides of the experimental design, specifically in the exposure of time (min), presenting an increase in curvature in the range of 5 to 20 minutes and 65 to 80

minutes. On the other hand, in the response surface there was an increase in the curvature in the ethanol concentration in the range 32─38 %, which also coincide with the maximum content of total polyphenols. The selection of the type of solvent is one of the most important factors to consider. In this experiment, ethanol was selected taking into consideration its potential use in the food industry and its wide use in the extraction of bioactive compounds (Pérez-Serradilla & Luque de Castro, 2011).

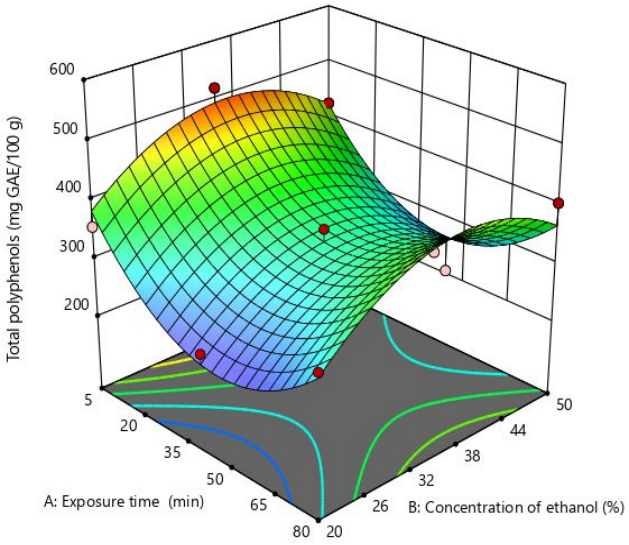


Figure.16. Response surface of concentration of total polyphenol versus the effects variables: A: Exposure time (min) ; B: Concentration of ethanol (%)

2.6.2.1.1. Statistical analysis

The effects experiments of the variables are performed according to the design matrix and the results of the analysis of variance (ANOVA) for the quadratic model are tabulated in the Table 22.

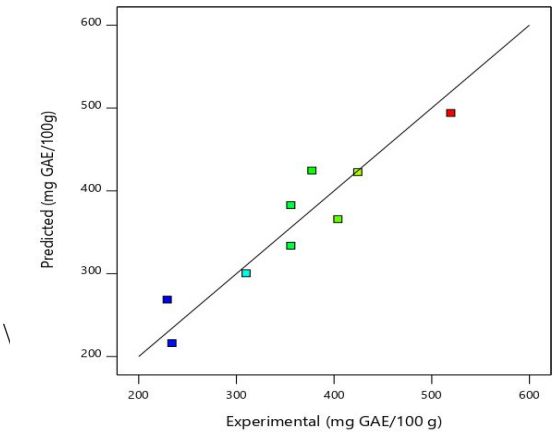
Concerning the results of this experiment, the predictive model obtained in the extraction of polyphenols from dry red grape pomace by means of conventional extraction using ethanol as solvent is shown in Figure 17. An acceptable level of predictability was presented, which is reflected in the linear regression analysis with a coefficient R^2 equivalent to 0.8, which means an acceptable linear response. The model F-value of 4.77 implies the model is not significant relative to the noise. There is an 11.43% chance that an F-value this large could occur due to noise. On the other hand, the probability $\rho < 0.1143$ implies that the model is not significant. Regarding the

independent factors introduced in the experiment (ethanol concentration and exposure time), they did not show significant effects on the extraction of total polyphenols, as shown in the table 18. where the *p* values are always higher than the critical value (0.05). The only aspect that can be highlighted is factor A^2, which is significant ($\rho < 0.0382$).

Table. 22. Effect of factors (single or their interactions) on the extraction of total polyphenols by conventional solvent method

Source	Sum of squares	df	Mean Square	F-value	p-value	
Model	59717.18	5	11943.44	4.77	0.1143	Not significant
A-Exposure time	7238.43	1	7238.43	2.89	0.1877	
B-Concentration of ethanol	4150.14	1	4150.14	1.66	0.2883	
AB	161.29	1	161.29	0.0644	0.8161	
A^2	31483.77	1	31483.77	12.57	0.0382	
B^2	16683.56	1	16683.56	6.66	0.0817	
Residual	7512.33	3	2504.11			
Cor Total	67229.51	8	11943.44			

The analysis of the interaction between the factors would have made it possible to evaluate the effect of their combination on the extraction of polyphenolic compounds, thus indicating the direction for future experiments. One reason that no significant variables were identified could also be due to the limited number of experiments predicted by the experimental design.



Std. Dev.	50.04	**R^2**	0.8883
Mean	356.61	**Adjusted R^2**	0.7020
C.V. %	14.03	**Predicted R^2**	-0.2864
		Adeq Precision	6.7999

Fig.17. Correlation chart of observed values/predicted values (total polyphenols (mg GAE/100 g dry weight) obtained from grape pomace and analysis of Variance (ANOVA) for the linear correlation test

Regarding the results of the statistical analysis in this experiment, the model obtained in the extraction of polyphenols from grape pomace by conventional solvent extraction is shown in figure 18. As observed at the R^2 values, the "predicted R^2" of -0.2864 was not in reasonable agreement with the "adjusted R^2" of 0.7020. Which implies that the overall mean may be a better predictor of the response than the current model. On the other hand, the "accuracy of adequacy or adequate precision" that is defined as the measure of the signal/noise ratio must be greater than 4 to be considered desirable. The ratio of 6.7999 suggested a suitable signal which means that this model can be used to navigate the design space.

2.6.2.2. Red wine lees

Table 23 summarizes the results of the experimental design for the extraction of phenolic compounds from red wine lees by means of conventional solvent extraction.

Table 23. Concentrations of total polyphenols extracted from red wine lees on the basis of the different combinations of conventional solvent method

Run	Exposure time (min)	Concentration of ethanol (%)	Total polyphenol (mg GAE/L)
1	80	20	506.3 ± 35.7^{ABC}
2	42.5	50	594.2 ± 54.5^{ABC}
3	42.5	35	754.5 ± 8.5^{AB}
4	80	35	787 ± 3.4^{A}
5	5	35	365.3 ± 57.9^{C}
6	80	50	515.9 ± 86.9^{ABC}
7	5	50	668.9 ± 221.5^{ABC}
8	5	20	438.8 ± 35.7^{BC}
9	42.5	20	414.7 ± 1.7^{C}

Values followed by different letters are significantly different according to the Tukey's HSD test ($p \leq 0.05$).

Statistically significant differences were identified between the amounts of polyphenolic compounds extracted in the different combinations of experiments. The concentration of phenolic compounds extracted from red wine less is in the range 365.3 ± 57.9 to 787 ± 3.4 mg GAE/L with a mean value of 560.6 ± 56.2 mg. An increase in the concentration of polyphenols was evidenced

when the ethanol concentration was established at 35% during an exposure time of 42.5 minutes, obtaining a maximum amount of polyphenolic compounds extracted of 787 ± 3.4 mg GAE/L.

As can be seen (Figure 18), in the response surface its curvature increases evidently in correspondence with the parameters of ethanol concentration and exposure time, which also coincide with the maximum concentration of polyphenols. It should be noted that there were no significant differences between the experimental runs that present an ethanol concentration of 35% and 42.5 minutes of operation and an ethanol concentration of 35% and 80 minutes of operation, obtaining a polyphenol concentration of 754.5 ± 8.5 and 787 ± 3.4 mg GAE/L, respectively.

Similar behavior was reported (Zhang et al., 2007) in extraction of lignans from flaxseed by means of a ethanol-water solution. The authors reported a positive effect when both the ethanol concentration (%) and exposure time (h) were increased, thus concluding 70% ethanol and 28 h of exposure were optimal conditions for the maximum lignin concentration obtained (8.97% (w/w lignans/defatted flaxseed powder).

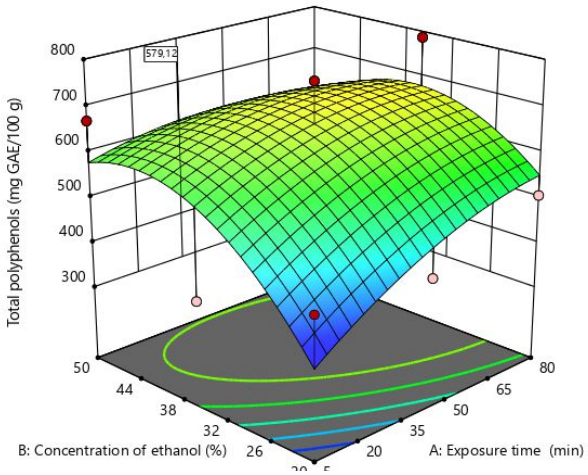


Figure.18. Response surface of concentration of total polyphenol versus the effects variables: A: Exposure time (min); B: Concentration of ethanol (%)

2.6.2.2.1. Statistical analysis

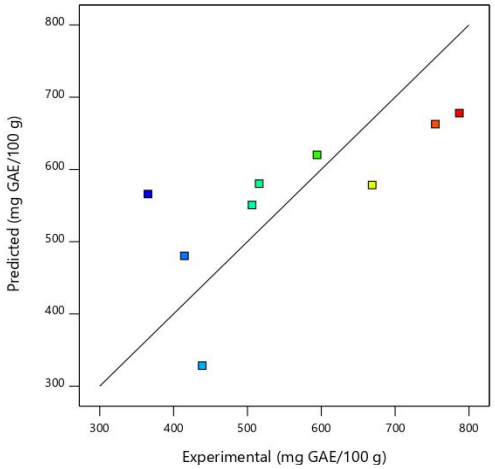
The effects experiments of the variables are performed according to the design matrix and the results of the analysis of variance (ANOVA) for the quadratic model are tabulated in the table 24. Regarding the results of this experiment, the predictive model obtained in the extraction of polyphenols from red wine lees by conventional extraction with ethanol is shown in figure 19.

A high predictive capacity was not presented, which is reflected in the linear regression analysis with a coefficient R^2 equivalent to 0.4914, which means a poor linear response. The F-model value of 0.5797 suggested that the model is not significant relative to the noise. There is a 72.31% chance that an F-value this large could occur due to noise. On the other hand, the probability $\rho < 0.7231$ implies that the model is not significant.

Table. 24. Effect of factors (single or their interactions) on the extraction of total polyphenols by conventional solvent extraction

Source	Sum of squares	df	Mean Square	F-value	p-value	
Model	88897.32	5	17779.46	0.5797	0.7231	Not significant
A-Exposure time	18832.20	1	18832.20	0.6140	0.4905	
B-Concentration of ethanol	29298.88	1	29298.88	0.9553	0.4005	
AB	12153.07	1	12153.07	0.3962	0.5737	
A^2	3323.25	1	3323.25	0.1084	0.7637	
B^2	25289.91	1	25289.91	0.8246	0.4308	
Residual	92012.67	3	30670.89			
Cor Total	1.809E+05	8	17779.46			

Regarding the independent factors introduced in the experiment (ethanol concentration and exposure time). They did not show significant effects on the extraction of total polyphenols, as shown in table 20, where the *p* values are always higher than the critical value of 0.05.



Std. Dev.	175.13	**R^2**	0.4914
Mean	560.62	**Adjusted R^2**	-0.3563
C.V. %	31.24	**Predicted R^2**	-4.7036
		Adeq Precision	2.4441

Figure.19. Correlation chart of observed values/predicted values (total polyphenols (mg GAE/100 g dry weight) obtained from red wine lees and analysis of Variance (ANOVA) for the linear correlation test.

Regarding the results of the statistical analysis in this experiment, the model obtained in the extraction of polyphenols from red wine lees by conventional solvent extraction is shown in figure 20.

On verifying the R^2 values, the "predicted R^2" of -4.7036 was not in reasonable agreement with the "adjusted R^2" of -0.3563. A negative Predicted R^2 implies that the overall mean may be a better predictor of the response than the current model.

The "accuracy of adequacy or adequate precision" that is defined as the measure of the signal-to-noise ratio must be greater than 4 to be considered desirable. The ratio of 2.4441 indicates an inappropriate signal and this model should not be used to navigate the design space.

2.6.3. Ultrasound Assisted Extraction

2.6.2.1. Red grape pomace

Table 25 summarizes the results of the experimental design for the extraction of phenolic compounds from red grape pomace exhausted by means of Ultrasound Assisted Extraction.

The acidification procedure involved the use of dilute hydrochloric acid solutions, whose impact, although minimal, must be considered in the perspective of developing a green protocol. For this reason, the experimental plan contemplated the use of non-acidified water in some experimental

combinations, in order to verify that acidification is advantageous and maximizes extraction performance.

Table 25. Concentrations of total polyphenols extracted (mean ±SD) from red grape pomace exhausted on the basis of different combination of ultrasound assisted extraction

Run	Concentration of acidified ethanol (%)	Acidified water	Volume of the solvent (mL)	mg GAE/100 g
1	10	Yes	5	903.9 ± 25.6^{A}
2	5	No	5	429.0 ± 10.7^{E}
3	5	Yes	10	776.5 ± 9.3^{BC}
4	10	Yes	10	738.1 ± 5.9^{BC}
5	5	Yes	5	610.1 ± 1.9^{D}
6	10	No	10	825.0 ± 8.0^{AB}
7	5	No	10	665.2 ± 73.9^{CD}
8	10	No	5	387.8 ± 15.9^{E}

Values followed by different letters are significantly different according to the Tukey's HSD test ($p \leq 0.05$).

2.6.2.1.1. Effect of variables on total phenols

Statistically significant differences were identified between the amounts of polyphenolic compounds extracted in the different combinations of experiments. The concentration of polyphenolic compounds extracted from red grape pomace is in the range of 387.8 ± 15.9 to 903.9 ± 25.6 mg GAE /100 g of dry weight with a mean value equivalent to 666.9 ± 18.9 mg GAE/100 g of dry weight.

The authors (Vergara-Salinas et al., 2015) studied the influence of a probe-assisted ultrasound treatment, with an acoustic frequency of 55 kHz and a temperature of 50 °C and for variable times up to a maximum of one hour; after this time, the average concentration value of extracted polyphenols equal to 771 ± 77.5 mg GAE /100 g of dry weight was obtained. A notable increase in the extraction of polyphenols was evidenced when there was the presence of acidified water and a maximum percentage of ethanol, obtaining a maximum amount of polyphenolic compounds extracted of 903.9 ± 25.6 mg of gallic acid/100 g of dry weight. As observed in the response surface (Figure 20), its curvature increases considerably in correspondence with the parameter of presence of acidified water and the percentage of ethanol, until reaching a maximum presence of acidified

water and a percentage of ethanol of 10%, which also agree with the maximum concentration of total polyphenols.

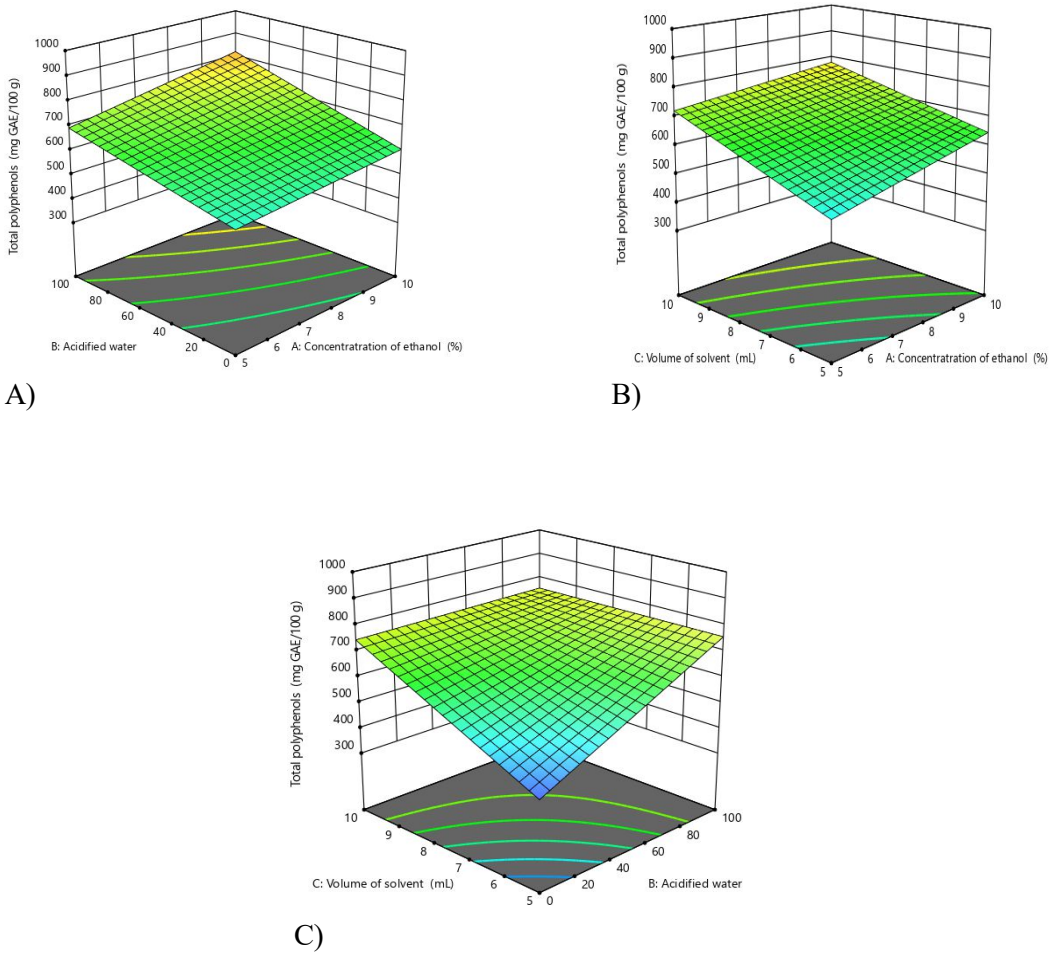


Figure.20. Response surfaces obtained from the experimental design used to optimize the extraction of polifenols from red grape pomace exhausted. A: Concentration of ethanol/Acidified water; B: Concentration of ethanol/Volume of solvent; C: Acidified water/Volume of the solvent

Regarding the interaction between the concentration and the effect of the presence/absence of acidified water (Figure 21-A), the increase of the first parameter has an effect on the increase of extracted polyphenols, reaching the maximum point of these compounds at around 10% of ethanol concentration. On the other hand, the tendency of the response surface is deduced by assessing the effect of the effect of the acidified water prevails over the concentration of organic solvent added to

the extraction mixture. The response surface increases its tendency evidently in correspondence with the presence of acidified water in the solvent used.

The interaction between ethanol concentration and solvent volume (Figure 21-B) has a similar trend with a curvature of the response surface indicating that for the concentration of ethanol at 10% the maximum point of total polyphenols is presented. It follows that a constant increase in the parameter "solvent volume" is obtained, which also has an evident effect on the increase in total polyphenols. The maximum point of total polyphenols is reached when the highest volume of solvent is used (10 mL). Comparing the effects of the interaction between the presence/absence of acidified water and the volume of solvent (Figure 21-C) it can be seen that in this case neither of them prevails over the effect of the increase in polyphenols, that is, it can be deduced that both parameters influence, with a very similar intensity, the increase in total polyphenol concentration. The maximum polyphenol concentration point is reached when the presence of acidified water and a maximum volume of solvents (10%) are present.

2.6.2.1.2. Statistical analysis

The effects experiments of the variables are performed according to the design matrix and the results of the analysis of variance (ANOVA) for the quadratic model are tabulated in the table 26.

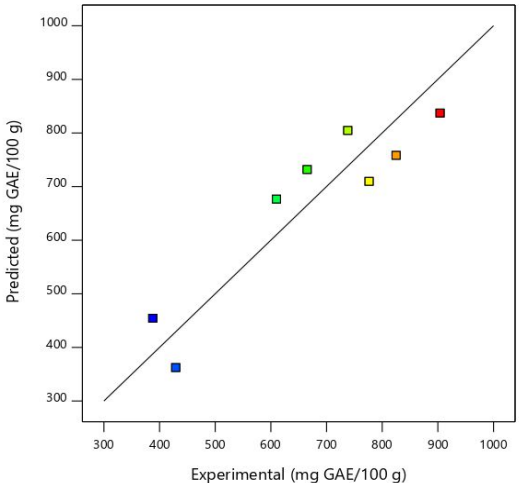
Regarding the results of this experiment, the predictive model obtained in the extraction of polyphenols from dried grape pomace by means assisted sonication is shown in figure 21.

An acceptable predictive capacity was presented, which is reflected in the linear regression analysis with a coefficient R2 equivalent to 0.8483, which means a good linear response. The model F-value of 8.46 suggested that the model is significant. There is only a 0.27% chance that an F-value this large could occur due to noise. Furthermore, the probability $\rho < 0.0027$ also valid the significant model. In the present experiment B, C and BC were significant factors, indicating a good synergy between the presence/absence of acidified water and the volume of the solvent on the results of the polyphenolic content.

Table. 26. Effect of factors (single or their interactions) on the extraction of total polyphenols by Ultrasound assisted extraction

Source	Sum of squares	df	Mean Square	F-value	p-value	
Model	4.008E+05	6	66803.75	8.46	0.0027	significant
A-Concentratration of ethanol	34982.70	1	34982.70	4.43	0.0647	
B-Acidified water	1.302E+05	1	1.302E+05	16.48	0.0028	
C-Volume of solvent	1.135E+05	1	1.135E+05	14.37	0.0043	
AB	4690.40	1	4690.40	0.5937	0.4607	
AC	4298.87	1	4298.87	0.5442	0.4795	
BC	1.131E+05	1	1.131E+05	14.32		
A^2	0.0000	0				
B^2	0.0000	0				
C^2	0.0000	0				
Residual	71099.13	9	7899.90			
Lack of Fit	71099.13	1	71099.13			
Pure Error	0.0000	8	0.0000			
Cor Total	4.719E+05		66803.75			

As checking the R^2 values, the "predicted R^2" of 0.5238 was not in reasonable agreement with the "adjusted R^2" of 0.7489. The "accuracy of adequacy or adequate precision" which is defined as the measure of the signal-to-noise ratio must be greater than 4 to be considered desirable. The ratio of 8.0775 suggested a suitable signal. According to the statistical analysis presented above, this model can be used to navigate through space.



Std. Dev.	88.88	**R^2**	0.8483
Mean	666.94	**Adjusted R^2**	0.7489
C.V. %	13.33	**Predicted R^2**	0.5238
		Adeq Precision	8.0775

Fig.21. Correlation chart of observed values/predicted values (total polyphenols (mg GAE/100 g dry weight) obtained from red grape pomace exhausted and analysis of Variance (ANOVA) for the linear correlation test

The response surface provides a deep analysis of the effect of the factors according to the corresponding model. However, the combination of the estimated effects allows to identify the values above which the extractability of the compounds of interest is optimized. For the present experiment the experimental design model provided an optimal combination of factors, which is shown in the table 27.

Table.27 Optimization of the extraction parameters by the experimental model

Factors	Optimal condition
Concentration of ethanol (%)	10
Acidified water	Yes
Volume of solvent (mL)	5

2.6.2.2. Red wine lees

Table 28 summarizes the results of the experimental design for the extraction of phenolic compounds from red wine lees by means of ultrasound assisted extraction

Table. 28. Concentrations of total polyphenols extracted (mean ±SD) from red wine lees on the basis of the different combinations of Ultrasound assisted extraction

Run	Concentration of acidified ethanol (%)	Acidified water	Volume of the solvent (mL)	mg GAE/100 g
1	10	Yes	5	391.1 ± 33.8^{C}
2	5	No	5	337.9 ± 1.9^{E}
3	5	Yes	10	431.7 ± 135.1^{B}
4	10	Yes	10	453.1 ± 24.7^{A}
5	5	Yes	5	340.3 ± 58^{D}
6	10	No	10	335.5 ± 29.6^{F}
7	5	No	10	302.6 ± 25.4^{G}
8	10	No	5	267.9 ± 45.8^{H}

Values followed by different letters are significantly different according to the Tukey's HSD test ($p \leq 0.05$).

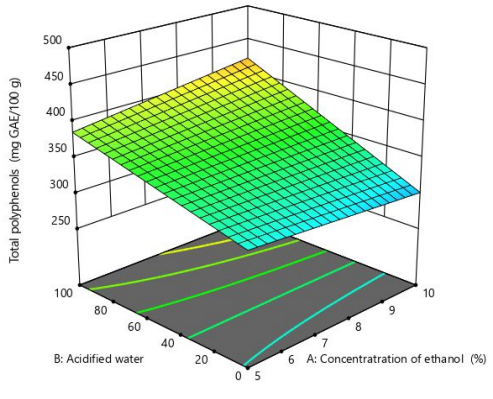
2.6.2.2.1. Effect of analysis on total polyphenols

Statistically significant differences were identified between the amounts of polyphenolic compounds extracted in the different combinations of experiments. The concentration of phenolic compounds extracted from the red wine lees is in the range of 267.9 ± 45.8 to 453.1 ± 24.7 mg of

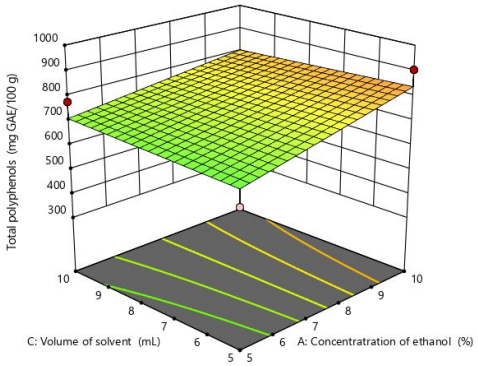
gallic acid/100 g of dry weight with an average value equivalent to 357.5 ± 44.34 mg of gallic acid /100 g of dry weight. A noticeable increase was evidenced regarding the extraction of polyphenols when the microwave operation power was to set to 350 Watts for 2 minutes (120 seconds) and 3 minutes (180 seconds) obtaining a maximum amount of polyphenolic compounds extracted of 2283.7 ± 115 and 2512.7 ± 12 mg gallic acid/100 g of dry weight, respectively.

According to the study developed by (Tao et al., 2014), in which an experiment was carried out by subjecting lees dissolved in 50% aqueous ethanol solution, to assisted treatment with an ultrasonic bath with a frequency of 40 kHz, an average value of 52 ± 31.56 mg was obtained, expressed in gallic acid within a range of 44 - 58.7 mg/L of total polyphenol concentration solution.

A notable increase in the extraction of polyphenols was evidenced when there was the presence of acidified water and a maximum percentage of ethanol, obtaining a maximum amount of polyphenolic compounds extracted of 453.1 ± 24.7 mg of gallic acid/100 g of dry weight. As observed in the response surface, its curvature increases evidently in correspondence with the parameter of presence of acidified water and the percentage of ethanol until reaching a maximum presence of acidified water and a percentage of ethanol of 10%, which also corresponds with the maximum concentration of total polyphenols.



A)



B)

C)

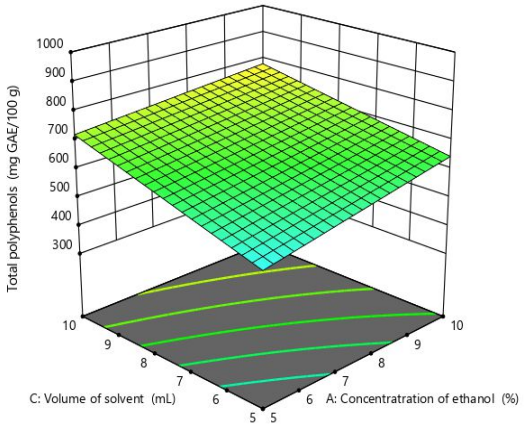


Figure.22. Response surfaces obtained from the experimental design used to optimize the extraction of polifenols from red wine lees. A: Concentration of ethanol/Acidified water; B: Concentration of ethanol/Volume of solvent; C: Acidified water/Volume of the solvent

Regarding the interaction between the concentration of ethanol and acidic water (Figure 22-A), the increase of the first parameter has an effect on the increase of extracted polyphenols, reaching the maximum point of these compounds around 10% ethanol concentration. However, it can be deduced from the trend of the response surface that the effect of acidic water prevails over the concentration of organic solvent with regard to the extractability of polyphenols from the raw lees. The response surface increases its tendency evidently in correspondence with the parameter of the acidic water, reaching a maximum using acidified water in the extraction mixture.

The interaction between the concentration of ethanol and the volume of solvent (Figure 22-B) also has a similar trend, in this case the curvature of the response surface informs that for the concentration of 10% of ethanol, the maximum point of polyphenols totals. It follows that there was a fairly constant increase in the second parameter (volume of solvent) which also has an evident effect on the increase in total polyphenols, the maximum point of total polyphenols is reached when the highest amount of solvent volume is used (10 mL).

When comparing the effects between the presence/absence of acidified water and the volume of solvent (Figure 22-C) it can be seen that, in this case, none of them prevails over the effect of the

increase in polyphenols, that is, it can be deduced that both parameters influence, with a very similar intensity, the increase in concentration of total polyphenols. The maximum concentration of polyphenols is reached when there is acidic water combined with the maximum volume of solvent (10 ml).

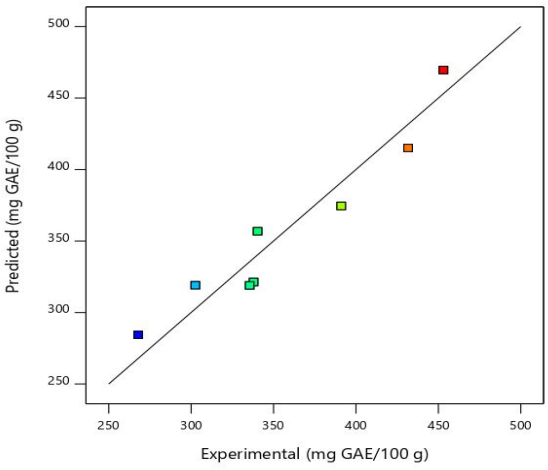
2.6.2.2.2. Statistical analysis

The effects experiments of the variables are performed according to the design matrix and the results of the analysis of variance (ANOVA) for the quadratic model are tabulated in the table 29. Regarding the results of this experiment, the predictive model obtained in the extraction of polyphenols from dried grape pomace by means of probe-assisted sonication is shown in figure 23. An acceptable predictive capacity was presented, which is reflected in the linear regression analysis with a coefficient R^2 equivalent to 0.9218, which means a good linear response. The model F-value of 17.67 suggested that the model is significant. There is only a 0.02% chance that an F-value this large could occur due to noise. In addition, the probability ρ <0.0002 also valid the significant model.

Table. 29. Effect of factors (single or their interactions) on the extraction of total polyphenols by ultrasound assisted extraction

Source	Sum of squares	Df	Mean Square	F-value	p-value	
Model	51577.91	6	8596.32	17.67	0.0002	significant
A-Concentratration of ethanol	308.41	1	308.41	0.6340	0.4464	
B-Acidified water	34647.34	1	34647.34	71.23	< 0.0001	
C-Volume of solvent	8616.59	1	8616.59	17.71	0.0023	
AB	2984.65	1	2984.65	6.14	0.0352	
AC	1354.84	1	1354.84	2.79	0.1295	
BC	3666.08	1	3666.08	7.54	0.0226	
A^2	0.0000	0				
B^2	0.0000	0				
C^2	0.0000	0				
Residual	4377.79	9	486.42			
Lack of Fit	4377.79	1	4377.79			
Pure Error	0.0000	8	0.0000			
Cor Total	55955.70	15				

In the present experiment B, C, AB and BC were significant factors, which indicates a good synergy between the presence/absence of acidified water and the concentration of ethanol and, presence /absence of acidified water and volume of solvent on the results of the polyphenolic content. As checking the R^2 values, the "predicted R^2" of 0.7527 was in reasonable agreement with the "adjusted R^2" of 0.8696. The "accuracy of adequacy or adequate precision" which is defined as the measure of the signal-to-noise ratio must be greater than 4 to be considered desirable. The ratio of 8.0775 suggested a suitable signal. According to the statistical analysis presented above, this model can be used to navigate through space.



Std. Dev.	22.05	**R^2**	0.9218
Mean	357.49	**Adjusted R^2**	0.8696
C.V. %	6.17	**Predicted R^2**	0.7527
		Adeq Precision	12.6955

Figure.23. Correlation chart of observed values/predicted values (total polyphenols (mg GAE/100 g dry weight) obtained from red grape pomace exhausted and analysis of Variance (ANOVA) for the linear correlation test

The response surface provides a deep analysis of the effect of the factors according to the corresponding model. However, the combination of the estimated effects allows to identify the values above which the extractability of the compounds of interest is optimized, and for the present experiment the experimental design model provided an optimal combination of factors that is shown in the table 30.

Table. 30. Optimization of the extraction parameters by the experimental model

Factors	Optimal condition
Concentration of ethanol (%)	10
Acidified water	Yes
Volume of solvent (mL)	10

2.6.4. Ultrasonic-Assisted Enzymatic Extraction

2.6.4.1. Red grape pomace

Table 31 summarizes the results of the experimental design for the extraction of phenolic compounds from fresh red grape pomace by means of ultrasound-assisted enzymatic extraction

Table. 31. Concentrations of total polyphenols extracted from fresh red grape pomace on the basis of the different combinations of Ultrasound assisted extraction

Run	Enzimatic dosage (mg/g^{-})	Cycles (No.)	Total polyphenols (mg GAE/100 g)
1	10	10	252.49 ± 0.5^{B}
2	20	7	333.97 ± 96.5^{AB}
3	10	7	313.10 ± 57.9^{AB}
4	20	10	319.92 ± 17.6^{AB}
5	30	4	271.75 ± 8.5^{B}
6	20	7	386.96 ± 51^{AB}
7	20	7	422.68 ± 109.5^{AB}
8	20	7	431.51 ± 24.4^{AB}
9	10	4	565.99 ± 10.2^{A}
10	30	10	430.31 ± 69.2^{AB}
11	30	7	379.73 ± 52.2^{AB}
12	20	7	530.66 ± 129.4^{A}
13	20	4	435.13 ± 2.4^{AB}

Values followed by different letters are significantly different according to the Tukey's HSD test ($p \leq 0.05$).

2.6.4.1.1. Effect of variables on total polyphenols

Statistically significant differences were identified between the amounts of polyphenolic compounds extracted in the different combinations of experiments. The concentration of phenolic compounds extracted from the red grape pomace is in the range of 252.49 ± 0.5 to 565.99 ± 10.2 mg of gallic acid/100 g of dry weight with an average value equivalent to 390.32 ± 49.8 mg of gallic acid /100 g of dry weight.

A noticeable increase was evidenced regarding the extraction of polyphenols when the number of cycles was to set to 4 with an enzymatic dosage of 10 mg g^{-1} obtaining a maximum amount of polyphenolic compounds extracted of 565.99 ± 10.2 mg gallic acid/100 g of dry weight

As observed in the response surface (Figure 24), it increases its curvature in an evident way in correspondence with the parameters number of cycles and dosage of the enzyme, until reaching a minimum of approximately 4 cycles and 10 mg g^{-1} of enzymatic dosage, which also coincides with the maximum concentration of total polyphenols.

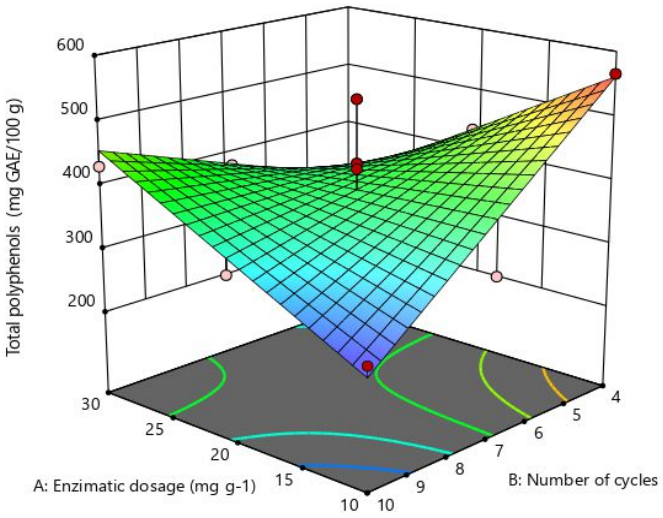


Figure 24. Response surface of concentration of total polyphenol versus the effects variables: A: Number of cycles; B: Enzimatic dosage

2.6.4.1.2. Effect of variables on total polyphenols

The effects experiments of the variables are performed according to the design matrix and the results of the analysis of variance (ANOVA) for the 2FI model are tabulated in the table 32.

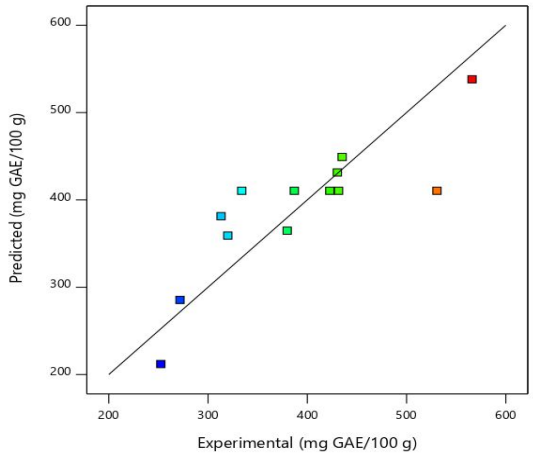
Regarding the results of the experiment, the predictive model obtained in the extraction of polyphenols from the dry red grape pomace by means of Ultrasound-Enzymatic extraction is shown in figure 25. A good predictive capacity was presented, which is reflected in the linear regression analysis with a coefficient R^2 equivalent to 0.6555, which means a good linear response. The model F-value of 5.71 suggested that the model is significant. There is only a 1.81% chance that an F-value this large could occur due to noise. In addition, the probability $\rho < 0.0181$ also validated the

model was significant. In the present experiment AB were significant factors, which indicates a well synergy between the enzimatic dosage and number of cycles on the results of the polyphenolic content. On verifying the R^2 values, the "predicted R^2" of 0.2860 was in reasonable agreement with the "adjusted R^2" of 0.5406.

Table. 32. Effect of factors (single or their interactions) on the extraction of total polyphenols by Ultrasound-enzimatic assisted extraction

Source	Sum of squares	df	Mean Square	F-value	p-value	
Model	68285.35	3	22761.78	5.71	0.0181	significant
A-Enzimatic dosage	412.92	1	412.92	0.1035	0.7550	
B-Number of cycles	12163.28	1	12163.28	3.05	0.1147	
AB	55709.15	1	55709.15	13.97	0.0046	
Residual	35893.63	9	3988.18			
Lack of Fit	15021.90	5	3004.38	0.5758	0.7220	not significant
Pure Error	20871.72	4	5217.93			
Cor Total	1.042E+05	12				

The "accuracy of adequacy or adequate precision " which is defined as the signal/noise ratio measure must be greater than 4 to be considered desirable (Muthukumar et al., 2003). The ratio of 9.3084 suggested an adequate signal. According to the statistical analysis presented above, this model can be used to navigate the design space.



Std. Dev.	63.15	**R^2**	0.6555
Mean	390.32	**Adjusted R^2**	0.5406
C.V. %	16.18	**Predicted R^2**	0.3860
		Adeq Precision	9.3084

Figure. 25. Correlation chart of observed values/predicted values (total polyphenols (mg GAE/100 g dry weight) obtained from red grape pomace exhausted and analysis of Variance (ANOVA) for the linear correlation test

The response surface provides a detailed analysis of the effect of the factors according to the corresponding model. However, the combination of the estimated effects allows for the identification of the values above which the extractability of the compounds of interest is optimized. For the present experiment, the experimental design model provided an optimal combination of factors which are presented in the table 33.

Table. 33. Optimization of the extraction parameters by the experimental model

Factors	Optimal condition
Enzimatic dosage (mg/g)	10
Number of cycles	4

2.6.5. Enzyme-extraction of polyphenols from grape pomace

2.6.5.1. Design of experiment and optimization of extraction parameters

As mentioned in the introductory section, several factors affect the activity of pectolytic enzymes, and the main purpose of the DoE is to guarantee the optimal activity range for the specific enzyme used in this study. The DoE was settled to include extreme temperature (i.e., higher 50°C) and pH (i.e., pH 2) to verify potentially limiting values. Furthermore, the effect of modulating factors pH and temperature over the three levels provided by DoE were investigated under the maximum incubation time (4h) and excluding the enzyme; results of these control experiments are summarized in table 34.

In the absence of enzymes, the concentration of total polyphenols obtained falls within the range 501-826 mg CE/100 g dry weight with average 623 ± 117.4 mg/100 g; tannins from grape pomaces were in the range 67 – 91 mg / 100 g of dry weight, with average 84 ± 7.6 mg/100 g of dry weight.

Table 34 - Effect of the incubation temperature and buffer pH (mean ±SD) on the extraction of polyphenolic compounds in the absence of enzymes.

Concentration of acidified ethanol (%)	**pH**	**Total polyphenols** *mg CE/100 g dw*	**Tannins (procyanidins)** *mg CE/100 g dw*
40 °C	2	518 ± 47	81 ± 2
	3	501 ± 10	78 ± 6
t= 4 h	4	524 ± 42	67 ± 10
45 °C	2	531 ± 15	87 ± 8
	3	577 ± 10	87 ± 8
t= 4h	4	698 ± 29	88 ± 11
50 °C	2	718 ± 30	91 ± 4
	3	713 ± 14	91 ± 0
t= 4 h	4	826 ± 8	85 ± 8

dw: dry weight; CE: (+)-catechin equivalents.

These preliminary control experiments confirm that when enzymatic activity is excluded, temperature plays a major role in polyphenolic extraction, with minimal impact on the availability of the grape tannins (procyanidins) (see Table 35).

Table 35. Iron reactive polyphenols and Tannins results (mean ±SD) of the experimental design.

Run	Enzyme dosage (mg/g)	pH	T (°C)	Time (h)	Iron-reactive polyphenols (mg CE/100g dw)	Tannins (mg CE/100g dw)	Tannins/total polyphenols (% w/w)
1	20	3	40	2	766 ±2^{M}	272 ± 10^{D}	35.5 ± 1.4^{B}
2	10	4	40	4	1389 ±13^{C}	319 ± 34^{C}	23.0 ±2.2^{CD}
3	20	3	50	3	973 ±27^{L}	123 ± 3^{H}	12.7 ± 0.6^{FGH}
4	10	3	50	4	1017 ±13^{K}	137 ± 2^{GH}	13.4 ± 0.4^{FG}
5	10	2	50	2	1163 ±48^{H}	136 ± 4^{H}	11.7 ± 0.2^{GHI}
6	10	2	45	2	1265 ±7^{FG}	437 ± 33^{A}	34.5 ± 2.4^{B}
7	10	3	45	3	1245 ±32^{G}	122 ± 15^{H}	9.8 ± 1.4^{I}
8	10	4	45	3	1467 ±39^{B}	159 ± 28^{FG}	10.9 ± 2.2^{HI}
9	30	4	50	2	1303 ±19DE	304 ± 7C	23.3 ± 0.2^{CD}
10	30	2	40	3	1062 ±15^{J}	361 ± 17^{B}	34.0 ± 1.1^{B}
11	20	2	50	4	1076 ±10^{J}	226 ± 17^{E}	21.0 ± 1.4^{D}
12	30	3	40	2	700 ±8^{N}	266 ± 3^{E}	38.0 ± 0.9^{A}
13	20	4	45	2	1533 ±4^{A}	356 ± 20^{B}	23.2 ± 1.4^{CD}
14	20	2	40	3	1121 ±6^{I}	165 ± 6^{F}	14.7 ± 0.5^{EF}
15	30	2	45	4	1284 ±13^{EF}	166 ± 4^{F}	12.9 ± 0.2^{FGH}
16	10	4	40	4	1424 ±11^{B}	361 ± 6^{B}	25.3 ± 0.2^{C}
17	30	4	50	3	1460 ± 18^{B}	159 ± 8^{FG}	10.9 ± 0.7^{HI}
18	30	3	45	4	1151 ± 18^{HI}	124 ± 4^{H}	10.7 ± 0.5^{HI}
19	20	4	45	4	1337 ± 35^{D}	223 ± 3^{E}	16.7 ± 0.2^{E}

dw: dry weight; CE: (+)-catechin equivalents. Values followed by different letters are significantly different according to the Tukey's HSD test ($p \leq 0.05$).

Polyphenols concentration ranged 694 – 1530 mg CE /100 g, meaning 0.6 to 1.5% of the original dry weight of pomaces; results are generally improved compared to the control samples (see Table 35)

Value within the range reported by the authors (Nayak, Bhushan, Rosales, et al., 2018) performing an aqueous 1:1 water ethanol extraction with 20:1 solvent-solid ratio at 25 °C from red grape pomace presenting a result of 801.66 mg GAE/100 g.

Other authors (Goula et al., 2016) reported a higher value, although still within the range obtained in this project. Using ultrasound technology at 20 watts and aqueous solvent (ethanol/water) for 10 minutes they achivied 957 mg GAE/100 g from red grape pomace.

The Lack-Of-Fit test (based on the F-test performed over replications) was used to verify the linearity of the two models in predicting the iron reactive polyphenols and tannins variables. The model showed high predictive capability, with improved linearity in predicting the total polyphenols (R^2 = 0.94, Figure 29) compared to tannins prediction (R^2 = 0.77, Figure 30). A slight deviation from linearity has been observed for the higher concentration levels of polyphenols (Figure 26), nevertheless, the test confirmed a linear correlation between provisional and experimental data (Prob > F = 0.5265); an increased dispersion of data was observed in the prediction of tannins (Figure 27) and the LOF test confirmed deviation of the model from linearity (Prob > F = < 0.0001).

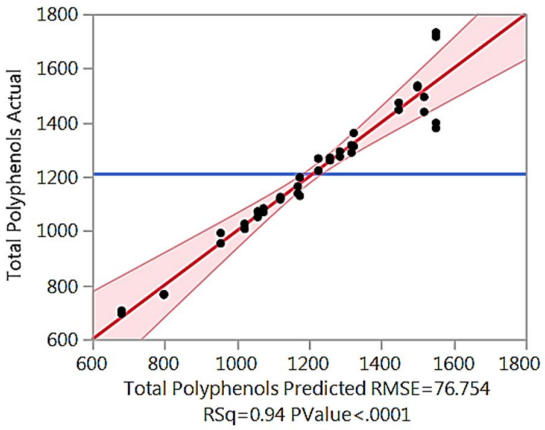


Figure 26. Correlation between predicted polyphenols (Y1) and experimental results (R^2 = 0.94, ρvalue <0.0001). A slight deviation from linearity was observed for polyphenols concentration > 1500 mg CE/ 100 g dw. The LOF test confirmed linearity of this regression (Prob > F = 0.5265).

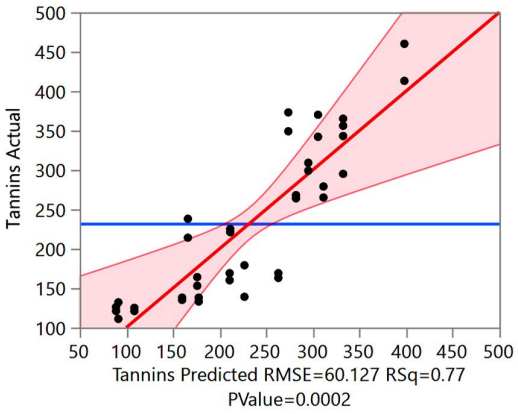


Figure 27. Correlation between predicted tannins (Y2) and experimental results (R^2 = 0.77, Pvalue = 0.0002). The model showed higher deviation from linearity as confirmed by the LOF test (Prob > F = <0.0001).

Results from DoE were evaluated by means of the analysis of the effects, to disclose the impact of linear, quadratic, and binary effects in the experimental outcomes. Table 36 reports the analysis for the iron reactive polyphenols (total polyphenols).

Table. 36. Outcomes from the analysis of the effects of experimental factors: pH, enzyme dosage, temperature, time, and their binary combinations in the extraction of total polyphenols (iron reactive polyphenols) using 95% confidence intervals.

Factors	**LogWorth**		**PValue**
pH*pH	5.303		0.00000
Enzyme dosage	3.780		0.00017
Enzyme dosage * Enzyme dosage	3.159		0.00069
pH	2.625		0.00237
Enzyme dosage *Time	1.539		0.02891
Enzyme dosage *Temperature	1.063		0.08656
Temperature*Time	0.893		0.12804
Time	0.872		0.13413
Time*Time	0.560		0.27524

Factors	LogWorth		PValue
pH*Time	0.404		0.39435
pH*Temperature	0.230		0.58943
Enzyme dosage *pH	0.207		0.62114
Temperature*Temperature	0.163		0.68692
Temperature	0.114		0.76908

The analysis of the effects of factors in the extraction of total polyphenols highlights that "enzyme dosage" and "pH", as well as their quadratic terms have a major effect on the amount of polyphenolic compounds in the extract ($p < 0.01$) than temperature, time and their respective quadratic effects ($p > 0.10$). If considering the binary effects, only the "enzyme dosage" and "time" combination significantly affects the extraction ($p = 0.02891$) within the selected ranges.

It has to be noted that a temperature increase might lead to an accelerated degradation of polyphenols in the extract, and this mechanism is strictly correlated to factors other than those taken into account in this DoE (i.e. the concentration of the oxidable substrate, the presence of catalysts, the oxygen uptake during the process). This effect is expected to be enhanced as the pH values increase from 2 to 4; nevertheless, the binary effect of temperature and pH did not significantly affect the total polyphenols content of the extract in this model.

The response surfaces for effects of the "pH", "temperature" and "time" independent variables vs the independent variable "enzyme dosage" are presented in Figure 28 – A, B, C. An increased enzyme dosage is requested at increasing pH values to gain higher extraction of polyphenols (A); lower temperature (B) and longer extraction time (C) showed positive effect on the enzyme activity. Nevertheless, a compromise is needed when considering the combination of factors other than enzymes. In particular, the oxidation onset of the polyphenolic compounds under high temperatures can be delayed by reducing pH values of the extraction buffers; at the same time, increasing temperatures could speed up the diffusion processes, which are responsible for an improved

extraction of bioactive compounds from vegetal tissues to a certain extent. Moreover, high pH seems to enhance the specific enzyme activity. All these stated, higher enzyme dosage (30 mg g^{-1}) and pH (4) are suggested in this model to maximize the polyphenols extraction under temperature values of 40°C and an incubation time of 3h. Results are in general agreement with previous experiments focused on the enzymatic-assisted extraction of flavan-3-ols (Stambuk et al., 2016).

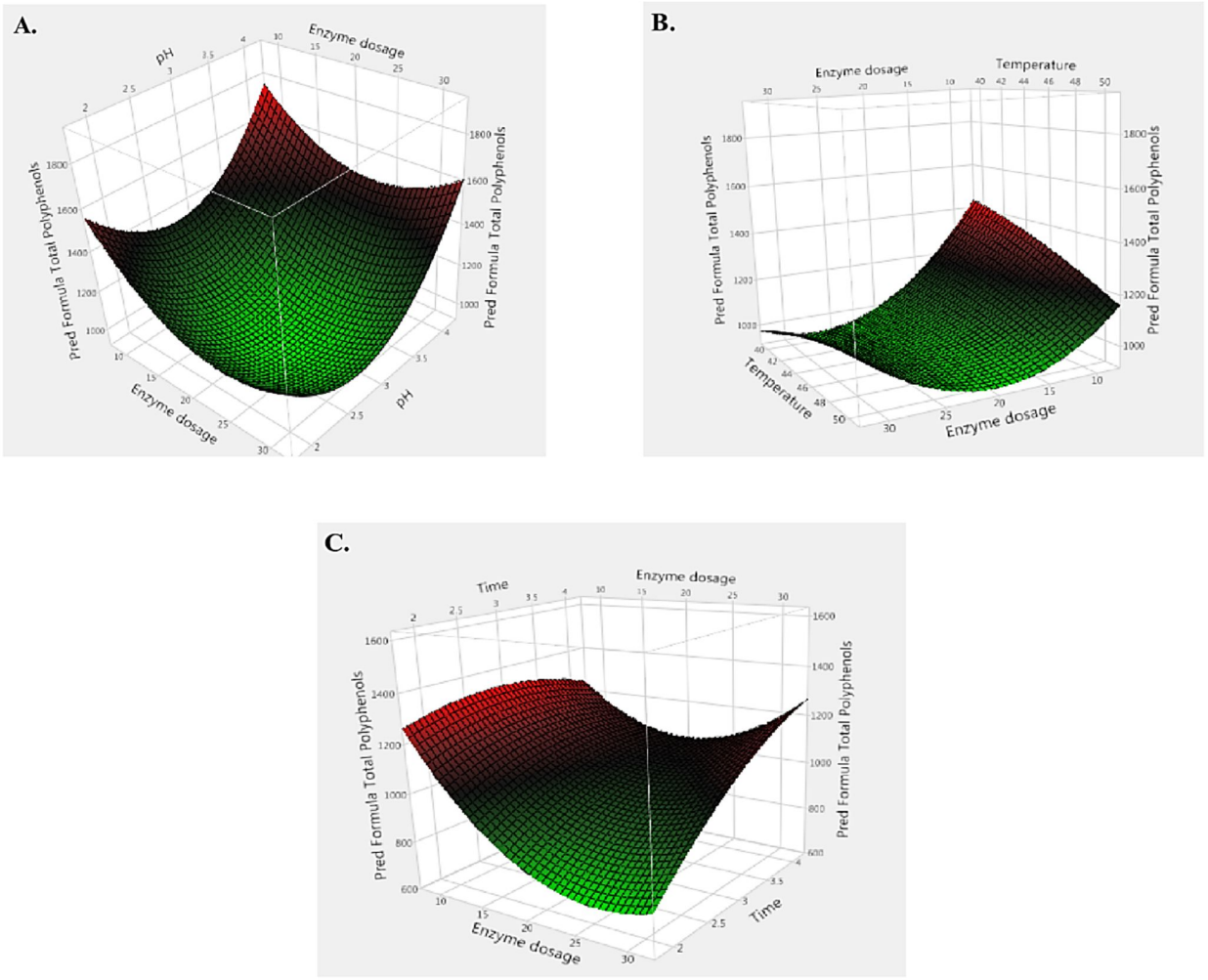


Figure 28. Response surfaces for the binary effects: A "Enzyme dosage*pH"; B "Enzyme dosage*temperature"; C "enzyme dosage*time.

Tannins constitute the most interesting fraction in terms of polyphenolic compounds which can be recovered from grape pomaces, due to their enhanced bioactivity, making them optimal reducing

agents, metal chelators and radical scavengers (Haslam, 1989; Okuda & Ito, 2011; Ricci et al., 2016). At the same time, tannins are the less available polyphenolic fraction, due to complex chemical interactions occurring between polyphenols macromolecules and vegetal cell walls constituents (polysaccharides, proteins), resulting in the formation of a tridimensional network and supramolecular. Previous studies have suggested that the extraction of tannins from crushed berries into wine is limited due to tannins binding to the cell walls through hydrogen bonding and hydrophobic interactions. In addition, the tannin-binding capacity of cell walls are strongly dependent on the tannins and polysaccharides structure and composition (Hanlin et al., 2010). It follows that cell wall changes induced by different events (i.e. the breaking /crushing of vegetal tissues, the macerating effect induced by organic solvents and, as for the present experiment, the usage of degradative enzymes) may reduce the tannin-binding capacity and facilitate their release in the extraction solvent.

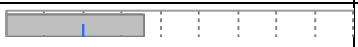
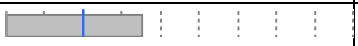
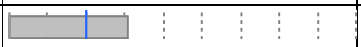
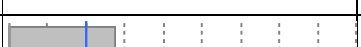
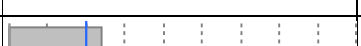
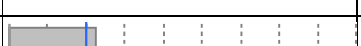
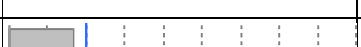
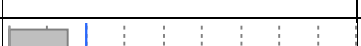
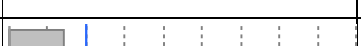
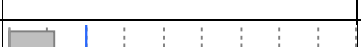
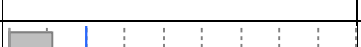
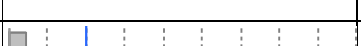
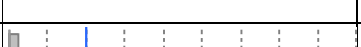
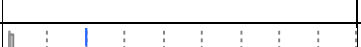
As previously observed in the control experiments (Table 30), in the absence of enzymes the temperature increase plays a role in enhancing the extraction of tannins through diffusion-regulated mechanisms; nevertheless, the mere increase of the temperature investigated in this study (40 to 50°C) produces a modest increase in the tannin content of the aqueous extract. Larger tannins are low-polar molecules, with reduced affinity towards aqueous extraction buffer; for this reason, the supporting the diffusion mechanisms.

Results from DoE showed the ability of pectolytic enzymes to enhance tannins extraction tot different extents compared to the control experiments (Table 31). Tannins concentration ranged 123 – 437 mg CE/100 g dry weight w, meaning 0.1 to 0.4% of the original dry weight of pomaces, with average 232 ±100 mg CE/100 g dry weight.

Similar value was found in the seeds of the fermented grape pomace after a water-ethanol extraction treatment (50-50%) with a value of 430 mg/100 g of tannins (Bosso et al., 2016).

The effect of experimental factors in the extraction of tannins (Y2) is analyzed in Table 37.

Table 37. Outcomes from the analysis of the effects of experimental factors: pH, enzyme dosage, temperature, time, and their binary combinations in the extraction of tannins (dependent variable Y2) using 95% confidence intervals.

Factors	**LogWorth**		**Pvalue**
pH*pH	3.599		0.00025
Enzyme dosage	3.513		0.00031
Enzyme dosage * Enzyme dosage	3.118		0.00076
pH	2.735		0.00184
Enzyme dosage *Time	2.412		0.00387
Enzyme dosage *Temperature	2.291		0.00511
Temperature*Time	1.698		0.02007
Time	1.559		0.02758
Time*Time	1.440		0.03629
pH*Time	1.181		0.06590
pH*Temperature	1.160		0.06917
Enzyme dosage *pH	0.458		0.34864
Temperature*Temperature	0.246		0.56763
Temperature	0.112		0.77208

The analysis showed a highly significant contribution of the “pH” factor (both linear term p = 0.00184 and quadratic term p = 0.00025) and “enzyme dosage” ((both linear term p = 0.00031 and quadratic term p = 0.00076) and a significant effect of the factor “time” in both linear (p = 0.02758) and quadratic (p = 0.03629) terms. The mere temperature contribution did not significantly affect the amount of tannins extracted, regardless its contribution in the binary combination with “enzyme dosage” (p = 0.00511). At the same way, the combined effect of “enzyme dosage” and “extraction time” was significant in the model proposed (p = 0.00387).

The combination of the factor “pH” with “enzyme dosage”, “temperature” and “time” did not show significant effects under these experimental conditions.

Generally, the contribution of factors involved in the extraction of tannins results in a more complex pattern. The enzymatic extraction of tannins exhibits enhanced efficiency when performed at the lower pH levels, which is the opposite of what is observed in the model of iron reactive polyphenols (total polyphenols).

A tentative explanation could be that an extensive activity of the enzyme, at the optimal pH activity encountered in the polyphenols extraction (4), might exert degradative effects on the tannins structure. If considering that grape tannins are condensed structure and are not susceptible to hydrolysis, we can hypothesize that the hydrolytic enzymes may react towards esterified structures (i.e. glycosylated and galloylated patterns which are commonly present in procyanidins) resulting in alteration in the structure of tannins; at pH values lower than optimal this activity might be reduced. A further hypothesis concerns the enhanced degradation of molecular networks involving tannins in the vegetal tissues, elicited by acidic conditions, with enhanced release of tannins in solution.

Hypothesis was confirmed by the authors who treated various wines by enzymatic means. They found that the enzyme-treated wines contain higher amounts of tannins than the control wines (which were not treated with enzymes). Its qualitative composition of tannins was different compared to the control wines. This may be due to easier extraction of higher molecular weight tannins as a result of increased enzyme-induced degradation of grape cell walls (Ducasse et al., 2010).

It can be inferred from (Figure 32), that if the effect of factors is considered separately, the mere enzyme concentration (A) has a less marked effect than the pH of the extraction buffer (B) in the tannins/total polyphenols ratio (which will hereinafter be referred to as relative tannins content, %); this effect is observed regardless the total polyphenols content of the extract. The response surface (Figure 29- C) informs that the absolute tannins content in the extract may be improved when combining higher enzyme dosages (30 mg g^{-1}) and pH values between 2-2.5. In brief, the pH-

dependent degradation of tannins might constitute a side-effect of the pectolytic enzyme action, and the abovementioned hypotheses deserve further investigations.

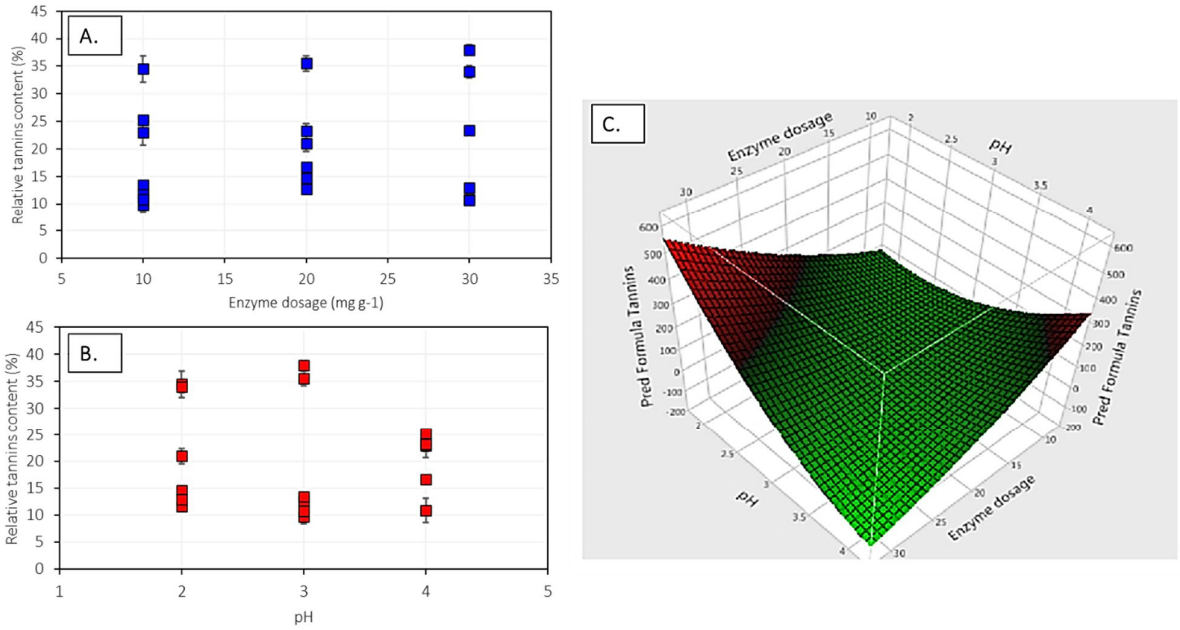


Figure 29. Linear effect of the enzyme dosage (A) and pH of the extraction buffer (B) in the relative tannins content (%), and effect of the binary combination of enzyme dosage*pH factors in the absolute amount of tannins in the extracts (C).

The experimental design provided the following values to enhance the tannins content in the extracts: enzyme dosage 30 mg g^{-1}, pH 2.5, temperature 45°C and extraction time 3.5 h, respectively.

2.6.5.2. Preparation of the enzymatic-assisted "bio-extract"

Optimal conditions obtained by DoE to maximize tannins extraction were applied to a batch of freshly-pressed grape pomaces; the aim of this experiment was (1) to confirm the extraction protocol settled by the experimental design and (2) to obtain a "bio-extract", an aqueous buffered solution enriched in bioactive polyphenolic compounds with antioxidant and (potential) antimicrobial activities. Tables 38 and 39 report the analytical parameters obtained under these

conditions. The control experiment (optimized factors with the exclusion of the enzymes) was carried out accordingly.

Table. 38. Polyphenolic content and antioxidant (reducing/antiradical) capacity of the enzymatic bio-extract (and related control experiment) obtained from fresh grape pomaces.

Source	**Sum of squares** *mg CE/ 100 g dw*	**Df** *mg CE/ 100 g dw*	**Mean Square** *% (10 dil)**	**F-value** *% (10 dil)**
Control	811 ± 21	206 ± 30	8 ± 0.4	11 ± 0.4
Enzymatic bio-extract	1798 ± 167	706 ± 5	17 ± 1.2	22 ± 1.3

*CE = (+)-Catechin equivalents.*Standard dilution to obtain optimal reagent-to-sample ratio*

Table. 39. Protein, simple sugars content and alcohol content of the enzymatic bio-extract (and related control experiment) obtained from fresh grape pomaces.

	Proteins *mg BSA/100 g dw*	**Alcohol content** (%)	**Simple sugars** (g/L)
Control	106 ± 0	< 0.3	< 0.5
Enzymatic bio-extract	44 ± 2	< 0.3	< 0.5

BSA = bovine serum albumin.

The control extract exhibits about 0.11% dw protein content, which is further reduced in the enzymatic extract (0.04%), possibly as a consequence of the hydrolytic activity of the enzyme on the amino acid units. The permanence of pomace in the extraction buffer did not elicit fermentation processes (alcohol content < 0.3%) due to the low pH value of the extract and the limited amount of reducing sugars (the latter being at the limit of sensitivity of the analytical methods used). The low sugar content in the extract is particularly important in the hypothesis of application of the bio-extract for antimicrobial purposes; nevertheless, targeted inhibition of microbial activities by the extract must be confirmed by further *in vitro* studies.

The enzymatic bio-extract exhibit enhanced total polyphenols content (1798 ± 167 mg CE/100 g dw) with respect to the control sample (811 ± 21 mg CE/100 g dw); moreover, the enzymatic extract retains a 40% relative tannins content against 25% in the control sample.

Antioxidant activity, expressed as the ability to reduce the iron (III) ion to less reactive iron(II) and as the ability to scavenge radical species, showed similar trend; results from both assays were almost doubled in the enzymatic bio-extract with respect to the control sample. Under the experimental conditions the enzymatic extract showed the ability to convert 17% of the initial Fe^{3+} ion and to remove 22% of the DPPH• radical in solution. According to the internal dataset developed in the Wine Science laboratory of the University of Bologna, the bio-extract shows typical antioxidant activity values observed in common table white wines (*data not reported*).

2.6.5.3. Hypotheses for the enzymatic bio-extract exploitation

The antioxidant, anti-inflammatory and antifungal properties of polyphenolic compounds, which are further enhanced in tannins, have been extensively reviewed in the scientific literature (Friedman, 2014; Landolfi et al., 1984); both in vitro and in vivo studies demonstrated that polyphenols may inhibit the oxidative damages induced in biological substrates by free radicals, and limit the extent of degenerative diseases (Pandey & Rizvi, 2009). Besides the pharmaceutical applications, polyphenols are broadly used in the nutraceutical, cosmetic and food industries, and further technological applications are elicited by their unique properties (animal feed, composite materials, dyestuff).

Wine industry by-products are gaining interest as a source of bioactive compounds, and in the last decade several scientific works have been devoted to exploring innovative strategies for the effective and sustainable recovery of polyphenols from grape pomace, and for their exploitation in the framework of virtuous chains and circular economy practices (Devesa-Rey et al., 2011).

Nevertheless, to meet the safety and quality requirements for the by-products recovery the content of agrochemical residues in raw materials must be below the threshold imposed by regulatory frameworks.

This is a critical aspect of the wine by-product valorization chain. Grape is particularly exposed to the risk of fungal contamination, which may irreversibly damage or reduce the quality of the

product at harvest. Phyto-pharmacological products used to overcome this limitation may persist in grape pomace to varying degrees (Communities, 2003).

Several green alternatives have been proposed to minimize the usage of phytochemicals and pesticides; among them, highly resistant grape cultivars with enhanced polyphenolic compounds biosynthesis (i.e., hybrid *Vitis* species) are gaining increasing interest, together with the application of polyphenols-enriched formulations to protect the grape bunches and vine leaves during ripening. These solutions originate from the primal role of polyphenolic compounds, which are synthesized in nature as secondary metabolites to protect plant against environmental stress (insects, pathogens, UV irradiation).

The enzymatic bio-extract proposed in this experiment could be considered in the framework of a virtuous circular process, where the bioactive extracts used to improve the resistance and health of vines are sourced from the vinification by-products. This could be an advantageous way to utilize a product which is routinely used in cellars (pectolytic enzymes), through a low-impact, highly sustainable extractive process.

This work moves from previous studies (Focaccia, 2019) and was carried out in the framework of the "Fondazione Giovanni Dalle Fabbriche" research project: "*Enzimi pectolitici per estrarre composti fenolici dalle vinacce*", both from the Wine Science group of the University of Bologna.

Future prospects provide a detailed analytical characterization of the composition of the extract, *in vitro* studies of antifungal/anti-microbial activities, and application of pilot scale treatments in case study.

2.7. Conclusions

There is a growing interest amongst the scientific community to develop and achieve the best conditions related to extraction technologies. In addition, there is a growing consumer demand for a market focused on the acquisition of products that improve the health and well-being of the population. In this context, the need is increasing for the efficient and sustainable recovery of compounds of high added value through sustainable methodologies and technologies with low impact on the environment. Important factors to consider include the type and amount of solvent, reduction of treatment time, reduction of high temperature exposure on the compounds of interest, and increase in yield are subjects of study.

This study provides a broad overview of the recovery of bioactive compounds such as polyphenols through a range of methodologies applied to the residues of the wine process; specifically on red grape pomace and red wine lees. For the first time, several extraction methods have been applied to the same grape pomace and wine less samples to highlight strengths and weaknesses of each methods.

In conclusion, this study showed that:

- The residues of the wine process, specifically red grape pomace and red wine lees are perfect candidates for the application of recovery methodologies and for the development of a circular economy.
- The microwave technique has proven to be an optimal methodology for the recovery of bioactive compounds, presenting excellent performance in the experimental phase. It is suggested to expand the range of the two conditions used so that an optimal performance can be found using higher maximum points.
- The enzymatic extraction technique was of considerable interest due to the significant yields of polyphenolic compounds obtained in addition to being a sustainable technique.

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CHAPTER 3

Nomenclature:

A.A: Antioxidant Activity

A: Surface of the membrane

CA: Cellulose Acetate

CE (%): Cleaning efficiency

CE: Catechin equivalents

CRL: Cabernet Sauvignon Wine lees

d.w: Dry weight

Da: Dalton

f: Apparent rejection coefficient

F: Feed solution

FI (%): Fouling index

HPLC: High Performance Liquid Cromatography

ICV: Ice cold water

Jp: Permeate flux

kDa: Kilo Dalton

Lp: hydraulic permeability

MF: Microfiltration

MGP: Mixed red grape pomace

mL: Milimeter

MRL: Mixed red wine lees

MWCO: Molecular Weight Cut Off

n.d: Non detected

NF: Nanofiltration

nm:Nanometers

NTU: Nephelometric Turbidity Units

OD: Osmotic destillation

P: Permeate solution

PEG: Polyethylene glycol

PVDF: Polyvinylidene fluoride

R (%): Rejection coefficient

R: Retentate

RPM: Revolutions per Minute

SD: Standard Deviation

Sec: Seconds

SRL: Sangiovese Red Wine Lees

TMP: Transmembrane pressure

TOC: Total Organic Compound

TP-FC: Total phenolic measured by Folin-Ciocalteu method

UF: Ultrafiltration

v/v: Volume/volume

VCF: Volume Concentration Factor

VRF: Volume Reduction Factor

μm: Micrometer

3.0. MEMBRANE TECHNOLOGY FOR THE SEPARATION OF BIOACTIVE COMPOUNDS

ABSTRACT

The technologies for separating and recovering bioactive compounds have been developed over the years to create tools that are increasingly versatile and efficient resulting in technologies that are both economically and technically advantageous in the food, chemical and pharmaceutical industries. Membrane technology has been at the forefront of the separation technologies and the great challenge for the membrane industry is to integrate multidisciplinary fields, overcoming technical problems and obtaining functional products.

The objective of this study was to assemble different types of membrane, as well as to make the membranes that would be used for the separation of bioactive compounds from the winemaking process, such as red and mixed grape pomace as well as red wine lees.

3.1. Introduction

In the field of membranes, a crucial moment was the preparation of the cellulose acetate membrane, described as a semi-permeable membrane operating under the principle of reverse osmosis and applying it to the desalination of sea water (Loeb, 1981). Since then, membranes have been developed with various materials, more homogeneous; with a higher permeate flow and high selectivity. In the 1970s the composite polymer membrane was developed for the gas separation of commercially feasible currents. At present, the principle of selectivity separates millions of cubic meters of gas. (Drioli & Romano, 2001).

Lately the processes of pressure-driven membranes such as microfiltration, ultrafiltration and nanofiltration are being applied to the treatment of agro-food wastewater. Having the membrane produces various advantages such as low cost, high separation efficiency, high productivity and easy handling (Cassano et al., 2011), avoiding the contamination of the product and preserving the biological activity of the compounds of interest (Drioli & Romano, 2001). Table. 1 describes the different bioactive compounds, type of membrane and its characteristics. In summary, the main compounds of interest to separate are phenolic compounds, processed by MF, UF and NF.

Along with the recovery of the compounds of interest, the membrane system is a key technology for the choice of a physicochemical, economic and non-destructive technique (Galanakis et al., 2015; Rahmanian et al., 2014). The separation capabilities of the microfiltration, ultrafiltration and nanofiltration membranes are related to the exclusion of size, influenced by parameters such as temperature, transmembrane pressure and the flow of food. (Salehi, 2014).

Table 1. Summary of different bioactive compounds and sources, types of membranes in the separation processes

Operation	From	Separated	Membrane	Pore size	Operating range	Membrane type	Reference
UF	Kiwi fruit	Compounds ascorbic, folic, citric, glutamic acids	Nadir®CO30 FM	30 kDa	1-11	Cellulose	(Cassano et al., 2008)
UF, OD	Blood orange juice	Phenolic compounds	DCQ III – 006C, Celvard ®	100 kDa – 30 nm (Darra et al., 2013)	2-13	Polysulphon, polyethylene	(Destani et al., 2013)
UF, NF	Distilled fermented grape pomace	Polyphenols	Nanomax 95, Nanomax 50, DL2540, GE2540, Inside Céram	250, 350, 150-300, 1000, 1000	-	PA/PS, PA/PS, TF, TF, Titania	(Díaz-reinoso et al., 2009)
UF, NF	Olive mil wastewater	Phenolic compounds, Hydroxynnamic acids	GR40PP, GR60PP, GR81PP, GR95PP, NF99	100 kDa, 25 kDa, 10 kDa, 2 kDa, 0.12 kDa	1-13, 1-13, 1-13, 1-13, 2-10	Polysulphone, polysulphone, polyethersulphone, polyethersulphone, polyethersulphone	(Galanakis et al., 2010)
MF	Wine	Proteins		0.2 µm	-	Cellulose	(Salazar et al., 2007)

Operation	From	Separated	Membrane	Pore size	Operating range	Membrane type	Reference
MF	Winery effluents	Phenolic compounds	V0.2, MFP5, Not supplied	0.2 µm, 0.5 µm, 0.4 µm	1-11, 1-11 -	PVDF, Fluoro polymer, polyimide	(Cassano et al., 2008)
MF, UF	Nixtamalization wastewaters	Phenolic compounds, calcium components, carbohydrates	CEP-1-E-4A, UFP-100-E-4A, UFP-1-E-4A	0.2 µm, 100 kDa, 1 kDa	2-14, 2-14, 2-14	Polysulfone, polysulfone, polysulfone	(Castro-Muñoz & Yáñez-Fernández, 2015)
NF	Orange press liquor	Polyphenols compounds	NF-70, NF-200, N30F, NFPES10	0.18 kDa, 0.3 kDa, 0.4 kDa, 1 kDa	2-11, 3-10, 2-11, 2-11	0.2 µm crosslinked aromatic polyamide + 0.46 µm polysulfone, polypiperazine amide thin-film composite, polyethersulphone, polyethersulphone	(Conidi et al., 2012)
UF	Xoconostle fruit (Opuntia joconostle) juice	Carbohydrates, betalain compounds, polyphenols compounds	UFP-100-E-4A	100 kDa	2-14	Polysulfone	(Castro-Muñoz et al., 2017)

The process of retention and filtration derives in two streams as shown in the figure 1. The two product streams, after the filtering process, are called permeate current and retention current. The permeate current is all the solutes that pass through the filtration membrane because of its low molecular weight compared to the pore of the membrane. The retention current is the solutes rejected by the membrane due to its high molecular weight. (Van Der Bruggen et al., 2003).

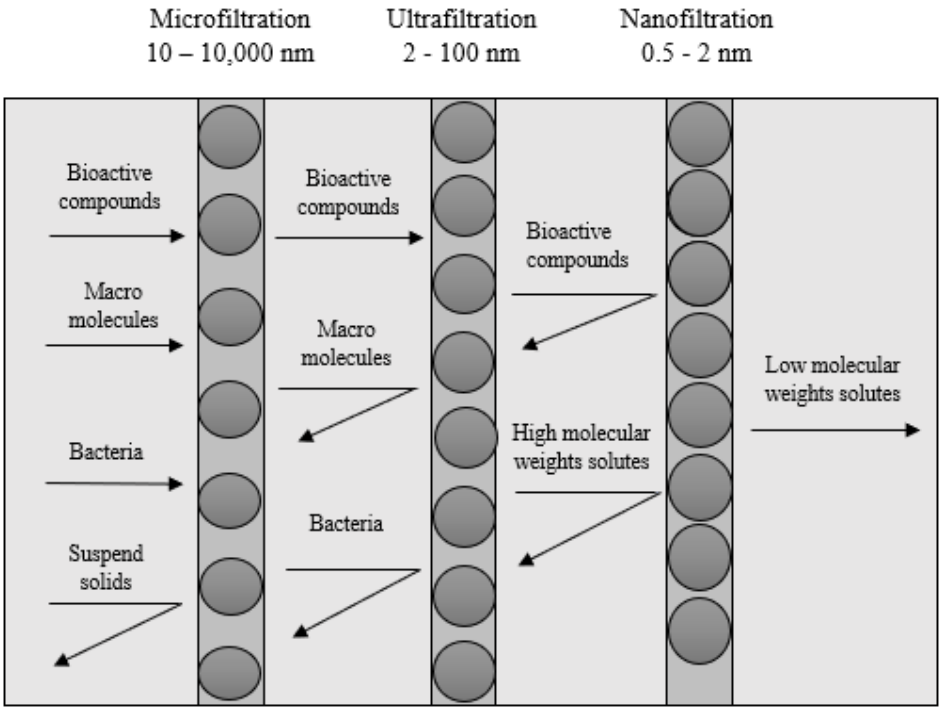

Figure 1. Operating principle permeation/retention of microfiltration membrane, ultrafiltration and nanofiltration.

Different studies have shown the efficiency of the membrane system in wastewater and juices. Research conducted by (Conidi et al., 2017) tested microfiltration and nanofiltration membranes with molecular weight nominal cut-off (MWCO), ranging from 1000 to 4000 Da, to purify biologically active compounds from clarified pomegranate juice. Various parameters including membrane productivity, phenolic compounds and antioxidant activity were evaluated. Once the membranes were probed with the two different transmembrane slices, concentration/diafiltration experiments were carried out in order to produce a retained stream rich in phenolic compounds and a permeate stream rich in glucose and fructose. The results showed yields of polyphenols and anthocyanins of 84.8% and 90.7% respectively. In addition, for the permeate flow, the diafiltration process produced yields of glucose and fructose of 90% and 93%, respectively. The conclusions were that the 2000 MWCO membrane was extremely useful due to its high productivity, retention

of phenolic and anthocyanin compounds and ability to obtain a permeate current rich in glucose and fructose.

The use of membrane processes for the recovery of bioactive compounds in the sub products of the wine industry is gaining strength in recent years, and various works have been developed on grape pomace and wine lees.

In the years 2002-2018 interesting research was developed with various types of sub products from the wine industry, such as fermentation of grape pomace, grape seeds and wine lees. The most common approaches were in the production of extracts from grapes, where different active compounds are known to be present, as shown in table 2.

Table 2. Various commercially interesting phenolic compounds present in grapes (Crespo & Brazinha, 2010)

Compounds	**Chemical family**	**Molecular weight (g/mol)**	**Concentration (mg/kg)**	**Location**
Anthocyanins - 3-monoglucosides - 3,5- diglucosides Higher number of sugars molecules attached	Flavonoids	~ 500 ~ 700 Higher than 1000	50600 ± 800 of total 3-monoglucosides anthocyanins	Skin of red grapes
Proanthocyanidins Monomeric flavan-3-ols - Catechins - Epicatechins Oligomers Dimers Trimers Tetramers Polymers	Flavonoids	~ 300 ~ 300 580 870 1160	2440[2] 1930[2] 2350[2] 840[2]	Mostly in grape seeds
Trans-Resveratrol	Stibenzenes	228	123.0 ± 5.1[1] ~ 20[3]	Mostly in red grapes skins

Membrane processes can be used in a versatile manner according to whether the objective is to obtain high molecular weight compounds or to extract low polymerization compounds. The use of different strategies for the optimization of this technology has been proposed. As is the case of the coupling of integrated membrane systems. As shown in figure 2. This strategy involves fractionation using microfiltration and ultrafiltration membrane systems in a sequence. The microfiltration membrane is used to eliminate the suspended solids that can induce a fouling in the membrane, the next sequential step is the use of the ultrafiltration and nanofiltration membrane, in this step the macro molecules are eliminated carrying out the polyphenol recovery in the permeate stream.

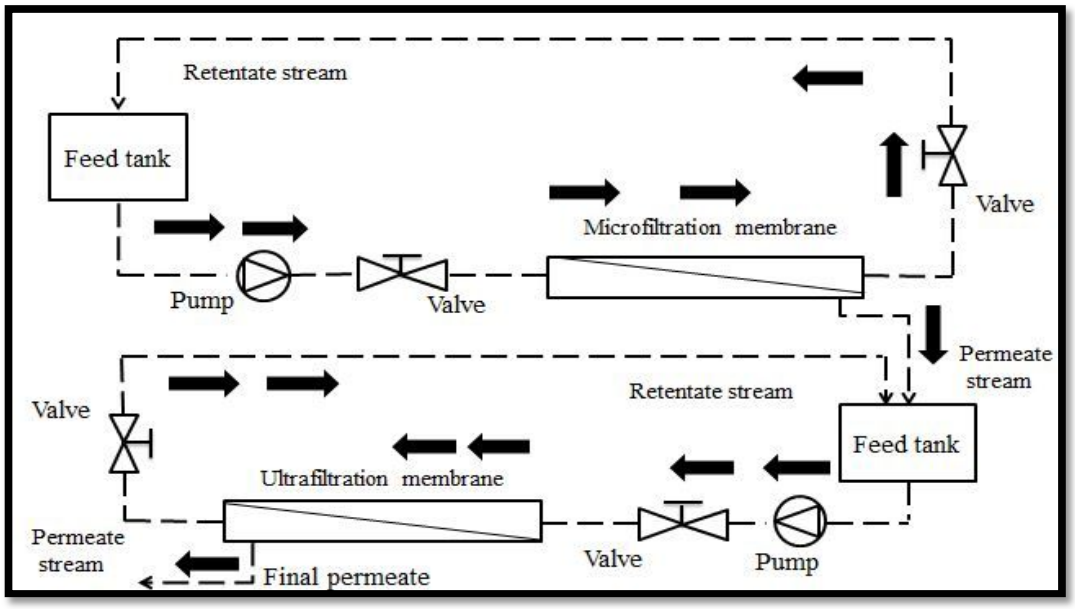

Figure 2. Integrated microfiltration and ultrafiltration membrane system.

An interesting work was the implementation of membrane sequences for the fractionation of proanthocyanidins of different degrees of polymerization, obtained from the seed of the grape. The first step was the extraction of the compounds of interest by means of a solvent. A suitable choice of solvent extraction was needed to prevent the extraction of proanthocyanidins of high polymerization, which are avoided due to their poor nutritional value, because of their lack of

intestinal adsorption properties. Thus, in this research 80/20 methanol was used as the most suitable solvent. After obtaining enough extract, it was introduced to the sequence of 4 membranes with different transmembrane cuts and materials. Several sequences were carried out during the filtration process. The first sequence (1) was to observe the transmembrane pressure, velocity of the crossflow, effect of the material and the transmembrane cut on the rejection current.

In this sequence, a polyamide membrane was used to remove the acids and aldehydes with low molecular weights, as well as to observe the characteristics of the membrane described above. It was observed that the optimal transmembrane pressure was 6, 0.2 and 5 bar for nanofiltration, microfiltration and ultrafiltration, respectively.

For sequence two (2), the order of the membranes was established according to the compounds to be retained. Therefore, it started from the polyamide nanofiltration membrane (60% $CaCl_2$ rejection) with a pressure of 6 bar, for the purpose of removing the low molecular weight compounds, later it was sought to retain the condensed polyphenols. Finally, the polyvinylidene fluoride microfiltration membrane (200 kDa) and a polysulphone ultrafiltration membrane (8 kDa) were used under the transmembrane pressures established in sequence one (1). During this sequence, a retentate rich in proanthocyanidins, with a low degree of polymerization, was obtained, specifically in the ultrafiltration process with 0.0347 mg/g seed and degree of polymerization of 2.19. (Santamaría et al., 2002).

The work published by Santamaría and collaborators in 2002, is an example of how the versatility of membrane processes, through correct sequences and established parameters in order to produces currents enriched with the products of interest.

A few years later in 2009 a study was published regarding the obtaining of fractions enriched in antioxidant compounds from fermented grape pomace, by means of the experimentation of four different membranes. The filtration and recovery process of the compounds of interest was carried out on each of the membranes individually, testing materials such as polyamide polysulphone, polytetrafluoroethylene and titania with transmembrane cuts of 250 to 1000 Da. The results showed

that all the membranes tested displayed similar rejections of total polyphenols and sugars, concluding that they were suitable for concentration purposes. The membrane that demonstrated the highest retention in the phenolic compounds was the membrane with a polyamide/polysulphone material with 250 Da and it showed an increase of 84.12% of polyphenolic compounds with respect to the initial extraction.

An important aspect to consider in the membrane process is the recovery of permeability by means of a cleaning protocol. The above-mentioned study also discussed that the type of membrane used was important as well as the number of cleaning cycles. They explained that cleaning cycles using a caustic solution of detergent at a temperature of 50 °C for 60 minutes per cycle was needed. They also, concluded that the membrane that achieved a total recovery of its permeability was inside ceramics with a material of titania and with a transmembranal cut of 1000 Da (Díaz-reinoso et al., 2009).

Another interesting study is the recovery of phenolic compounds from wine sludge and its separation from other extracted components. The research was undertaken with two hydro ethanol extracts and three types of membranes (polysulfone 100 kDa and 20 kDa, and fluoropolymer membrane 1 kDa). The suitability of the fluoropolymeric membrane of 20 kDa allowed the retention of 60% of polyphenols and sugars. The use of a nanofiltration membrane of 1 kDa was successful in separating different phenolic classes such as hydroxycinnamic acids, flavonols and anthocyanins (Galanakis et al., 2013).

In 2015 a study focused on the extraction, purification and adsorption/desorption of phenolic compounds from grape pomace (Merlot variety). The extraction was achieved with an optimization of the percentage of ethanol-water of 50-49%, acidity of 1% HCL 1 N and 15 minutes of agitation obtaining a concentration of 440 mg/L of polyphenolic content. Next an ultrafiltration ceramic membrane with a 100 nm pore diameter was used for the removal of suspended solids, leading to a significant reduction of 35% polyphenolic compounds and 50% carbohydrate content.

Subsequently, the clarified solution obtained from the ultrafiltration process was introduced to the nanofiltration membrane of polyamide with a transmembrane cut of 470 Da. In this step, 95% polyphenol content and 80% carbohydrate content were retained due to polyphenolic compounds and monosaccharides having lower molecular weights than the transmembrane cut used in the membrane (Zagklis & Paraskeva, 2015)

Another study demonstrated the suitability of this technology. (Giacobbo et al., 2015) reported a vacuum aqueous extraction method with various dilution factors associated with the microfiltration process for the polyphenol recovery from wine lees. The results showed that in the highest dilution factor there was higher productivity in the membrane with a polyphenol recovery rate of 12%. In addition, the membrane with the largest pore size exhibited lower permeate fluxes and presented a greater incrustation of the high molecular weight compounds.

One of the most recent works published is the use of nanofiltration for the fractionation of polyphenolic compounds from grape pomace extracts. The parameters of the transmembrane pressure, tangential velocity, temperature and the transmembrane cut of nine membranes were investigated. The evaluation of the rejection coefficient in several polyphenol families was an important factor to determine the effectiveness of the membrane treatment. Membranes having a transmembrane cut-off range between 500 to 1000 Da were able to recover the polymer proanthocyanidins in the concentrate stream and separate them from the phenols that passed through the membrane to the permeate stream. The filtration sequence included two membranes with a transmembrane cut of 500-1000 and 1000 Da, where the first three membranes have a polyamide material and the last membrane has a fluoropolymer material, respectively. The study also demonstrated that the membrane comprised of polyamide material and with a transmembrane cut-off range of 300-600 Da is useful for fractionating families of monomeric phenols. These results show that the separation of proanthocyanidinic polymers and monomeric phenols is governed by the pore size of the membranes used. Regarding fouling of the membrane, several factors affect the

deposit of cakes on the membrane, for example hydrophobicity membranes and the presence of polysulfone material present a high fouling (Yammine et al., 2019).

The functionality of the membrane process is not only limited to the recovery of compounds by the side of a stream obtained by the process, but it is possible to obtain two compounds of interest from different chemical families separately. This is the case in a study carried out by (Giacobbo et al., 2017). The assembly of a set of ultrafiltration and nanofiltration membranes helped the fractionation of polyphenols and polysaccharides present in the wine lees from the second racking from the Merlot variety. The ultrafiltration step (with a material membrane module and a transmembrane cut) was able to separate the polysaccharides from the polyphenols obtaining the retentate stream rich in polysaccharides while the polyphenols were preferentially permeated into the permeate stream. Subsequently, by means of a nanofiltration membrane, polyphenols from the previous stream of the ultrafiltration process, were successfully retained. Therefore, the concentration of the polyphenolic compounds could be achieved by means of nanofiltration, thus achieving a solution with high antioxidant activity.

Over the years, the versatility and efficiency of the membrane system in recovering compounds of interest on agro-industrial by-products and waste from the wine industry has been demonstrated. The transformation and addition of added value, drives the field of membrane technology development and the researchers who are involved in developing, improving, and integrating more efficient processes. The great challenge for the membrane industry is to integrate the multidisciplinary field, overcoming technical problems such as the concentration polarization and the different fouling phenomena, which are key to converting this technology into a significant alternative with important benefits.

3.2. Materials and methods

3.2.1. Description of the samples

The following samples were used:

- Mixed red wine lees (MRL), obtained by raking after fermentation of must from different red grape varieties, was used for microfiltration (MF) and nanofiltration (NF) experiments.

- Red wine lees (SRL), obtained from the Sangiovese red grape variety, was used for ultrafiltration (UF) experiments.

- Red wine lees (CRL) obtained from the Cabernet Sauvignon red grape variety was used for nanofiltration (NF) experiments.

- Mixed red grape pomace (MGP), comprising of 60% Cabernet Sauvignon, 30% Sangiovese and 10% Syrah, was used for the nanofiltration experiment.

All samples were kindly provided by Cantina di Terre Naldi (Faenza, Emilia-Romagna, Italy) and were stored at -20°C. Samples MRL and MGP were dried at 50 °C for 24 h before use.

3.2.2. Microfiltration and nanofiltration experiments for the mixed red wine lees (MRL)

3.2.2.1. Microfiltration and nanofiltration membranes

The mixed red wine lees were pre-treated by a microfiltration process using a PVDF membrane with a 0.15 µm membrane pore size. The clarified solution was treated using three different flat sheet membranes with a molecular weight limit in the range of 150-1000 Da. The filtration experiments were carried out at a transmembrane pressure of 24 bar and at an operating temperature of 25 °C. The main characteristics of the selected membranes are reported in Table 3.

Table.3. Characteristic of the microfiltration (MF) and nanofiltration (NF) membranes

Membrane Type	**FSM0 15PP**	**ETNA 01PP**	**NTF50**	**DK**
Manufacturer	Alfa Laval	Alfa Laval	Alfa Laval	GE Osmonics
Membrane material	PVDF	Composite fluoro polymer	aromatic/aliphatic polyamide	cross-linked aromatic polyamide
Configuration	Flat-sheet	Flat-sheet	Flat-sheet	Flat-sheet
Pore size (μm)	0.15	-	-	-
Nominal MWCO (Da)	-	1000	150	150-300
pH operating range	1-11	1-11		3-9
Max. operating temperature (°C)	65	60	50	55
Max. operating pressure (bar)	10	10	55	41
Membrane surface area (cm^2)	14.6	14.6	14.6	14.6

3.2.2.2. Experimental set-up and procedures

Dead-end filtration experiments were performed in a stainless-steel stirred cell filtration system (SterlitchTM HP 4750, Kent, WA, USA) with an effective membrane area of 13.85 cm^2 and a volume capacity of 300 mL and operating pressure capacity of 68.9 bar.

The stirring cell assembly consists of three main components: 1) body with removable top and bottom; 2) stir bar assembly coated with Teflon to provide mixing; 3) porous stainless-steel membrane support disc.

A nitrogen cylinder equipped with a two-stage pressure regulator was connected to the top of the stirred cell to supply the desired pressure for the corresponding filtration experiments. The configuration scheme is presented in Figure 3 and schematic representation of the experimental procedure with samples obtained for each membrane (figure 4) are reported.

Figure. 3. Stirred cell rig for microfiltration and nanofiltration membranes

A magnetic stirrer attached to the surface of the membrane provided a constant agitation speed of 350 rpm. For the microfiltration experiment, the volume of the feeding solution was 250 mL, which was concentrated to 178.61 mL until reaching a final volume reduction factor (VRF) of 3.5 (the volume reduction factor is defined as the ratio between the volume between the initial feed volume and the final retentate volume (Defines as the fraction that is rejected due the dimension of the compounds being larger than the dimension of the pore of the membrane). For the nanofiltration experiment, the volume of the feeding solution ranged from 65 to 100 mL; this volume was concentrated to obtain a range from 46 to 76 mL.

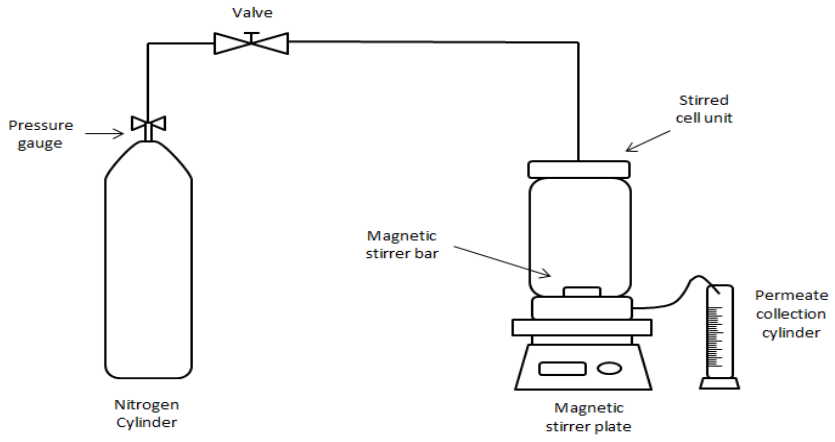

Figure 4. Schematic diagram of the stirred cell rig

3.2.3. Ultrafiltration experiments for Sangiovese wine lees (SRL)

3.2.3.1. Membrane, preparation and ultrafiltration process

Permeation experiment was carried out with a laboratory-made cellulose acetate prepared as reported by (Kunst & Sourirajan, 1974). The casting solution composition of the CA38 membrane is shown in table 4. All reagent grade chemicals were supplied by Merck (Hohenbrunn, Germany).

Table 4. Film casting conditions of membranes

Membrane	**CA38**
Casting solution (wt %)	
Cellulose acetate	17
Acetone	45
Formamide	38
Casting conditions	
Temperature of coagulation bath solution (°C)	0
Temperature of atmosphere (°C)	20-25
Solvent evaporation time (sec)	30
Gelation medium	Ice cold water (1-2 h)

The cellulose acetate (CA) membranes are prepared by the wet reverse phase. This technique requires the preparation of a polymeric solution of foundry, which after 24 hours of homogenization, is spread on a glass plate, then evaporation is undertaken for 30 seconds. The cast films are immersed in a pre-coagulation bath for about 5 minutes at 0 °C, then immersed in the coagulation bath for 2 hours at a temperature of 0 °C to obtain the final membrane films (Figure 5 and 6).

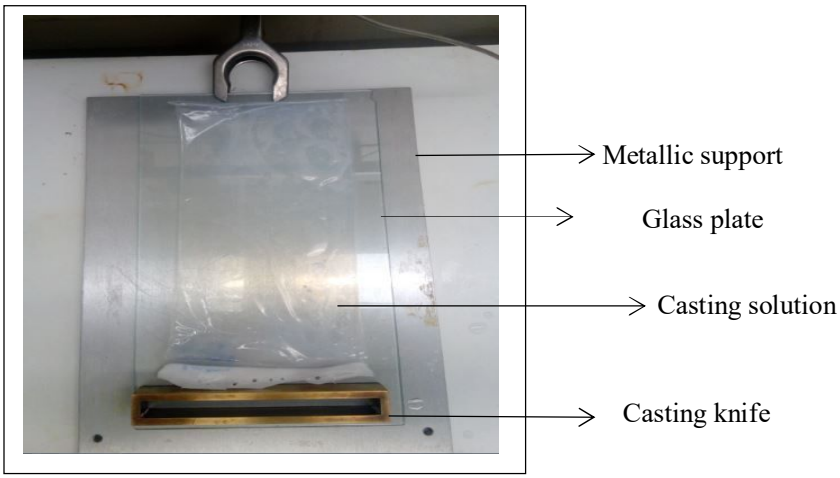

Figure. 5. Cellulose acetate spread over glass plate

Acetate cellulose membranes should be stored at 4 °C in a 10% (V/V) solution of ethanol to avoid contamination (Kunst & Sourirajan, 1974).

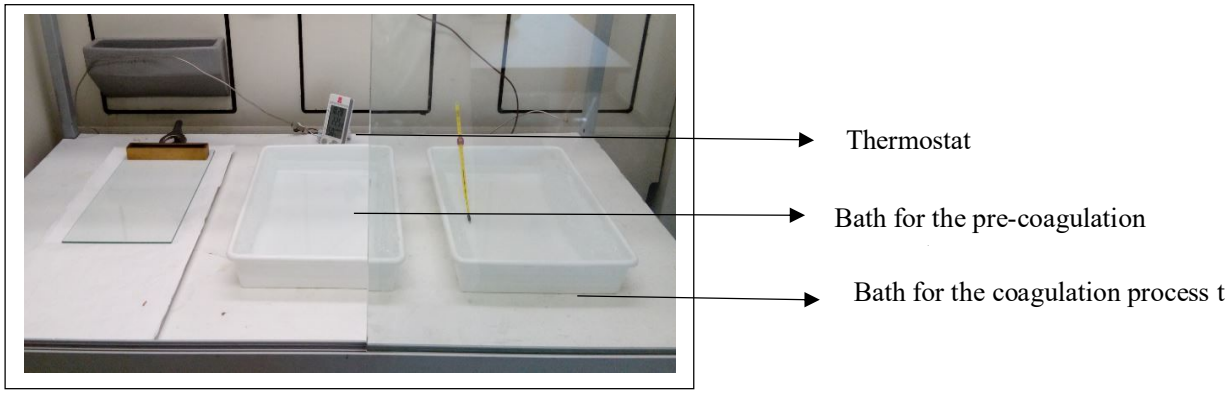

Figure 6. Coagulations baths for the preparation of the cellulose acetate membrane

The membrane MWCO was evaluated by means of permeation data quantified with solutions of polyethylene glycols and dextran of increasing molecular weight at a concentration of 600 ppm.

The apparent rejection of the solute (*f*), was evaluated in terms of the total organic carbon (TOC) through the following equation (1):

$$f = \left(\frac{C_{feed} - C_{permeate}}{C_{feed}}\right) \qquad (1)$$

Where C_{feed} and $C_{permeate}$ are the TOC content in the feed and in the permeate, respectively.

3.2.3.2. Experimental set-up and procedures

The permeation experiments were carried out in the laboratory filtration unit illustrated in Figure 7 and 8. The ultrafiltration system consists of: feed tank, pump, potentiometer, pressure gauge, heat exchanger, pressure regulating valve, permeate collector and concentrate collector. The feed tank has a maximum capacity of 500 mL and is covered by the heat exchanger which allows a control of the temperature of the recirculating solution (feed solution). The pump is installed with a potentiometer that together with the pressure regulation valve establishes the recirculation flow of the feeding solution.

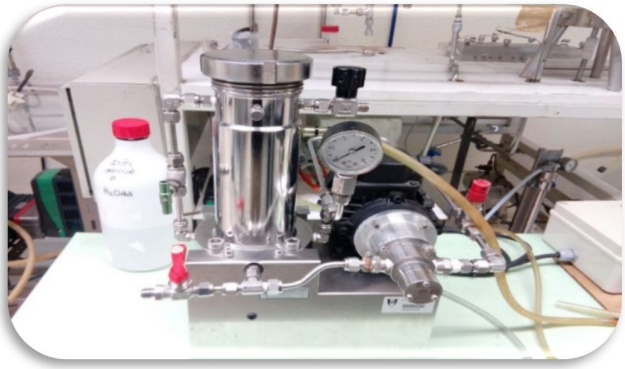

Figure 7. Ultrafiltration system - Celfa P – 28

The permeated solution is collected, while the solution that does not pass through the membrane (retentate fraction) can be either collected in the concentrate collector (if it is open) or recirculated back to the feed tank, (valve of concentrate collector closed) (Figure 7).

Before using the ultrafiltration system, it was necessary to calibrate it. For this purpose, the feed source was filled with water and the permeate cell was sealed.

A mass of water was collected in a certain time determined at different pressures and pumping powers to calculate the volumetric flow (cm^3/s). By correlating the volumetric flow with the power of the pump at each pressure, it was possible to determine the conditions required to obtain a certain volumetric flow. The calibration curves of the volumetric flow rates versus the power of the pump are provided in annex I.

Permeation experiments were performed in a flat sheet laboratory cell, with a membrane surface area of $2.5 \times 10^{-3} m^2$. The ultrafiltration process was performed in concentration mode, where the recirculation of the concentrate to the feeding tank and a continuous collection of the permeate was carried out. The initial volume of the feeding solution was 500 mL and the stabilization time for each experimental run was 30 minutes.

It should be noted that the rejection coefficients were calculated at 1 bar pressure. The sample collected from the permeate stream was taken for the study of the variation in the permeate flow and respective chemical analyzes. At the end of each experimental run, the membrane was washed with deionized water. Feeding samples were taken at the beginning and at the end of the experiment.

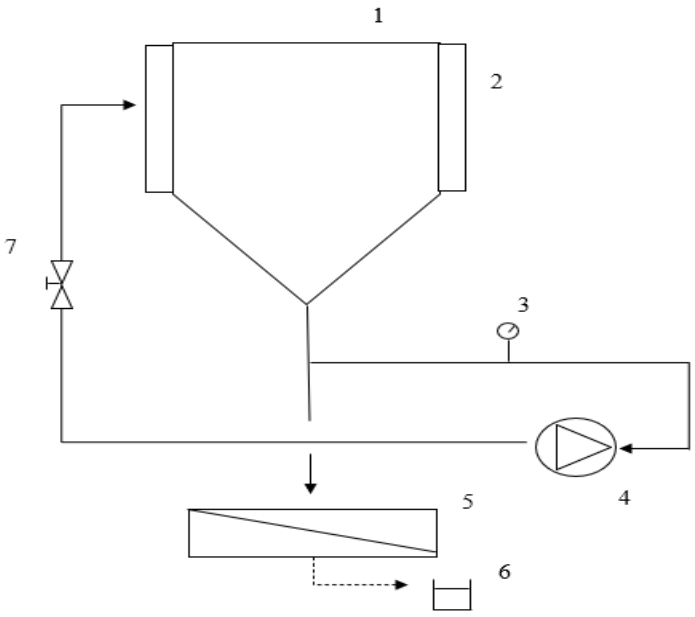

Figure 8. Schematic of lab-scale filtration unit 1. Feed tank, 2. Heat changer, 3. Manometer, 4. Pump, 5. Membrane, 6. Permeate, 7. Pressure regulation valve Δ

The membrane was placed in the membrane module and was subsequently characterized in terms of hydraulic permeability with the transmembrane pressure (TMP) of 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0, 2.25 and 2.5 bar with a flow rate of 0.55 L/min. The hydraulic permeability of the

membrane (Lp) was obtained as the slope of the straight line from that plot. Moreover, the membrane was characterized in terms of rejection coefficients to the following reference solutes: Polyethylene glycol of 1000, 2000 and 35000 Daltons and Dextran 40000 Dextran.

The experiments were carried out in batch concentration mode with a recirculation of the retentate into the feed tank. The operating conditions were the follows: temperature 25 ± 1° C; TMP: 2 bar. Approximately 500 mL of red wine lees was used in the UF experiment.

3.2.4. Ultrafiltration experiments for Sangiovese wine lees (SRL)

3.2.4.1. Membrane used, preparation and characterization in the ultrafiltration process

Permeation experiments were performed with three laboratory-made cellulose acetate membranes prepared as reported below, and one commercial membrane (NF90) supplied by DowFilmtec (USA). The nanofiltration system is presented in the Figure 9.

Three laboratory made membranes were performed according to the phase inversion method reported by (Kunst & Sourirajan, 1974). Table 5 presents the casting solution composition of the CA400-22, CA316-70 and CA316 membranes, and table 6 gives the characteristics of the NF90 commercial membrane. All reagent grade chemicals were supplied by Merck (Hohenbrunn, Germany).

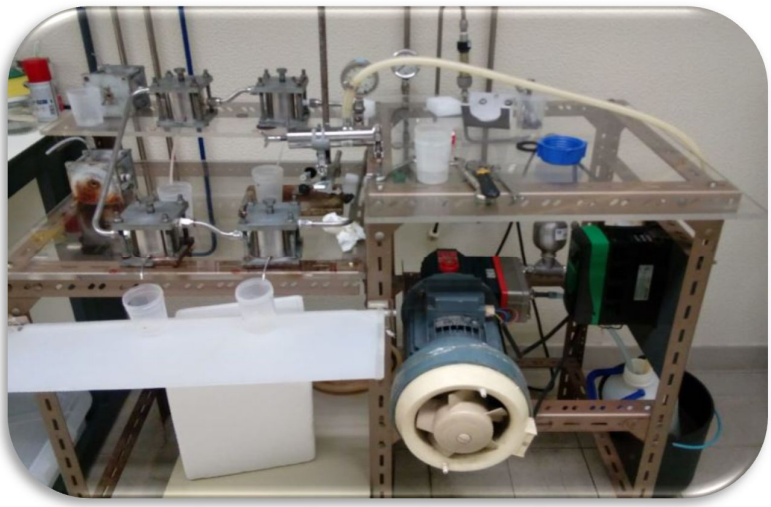

Figure.9. Nanofiltration system

Permeation experiments were performed in flat sheet laboratory cells with a membrane surface area of 13.2 x $10^{-4}m^2$ using three cellulose acetate membranes CA316-70, CA400-22 and CA316 manufactured in the laboratory and the NF90 commercial membrane supplied by DowFilmtec (USA).

Table 5. Film casting conditions of nanofiltration membranes

Membrane	**CA316**	**CA316-70**	**CA400-22**
Casting solution (wt %)			
Cellulose acetate 398	17	17	17
Acetone	69.2	69.2	61
Formamide			22
Magnesium perchlorate Mg $(ClO_4)_2$	1.45	1.45	
Casting conditions			
Temperature of coagulation bath solution (°C)	0	0	0
Temperature of atmosphere (°C)	20-25	20-25	20-25
Solvent evaporation time (min)	1	0.5	0.5
Gelation medium	ICV (1-2 h)	ICV (1-2 h)	ICV (1-2 h)
Annealing conditions			
Annealing time (min)		11	
Annealing temperature (°C)		70	

ICV: Ice cold water

The experiments were carried out in total recirculation mode, where the permeate and retentate streams were recirculated to the feed tank. The initial volume of the feed solution for the experiment was 5 L; the stabilization time for each experimental run was 30 minutes and the rejection coefficients were evaluated at the pressure of 20 bar. Samples of the permeate streams were taken from the four membranes cells to study the variation in the permeation fluxes and chemical analysis. Between each run, the membranes were washed with deionized water. Feed samples were taken at the beginning and at the end of the experiment.

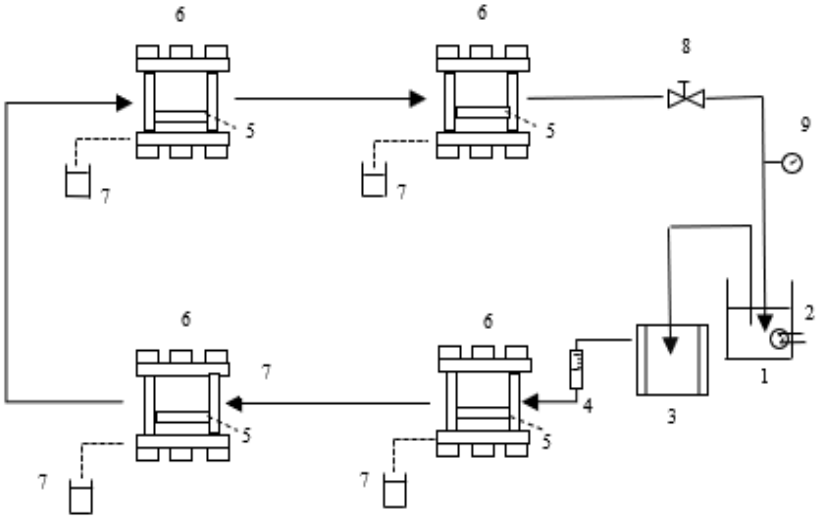

Figure.10. Nanofiltration system, 1.Feed tank, 2. Heat changer, 3. Pump, 4. Rotameter, 5. Membrane, 6. Membrane cell, 7. Permeate sample, 8. Pressure – regulation valve, 9. Manometer

Table 6. display the characteristics of the NF90 commercial membrane. The membranes were placed in the membrane cells shown in Figure 10 and were characterized in terms of hydraulic permeability (Lp), with a feed flow rate of 0.8 L/min at a transmembrane pressure (TMP) of 5, 10, 15 and 20 bar and in terms of rejection coefficients to reference solutes, salts and solvent which are: D – (+) glucose, raffinose, polyethylene glycol (PEG 1000 Da), sodium chloride, sodium sulphite and ethanol.

Table 6. Characteristics of selected sheet flat NF90 commercial membrane

Membrane type	**NF90**
Manufacturer	DowFilmtec
Membrane material	Polyamide - TFC
MWCO (Da)	200-400
Max. pressure (bar)	41
Max. temp. (°C)	35
pH operating range	2-11

3.2.4.2. Membrane compaction

The membrane compaction was carried out on all the membranes belonging to the UF process of Sangiovese wine lees and the NF process of Cabernet Sauvignon wine lees.

The compaction process allowed the adaption of the membrane to the module space, without altering its structure.

The membrane adaptation process is performed by recirculating water in the filtration system at a pressure that is 20 percent higher than the maximum operating pressure.

$$P_{Compactation} = 20\% * P^{max}_{operating} \quad (3)$$

The operation pressure for the compaction in the UF process on the Sangiovese wine lees was of 3 bar while for the NF process on the Cabernet Sauvignon wine lees was of 35 bar. The operation time for each process was around 2-3 hour.

3.2.4.3. Membrane parameters

The following membrane parameters were used for the UF process on Sangiovese wine lees and NF process on Cabernet Sauvignon wine lees except for the MF and NF process on the mixed red wine lees, which carried out all the parameters plus the volume reduction factor (VRF) parameter.

The VRF was defined as the radius between the initial feeding volume and the resulting retentate volume according to the following equation (2):

$$VRF = \frac{V_f}{V_r} \quad (2)$$

where V_f and V_r are the feed and retentate volumes, respectively.

The apparent rejection coefficient of each solute was obtained by the follow equation (3):

$$f = \left(\frac{C_f - C_p}{C_f}\right) \quad (3)$$

Where C_b and C_p are the solute concentrations in the bulk feed solution and permeate solution, respectively.

The permeate flux (Jp), which is expressed as L/m^{2}h, was calculated by measuring the collected permeate mass at established time intervals using a digital scale as follows (4):

$$J_p = \frac{\mathrm{W_p}}{A * \mathrm{t}} \qquad (4)$$

where W_p is the mass of permeate collected during the established operating time (t) and A is the surface of the membrane.

The rejection of the selected membranes towards specific compounds was calculated with the following formula:

$$R\ (\%) = \left(1 - \frac{C_p}{C_f}\right) * 100 \qquad (5)$$

where $C_p$and C_f are the measurements of the concentration of specific compound in the permeate and feed streams, respectively.

In order to determine the fouling index (FI) of the investigated membranes the follow equation (6) was applied, by measuring the hydraulic permeability (Lp) before and after the nanofiltration processes:

$$FI\ (\%) = \left(1 - \frac{Q_{f1}}{Q_{f0}}\right) * 100 \qquad (6)$$

Where the Q_{f0} and Q_{f1} are the pure water permeability before and after the nanofiltration processes, respectively.

After the filtration process, the membranes were washed with deionized water for 30 minutes and their permeability with deionized water was measured. The cleaning efficiency (CE) was evaluated according to the following equation (7):

$$\mathrm{CE}\ (\%) = \left(\frac{Q_{f2}}{Q_{f0}}\right) * 100 \qquad (7)$$

Where the Q_{f2} is the water permeability after the cleaning process and Q_{f0} is the water permeability before the nanofiltration processes with the grape pomace.

3.2.5. Analytical evaluations

Feed (F) and the permeate (P) solutions of solutes, salts and solvent were analyzed for the following parameters: Total organic carbon (TOC, TOC-LCPH Shimadzu carbon analyser, (Duisburg, Germany) conductivity ($\mu S\ cm^{-1}$ at 25 °C, Crison 525 conductivity mete (Barcelona, Spain).

Feed (F) permeate (P) and retentate (R) samples obtained from the nanofiltration experiment were immediately refrigerated and kept at 4 °C until analysed. Grape pomace samples were analysed in terms of total phenols, proanthocyanidins content, antioxidant activity, sugars (glucose and fructose), pH and turbidity.

Total phenols were quantified by means of the Folin-Ciocalteu method (TP-FC) using a calibration curve with gallic acid as standard and expressed in milligrams of gallic acid equivalent per gram of dry matter (mg GAE/100g). The TP-FC method (Ribéreau-Gayon et al., 1976) is based on the redox reaction of phenolic compounds with a mixture of phosphotugnstic ($H_3PW0_{12}0_{40}$) and phosphomolybdic ($H_3PM0_{12}0_{40}$) acids in an alkaline medium to create a blue complex. The reaction was performed by mixing a 0.250 mL sample aliquot, 0.5 mL of Folin-Coicalteu reagent (diluted 1:4) and 1.75 mL of a 15% sodium carbonate solution. After 1 hour, the absorbance was read using an UV-vis spectrophotometer (UV-1700 Shimadzu) at a wavelength of 725 nm.

The quantification of proanthocyanidins (mg/L) was carried out by means of a spectrophotometric assay with the 4-dimethyl-amino cinnamaldehyde reagent (DMAC) according to literature (Y. Wang et al., 2016).

For the analysis of sugars, d-glucose and d-fructose (mg/L) were determined by the enzymatic assay (Megazyme, Chicago, USA). The analysis is based on the sequential reactions that begin after the addition of hexokinases (HK) and phosphoglucose isomerase (PGI), for the determination of glucose and fructose, respectively. The amount of NADPH formed throughout the reactions, which is measured by increasing the absorbance at a wavelength of 340 nm, is stoichiometric with the amount of d-glucose and d-fructose present in the sample.

The pH value was measured with a HANNA 209 pH meter (Merck, Germany); which was calibrated with buffer solutions of pH 4.0 and 7.0.

Turbidity was measured using a compact Aqualytic® infrared turbidity meter (AL250T-IR, Germany) with a measurement range of 0.01 to 1110 nephelometric turbidity units expressed in NTU and a detection limit of 0.01 NTU.

The antioxidant activity was measured using the method reported by (Re et al., 1999) which is based on the ability of antioxidants to interact with the ABTS radical, decreasing its absorbance to 734 nm. Briefly, a radical solution was prepared, which is based on 7 mM ABTS (2,2'-azino-bis-(-3-ethylbenzothiazoline-6-sulfonic acid)) and 2.45 mM sodium persulfate, then solution was left in the dark at room temperature for 16 hours before use. Subsequently, the ABTS stock solution was added in acetate buffer to an absorbance range of 0.68 to 0.70 at a wavelength of 734 nm. For analysis, 2.9 mL of the ABTS reagent solution was mixed with 0.1 mL microliters of the sample and the blank was prepared by adding 2.9 mL ABTS reagent solution to 0.1 mL distilled water. After 300 minutes the absorbance was read by using an UV-vis spectrophotometer (Agilent cary 60) at 734 nm.

Antioxidant activity (AA) was calculated as the percentage of inhibition of absorbance according to the follow equation (8):

$$AA\ (\%) = \left(\frac{R_0 - R_1}{R_0}\right) * 100 \qquad (8)$$

Where R_0 is the absorbance value of the blank and R_1 is the absorbance of the extract sample.

Results have been reported as percentage of the radical scavenging of the sample.

Another method used to verify the antioxidant activity reported by (Brand-Williams et al., 1995) is by using the stable DPPH radical (2,2-diphenil-1-picridazil) characterized by an intense purple red color, which bleaches when reduced in the presence of a molecule capable of neutralizing by transfer of proton or electron. By spectrophotometric measurement at 517 nm of the change in absorbance of the DPPH solution in the presence of an antioxidant it is possible to quantify the

sample's ability to remove free radicals. The result is expressed as DPPH IC50 which represents the quantity of antioxidant capable of reducing 50% of the initial concentration of DPPH. In brief, the DPPH reagent was dissolved in pure methanol, after which it was brought to an absorbance of 0.7 (± 0.010). The solution was kept refrigerated at 4 °C before use. For analysis, 2.9 mL of the DPPH solution was mixed with 0.1 mL of the sample and the blank was prepared by adding 2.9 mL DPPH solution to 0.1 mL distilled water. Subsequently, the solution was stirred and kept in the dark at room temperature. After 1 hour it was read on a spectrophotometer (Agilent cary 60, Santa Clara, CA, USA) at a wavelength of 517 nm against methanol. The result was expressed in terms of percentage of radical removal according to the following equation (9):

$$R = \left(\frac{B_1 - B}{B_1}\right) * 100 \qquad (9)$$

where $_{B1\ is}$ the absorbance of the radical in the absence of the antioxidant and B_2 is the absorbance after adding the antioxidant.

The total polysaccharides are measured by the phenol sulfuric method (Dubois et al., 1956). (Segarra et al., 1995) proved the applicability of this method in polysaccharide solutions of wines obtained by using the ethanol precipitation, as described above.

To 400 uL of the sample, 400 uL of a phenol solution (5 wt%) and 2 ml of concentrated sulfuric acid (98 wt%) are added and then stirred. The reaction between the sugars and the phenol in the acid medium, leads to the formation of brownish-yellow compounds. After 40 minutes the absorbance was read in a spectrophotometer at a wavelength of 495 nm.

The determination of the proteins was carried out by isolating the polysaccharides through the HCL-ethanol solution and subsequently they were centrifuged according to Guadalupe et al. (2007). Then, the separation of mannoproteins was carried out according to the method described in resolution OENO 26/2004.

Finally, the mannoproteins were quantified by the enzymatic assay (Megazyme, Chicago, USA).

3.2.5.1. Phenolic compounds by HPLC for the red mixed wine lees

HPLC quantitative analyses of target antioxidant phenolics were performed on feed, permeate and retentate samples after microfiltration and nanofiltration treatment.

Feed, permeate and retentate were previously filtered with a capsule nylon filter of 0.45 μm in order to remove suspended particles. HPLC analyses of phenolic compounds were performed using a HPLC system equipped with a GP50 pump and a PDA-100 detector. Analyses were carried out with a C18 column (250 mm X4.6 I.D, 5 μm particle size) (InertSustain). The solvent system used was a gradient of eluent A (5% acetic acid) and eluent B (80% acetonitrile). The elution of the gradients was carried out in the following manner: 0 to 30 min, linear gradient from 0% to 5% of the B eluent; 30 to 50 min, linear gradient from 5% to 10% of eluent B; 50 to 70 min, linear gradient from 10% to 11% of eluent B; 70 to 82 min, linear gradient from 11% to 15% of eluent B; 82 to 95 min, linear gradient from 15% to 60% of eluent B; in an additional 14 minutes, the column was reconditioned with the initial eluent. The flow rate of the eluents was 0.5 mL/min.

3.2.5.2. Phenolic compounds by HPLC for Sangiovese and Cabernet Sauvignon wine lees

The monomeric phenolic fraction and anthocyanins were analyzed using Agilent 1290 Infinity LC System with ultraviolet/visible (UV/VIS) Diode Array detection (DAD) (Agilent, Palo Alto, CA, USA). The monomeric phenolic fraction were analyzed using the follow mobile phases: Acetic acid (5%) and distilled water (defined as solvent A) and acetonitrile (80%) and distilled water (defined as solvent B). The anthocyanins were analyzed using the following mobile phases: water/methanol (70/30; v/v) containing 6 mL / L of 70% perchloric acid (defined as solvent A) and water/methanol (25/75; v/v) containing 6 mL / L of 70% perchloric acid (defined as solvent B). The flow rate is 0.9 mL/min. Anthocyanins were recorded at 530 nm and the proportions of solvent B is 0 minutes, 0%; 23 minutes, 25%; 51 minutes, 70%; 60 minutes, 100%; 65 minutes, 0%; according to the methods described by (Ivanova-Petropulos et al., 2015). Results were expressed using calibration curves of the reference standard polyphenolic compounds. Standard polyphenolic

monomers and malvidin-3-O-glucosyde used for HPLC calibration were purchased from Extrasynthese (Genay, France).

3.2.5.3. Total Organic Carbon (TOC)

The organic solutes concentrations of MWCO samples were determined in terms of total organic content (TOC), using a Total Organic Carbon Analyzer (Shimadzu, TOC-V CSH, Duisburg, Germany). The Cb (Water sample) and Cp (Sample to analyze) were acidified with hydrochloric acid 2M to a pH lower than 2 for 10s and sparge gas was bubbled, to purge the volatile inorganic carbon. After that, the samples were introduced into the combustion tube, which was filled with an oxidation catalyst and heated to 680°C. As a result, the components in the sample were converted into carbon dioxide. The sample was then carried to a non-dispersive infrared (NDIR) gas analyzer, where the carbon dioxide was measured as a peak. To relate the peak area with organic carbon concentration, a calibration curve of TOC content in different solution of potassium hydrogen phthalate in function of the area of the peaks detected was acquired. Figure 11 shows the Total Organic Carbon Analyzer.

The TOC content of each sample was obtained by the difference between the TOC content of the sample and their blank. The blank solution consisted of the deionized water used in the preparation of the dextrans solutions.

Figure 11. Total Organic Carbon Analyzer TOC-V CSH

3.2.6. Statistical analysis

Data are presented as the mean values ± standard deviation (SD) obtained from three replicate analyzes. ANOVA one-way analysis of variance with significance $\rho \leq 0.05$ was performed and Tukey's HSD post-hoc test was carried out using Minitab ® 17.1.0 statistical software (Minitab, Ltd. United Kingdom).

3.3. Results and Discussion

3.3.1. Microfiltration and nanofiltration experiments for the mixed red wine lees (MRL)

3.3.1.1. Chemical composition of red wine lees (MRL)

Table 7 shows the physicochemical composition of the red wine lees extract obtained during the microwave process (Section 2.2.3; Chapter 2) and introduced into the membrane system.

The pH is around 2.0, which is due to the hydrochloric acid used in the microwave extraction process. The total polyphenol content of the extraction, at a wavelength of 750 nm, resulted in 933 mg GAE/L; while when it was analyzed at wavelength of 280 nm it presented a higher result of 1939 mg GAE/L.

Table.7. Physicochemical characteristics (mean ±SD) of red wine lees extract

Parameter	Value
pH	2.0 ± 0.1
Turbidity (NTU)	218.0 ± 1.4
Total phenols at 280 nm (mg gallic acid/L)	1938.7 ± 13.3
Total phenols FC (mg gallic acid/L)	933.2 ± 9.4
Glucose (mg/L)	580.0 ± 11.6
Fructose (mg/L)	30.0 ± 0.6
Proanthocyanidins (mg catechin/L)	6.9 ± 0.3
Flavanol catechin derivative (mg/L)	183.9 ± 3.6
Gallic acid (mg/L)	18.1 ± 0.3
(+)–Catechin (mg/L)	3.1 ± 0.1
Syringic acid (mg/L)	36.1 ± 0.7
Gallocatechin derivate (mg/L)	20.7 ± 0.4
Polymeric phenolics (mg/L)	235.4 ± 4.7

This can be explained by assuming that after fermentation the wine lees have a high protein content, which are protein residues, and these could be detected at 280 nm causing interference and overestimations. On the other hand, five phenolic compounds were identified and reported, which are derived from flavanol catechin, (+) - catechin, gallic acid, syringic acid and derivative of gallocatechin. Similarly, gallic acid and (+) - catechin were detected by LC-MS in wine lees after microwave-assisted solid-liquid extraction (Delgado De La Torre et al., 2015b).

The extract contained 610 mg/L of total sugars (glucose and fructose) which, when compared with another previous study, show a disparity. In fact, (Galanakis et al., 2013) reported a concentration of 1065 mg/L and 3910 mg/L in diluted hydroethanol extracts and concentrated cellar sludge, respectively. However, the sugar concentration in lees can have great variability, because it depends on the vinification process adopted and as a consequence, on the residual non-fermented sugars.

3.3.1.2. Analysis of permeate flux and membrane fouling

Figure 12 shows the temporal evolution of the permeate flux and VCF in the clarification of the wine lees extract with the MF membrane.

The initial permeate flux of approximately 12 L/m^2h gradually declined to a steady state value of approximately 2.5 L/m^2h. In particular, the permeate flow behavior over time is characterized by a rapid decrease in the permeate flow in the first 50 min of the process, which represents a 50% reduction in relation to the initial permeate flow, followed from a second period, until it reaches a VCF of 1.7, corresponding to a smaller decrease in the permeate flow and a third period characterized by a small decrease in the permeate flow to a steady state value.

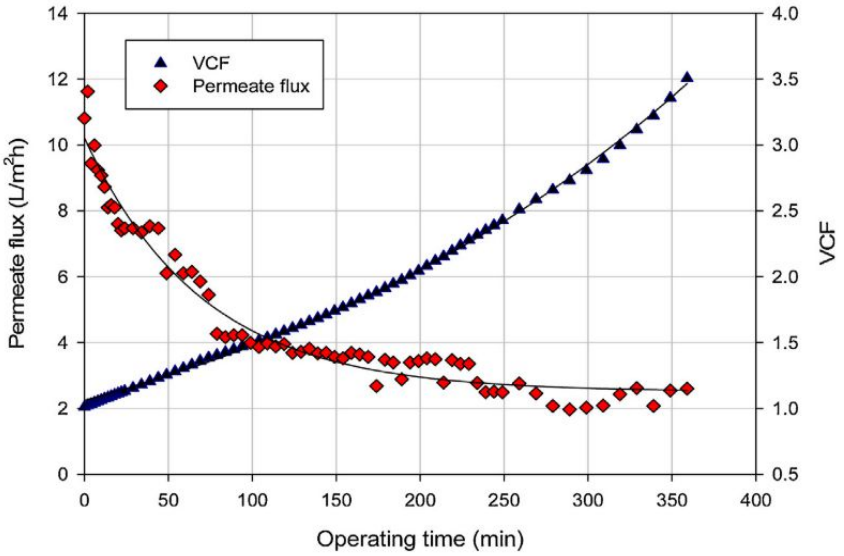

Figure12. Microfiltration of red wine lees. Time evolution of permeate flux and VCF.

This behavior can be attributed to different phenomena including polarization of the concentration on the membrane surface, fouling of the membrane and an increase in the concentration of solutes in the retentate stream. In fact, as the feed concentration increases, the concentration polarization becomes more severe. Higher volumes of solutes are directed to the surface of the membrane, resulting in a thicker cake layer. Similar trends have been reported in the clarification of fruit juices (A. Cassano et al., 2007) and plant extracts (Chhaya et al., 2012) using UF membranes.

The clarified extract of mixed red wine lees (MRL) was processed with three selected membranes and the permeate flux gradually decreased, increasing the VCF until reaching a stable value (Figure 13). A strong correlation was seen between the permeate flux and the MWCO of the selected membranes. In particular, the ETNA 01PP membrane, with the highest MWCO, presented a higher permeate flux compared to the other two membranes. The initial permeate flow of approximately 42.5 L/m^{2}h decreased by approximately 50% when the final VCF was reached.

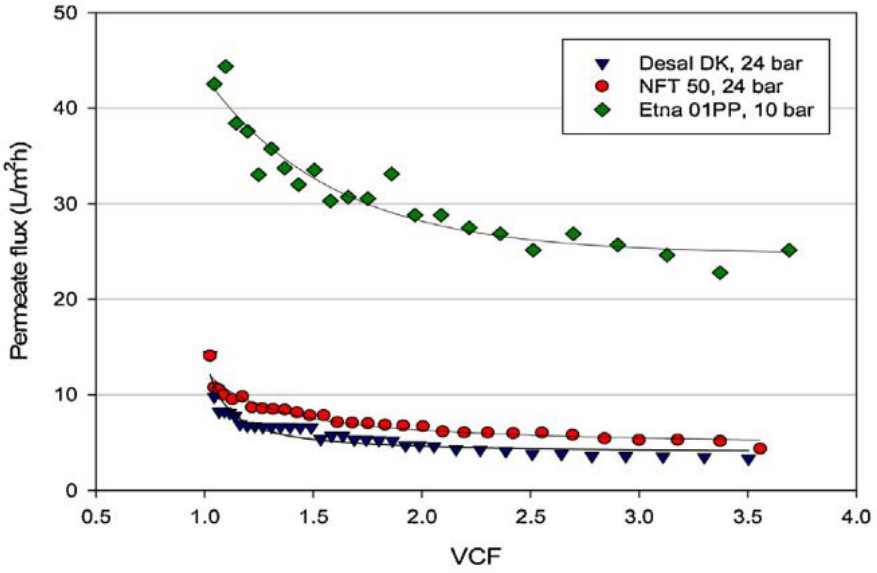

Figure 13. Nanofiltration of microfiltered red wine lees. Permeate flux as a function of VCF.

The Desal DK and NFT50 membranes both presented a similar productivity with an initial permeate flux of 14 kg/m^{2}h and steady state values of 3.5 and 5 kg/m^{2}h, respectively.

The observed differences in flow results can be explained by assuming small differences in hydrophilicity when these membranes are exposed to water and ethanol solution. In fact, according to the data from the contact angle measurements reported in the literature (Petrinić et al., 2007), the NFT-50 membrane is characterized by greater hydrophilicity, which has a contact angle of 21 ± 0.9, compared to the Desal DK with a contact angle of 28.1 ± 4.8.

Both membranes contain polar amide groups in their polymeric structure which can interact with molar solvents through hydrogen bonds (Vieira et al., 2018).

The fouling index of the selected membranes was calculated by the water permeability of the membranes and the measurement after treatment of the clarified extract.

According to the measurements of this parameter, the NFT50 membrane showed the highest fouling index with 32.1%, followed by the ETNA 01PP and Desal DK membranes with 18.4% and 15.5%, respectively. Even so, it should be emphasized that the reduction of the permeate number as well as the fouling number of nanofiltration (NF) membranes are minimized due to the preliminary

clarification step. It should also be noted that the MF membrane eliminated most of the initial turbidity of the feed solution with a removal of around 77%, producing a transparent permeate in which glucose and most of the phenolic compounds were recovered, as reported in table 8.

Table.8. Hydraulic permeability values and fouling index of the membranes on processing of extract of red wine lees

	Type of Membrane			
	FSM0 15PP	ETNA01 PP	Desal DK	NTF50
L_{w0} (L/m^2h)	37.3	22.3	4.5	13.4
L_{w1} (L/m^2h)	18.6	18.2	3.8	9.1
Fouling index (%)	50.1	18.4	15.5	32.1

The fouling index of the MF membrane was approximately 50% as a result of the larger pore size of the membrane compared to those of the NF membrane, which consequently leads to a greater blockage of pores (Benítez et al., 2006; Hwang et al., 2008).

3.3.1.3. Membrane selectivity analysis – Red wine lees extract microfiltration

The physicochemical composition of the feed, permeate and retentate streams obtained in the MF from the extract of red wine lees is reported in table 9.

The pH analysis of the permeate and retentate samples obtained in the MF of the extract of red wine lees presented minimal changes with respect to the feed solution, which can be attributed to the variability of the sample measurement. On the other hand, a removal of the turbidity of the extract from 218 to 55.1 NTU was evidenced. Therefore, it was deduced that the extract is characterized by a high concentration of high molecular weight particles that are retained by the MF membrane, forming an encrusting layer on the surface of the membrane (Ripperger & Altmann, 2002).

According to the data in the Table 9, most of the phenolic compounds and sugars are recovered in the clarified extract.

Table.9. Physico-chemical characteristics of feed, permeate and retentate samples (mean ±SD) obtained in the clarification of red wine lees extract with the MF membrane

Parameter	Feed	Permeate	Retentate
pH	2.0 ± 0.1^{A}	1.86 ± 0.1^{B}	1.94 ± 0.1^{AB}
Turbidity (NTU)	218.0 ± 1.4^{B}	55.1 ± 1.3^{C}	416.0 ± 2.8^{B}
Total polyphenol at 280 nm (mg gallic acid/L)	1938.7 ± 13.3^{B}	1492.1 ± 6.5^{C}	3116.6 ± 171.0^{A}
Total polyphenol FC (mg gallic acid/L)	933.2 ± 9.4^{B}	857.0 ± 14.9^{B}	4662.5 ± 224.8^{B}
Glucose (mg/L)	580.0 ± 11.6^{A}	570.0 ± 11.4^{A}	-
Fructose (mg/L)	30.0 ± 0.6^{A}	28.0 ± 0.5^{B}	-
Proanthocyanidins (mg catechin/L)	6.9 ± 0.3^{B}	3.9 ± 0.6^{B}	33.5 ± 1.2^{A}
Flavanol catechin derivative (mg/L)	183.9 ± 3.6^{A}	144.8 ± 2.9^{B}	45.1 ± 0.9^{C}
Gallic acid (mg/L)	18.1 ± 0.3^{A}	16.5 ± 0.3^{B}	14.2 ± 0.3^{C}
(+)–Catechin (mg/L)	3.1 ± 0.1^{AB}	2.7 ± 0.1^{B}	3.4 ± 0.1^{B}
Syringic acid (mg/L)	36.1 ± 0.7^{B}	34.8 ± 0.7^{B}	41.2 ± 0.8^{B}
Gallocatechin derivate (mg/L)	20.7 ± 0.4^{B}	20.4 ± 0.4^{B}	23.8 ± 0.4^{B}
Polymeric phenolics (mg/L)	235.4 ± 4.7^{B}	214.6 ± 4.3^{C}	1854.1 ± 37.1^{A}

Different superscript letter in properties are significantly different according to the Tukey's HSD test ($p \leq 0.05$).

The retention of the MF membrane towards specific compounds is illustrated in Figure 14.

Despite the high fouling index of the MF membrane of 50.1%, the retention of most phenolic compounds including gallic acid, (+) - catechin and the derivative of gallocatechin, is less than 10%. This can be attributed to the large pore diameter of the MF membrane that allows the diffusion of low molecular weight compounds (290.3 Da and 170.1 Da for (+) - catechin and gallic acid, respectively.

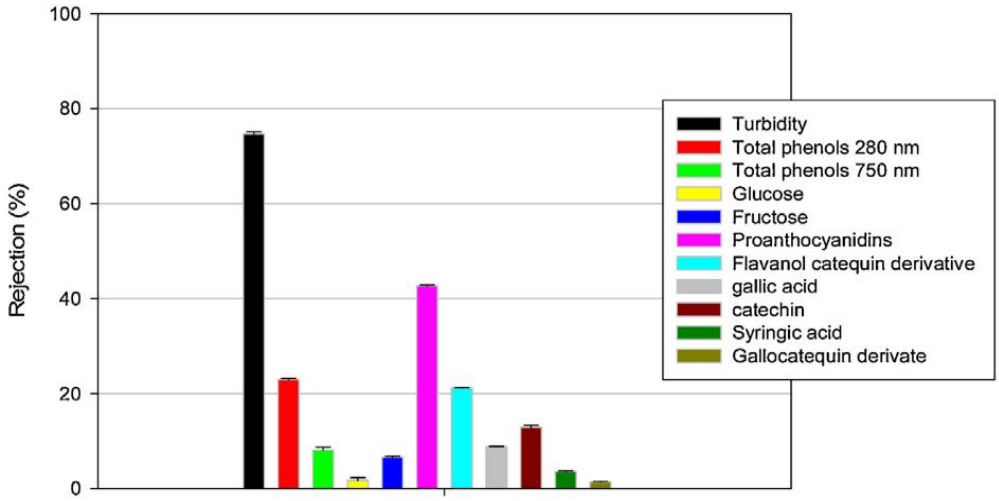

Fig.14. Rejection of the MF membrane towards specific compounds of red wine lees

There was a low retention in polyphenolic compounds at 280 nm (23% reduction) which was much lower presented by the authors (Giacobbo et al., 2015). They reported a 78% and 38% reduction of polyphenols in the microfiltration process (using a sheet flat PVDF with a pore size of 0.2 μm) of red wine lees with pre-filter vacuum treatment and without filtration treatment, respectively.

Similarly, PVDF hollow fiber membranes with a pore size of 0.13 μm showed a low retention of phenolic compounds (3.1%) in the clarification of cattle juice (Galiano et al., 2016). The retention of the MF membrane towards proanthocyanidins was approximately 42%. This phenomenon can be attributed to the interaction with other types of specific compounds of the treated extract or compounds that cause the fouling phenomena.

3.3.1.4. Analysis of the selectivity of the NF membranes on the clarified extract

The physical-chemical characterization of the permeate samples produced in the treatment of the clarified extract with UF and NF membranes is reported in table 10.

Figure 15 shows the rejection coefficients for different compounds for the selected membranes.

The rejection trend towards the detected compounds was seen as follows: ETNA 01PP> Desalination DK> NTF50.

Therefore, the rejection of selected membranes towards specific compounds was strongly correlated with their MWCO. All the selected nanofiltration membranes made it possible to further reduce the turbidity of the clarified extract. The DK and NFT50 membranes removed about 80% with respect to turbidity, while with the ETNA 01PP the removal was approximately 50%.

Table.10. Physico-chemical characteristics of feed and permeate (mean ±SD) samples obtained in the treatment of clarified red wine lees extract with NF membranes

Parameter	Feed	Permeate ETNA01 PP	Permeate Desal DK	Permeate NFT50
pH	1.86 ± 0.1^{B}	1.77 ± 0.1^{C}	1.94 ± 0.1^{A}	1.88 ± 0.1^{B}
Turbidity (NTU)	55.1 ± 1.3^{A}	27.3 ± 1.9^{B}	12.1 ± 0.6^{C}	10.3 ± 0.5^{C}
Total polyphenol at 280 nm (mg gallic acid/L)	1492.1 ± 6.5^{A}	1398.3 ± 51.0^{A}	1107.9 ± 39.5^{B}	768.8 ± 39.3^{C}
Total polyphenol FC (mg gallic acid/L)	857.0 ± 14.9^{A}	782.2 ± 30.5^{A}	517.6 ± 24.5^{B}	380.9 ± 13.1^{C}
Glucose (mg/L)	570.0 ± 11.4^{A}	470.0 ± 9.4^{B}	340.0 ± 6.8^{C}	280.0 ± 5.6^{D}
Fructose (mg/L)	28.0 ± 0.5^{A}	23.0 ± 0.4^{B}	16.7 ± 0.3^{C}	13.7 ± 0.2^{D}
Proanthocyanidins (mg catechin/L)	3.9 ± 0.6^{A}	2.8 ± 0.1^{AB}	2.1 ± 0.1^{B}	n.d.
Flavanol catechin derivative (mg/L)	144.8 ± 2.9^{A}	118 ± 2.3^{B}	99.8 ± 2.0^{C}	84.0 ± 1.7^{D}
Gallic acid (mg/L)	16.5 ± 0.3^{A}	13.4 ± 0.4^{B}	7.0 ± 0.1^{C}	4.7 ± 0.1^{D}
(+) – Catechin (mg/L)	2.7 ± 0.1^{A}	2.0 ± 0.03^{B}	1.8 ± 0.04^{B}	0.4 ± 0.01^{C}
Syringic acid (mg/L)	34.8 ± 0.7^{A}	27.6 ± 0.5^{B}	19.6 ± 0.4^{C}	9.9 ± 0.2^{D}
Gallocatechin derivate (mg/L)	20.4 ± 0.4^{A}	18.3 ± 0.3^{B}	11.1 ± 0.2^{C}	5.6 ± 0.1^{D}
Polymeric phenolics (mg/L)	214.6 ± 4.3^{A}	193.0 ± 3.8^{B}	171.6 ± 3.4^{C}	123.8 ± 2.5^{D}

Different superscript letter in properties are significantly different according to the Tukey's HSD test ($p \leq 0.05$).

The ETNA 01PP membrane also presented the lowest rejection towards phenols at 280 nm and 750 nm was 6.3% and 7.7%, respectively; and for glucose and fructose the rejection was 17.5% and 17.9%, respectively.

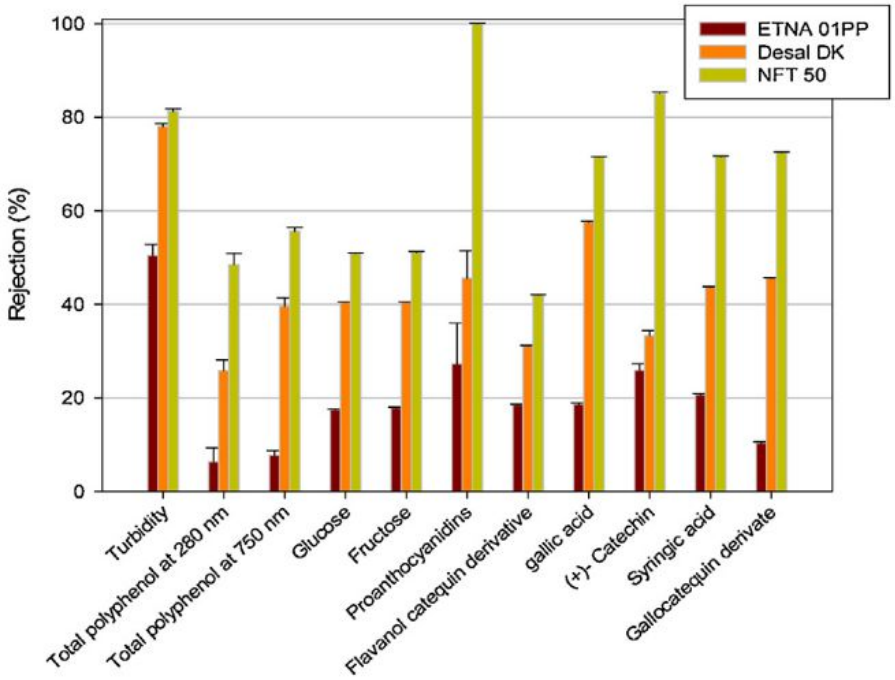

Figure 15. Rejection of selected membranes towards specific compounds of clarified red wine lees extract

On the other hand, the retention towards free polyphenols of low molecular weight such as (+) - catechin, syringic acid, gallo catechine and flavonol catechin derivatives, was less than 25%.

Similar values were found in the treatment of clarified wastewater from oil mills using the same membrane (Cassano et al., 2013).

On the other hand, the DK membrane, with a membrane pore cut-off of 150-300 Da, presented a retention index higher than 30% for most of the phenolic compounds (Flavanolo catechin, (+) - catechin, syringic acid and derived gallic catechin derivated). The NFT50 membrane exhibited the highest retention towards all compounds. However, for all phenolic compounds detected by HPLC, the rejection was greater than 70% (excluding the flavonol derivative catechin with a rejection of 42%).

The proanthocyanidins were completely retained by NFT50 membrane. Concerning the literature, a similar retention was found for phenolic compounds in the treatment of the aqueous extract of mate (Ilex paraguariensis A. St. Hil) using a polyamide membrane with the same MWCO (150-300 Da) in a spiral-wound configuration (HL2521TF, Osmonics) (Negrão Murakami et al., 2011).

For these compounds, the observed rejection of the NFT50 membrane can be attributed, in addition to the steric hindrance, to the adsorption phenomena that could be governed by various phenomena such as polar interactions, van der waals-type interactions and electron donor-acceptor interactions (Ulbricht et al., 2009).

3.3.2. Ultrafiltration experiments for Sangiovese wine lees (SRL)

3.3.2.1. Membrane characterization

In this experiment test, conditions were as follow: feed solute concentration: 600 ppm; temperature: 25 ± 1 °C; feed flowrate: 0.55 L/min; pressure: 1 bar. The UF membrane showed that the rejection percentage was related to the increasing molecular weight of the reference solutes (table 11).

Table 11. Rejections of UF membrane to specific molecular weights

Membrane type	PEG 10000 (%)	PEG 20000 (%)	PEG 35000 (%)	Dextran 40000 (%)
CA38	94	91.6	99.5	98.8

The pure water permeation permeate flux was measured at different transmembrane pressures from 0.25 to 2.25 bar. The permeate flux was plotted as a function of ΔP and the membrane hydraulic permeability was obtained as the slope of the straight line from that plot. The curve-fitting of plot log (f/(1-f)) as a function of the solute molecular weight was intersected by the 99% rejection line and yielded the molecular weight cut-off of 35000 Da to the CA38 membrane (Figure 16).

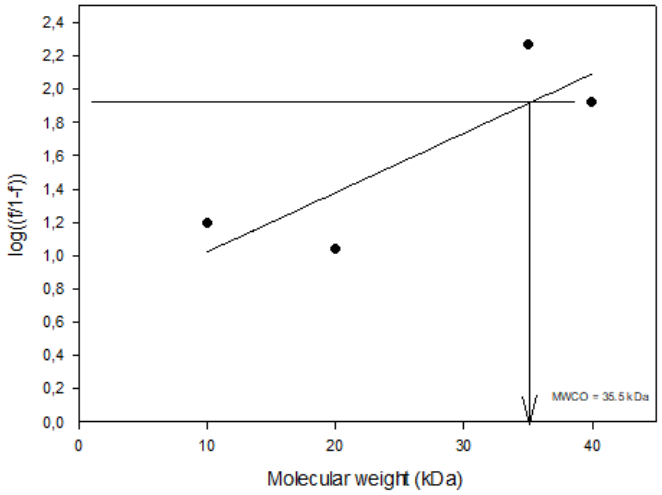

Figure. 16. Molecular weight nominal cut-off (MWCO) determination for the CA38 membrane

3.3.2.2. Chemical composition of Sangiovese red wine lees

Table 12 shows the physicochemical composition of the Sangiovese red wine lees. The pH value detected in this experiment (pH = 3.7) is at the limit of the acidity values normally detected in wine lees (3.8 < pH < 6.8). However, the pH value will depend on the wine production process (Bustamante et al., 2008). In our experiment the total polyphenols content was 655.4 mg GAE/L; this result is in agreement with studies carried out by other researchers in wine lees of different varieties with value range 400-1000 mg GAE/L (Giacobbo et al., 2015; Lužar et al., 2016). The

wine lees contained 358.2 of polysaccharides expressed in mg glucose/L. A different result was obtained by (Giacobbo, Bernardes, et al., 2013) reporting 49.8 mg glucose/L in the second racking from Syrah wine lees.

The catechin content evidenced in the Sangiovese lees in this experiment was 14.8 mg EC/L A similar result of 6.9 mg EC/L was found in the mixed red wine lees (MRL) (See section 3.3.1.1; Chapter 3)

The concentration of mannoproteins in Sangiovese wine lees was 0.39 g/L. The production and release of mannoproteins during wine fermentation depends on the yeast strain (Vidal et al., 2003).

The Sangiovese wine lees contained 0.718 mg/L of total sugars, including glucose and fructose. (Galanakis et al., 2013) obtained significantly different results, which reported total sugar concentrations of 1.065 mg/L and 3.91 mg/L in diluted and concentrated hydro-ethanol extracts of wine lees, respectively. However, the concentration of total sugars can vary in wine lees because it depends on the vinification process adopted and therefore, on the residual unfermented sugars.

Table.12. Physico-chemical characteristics (mean ±SD) Sangiovese wine lees

Parameter	**Feed**
Turbidity (NTU)	1000 ± 0.0
pH	3.7 ± 0.0
Polysaccharides (mg glucose/L)	358.2 ± 87.3
Total polyphenols (mg GAE/L)	655.4 ± 13.6
AC ABTS (% scavenging)	66.4 ± 0.0
AC DPPH (% scavenging)	56.4 ± 0.2
Proanthocyanidins (mg CE/L)	14.8 ± 1.3
Mannoproteins (g/L)	0.39 ± 0.1
Glucose (mg/L)	0.67 ± 0.1
Fructose (mg/L)	0.048 ± 0.2

As is reported in table 13, a total of 14 phenolic compounds were identified by HPLC analysis: Protocatechuic acid, vanillic acid, gallic acid, syringic acid, catechin, epigallocatechin gallate, epicatechin, quercetin aglycone, fertaric acid, coutaric acid, trans-caftaric acid, cis-caftaric acid,

chlorogenic acid and caffeic acid. Similarly, gallic acid, (+) - catechin, and epicatechin were detected by MS/MS in the wine lees (Delgado De La Torre et al., 2015a; Pomar et al., 2005).

Table.13. Phenolic profile compounds by HPLC on the feed stream.

Phenolic compounds	**Feed**
Protocatechuic acid (*mg/L GAE*)	10.0
Vanillic acid (*mg/L GAE*)	5.9
Gallic acid (*mg/L*)	47.1
Syringic acid (*mg/L*)	2.2
(+) – Catechin (*mg/L*)	7.7
(-) – Epigallocatechin gallate (*mg/L CE*)	4.9
(-) – Epicatechin (*mg/L*)	10.4
Quercetin aglycone (*mg/L*)	47.7
Fertaric acid (*mg/L CUE*)	3.6
Coutaric acid (*mg/L CUE*)	3.5
Trans – Caftaric acid (*mg/L CAE*)	12.6
Cis – Caftaric acid (mg/L)	3.2
Chlorogenic acid (*mg/L CAE*)	2.3
Caffeic acid (*mg/L CAE*)	7.1

Legend: GAE: Gallic acid-equivalents *CE*: (+) – Catechin-Equivalents*: QuE*: Quercitin Aglicone-Equivalents*; CUE*: *p*-Coumaric Acid-Equivalents*; MvE*: Maldivin – 3 – O – Glucoside – Equivalents

3.3.2.3. Filtration experiments

The SRL sample was previously centrifuged at 4000 RPM to obtain a solution adapted for processing in the nanofiltration system. The centrifuged solution was successively ultrafiltered using a flat sheet membrane. The membrane code and specifications are reported in table 1.

Figure 17. Shows the behaviour of the permeate flux and VRF flux in the ultrafiltration treatment of Sangiovese wine lees. The initial permeate flow was approximately 7.82 L/m^{2}h and gradually decreased until reaching a steady state value of approximately 5.25 L/m^{2}h.

The behavior of the permeate flow through the operation time of the filtration treatment is characterized by a rapid decrease in the permeate flow in the first 245 minutes of the process with a reduction of 21.23% with respect to the initial permeate flow followed (with approximate VRF value of 1.17). Subsequently, a second period is presented until reaching a VRF of 1.19, which corresponds to a greater decrease in the permeate flow until reaching a stationary period.

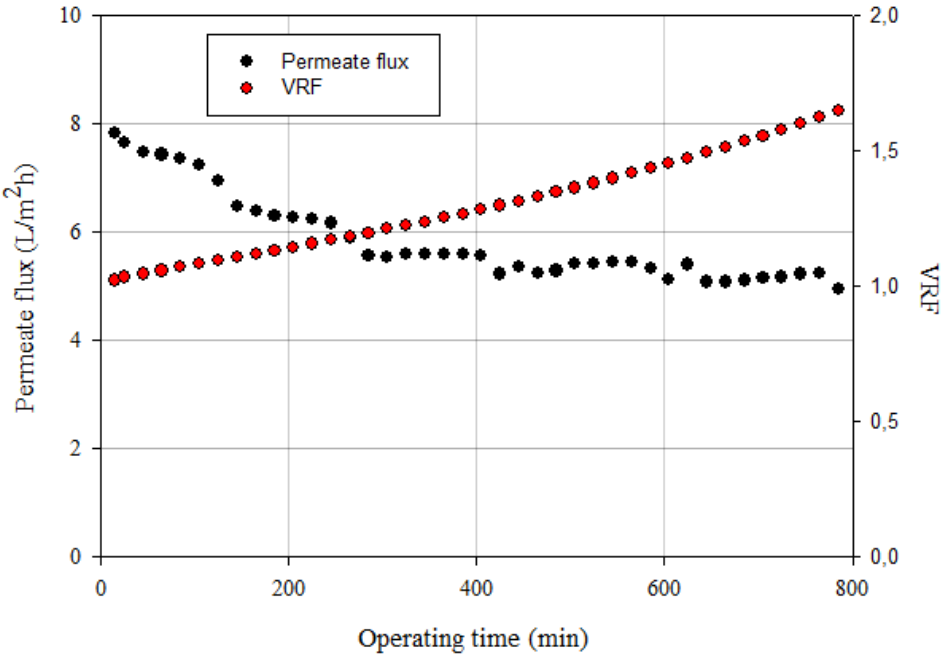

Figure 17. Behaviour of the permeate flux and VRF as a function of the operation time (Operation conditions)

This behavior in the permeate flow can be attributed to different phenomena including membrane fouling, concentration polarization and an increase in the concentration of solutes in the retentate stream (Cassano et al., 2007). It was observed that, as the feed concentration increases, the concentration polarization becomes more severe. Accumulation of solutes occurs towards the membrane surface, resulting in a thicker layer. Similar behavior reported by the authors in the ultrafiltration treatment in xoconostle juice (Opuntia joconostle) by means of a polysulfone hollow fiber membrane showing two periods in the process: Rapid decrease in permeate flow and stationary period (Castro-Muñoz et al., 2017). It should be noted that the stationary period represents the non-variation of the permeate flux as a function of the operating time.

The ultafiltration process was achieved close to a VRF of 1.64. After this, the ultafiltration process was stopped. The feed stream volume was 500 mL; the final streams recovered after UF processing were 192 and 237 mL for permeate and retentate, respectively. This means, 38.4% of the initial Sangiovese wine lees was recovered as clarified solution.

3.3.2.4. Fouling index and cleaning efficiency

Related to the hydraulic permeability, fouling index and cleaning efficiency values are reported in table 14. The hydraulic permeability of the membrane was close to 23.45 L/m^2h, however it decreased after the ultrafiltration process to 9.80 L/m^2h. The fouling index of the membrane was about 41.8%. The fouling index was similar to that reported by (Cassano et al., 2007) whilst researching the ultrafiltration of kiwifruit juice (32%). The fouling of the membrane is influenced by different factors such as the physicochemical composition of the solution, the phenomenon of polarization concentration, adsorption effects (solute-solute and solute-membrane), characteristics of the membrane (MWCO, configuration model, material hydrophobicity, porosity, and surface charge), operation conditions (TMP, flow rate and temperature) and electrostatic interactions (Boussu et al., 2006).

Table. 14. Hydraulic permeability values, fouling index and cleaning recovery of the membranes on ultrafiltration processing from Sangiovese wine lees

	Membrane type
	CA38
Q_{f0} before (L/m^2h)	23.45
Q_{f1} After (L/m^2h)	9.80
Q_{f2} (L/m^2h)	10.06
Fouling index (%)	41.8
Cleaning recovery (%)	42.9

3.3.2.4. Membrane selectivity – Ultrafiltration of red wine lees

The physicochemical composition of feed, permeate and retentate streams obtained in the UF of Sangiovese wine lees is reported in Table 15. The analysis of the pH value of the samples of the permeate and retentate streams obtained in the UF of Sangiovese wine lees indicated a minimal change numerically and without significant differences, with respect to the feed solution attributed to the variability of the measurement of the samples. The ultrafiltration process removed 98.5% of the total turbidity present in the feed solution compared to the permeate solution (from 1000 to 1.5 NTU). Suspended solids in the feed solution could be related to residual yeasts, lactic bacteria from alcoholic and malolactic fermentation and to residual cells (Vernhet & Moutounet, 2002).

According to the data in table 15 the highest number of phenolic compounds, bioactive compounds that favor antioxidant activity and sugars are recovered in the retentate stream.

Table 15. Physico-chemical characteristics (mean ±SD) of feed, permeate and retentate sample obtained in the treatment of red wine lees with UF membrane.

Parameter	**Feed**	**Permeate**	**Concentrated**
Turbidity (NTU)	1000 ± 0.0^{A}	1.5 ± 0.2^{B}	1000 ± 0.0^{A}
pH	3.7 ± 0.0^{A}	3.6 ± 0.0^{AB}	3.7 ± 0.0^{B}
Polysaccharides (mg glucose/L)	358.2 ± 87.3^{A}	26.3 ± 4.4^{B}	635.9 ± 24.3^{C}
Total polyphenols (mg GAE/L)	655.4 ± 13.6^{A}	382.6 ± 25.1^{B}	715.9 ± 44.5^{C}
AC ABTS (% scavenging)	66.4 ± 0.2^{B}	40.7 ± 1.0^{C}	70.2 ± 0.6^{A}
Proanthocyanidins (mg CE/L)	14.8 ± 1.3^{A}	5.8 ± 0.3^{B}	16 ± 0.3^{A}
Mannoproteins (g/L)	0.39 ± 0.1	n.d	0.24 ± 0.1^{B}
Glucose (g/L)	0.67 ± 0.0^{A}	0.60 ± 0.0^{B}	0.65 ± 0.2^{AB}
Fructose (g/L)	0.04 ± 0.2^{B}	0.04 ± 0.1^{B}	0.05 ± 0.2^{A}

Different superscript letter in properties are significantly different according to the Tukey's HSD test ($p \leq 0.05$).

On the other hand, the rejection towards polysaccharides is around 92% with the UF membrane. A similar value was reported by (Giacobbo et al., 2015) using Merlot wine lees with around 95% of rejection. In the same work cited, the authors reported a retention of 74.7% towards the polyphenolic content using polyimide hollow fiber membrane with 0.4 μm pore size. In the present research, a lower retention of the polyphenolic content was evidenced (Approximately 44%).

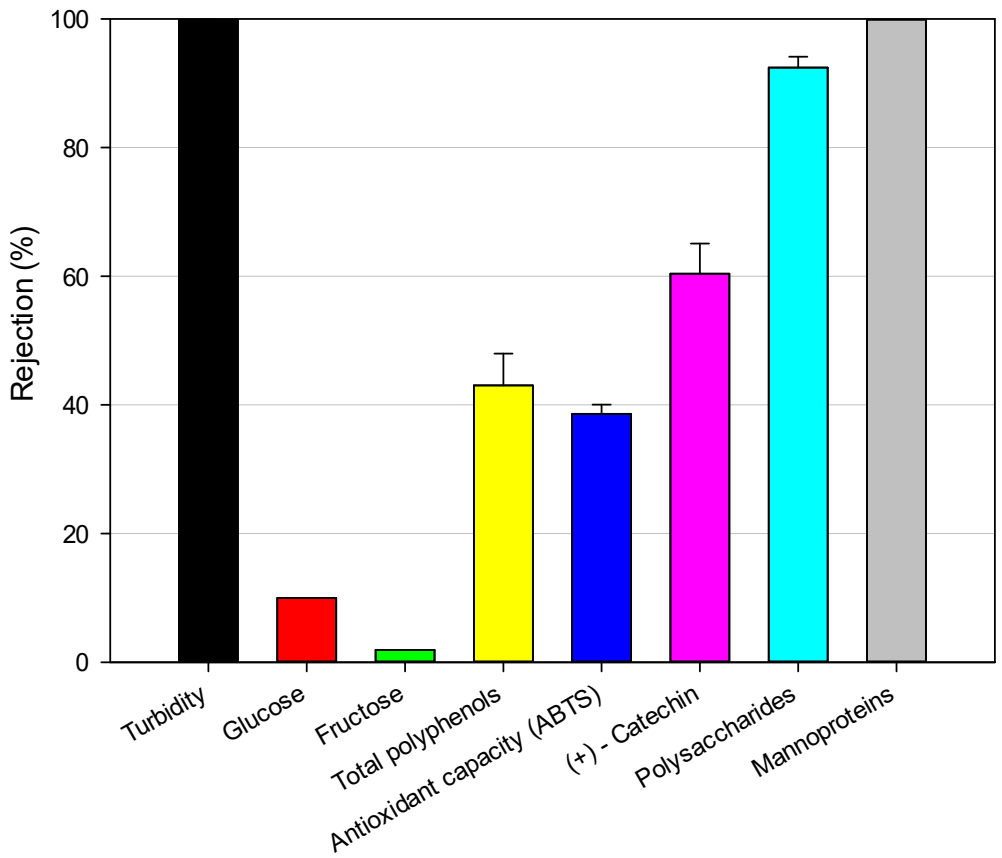

Figure 18. Rejection of the ultrafiltration membrane towards compounds of Sangiovese wine lees

The retention of the UF membrane towards the specific compounds is shown in figure 18.

Regarding the percentage of retention of polyphenols, similar result was reported by (Streit et al., 2009) using a leather effluents solution enriched with salts and organic compounds. The authors used a fluoro polymer composite UF membrane with a molecular weight cut-off (MWCO) of 1000 Da, which exhibited 45% rejection towards tannins (polyphenolic compounds).

Regarding the rejection of polysaccharides, (Giacobbo et al., 2013) reported a value of about 90% from wine lees using a polyethersulphone membrane with a molecular weight cut-off (MWCO) of 7600 Da. These results are in line with those obtained in the present investigation (92% rejection). This indicates that, apparently, ultrafiltration membranes are preferable for permeation of polyphenol compounds and reject polysaccharides regardless of the manufacturing material.

The UF membrane presented 100% rejection towards mannoproteins. On the other hand, a preferential permeation towards phenolic compounds and monomeric anthocyanins was evidenced with a rejection of less than 25%

This behavior makes possible a fractionation of polyphenols and polysaccharides through ultrafiltration, in this way the retentate stream is rich in mannoproteins and polyssacharides while the permeate stream is rich in anthocyanins and other phenolic compounds.

Table 16 shows the phenolic compounds and anthocyanin monomers found in the three streams of the UF process. HPLC analysis revealed a small increase in the concentration of phenolic compounds and anthocyanin monomers on the retentate side; Except for caffeic acid, (+) - catechin, Cyanidin-3-O-glucoside, petunidin-3-O-glucoside, peonidin-3-O-glucoside and malvidin-3-O-glucoside which showed a small decrease. Moreover, permeate and retentate fractions presented concentration in flavonoids similar to previous studies (Conidi et al., 2011) carried out on bergamot juice using a UF hollow fiber polysulfone membrane with a nominal MWCO of 1 kDa.

Despite the high fouling index of 41.8%, the retention of some phenolic fractions (syringic acid, (+) - catechin, (-) – epigallocatechin, quercetin aglycone), and some anthocyanin monomers (yanidin-3-O -glucoside, peonidin -3-O-glucoside and malvidin-3-O-glucoside) is less than 5%, while other phenolic fraction compounds (vanillic acid, gallic acid, fertaric acid and courtaric acid and petunidin-3-O- glycoside) are in the range of 5-10%. This can be attributed to the large pore diameter of the UF membrane (35000 Da) which allows diffusion of low molecular weight compounds. It should be noted that these compounds normally have a molecular weight of less than 1000 Da (Garcia et al., 1999; He et al., 2016).

Table.16. Phenolic profile of Feed, Permeate and Retentate sample obtained in the treatment of Sangiovese wine lees with UF membrane.

Polyphenols compounds	Feed	Permeate	Retentate	Rejection (%)
Non-Flavonoids				
Benzoic acids				
Protocatechuic acid (*mg/L GAE*)	10.0	8.3	10.4	17
Vanillic acid (*mg/L GAE*)	5.9	5.6	5.9	5.08
Gallic acid (*mg/L*)	47.1	42.4	47.4	9.97
Syringic acid (*mg/L*)	2.2	2.4	2.9	0.0
Hydroxycinnamic acids				
Fertaric acid (*mg/L CUE*)	3.6	3.4	3.9	5.55
Coutaric acid (*mg/L CUE*)	3.5	3.2	3.7	8.57
Trans – Caftaric acid (*mg/L CAE*)	12.6	10.5	13.3	16.66
Cis – Caftaric acid (mg/L	3.2	2.4	4.1	25
Chlorogenic acid (*mg/L CAE*)	2.3	2.1	2.4	17
Caffeic acid (*mg/L CAE*)	7.1	6.5	6.8	5.08
Flavonoids				
Flavan-3-ols				
(+) – Catechin (*mg/L*)	7.7	7.4	7.0	3.89
(-) – Epigallocatechin gallate (*mg/L CE*)	4.9	4.7	5.3	4.08
(-) – Epicatechin (*mg/L*)	10.4	9.1	11.7	12.50
Quercetin aglycone (*mg/L*)	47.7	56.7	47.7	0.0
Anthocyanins				
Cyanidin-3-O-glucoside (*mg/L MvE*)	0.7	0.9	0.5	0
Petunidin-3-O-glucoside (*mg/L MvE*)	1.1	1.0	0.9	9.1
Peonidin-3-O-glucoside (*mg/L MvE*)	1.7	1.9	1.4	0
Malvidin-3-O-glucoside (*mg/L*)	13.7	13.2	11.6	3.6

Legend: GAE: *Legend: GAE*: Gallic acid-equivalents; *CE*: (+) – Catechin-Equivalents*; CUE*: *p*-Coumaric Acid-Equivalents; *QuE*: Quercetin Aglicone-Equivalents; *CAE*: Quercetin Aglicone-Equivalents; *MvE*: Maldivin – 3 – O – Glucoside – Equivalents

Furthermore, these results are in line with Conidi et al. (Conidi et al., 2017) as reported a 6.9 retention of cyanidin 3-O-glucoside using a composite fluoro polymer flat sheet UF membrane with a MWCO of 1000 Da of pomegranate juice clarification.

3.3.2. Nanofiltration experiments for mixed grape pomace (MRL)

3.3.2.1. Membrane characterization

The rejections of the NF membranes towards a set of reference solutes, salts and ethanol decreased as follows (Table 17):

NF90> CA316-70> CA316> CA400-22

The NF90 and CA316-70 membranes presented rejections of 99% and 97% sodium sulfate, respectively, and 95% and 77% sodium chloride, respectively.

On the other hand, the membranes CA400-22 and CA316 presented lower rejections to sodium sulfate, with 47% and 86%, respectively, and sodium chloride, with 10% and 27%, respectively, but there is still a differentiation between the two salts as is characteristic of NF membranes.

The result reflects the characteristic behavior of NF membranes. The water permeability data coincided with the typical values of the NF membranes, with the CA400-22 membrane exhibiting the highest value (8.34 L/m^2hbar) and the NF90 membrane giving the lower (3.75 L/m^2hbar).

Table.17. Rejections (expressed as %) of NF membranes for specific solutes, salts and ethanol

Membrane type	Saccharose	Glucose	Raffinose	PEG	NaCl	Na_2SO_4	Ethanol
NF90	100	100	100	99	95	99	50
CA316-70	98	95	98	89	77	97	7
CA316	70	50	77	55	27	86	1
CA400-22	16	11	21	20	10	47	2

3.3.2.2. Chemical composition of grape pomace extract

Table. 18. Shows the chemical composition of grape pomace extract.

Phenolic compounds were extracted from grape pomace by means of an optimized enzymatic treatment assisted by ultrasound, from section 2.2.5 of chapter 2 in the present thesis.

Table.18. Physico-chemical characteristics and antioxidant activity (mean ±SD) of red grape pomace extract.

Parameter	Value
Turbidity (NTU)	169 ± 0.5
pH	4.0 ± 0.1
Total polyphenolics FC (mg GAE/100 g dw)	260 ± 10.3
AA DPPH (% scavenging)	16 ± 0.3
AA ABTS (% scavenging)	41 ± 3.5
Proanthocyanidins (mg CE/100g dw)	49 ± 6.2
Glucose (mg/ 100 g)	46 ± 0.0
Fructose (mg/100 g)	403 ± 0.1

Legend: GAE: Gallic acid-equivalents *CE*: (+) – Catechin-Equivalents
FC: Folin-Ciocalteu; dw: dry weight marc; AA: antioxidant activity.

The red grape pomace extract had a pH of 4.0 due to the use of the buffer solution containing succinic acid (C4H6O4) and sodium borate (Na2B4O7 * 10H2O). The total phenol content in the extract was 260 mg GAE/100 g using a solid-solvent ratio of 1:80 (w/v). Similar values were obtained by (Nayak, Bhushan, & Rodriguez-Turienzo, 2018). They reported a total phenol content of 427.9 mg GAE/100 g for water extraction (solid-solvent ratio of 1:20 p/v) compared to the upper value of 801.6 mg GAE/100 g obtained by the same author with water-ethanol extraction (solid-solvent ratio 1:20 p/v) of the Cabernet Sauvignon grape pomace. Differences in the extraction performance of polyphenolic compounds from grape pomace can be discussed in light of several technological factors, including grape variety, soil management, winemaking conditions, solvent type (aqueous, organic) and extraction technology (Monagas et al., 2003).

Red grape pomace extract contained 46 mg/100 g dw and 403 mg/100 g dw of glucose and fructose, respectively. The glucose content in the grape pomace was low (0.046% dw), with an increase in the extracted fructose (0.40% dw); both sugars were several orders of magnitude lower than previous reports after (Pedras et al., 2020) where glucose and fructose obtained in red grape pomace extract were 2.8% dw and 3.1% dw, respectively.

The content of proanthocyanidins in the extract was 49.0 mg CE/100 g of dry weight and higher values (in the range of 100-250 mg of catechin /100 g) were reported for different varieties of grape

pomace, (de la Cerda-Carrasco et al., 2015). It should be noted that the values estimated by these authors include monomeric, oligomeric and polymeric flavan-3-ols and that the total polyphenols obtained by stepwise methanol/water and acetone/water extraction were 10 orders higher than those obtained by assisted enzymatic extraction (de la Cerda-Carrasco et al., 2015).

The antioxidant activity can be considered as strong, intermediate or weak when the DPPH radical scavenging activity is above 70%, between 50 and 70% and below 50%, respectively (Zhu et al., 2019). According to the aforementioned ranking, the red grape pomace extract had a weak antioxidant activity measured by the DPPH radical scavenging test (16.5 ± 0.39%) and showed increasing efficacy when ABTS was used as a radical of reference (41.2 ± 3.52%). In both cases, the relatively low content of polyphenolic compounds in the extract represented a limiting factor for its application as an antioxidant; This limitation could be overcome by careful selection of raw materials and optimization of extraction parameters.

3.3.2.3. Productivity of the membrane

Table. 19. reports the average permeate fluxes (Jp) of the nanofiltration of the grape pomace extract at 20 bar with the four NF membranes. The CA400-22 membrane showed the highest permeate flux with a value of 50.58 L/m^2h, while the CA316 and CA316-70 membranes showed quite similar permeate flux values of 44.44 and 43.38 L/m^2h, respectively; on the other hand, the NF90 membrane showed the lowest permeate flux (26.09 L/m^2h). These results are in agreement with the rejection values measured for different solutes and water permeability data. In particular, a strong correlation can be inferred between membrane shear and permeate flux values, although the chemical nature of the membrane material plays a key role in membrane performance.

It is worth mentioning that commercial polymeric membranes such as DL2540 and GE2540 (from Osmonics) with a cut-off of 150-300 and 1000 Da, respectively, produced much lower permeate fluxes (in the order of 25 and 5 L/m^2h) when processing. aqueous extracts of fermented distilled grape pomace (Díaz-reinoso et al., 2009).

Table.19. Average permeate flux for selected membranes in the nanofiltration of red grape pomace extract.

Membrane type	J_p (L/m^2h)
NF90	26.09
CA316-70	43.38
CA316	44.44
CA400-22	50.58

3.3.2.4. Fouling index and cleaning efficiency

The fouling index is another factor that must be considered in the processing of natural extracts by membrane technology and in the selection of a membrane for a specific application, since fouling represents a decrease of the permeate flux versus the operating time, reducing the productivity and shortening membrane life time (Nilsson, 1990).

The fouling index for the investigated membranes was calculated on the basis of water permeability measured before and after the nanofiltration of the red grape pomace extract. According to the results in Table 20, the NF90 membrane showed the highest fouling index, which is 40.53%, followed by the CA316-70 membrane with a 35.97%, the CA400-22 membrane with a 23.38% and lastly the CA316 membrane with a 13.39%. The highest fouling index reported for the NF90 membrane could be explained due to the adsorption of organic compounds on the membrane surface through the possible formation of hydrogen bonds between the membrane polymer (polyamide) and organic compounds. Furthermore, according to the results reported by (Arsuaga et al., 2010) the adsorption of phenolic compounds on the membrane could be promoted by the hydrophobic interactions with the membrane material, playing an important role in the retention of solutes, a higher flux decline and a high fouling index. On the other hand, the permeate fluxes of UF and NF membranes may be severely decreased when treating low molecular weight hydrophobic solutes. In addition, the extent of the permeate fluxes reduction is affected by the hydrophilic/hydrophobic properties of the membrane material and the concentration of the solutes

(Jönsson & Jönsson, 1995). Accordingly, the three laboratory-made cellulose acetate membranes showed a low fouling index when compared with the NF90 membrane.

Table. 20. Hydraulic permeability in NF/cleaning cycles, fouling index and cleaning efficiency of selected membranes.

	Membrane type			
	CA316	CA316-70	CA400-22	NF90
W_{p0} (L/m^{2}hbar)	4.63	4.42	8.34	3.75
W_{p1} (L/m^{2}hbar)	4.01	2.83	6.39	2.23
W_{p2} (L/m^{2}hbar)	4.14	3.54	6.72	2.26
Fouling index (%)	13.39	35.97	23.38	40.53
Cleaning efficiency (%)	89.41	80.10	80.57	60.26

W_{p0}, water permeability before the NF of grape pomace extract; W_{p1}, water permeability after the NF of grape pomace extract; W_{p2}, water permeability after cleaning with distilled water.

A high recovery of hydraulic permeability (89.41%) was noticed for the CA316 membrane after cleaning with distilled water. Similar results were observed for the CA316-70 and CA400-22 membranes, with recoveries of 80.10% and 80.57%, respectively. Based on the highest fouling index measured for the NF90 membrane, the cleaning efficiency for this membrane was the lowest with a recovery of initial water permeability of 60.26%. In general, an incomplete recovery of hydraulic permeability in membranes can be attributed to irreversible fouling, which is a phenomenon associated with the absorption of phenolic components on the surface of the membranes (Sotto et al., 2013).

3.3.2.5. Membrane selectivity analysis

The physicochemical parameters of the NF permeate samples compared to the red grape pomace extract are reported in Table 21. Minimal changes in the pH values were noted in all permeate fractions compared to the pomace extract of red grape. Although the grape acids (malic and tartaric acid) are associated with a pH generally in the range of 3.2 and 4.0 (Moreno & Peinaldo, 2012); the

pH of this extract is due to the preparation of the buffer solution with succinic acid (C4H6O4) and sodium borate ($Na_2B4O7 * 10H_2O$), setting the pH value of 4.0.

All the membranes used allowed a significant reduction in turbidity of around 95%. In particular, the NF90 membrane allowed a reduction in turbidity of 99.12%. In addition, the NF90 membrane showed the highest rejection coefficient towards total polyphenols and antioxidant activity. The rejection of total polyphenols was 97%; for antioxidant activity; the rejections were 74% and 100% in relation to the DPPH and ABTS analysis, respectively. The authors (Cassano et al., 2011) noted similar results in the treatment of oil mill wastewater with the same membrane, obtaining a rejection of total polyphenols of 93%. Polyphenol rejection coefficients greater than 92% were also measured by other authors (Giacobbo et al., 2018) in the treatment of winery wastewater generated in the second transfer of red wine production with a polyiperazine membrane, with a cut-off molecular weight of 300 Da (NF270, from DOW-Filmtec, Edina, MN).

Table. 21. Physico-chemical characteristics of feed and permeate samples (mean ±SD) obtained in the NF of grape pomace extract.

Parameter	**Feed**	**Permeate**			
		CA316	**CA316-70**	**CA400-22**	**NF90**
Turbidity (NTU)	169 ± 0.5^{A}	2.9 ± 0.4^{C}	2.2 ± 0.5^{C}	7.2 ± 0.9^{B}	1.5 ± 0.4^{C}
pH	4.0 ± 0.1^{A}	3.7 ± 0.1^{AB}	3.7 ± 0.2^{B}	3.8 ± 0.1^{AB}	3.6 ± 0.0^{B}
Total polyphenol FC (mg GAE/100 g)	260 ± 10.3^{A}	54 ± 4.1^{B}	50 ± 15.1^{B}	70 ± 2.7^{B}	9.1 ± 6.3^{C}
AA DPPH (% scavenging)	16 ± 0.3^{A}	6.4 ± 0.4^{CD}	8.9 ± 2^{BC}	11 ± 0.9^{B}	5.6 ± 0.3^{D}
AA ABTS (% scavenging)	41 ± 3.5^{A}	3.5 ± 0.1^{C}	3.8 ± 0.8^{C}	13.9 ± 0.7^{B}	n.d
Proanthocyanidins (mg CE/100 g dw)	49 ± 0.7^{A}	n.d	n.d	4.0 ± 1.7^{B}	n.d
Glucose (mg/100 g)	46 ± 0.0^{A}	5.7 ± 0.0^{D}	n.d	37 ± 0.0^{B}	12 ± 0.0^{C}
Fructose (mg/100 g)	403 ± 0.1^{A}	98 ± 0.2^{C}	n.d	354 ± 0.4^{B}	27 ± 0.0^{D}

Different superscript letter in properties are significantly different according to the Tukey's HSD test ($p \leq 0.05$). FC: Folin-Ciocalteu; GAE: gallic acid equivalents; CE: (+)-catechin equivalents; dw: dry weight pomaces; AA: antioxidant activity.

Figure 19 shows the rejections of the NF membranes to the different compounds of the grape pomace extracts. The CA400-22 membrane showed a rejection of around 73% to total polyphenols

and a rejection of around 60-70% to antioxidant activity. This membrane showed an almost total retention towards proanthocyanidins (around 93%) and allowed the recovery of most of the glucose and fructose in the permeate stream (rejection values of 19.5% and 12.5%, respectively) obtaining a permeate rich in sugars. Therefore, this membrane offered the best performance in terms of separation between sugars and phenolic compounds. On the other hand, the membranes CA316 and CA316-70 presented similar retention capacities for total phenols (greater than 80%) and antioxidant capacities (greater than 90% in the ABTS test). In addition, they performed well for glucose and fructose, with high retention values of 87% and 100% for the CA316 membrane and a total retention of both compounds for the CA316-70 membrane.

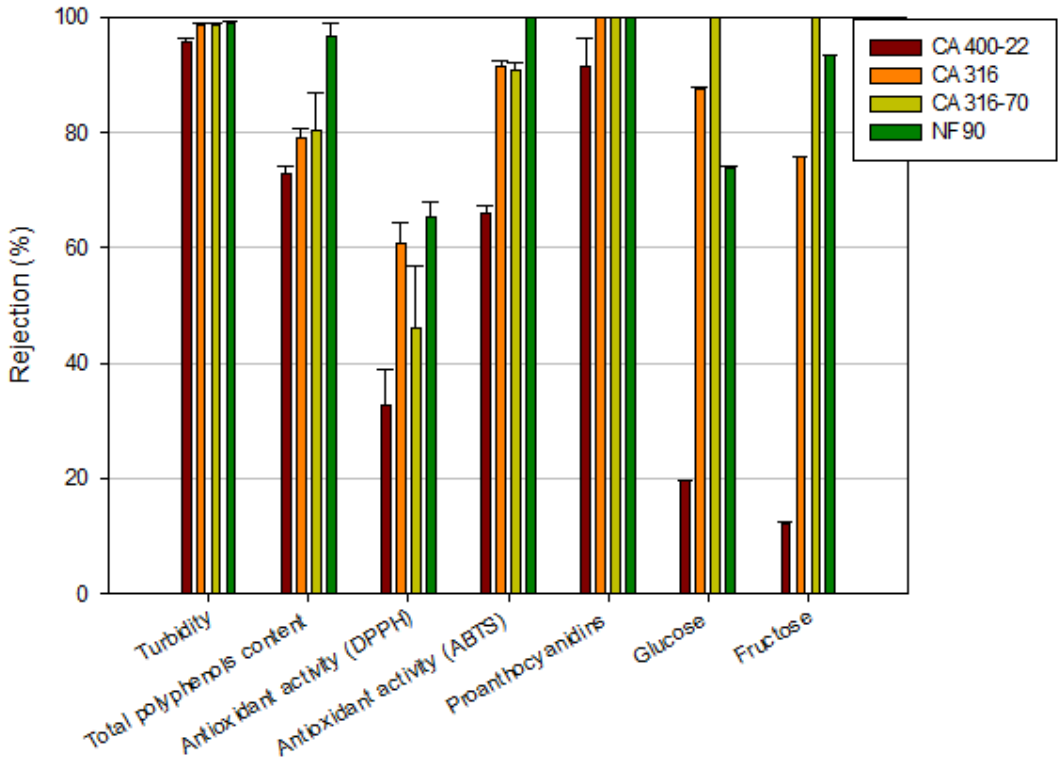

Figure 19. NF rejections to specific compounds and characteristics of grape pomace extract. Membrane: CA316, CA316-70, CA400-22 and NF90.

(Galanakis et al., 2013) also measured similar retention values for sugars and phenolic compound in the treatment of winery sludge with a 1 kDa NF fluoropolymer composite membrane (ETNA 01PP

from Alfa Laval, Nakskov, Denmark). This membrane successfully separated hydroxycinnamic acid derivatives from anthocyanins and flavanols into dilute and concentrated extracts, respectively.

3.3.3. Nanofiltration experiments for Cabernet Sauvignon wine lees (CRL)

3.3.3.1. Membrane characterization

Regarding the rejection of the NF membranes towards the set of solutes, salts and solvent references shown in table 22, the NF90 membrane presented the highest rejection followed by the CA316-70, CA316 and CA400-22 membranes.

The NF90 and CA316-70 membrane showed 99% and 97% rejections towards sodium sulfate, respectively. In addition, these same membranes presented 95% and 77% rejection towards sodium chloride, respectively.

On the other hand, the membranes CA400-22 and CA316 presented the lowest rejections towards sodium sulfate of 47% and 86%, respectively. While, in the sodium chloride solute it was 10% and 27%, respectively. These results are characteristic of the behavior of NF membranes.

Table 22. Rejections of NF membranes to specific solutes, salts, and ethanol (Operation conditions: feed solute concentration, 600 ppm; temperature, 25 ± 1 °C; feed flow rate, 0.8 L/min; pressure, 20 bar).

Membrane type	Saccharose (%)	Glucose (%)	Raffinose (%)	PEG (%)	NaCl (%)	Na_2SO_4 (%)	Ethanol (%)
NF90	100	100	100	99	95	99	50
CA316-70	98	95	98	89	77	97	7
CA316	70	50	77	55	27	86	1
CA400-22	16	11	21	20	10	47	2

3.3.3.2. Chemical composition of Cabernet Sauvignon wine lees

Table 23. shows the physicochemical composition of the Cabernet Sauvignon wine lees (CRL), which presented a pH of 3.8, similar to values reported in literature (Giacobbo et al., 2013).

The total phenolics content in the wine lees was 384.1 mg GAE/L. A similar value was reported by (Galanakis et al., 2013) where they obtained 476 mg GAE/L in the aqueous extract of wine lees.

Even so, the differences in the performance of the polyphenol content are considered to be due to different technological factors, including the variety of the grape, winemaking conditions and soil management (Monagas et al., 2003).

The wine lees contained 1.67 g/L and 0.06 g/L of glucose and fructose, respectively. The glucose content in the wine lees was higher than fructose; however both sugars were several orders of magnitude lower than previous reports by (Arboleda Mejia et al., 2019) where the glucose and fructose contents obtained in the mixed red wine lees were 5.8 g/L and 0.3 g/L, respectively.

The content of proanthocyanidins in the wine lees was 12.2 ± 0.36 mg CE/L. The same authors cited above reported a similar proanthocyanidin content of 6.9 mg CE/L for mixed red wine lees.

Regarding the antioxidant activity, with a radical scavenging activity value of 62.2% ± 1.9, it is positively correlated with the concentration of polyphenolic compounds (Floegel et al., 2011).

Table 23. Physicochemical characteristics (mean ±SD) of Cabernet Sauvignon wine lees

Parameter	**Value**
Turbidity (NTU)	1000 ± 0.0
pH	3.81 ± 0.07
Total polyphenolics FC (mg GAE/L)	384.1 ± 5.5
AA ABTS (% scavenging)	62.9 ± 1.9
Proanthocyanidins (mg CE/L)	12.2 ± 0.36
Polysaccharides (mg glucose/L)	185 ± 32
Mannoproteins (g/L)	0.11 ± 0.0
Glucose (mg/100 g)	1.67 ± 0.02
Fructose (mg/100 g)	0.06 ± 0.01

FC: Folin-Ciocalteu; GAE: gallic acid equivalents; CE: (+)-catechin equivalents; dw: dry weight pomaces; AA: antioxidant activity.

3.3.3.3. Membrane productivity

Table 24. reports the average permeate fluxes (J_p) of the Cabernet Sauvignon red wine lees nanofiltration at 20 bar with the four NF membranes.

The CA316-70 membrane showed the highest permeate flux with a value of 12.46 L/m^{2}h, while the NF90 showed the lowest permeate flux with a value of 3.72 L/m^{2}h. It is worth noting that the membranes CA400-22, CA316 and CA316-70 presented very similar values. The NF90 membrane presented good concordance in relation to the rejection values of solutes, salts and solvent.

Table.24. Average permeate flux for selected membranes in the nanofiltration of Cabernet Sauvignon wine lees (operating conditions: pressure, 20 bar; temperature, 25 ± 1 ◦C).

Membrane Type Jp (L/m2 h)	Jp (L/m^2 h)
NF90	3.72
CA316-70	12.46
CA316	12.28
CA400-22	11.25

The CA316-70 membrane showed the highest permeate flux with a value of 12.46 L/m^2h, while the NF90 showed the lowest permeate flux with a value of 3.72 L/m^2h. It should be noted that the membranes CA400-22, CA316 and CA316-70 presented very similar values. The NF90 membrane presented good agreement in relation to the rejection values of solutes, salts and solvent. However, it should be noted that the same was not true when comparing the CA400-22, CA316 and CA316-70 membranes, in reference to their rejection values of solutes, salts and solvent. This is possibly due to the low permeate flux values where the difference in fluxes between these three membranes can be noticed. High values of permeate flux in the same membranes were identified by the authors (Arboleda Mejia et al., 2020) where it is possible to observe a concordance between the permeate fluxes and the rejection values of solutes, salts and solvent.

3.3.3.4. Fouling index and cleaning efficiency

The table 25. Shows the hydraulics permeabilities, fouling index and cleaning efficiency of the selected membranes. One of the important factors in the performance and hence in the selection of the membrane for a specific application is the fouling index, which is represented as a decrease in the permeate flux versus the operating time, reducing the productivity and shortening the life of the membranes (Nilsson, 1990).

The fouling index for the investigated membranes was calculated based on the water permeability measured before and after the nanofiltration treatment of the Cabernet Sauvignon wine lees.

According to the results presented in table 24, the NF90 membrane showed the highest fouling index with a value of 42.28% followed by the CA400-22 membrane with 23.84%, the CA316-70 membrane with 9.57 % and finally the CA316 membrane with 8.63%.

Table. 25. Hydraulic permeability in NF/cleaning cycles, fouling index and cleaning efficiency of selected membranes.

	Membrane type			
	CA316	CA316-70	CA400-22	NF90
W_{p0} (L/m^{2}hbar)	3.94	3.24	6.92	3.24
W_{p1} (L/m^{2}hbar)	3.60	2.93	5.27	1.87
W_{p2} (L/m^{2}hbar)	3.65	3.48	5.77	1.88
Fouling index (%)	8.63	9.57	23.84	42.28
Cleaning efficiency (%)	92.64	100	83.38	58.02

W_{p0}, water permeability before the NF of grape pomace extract; W_{p1}, water permeability after the NF of Cabernet Sauvignon; W_{p2}, water permeability after cleaning with distilled water.

The highest fouling index reported for the NF90 membrane could be explained due to the adsorption of organic compounds on the surface of the membrane through the formation of hydrogen bonds between the polymeric membrane and organic compounds. In addition, the adsorption of polyphenolic compounds on the membrane surface could promote hydrophobic interactions with the membrane material, which plays an important role in the retention of solutes, a high fouling index and a large decrease in the flux of permeated. Furthermore, the permeate flux of the NF membranes must be governed by the decrease in the treatment of hydrophobic low molecular weight solutes (Arsuaga et al., 2010; Jönsson & Jönsson, 1995). The three laboratory-made cellulose acetate membranes showed a low fouling index compared to the NF90 membrane.

A high recovery of the hydraulic permeability of the value of 100% was observed in the CA316-70 membrane after cleaning with distilled water. Results higher than 80% regarding water permeability were observed in the CA316 and CA400-22 membranes. According to the highest fouling index which was presented by the NF90 membrane, the cleaning efficiency for this membrane was the lowest with a recovery of water permeability of 58.02%. Different phenomena can explain an incomplete recovery of the hydraulic permeability of the membrane, such as irreversible fouling

which is governed by the absorption effect of phenolic compounds on the surface of the membranes (Sotto et al., 2013).

3.3.3.5. Analysis of membrane selectivity – Nanofiltration of Cabernet Sauvignon wine lees

The physicochemical parameters of the feed and permeate streams obtained from the NF treatment are reported in table 26.

Minimal changes in pH values were noted in all permeate and feed fractions. This result is in agreement with others authors (Giacobbo et al., 2015) when analyzing the feeding solution of the Merlot wine lees where a change in pH in the permeate solution from 3.78 to a value of 3.92 measured during the microfiltration treatment using a polyamide membrane is reported.

Table 26. Physicochemical characteristics of feed and permeate samples (mean ±SD) obtained in the NF of grape pomace extract.

Parameter	Feed	Permeate NF90	Permeate CA316-70	Permeate CA316	Permeate CA400-22
Turbidity (NTU)	1000 ± 0.0^{A}	22.73 ± 5.11^{B}	0.58 ± 0.14^{C}	0.87 ± 0.04^{C}	0.74 ± 0.24^{C}
pH	3.81 ± 0.07^{C}	3.75 ± 0.02^{C}	3.13 ± 0.06^{D}	3.84 ± 0.0^{C}	4.27 ± 0.06^{A}
Total polyphenols FC (mg gallic acid/L)	384.1 ± 5.5^{A}	22.9 ± 2.4^{D}	97.9 ± 4.1^{C}	108.8 ± 4.1^{C}	128.7 ± 7.9^{B}
AA ABTS (% scavenging)	62.9 ± 1.9^{A}	14.5 ± 0.6^{C}	45.8 ± 6.5^{B}	44.1 ± 4.7^{B}	50.75 ± 0.2^{AB}
Proanthocyanidins (mg CE/L)	12.2 ± 0.36^{A}	n.d	8.87 ± 0.08^{B}	11.9 ± 0.65^{A}	11.3 ± 0.45^{A}
Polysaccharides (mg glucose/L)	185 ± 32^{B}	6 ± 9.1^{C}	4.8 ± 5^{C}	0.02 ± 1.1^{C}	1.4 ± 1.7^{C}
Mannoproteins (g/L)	0.11 ± 0.0^{A}	nd	nd	0.005 ± 0.01^{B}	0.004 ± 0.02^{B}
Glucose (g/L)	1.67 ± 0.02^{A}	n.d	0.12 ± 0.01^{C}	0.003 ± 0.01^{D}	1 ± 0.03^{B}
Fructose (g/L)	0.05 ± 0.01^{A}	n.d	0.005 ± 0.01^{C}	0.001 ± 0.01^{C}	0.03 ± 0.02^{B}

Different superscript letter in properties are significantly different according to the Tukey's HSD test ($p \leq 0.05$). FC: Folin-Ciocalteu; GAE: gallic acid equivalents; CE: (+)-catechin equivalents; dw: dry weight pomaces; AA: antioxidant activity.

All the membranes used allowed a significant reduction in turbidity of about 97%, with the CA400-22 membrane reaching a reduction of 99.4%. It should be noted that, although all the membranes presented high removal of turbidity, the NF90 membrane also presented a high rejection coefficient for total polyphenolic compounds (96.15%) and antioxidant activity (94%).

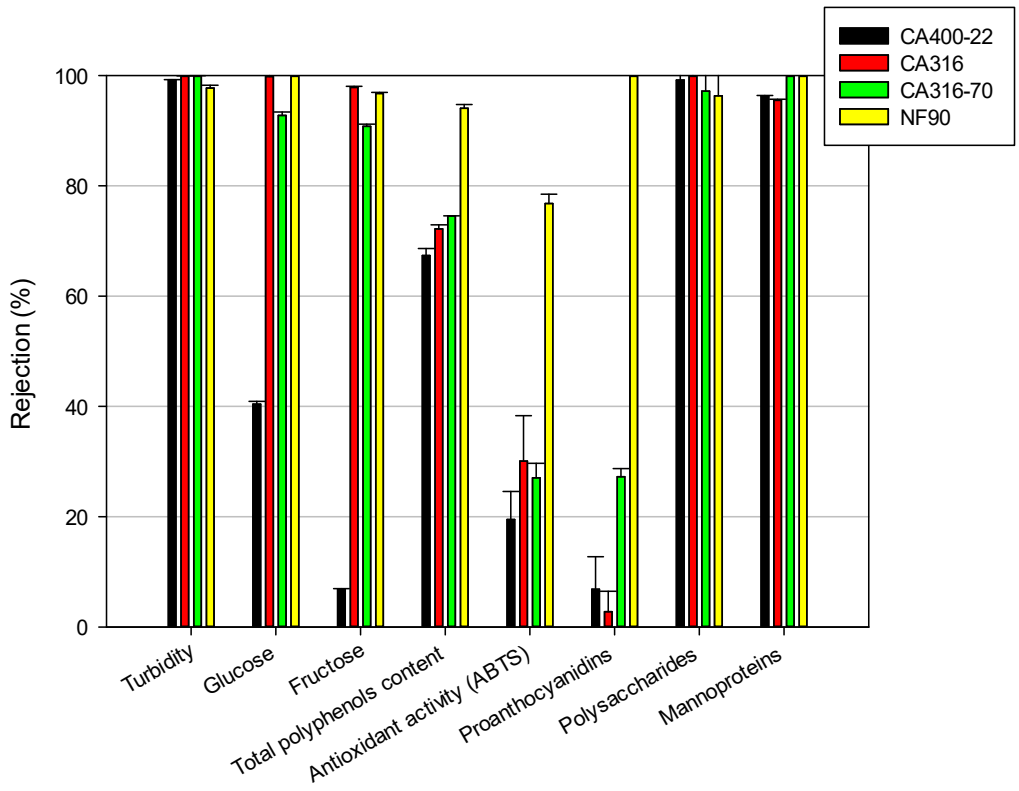

Figure 20. NF rejections to specific compounds and characteristics of grape pomace extract. Membrane: CA316, CA316-70, CA400-22 and NF90.

Figure 20 shows the NF membranes rejections to the different compounds from Cabernet Sauvignon wine lees. The CA400-22 membrane presented a proanthocyanidin rejection coefficient and antioxidant activity by ABTS of 6% and 19.4%, respectively.

Likewise, the CA400-22 membrane showed a rejection coefficient for glucose and fructose of 40% and 44.6%, respectively.

The CA316 and CA316-70 membranes gave rejection coefficients of 72.1% and 74.5%, respectively, for polyphenolic compounds, and 99.7% and 92.7%, respectively, for total sugars.

Galanakis et al found similar results (Galanakis et al., 2013), and reported a rejection coefficient of 81% for polyphenolic compounds and 74% for total sugars using a 100 kDa polysulfone membrane, during the ultrafiltration process of Cypriot wine lees.

Table 27. Presents the phenolic compounds and anthocyanin monomers found by HPLC in the feed and permeate from the NF process.

All membranes showed a rejection coefficient greater than 80% for two classes of flavonols: Myricetin and Quercetin aglycone. This is due to the fact that these two compounds have a high molecular weight (Myricetin = 318 Da and Quercetin aglycone = 302 Da) compared to the other types of flavonol compounds, which have a significantly lower molecular weight and consequently a low range of rejection coefficient (from 0.6 to 19.9%). However, this was not seen in the CA316 membrane, which presented a high rejection coefficient for all flavonoids. Furthermore, electrostatic interactions between the membrane surface and solutes can enhance the membrane selectivity of NF processes (Galanakis, 2015).

Regarding benzoic acid, the CA316 membrane obtained the highest rejection coefficient which ranged from 74 to 100%. On the other hand, the CA400-22 membrane showed the lowest rejection coefficient with a range from 48 to 76%.

Concerning hydroxynamic acids, the CA316 membrane presented the highest rejection coefficient belonging to several species with a range from 65.2 to 100%. The functionality of streams enriched with hydroxynamic acids is evidenced in their use in foodstuff as antioxidants (Galanakis et al., 2013).

Table 27. Phenolic profile compounds by HPLC on the Feed (F), Permeate (P) and Retentate (R)

Phenolic compounds	Feed	Permeate CA400-22	Permeate CA316	Permeate CA316-70	Permeate NF90
Non flavonoids					
Benzoic acids					
Protocatechuic acid (*mg/L GAE*)	7.1	3.7	1.5	3.4	3.8
Vanillic acid (*mg/L GAE*)	6.0	4.6	1.5	5.1	4.9
Gallic acid (*mg/L*)	17.9	4.1	n.d	1.0	3.4
Syringic acid (*mg/L*)	2.9	1.4	n.d	1.1	1.3
Flavan-3-ols					
(+) – Catechin (*mg/L*)	4.6	3.7	0	3.7	4.4
(-) – Epigallocatechin gallate (*mg/L CE*)	2.1	1.9	0	2.1	2.2
(-) – Epicatechin (*mg/L*)	22.5	18.8	1.9	18.3	20.2
Quercetin aglycone (*mg/L*)	25.4	4.0	n.d	n.d	1.7
Myricetin *(mg/L QuE)*	22.6	6.4	n.d	0.1	4.5
Flavonoids					
Hydroxycinnamic acids					
p – Coumaric acid	4.1	4.0	1.4	1.5	1.4
Fertaric acid (*mg/L CUE*)	2.2	1.3	n.d	1.2	1.2
Coutaric acid (*mg/L CUE*)	1.8	1.2	n.d	n.d	1.2
Trans – Caftaric acid (*mg/L CAE*)	4.6	1.7	n.d	1.2	1.4
Cis – Caftaric acid (mg/L)	1.4	1.3	n.d	1.2	1.2
Chlorogenic acid (*mg/L CAE*)	1.9	1.3	n.d	1.3	1.3
Caffeic acid (*mg/L CAE*)	9.6	7.6	1.9	1.9	8.5
Anthocyanins					
Malvidin-3-O-glucoside *(mg/L)*	7.4	2.3	n.d	n.d	1.1

Legend: GAE: Gallic acid-equivalents; *CE*: (+) – Catechin-Equivalents*; CUE*: *p*-Coumaric Acid - Equivalents; *QuE*: Quercetin Aglicone-Equivalents; *CAE*: Quercetin Aglicone-Equivalents

Table 28. presents the rejection coefficient of NF membranes towards phenolic compounds from Cabernet, The Sauvignon wine lees, The CA400-22 membrane appears to be favorable to the separation of some hydroxyinamic acid species from anthocyanin species. Likewise, the membrane presented a low rejection coefficient for hydroxynamic acid and a high rejection coefficient for anthocyanins. Moreover, a permeate stream rich in hydroxynamic acids with a low anthocyanin concentration was obtained from this membrane.

Table 28. Rejection coefficient of NF membranes towards phenolic compounds from Cabernet Sauvignon wine lees

Phenolic compounds	Rejection (%)			
	CA400-22	**CA316**	**CA316-70**	**NF90**
Non flavonoids				
Benzoic acids				
Protocatechuic acid (*mg/L GAE*)	48.7	79.4	52.5	47
Vanillic acid (*mg/L GAE*)	23	74.7	14	5.6
Gallic acid (*mg/L*)	76.9	100	94.2	80.8
Syringic acid (*mg/L*)	52	100	62.2	57
Hydroxycinnamic acids				
p – Coumaric acid	1.3	65.2	64.5	65
Fertaric acid (*mg/L CUE*)	39.8	100	44.4	44.4
Coutaric acid (*mg/L CUE*)	33.9	100	100	33.8
Trans – Caftaric acid (*mg/L CAE*)	63.5	100	73.7	69.7
Cis – Caftaric acid (mg/L	6.6	100	16.2	16.2
Chlorogenic acid (*mg/L CAE*)	28.5	100	30	29.9
Flavonoids				
Flavan-3-ols				
(+) – Catechin (*mg/L*)	19.9	100	19.7	3.1
(-) – Epigallocatechin gallate (*mg/L CE*)	8.1	100	0.6	1.8
(-) – Epicatechin (*mg/L*)	16.6	91.6	18.5	10.1
Quercetin aglycone (*mg/L*)	84.1	100	100	93.1
Myricetin	71.1	100	99.5	80.2
Anthocyanins				
Malvidin-3-O-glucoside *(mg/L)*	69.1	100	100	85.1

Legend: GAE: Gallic acid-equivalents; *CE*: (+) – Catechin-Equivalents*; CUE*: *p*-Coumaric Acid-Equivalents; *QuE*: Quercetin Aglicone-Equivalents; *CAE*: Quercetin Aglicone-Equivalents

The CA316 membrane showed the highest rejection coefficient for all polyphenolic compounds and anthocyanin monomer. On the other hand, the CA400-22 membrane presented the lowest ones.

The CA400-22 membrane has the largest gap between the rejection coefficient for polysaccharides and polyphenols. These results clearly show an opportunity for the fractionation of polyphenolic compounds and polysaccharides. This suggests that concentration by means of the NF system using the CA316 membrane produces a stream rich in polysaccharides and polyphenols. On the other hand, the permeate obtained with the CA400-22 membrane produces a stream rich in polyphenolic compounds but lacking in polysaccharides.

3.4. Conclusions

Microfiltration and nanofiltration for bioactive compounds from mixed wine lees

The integration of membrane processes has been developed in order to take advantage of the versatility that the membrane system offers for the treatment of agro-industrial waste.

In this study, the recovery of phenolic compounds from red wine lees by membrane processes that had been previously treated by microwave extraction, was investigated.

- The hydroalcoholic extracts were clarified by microfiltration and then processed with polymeric membranes that had different cut-offs. Among the selected membranes, ETNA 01PP showed the highest productivity under certain operating conditions, but a lower retention of phenolic compounds and sugars compared to the other membranes.
- On the other hand, the NFT-50 membrane gave retention coefficients higher than 70% for all the low molecular weight free phenolics detected. None of the selected membranes showed a preferential rejection of phenolic compounds over sugars. Therefore, the processing of membranes is oriented to the concentration of bioactive compounds. Based on these results, an integrated process based on the combination of microwave extraction, microfiltration (PVDF membrane, 0.15 μm) and nanofiltration (polyamide membrane, 150 Da) is considered to be a practical approach for the production of concentrated fractions of bioactive compounds from red wine lees, with potential health benefits in the pharmaceutical, cosmetic and food industries.

Ultrafiltration treatment for the bioactive compounds from Sangiovese wine lees

- Regarding the UF Sangiovese wine lees, the results showed a high selectivity of the CA38 cellulose acetate membrane to retain polysaccharides compounds (retention about 95%) and

allow the passage of polyphenolic compounds (retention about 40%), furthermore rich streams of these compounds were evidenced.

- The cellulose acetate membrane (CA38) showed a low retention towards several phenolic compounds. Specifically, syringic acid, (+) – catechin, (-) – epigallocatechin and quercitin aglicone, all presented a retention lower than 5%. On the other hand, the same membrane presented a rejection towards the vanillic acid, gallic acid, fertaric acid and courtaric acid in the range of 5-10%.

The results obtained in this experiment show that the ultrafiltration treatment using the CA38 cellulose acetate membrane (made in the laboratory) is a low-cost alternative for the effective separation of polysaccharides and various polyphenolic species, obtaining two rich streams of these compounds.

Nanofiltration treatment for the bioactive compounds from red mixed grape pomace

In this experiment, the sustainable valorization of grape pomace through membrane processing is proposed. Through the preparation, characterization and evaluation of cellulose acetate membranes in flat sheet configuration, to determine their selectivity towards phenolic compounds, performance and comparison with a commercial nanofiltration membrane.

The CA400-22 membrane exhibited low glucose and fructose retention values 19.5% and 12.5%, respectively) and rejection of phenolic compounds and proanthocyanidins of 73% and 92%, respectively. Therefore, this membrane is a suitable candidate for the fractionation of phenolic / sugar compounds. Furthermore, its productivity was the highest under selected nanofiltration extract processing operating conditions. The results obtained show that the treatment of NF with cellulose acetate membranes could be a useful and sustainable approach for the recovery of valuable fractions of grape pomace, for the production of innovative formulations with specific requirements, in both the pharmaceutical and food industries.

Nanofiltration treatment for the bioactive compounds from red mixed grape pomace

The membranes CA316-70, CA316 and CA400-22 all presented high productivity in the permeate flow compared to the NF90 membrane. However, all the membranes presented a rejection greater than 95% towards polysaccharides. The fraction obtained from the CA400-22 membrane presented the highest content of polyphenolic compounds and a near absence of polysaccharides, in addition to a high productivity in the permeate flux.

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Microwave-assisted extraction and membrane-based separation of biophenols from red wine lees

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ABSTRACT

Winemaking generates huge quantities of waste streams with high added value which can be recycled for the production of innovative products in different market areas. In particular, wine lees contain high concentration of bioactive molecules which can be exploited to obtain extracts or semifinished products of interest for food, nutraceutical and pharmaceutical applications.

In agreement with a biorefinery approach applied to winemaking, this work aimed at evaluating the potential of an integrated process, based on a combination of microwave-assisted extraction and membrane-based operations, for the recovery of phenolic compounds from red wine lees before using biomass for energy purposes.

Specifically, the hydro-alcoholic extract was clarified by microfiltration (MF) and then processed with three different polymeric membranes: an ultrafiltration (UF) membrane with a molecular weight cut-off (MWCO) of 1000 Da (Etna 01PP) and two nanofiltration (NF) membranes with MWCO of 150–300 Da (NFT50 and Desal DK).

The performance of selected membranes was measured in terms of permeate flux, fouling index and retention of phenolic compounds and sugars. Experimental results indicated that all these parameters are mainly affected by membrane material and pore size. All tested membranes did not show a preferential rejection of phenolic compounds over sugars. Among the selected membranes the NFT50 presented the highest retention towards phenolic compounds allowing to maximize the recovery of these compounds in the retentate fraction.

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1. Introduction

The winemaking industry produces a large amount of organic and inorganic wastes in a short period of time creating serious environmental problems due to their composition (Meyer et al., 1998). The most significant residues are represented by grape pomace (a solid material separated from the juice in the pressing step), grape seeds and lees (residues formed at the bottom of wine containers after fermentation, during storage or after wine filtration). The worldwide production of grapes in 2017 was of about 79.8 mt (OIV, 2018); it has been estimated that for each tonne (t) of processed grapes are generated 0.13 t of marc, 0.06 t of lees, 0.03 t of bunches and 1.65 m^3 of wastewater (Oliveira and Duarte, 2016).

Conventional treatments of winery wastes are increasingly expensive, requiring significant amounts of effort, resources and energy for a safe discharge into the environment (Zacharof, 2017). Therefore, wine industries are strongly motivated in finding new technological approaches able to decrease the impact of agro-industrial residues on

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Article

Recovery of Phenolic Compounds from Red Grape Pomace Extract through Nanofiltration Membranes

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Abstract: The winemaking process generates a large amount of residues such as vine shots, stalks, grape pomace, and wine lees, which were only recently considered for exploitation of their valuable compounds. The purpose of this work was to investigate the performance of nanofiltration for the recovery of phenolic compounds, with bioactive capacity like antioxidant, from red grape pomace extract. Four membranes were compared in this study—three cellulose acetate (CA series: lab-prepared by phase inversion) and one commercial (NF90). All membranes were characterized for their hydraulic permeability and rejection coefficients to reference solutes like saccharose, glucose, raffinose, polyethylene glycol, sodium chloride, and sodium sulfate. Permeation flowrates and rejection coefficients towards total phenolics content, antioxidant activity, proanthocyanidins, glucose and fructose were measured in the nanofiltration of grape pomace extract using selected operating conditions. Among the investigated membranes, the CA400-22 exhibited the highest permeate flux (50.58 L/m^2 h at 20 bar and 25 °C), low fouling index (of about 23%), the lowest rejection coefficients towards the reference solutes and the best performance in terms of separation between sugars and phenolic compounds. Indeed, the observed rejections for glucose and fructose were 19% and 12%, respectively. On the other hand, total phenolics content and proanthocyanidins were rejected for 73% and 92%, respectively.

Keywords: antioxidants; biorefinery; process optimization; fractionation; sustainability; winemaking exploitation

CHAPTER 4

Nomenclature:

A.A: Antioxidant activity

A.A: Antioxidant activity

A1: Permeate stream obtained from CA316 membrane

A2: Permeate stream obtained from CA400-22 membrane

A3: Permeate stream obtained from CA316-70

A4: Retentate stream obtained from the NF process

A_w: Water activity

BBC: Bioaccessible bioactive compounds

CCD: Central composite design

CE: Catechin equivalents

DoE: Design of Experiments

dw: Dry weight

DY: Drying yield

HPLC: High Performance Liquid Cromatography

MD: Maltodextrin

ME: Microencapsulation efficiency

Mo: Moisture content

NF: Nanofiltration

PR: Polyphenols recovery

PR: Polyphenols retention

SB: Surface bioactive compounds

SBC: Surface bioactive compounds

SD: Standar deviation

SGF: Simulated gastric fluid

SIF: Simulated intestinal fluid

SSF: Simulated salivary fluid

TBC: Total bioactive compounds

Tg: Transition temperature

TPC: Total polyphenol content

v/v: Volume/volume

µM: Micromol

µm: Micrometro

4.0. MICROENCAPSULATION TECHNOLOGY FOR BIOACTIVE COMPOUNDS

Abstract Polyphenolic bioactive compounds were recovered by ultra and nanofiltration of Cabernet sauvignon wine lees (first racking) and encapsulated with maltodextrin, to obtain a spray dried micropowder with enhanced nutritional value. The spray drying process was optimized by means of a quadratic factorial design (Central Composite Design, CCD), using a commercial grape extract as the reference source for bioactive polyphenols. The maltodextrin (carrier) and the inlet temperature of the spray drier were the independent variables. Optimized conditions were then applied to obtain microencapsulated powders from permeates and retentate derived from the filtration process (see chapter 3). The microcapsules were characterized according to the drying yield (DY) and moisture (Mo), total bioactive compounds (TBC), surface bioactive compounds (SBC), microencapsulation efficiency (ME), polyphenols recovery (PR), antioxidant activity (AA) and bioaccessible bioactive compounds (BBC). Furthermore, the stability of the bioactive microcapsules under optimal conditions was investigated using the stress-heat test (isothermal conditions 50°C for 22 days) and the degradation rate constants of the TBC and AA were also evaluated. Ultimately an *in-vitro* simulated digestion was performed under physiological conditions to investigate the nutritional value of microencapsulates powders.

4.1. Introduction

Microencapsulation is defined as the process in which the microparticles or droplets are surrounded by a wall material and its acts as a physical barrier between the core and the other materials present in the product. The wall material could be either pure and homogeneous or a mixture and heterogeneous. The wall material is also designated as packing material, coating material, capsule, membrane, external phase or carrier. This core material is also named as core actives, coating material, internal phase or fills payload. (Fang & Bhandari, 2010).

The main objectives of microencapsulation are the following (Shahidi et al, 1993):

- Adaptation of the release of core material

- Protect the core material from undesirable environmental conditions (such as light, humidity and oxygen), thus reducing its reactivity with the external environment.

- Suppress the unwanted aroma or flavor of the core material

- Modification of the physical characteristics of the original material for easy handling

- Dilution of the wall material when it should be used in small quantities

- Separate the components of the mixture that can react easily

Various techniques used for microencapsulation process in food industry include spray drying, spray chilling, air suspension coating, extrusion, spray cooling, fluidized bed coating, centrifugal extrusion, freeze drying, coacervation, co crystallization, lipose entrapment, interfacial polymerization, molecular inclusion (Kandansamy & Somasundaram, 2012).

Microencapsulation by spray drying involves four steps which are preparation, homogenization, atomization and dehydration (Shahidi et al., 1993).

Spray drying techniques were used for the first time in the mid-nineteenth century when the process of drying eggs was investigated, specifically in 1865. Its use on an industrial scale started in the 1920s and the first products manufactured by this technique were milk and powdered detergent. Since then, its use has been widely used by all the processing industries with extensive application in the food, pharmaceutical and chemical industries (Pereira Silveira et al., 2009).

The spray drying process consists of transforming a product from a fluid to a solid state in powder form, by means of the dispersion of the microdroplets of the product inside a chamber in which it comes into contact with hot air. Spray drying results from the application of energy, which current on the liquid, to the point of causing its rupture and disintegration. This divides it into millions of individual particles, thus creating a mist or dew of microdroplets. The main advantages of this type of drying process are its high performance and a reduction in the exposure time of the product to high temperatures (Finney et al., 2002; Rodríguez-Hernández et al., 2005). Figure 1 shows the process of spray drying.

Figure 1. Display the process of spray drying. This process is a continuous operation that can be described in three stages:

- First stage: Atomization is carried out where the liquid feed is transformed into microdroplets, which can be called "spray" when creating the droplets. The surface of the fluid increases considerably, which is important in ensuring rapid and efficient evaporation of moisture from the drying droplet.

- Second stage: Microdroplets come into contact with the drying medium (sprayed air). Hot dry air is generally used as the drying medium.

In the case of very sensitive or reactive materials, it is possible to use nitrogen as a drying medium.

In this stage, the best contact conditions between the microdroplets and the air must be achieved, and consequently the formation of particles inside the drying chamber will occur.

- Third stage: This phase gives rise to evaporation or drying and therefore the formation of particles within the drying chamber. The evaporation of water from the microdroplet or dry particle occurs

on the surface. The particle is cooled by the evaporating water. During this phase of the spray drying process, speed is constant, and a solid structure develops inside the drying drop and water transport begins through the capillaries to the surface of the particle. At the end of this phase, the moisture content on the surface of the microdroplet has decreased to the moisture content that develops when it comes into contact with saturated humidified air.

In this phase of the spray drying process, the moisture content on the surface of the particle decreases further to a state of equilibrium with the drying material. Heat conduction in the product takes place at this stage and adversely affects the drying speed. The moisture bound by sorption begins to be removed from the microdroplet. It is noted that the drying rate decreases even more due to a decrease in the vapor pressure difference between the interior of the microdroplet and the drying material.

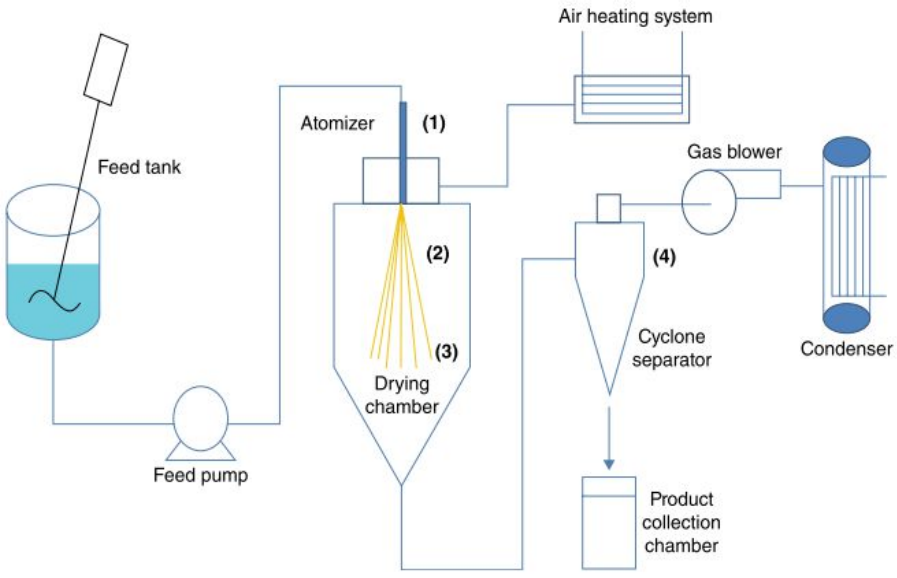


Figure. 1. Process of spray drying. 1) Atomization; 2) Spray-hot air contact; 3) Evaporation of moisture; 4) Product separation (Anandharamakrishnan & Ishwarya, 2015)

The product can be recovered directly from the bottom of the drying chamber or in the case of fine particles by separating the air with cyclones. There are a number of variables to consider in the spray drying process including the chemical and physical characteristics of the feed, the design and

the operation of the dryer, as well as how the products are formed in a powder state, either granulated or agglomerated (Drusch & Diekmann, 2015).

4.1.1. Contact of the microdroplets with the drying material

At the time of atomization during the spray drying process, the microdroplets of liquid come into contact with the drying medium. The most common drying medium is hot air at an inlet temperature between 150 ° C and 200 ° C. In some applications an inert gas such as nitrogen is used. Inert gas avoids the risk of explosion when flammable or when explosive organic solvents are involved, as well as oxidation in the case of products sensitive to contact with oxygen. In this case, a closed system is required in which an inert gas is used. In the case of hot air in an open system, the filtered air can be exhausted to the atmosphere.

The contact between the microdroplet and the drying material determines the drying kinetics and the properties of the dust particles. In this context, the positioning of the atomizer unit and the air supply and consequently the air flow pattern within the drying chamber, are all important critical points.

The main advantages for the use of microencapsulation technology include:

- Protection of the product from the environment (Temperature, humidity, UV radiation and interaction with other materials)
- Protection of the environment against any dangerous or toxic agents
- Decrease in the rate of evaporation or transfer of the core material to the experimental environment.
- Dry handling: conversion of sticky liquids and solids into powders.
- Masking of any undesired properties of the active component such as taste, odor, pH or catalytic activity.
- Control of the release rate of the core material under the desired conditions.

For food matrices a significant advantage of microencapsulation is that it protects sensitive food components from other food ingredients during storage. Protection against nutritional loss and can even be used as an added value product to other food matrices after processing. Other benefits include the incorporation of unusual or prolonged release mechanisms in the formulation and masking or preserving of flavors and aromas. Finally, an additional attraction is provided for the exhibition and commercialization of food products, which provides greater flexibility and control in the development of food (Ré, 1995).

Encapsulation involves incorporating various ingredients into a capsule approximately 5 to 300 microns in diameter. The capsule can be made of sugars, gums, proteins, natural and modified polysaccharides, lipids and synthetic polymers. Figure 2 display different morphology of the microcapsules.

Encapsulation can be in many different forms such as a single membrane liner, a spherical or irregular shaped wall or membrane, a multi-walled structure with walls of the same or different compositions or numerous cores within (Gibbs et al., 1999).

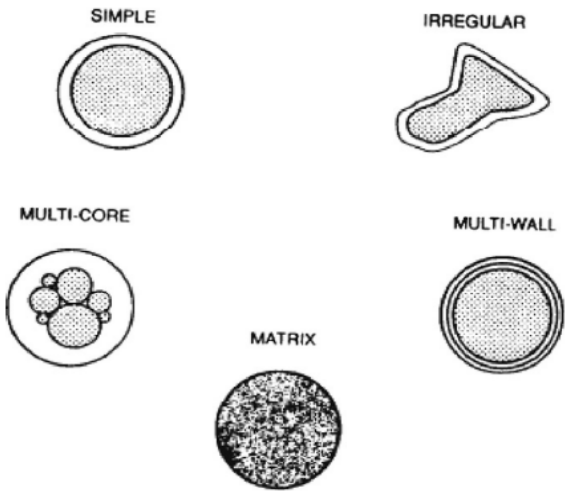


Figure. 2. Morphology of different types of microcapsules (Gibbs et al., 1999)

4.1.2. Characteristic of operating conditions

To obtain good efficiency in the microencapsulation process and even if the wall material is adequate, it is required to use optimal spray drying conditions.

The main spray drying factors to be optimized are the inlet and outlet temperatures of the drying medium (usually air). The feeding temperature modifies the viscosity of the emulsion, its fluidity and consequently its homogeneous spraying capacity. When the temperature of the feed is increased, the viscosity and the size of the droplets decrease, even so the high temperatures can cause volatilization or degradation of some thermosensitive compounds. The feed rate delivered to the atomizer is adjusted to ensure that each sprayed microdroplet reaches the desired level of dryness before it comes into contact with the surface of the drying chamber. Further, it is important to consider the proper setting of the air inlet temperature and flow rate. In fact, the inlet temperature of the drying medium is directly proportional to the drying speed of the microcapsule and the final water content (Liu et al., 2004; Zbicinski et al., 2002).

When the inlet temperature of the drying material is low, the low evaporation rate causes the formation of microcapsules with membranes of high density, high water content, poor fluidity and ease of agglomeration. Even so, when a high inlet temperature occurs in the drying material it causes excessive evaporation and results in cracks in the membrane that induce a subsequent premature release and a degradation of the encapsulated compound of interest.

The inlet temperature of the drying material is determined by two factors which are: 1) Inlet temperature of the drying material that can be used without damaging the product or creating operational risks and 2) the comparative cost of the sources of drying (Gharsallaoui et al., 2007; Zakarlan & King, 1982). The temperature at the end of the drying zone (Air outlet temperature) obtained under certain conditions can be considered as the dryer control index. It should be noted that it is quite difficult to predict the outlet temperature in advance for a given product because it depends on the drying characteristics of the material. As opposed to the air inlet temperature, the air outlet temperature cannot be controlled directly, as it depends on the air inlet temperature. In Table

1 the experimental conditions and the target compounds encapsulated by spray drying technique are given.

The best conditions for the spray drying process include a compromise between high air temperature, high concentration of solids in the solution and easy spraying and drying without expansion and cracking of the final particles

The main limitation of the spray drying technique in the microencapsulation of compounds of interest is the limited number of wall materials available and that they must have good solubility in water. Another limiting aspect that must be taken into account, is the application of any additional process on the powder obtained after spray drying process, such as agglomeration (Gharsallaoui et al., 2007).

Table 1. Experimental conditions and target compounds in the spray drying process

Feed solution	**Encapsulated compound**	**Wall material**	**Air inlet temperature (°C)**	**References**
Cactus pear (Opuntia ficus-indica)	Phenolic, indicaxanthins, betacyanins	Maltodextrin- inulin	140	(Saénz et al., 2009)
Cactus Pear Juice (Opuntia streptacantha)	Vitamin C	Maltodextrin	205 1930[2]	(Rodríguez-Hernández et al., 2005)
lemongrass oil	Oil	Gum arabic, cassava, corn maltodextrins, octenyl succinic anhydride	170	(Carvalho et al., 2019)
Raisin Juice Concentrate		Maltodextrin	110	(Papadakis et al., 2006)
Cranberry juice	Phenolic compounds, anthocyanin	Arabic gum, maltodextrin	185	(J. Zhang et al., 2020)
Pomegranate juice	Phenolic compounds, Anthocyanins	Maltodextrin soybean protein isolates	120-153	(Robert et al., 2010)
Wine lees	Phenolic compounds	Maltodextrin, aerosil-200	150	(Pérez-Serradilla & Luque de Castro, 2011)

4.1.3. Wall material selection

An important step for the successful development of the microcapsules through the spray drying process is the appropriate selection of the wall material because it must meet required criteria such as mechanical resistance, compatibility with the food product, thermal release or appropriate dissolution and suitable particle size. The selection of wall materials for microencapsulation by spray drying has traditionally involved trial and error tests in which the microcapsule is formed.

These tests are evaluated by assessing a number of factors that help to determine the encapsulation efficiency including, the stability in different storage conditions, the degree of protection provided to the encapsulated compound of interest and the observation of the surface by means of scanning electron microscopy (Pérez-Alonso et al., 2003).

4.1.4. Microcapsule Wall Materials Type

Numerous materials are commercially available for utilization as encapsulating agents. The most commonly used are:

- Carbohydrates (starch, maltodextrin, corn syrup, cyclodextrins)
- Cellulose ethers and esters (Carboxyl methylcellulose, methylcellulose, ethyl cellulose)
- Gums (acacia gum, agar, sodium alginate)
- Lipids (wax, paraffin, fats, oils)
- Proteins (gelatin, soy protein, whey protein)

4.1.4.1. Carbohydrates

Carbohydrates are widely used as encapsulating agents and are considered good encapsulating agents because they exhibit low viscosities with high solid contents and good solubility. However, most carbohydrates lack the interfacial characteristics required, resulting in a low microencapsulation efficiency and are therefore regularly associated with other encapsulating materials such as proteins or gums. Some hydrolyzed starches such as maltodextrin or corn syrup and modified starches, are widely used for encapsulation by spray drying due to their aqueous

solubility, low viscosity and ease of drying. Maltodextrins and corn syrup solids generally do not result in good retention of volatile compounds during the drying process due to their poor film-forming ability (Gharsallaoui et al., 2007; Ré, 1995).

4.1.4.2. Gums

Gums have been widely used due to their emulsion stabilizing characteristics. One of the best-known gums is gum arabic, which is considered one of the best because of its emulsifying properties. Gum arabic is a polymer that consists of D-glucuronic acid, L-rhamnose, D-galactose and L-arabinose, and with approximately 2% protein, which is attributed to emulsifying properties since it acts as an interface between the oil and the water. The film-forming characteristics come from the arabinogalactan fraction of the gum. the low viscosity and consequently the high solubility of this portion is probably responsible for the barrier film that forms after evaporation of water during drying (Drusch & Diekmann, 2015; Gharsallaoui et al., 2007). Despite these desirable characteristics, high costs, limited supplies, and quality in variations have restricted the use of gum arabic for the purpose of encapsulation and have led researchers to search for alternative microencapsulant materials such as mesquite gum (Beristain et al., 2001).

4.1.4.3. Proteins

The desirable functional characteristics of proteins make them a good coating material for spray drying microencapsulation. A significant benefit of proteins is their ability to bind to flavoring compounds. The proteins most widely used for the encapsulation of ingredients at the food level are milk protein (also known as whey) and gelatin (Landy et al., 1995).

4.1.5. Gastrointestinal Digestion for bioactive compounds

Many food industries such as the wine industry have a negative environmental impact due to the presence of residual phenols in their waste originating from the biological raw material used. These compounds considerably increase the biochemical and chemical oxygen demands with adverse

effects on the ecosystems within the discharge areas. Furthermore, solid residues used for obtaining fertilizers, often have relatively high levels of phenolic compounds, which are a problem due to their inhibition of germinal properties. However, polyphenols from wine process residues (grape pomace and wine lees) have many beneficial effects on human health such as cardioprotective, anti-inflammatory, antifungal, antimicrobial and anticancer properties (Brezoiu et al., 2019; Paulino et al., 2016). Thus, phenolic compounds can be considered value added by-products which motivate their extraction from industrial wastes. In fact, these residues could be an alternative source for obtaining natural antioxidants that are considered completely safe when compared to synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), which are used in the food industry and have undesirable effects on enzymes within human organs (Negro et al., 2003).

Grapes have one of the highest sources of phenolic compounds in fruits. From a qualitative point of view, grape polyphenols belong to different classes distributed in each part of the fruit. The skin contains the highest amount of polyphenols and in particular condensed tannins, flavonols and monomeric flavanols, phenolic acids and resveratrol. In red grape varieties, it has been reported that the skin contains anthocyanins, which are responsible for the color of the berries. The main components of the pulp are phenolic acids and monomeric flavonoids such as flavonoids (Manach et al., 2004; Mané et al., 2007; Pinelo et al., 2006).

Under in vivo conditions, polyphenols from the human diet must be removed after gastrointestinal digestion. The nature of the extractable phytochemicals, their stability and antioxidant activity depend on many factors, such as the food matrix, pH, temperature, the presence of inhibitors or enhancers of absorption, the presence of enzymes, the host and other related factors (Tagliazucchi et al., 2010). The bioavailability and metabolic fate of polyphenols is one of the main issues to be considered. The bioavailability of a dietary compound depends on its digestive stability, its release from the food matrix and the efficiency of its transepithelial passage. Bioavailability, which means

the release of the food matrix, differs greatly from one polyphenol to another and for some compounds depends on the dietary source. The absorption of phenolic compounds is considered low, not exceeding plasma concentrations of 10 μM. This low absorption can be partially attributed to the chemical structures of different polyphenols that determine their intestinal absorption. The polyphenols that are best absorbed in humans are isoflavones and gallic acid, followed by catechins, flavonones and quercetin glucosides. The polyphenols that are least absorbed in humans are proanthocyanidins, galloylated tea catechins and anthocyanins. (Manach et al., 2005b).

4.2. Materials and methods

4.2.1. Materials and chemicals

The extract used to set-up the experimental design was a commercial grape tannin from Laffort (Bordeaux, France); it was supplied as a liophilized powder and directly dissolved in Milli-Q water at the 1 g/L concentration. The Milli-Q water was obtained from a Milli-Q water purification system (Millipore Corp., Bedford, MA, USA).

Maltodextrin Dridex 13-17 (MD 13-17 DE, Merk Group, Darmstadt, Germany) was used as the natural carrier for microencapsulation of the bioactive polyphenols.

The gallic acid and (+)-catechin standards were used for quantitation, as well as the Folin-Ciocalteu phenol reagent, anhydrous sodium carbonate and the standard polyphenols used for HPLC calibration were purchased from Sigma–Aldrich (St. Louis, USA).

4.2.2. Polyphenolic profile and antioxidant activity of grape extract and wine lees filtrates

Samples obtained from wine lees filtration (both permeates and retentate) were collected and analyzed in terms of total bioactive compounds, anthocyanins and simple phenolic (monomers) fraction. Results were presented and contextually discussed in the Chapter 3 (section 3.3.3).

The grape extract (1 g/L concentration in 12% hydroalcoholic solution) used to build the experimental design for optimal spray drying conditions, was analyzed in terms of total bioactive

compounds (Ribéreau-Gayon et al. 1976), with results expressed as mg gallic acid equivalent/g of the powder (mg GAE/g dw); iron-reactive polyphenols and proanthocyanidins (Harbertson et al., 2002), with results expressed as mg (+)-catechin equivalent/g of the powder (mg CE/g dw).

Polysaccharides in the grape extract were measured according to the colorimetric method described by (Segarra et al., 1995).

The antioxidant activity (AA) was determined by means of the 2,2 -azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS•+) – based colorimetric assay, according to the method described by (Re et al., 1999); results were expressed as (%) scavenging activity.

4.2.3. Central Composite Design of Experiment (CCD) applied to the spray drying process

A Mini Spray Dryer B-191 (Büchi Laboratoriums-Technik, Flawil, Switzerland) equipped with a 0.7 mm nozzle was employed for obtaining microencapsulation of the grape-derived polyphenols. The following operational parameters were kept constant throughout the experiments: aspiration rate 100 %, compressed air flow 800 NI/h, pressure 50 mbar, percentage of the peristaltic pump 20%. Oulet temperatures in the experimental design were 79, 100 and 124 °C, corresponding to inlet temperatures of 110, 135 and 160 °C, respectively.

Central Composite Design of Experiment (CCD) including two replications in the central point, was used to optimize the spray drying conditions (Design Expert 11.0v, USA). Table 2. shows the experimental design included 10 experiments and their replications, with an overall 20 determinations. Variable factors were: inlet temperature of the spray drier (Ti, variability range 110 – 160°C) and maltodextrin concentration levels (MD 13-17 DE, variability range: 5-15 g/100 mL). MD was added directly in the grape tannin solution at the different concentration levels provided by the experimental design (5%, 10%, 15%, respectively). A large excess of natural carrier was applied for ensuring high microencapsulation efficiency; the carrier/extract ratios varied between 5:0.1, 10:0.1 and 15:0.1 (i.e., 5 g of carrier per 0.1 g of grape extract, thereof containing 67.7% total polyphenols).

Optimal conditions from the experimental design were applied to the grape extract and to the wine lees filtrates (samples obtained from the permeate streams of the membranes CA316 (A1), CA400-22 (A2) and CA316-70 (A3) and, retentate (A4); See section 3.3.3. Chapter 3).

Table 2. the central composite design (CCD) experiment used to optimize the spray drying process parameters.

Observations	Maltodextrin (g/100 mL)	Temperature (°C)
1	15	135
2	10	135
3	10	135
4	15	110
5	10	110
6	15	160
7	10	160
8	5	135
9	5	110
10	5	160

The equipment was carefully washed with water between different spray drying processes. All spray-dried powders were collected, weighed, sealed in plastic vials and immediately used for the analytical determinations. Figure 3. summarizes the spray drying process.

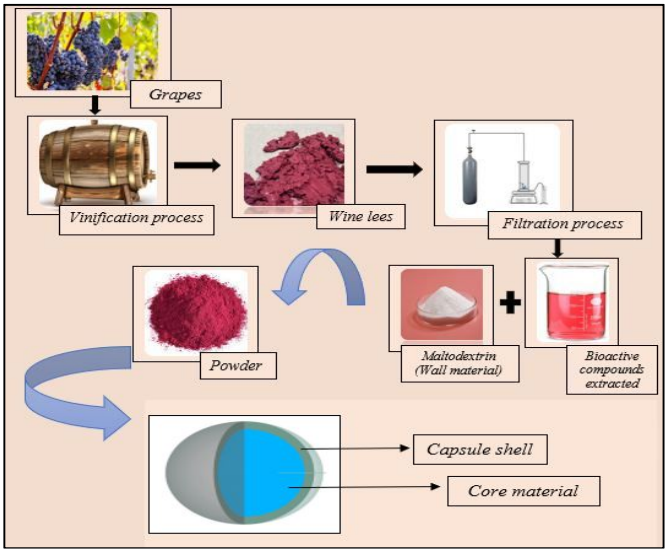


Figure. 3. Schematic representation of the spray drying experiment.

Optimal conditions from DoE were applied to the spray drying of the grape extract and to the wine lees filtrates in maltodextrin. For the spray drying of ethanolic compounds (wine lees filtrates) a Mini spray drier Büchi B290 equipped with Inert loop B295 (condenser), and a 0.7 mm nozzle was used. The following conditions were applied for analysis: 6 mL/min feed rate (20 %), 100% aspiration. The analysis was performed under a 600 NL/h dry nitrogen flow to provide enhanced protection against undesirable oxidations (residual oxygen levels during the experiments: 0.35 ± 0.10 %). Outlet temperatures ranged 63 – 70°C in these experiments.

4.2.4. Analysis of the spray-dried powders

4.2.4.1. Drying yield (DY)

The spray drying yield was evaluated following the procedure reported by (Fazaeli et al., 2012) and expressed as the percentual ratio between the total mass of product recovered by the mass of extract fed to the system (dry basis).

4.2.4.2. Moisture content (Mo)

The moisture content was determined using a method inspired by the official AOAC method (AOAC, 1990), with minor modifications. Duplicate samples of microencapsulate powder (1 g each) were weighed and then dried in a vacuum oven at 50 °C. The monitoring of weight loss was repeated on a daily basis; the Moisture content (Mo) was expressed as the percentage of weight reduction at the time when constant weight was obtained (1), according to (Mohammed et al., 2017):

$$Moisture\ (\%) = \left(\frac{W1 - W2}{W1}\right) * 100 \qquad (1)$$

With:

W1 = weight of the sample before oven-dried (g)

W2 = weight of the sample after oven-dried (g)

4.2.4.3. Total bioactive compounds (TBC)

The analysis of total bioactive compounds (TBC) in the dried powder was performed in line with the work by (Robert et al., 2010) with minor modifications: briefly, the microcapsules were destructed by adding 25 mg of the dried powder in 1 mL of methanol: acetic acid: water solution (50:8:42 v/v/v). Microcapsules were dissolved by vortex (1 min) followed by ultrasonication (20 min); the procedure was repeated twice. Samples were then centrifuged at 14500 rpm for 5 minutes, after which the supernatant was collected and filtered using a 0.22 µm cellulose acetate syringe filter.

The TBC value was determined using the Folin-Ciocalteau method according to (Ribéreau-Gayon et al., 1976) and results were expressed as mg GAE/100 mg microencapsulated powder.

4.2.4.4. Surface bioactive compounds (SBC) and microencapsulation efficiency (ME)

The analysis of surface bioactive compounds (SBC) in the dried powder was undertaken following the procedure reported by (Robert et al., 2010) with minor modifications: briefly, 25 mg of microcapsules were added to 1 mL of a mixture of ethanol and methanol (1:1 v/v); samples were vortexed for 1 min, before being filtered using a 0.22 µm cellulose acetate syringe filter. The SBC value was determined using the Folin-Ciocalteau method (Ribéreau-Gayon et al., 1976) and results were expressed as mg GAE/100 mg microencapsulated powder.

The following equations were applied to obtain SBC and ME percentages (Robert et al., 2010):

$$SBC\ (\%) = \left(\frac{BC}{TBC}\right) * 100 \qquad (2)$$

$$ME\ (\%) = 100 - SBC\ (\%) \qquad (3)$$

4.2.4.5. Polyphenols retention (PR)

Polyphenols retention (PR) after spray drying was calculated according to the method described by (Fang and Bhandari., 2011), based on dry matter measurements (4):

$$PR\ (\%) = \left(\frac{TBC\ (\%) In\ spray\ dried\ powder\ \left(\frac{mg}{100\ mg}\right)}{TBC\ (\%) in\ feed\ solution\ \left(\frac{mg}{100\ mg}\right)}\right) * 100 \qquad (4)$$

4.2.4.6. Antioxidant activity (AA)

The antioxidant activity of the dried powder was evaluated by means of the ABTS•+ colorimetric assay (Re et al., 1999) following preliminary treatment of the microcapsules described in Section 4.2.4.3. Results were expressed as percentage scavenging activity, using the following equation (5):

$$AA\ (\%) = \left(\frac{Abs - 734nm_{reagent\ blank} - Abs - 734nm_{sample}}{Abs - 734nm_{reagent\ blank}}\right) * 100 \qquad (5)$$

4.2.4.7. Storage stability evaluation

Microcapsules obtained with the optimal conditions were assessed for their stability under accelerated aging conditions. In detail, 1 gram of each of the three replicates was placed in a plastic vessel and stored at 50 ± 1 °C for 22 days. Aliquots (25 mg each) of the microencapsulates were collected every two days and measured according to TBC (mg/100 mg) and AA (%) values. The kinetic rates of TBC and AA decrease over time can be written as (6):

$$\frac{-d[\mathrm{A}]}{dt} = k[\mathrm{A}] \qquad (6)$$

Rearrangement yields the following (7):

$$\frac{d[\mathrm{A}]}{[\mathrm{A}]} = -\,k\,\mathrm{dt} \qquad (7)$$

To obtain a linear equation we integrate the Eq. (7) to obtain (8):

$$\ln[\mathrm{A}] = -kt + C \qquad (8)$$

Considering the general equation of a straight-line y = mx + b, we consider the y-value is ln [A], m equals negative k, the x-value is t, and the y-intercept is ln $[A]_o$.

A plot of ln [A] versus t is a line with slope corresponding to negative k (first-order kinetic rate).

4.2.5. Water activity (A_w)

The water activity (A_w) of the microcapsules from Cabernet Sauvignon wine lees, following the spray drying, was measured using a water activity meter AquaLab 3T (Pullman, Washington) at 25 °C. Samples (1-2 g of each) were analyzed in triplicate and A_w were reported as mean values.

4.2.6. Digestion experiment

Microcapsules used for the digestion experiment were obtained from the Cabernet Sauvignon wine lees permeates and retentate, obtained by means of nanofiltration process with acetate cellulose and commercial membranes (See the section 3.3.3. Chapter 3). The *in vitro s*imulated gastro-intestinal digestion of the bioactive compounds encapsulated from wine lees filtrates was carried out according to the methodology reported by (Carri et al., 2014) whereby physiologically relevant simulated conditions were applied to achieve various endpoints, which included the oral, gastric and intestinal phase. The enzyme products were provided by Sigma-Aldrich (St. Louis, US).

4.2.6.1. Oral phase

1.8 g of the microencapsulated bioactive compounds from Cabernet Sauvignon wine lees was mixed with 1.26 mL of simulated salivary fluid (SSF) electrolyte stock solution (SSF corresponding constituent: K^+, Na^+, Cl^-, H_{2PO}^4, HCO^3, Mg^2, NH_4 and Ca^2 with concentrations of 18.8, 13.6, 19.5, 3.7, 13.7, 0.15, 0.12 and 1.5 mmol L^{-1}, respectively) and ground together. Subsequently, 0.18 mL of salivary α-amylase solution of 1500 U mL^{-1} was prepared in an SSF electrolyte stock solution, followed by 9 mL of 0.3 M $CaCl_2$ and 0.351 µL of water and mixed thoroughly.

4.2.6.2. Gastric phase

10 mL of the oral phase sample was taken and mixed with 7.5 mL of simulated gastric fluid (SGF) electrolyte stock solution (SGF corresponding constituent: K^+, Na^+, Cl^-, H_{2PO}^4, HCO^3, Mg^2, NH_4 and Ca^2 with concentrations of 7.8, 72.2, 70.2, 0.9, 25.5, 0.1, 1.0 and 0.15 mmol L^{-1}, respectively). Then, 1.6 mL of a 25000 U mL^{-1} pepsin stock solution portion prepared in a SGF electrolyte stock

solution (porcine gastric mucosa pepsin, Sigma-Aldrich) was added. To reach pH 3.0, 5 mL of 0.3 M CaCl2, 0.2 mL of 1 M HCl and 0.695 mL of water were used.

4.2.6.3. Intestinal phase

20 mL of the solution obtained from the gastric digestion process was taken and mixed with 11 mL of simulated intestinal fluid (SIF) electrolyte stock solution (SIF corresponding constituent: K^+, Na^+, Cl^-, H_{2PO}^4, HCO^3, Mg^2 and Ca^2 with concentrations of 7.6, 123.4, 55.5, 0.8, 85, 0.33 and 0.6 mmol L^{-1}, respectively). Following this, 5.0 mL of an 800 U mL^{-1} pancreatin solution was made up in a prepared electrolyte stock solution (SIF), based on trypsin activity (porcine pancreatin, Sigma-Aldrich), 2.5 mL of fresh bile (160 mM in fresh bile) (Bile, Sigma-Aldrich), 40 µL of 0.3 M CaCl2, 0.15 mL of 1 M NaOH were used to reach a pH of 7.0 and 1.31 mL of water.

4.3. Statistical analysis

All experiments were conducted in duplicate. Results from the CCD Experiment, including the analysis of significant effects at $p < 0.05$ and the surface response methodology, were performed using the Design Expert software (Stat-ease, Minneapolis, USA). The Microsoft Excel program (Microsoft Corporation, Washington, USA) was used to process results from the analytical determinations (expressed as mean values ± SD) as well as for the kinetic studies of results from the stability test.

4.4. Results and discussion

4.4.1. Grape extract characterization and bioactive content

Table 3. shows the proximate compositional information of the botanical extract used to develop the CDD experiment. Polyphenols constitute approximate 68% of the dry weight of the extract; most of these compounds (98.5% of the total polyphenols content) were characterized as iron-reactive polyphenols, which represent the most effective fraction against metal catalysts and free radical species. Tannins (procyanidins) make up 38.3% of the total polyphenols in the extract. The residual

dry weight fraction is dominated by polysaccharides, which are possibly derived from the structural tisses of grapes and constitute approx. 25.4% of the total dry weight of the extract.

Table. 3. Composition of the liophilized grape pomace extract used in the CCD experiment.

TPC (mg GAE/g dw)	677 ± 34
Iron-reactive polyphenols (mg CE/g dw)	667 ± 15
Tannins (mg CE/g dw)	259 ± 6
Polyphenolic substances/dry weight (%)	67.7
Tannins/TPC (%)	38.3
Polysaccharides (mg Glu/g dw)	254 ± 37
Radical scavenging (% ABTS•+ scavenging)	57.3 ± 5.6

GAE: Gallic acid equivalents; CE: (+)-Catechin equivalents; Glu: Glucose equivalents.

According to previously published research, we can consider the relevant content of complex sugars in the extract, as a potentially advantageous condition for the spray drying experiment. In fact, polysaccharides from grapes (primary pectins) are likely to interact with hydrophobic compounds (i.e. tannins) resulting in colloidal systems with high retention of polyphenolic compounds (Carvalhoet al., 2006). Moreover, previous literature has highlighted the potentially beneficial effect of maltodextrins/pectin matrix systems in improving organoleptic properties and the stablity of powders derived from spray drying technology (Sansone et al., 2011).

4.4.2. Microencapsulation of grape polyphenols

Table 3 displays results from the experimental design. The relevant parameters accounting for the effectiveness of the spray-drying process were considered and they will be discussed in this section.

It was decided to undertake the moisture content (Mo) analysis separate from the experimental design and therefore it was not included in the model. The “Mo” is an important variable when assessing the shelf life of powders, due to its relationship with the drying efficiency, powder flowability, stickiness, and storage stability (Mahdavi et al., 2016). Mo in the microencapsulates obtained from DoE ranged 0.85 to 2.80% with average 1.40 ± 0.57% value. These values were generally lower than those reported in the literature and even lower than results from experiments performed under dehumidified air conditions (Mohammed et al., 2011; Mahdavi et al., 2016; Goula

and Adamopoulos, 2005). The results were more in the range of values obtained by (Tan et al., 2015) where they used a combination of maltodextrin and gum Arabic as the encapsulating agent.

The low moisture content positively affects the physico-chemical properties of the microencapsulates limiting the ability of water to act as a plasticizer and to reduce the glass transition temperature (Tg). This is important for the following reasons:

(1) During the spray drying process, to ensure reduced surface stickiness of the droplets of sugar-enriched formulations during spray drying, thus enhancing the process yield and improving processability, handling properties and stability of powders (Adhikari et al., 2005; Roos, 2002).

(2) During storage of the microencapsulates, in fact, when the storage temperature falls above the Tg value of the powder, the amorphous phase of the powder become predominant, the molecular mobility of the matrix and the reactants are accelerated, and physico-chemical modifications (collapse, caking, agglomeration, browning and oxidation) are accelerated (Bhandari & Howes, 1999).

Regarding DoE, the response surface analysis was applied to guarantee optimal microencapsulation of polyphenols considering the linear, quadratic and cross-product forms for selected independent variables (encapsulating agent and temperature) at $P \leq 0.05$ levels, for each system. Figure 4a–e shows the graphs obtained with the response surface methodology for the CCD experimental design. The effect of the encapsulating agent and inlet temperature on selected properties of microencapsulated is shown.

The drying yield (DY, Figure 4a) of the process was not significantly affected ($p > 0.1$) by the factors selected in this DoE, i.e., the concentration of the maltodextrin as the carrier agent and the drying inlet temperature, for both linear and quadratic terms of the model. The yield of the spray drying process (DY) ranged 44.6 – 63.4 %, with average 56.6 ± 6.2 % value (Table 4); absolute values of powders obtained after spray drying followed an obvious trend related to the initial carrier concentration levels (maltodextrin: 5-10-15 g/100 mL) used in the experiments. In general terms,

the loss rates with respect to the initial solid matter fed to the spray drier ranged 32.2 – 46.5 %, with average 36.9 ± 6.7% decrease.

As shown in previous spray drying experiments (Tolun et al., 2016), part of the micro-powders obtained were possibly retained into the spray drying chamber and to the cyclone walls, to an extent which is dependent on the water retention during the drying process and final moisture content of the powder (Roos, 2002). In this experiment, the retention effects were not analysed neglected and all the experimental outcomes were established based on the spray dried material which was allowed to reach the collector cabin.

The amount of polyphenolic compounds retained after the spray drying process (PR, Figure 4b) was not significantly affected by either the carrier concentration, the inlet temperature or their combined effects, in this case study. However, the total bioactive content (TBC, Figure 4c) of the dried powders in this model ranged from 0.30 – 1.04 % of the dry weight of the microencapsulated powders (Table 3) and was significantly affected by the quadratic term of the content of wall material (p = 0.041). The other linear and quadratic terms failed to give a significant influence on the total bioactive content ($p > 0.05$). Therefore, the encapsulating agent was the most significant variable for maximizing the total polyphenols content of the powders under the experimental conditions, and it was connected to a dilution effect observed when the maltodextrin concentration was raised. The same result was obtained by Robert et al. (2010) in the microencapsulation of pomegranate juice with maltodextrin as the carrier agent, and by (Mishra et al., 2014) in the encapsulation of *Emblica officinalis* (amla) extracts.

In general, maltodextrin is a widespread biopolymer used as the carrier for polyphenolic encapsulation, as it has several advantageous properties including low viscosity at high solid contents, good solubility and notable heat protection capacity, long-term resistance, as well as having a pleasant flavor (Gharsallaoui et al., 2007, Kha et al., 2010, Robert et al., 2010). The usage of an emulsifier is not envisaged to achieve optimal spray drying of polyphenolic compounds, due to their relative hydrophilicity; nevertheless, it has been demonstrated that the combined used of

maltodextrin with biopolymers as gum Arabic and soy/whey proteins, might improve the encapsulation especially for hydrophobic polyphenols like flavan-3-ols and tannins (Busch et al., 2017; Tan et al., 2015; Tolun et al., 2016). Some authors performed comparative studies between polysaccharidic and protein -based carriers and reported that the retention of negatively charged polyphenols may be improved by using an aminoacidic polyelectrolyte carrier with higher affinity, i.e., soybean protein isolate, while the use of maltodextrin can increase the encapsulation efficiency of positively-charged anthocyanins (Robert et al., 2010; Kim and Morr, 1996). In this perspective, future studies will enable the attainment of improved results with respect to the present experiment.

Surface bioactive compound (SB) content, i.e., bioactive compounds which were not effectively encapsulated, constitute a minor fraction of the total bioactive content, in the range 0 – 9.22% of TBC. These can be considered very low values corresponding to high microencapsulation efficiencies (ME > 90%) for all experiments, regardless the carrier concentration and the inlet temperature values. In summary, none of the factors or level combinations analyzed in this experiment showed significant effect on SBC and ME ($p > 0.10$).

The ME (Figure 4d) is a key parameter for optimal spray drying processes, guaranteeing the stability and controlled release of the bioactive compounds (Ozkan et al., 2019). Previous studies on the encapsulation of phenolic compounds in maltodextrin showed lower performances with respect to this parameter; typical ME values ranged 65-77% (Paini et al., 2015), 53.5-71.0% (Robert et al., 2010), 69.6-73.4% (McNamee et al., 2001), 69-75% (Pasrija et al., 2015) and 18.4 -45.0% (Sun-Waterhouse et al., 2013) under relevant experimental conditions.

Comparable ME values to those reported in Table 3 were reached in previous experiments by (Mahdavi et al., 2016), with optimal 92.8 % ME, and also by (Zhang et al., 2007), where ME ranged between 97.9 – 99.7%. In both cases, the encapsulation was performed using combined maltodextrin – gum Arabic as the carrier agent. In the present study, where a single encapsulating agent was used, the high ME yield achieved may be due to the large excess of maltodextrin found (see section 4.2.3 for experimental details). This resulted in a limited polyphenolic content per unit

weight (up to 1.04 % of the dry weight of the powder) in favor of an increased microencapsulation yield.

The antioxidant activity (AA, Figure 4e) displayed by microencapsulates ranged 27.6-93.1 %, expressed as the ability to scavenge the synthetic ABTS•+ radical (Table 4), and it was significantly affected by the experimental factors selected in this DoE: main contributions arose from the quadratic term of the carrier (maltodextrin) concentration in the feed solution ($p = 0.003$), followed by a significant contribution of the maltodextrin – inlet temperature binary combination ($p = 0.0156$) and of both linear ($p = 0.025$) and quadratic ($p = 0.014$) terms of the inlet air temperature. Linear term of maltodextrin concentration level failed to significantly influence the antioxidant activity of the spray dried powders.

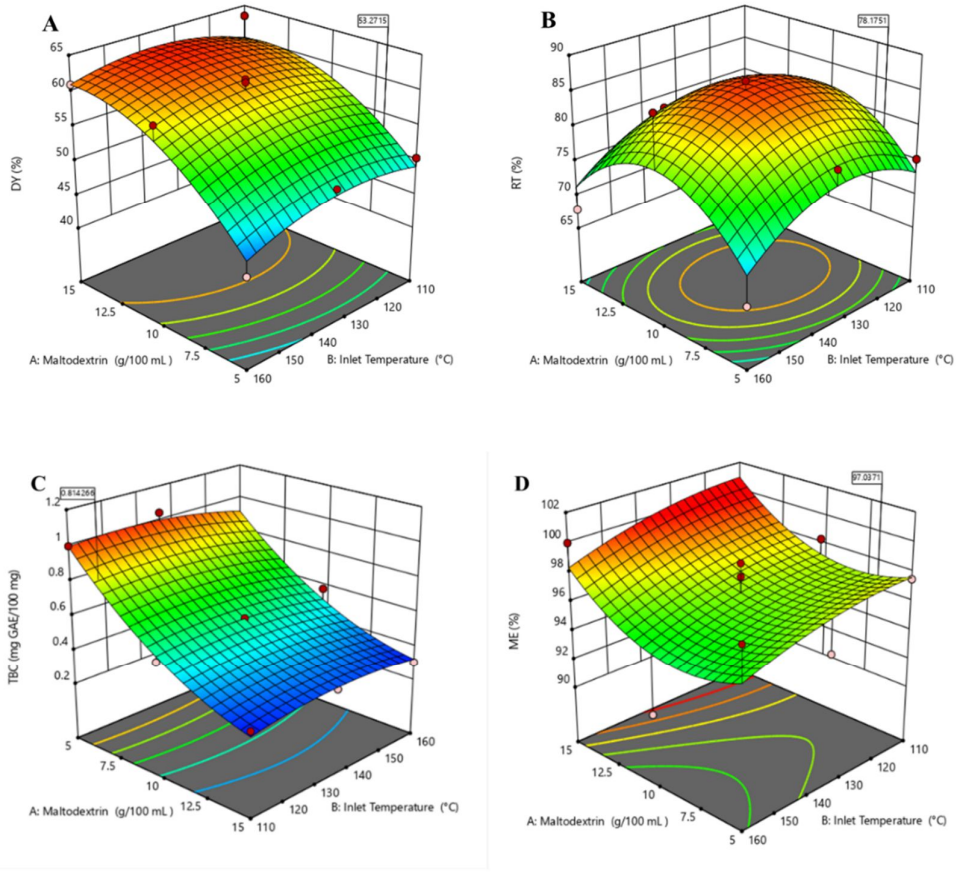
Due to high microencapsulation efficiency, the microcapsules revealed good antiradical performances with peak values when the higher active compound/carrier ratio occurred (i.e., maltodextrin level 5 g/100 mL). Previous studies (Tuyen et al., 2010; Souza et al., 2014; Silva et al., 2013) showed that the MD concentration in the feed solution displayed significant effect on the antioxidant activity of microencapsulates, and it decreased significantly when the concentration of the carrier material was increased.

The present experiment highlighted chemical inertia of the maltodextrin wall material with respect to the redox-reducing properties involving the encapsulated bioactive compounds: targeted analyses on aqueous solutions containing maltodextrin at the different concentration levels (i.e., 5 - 15 g/100 mL). This proved that the carrier did not display any ability to neutralize the ABTS•+ radical as such, showing a radical scavenging activity < 2% in all cases, and confirming previous results from the literature (Sahin-Nadeem et al., 2013; Mishra et al., 2014).

The inertness of maltodextrin with respect to reducing and redox mechanisms is possibly the basis for the enhanced antiradical and storage stability of the maltodextrin microencapsulated. Higher retention of their antioxidant properties during storage was observed compared to performances by different carrier agents, i.e., HP-β-cyclodextrin and Arabic gum (Wilkowska et al., 2016).

Considering all of the above points, it has been concluded that on the one hand microcapsules have a protective action towards the inner polyphenolic compounds, and on the other hand they do not trigger side-reactions with them, preserving their original bioactivity in time.

Figure 4a–e presents the graphs obtained with the response surface methodology for the CCD experimental design. The effect of the encapsulating agent and inlet temperature on selected properties of microencapsulated is shown.



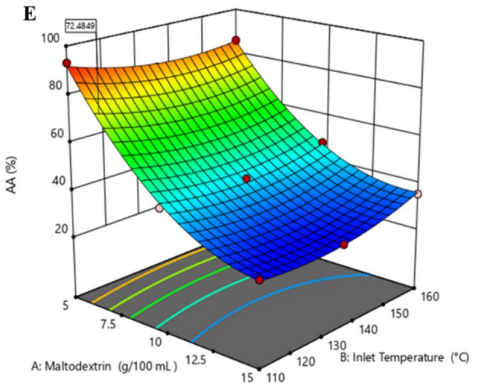


Figure. 4. Response surfaces obtained for the CCD experimental design showing the effect of the maltodextrin concentration and inlet temperature on selected properties of microencapsulated. A. Drying yield (DY, %); B. Polyphenols retention (PR, %); C. Total bioactive compounds (TBC, mg GAE/100 mg dw); D. Microencapsulation efficiency (ME, %); E. Antioxidant activity (AA, %).

The following optimal conditions were settled by DoE to optimize performances of the spray drying process: (a) Maltodextrina concentration: 7 g/100 mL; (b) Inlet temperature: 110°C. These conditions were applied to the same grape extract and to selected samples from the wine lees filtration experiment (See the section 3.3.3. Chapter 3).

Table. 4. Experimental outcomes from the CCD experiment. Results are expressed as the mean value over two replicates (±SD).

Observation	**Maltodextrin**	**Temperature**	**Spray dried powder**	**Mo**	**DY**	**TBC**	**SBC**	**SBC**	**ME**	**PR**	**AA**
Nr	*g/100 mL*	*°C*	*g (fw)*	*%*	*%*	*mg GAE/100 mg dw*	*mg GAE/100 mg dw*	*%*	*%*	*%*	*%*
1	15	135	9.50 ± 0.13	1.27 ± 0.09	59.4 ± 0.8	0.35 ± 0.01	0.003 ± 0.000	0.86 ± 0.08	99.5 ± 0.6	78.4 ± 2.3	27.6 ± 3.1
2	10	135	6.73 ± 0.32	0.99 ± 0.01	61.2 ± 2.9	0.54 ± 0.02	0.007 ± 0.002	1.37 ± 0.32	98.6 ± 0.3	80.8 ± 3.0	41.3 ± 2.02
3	10	135	6.78 ± 0.05	0.89 ± 0.06	61.6 ± 0.4	0.58 ± 0.01	0.013 ± 0.002	2.28 ± 0.38	97.7 ± 0.4	86.4 ± 1.2	43.6 ± 1.2
4	15	110	10.15 ± 0.06	1.13 ± 0.06	63.4 ± 0.4	0.34 ± 0.01	0.000	0.0	100.0 ± 0.0	76.8 ± 2.7	28.7 ± 2.9
5	10	110	5.91 ± 0.43	2.41 ± 0.55	53.7 ± 3.9	0.52 ± 0.01	0.009 ± 0.00	1.72 ± 0.02	98.3 ± 0.0	77.2 ± 0.9	44.3 ± 2.3
6	15	160	9.75 ± 0.88	0.89 ± 0.05	60.9 ± 5.5	0.30 ± 0.00	0.000	0.0	100 ± 0.0	67.8 ± 0.0	35.3 ± 2.4
7	10	160	6.57 ± 1.76	1.36 ± 0.45	59.7 ± 16.0	0.58 ± 0.02	0.053 ± 0.006	9.22 ± 0.62	90.8 ± 0.6	86.3 ± 3.7	47.6 ± 3.4
8	5	135	3.06 ± 0.13	1.48 ± 0.30	51.0 ± 2.2	1.04 ± 0.08	0.053 ± 0.020	5.15 ± 2.62	94.8 ± 2.6	78.7 ± 6.3	82.5 ± 1.1
9	5	110	3.03 ± 0.12	2.34 ± 0.26	50.4 ± 2.0	1.00 ± 0.02	0.025 ± 0.002	2.47 ± 0.16	97.5 ± 0.2	75.2 ± 1.4	93.1 ± 1.2
10	5	160	2.68 ± 0.49	1.24 ± 0.33	44.6 ± 8.1	0.87 ± 0.01	0.017 ± 0.000	2.01 ± 0.01	98.0 ± 0.0	65.5 ± 0.5	85.8 ± 2.4

Mo: Moisture content; DY: drying yield; TBC: Total bioactive compounds; SBC: Surface bioactive compounds; ME: Microencapsulation efficiency; PR: Polyphenols retention; AA: Antioxidant activity; GAE: Gallic acid equivalents.

4.4.3. Application of optimal spray drying parameters to the grape extract: characterization of the microencapsulates and stability test

Table 5. shows the experimental results for microencapsulates obtained under optimized spray drying conditions; the commercial grape extract was dissolved in the same concentration of the experimental design (1 g/L) to verify performances of the CCD outcomes.

Table 5. Characterization of the spray dried powder obtained (mean ±SD) with optimal process parameters settled. (Maltodextrin: 7 g/100 mL; inlet temperature: 110°C).

Mo	**DY**	**TBC**	**SBC**	**SBC**	**ME**	**PR**	**AA**
%	%	mg GAE/ 100 mg dw	mg GAE/ 100 mg dw	%	%	%	%
1.40 ± 0.24	70.0 ±2.7	0.73 ± 0.06	0.056 ± 0.038	7.6 ± 5.3	92.4 ± 5.3	87.4 ± 7.7	61.8 ± 1.7

Mo: Moisture content; DY: drying yield; TBC: Total bioactive compounds; SBC: Surface bioactive compounds; ME: Microencapsulation efficiency; PR: Polyphenols retention; AA: Antioxidant activity; GAE: Gallic acid equivalents.

The microencapsulated powder showed an average 29.1 ± 2.7% reduction of solid matter with respect to the solid content of the feed solution (approx. 7 g considering the maltodextrin content), and, contextually, a high yield of the drying process (DY 70.0 ± 2.7 %, Table 5). At a glance, this result is contradictory compared to the experiment 4 of the CCD, where the higher maltodextrin level, 15 g/100 mL of the feed solution provided best performances in terms of drying yield (63.4 ± 0.4 %, Table 4). It should also be noted that the maltodextrin level has not displayed significant effects on the DY according to the CCD experimental outcomes. Average solid matter recovery in the CCD experiments (56.6 ± 6.2 %, Table 4), showed a stochastic trend which was connected to the technological performances of the spray drier, in particular to the amount of solid matter which was allowed to reach the collector cabin, more than to the experimental conditions settled. The moisture content (Mo) of the microencapsulates (1.40 ± 0.24%, Table 5) was found to align

with average values observed along the CCD experiment (1.40 ± 0.57%), hence having a potentially positive impact in the storage stability (See the section 4.4.2 of this present chapter).

The TBC value (0.73 ± 0.06 mg GAE/100 mg dw, Table 5) fell within the limits set by the experimental design (0.30 – 1.04 %), and it was slightly reduced with respect to the higher values obtained within the DoE (Table 4). This is a compromise solution between ensuring high microencapsulation efficiency (higher maltodextrin contents) and maximizing the content of bioactive compounds in the total dw of the powders (guaranteed by lower maltodextrin contents). The ME parameter was 92.4 ± 5.3% in the optimized spray drying process and was noted to be an improvement with respect to previous literature where maltodextrin was used as the carrier agent (See the section 4.4.2 of this present chapter). The antioxidant activity displayed by microcapsules showed satisfactory results (61.8 ± 1.7%, as shown in Table 5), which appear to be aligned with the availability of polyphenols (TBC value) in the powders.

The retention of polyphenols (RP) during the spray drying process was high in this experiment (87.4 ± 7.7%, as shown in Table 5); nevertheless, the experiments failed to minimize the surface bioactive compounds (SBC 7.6 %), meaning that not a negligible fraction of polyphenols are not embedded as core compounds, but simply adsorbed to the surface of maltodextrin capsules. The high standard deviation (± 5.3%) informed the limited control over the amount of encapsulated polyphenols under these experimental conditions. The SB compounds do not give an advantage to supporting the protective effect of the carrier material. For this reason, a detailed study on the stability of microcapsules was set-up to predict storage stability and retention of bioactive properties of the powders obtained.

At this proposal, Figure 5 shows the natural log of the total bioactive compounds measured in the microencapsulates, monitored while maintained at a temperature of 50°C to induce accelerated aging (22 days). The degradation kinetic, resulting in a decrease of TBC content over time, fits well into a first order kinetic equation; both kinetic law and the kinetic rate value (kinetic rate observed, $K_{obs} = 1.05 \times 10^{-2} \pm 0.10 \times 10^{-2}$ days^{-1}) resemble previous findings reported in the literature (Robert

et al., 2010; Tolun et al., 2016), regardless of the fact that in both these previous studies the storage temperature was maintained at 60°C. Despite the good linearity of the graph (R^2 = 0.949, as shown in Figure 5a), it was observed that a second-order polynomial curve provided an enhanced fitting of experimental data (R^2 = 0.987, as shown in Figure 5b). This is consistent with previous observations, in fact, (Tolun et al., 2016) reported that the pseudo-first order degradation graph of polyphenolic compounds showed two distinctive steps: the second slope represented a second pseudo-first order step with a slower rate than the first slope. The authors concluded that this peculiar trend was the consequence of degradative effects involved in the surface bioactive compounds (SBC in this study), corresponding to a faster rate, followed by a slowing down of the curve in correspondence to degradation of the polyphenolic compounds (Tolun et al., 2016). This is a further confirmation of the protective effect displayed by the carrier material on the encapsulated bioactives. The limited SBC content observed in this study did not allow for the appreciation of the significant differences in the SBC degradation over time in the accelerated aging test (*data not shown*). For this reason, we assumed that the first-order linear equation could provide satisfactory kinetic description to account for the shelf life of microcapsules.

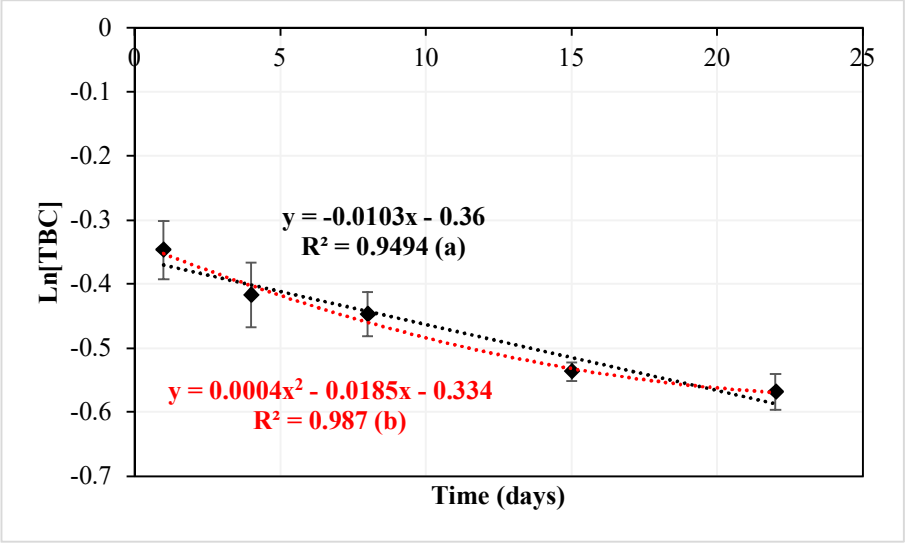


Figure. 5. First-order TBC degradation of microcapsules obtained under optimal conditions and stored at 50 ± 1°C.

Figure 6. highlights a similar trend for the reduction of the microcapsules' bioactivity over time and under heat-stress conditions. The reduction of the ABTS•+ radical scavenging capacity followed a first order kinetic (kinetic rate observed, K_{obs} = 2.37 x 10^{-2} ± 0.99 x 10^{-2} $days^{-1}$), and the same observations can be applied (Figure 6-a, b) about the potential two-step mechanism involved in the thermal degradation (see Figure 5-a and b for comparison). In absolute terms, the thermal treatment induced a percentage reduction of the antiradical capacity of 31.4 ± 4.4 % with respect to the values obtained at the time zero, meaning that approx. 70% of the original antiradical capacity is retained under extreme storage conditions.

We can conclude that when using an excess of carrier material in the spray drying procedure, it might result in an enhanced encapsulating efficiency and improved protection of the polyphenolic compounds encapsulated, with retention of their bioactive properties over time. Previous studies confirmed that samples of spray dried polyphenols showed good stability during storage in comparison to alternative storage methods (freeze-drying or simple aqueous extraction), and that the improved durability was due to the presence of an inert carrier acting as a protective agent (de Souza et al., 2014).

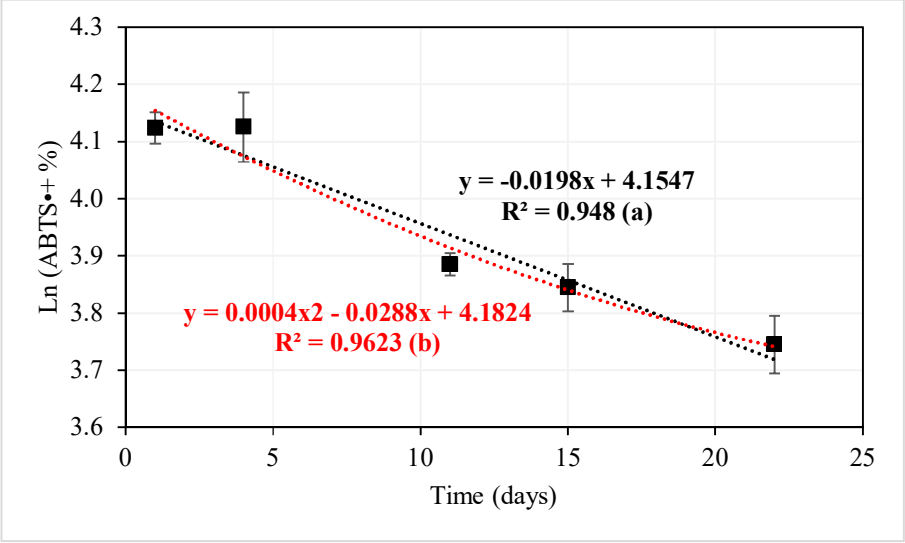


Figure 6. First-order antiradical activity (AA) decrease of microcapsules obtained under optimal conditions and stored at 50 ± 1°C.

4.4.4. Application of optimal spray drying parameters to the wine lees filtration products: characterization of the microencapsulates and *in vitro* digestion experiment

After settling on the optimal parameters in the microencapsulation of a commercial grape extract, a further experiment involved the encapsulation of polyphenol-rich solutions, which were obtained from the wine lees filtration experiment (see the section 3.3.3. Chapter 3). This experiment aimed at hypothesizing a virtuous chain for the recovery of a by-product from the wine industry (wine lees), obtaining a food supplement with high nutraceutical value. The retentate (A4) of the filtration process (see the section 3.3.3. Chapter 3) contained 587 ± 22 mg GAE/L, which resembled the total polyphenols content of the grape extract used for DoE (677 ± 34 mg GAE/L) which was obtained dissolving 1 g of the powder in 1 L water. Permeates which were selected for the spray drying experiment (Samples obtained from the permeate streams of the membranes CA316 (A1), CA400-22 (A2) and CA316-70 (A3) (see the section 3.3.3. Chapter 3) exhibit TPC values ranging 71-102 mg GAE/L.

The samples contained approximate 12% ethanol (v/v), which was trapped into a condenser during the drying process. Table 6. summarizes the experimental results by applying optimal parameters to the spray drying of the wine lees permeates/retentate and using maltodextrin as the carrier agent.

Table 6. Characterization of the spray dried powder (mean ±SD) obtained by processing wine lees permeates and retentate with maltodextrin under optimal spray drying conditions (see 4.4.2-4.4.3 for optimization of the experiment).

Sample	Sample code	Mo (%)	DY (%)	TBC mg GAE/100 mg dw	SBC (%)	ME (%)	PR (%)	AA (%)	Aw (25 °C) (%)
CA316	(A1)	1.52	73.9	0.10 ± 0.01	30.6 ± 10.4	69.4 ± 10.4	82.7 ± 4.9	8.5 ± 2.8	0.313 ± 0.0
CA400-22	(A2)	0.81	69.1	0.09 ± 0.01	34.5 ± 16.9	65.5 ± 16.9	57.4 ± 5.0	7.8 ± 1.1	0.303 ± 0.0
CA316-70	(A3)	1.27	62.9	0.08 ± 0.01	5.8 ± 2.9	94.2 ± 2.9	81.7 ± 7.5	7.2 ± 1.7	0.362 ± 0.02
Retentate	(A4)	1.32	82.0	0.57 ± 0.03	8.2 ± 1.3	91.8 ± 1.3	68.1 ± 3.1	30.5 ± 2.7	0.272 ± 0.0

Mo: Moisture content; DY: drying yield; TBC: Total bioactive compounds; SBC: Surface bioactive compounds; ME: Microencapsulation efficiency; PR: Polyphenols retention; AA: Antioxidant activity; GAE: Gallic acid equivalents; A_w: water activity.

According to the results, microencapsulates of samples CA316 (A1), CA400-22 (A2), CA316-70 (A3) and retentate A4 retained a low moisture content, ranging 0.81-1.52% with an average of 1.23%. The average moisture content was aligned with the result obtained (1.40%) in the optimization of the spray drying conditions with microencapsulation of the commercial grape extract (Table 5). The low moisture content ensures the stability of microcapsules over time if adequate storage conditions are applied; in fact, micropowders with low moisture content are more hygroscopic and their capacity to absorb humidity from the environment is related to the water concentration gradient occurring between the product and air interface (Tonon et al., 2009).

The drying yield of the wine lees microencapsulated ranged 62.9-82.0% with average 71.9%; in particular, the mean drying yield value are aligned with results reported in the paragraph 4.4.3 with optimized spray drying conditions (Table 6).

The TBC values of the microcapsules ranged 0.08 - 0.57 mg GAE/100 mg dw, with an average value of 0.21 ± 0.24 mg GAE/100 mg dw. It is worth noting that the trend in the concentration of polyphenols in the powders obtained (Table 6), produced similar concentrations of polyphenols contained within permeates and retentate from Cabernet Sauvignon wine lees, obtained in the nanofiltration process (See Table 26; Section 3.3.3.5; Chapter 3).

The encapsulation efficiency (ME) in the spray dried powder obtained by processing the wine lees ranged between 65.5 - 94.2 %, with a mean value of 80.22 ± 14.8 %. The polyphenols retention (PR) was within the range 57.4-82.7 with respect to the solution fed to the drying system, with a mean value of 72.4 ± 12.0% (Table 5). Among the samples, spray drying of CA316 (A1), CA316-70 (A3) and retentate (A4) resulted in microcapsules with higher ME and PR; however, the sample CA400-22 (A2) presented a low retention of polyphenols (57.4%) resulting in higher surface bioactive compounds level (SB 34.5%, as present in Table 6). In general, the bioactive compounds retained in the surface bioactive compounds (SBC) ranged between 5.8 - 34.5% with a mean value of 19.7 ± 14.8%. If considering the presence of a condensing system to trap the ethanol during spray

drying, it could be hypothesized that a partial migration of polyphenolic compounds towards the outer surface of the capsule is accelerated by solvent evaporation during the drying process.

The value of the antioxidant activity (A.A) of the microcapsules ranged between 7.2 - 30.5%, with a mean value of 13.5 ± 11.3% (Table 6); AA results are aligned with the total availability of polyphenolic compounds in the micropowders (TBC value).

The water activity (Aw) for the spray dried powder obtained by processing wine lees permeates and retentate ranged between 0.272 - 0.362 with a mean value of 0.3125 ± 0.03. Results were evaluated according to the food stability diagram in terms of water activity values reported by Labuza (Labuza, 1972); all values fall below the maximum acceptable value to prevent the decomposition of food matrices by microorganisms, together with biological and chemical reactions such as the Maillard reaction (non-enzymatic browning) and enzymatic activities. It can be concluded that the Aw values, together with the reduced moisture content exhibited by micropowders, might ensure good stability over time under suitable storage conditions.

The CA316 (A1), CA400-22 (A2), CA316-70 (A3) and the retentate (A4) microcapsule powders were subjected to a gastric digestion process at different endpoints (see section 4.2.6 of this present chapter). Table 7 shown the bioaccessible bioactive compounds (BBC) and the antioxidant activity (AA) as measured in the synthetic juices solution following the *in vitro* digestion process.

Table 7. Results from the in vitro digestion experiment (mean ±SD) on the microcapsules obtained from the wine lees filtrates.

Sample	**Sample code**	**BBC** (mg GAE/ 100 mg dw)	**AA** (%)
CA316	(A1)	0.07 ± 0.00	18.8 ± 4.0
CA400-22	(A2)	0.07 ± 0.02	18.0 ± 1.1
CA316-70	(A3)	0.04 ± 0.0	17.0 ± 3.2
Retentate	(A4)	0.36 ± 0.03	61.3 ± 6.5

BBC: Bio-accessible bioactive compounds; AA: antioxidant activity

One of the main issues related to the beneficial effects of polyphenols is their bioavailability. This factor depends on their digestive stability and release from the food matrix, which is known as bioaccessibility, and the efficiency of its transepithelial passage (Manach et al., 2005a).

After the three-step digestion process (oral, gastric and intestinal) the bioaccessibility (BBC, expressed as mg GAE/100 mg dw of microcapsulated) for the four samples was as follows: A1 (0.07 ± 0.0), A2 (0.07 ± 0.02), A3 (0.04 ± 0.0) and A4 (0.36 ± 0.03). Consequently, the digestion process determined a reduction of the total bioactive compounds (TBC) of 30%, 22.2%, 50% and 36.8% in the four samples, respectively. However, an increase in antioxidant activity (ranging from 100% to 136% with respect to AA of TBC samples) was registered (Table 7).

Similar behavior was reported by the authors (Tagliazucchi et al., 2010) after evaluating the content of different compounds extracted from the grape, after simulating the gastric and intestinal phase regarding the increase of the bioaccessibility of catechin after gastric digestion and a 20.5% increase of the bioaccessibility of polyphenol content after intestinal digestion.

4.5. Conclusions

The experimental design applied to the commercial grape extract allowed for the determination of the best operating conditions in the microencapsulation process. The results show a high efficiency of encapsulation of polyphenolic compounds and a good anti-radical behavior when the active compound/carrier ratio of 5 g of maltodextrin/100 mL is present. Furthermore, the low water content positively affects the microcapsules, thus reducing the glass transition temperature (Tg) and consequently avoiding accelerated oxidation modifications and abnormal formations.

Concerning the optimal drying conditions applied on wine lees filtration products, the inlet temperature of 110 °C and maltodextrin concentration of 7g/100 mL was adequately effective in the microencapsulation efficiency and retention of polyphenolic compounds. In addition, a low humidity and water activity might ensure a good stability over time, under suitable storage conditions.

The digestion *in vitro* process of the microcapsules through the different endpoints has provided information about the bioaccessibility of bioactive compounds. Our results suggest that although a reduction in the bioaccessibility of polyphenolic compounds was evidenced (Average reduction of 34.7%), the bioaccessibility of antioxidant compounds is not affected, suggesting their potential health benefit and thereby justifying their valorization.

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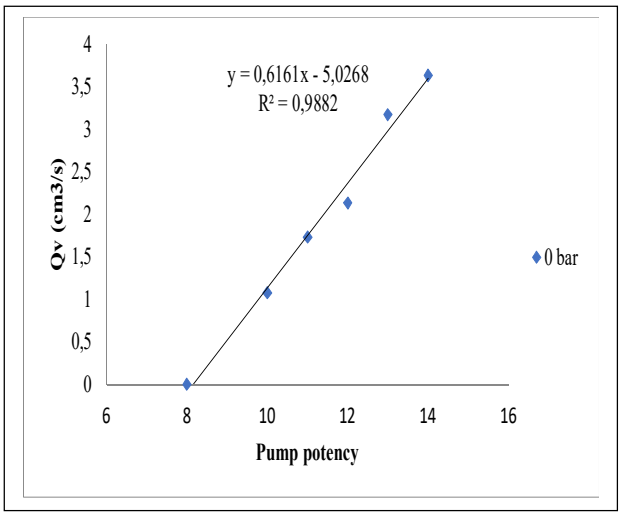
ANNEX 1.

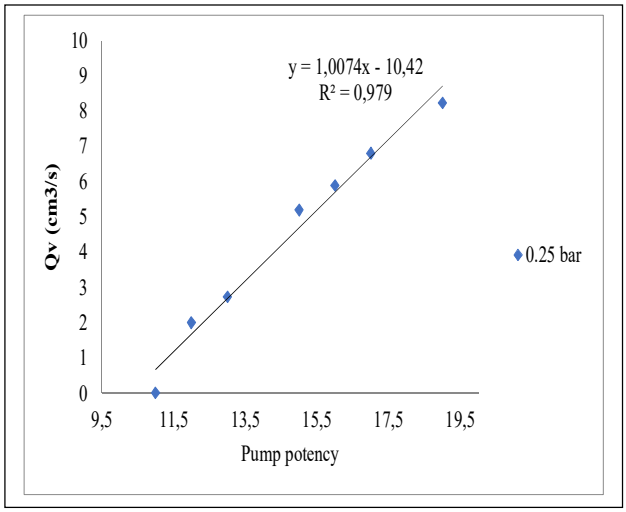
Calibration curve of the volumetric flow (cm^3/s) vs pump potency of the lab scale ultrafiltration scale CELFA P28 UF.

a) Qv VS pump potency at 0 bar; b) Qv VS pump potency at 0.25 bar; c) Qv VS pump potency at 0.5 bar; d) Qv VS pump potency at 0.75 bar; e) Qv VS pump potency at 1 bar; f) Qv VS pump potency at 1.25 bar; g) Qv VS pump potency at 1.5 bar; h) Qv VS pump potency at 1.75 bar; i) Qv VS pump potency at 2.0 bar; j) Qv VS pump potency at 2.25 bar; k) Qv VS pump potency at 2.25 bar.

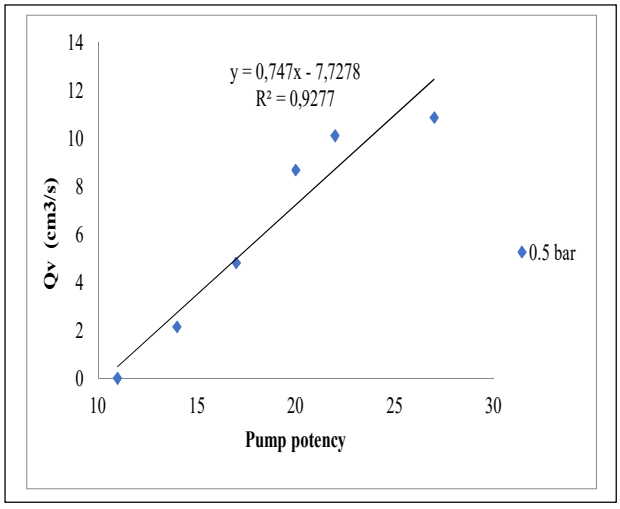
a)



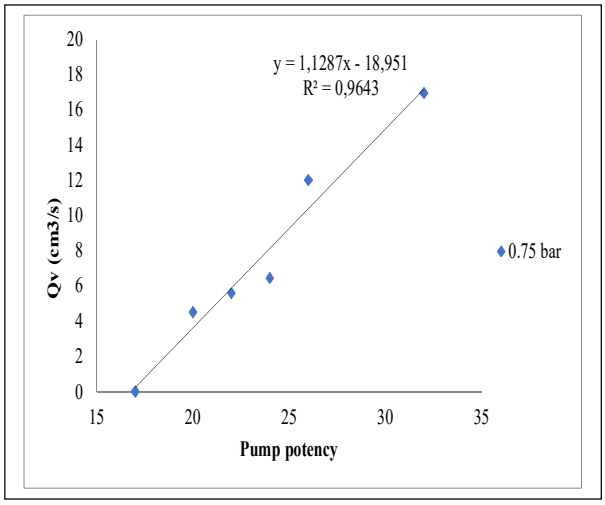
b)



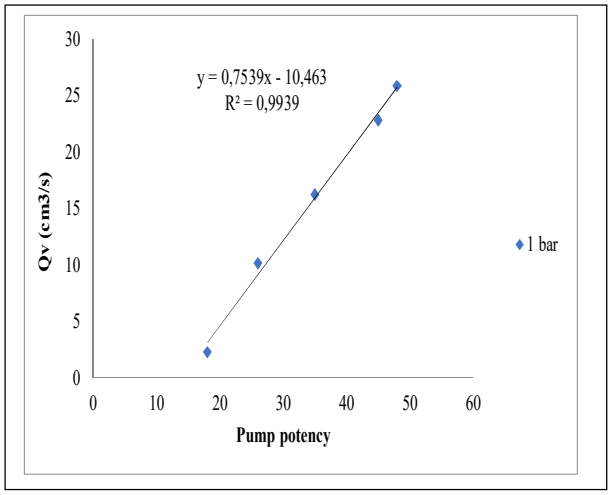
c)



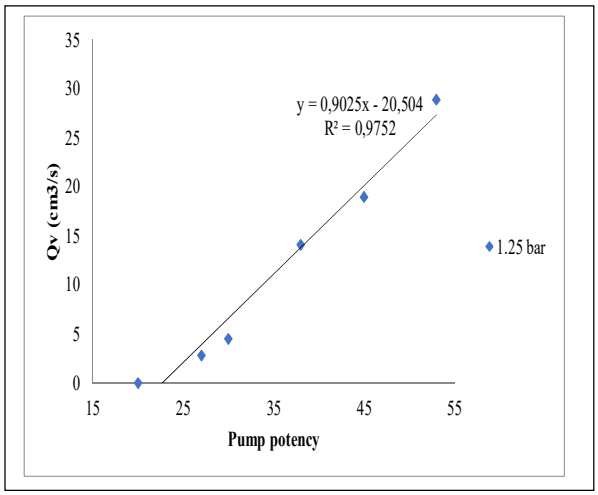
d)



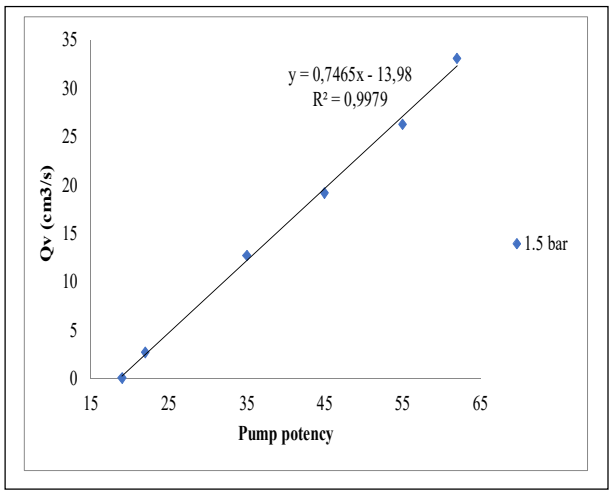
e)


y = 0,7539x - 10,463
R² = 0,9939
Qv (cm3/s)
Pump potency
1 bar

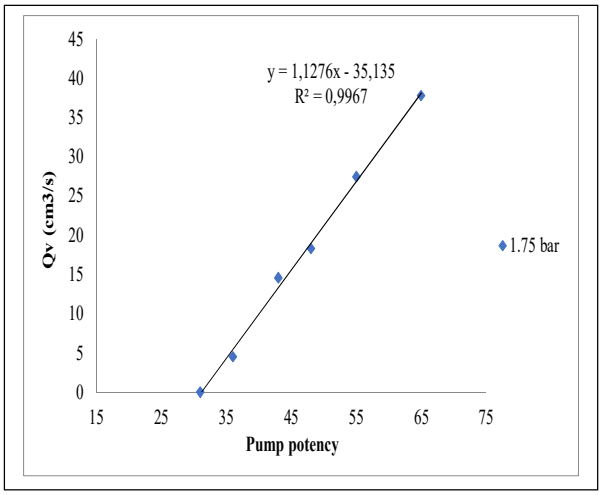
f)


y = 0,9025x - 20,504
R² = 0,9752
Qv (cm3/s)
Pump potency
1.25 bar

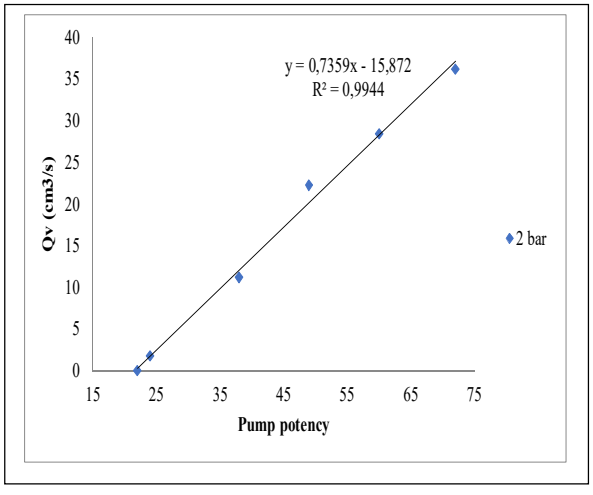
g)


y = 0,7465x - 13,98
R² = 0,9979
Qv (cm3/s)
Pump potency
1.5 bar

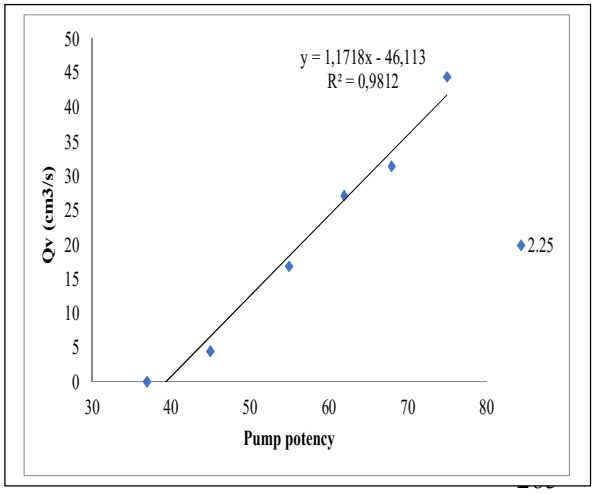
h)


y = 1,1276x - 35,135
R² = 0,9967
Qv (cm3/s)
Pump potency
1.75 bar

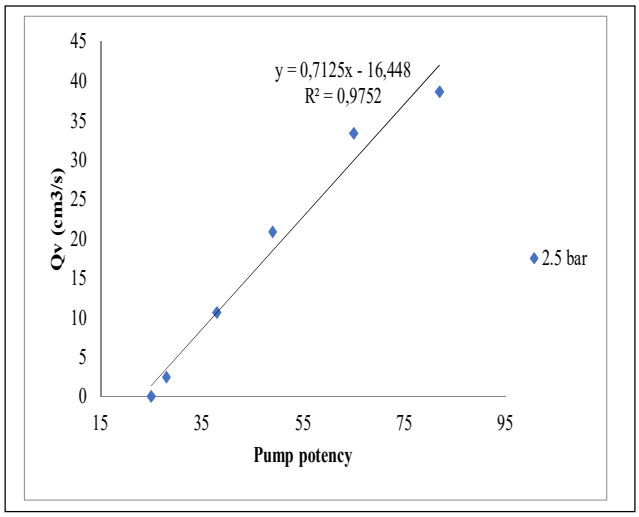
i)


y = 0,7359x - 15,872
R² = 0,9944
Qv (cm3/s)
Pump potency
2 bar

j)


y = 1,1718x - 46,113
R² = 0,9812
Qv (cm3/s)
Pump potency
2.25

k)


y = 0,7125x - 16,448
R² = 0,9752
Qv (cm3/s)
Pump potency
2.5 bar
45
40
35
30
25
20
15
10
5
0
15
35
55
75
95